

Effect of zein additive on perfume evaporation

A. Cabin-Flaman^{*,†} , A. Delaune^{*,†}, P. Poutrain[†], Y.G. Gangwe Nana[†], B. Jourdain[†], D. Gibouin^{*,†}, J.P. Paris[‡], S. Trestour[‡], J.M. Seigneuret[§], A. Léopoldès de Vendômois[§], P. Cosette[¶], J.J. Etienne^{**} and C. Ripoll[†]

^{*}Groupe de Physique des Matériaux, UMR 6634 CNRS, Faculté des Sciences et Techniques, [†]Département de Biologie, Faculté des Sciences et Techniques, Université de Rouen Normandie, Mont-Saint-Aignan, [‡]Payan Bertrand S.A., Grasse, [§]Alban Muler International, Vincennes,

[¶]Département de Chimie, Plateforme Protéomique PISSARO, UMR 6270 CNRS, Faculté des Sciences et Techniques, Université de Rouen Normandie, Mont-Saint-Aignan, and ^{**}ATN Conseil, Vincennes, France

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Abstract

OBJECTIVE: Zein is known to have filmogen properties. We wanted to show if a zein film containing eugenol (eugenol as model) would retain the fragrances, slow their evaporation and therefore produce a long-lasting perception of perfume.

METHODS: We added corn zein to eugenol in a hydro-alcoholic solution to form a film *in vitro* and at the surface of the human skin. We have studied the trapping and release of eugenol from zein film by GC/MS. Also we labelled eugenol with deuterium to image specifically its distribution in the zein film using Secondary Ion Mass Spectrometry technique (NanoSIMS 50). Finally, we applied the zein/D-eugenol formulation onto skin to image the eugenol location on and in skin by SIMS (Secondary Ion Mass Spectrometry).

RESULTS: We showed that eugenol evaporation from zein film can be divided in three periods. The first period (≤ 2 h) corresponds to the simultaneous solvent and eugenol evaporation occurring during film formation. The second period corresponds to the continuous and slow eugenol evaporation during a few hours (about 10 h) but not to its completion. The third period (at least up to 48 h) results from the trapping of eugenol in zein film. After 24 or 48 h, trapped eugenol can be released and evaporated under mechanical deformations of the film. Moreover we showed that zein addition does not favour the eugenol penetration into viable epidermis which may cause allergenic cutaneous reaction.

CONCLUSION: The zein additive is safe to use, does not impact the olfactory perception, allows a better perception of the fragrance (long-lasting effect) in a more protective way and can be used in perfume.

Résumé

OBJECTIF: La zéine est connue pour ses propriétés filmogènes. Nous avons voulu montrer si un film de zéine contenant de l'eugénol (comme fragrance modèle) peut retenir les fragrances, ralentir leur évaporation et engendrer ainsi une perception prolongée du parfum (effet long lasting) si la formulation devait être appliquée sur la peau humaine.

METHODES: Nous avons ajouté de la zéine de maïs à une solution hydro-alcoolique d'eugénol pour former un film *in vitro*. Nous

avons étudié le piégeage et la libération de l'eugénol à partir du film de zéine formé en GC/MS. Puis nous avons marqué l'eugénol au deutérium pour obtenir des images de sa distribution dans un film de zéine en utilisant la technologie Secondary Ion Mass Spectrometry (NanoSIMS 50). Enfin, nous avons appliqué le mélange zéine/D-eugénol à la surface de la peau et étudié la localisation de l'eugénol par SIMS sur et dans la peau.

RESULTATS: Nous montrons que l'évaporation de l'eugénol à partir d'un film de zéine se déroule en trois phases. La première phase (≤ 2 heures) correspond à l'évaporation simultanée du solvant et de l'eugénol pendant la formation du film. La seconde phase correspond à une libération continue et faible de l'eugénol durant quelques heures (une dizaine d'heures environ); la libération n'est alors pas totale. La troisième période, qui peut aller au moins jusqu'à 48 heures, correspond à un piégeage de l'eugénol dans le film de zéine; eugénol qui peut être libéré et évaporé sous l'effet de déformations mécaniques du film. De plus, nous montrons que l'ajout de zéine ne favorise pas la pénétration de l'eugénol dans l'épiderme viable, ce qui aurait pu augmenter le risque de réaction cutanée indésirable.

CONCLUSION: L'utilisation de zéine comme additif n'altère pas la perception du parfum, permet une perception prolongée du parfum (effet long lasting) sans risque pour la santé. La zéine peut donc être utilisée dans une formulation cosmétique.

Introduction

It is well-known that a thin layer of a solution of natural prolamin such as zein forms a film after solvent evaporation. Corn zein is an amphiphilic protein, biodegradable and has excellent film forming properties. It is extensively used in a variety of scientific and industrial domains such as tissue engineering, pharmacy, food chemistry and cosmetics [1–5]. Most of these uses are based on the release of active substances that have been added to a zein film [6]. These substances include salicylic acid or acetylsalicylic acid [7], lysozyme or catechin [8], nisin [9], phenolic compounds such as thymol, carvacrol or eugenol [10–13].

Since zein is soluble in hydro-alcoholic support and does not alter significantly the olfactory perception [14] it is well suited to perfumery and cosmetics. The aim here is to add corn zein to a model fragrance (hydro-alcoholic solution of eugenol) in order to form a film at the surface of the skin. Such a film could retain the fragrances ingredients and slow their release and evaporation and therefore produce a long-lasting olfactory of the perfume. As a

Correspondence: Armelle Cabin-Flaman, Groupe de Physique des Matériaux, UMR 6634, Faculté des Sciences et Techniques, Avenue de l'Université, BP12 76801 Saint-Etienne du Rouvray, France. Tel.: (33) 2 35 52 29 78; fax: (33) 2 32 95 50 32; e-mail: Armelle.cabin@univ-rouen.fr

possible drawback, however, such a retention could favour the diffusion of the fragrance into the viable epidermis and thereby increase the risk of allergenic reaction.

We therefore investigated whether the presence of a film of zein increases the concentration of fragrances compounds in the different skin tissues. For this purpose, we used eugenol as a model fragrance due to it being a compound of essential oils, commonly used in perfumes and easy to quantify. We have labelled eugenol with deuterium in order to specifically image its distribution in the zein film and in the skin and using Secondary Ion Mass Spectrometry (NanoSIMS 50). We also studied the release and trapping of eugenol from the zein film.

Materials and methods

Chemicals

Palladium/Carbon powder was purchased from VWR. All other chemicals were purchased from Aldrich (purity > 98%).

Zein production and characterization

Zein was produced by Alban Muller International as previously published [14]. Briefly, zein was extracted with ethanol (70%) at 50°C, nanofiltered using an organic membrane with a weight-cut-off of 6 kDa and dried by zeodration.

The composition of zein extract was analysed by SDS-PAGE in reducing conditions with 10 µL of zein solution (2% in 70% EtOH/H₂O) in 12% acrylamide separating gel at 16 mA and running at 32 mA; after fixation, gels are stained with silver nitrate. This analysis was confirmed by HPLC profile. Briefly 10 µL zein (0.125% w/v) was injected in Chromolith Performance RP-18 (Merck, France) with phosphoric acid 0.2% v/v in acetonitrile or in water at 35°C at 1.8 mL min⁻¹ and detected at 277 nm.

The identity of zein polypeptide was controlled by LC-MS-Q-ToF as following: Trypsin digestion of protein bands excised from SDS-PAGE was performed overnight. Peptides were analysed using a 6520 Accurate-Mass Q-TOF LC/MS (Agilent, United States) equipped with a HPLC-chip cube interface. For the protein identification, MS/MS peak lists were extracted, converted into mzf format files and compared with the protein database using the MASCOT (MatrixScience, England) Daemon search software.

Zein iodination

In case of SIMS imaging of skin samples, to specifically distinguish the zein film from the biological tissues we iodinated zein as follows: 2331 µL of a solution containing zein at 15 mg mL⁻¹ in 70% EtOH, 105 µL of NaI at 50 mg mL⁻¹ of 0.05 M cacodylate buffer pH 4.0, 994 µL of 0.5 M cacodylate buffer/70% EtOH pH 7.4 and 70 µL of ChloraminT at 50 mg mL⁻¹ of 0.05 M cacodylate buffer pH 4.0 were added. After the brown colour appearance, reaction medium was dialysed (molecular-weight-cut-off of 3500 Da) against water overnight. Zein precipitate was separated by a short centrifugation at 32 500 g and the solid phase was freeze-dried to obtain iodinated zein.

Eugenol deuteration (D-eugenol)

To detect eugenol using SIMS imaging we have deuterated eugenol as follows: 11.3 g of D₂O, 16.4 mg of eugenol and 8 mg of Pd/C

were introduced in a metallic (Ni 56% and Cr 22%) reactor [15]. After 2 h at 250°C to obtain 300 bars, the reactor was cooled in ice. Deuterated product was recovered by liquid-liquid extraction with methylene chloride which was then evaporated under N₂ flux leaving deuterated eugenol. The number of substituted hydrogen atoms was estimated by Gas Chromatography coupled to Mass Spectrum GC/MS with five independent assays. The mean deuterium percentage was 43% ± 5%. In our conditions, the deuteration step leads to a mix of four deuterated compounds: deuterated eugenol, cis and trans deuterated isoeugenol and deuterated dihydroeugenol in variable proportions. These molecules are chemically similar to eugenol and have similar properties. For these reasons, we chose the name D-eugenol for the resulting mixture of deuterated compounds.

GC/MS analysis for eugenol and D-eugenol released from zein film

Zein films containing eugenol or D-eugenol were made with 190 µL of a solution containing 2% zein/eugenol (0.2; 0.9; 1.7; 3.7 and 5.7 mg of eugenol per zein film) or 190 µL of a solution containing 1% zein/D-eugenol (1.9 mg per zein film). They were deposited in laboratory-made hydrophobic plastic moulds. After 2, 24 or 48 h of drying at 37°C, the films were carefully peeled off. With this process, films took the form of a lens 1.7 cm in diameter; the masses and surfaces of which were very reproducible (3.8 ± 0.1 mg and 2.28 cm² ± 0.05 for 2% zein films).

The peeled films were then introduced in a vial containing 1 mL of methylene chloride to extract the eugenol from the films. Extracted eugenol or D-eugenol were quantified by GC/MS analysis in with internal standard calibration (1,4-dibromobenzene). Analyses were carried out using an Agilent 7890 A/5975 C system equipped with a VF-WAXms column and operated using the following conditions: carrier gas: helium; constant flow: 0.8 mL min⁻¹; injector temperature: 250°C; injected volume: 1 µL and mode : split 1 : 50. GC oven temperature was set to 60°C and increased to 250°C with a rate of 10°C min⁻¹. Transfer line temperature was 260°C. Electron impact mass spectra was recorded at 70 eV with full scan mode (mass-to-charge ratio (m/z) range from 40 to 400 amu) or with selected ion monitoring mode.

SEM and SIMS analysis for zein film structure and eugenol localization

For SEM imaging, films obtained without eugenol and dried 24 h at 37°C were carefully peeled off from their plastic moulds with tweezers and observed by SEM (Zeiss Evo40, Carl Zeiss SAS, division microscopy, France) under 10 kV after carbon coating.

For SIMS analysis, zein films containing 1% eugenol or 1% D-eugenol (w/v) were made with 190 µL of a solution containing 2% zein deposited in laboratory-made hydrophobic plastic moulds. Films obtained after 24 h drying at 37°C were carefully peeled off from their plastic moulds with tweezers and directly introduced in gelatin moulds containing LRWhite before hardening at 60°C overnight. Sections of 200 nm in thickness were analysed with a NanoSIMS 50 (Cameca, France) analyser. The principle of this analysis is the sputtering of the sample by a rastering Cs⁺ primary ion beam under vacuum. The sputtered matter composed of ions (the secondary ions) is analysed in a double-focusing mass spectrometer. The magnet of the instrument is equipped with five detectors (electron multipliers) allowing the simultaneous counting of five different secondary ions. By the scanning mode, 2D images of the five

ion-masses can be simultaneously obtained. The elemental film structure was always confirmed by carbon, nitrogen and sulphur images at the ^{26}C and ^{32}S respective masses under the following conditions: 1 pA Cs^+ primary beam and D1-3 diaphragm. To know the density of zein matter in sample we opted to calculate the ratio of CN^- signal as CN^- mean value in the area of interest divided by the CN^- value of the second area of interest. Images of zein films were also acquired at masses ^1H and ^2H (to localize the deuterium and then D-eugenol) in the same conditions as described above. When necessary, two or more successive images were sequentially acquired by two or more successive planes.

For practical reasons, the isotopic fraction (or atomic abundance), $A_{2\text{H}}$, is expressed as the following equation:

$$A_{2\text{H}} = ({}^2\text{H}/[{}^2\text{H} + {}^1\text{H}]),$$

where ^1H and ^2H are the number of secondary ^1H or ^2H ions counted by detectors. The ^2H enrichment values per pixel, $E_{2\text{H}}$, are expressed as the ^2H isotopic fraction in each pixel compared to the natural ^2H isotopic fraction (0.000115) as reference (also named natural deuterium abundance), by the following equation:

$$E_{2\text{H}} = A_{2\text{H}}/0.000115.$$

SIED analysis

In all cases, ^1H and ^2H SIMS images were analysed with Significant Isotopic Enrichment Detection (SIED) laboratory-made software [16] to quantify isotopic signal. SIED performs the statistical analysis of the isotopic fraction from SIMS isotopic images. Briefly, for each pixel of a ^1H and ^2H SIMS images, SIED software automatically computes the ^2H isotopic fraction, $A_{2\text{H}}$ and the probability, the p -value, that this fraction is not different to a chosen reference isotopic fraction (the natural isotopic fraction 0.000115 or in some cases the mean isotopic fraction of the imaged sample). A P -value that is below 0.05 (using a Bonferroni correction for multiple test) means that the measured isotopic fraction is significantly different from the reference isotopic fraction in the analysed pixel. In this case, the ^2H enrichment is significant and the pixel is collared in blue in the ^1H image (which is chosen as the structural reference image). When $0.1 > P\text{-value} > 0.05$, the pixel-enrichment is high and close to be significant thus the pixel is displayed in red. This representation enables easy identification of the significant deuterium-enriched pixels and thus areas on a structural image but does not show the magnitudes of the enrichments.

To show the ^2H enrichment magnitude in an image, we opted for another representation where only significantly enriched pixels are shown in a grey image. In this representation, when the enrichment is not significantly higher than 1 (or isotopic fraction is not significantly higher than the natural ^2H isotopic fraction 0.000115) pixels are black and conversely pixels that are significantly enriched (significantly higher than 1) are displayed in grey. The grey level that is proportional to the ^2H enrichment magnitude and so reflects the deuterium concentration which in turn reflects the D-eugenol concentration in each pixel.

SIMS analysis for films structure and eugenol localization on skin surface

Five microlitre of 1% w/v iodinated zein solubilized in 70% hydro-alcoholic solution with 1% D-eugenol were deposited onto the

surface of fresh human skin pieces (abdominal surgical wastes; gift from CHU Rouen) maintained on DMEM medium culture in a six-well plate at 34°C . After 24 h, 1 mm³ skin pieces were cut and fixed during 4 h in 25% glutaraldehyde vapours in a closed chamber at 37°C , dehydrated with ethanol increasing concentrations during 6 h and embedded in LRWhite medium resin, hardened at 60°C overnight and cut (200 nm thick sections) for NanoSIMS 50 analysis.

Results and discussion

Zein characterization

Four distinct forms of zein (α , β , δ and γ), soluble in hydro-alcoholic solutions, are known in corn. Their molecular weight depends on corn variety and genotype, soil and climatic conditions for the cereal growth [17–19]. The molecular weight depends also on the process of extraction including the nature of the used solvent [2,3,20–22]. The molecular weights of α zein (70–85% of total zein) and γ zein (10–20% of total zein) are between 19 and 24 kDa and 16 and 27 kDa respectively. In electrophoresis two isoforms of α zein are revealed and called ‘Z19 and Z22’ according to their most frequently encountered molecular weights 19 and 22 kDa respectively [19,22].

In our electrophoretic analysis in reducing conditions, two major bands ($42\% \pm 5\%$ of the total band intensity) of zein were detected in the range 18 and 25 kDa with mean values at 19 and 24 kDa (not shown). The two corresponding proteins were identified as α -zein by LC-MS-MS-Q-ToF analysis (data not shown) and were classified in Z19 and Z22. Note that in our SDS-PAGE profile, a band ($17\% \pm 2\%$ of the total band intensity) is often present in the range 53–58 kDa. This band most likely corresponds to α -zein multimers [17,23,24] and not to a distinct protein because only one peak is visible in HPLC (data not shown).

Eugenol evaporation from zein film

We investigated the eugenol content of the film after 2, 24 and 48 h of free simultaneous evaporation of the hydro-alcoholic solvent and eugenol (drying process). We chose 2 h as the shorter time because it is the time at which mechanical strength of the film is high enough to be manipulated (duration of film formation).

To estimate the amount of eugenol that has been evaporated during and after zein film formation, eugenol was extracted from the film with methylene chloride (a high solubility for eugenol) and its concentration was measured by GC/MS. Figure 1 shows the decrease of the amount of extracted eugenol from the film as a function of time. The proportion of eugenol extracted is the same after 24 and 48 h suggesting that the evaporation of eugenol was negligible after 24 h. The proportion of eugenol extracted is approximately $74\% \pm 2\%$; $67\% \pm 6\%$; $48\% \pm 3\%$; $26\% \pm 1\%$ and $28\% \pm 1\%$ for the initial amount of eugenol 0.2; 0.9; 1.7; 3.7 and 5.7 mg per zein film respectively. Therefore whatever its initial concentration it remains a substantial amount of eugenol in the film even after a long time.

On the basis of the shape of the curves of Fig. 1, however, we estimated that the evaporation of eugenol was already negligible after about 10 h of drying.

These results suggest that there are two pools of eugenol in zein film: one pool that we call free-eugenol which can be

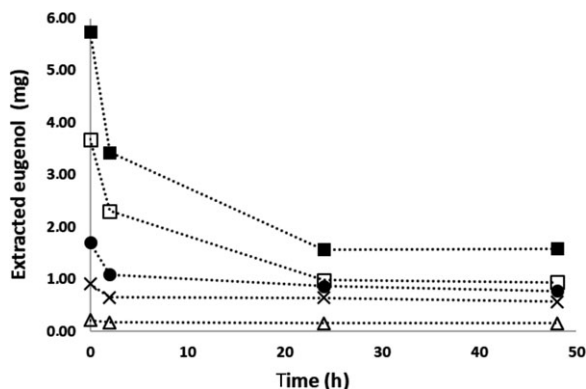


Figure 1 Kinetics of eugenol release from zein films. 190 μ L of 2% zein with eugenol at (Δ): 0.21; ($\times$): 0.91; ($\bullet$): 1.71; ($\square$): 3.67 and ($\blacksquare$): 5.74 mg were dried at 30°C during 2, 24 and 48 h and introduced in vials containing 1 mL of methylene chloride. Concentration of eugenol in methylene chloride was measured by GC/MS.

spontaneously evaporated during approximately the 10 first hours and another pool that we call trapped-eugenol which is kept in zein film. This is in agreement with the reported results on the kinetics of release of eugenol or thymol showing that evaporation occurs in short delays [25] and is not total [10,26].

Moreover, we observed that the stretching of a zein film that has been dried during more than 10 h produces a release of trapped-eugenol as attested by the characteristic smell of this compound.

Indeed if such a film containing a fragrance compounds is formed on the surface of the skin, the natural movements of the body will stretch it and therefore could release the fragrances and confer olfactive long-lasting properties to the perfume.

Localization of eugenol in zein film

Zein films without additive are hard, stiff and brittle [27]. In contrast we observed that the zein film after addition of eugenol crept under stretching. This property is conserved even after the evaporation of the free-eugenol. Film creeping increases with concentration of added eugenol (not shown). Creeping could be attributed to the location of eugenol between zein molecules. This location would induce modifications of intermolecular polymer association [28].

Indeed, it has been reported that addition of different molecules modifies the zein film structure and reinforce the tensile strength [24,25,29,30–34]. It is therefore likely that the trapped-eugenol changes the zein film structure. We therefore decided to specifically image the film and the eugenol location using NanoSIMS 50.

In absence of eugenol, zein film is unambiguously imaged using CN^- secondary ion detection (mass 26, Fig. 2A) and S^- secondary ion detection (mass 32, Fig. 2B). Indeed only zein contains nitrogen and sulphur and therefore can produce CN^- and S^- secondary ions whilst the embedding resin, which is devoid of these elements, cannot produce them and therefore appeared in black in these SIMS images. Essentially three areas are visible in zein films: (1) an 'internal' area that constitutes the core of the film where CN^- and S^- signals are high, (2) an 'air-edge' area (upper edge in Fig. 2A) that was in contact with air during the drying of the film and

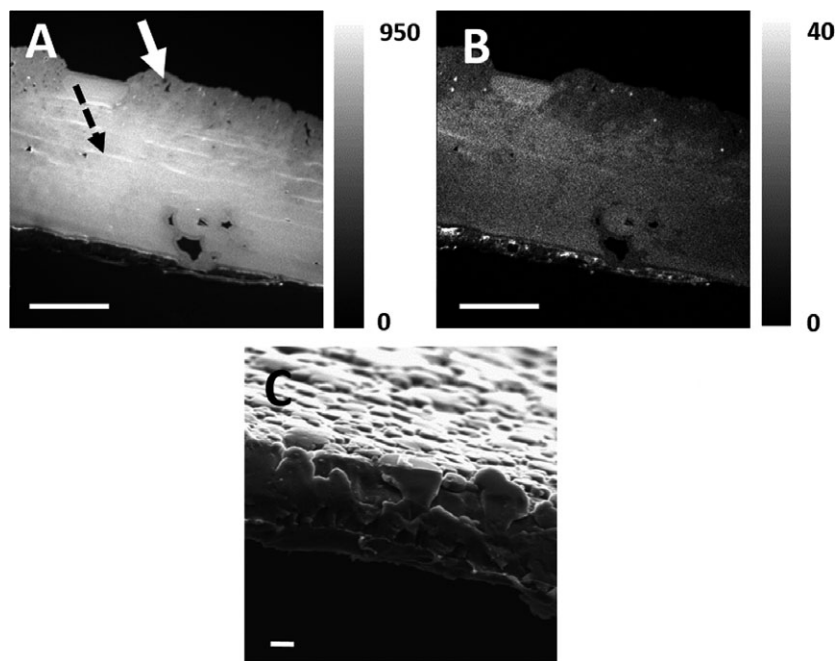


Figure 2 Structure of zein film after 24 h drying at 37°C (transversal sections). Film prepared with 5% zein is imaged by NanoSIMS 50 at ^{26}CN mass (A) and ^{32}S mass (B), 10 ms per pixel and 512×512 pixels. Vertical grey scale: The number of secondary ions counted per pixel. White arrow: cavities near the surface of film in contact with air during drying. Black arrow: lines of compaction. (C) SEM analysis of another zein film than (A,B). Bar scale: 5 μ m (A,B), 10 μ m (C).

where these signals are low and (3) a 'support-edge' area (lower edge in Fig. 2A) that was in contact with the plastic mould where these signals are also low. The core seems to be not uniform as revealed by narrow parallel areas (dashed black arrow Fig. 2A) particularly rich in nitrogen and sulphur (Fig. 2A,B). We interpret these differences in secondary ion signal intensities as coming from density differences of zein in these areas. We estimated these differences measuring the following CN^- ratios: air-edge area/internal area = 0.7 and support-edge area/internal area = 0.4. These results show that the internal area has a higher density than the two edge areas. This observation might be explained by a progressive and asymmetric compaction of zein during the film formation. The lower compaction in air-edge area could be mainly attributed to the rapid ethanol evaporation that prevents the organization of zein that remains in a loose structure [35]. It has also been shown that the organization of the hydrophilic and hydrophobic domains

of zein molecules near the film surface depends on the hydrophobicity of medium in contact with this surface [31,36].

In zein films, a few cavities are visible (very low CN^- and S^- signals, white arrow in Fig. 2A). Very small cavities are mainly located in the air-edge area and might result of the void spaces formed during the drying by the agglomeration of spherical zein particles [35] (Fig. 2C).

In presence of 1% eugenol, the three areas that we have observed on SIMS images of films without eugenol are not visible. In contrast, numerous cavities are present; they appear in black in the image due to their low sulphur content (Fig. 3A). These cavities confer a sponge-like structure to the films. The mean diameter of these cavities in zein films was $2 \pm 2 \mu\text{m}$ after 24 h of drying and represents 10.7% of the film thickness. This structure was observed whatever the nature of the support used for film formation (not shown). It has been previously shown that supercritical CO_2 , gallic acid [37],

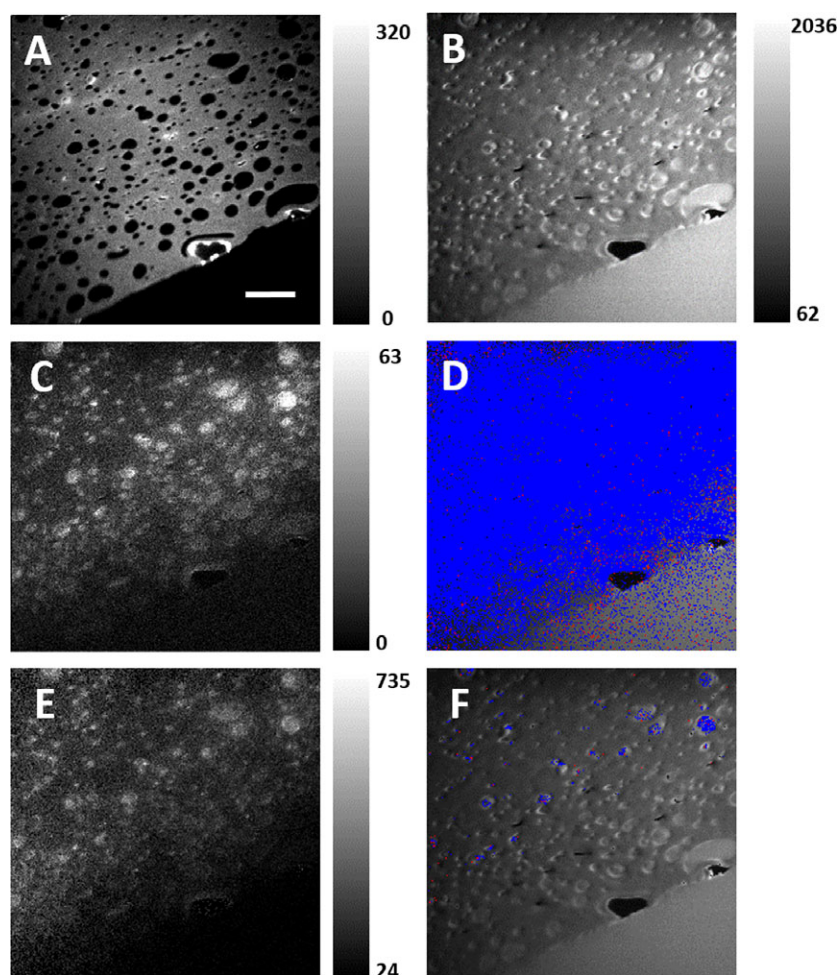


Figure 3 Localization of deuterated eugenol into zein film. Film prepared with 1% zein and 1% D-eugenol is imaged by NanoSIMS 50 at ^{32}S mass (A), ^1H mass (B) and ^2H mass (C). (D,F) Images analysed with Significant Isotopic Enrichment Detection software where ^2H isotopic fraction is significantly greater than the natural abundance (D) (blue: $P < 0.05$, red $0.05 < P < 0.1$) or greater than the mean isotopic fraction of the imaged sample (F). (E) Image of the deuterium enrichment where the isotopic fraction is significantly higher than the natural abundance. Vertical grey scale: The number of secondary ions counted per pixel in (A–C); or the ^2H enrichment value $E_{2\text{H}}$ per pixel (E). Scale bar: $8 \mu\text{m}$; (A–C): 40 ms per pixel, 256×256 pixels; (D–F): $39 \times 39 \mu\text{m}$, 40 ms per pixel, 250×250 pixels.

thymol [10,11,34] or oleic acid [38] also induce cavities formation in zein films, the size of which depends on the nature of added compounds [13] and on their concentration [10].

To investigate the eugenol distribution in cavities and in the core of the zein films we used SIMS imaging of deuterated eugenol (D-eugenol). Indeed eugenol which is only constituted of H, C and O atoms cannot be distinguished from other organic compounds such as zein in SIMS analysis if it is not isotopically labelled. We have labelled eugenol with deuterium (D/H substitution 45%). To obtain a reliable localization and quantification [16,38,39] of D-eugenol we determined the deuterium isotope fractions $^2\text{H}/[^1\text{H} + ^2\text{H}]$ from the corresponding ^1H (Fig. 3B) and ^2H (Fig. 3C) images and calculated the ^2H enrichment $E_{2\text{H}}$ per pixel.

To ensure that the ^2H enrichment was statistically significant we used as explained in materials and methods the SIED software [16]. Figure 3D shows in blue (P -value < 0.05) or in red ($0.05 < P$ -value < 0.1), the pixels that are significantly enriched compared to the ^2H natural abundance whatever the enrichment degree. Almost all the pixels are in blue except near the edge of the film and in resin. Figure 3E shows only the pixels for which the ^2H enrichment is statistically significant. Comparison of Fig. 3A,E shows that the areas of highest enrichment correspond to the cavities.

Nevertheless, there is a significant enrichment in the whole film as shown in Fig. 3D. To ensure that the differences of enrichments between cavities and the core of the film are statistically significant, we compared the ^2H isotopic fraction of every pixel to the mean ^2H isotopic fraction of the image. The image of Fig. 3F is an overlay of

the ^1H image and an image showing in blue the pixels that have ^2H isotopic fraction significantly higher than the mean isotopic fraction of the image. This image shows that the most enriched areas are the cavities located far from the edge. Enrichment differences between the cavities and the core however are mainly statistically non-significant near the edge. We found (in cavities + core) a mean enrichment $E_{2\text{H}}$ of 100–140 folds in the centre of the film (we calculated that an enrichment of 100 corresponds to 2500 molecules of D-eugenol per pixel). Near the edge of the film, however, there is a decreasing gradient of D-eugenol down to 20-folds. This gradient is probably due to evaporation and diffusion of eugenol out of the film.

We interpreted these observations with the following model. During the film formation the ethanol is evaporated more rapidly than water and therefore there is a progressive increase in water content that induces partial demixing (phase separation) of eugenol. This generates very small droplets of eugenol spread in the whole film thickness. These droplets are trapped and form cavities in the zein film. As the demixing is probably only partial, eugenol is also contained in the core of the film.

D-eugenol trapping by zein film on skin surface

As already stated in the introduction, the aim of this work was to add zein to a perfume in order to prevent the diffusion of the allergenic compounds contained in the perfume into the skin. Residual fragrance compounds in zein could give the potential issue of

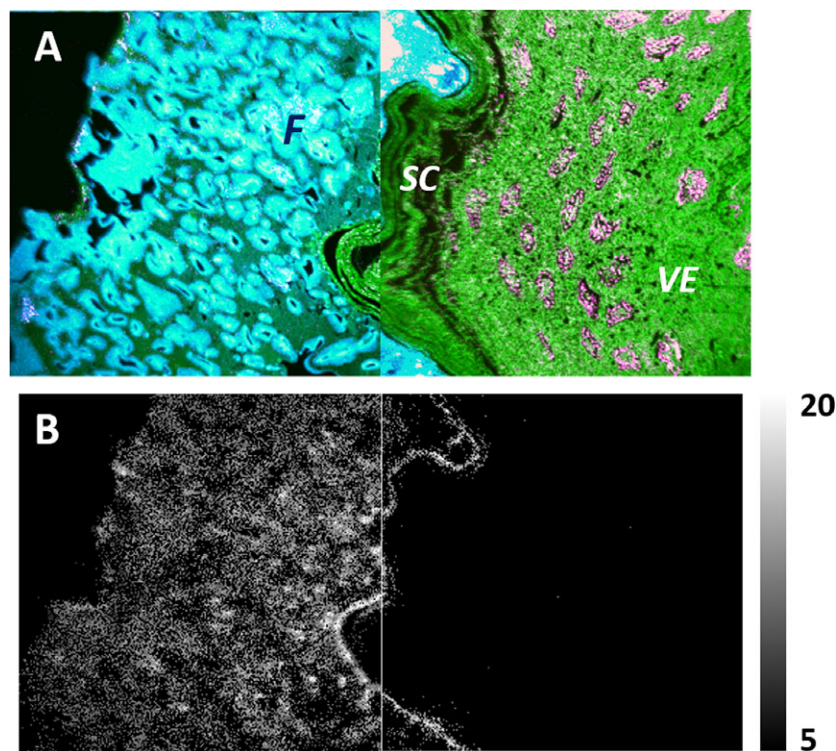


Figure 4 SIMS images of zein film at the skin surface. (A): Colour image reflects the elemental composition of different structures: zein film by I^- signal in blue, compounds containing C and N atoms by CN^- signal in green and nuclei by PO_2^- in pink. (B): The deuterium enrichment due to the presence of Deugenol underlines the zein film (F) at the surface of the skin. (SC) stratum corneum; (VE) viable epidermis. (A): $40 \times 40 \mu\text{m}$, 256×256 pixels and (B): $39 \times 39 \mu\text{m}$, 250×250 pixels, 20 ms per pixel and sum of 3 successive planes. Vertical grey scale: ^2H enrichment value $E_{2\text{H}}$ per pixel.

facilitating the diffusion of the fragrance into the viable epidermis and thereby increasing the risk of allergenic reaction. We therefore investigated the eugenol localization in different tissues of skin using NanoSIMS 50. A hydro-alcoholic solution containing iodinated zein and deuterated eugenol (as model allergenic compound) [40,41] was directly applied on human skin (see Materials and methods) and left to dry for 24 h. The film formed and the skin were then observed by SIMS (Fig. 4).

Iodinated zein was used to discriminate molecules of zein from other molecules such as those of *stratum corneum* in the skin which do not contain iodine. Figure 4A shows an overlay of three images: (1) CN^- (green) as a marker of nitrogen containing biomolecules, (2) PO_2^- (magenta) as a marker of nucleus and thus of viable epidermal cells and (3) I^- (cyan) as a marker of iodinated zein and thus of the film. In this overlay the film and epidermal tissues are clearly distinguished. In the film the iodine distribution is not uniform and could correspond to zein aggregates. The film, however, has not a so marked sponge-like structure as observed in the case of non-iodinated zein (Fig. 3A); this is probably due to different demixing properties of eugenol in the iodinated film in our experimental conditions. Figure 4B shows the ^2H enrichment of the sole significantly enriched pixels. The ^2H enrichment is almost completely visible in the sole film; some enrichment, however, is found along the first external layer of *stratum corneum*. The inner part of the *stratum corneum* and the other tissues of the skin show no enrichment (Fig. 4B). These results show the following: (1) D-eugenol is still present in zein film after 24 h on skin and therefore zein film has inhibited the fragrance evaporation, (2) zein film does not increase the diffusion of allergenic eugenol into the viable epidermis. This latter point is in agreement with our previous finding that zein could be used as a barrier against allergenic fragrances applied on skin to improve safety for users [14].

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Conclusion

We showed in this work that the olfactive perception of a perfume containing zein could be divided in three periods.

The first period is of short duration (≤ 2 h) and corresponds to the formation of the film during which eugenol evaporation is proportional to eugenol initial concentration. During the second period, after the film formation, eugenol evaporates continuously during a few hours but not to completion. This evaporation is slowed down compared to that would be observed in absence of zein, as expected. The third period (ranging approximately from 10 to at least 48 h) results from trapping of eugenol in zein film. This trapped eugenol can be released and evaporated after mechanical deformations of the film due to movement of the film, for example. Moreover, we showed that zein addition does not give rise to the eugenol penetration into viable epidermis. Therefore the zein additive is safe to use, does not impact the olfactory perception and allows to a better persistence of the fragrance in a more protective way.

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Conflicts of interest

None declared.

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