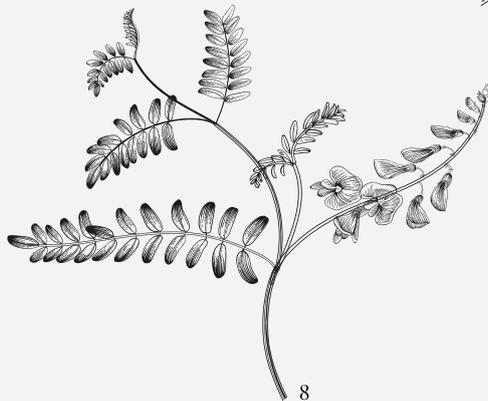
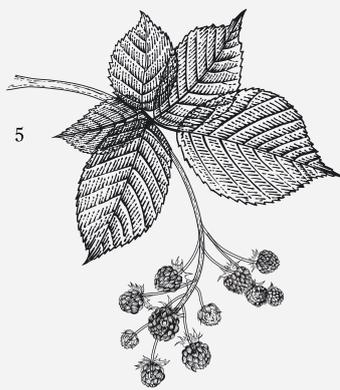
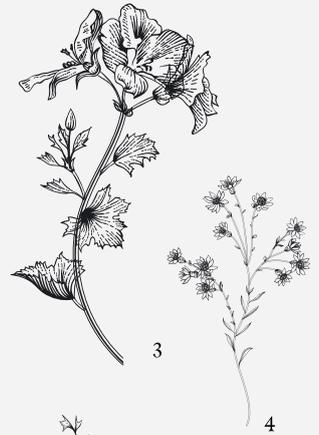




SAFE USE OF BOTANICALS IN BEAUTY PRODUCTS



FOREWORD

At P&G, product safety is paramount. Ensuring the safety of our products for the people who use them is at the heart of what we do, and we have over one hundred in-house experts who are devoted to the safety of all our products and ingredients.

As the popularity of products with botanical ingredients has increased, P&G has worked hard to develop a robust safety approach which allows these complex mixtures of natural materials to be assessed and used. Contrary to popular belief, 'natural' does not automatically mean 'safe'.

Safe use of botanical ingredients is dependent on understanding the identity and composition of their chemical components, the complex molecules and proteins that give rise to the product performance and experience enjoyed by consumers worldwide.

However, understanding this complexity can be challenging; plants can vary depending on local growing conditions, botanical extracts can be prepared in numerous ways, and many botanical ingredients have

not been properly analysed and assessed against the standards applied to other non-botanical ingredients, their application based on hundreds of years of traditional use.

P&G's safety scientists are passionate about their contribution to the expert safety community. They have spent over a decade working collaboratively to address the different challenges associated with ensuring the safe use of botanical ingredients and have shared their work through numerous peer-reviewed publications and presentations.

This white paper presents guidance for assessing the safe use of botanicals in beauty products, with historical case examples, practical tools and background knowledge. P&G hopes this work will help ensure all botanicals used in beauty products will be safely enjoyed by consumers throughout the world.

To find out more about P&G's commitment to Responsible Beauty, visit: <https://us.pg.com/responsible-beauty/>.



INTRODUCTION

Botanical ingredients or extracts are naturally-occurring complex chemical mixtures from plants (Roe et al., 2018). They can be sourced from the whole plant or from specific parts of the plant such as the leaves, roots, flowers, fruits, seeds, or berries. Botanicals are typically used as extracts, oils or isolated by more complex processes such as fermentation (Troyano et al., 2011).

Beauty products often include botanicals because of their innate ability to moisturise, cleanse and perfume or due to claimed qualities such as antioxidant, anti-inflammatory and antimicrobial properties (Corazza et al., 2013).

A popular consumer belief is that natural ingredients are intrinsically 'safe'. However, there are many recorded cases where beauty products containing botanicals have caused serious adverse events such as irritation, allergic contact dermatitis (inflammation and chronic sensitivity of the skin), photosensitisation (skin reactions to light), contact urticaria (localised swelling and redness of the skin) and difficulty breathing (respiratory reactions) (Corazza et al., 2013; Kiken and Cohen, 2002). Often these adverse events are the result of including botanicals without proper consideration for identification, processing or exposure.

Between 2004 and 2010, a mail-order soap known as 'Cha no Shizuku' was sold to 4.6 million customers in Japan (Iwamoto et al., 2012). More than 2,100 individuals who used the soap went on to develop allergic symptoms after ingesting natural wheat proteins (Yagami et al., 2017), with some cases of extreme hypersensitivity and anaphylaxis. In these cases, the development of extreme Type I sensitivity observed

in individuals were linked to the presence of large, hydrolysed wheat proteins used within the product (Burnett et al., 2018). Sales were swiftly discontinued in May 2011 (Yagami et al., 2017).

Botanicals can be safely used in beauty products, provided appropriate care is taken to ensure that the correct botanical is used, and enough toxicological data are available to understand the risks and develop adequate safety margins (Troyano et al., 2011). This is the same standard applied to other ingredients used in beauty products, including synthetics.

In this white paper, we describe the three steps that P&G carry out to ensure the safe use of botanicals in their beauty products. These include:



Identification of the plant, the method of botanical preparation and extraction



Assessment of the daily dose exposure to the botanical and safety margin



Analysis of the related botanical safety data, including any adverse events identified through post-market surveillance and development of a robust safety margin



UNDERSTANDING BOTANICALS - IDENTITY, QUALITY AND PURITY

A robust understanding of botanical ingredients will help select the right one for use in a beauty product and help to minimise the risk of potential adverse events. Natural biological variation, and the different methods of processing, can impact the properties of the desired botanical ingredient and its subsequent functional role. Identifying, establishing and maintaining relationships with trusted botanical suppliers, is essential to ensuring high-quality, reproducible botanical ingredients with consistent safety profiles for use in beauty products.

Botanicals are rarely bought or sold in their raw form; they tend to be supplied in waxes, oils or aqueous solutions, depending on how they have been prepared (Lesage-Meessen et al., 2015) and their intended use. Due to these variables, it is important to understand details about the plant from which the botanical is derived as they may not be readily apparent. A quality supplier will be able to provide information about the plant genus and species of origin, the part of the plant, and the method used to prepare the botanical ingredient. This information is paramount for a thorough safety assessment.

Ensuring quality and botanical fingerprinting

The need for quality suppliers is recognised throughout industry, as it is possible for botanical ingredients to be contaminated or adulterated with cheaper materials. Many intentional or non-intentional adulterants can have serious safety issues (Roberts et al., 2019). For example, grape seed extract, commonly used as a dietary supplement due to its claimed antioxidant effects, can be replaced or supplemented by peanut skin or pine bark. These natural extracts all contain different proanthocyanidins, but the product may not provide the consumer with the intended benefit without the specific type and amount of proanthocyanidin provided by grape seed extract. Some adulterants may be safe but fall short of the intended functional role of the botanical ingredient.

Consequently, purchasers need to be aware of the common adulterants and build relationships with quality suppliers to ensure the botanicals bought are of known purity. Industry associations such as the American Botanical Council keep purchasers up to date on the most common adulterants and circulating contaminant trends.



Another way to ensure the quality of ingredients provided by a supplier is to test a sample of the botanical for signature chemical markers – a botanical ‘fingerprint’ (see also example in Figure 1). This chemical fingerprint can be compared to known reference standards using techniques such as nuclear magnetic resonance spectrometry (¹H-NMR), high performance liquid chromatography with a UV light detector (HPLC-UV), gas chromatography (GC) and thin-layer chromatography (TLC). GC, coupled with time-of-flight mass spectrometry (ToFMS), has been used to characterise the chemical signatures of different types of tea, and benchmark markers of quality for the tea production chain. TLC has been used to verify the chemical signatures of some essential oils, and to evaluate the quality of the finished product after development (Narasimhaji et al., 2019). The combination of GC and TLC have been shown to reliably verify the contents of botanical fingerprints in both a qualitative and quantitative manner, allowing authentication of the botanical ingredient (Narasimhaji et al., 2019).

It is possible to use suppliers of United States Pharmacopoeia-grade ingredients or International Organization for Standardization (ISO) ingredients like essential oils, which must fall within known ranges

for their perfume-like properties. Essential oils are one example of a botanical material that have been well-characterised, with defined chemical constituent ranges widely published.

Botanical fingerprinting can also be performed by botanical experts such as at the Royal Botanic Gardens, Kew, London. The Royal Botanic Gardens, Kew, has the most diverse botanical resource collection in the world, including 7 million dried plants, 1.25 million dried fungi and 50,000 plant DNA samples, as well as an extensive seed bank. The Royal Botanic Gardens, Kew, can assist with the selection of the best sources of sustainably grown quality plants for optimum bioactivity. Their scientists can also use a range of different analytic methods - ¹H-NMR, liquid chromatography-mass spectrometry (LC-MS) and gas chromatography-mass spectrometry (GC-MS) - to analyse samples against their diverse plant collections, looking for comparable botanical fingerprints as well as potential adulterants. P&G works collaboratively with the Royal Botanic Gardens, Kew, to identify plant extracts and ensure the purity of botanical samples for certain botanical extracts. P&G also completes rigorous and frequent Good Manufacturing Practice Assessments (GMPA) on its suppliers.

Figure 1 Aloe vera - quality and botanical fingerprinting



Aloe vera (L.) Burm. f. (also known as *Aloe barbadensis* Miller) is a succulent plant with a long history of claimed health benefits. The gel within its leaves is widely used in beauty products for its moisturising or hydrating effects to both skin and hair. Due to the worldwide demand for this ingredient, purchasers need to understand how the botanical composition can vary, in order to monitor the quality of ingredients provided by suppliers so that their beauty product produces consistent benefit for their customers.

Three main factors affect the botanical composition of aloe gel extracts; growing conditions, time between harvesting and preparation, and method of processing. These factors affect the ratios of chemical components, including sugars, enzymes, organic acids and polysaccharides, such as acemannan, the main bioactive ingredient (Marsh and Simmonds, 2020).

For beauty products, dry preparations of aloe are preferred for ease of transportation and product stability, but they can be adulterated with substances such as maltodextrin. In a study comparing commercially available aloe powders from leading international suppliers, a multi-technique approach was taken to determine the quality and authenticity of aloe powder versus fresh aloe gel. ¹H-NMR was used to identify the essential components of the aloe gel, which provide the characteristic signals that can be considered the ‘fingerprint’ of aloe. The same technique was also used to distinguish between the expected aloe fingerprint and any potential adulterants, such as maltodextrin. HPLC-UV provided information about the freshness of the product and the time between the harvest and processing.

The study found the quality of the powdered samples to be inconsistent and, in some cases, very poor. Some showed evidence of degradation and bacterial fermentation, and only three of the nine samples analysed contained satisfactory amounts of the bioactive ingredient (Bozzi et al., 2006).

DETERMINING CONSUMER EXPOSURE OF BOTANICALS FROM BEAUTY PRODUCTS

Beauty products are typically applied to the skin. Exposure tends to be low as ingredients often remain on the skin and would have to be absorbed through the skin to enter the body; or enter the body through an indirect and unintended route.

Despite this low exposure, all ingredients, including botanicals, have the potential to cause adverse effects and so a quantitative toxicological risk assessment is necessary to ensure product safety (Corazza et al., 2013). Understanding realistic human exposure to botanicals through beauty products is vital for a proper evaluation of the ingredients used.

Various regulatory agencies and authoritative bodies may have standard methods to assess exposure (i.e. Europe's Scientific Committee on Consumer Safety [SCCS]). Best practice should be to consider any appropriate regulatory agency approach and evaluate the following factors based on consumer observation, diary studies, and other means to monitor consumer exposure:

Exposure: Most beauty products are typically applied, so the main routes of exposure are dermal (rubbing in moisturiser to the skin or shampoo to the hair) or through inhalation (breathing in deodorant sprays or aerosols). Ocular (via the eyes) exposure can be considered accidental, as is not the intended area



of use, but is important to note for products used on the face, such as facial moisturiser and eye cream. Ingestion is the final potential route of exposure and tends to relate more to food or dietary supplements.

Habits: Knowing how frequently a product is used is essential in establishing the potential level of human exposure. This includes how much of a product is being used during one application, the number of times in a day the product is applied, the number of days it is applied for, and whether it is a product to be used chronically; which is often the case for most beauty products. Consumers may also use multiple products from the same brand, resulting in cumulative exposure to the same ingredients over time.



Duration: 'Leave-on' products such as moisturisers will remain on the skin longer and so human exposure is higher than compared with 'rinse-off' products like shampoo. However, despite 'rinse-off' products seemingly staying on skin for a short time, residual traces of the product can remain and be found days after use.



By considering these different factors, the exposure of a botanical can be calculated and used for safety analyses; Figure 2 (SCCS, 2012).

Figure 2 Beauty botanical ingredient A



A beauty product contains 4% of the botanical ingredient A.

This ingredient contains 20% plant extract. The plant extract was made by adding 1 kg of plant to 9 kg solvent via maceration. The following method was used to calculate the **overall concentration** of plant in the product:

Concentration in the product = Amount used in the product × concentration of the ingredient

Concentration in the product = 4% × 10% × 20%

*Concentration in the product = **0.08% (0.0008)***

Consumer observation showed that 15 g of the product was used once per day. Only 1% of the product was retained (not washed off).

To calculate the **daily exposure** to the botanical in the product, the following method was used:

Dermal exposure per day = concentration in the product × amount used (per day) × retention

Dermal exposure per day = 0.0008 × 15 g × 0.01

*Dermal exposure per day = **0.00012 g or 120 µg***

SAFETY ASSESSMENT

Once the botanical has been identified, checked, and the daily exposure calculated, the next step of analysis is the safety assessment. There are three parts to a thorough safety assessment, and each outcome needs to be acceptable for the botanical to be used in a beauty product.

The three parts to a botanical safety assessment are:



Systemic safety: assessing botanical safety inside the body through three tiers of evaluation (see Figure 3)



Skin safety: assessing the potential for skin irritation, delayed skin allergy (Type IV allergy) and phototoxicity (skin reactions to light)



Immediate allergy safety: assessing the potential for immediate, food-type allergies (Type I allergy) such as hives, asthma and anaphylaxis

Systemic safety

Tier 1 – is the botanical amount below a threshold that is safe for exposure inside the body?

The idea for a threshold under which exposure to a chemical would not result in any toxicity was first proposed in 1967, and later developed in 1996 into the Threshold of Toxicological Concern (TTC) (Munro et al., 1996). TTC is a principle commonly used in food safety assessment which refers to the possibility of establishing a human exposure threshold value for ingredients below which there is no appreciable risk to human health (More et al., 2019). Based on the ‘safe’ doses for known substances in food, this corresponds to a maximum dietary exposure of 0.15 μg per person per day. (Mahony et al., 2020; Munro et al., 1996).

This is remarkably similar to the current challenge with botanicals; potentially harmful chemical constituents are known to be present in some plants, but not all

may have been tested for safety. A study was carried out to apply this TTC approach to the concentration of natural chemical constituents in botanicals. Based on an extensive literature analysis, a maximum TTC of 10 μg per person per day of botanical plant material, based on dry weight, was determined to be sufficiently protective (Mahony et al., 2020).

As discussed in the earlier example (see Figure 2), a rinse-off product containing 4% of botanical ingredient A, corresponded to a total daily exposure of 120 μg per day. This value is over the TTC limit. Determining whether a botanical ingredient is above or below this threshold is known as a Tier 1 systemic safety assessment. If the value is found to be above 10 μg per person per day, then subsequent Tier 2 or Tier 3 assessments are required.

To note, TTC can be used for botanical plants and some extracts. TTC cannot be applied to extracts that are selectively or wholly concentrated as this alters natural ratios of chemical constituents found in the plant (Mahony et al., 2020). For example, it would not be applicable to use TTC on a selectively concentrated lavender extract that has been processed to contain significantly higher levels of linalool, but where all other chemical constituents remain the same or at lower concentrations than what is found in the naturally-occurring botanical. Further, it would not be applicable to use TTC on a 200x concentrated aloe vera extract in which all of the chemical constituents are at a 200-fold higher concentration, but still in their ratios, than what is found in the naturally-occurring botanical. Therefore, the value of 10 μg per person per day should not be applied to concentrated botanical materials like essential oils, or for botanicals with a known history of toxicity through preclinical or clinical data.

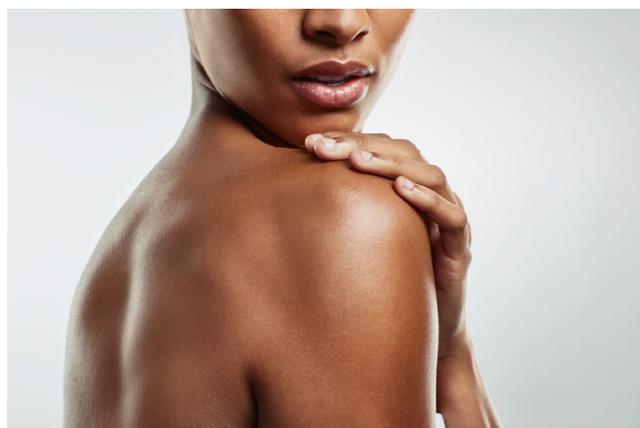
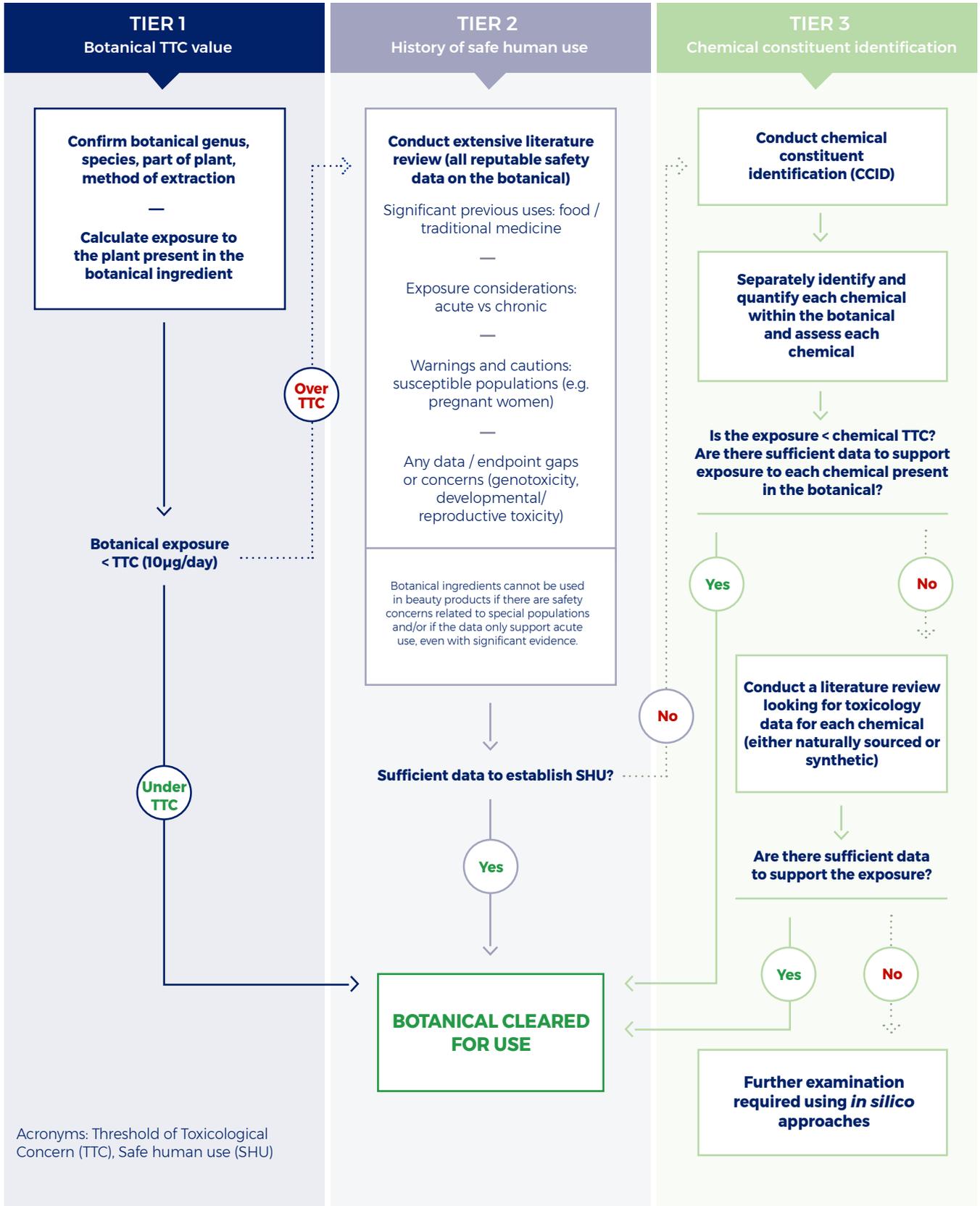


Figure 3:
Systemic safety assessment tiers for botanical ingredients in beauty products



Tier 2 – is there enough data on the botanical to confirm safe human use?

If the daily systemic exposure for the botanical ingredient is found to be higher than 10 µg per person per day, then Tier 2 assessment is required. This step involves scrutinising all reputable published data on the botanical ingredient for known hazards, to show that within reasonable and practical margins, it has a history of safe human use (SHU).

Data should be collected about the plant, different methods of preparation, common usage, any safety endpoint data (e.g. liver toxicity, genotoxicity or carcinogenicity), epidemiology, and from current or historical clinical studies, including surveillance reports from adverse event databases such as the [World Health Organisation's VigiAccess™](#). If robust data are found to support safe dietary use in humans or safe chronic use as a traditional medicine, then the botanical can be used in the beauty product (Galli et al., 2019).

However, if data are not found, or if safe chronic human use is only available in a specific population, then the botanical cannot be used before it has been further evaluated. As beauty products are available for anyone to purchase, and the beauty market is not governed on a country-by-country basis, the use of a botanical in a beauty product should be proven safe for everyone, including sub-populations assumed to be more sensitive – children, pregnant women and the elderly.

If the botanical product does not have a proven history of SHU, then it is necessary to perform a Tier 3 assessment.

Tier 3 – are the individual botanical chemical constituents safe?

If the botanical exposure is above the TTC and a history of SHU data is not available, it is necessary to quantify and assess the botanical at a chemical constituent level (Baker and Regg, 2018). For chemical constituent identification (CCID), the botanical is separated into its constituent parts, and each of these chemicals are separately assessed for safety using the chemical TTC or available toxicology data.

Chemical constituent identification

Several established methods are available to determine the constituents within a complex mixture such as botanicals, including high- and ultra-high-performance liquid chromatography (u/HPLC) (Guldiken et al., 2018), high-resolution mass spectrometry (HRMS), ultraviolet (UV) detection, and charged aerosol detection (CAD), Figure 4, (Baker and Regg, 2018). HPLC is a robust, yet versatile technique, commonly used to isolate constituents in natural products through a series of mobile and stationary phases (Sasidharan et al., 2011). HRMS can be used to quickly assign a molecular formula to constituents, especially in the absence of reference standards, which can be cross-referenced with online databases (Baker and Regg, 2018). Tandem mass spectrometry (MS/MS) studies are able to provide structural clues to support conclusions by comparing against fragmentation of known molecules in the same class, and aiding comparisons when using databases such as [mzCloud](#) (Baker and Regg, 2018). UV detection is a common technique for identifying unknown chemicals, and is dependent on the presence of a chromophore, a part of the chemical's molecular structure that absorbs UV light. This absorption can be used to identify atomic structures and bond characteristics of specific chemicals. In components without a chromophore, a CAD detector can be used (Baker and Regg, 2018). This method uses polar charge, rather than light absorption, to identify molecular components.

Figure 4

Ginkgo biloba leaf – identifying chemical constituents



There are various methods to characterise complex botanical mixtures, but in the absence of reference standards, it can be difficult to quantify individual chemical constituents.

Using Ginkgo biloba leaf as an example, Baker and Regg (2018) demonstrated an approach that combines the different strengths of multiple instruments to allow the separation and identification of complex botanical mixtures. Once separated by uHPLC, the components were individually identified and quantified by UV, CAD and HRMS, allowing for a direct correlation between identity and relative ratio of different chemicals in the botanical mixture.

This technique may provide a platform for enabling *in silico* (computer modelling) of safety assessments and providing quality assessments of botanicals without suitable reference standards.

Some botanical ingredients, like essential oils used for flavours and aromas, are well characterised, so it may not be necessary to undertake CCID as published data on its chemical constituents may be sufficient to decide on safety.

While these techniques are very good at identifying the chemical constituents in a botanical ingredient sample, they may not be able to isolate everything (Baker and Regg, 2018). For instance, during chromatography, some of the complex mixture can be lost due to volatility or through elution (it remains within the stationary phase and does not pass through to the detector). In addition, some chemical constituents may be present in such small quantities that they are below the limit of detection (LOD) of the analytical method. If feasible, the LOD for the analytical method is set at the chemical TTC level. Although the resulting data may not be a fully comprehensive representation of the complete botanical mixture, it is usually sufficient to establish the main constituents in a sample. Through the optimisation of techniques used to separate similar constituents in a complex mixture, and the ability to present them to detectors separately for identification, data interpretation and quantification can be facilitated.

Once separated and identified, the chemical constituents must be quantified for safety.

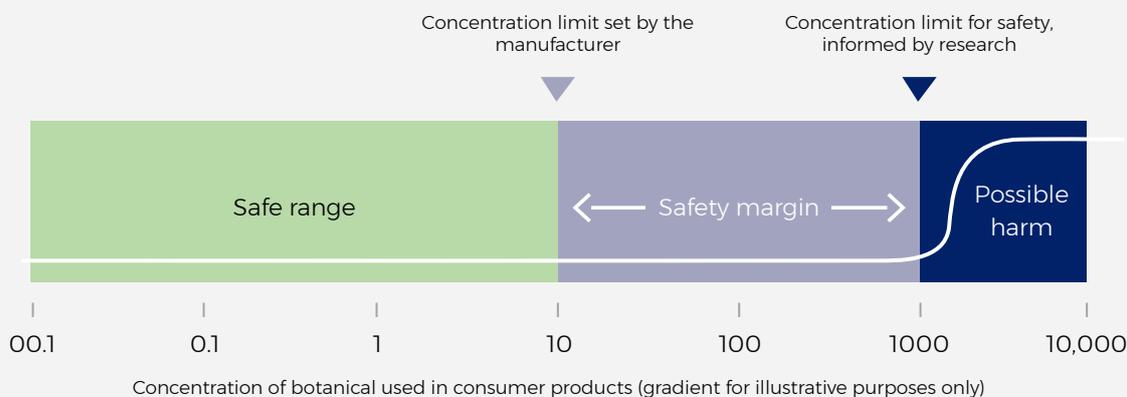
Final steps for systemic safety assessment

From the literature review in Tier 2, a safe dose range for the botanical ingredient can be calculated. The safety endpoints are ranked and the toxic effect that occurs at the lowest dose, the 'critical effect' is identified for the botanical. This will be the hazard which will be used to calculate the safe range for the entire botanical ingredient. To the 'critical effect' dose, an additional 'uncertainty factor' safety margin is added by reducing the safe dose level by at least two orders of magnitude (SCCS, 2012). This is the maximum botanical dose (see Figure 5).

If data were insufficient for Tier 2 and a Tier 3 chemical constituent identification was done, a safe dose range will need to be calculated for each of the chemical constituents identified based on the 'critical effect'. As in the Tier 2 botanical example, the process of identifying the maximum dose would be the same as noted for the whole extract, but this time done at the constituent level (SCCS, 2012).

Once the botanical and/or its chemical constituents have completed the systemic safety assessment, the next step is the site of contact assessment.

Figure 5:
How a safe range for a botanical ingredient is calculated



All botanical ingredients in a beauty product are evaluated and confirmed to be well within the safe range for all relevant toxicological endpoints

Skin safety

Most beauty products are applied to the skin as the 'site of contact'. Site of contact analysis includes skin allergy (Type IV contact sensitisation), irritation and phototoxicity through dermal exposure (Troyano et al., 2011). As a rule, P&G does not formulate products with botanicals that are known to cause irritation.

An important factor to consider is Type IV allergy or delayed dermal contact sensitisation, similar to that observed by poison ivy. If an individual experiences sensitisation or becomes allergic to a product or ingredient, this usually persists for life. All ingredients in a beauty product, including botanicals, are evaluated for contact sensitisation potential (see Figure 6).

The initial literature search on the botanical will include any data relevant to contact sensitisation. When available, these data will be used in the Quantitative Risk Assessment (QRA) approach to evaluate safety for skin sensitisation (Kimber et al., 2017). When relevant data are not available for the botanical, a maximum threshold value, based on previous data, is needed to perform a skin sensitisation risk assessment. A suggested benchmark for dermal sensitisation is a dose of $10 \mu\text{g per cm}^2$. This value was selected based on data from most potent skin sensitisers and is a protective value reflecting what could be the 'worst-case scenario' (Troyano et al., 2011). A botanical in its natural form, not in a concentrated

form, should be below this benchmark value. An additional uncertainty factor is not added to this benchmark value as the natural extract will represent a much smaller fraction of the whole botanical component (Troyano et al., 2011). The $10 \mu\text{g per cm}^2$ benchmark provides assurance of negligible contact allergy risk and can also be used for dermal irritation, photoallergy potential and phototoxicity (Troyano et al., 2011). Human Repeat Insult Patch (HRIPT) studies should only be used for confirmatory purposes once the botanical exposure relevant to the contact sensitisation endpoint is determined to be safe.



Figure 6 Essential oils – the need for safety evaluation



Essential oils are widely used botanical ingredients, but at least 79 of them are known to cause irritation leading to inflammation of the skin (de Groot and Schmidt, 2016).

In 2016, essential oil use was examined in adults at a Minnesota State Fair. Common reasons for using essential oils included a desire for alternative treatments, a belief that essential oils were safer than traditional therapies, or that they had turned to essential oils after the failure of standard therapies. The survey also reported that in addition to physical conditions such as musculoskeletal, upper respiratory and skin complaints, they were also being used for emotional reasons such as anxiety and depression (Goodier et al., 2019).

Typical adverse events reported included rash, difficulty breathing, burning sensation, allergy and discomfort during urination after oral ingestion (Goodier et al., 2019). With the apparent rise in popularity of essential oils, quality ingredients and appropriate safety limits are critical to prevent a corresponding rise in contact allergic reactions

IMMEDIATE ALLERGY SAFETY

Type I allergy is where the human body produces IgE allergic antibodies in response to a foreign substance (an 'allergen'), leading to an inflammatory response and the development of symptoms such as conjunctivitis, hives, asthma, or anaphylaxis. This can be triggered via four main routes of exposure to an allergen; inhalation, ocular, dermal and systemic (Krutz et al., 2019).

Allergens are often specific proteins, and each protein can be assigned a level of allergenic potential. A protein can be considered to have a low allergenic potential if there is evidence of ongoing human exposure without reports of an allergic reaction (Krutz et al., 2019). Databases such as [Allergome](#) can also be used to understand whether the structure of particular proteins contains any IgE-binding antigens, the lack of which would provide good evidence for the lack of allergenicity. Proteins with a higher molecular weight (>2.5 kDa, or 20-30 amino acids) should be considered potential allergens unless there is significant evidence to the contrary.

A pragmatic approach to reducing this effect is avoiding known sources of allergens. If possible, botanicals with a low protein content should be used, unless the protein is the functional bioactive

component (Troyano et al., 2011). For example, P&G generally avoid using globally recognised food allergens, such as tree nuts, celery, and sesame seeds, in their beauty products (Troyano et al., 2011), as they could elicit a response in individuals already sensitised (allergic) to these foods.

One of the most common routes of exposure by which beauty products can cause Type I allergy is through direct inhalation of aerosols, such as deodorant and hair spray. It is also possible to indirectly inhale more viscous topical products, such as shampoos, conditioners, and body washes, when small amounts combine with fine water vapour in the shower. There have also been cases where residual topical body moisturisers, applied to the body the day before, have atomised off the skin in the shower the next morning and been inhaled (Kelling et al., 1998).

Botanical ingredients intended for use in direct spray products and/or used (e.g. shampoo) or washed off in the shower (e.g. body lotion) are tested for total protein content and the resulting protein exposure must be lower than the allergy benchmark before they are included in a beauty product. Proposed protein exposure should be below the protective allergy benchmark of 0.1 ng/m³ (Troyano et al., 2011).



Methods for quantifying protein amount

Kjeldahl nitrogen analysis

Developed in 1883 by Johann Kjeldahl, this method quantifies the amount of protein in a botanical by breaking down the sample with strong acids and analysing the amount of nitrogen released. This process is suitable for botanical oils, waxes and aqueous solution samples and is internationally recognised (Neilson, 2019). The use of the Ion Selective Electrode Detection system can increase the sensitivity of the process to ~5ppm nitrogen. The nitrogen results are multiplied by the factor 6.25 based on an AOAC compendia method to convert the results into total protein (Troyano et al., 2011). Many botanicals may contain other non-protein nitrogenous chemical constituents and require a technique with a greater degree of precision. The Food and Agriculture Organization (FAO) of the United Nations recently recommended the use of amino acid analysis instead of the Kjeldahl method, but they also noted that amino acid analysis often requires the use of sophisticated instrumentation, which may not be readily available (Neilson, 2019).

Amino acid analysis

An amino acid analysis is used to quantify the amino acid composition of an aqueous solution sample and estimates the molecular mass of a protein (Neilson, 2019). First, the sample is hydrolysed under acidic and high-temperature conditions to break the proteins down into amino acids, and then these amino acids are separated using chromatographic techniques (Neilson, 2019; Troyano et al., 2011). Ion-exchange chromatographic separation with post-column ninhydrin derivatisation has been shown to provide reliable data for the analysis of amino acids (Troyano et al., 2011). Once the sample has been hydrolysed and separated, each separate amino acid is detected and quantified. The estimated protein concentration of the botanical sample can then be calculated:

$$\frac{(\text{total mass of recovered amino acids})}{(\text{total mass of the sample})} = \text{total protein concentration (\% weight)}$$

Molecular weight distribution

Molecular weight distribution is used to analyse proteins in a botanical by relative mass. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) or size-exclusion chromatography (SEC) can be used to analyse protein chains longer than 2.5 kDa (Neilson, 2019; Troyano et al., 2011). Aqueous

samples are usually analysed at three different concentrations, the desired concentration (i.e. 0.1%), ten times more concentrated (1%), and ten-fold diluted (0.01%). If the protein is not visible in either the desired or diluted concentrations, it can be concluded that the concentration of protein >2.5kDa in size is acceptable (Troyano et al., 2011). SEC is an alternative method of determining protein molecular weight distribution, through the separation of molecules based on their size using a column, where the largest molecules are eluted first. Despite it having a lower resolution than SDS-PAGE, SEC can be automated, and can also quantify based on comparison with the UV absorbance of protein standards (Neilson, 2019).

Methods for characterising protein structure for potential allergenicity

Proteomics and bioinformatics

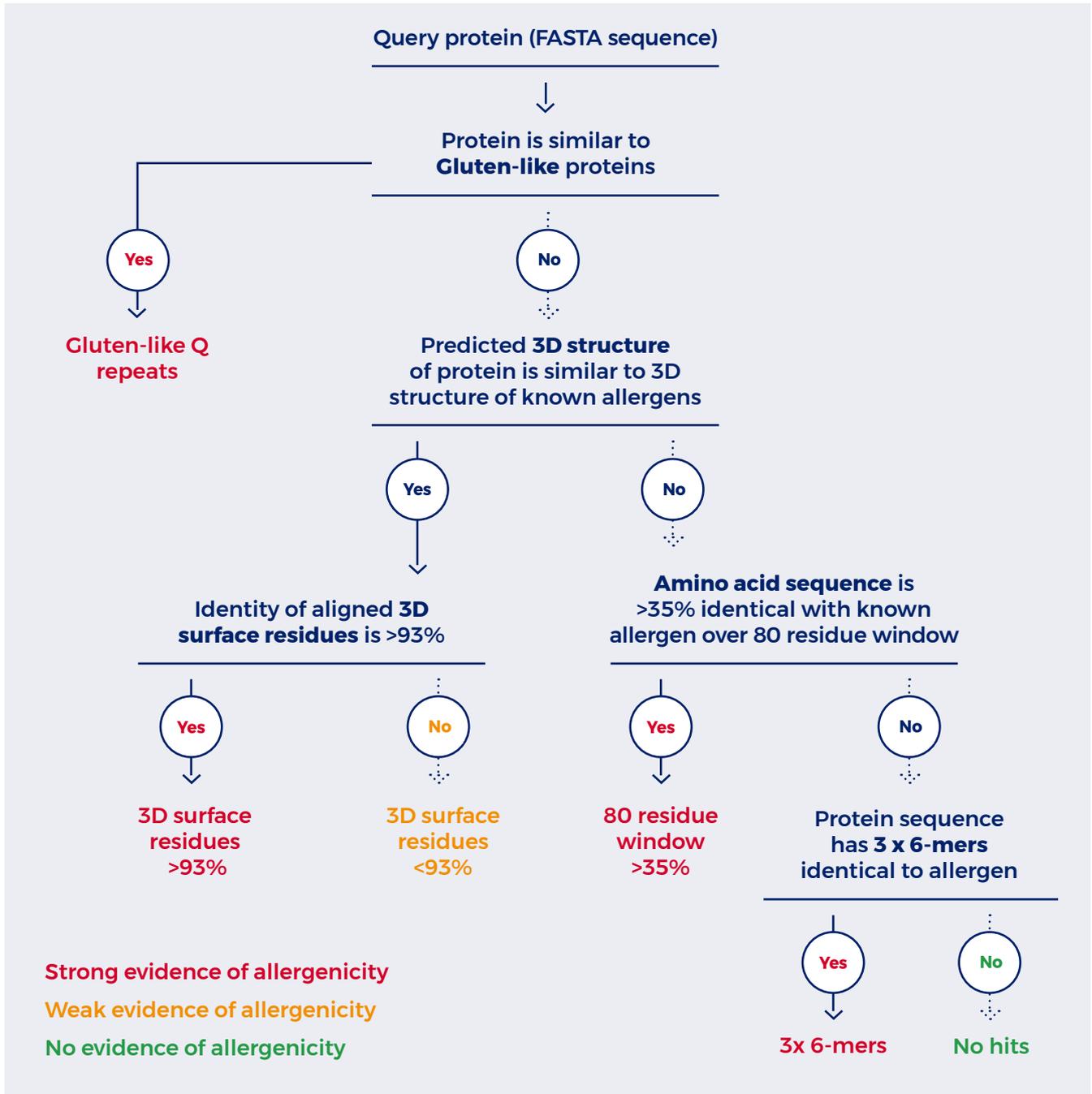
Recent approaches to the structural characterisation of proteins in a botanical include proteomics and bioinformatics analysis.

Proteomic analysis maps the specific protein sequence. Databases such as [NCBI](#) and [UniProt](#) can then be used to identify specific protein sequences associated with allergenicity. Their 3D protein structure can then be further analysed in order to characterise structural or molecular features relating to allergenicity (Maurer-Stroh et al., 2019). The database [AllerCatPro](#) also stores information about known allergens, and can be used to compare and predict whether proteins are likely to have a high or low allergenic potential (Krutz et al., 2019) (see Figure 7).

Bioinformatics expand on this by collating large amounts of existing data on known allergens (Krutz et al., 2019). The protein sequence to be analysed can be added to the database in the form of a 'FASTA' file, a text-based format for representing amino acid sequences. The database is then able to assess the sequence to identify any similarity to known allergens. If there is a similarity, there is a high chance of allergenicity. If there are no matches, the 3D structure of the protein is compared to that of other known allergens. If similar structures are identified, the sequences of each are then compared to indicate the likelihood of the sample being an allergen.

Once the three parts of the safety assessment have concluded and the botanical has been cleared, it can be used in a beauty product.

Figure 7:
Proteomic and Bioinformatic analysis:
assessing allergenic potential



SUMMARY

The common belief that natural ingredients, such as botanicals, are intrinsically 'safe' to use in beauty products is false, as supported by the examples presented in this white paper. However, botanicals can be safely used in beauty products, provided appropriate care and a range of analyses are undertaken including accurate identification of the botanical, followed by defining consumer exposure, and a tiered safety assessment. This consumer exposure and safety assessment follows the same standards applied to man-made beauty ingredients. Through these extensive analyses and safety assessments, the risk of potential adverse events can be minimised.

Aside from the details discussed in this white paper, further analyses may also need to be carried out to

ensure product safety. One such example is the assessment of aggregate exposure needed for all ingredients in a beauty product (Tozer et al., 2015; Tozer et al., 2019). Another area of further study may include the natural variations of chemical constituents within a single species, and in different parts of the same plant, depending upon the method of processing.

P&G strives to continually collaborate and share information on the safe use of botanical ingredients in beauty products with industry, manufacturers, suppliers and retailers, based on over a decade of research in this area.

For more information visit P&G's Responsible Beauty website <https://us.pg.com/responsible-beauty/>.



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