Anti-ageing actives and technologies behind them

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Technology is an essential part of every industry, and the personal care sector is no different. For consumers, this comes in the form of apps which allow the user to 'try on' virtual makeup looks or hair styles by using facial mapping technology[1] as well as diagnostic tools, which give personalised skin care recommendations based on the condition of and changes in the user's skin.[2,3] For raw material suppliers, however, new technologies are valuable tools in identifying, extracting and studying new active materials.

At the beginning of development, computational analysis can be used to analyse biological extracts, and to identify whether they have any potential efficacy in the skin. In the case of Sirtalice (INCI: Bacillus Ferment), computational analysis identified a potential activity on the sirtuin-3 gene, which would increase cellular energy. This process can also be used to analyse and identify the properties of synthetic compounds. By using bioinformatics analysis in conjunction with *in silico* modelling techniques, the molecular structure of novel peptides, such as Munapsys (INCI: Acetyl Hexapeptide-1), can be computationally designed based on the potential mechanisms identified.

Advances in genetic technologies are also implemented in protein production, by inducing transient expression of the desired protein in plants which produces safer, more efficacious proteins than similar products that use alternative production methods. Natural materials can also benefit from advances in biotechnology, such as enzymatic enrichment of extracts to improve their efficacy.

While technology is often used in the development of novel active materials, manufacturers have not forgotten traditional active ingredients. Biogenic Gentinol-200 (encapsulated retinol) is one such example of an already well known material which has benefitted from the application of new techniques.

Enhancing traditional anti-ageing materials

Retinol (vitamin A) was first used in the treatment of photo-ageing in the early 1980s,[4] and remains a popular choice in 2019, due to its proven high efficacy.[5] Its dual action stimulates collagen synthesis while simultaneously reducing the UV-induced production of collagen-reducing enzymes, which makes it extremely efficacious at smoothing fine lines and wrinkles, as well as preventing formation of wrinkles caused by UV irradiation.[6]

However, retinol is notoriously difficult to stabilise in cosmetic products, as it is sensitive to light, moisture, heat, oxygen, pH and heavy metals.[6-8] The composition of cosmetic emulsions containing retinol must be carefully considered, and products often contain a large oil phase[8] with additives such as UV filters to protect the active from degradation.[6-8]

In order to simplify the formulating process, a stabilised retinol material has been developed which encapsulates retinol in a novel polymeric micellar system.[9] The oil soluble active is surrounded by an inner layer of lipophilic stabiliser, a polymer capsule and then a hydrophilic stabiliser, to render it water soluble and protected against degradation. The product is then further stabilised in solution using antioxidants.

The stabilised retinol was tested at 25°C and 40°C for 24 weeks against two alternative stabilised retinol products (A and B). The materials were added to an oil in water emulsion at pH 5.5, each with a 0.11% retinol content, which was then monitored by HPLC every 4 weeks. After 24 weeks at 40°C, the retinol content in samples A and B contained 52% and 38% of the initial retinol level respectively, however the sample containing the stabilised retinol contained 74%. The same was seen at room temperature, with samples A and B retaining 70% and 50% respectively, compared to 92% for the stabilised retinol sample.

Using a stabilised version of retinol allows more freedom in development, as the formulatory constraints are significantly reduced, and the formulator can be confident that the efficacy of the final product will remain consistent throughout its shelf life. This is particularly important for retinol, as it has a strong reputation for high efficacy among consumers.[10]

Retinol is also well-known as an irritating material, particularly on sensitive skin, and cosmetic researchers have been searching for a milder alternative. It was not until the year 2000 that peptides began to establish their own reputation for high efficacy, without the irritant and inflammatory side effects.[11]

Advances in peptide and protein development

Protein sources have been used for cosmetic purposes since the ancient civilisations - Cleopatra famously bathed in

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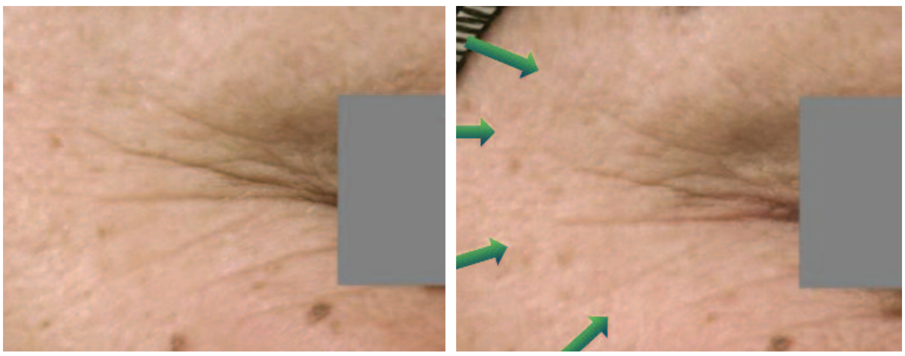

Figure 1: Results from *in vivo* test: application of cream containing 3% Acetyl Hexapeptide-1 twice a day for 14 days.

Figure 2: Results of *in vivo* test: application of gel cream containing 1.5% Nicotiana benthamiana sh-Oligopeptide-2 twice a day for 28 days.

donkey milk to preserve her youthful skin[12] – and synthetic peptides have been used in skin care products since the late 1980s.[13] Cosmeceutical peptides are used in anti-ageing products for collagen stimulation and wrinkle smoothing, and in other products for antioxidative, antimicrobial and skin whitening effects.[13]

Traditionally, natural peptides have been manufactured through partial hydrolysis of plant or animal proteins to give undefined peptide mixtures.[14] More recently, novel peptides have been designed *in silico*, where the peptide structure is optimised by identifying the lowest energy interactions between amino acids and the biological compounds being targeted. This has enabled the design of peptides which have highly specific and efficacious interactions with cellular mechanisms. These peptides are then synthesised in the solid phase to create highly potent and defined cosmetic ingredients.

This process was used to produce Acetyl Hexapeptide-1, through identifying the optimum amino acid sequence to target presynaptic muscle contraction processes, for a Botox-like activity.

The pre-synaptic muscle contraction mechanism requires Munc-18 proteins to bind to syntaxin, then SNAP-25 and VAMP, forming a SNARE complex. The VAMP protein is attached to a vesicle containing Acetylcholine (ACh), which when the SNARE complex is formed, fuses with the neuron membrane, releasing ACh into the synapse. ACh then binds to receptors in the post-synaptic muscle tissue, releasing Ca^{2+} ions, which cause the muscle to contract.[15]

The *in silico* design process identified Acetyl Hexapeptide-1 as the ideal peptide sequence to compete with Munc-18 to bind with syntaxin in the pre-synaptic mechanism to reduce Munc-18/syntaxin binding. *In vitro* efficacy tests supported the hypothesis, with a reduction of up to 37% for Munc-18/syntaxin binding. Consequently, ACh release is also reduced, by up to 35%.

Computational analysis of the material also identified potential post-synaptic activity. Further *in vitro* testing confirmed that Acetyl Hexapeptide-1 binds to the post-synaptic receptors, disrupting the ACh-Receptor cluster and reducing Ca^{2+} release, which is required for muscle contraction. This is the first material available which modulates both the pre- and post-synaptic muscle contraction processes. This dual action approach gives the material enhanced performance in reducing expression wrinkles and lifting labial commissures, and this activity has been substantiated through *in vivo* studies[16] (see Fig 1).

Solid phase synthesis is expensive and time consuming, therefore *in silico* design is an essential tool to ensure the time and money is well spent. Thanks to advances in genetic technologies, other more cost effective production methods have been developed. Bacterial and yeast cultures are now commonly used for the production of naturally occurring proteins; the genetic material of the host bacteria is modified, causing it to produce the desired material.[17] However, proteins produced in prokaryotic cells are not ideal for use in humans. Prokaryotes do not carry out the same post-translational modifications that occur in eukaryotic cells, and therefore the proteins do not have the specific structure required for interactions in eukaryotic cells. This leads to lower efficacy and also decreased safety, as the interactions that occur could induce an immune response.[18, 19] In the early 1990s, the pharmaceutical industry began developing agroinfiltration methods to overcome this issue, using vectors to insert genetic material into plant cells, to stimulate transient expression of the required proteins.[17] The post-translational modifications which occur in plants are comparable to those in humans, and the proteins are functionally identical. They therefore have superior performance and are safer for human use, as their activity is analogous to human protein interactions. Furthermore the process is more cost effective.[17, 20] This non-GMO method has been adapted to produce proteins for the cosmetics industry, using *Nicotiana benthamiana* plants as biofactories for cosmetic active ingredients.[21]

N. benthamiana is an ideal host for transient expression vectors due to its unique adaptability. In the wild it is extremely resistant to environmental changes, and has survived for around 750,000 years. In biofactories, the plant incorporates synthetic transcripts and modulates the expression of the desired proteins efficiently. The plant also gives a high biomass yield with a high protein level, allowing short production cycles and a high yield.

In order to produce the proteins in this way, the plants are cultivated in a pesticide free, closed environment with controlled conditions. Once the plants are ready, the vectors containing the genetic material required to produce the peptide are mechanically introduced through the leaves. The vectors enter the cytoplasm of the plant cells, and extend through to the adjacent cells via the plasmodesmata. The genetic material is then translated into human protein by the plant, without entering the nucleus or modifying the plant's own genetic material. The proteins then undergo post-translational modifications, at which point the plants are ready to be harvested, and the peptides can be isolated and purified. The complete process takes around 10 days, and yields a high quality, highly efficacious product.

Nicotiana benthamiana sh-Oligopeptide-2 is a plant-produced protein analogous to Insulin Growth Factor-1 (IGF1). Growth Factors are naturally present in the skin and are efficient at rejuvenating mature skin but their numbers decrease with age.[22] Nicotiana benthamiana sh-Oligopeptide-2 can be applied topically to boost the rejuvenation processes. IGF-1 is activated upon UV exposure, and works against the damaging effect of UV radiation by controlling the repair of damaged DNA, removing severely damaged cells and activating proteasomes. The proteasomes recycle UV-damaged proteins into peptides which can be reused elsewhere. When IGF-1 levels are reduced, for example in more mature skin, the proteasomes are not activated and the damaged compounds accumulate in the cells causing further damage.

In vitro efficacy tests showed that application of Nicotiana benthamiana sh-Oligopeptide-2 to the skin increases

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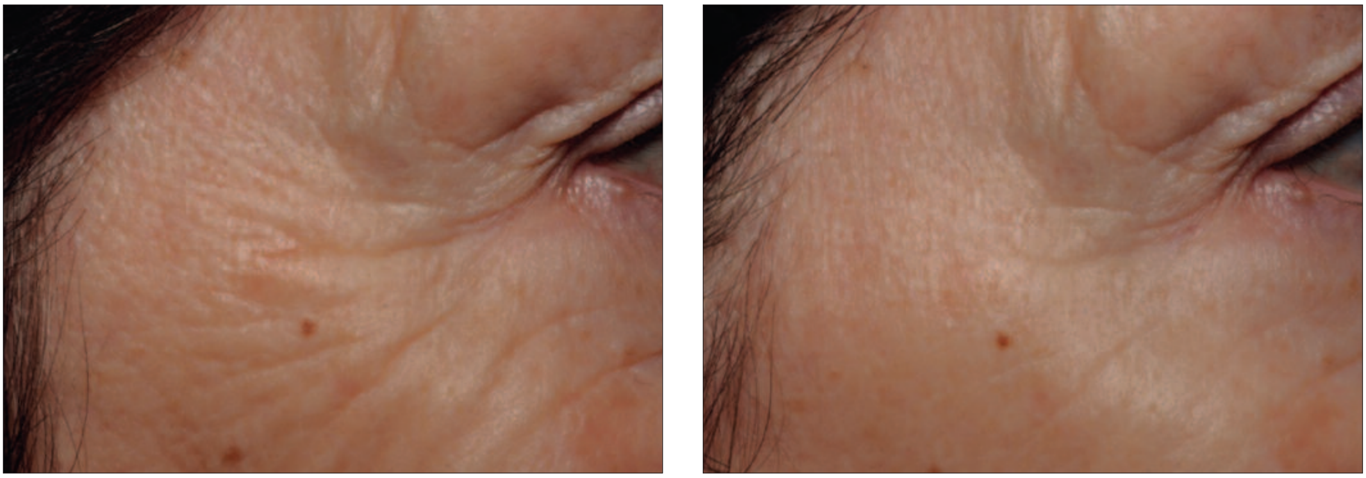

Figure 3: Results of *in vivo* test: application of a cream containing 3% Vitis vinifera grape juice extract twice a day for 56 days.

proteasome activity by 39% compared to non-treated samples, and also reduces carbonylated proteins to the original level prior to UV-irradiation. Nicotiana benthamiana sh-Oligopeptide-2 also reactivates the epidermal basal layers of the skin by increasing expression of cytokeratin 14 by up to 68%. These activities work to restore and preserve skin integrity, and are supported by the *in vivo* test results, where eye contour wrinkles were reduced by 35%.

Enhancing natural extracts

Biologically active proteins are not only used as ingredients for final products; enzymes can be extremely efficient in the production of anti-ageing ingredients. Enzymes are biologically active proteins which catalyse chemical reactions inside cells. They are highly specific molecules, and catalyse only one type of reaction, often much faster and with higher yields than is possible to achieve without using the enzyme.

Using enzymes in large scale manufacturing can reduce the energy, time and cost of production, and can generate highly specific molecules which often cannot be made through traditional synthetic routes.[23] One such molecule is δ-viniferin, a dimer form of resveratrol, which exhibits increased anti-inflammatory and anti-ageing properties compared to its monomer.[24-26]

Vitis vinifera grapevines contain high levels of resveratrol as part of their fungal defence mechanism. When the grapevines are infected by the *Botrytis cinerea* fungus, resveratrol is naturally converted into δ-viniferin by the laccase enzyme produced by the fungus. The antifungal properties of δ-viniferin make it toxic to *B. cinerea*, and therefore low concentrations are found in nature, as the fungus does not survive long enough to produce larger amounts. However, this limitation can be overcome by using direct enzymatic bioconversion technology.[26]

First, laccase is isolated from the *B. cinerea* fungus and purified. The enzyme is then mixed with Vitis vinifera grape juice extract, which has a high concentration of resveratrol, and the conversion into δ-viniferin is monitored by High Pressure Thin Liquid Chromatography (HPTLC) until the desired concentration is achieved. The enzyme is then deactivated via heat denaturation and the product is purified by filtration, before being added to the carrier.[26]

The resulting Vitis vinifera grape juice extract has enriched levels of δ-viniferin, and exhibits enhanced anti-ageing performance. *Ex vivo* studies show that at 0.5%, enriched *Vitis vinifera* grape juice extract increases collagen synthesis by up to 35%. The extract also inhibits the release of collagenase, which further protects the newly synthesised collagen. To support these findings, *in vivo* studies were carried out using 3% of the enriched extract on 22 volunteers, and the results showed significant smoothing of wrinkles.

Bioactive compounds with anti-ageing properties are often found in plant extracts, and these materials have long been used in cosmetics. In the search for new extracts and materials, the industry has recently taken an interest in marine species. With over 70% of the world's surface covered by oceans, the marine environment offers a vastly diverse range of organisms, and a rich source of natural, biologically active compounds.[27]

In 2010, 250 scientists embarked on the Malaspina Circumnavigation Expedition, which took them across 5 continents with the purpose of exploring the biodiversity in the world's oceans. Over 350 marine water samples were collected at varying depths, and more than 120 previously unknown bacterial strains were isolated.[28] These samples were examined to identify potential anti-ageing compounds using cutting edge computational analysis.

The RNA sequences are determined using transcriptomic methods, and bioinformatics analysis of these sequences allows the genes to be categorised into the relevant metabolic pathway. This data can then be used in one of two ways: to identify the mechanism of action of a particular compound, or to identify the best active materials for a defined mechanism of action. The mechanism of action is then confirmed through efficacy testing.

Using this process, a particular

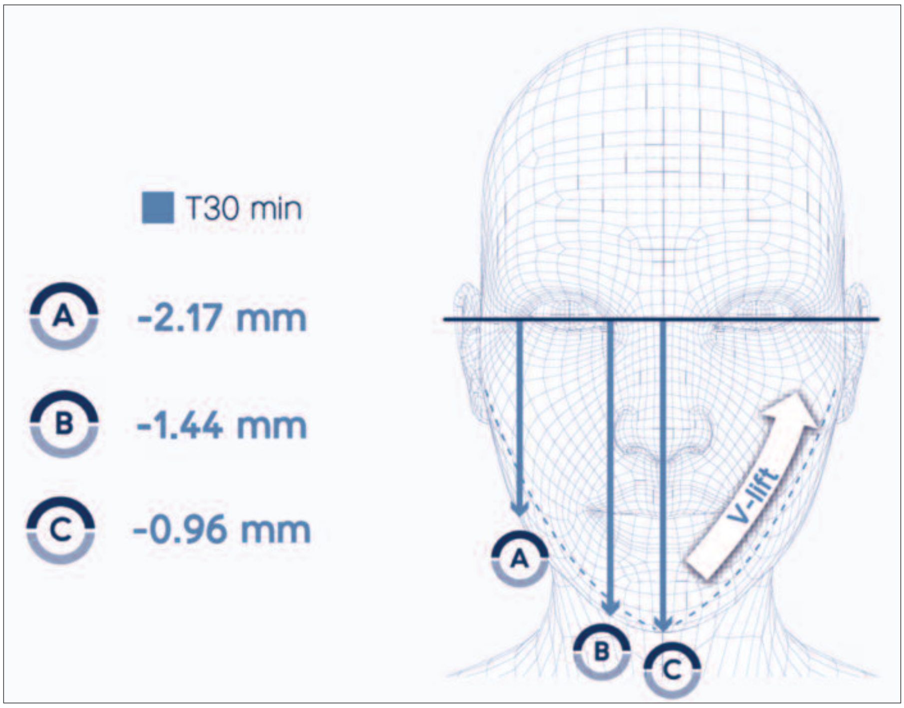

Figure 4: Diagram of V-Lifting effect.

microorganism was identified from one of the Malaspina 2010 expedition samples, which was collected at a depth of 3400 m in the Indian Ocean near Madagascar. In order to obtain the active material, the microorganism is fermented and proteins extracted to create a cosmetic ingredient with the general INCI name Bacillus Ferment, which offers revolutionary anti-ageing properties.[29]

The effects of Bacillus Ferment on the regulation of 600 genes which are expressed in the skin were studied using bioinformatics analysis. Among other activities, the sirtuin-3 gene was identified as a key target. Sirtuin-3 controls the synthesis of Adenosine Triphosphate (ATP) and increases cellular antioxidant activity when it is expressed, thereby modulating oxidative stress. A genetic link between sirtuin-3 levels and longevity is well known. The Bacillus Ferment was shown through *in vitro* efficacy testing to up-regulate the sirtuin-3 gene by 10%, thereby increasing ATP levels by 32% and reducing Reactive Oxygen Species (ROS) by 4%.

The material also exhibited immediate anti-ageing activity in *in vivo* tests, with a 13% reduction in wrinkle depth after just 30 minutes. A visible, 50.6% reduction in wrinkle depth was recorded in one volunteer.[30] The material also exhibited a V-reshape lifting effect of the face contour after 30 minutes, with an average of 2.17 mm lift at point A after 30 minutes.[29]

Reducing technology for conservation

While marine biotechnologies are leading to exciting new discoveries and materials, other marine based extraction methods are going back to basics in an effort to conserve the environment.

The Island of Ouessant in the Iroise Sea is part of a UNESCO biosphere reserve, due to its unique temperature conditions. The area exhibits pockets of cold water surrounded by warmer waters, which are unaffected by the North-South gradient, and support a high biodiversity of flora and fauna. The area is particularly at risk from rising sea temperatures, and is therefore controlled by strict conservation policies.[31]

Ascophyllum nodosum is a species of algae which is harvested from Ouessant Island, and exhibits excellent anti-ageing performance in cosmetic products. Responsible harvesting of the algae is carried out by just two accredited people, who collect the algae by hand, and on a rotation, to allow optimal regeneration of the species and minimal disruption to the environment.[32]

The extract is a concentrated form of fucoidans, obtained by aqueous extraction of the intracellular polysaccharides of Sea Rockweed by ultrafiltration. It specifically targets pollution-induced signs of ageing, by inhibiting the Aryl-Hydrocarbon Receptor (AhR) pathway mechanism. This pathway is activated when pollutants, such as heavy metals, are detected at a cutaneous level. This triggers the overexpression of the genes which stimulate production of inflammatory mediators, and increases tyrosinase activity which in turn increases melanin production. Prolonged exposure to pollutants leads to prematurely aged skin caused by redness, breakdown of barrier function, hyperpigmentation and a plethora

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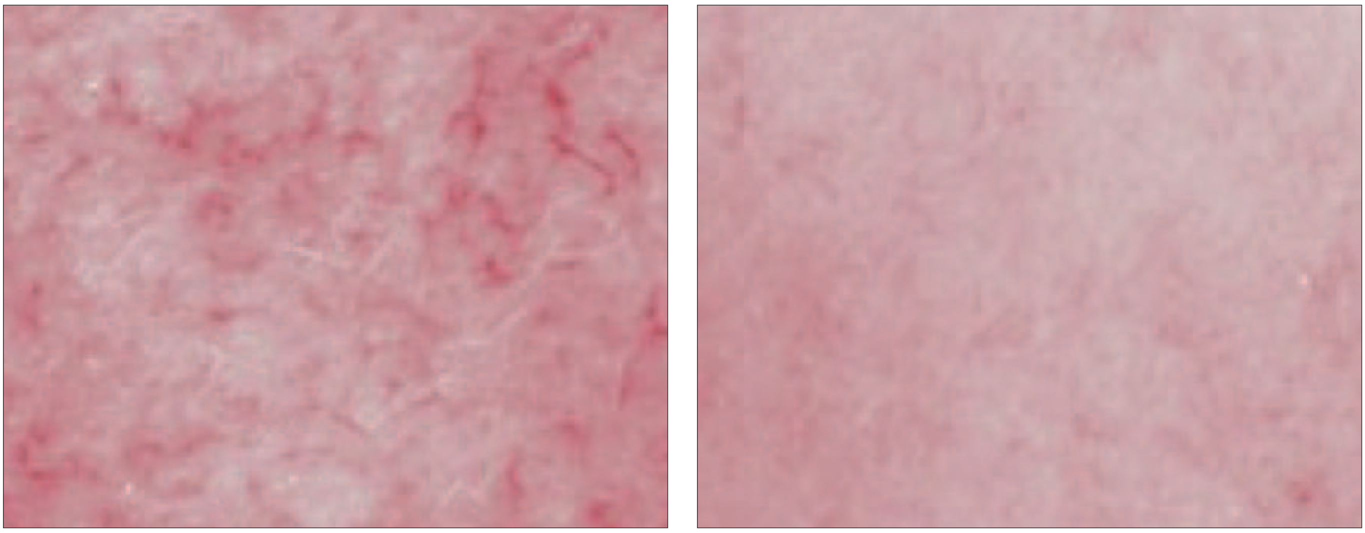

Figure 5: Redness study.

of other symptoms. Ascophyllum nodosum extract inhibits the AhR pathway, and in particular inhibits tyrosinase activity, thereby reducing hyperpigmentation.

An *in tubo* test showed that Ascophyllum nodosum extract reduces tyrosinase activity by 43% when used at 0.1%, increasing to a 76% reduction if 5% extract is used. *Ex vivo* tests showed a significant reduction in AhR expression when 3% Ascophyllum nodosum extract is applied, as the extract neutralises the pollutants. This is confirmed by testing the effects of the extract with no pollutants, where the AhR expression is unchanged. As well as this, a 69% decrease in oxidised proteins was observed with 1% extract, indicating that Ascophyllum nodosum extract provides protection against pollution induced oxidation and preserves protein function. An *in vivo* study on 10 volunteers, who suffer with skin redness and live in urban environments, showed that topical application of a cream with 3% Ascophyllum nodosum extract reduced cutaneous redness by 9% (Fig 5).

Conclusion

While Invincity (INCI: Ascophyllum nodosum Extract) offers a unique story and involves minimal technology, the role of technological advances in cosmetics has undeniable potential. Computational advances will continue to improve the development process of new cosmetic materials, with faster identification and improved methods of efficacy testing. Progress in chemical and bio-technologies will allow us to stabilise and increase the efficacy of actives ,for example through increasing the level of δ-viniferin in Viniderm (INCI: Vitis vinifera Grape Juice Extract), and to stabilise actives which are difficult to formulate with which are difficult to formulate with, and this in turn will improve product development and manufacturing processes.

Technology also has a part to play in improving sustainability. The transient expression of genes in plant-based Scelleye (INCI: Nicotiana benthamiana sh-Oligopeptide-2) reduces the need for synthetic manufacture of peptides. All of these advances will contribute to the end product, which, most importantly, enhances the consumer's experience. PC

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