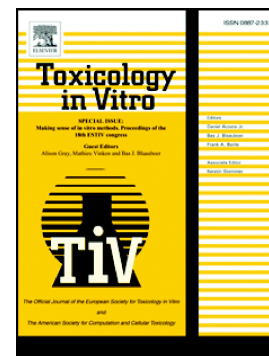


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***In vitro* measurement of skin sensitization hazard of mixtures and finished products : Results obtained with the SENS-IS assays.**

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ABSTRACT

Product safety evaluation in the EU is based on data mainly obtained on individual ingredients. However, mixture effects have been demonstrated in numerous skin sensitization studies due to the presence of irritating chemicals or to modification of dermal absorption. To evaluate the ability of the SENS-IS assay to detect such mixture effects, we performed three sets of experiments: First, the importance of the vehicle on absorption of individual ingredients was evaluated by testing the effect of commonly used cosmetic preparations on the sensitizing potential of 3 chemical allergens and 2 fragrance blends. The sensitizing potential of the 3 allergens was significantly reduced when tested in microemulsion while the “cleansing water” preparation significantly increased it. Water in oil, oil in water or oil preparations had significant but more moderate (enhancing or reducing) effects on the skin sensitization potency of the tested chemicals. We then analyzed the influence of irritants (SDS and Lactic acid) on the sensitizing potency of various allergens. The SENS-IS assay detected an enhancement of the potency of some allergens when mixed with non-irritating concentrations of irritant chemicals. We also tested the influence of mixing different sensitizers to analyze the effect of mixtures on the sensitization threshold. Some mixtures of chemicals, at doses that did not induce a positive signal in the SENS-IS assay alone, became positive, indicating a mixture effect. Finally we tested commercially available finished cosmetic products to find out that they were not all negative. These results indicate that the SENS-IS assay is a valuable source of information when analyzing mixture component effects and finished products.

Introduction

Allergic contact dermatitis is an adverse human health effect caused by dermal exposure to chemicals with skin sensitizing properties. Therefore, different European regulations for safety assessment of chemicals, such as Registration, Evaluation and Authorization of Chemicals (Regulation [EC] No 1907/2006) and the Cosmetic European Regulation [EC] No 1223/2009 require that a hazard characterization of the skin sensitization properties of chemicals must be performed. The development of *in vitro* alternative approaches (Hoffmann et al., 2018) has allowed substituting animal assays by a combination of suitable *in vitro* approaches to obtain adequate information. Nevertheless, there are some limitations to the use of *in vitro* assays : chemicals with a poor solubility or high log P can be difficult to measure (Hoffmann et al., 2018). The metabolic capacity of the test system should also be taken into account. In addition, the majority of the *in vitro* sensitization assays are not quantitative and thus cannot deliver the information necessary to estimate safe exposure levels. Indeed, a quantitative risk assessment (QRA) approach needs assays providing quantitative information to determine the maximum concentration levels acceptable in consumer products. Besides, for the safety assessment of mixtures, a QRA approach based solely on quantitative data obtained with single chemicals may not be directly applicable due to mixture component effects.

Most finished (commercialized) products contain mixtures of different chemicals and consumers may thus be exposed to combinations of vehicles, irritants and sensitizers (Uter et al., 2014a; 2014b; 2013). To accurately evaluate the resulting human safety risk, it is necessary to use or develop assays that can provide information on the possible interactions of the components of such mixtures, since such interactions may influence the response to single substances.

Usually, this is done by comparing the biological response observed with the mixture to the response predicted by dose addition calculation (Berenbaum, 1985). During skin sensitization process, interactions between ingredients may influence mainly two pathways : hapten formation and elicitation of epidermal danger signals. Immunogenic haptens are generally produced through covalent binding of a chemical to proteins (Martin et al., 2011; Natsch 2009) resulting in a new chemical structure (the hapten) specific for each chemical. Mixtures interactions at this level seem quite rare but may nevertheless explain the puzzling “quenching” effect (Basketter, 2000). In contrast, the elicitation of epidermal danger signals is not or less chemical specific and can result from co-exposure to other chemicals present in the applied mixture, including irritants (Smith et al., 2002) and skin sensitizers (Esser et al., 2012). Therefore, exposure to a product containing, for example, a mixture of skin sensitizers and irritants might induce a stronger than expected biological effects due to increased danger signals generated by the different components. Indeed, numerous animal (Cumberbatch et al., 1993) and human (McLelland et al., 1991) studies have shown enhancing effects of the combined exposure to a skin sensitizer and an irritant. Experimental evidence for enhanced sensitization potency has been found as well in the case of mixture exposure to skin sensitizers, (Bonefeld et al., 2011; Jowsey et al., 2008). Moreover, the vehicle use may also influence the potency as shown using the LLNA (Anzai et al., 2010).

We have developed the SENS-IS assay which uses the Episkin[®] large model, a human 3D reconstructed epidermis, as a test system. The different tested products and ingredients are directly applied on the surface epidermis to mimic the “in use” situation (Cottrez et al., 2015). The read out of the assay uses a genomic signature to distinguish non sensitizers, from irritant or sensitizers. The SENS-IS assay has been

shown to measure skin sensitization hazard and potency by detecting the minimal positive dose tested with a very good accuracy (Cottrez et al., 2016), similar to the LLNA data.

In this paper, we present results obtained using the SENS-IS assay to illustrate the ability of the assay to detect modification of the sensitizing potency of chemicals mixed with different components. The following influences were analyzed: (i) the effects of cosmetic formulations on the potency of different sensitizers to analyze the influence of skin penetration and bioavailability (Anderson et al., 2011) (ii) the effects of cosmetic formulations on the potency of fragrance blends to analyze whether the measured modifications could also be observed with more complex mixtures (iii) the effects of various irritants on the potency of skin sensitizers to evaluate the influence of combined irritation on the potency of sensitizers (Smith et al., 2002, Esser et al., 2012) (iv) the analysis of the influence from multiple different sensitizers challenges on the overall measured potency and finally (v) the analysis of commercially available cosmetic finished products.

2. Materials and Methods

2.1 Chemicals and mixture preparation

Hydroxycitronellal (OHC) (CAS # 107-75-5, purity $\geq$ 95%), alpha-hexyl cinnamaldehyde (HCA) (CAS# 101-86-0, purity $\geq$ 84%), Glyoxal (CAS# 107-22-2, purity 40% solution in H₂O) Dihydrocoumarin (CAS # 107-75-5, purity $\geq$ 99%), Isoeugenol (CAS # 97-54-1, purity $\geq$ 98%), cinnamaldehyde (CAS # 14371-10-9, purity $\geq$ 99%), hydroxyisohexyl 3-cyclohexene carboxaldehyde (HICC-Lyral, CAS

#31906-04-4; 51414-25-6 purity $\geq$ 97%), 1,4-phenylene diamine (CAS # 106-50-3, purity $\geq$ 99%) and methyl dibromo glutaronitrile (CAS # 35691-65 -7) were obtained from Sigma-Aldrich (Lyon, France). Gibco Phosphate buffer saline (PBS) 1x was obtained from Thermofisher scientific France, Pan Biotech's DMSO was obtained from Dutcher France, Olive oil was obtained from Puget France and dipropylene glycol (CAS # 14371-10-9, purity $\geq$ 99%), purity $\geq$ 99%) was obtained from Sigma-Aldrich France.

Fragrance blends coded FM and RN, previously tested positive by the SENS-IS assay were provided by Pierre Fabre laboratories. For FM, the main sensitizing constituents were benzyl salicylate, linalool and octahydro tetramethyl acetone and for RN, linalool and octahydro tetramethyl acetone.

Five Cosmetic preparations, described in Table 1, "oil", "water in oil", "oil in water", "cleansing water" and "microemulsion" incorporating the tested ingredient at different concentrations were prepared at Pierre Fabre laboratories.

The different commercially available finished products from different brands are described in Table 2. They were directly obtained from retail stores.

2.2 The SENS-IS test protocol

The SENS-IS assay protocol has been described in (Cottrez et al., 2016). A brief outline (derived from the original publication) is described below:

A) Cell culture

The reconstructed epidermis, Episkin[®] large model was purchased from Skinethic, Lyon, France. These models are differentiated from human keratinocytes isolated

from healthy donors to form a fully stratified and differentiated epidermis covered by a stratum corneum (Cotovio et al., 2005).

The Episkin cultures are shipped onto a nutritive gel for transportation to maintain viability. In accordance with the manufacturer's instructions, epidermises were transferred to sterile 12-well culture dishes containing 2ml/well of pre warmed maintenance medium (DMEM/Ham's F12) supplied with the kit.

B) Treatment protocol

At day 14, liquid or viscous test preparations ($30 \pm 3 \mu\text{l}$) are applied with a micropipet onto the stratum corneum of the epidermis. The tissues are then incubated with the test preparation for 15 min at room temperature and washed with 25 ml PBS per epidermis. After washing, the tissues are incubated at 37°C, 5% CO₂ for 6 hours.

C) Total RNA extraction

Each epidermis model is transferred into a vial for snap freezing in liquid nitrogen. Total RNA is extracted using Qiazol reagent and RNeasy Mini Kit (Qiagen, Courtaboeuf, France). Briefly, the epidermis model is submerged in 1 ml Qiazol reagent and homogenized using two steel beads with a TissueLyser (Qiagen). After centrifugation, the supernatant is collected, completed with 0.2 ml of bromochloropropane (Sigma, Lyon, France) and vortexed. After centrifugation at 12'000 g for 15 min at 4 °C, the aqueous phase is collected and added to 1 ml of ethanol, immediately mixed by pipetting and loaded onto a RNeasy spin column placed in a 2 ml collection tube. Total RNA is then extracted according to the manufacturer's instructions.

D) Quantitative Reverse Transcription-Polymerase Chain Reaction (RT-PCR) Analysis

Reverse transcription of total RNA is performed using 5 µg total RNA in 100 µl final volume using Random Primers and SuperScript III Reverse Transcriptase (Invitrogen, Saint Aubin, France), according to the manufacturer's instructions. Quantitative RT-PCR is performed using SYBR Green Real-time PCR Master Mix (ROCHE Diagnostic, Grenoble, France) with 0.4 µM of each oligonucleotide primer in a total volume 10 µl. The reaction is performed in an LC480 System (ROCHE Diagnostics). The amplification program consists of one cycle at 95 °C with a 5 min hold, followed by 40 amplification cycles (95 °C for 10 s and, annealing at 60 °C for 10 s) and completed by a final 72 °C step for 10 s.

The relative amount of each transcript is normalized to the amount of the mean expression levels of 3 house-keeping genes transcripts (Glucuronidase β , β 2 microglobuline, and « non-POU domain containing, octamer-binding » (NONO)).

E) Prediction model

The genes expressed in each sample are measured through an absolute quantification analysis using the second derivative maximum method with the software provided by ROCHE Diagnostics. The level of tissue destruction is evaluated with the Cycle Threshold (CT) value of the HSPAA1 gene. A sample CT value > 120% of the corresponding value obtained for the vehicle control indicates an unacceptable level of tissue damage. Moreover, irritation signals are analyzed by measuring the expression level of 23 genes (TNFSF9, IL-8, IL-1A, PLAUR, IL-7R, JUN, TNFA, RELT, IER3, IL-4R, ICAM2, CCL20, COX2, MMP10, MKP1, GM-CSF, IL-23, IL-24, ICAM1, CXCL1, MMP3, MMP9, EGR1). If > 20 genes are over-expressed (> 1.25 x control vehicle value) the sample is rejected and the chemical tested at a lower concentration. A test item is considered as a sensitizer (positive)

when at least 7 genes from either the REDOX or the SENS-IS gene lists are over-expressed ($> 1.25 \times$ control vehicle value) (Cottrez et al., 2015). The lowest test item concentration fulfilling these criteria is then used for potency classification as follow : A chemical is classified as an extreme, a strong, a moderate or a weak sensitizer if found positive at 0.1%, 1%, 10% or 50% respectively. If negative at all tested concentrations, it is considered as a non-sensitizer.

2.3 Dose-range finding study for Individual chemicals

Each chemical was tested for solubility in 4 different vehicles i.e. PBS, DMSO, Olive oil and dipropylene glycol. To select the more appropriate vehicle for the testing, the chemicals were tested at 10% v/v in the SENS-IS assay in the vehicles in which they were soluble. The vehicle that gives the highest number of positively regulated genes was selected. In order to find the lowest positive concentration for each test chemicals, they were diluted at different concentrations into the selected vehicle.

2.4 Evaluation of the effects of cosmetic formulations on the potency of different sensitizers.

Three sensitizers were tested in the appropriate vehicle (Olive oil for cinnamaldehyde and isoeugenol and DMSO for hydroxycitronellal). The chemicals were also incorporated into 5 different cosmetic formulations (Table 1) at different concentrations i.e. 10%, 5%, 2%, 1% and 0.1% (v/v). The three ingredients were then tested onto the Episkin[®] large model in parallel, using either the selected vehicle or the different cosmetic formulations to compare the number of overexpressed genes in the 2 different groups of genes (SENS-IS or ARE) in the same experiment and the same batch of episkin.

2.5 Evaluation of the effects of cosmetic formulations on the potency of fragrance blends

For the 2 different fragrance blends, the same protocol as for the ingredients was followed using 3 different cosmetic formulations i.e. “oil”, “cleansing water” and “microemulsion”.

2.6 Evaluation of the effects of various irritants on the potency of skin sensitizers.

For the analysis of the irritant component effects onto the skin sensitization potency (i.e. the minimal positive dose) six different sensitizers were tested in DMSO at sensitizing concentrations (1% for cinnamaldehyde, isoeugenol, 10% for hydroxycitronellal and 50% for alphahexyl cinnamaldehyde, Glyoxal and dihydrocoumarin.) and non-sensitizing concentrations, i.e. concentrations that did not induce a positive signal in the SENS-IS assay (0.1% for cinnamaldehyde, isoeugenol, 1% for hydroxycitronellal and 10% for alphahexyl cinnamaldehyde, Glyoxal and dihydrocoumarin.) The irritant component effect was measured by addition of 1% of SLS or lactic acid. The different solutions obtained were then placed onto the Episkin[®] large model and the number of overexpressed genes in either the SENS-IS (hydroxycitronellal, alphahexyl cinnamaldehyde) or the ARE (cinnamaldehyde, isoeugenol, Glyoxal and dihydrocoumarin) groups of genes measured.

2.7 Analysis of the influence of mixing sensitizers on the skin sensitization potency of mixtures.

To analyze the ability of the SENS-IS assay to detect the influence of mixing different sensitizers on the potency of a mixture, we used 2 groups of sensitizers for which additive or synergistic effects of their mixtures was already published. This was the case for a mixture of isoeugenol, cinnamaldehyde and HICC (Bonefeld et al., 2011),

and for a mixture of 1,4-phenylene diamine (PPD) and methyldibromo glutaronitrile (Jowsey et al., 2008a).

For the 2 different analysis, we prepared 3 mixtures in DMSO with high, medium and low potency. High-potency mixture : isoeugenol 10%, cinnamaldehyde 10% and HICC 25%, Medium-potency mixture : isoeugenol 1%, cinnamaldehyde 1% and HICC 10% and low-potency mixture : isoeugenol 0,2%, cinnamaldehyde 0,2% and HICC 2%. Similarly for PPD and BBMG mixtures, high potency was 10% PPD and 10% BBMG, medium potency was 1% PPD and 10% BBMG and low potency was 0,2% PPD and 0,2% BBMG.

These different mixtures and the corresponding chemicals were then tested onto the Episkin[®] large model and the number of overexpressed genes in both the SENS-IS and the ARE groups of genes measured.

2.8 Evaluation of the hazard of cosmetics finished products using the SENS-IS assay.

Cosmetic products were assayed by applying 30 µl of the pure product directly onto the Episkin[®] large model. Two protocols were used : for the first one, similar to the protocols for pure ingredients, the tissues are washed with PBS fifteen minutes after application followed by a post incubation for a total of 6hrs after application. For the second one, the epidermises are washed only 6 hrs after application of the products, just before collection of the tissues for gene expression analysis.

Results

Dose-range finding study for the Individual chemicals

The results of the dose-range finding experiment with various concentrations of the different tested chemicals are plotted in Figure 1. As previously described, positivity is obtained when more than 7 genes are over expressed ($> 1.25 \times$ control vehicle value) into either the SENS-IS or the ARE group of genes (Cottrez et al., 2015). Accordingly, Glyoxal diluted in PBS was positive up to 50% (20% true concentration) by enhancing more than 7 genes in the ARE group of genes, whereas alphahexyl cinnamaldehyde (HCA) diluted in Olive oil (OO) and dihydrocoumarin diluted in DMSO were positive up to 25% by enhancing more than 7 gene in the SENS-IS group for HCA or in the ARE group for dihydrocoumarin. Finally Hydroxycitronellal (OHC) diluted in DMSO was positive up to a concentration of 5% while Isoeugenol and cinnamaldehyde, diluted in OO, enhanced more that 7 genes in the REDOX group of genes up to a concentration of 1%.

Effects of cosmetic formulations on the potency of different sensitizers

The effect of different cosmetic formulations on the sensitizing potency was analyzed as described under 2.4. The 3 skin sensitizers (cinnamaldehyde, isoeugenol and hydroxycitronellal) were prepared at serial dilutions, i.e. 10%, 5%, 2%, 1% and 0.1% (v/v) into 5 different cosmetic preparations, “oil”, “water in oil”, “oil in water”, “cleansing water” and “microemulsion” (Table 1, Figure 2). As expected the five cosmetic formulations were negative (not sensitizing) in the SENS-IS assay. Cleansing water induced the overexpression of some of the genes used in the

“irritation signature” but not to a level that would classify it as irritant (data not shown). As compared to the results obtained with the vehicle used in the Figure 1 (dashed lines in Figure 2), DMSO for hydroxycitronellal and olive oil for cinnamaldehyde and isoeugenol, the “oil” and “oil in water” cosmetic preparations did not change the minimal positive dose for cinnamaldehyde and isoeugenol (1%) and minimally changed it for OHC, from 5% to 10% for the “oil” preparation and from 5% to 2% for the “oil in water” preparation (Fig 2A and D).

On the other hand, “water in oil” emulsion which enhanced the sensitizing potential of OHC up to 1% (5 fold less from 5% to 1%) and “cleansing water” which enhanced the sensitizing potential of OHC up to 1% (10 fold less) and the sensitizing potential of Cinnamaldehyde and isoeugenol up to 0,1% (10 fold less from 1% to 0.1%) (Fig 2B and C) showed a clear potentiating effect on the sensitizing risk of cosmetic ingredients. It cannot be excluded that the observed enhancing effect could be higher than 10 fold since lower negative concentrations (below 0.1% or in between 1 and 0.1%) were not tested.

In contrast, microemulsion showed a marked dampening effect on the sensitizing potential of all three sensitizers (Fig 2E) since none were detected as sensitizers when tested at 10% (more than 10 fold more for Cinnamaldehyde and isoeugenol).

Effects of cosmetic formulations on the potency of fragrance blends

To confirm the enhancing or inhibiting effect of the different cosmetic formulations we tested 2 different fragrance blends containing at least 10 different fragrance ingredients. These two fragrance blends were selected because they were tested positive for sensitization in the SENS-IS assay when diluted up to 5% in Olive oil (v/v) (Figure 3). The two fragrance blends were tested at serial dilutions, i.e. 10%, 5%, 2%,

1% and 0.1% (v/v) into “cleansing water” (Fig 3A), “Oil” (Fig 3B) and “microemulsion” (Fig 3C) cosmetic preparations. If the “Oil” cosmetic preparation did not modify the sensitizing potential of the fragrance blends tested in Olive oil (dashed bars), cleansing water showed an enhancement of the sensitizing potential of the two fragrance blends (at least 2 fold from 5% to 2%) (Fig 3A). In contrast, microemulsion showed a decrease (a least 2 fold) of the sensitizing potential of the two tested fragrance blends (Fig 3C). Since the most potentiating cosmetic formulation, the cleansing water, had both a penetrating and an irritating effect (it contains a surfactant) we therefore decided to measure the effect of different types of irritant on the potency of skin sensitizers.

Effects of various irritants on the potency of skin sensitizers.

The potentiating effects of irritant on skin sensitization has been described in numerous studies (Pedersen et al., 2004b). However, since these studies were mainly performed with detergents, the balance between skin irritation and barrier injuring action of detergents on the enhanced skin sensitizer potency (measured) was not clearly stated. Therefore, we selected, in addition to SLS, a second irritant, Lactic acid, which has a different irritating action onto the skin (Basketter and Griffiths, 1993). Six different sensitizers, CinA, ISE, OHC, alpha hexyl cinnamaldehyde (HCA), Glyoxal and Dihydrocoumarin (DHC) were tested at sensitizing doses (1% for CinA and ISE ; 10% for OHC and Glyoxal, and 50% for HCA and DHC) and non sensitizing doses (0.1% for CinA and ISE ; 1% for OHC and Glyoxal, and 10% for HCA, and DHC) (Figure 4). The non sensitizing doses were also tested in combination with non irritating doses of SLS (1%) and non irritating doses of lactic acid (1%). The addition of a sub-irritating dose of either SLS or lactic acid resulted in modification of the

measured sensitization potency (the minimal positive dose) from 1% to 0.1% for CinA and ISE, from 10% to 1% for OHC and from 50% to 10% for HCA. In contrast addition of an irritant did not result in a similar enhancement in the sensitizing potency of DHC and Glyoxal, suggesting that the synergistic effects of irritant on skin sensitization potency could be dependent on the type of sensitizer used.

To evaluate the effects of irritant onto the potency of skin sensitizer on a more complex mixture of fragrance, RN and FM, the two fragrance blends used above, were tested at non sensitizing concentration (1% in DMSO) in absence, or presence of low doses of irritants, SLS and lactic acid at 1%. Similarly to CinA, ISE, OHC and HCA, addition of irritant enhanced the sensitizing potential of the two different fragrance blends (Figure 5).

Analysis of the influence of mixing skin sensitizers on the resulting potency of the mixture.

To evaluate the ability of the SENS-IS assay to measure the influence of mixing different skin sensitizers on the potency of the resulting mixture we used 2 different mixtures for which a mixing enhancing effect was measured in the LLNA assay (Bonefeld et al., 2011; Jowsey et al., 2008a).

The mixtures of isoeugenol, cinnamaldehyde and HICC were all detected positive in the SENS-IS assay (Table 3), even though the low potency mixture contained the 3 sensitizers at doses below their positive threshold i.e. 0.2% for cinnamaldehyde and isoeugenol and 2% for HICC, suggesting that at least an additive effect in the potency of the resulting mixtures was measured as previously described (Bonefeld et al., 2011). Similarly, for a mixture composed of PPD and BBMG at 0.2% in DMSO a positive result was obtained with the mixture, whereas the chemicals tested at 0.2%

alone were negative, showing that an enhancing effect of the mixture was detected. These results show that the SENS-IS assay has the potential to detect as positive a mixture composed of different sensitizers at a dose below their individual No-Expected-Sensitizing-Induction-Level (NESIL).

Measurement of the skin sensitizing hazard of commercially available cosmetic finished products using the SENS-IS assay.

We used the protocol described in 2.8 to test 25 commercially available cosmetic products from 18 different brands (Table 2). Two were dedicated to babies, two soaps (liquid or foam), three make-up removers, one shaving gel, one shampoo and sixteen body or facial skincare. Most of the products were negative and the two products for babies completely negatives (less than 2 sensitization biomarkers overexpressed). However, five finished products were detected as sensitizers, a shaving gel, two skin care products from the same brand, one skin make-up remover and one soap, with one extremely positive (the shaving gel). However, it has to be noted that three of the positive products were rinsed-off products, the shaving gel, the soap and the make-up remover. We also analyzed the irritation hazard for the different products. Not surprisingly all the soaps were detected as irritant except the ones dedicated to babies. One make-up remover was also irritant and sensitizer. These results suggest that the SENS-IS assay could be a good asset for testing complex commercially available cosmetic finished products.

Discussion

We ({Cottrez:2015jx}){Cottrez:2016hk} and others ({Clouet:2017ft} {Petry:2018kn} {Hoffmann:2018iy}) had shown that the SENS-IS assay was reliable for detecting safety hazard and measuring skin sensitization potency of chemical ingredients. Here we show that the SENS-IS assay is also performant for detecting skin sensitizing danger in complex mixtures and final products. This suggests that an *in vitro* assay using human reconstituted epidermis can bring pertinent informations for the safety evaluation of cosmetic products.

To date, the safety evaluation of cosmetic finished products is mostly based on information of the sensitization potency of individual ingredients. Mixtures are classified as skin sensitizers when an identified skin-sensitizing ingredient is equal to or exceeds 1% in the product. Besides, the current regulation requires disclosing the presence of a classified sensitizer in the Material Safety Data Sheet (MSDS) when present at more than 0.1% in the product (ECHA 2015).

Another way to evaluate the safety of cosmetic ingredients and products uses the Quantitative Risk Assessment (QRA) (Basketter and Safford, 2016), originally developed for fragrances (Api et al., 2008). In this case, the threshold amount, for a given allergen to induce skin sensitization, is also evaluated by its individual sensitization potency. However, the threshold may also be impacted by the composition of the product or mixture (Kimber et al., 2008). Therefore, the highest dose not inducing skin sensitization, the No-Expected-Sensitizing-Induction-Level (NESIL), is adjusted by uncertainty factors (Sensitization Assessment Factors (SAFs)) (Felter et al., 2002) in order to calculate an acceptable exposure level (AEL). Among these uncertainty factors the role of the vehicle/matrix in which the product is presented to the skin must be evaluated.

To measure the impact of the matrix on the potency of different sensitizers, 3 sensitizing ingredients (cinnamaldehyde, isoeugenol and hydroxycitronellal) and 2 perfume blends were incorporated into different cosmetic formulations (Table 1) and tested with the SENS-IS assay. The enhancing effect observed with the “cleansing water” formulation can be explained by the presence of irritants (see below) and detergents (Pedersen et al., 2004a) into the preparation that may therefore enhanced the amount of ingredient that will penetrate into the epidermis. Similarly the “water in oil” formulation must have enhanced the amount of hydroxycitronellal that entered into the epidermis, therefore decreasing the amount of ingredient needed to induce a positive sensitization signal. In contrast to the “cleansing water or the “water in oil” formulations, the “microemulsion” inhibited clearly the hazard of the mixtures. This shows that the SENS-IS assay can evaluate the influence of the matrix on the skin sensitization potency of ingredients. Previously, using the LLNA assay, the only other assay that allows a quantitative assessment of relative potency, an estimation of the influence of different vehicles was analyzed (Jowsey et al., 2008b). Although these analysis were performed with more simple vehicles compared to the cosmetic formulations (Table 1), the conclusions were also that an at least 10-fold difference in induction between vehicles could be measured.

Another factor that has been shown to enhance skin sensitization potency is the induction of skin inflammation by irritant (Cumberbatch et al., 1993) (Pedersen et al., 2004a) (Pedersen et al., 2004b). We therefore analyzed in the SENS-IS assay the influence of 2 different irritants, SLS and lactic acid at 1%, on the minimal positive dose of several skin sensitizers. We observed, for most of the sensitizers tested, an enhancement of their sensitizing potency. At least a 10 fold increase in the potency was observed for CA, IE (from 1% to 0.1%), and OHC (from 10% tp 1%). A 5-fold

increase for HCA from 50% to 10% was also shown. Interestingly, the potentiating irritant effect was not observed with other sensitizers like dihydrocoumarin tested at 10% or glyoxal at 10%. This result suggests that inflammation induced by irritant is not always associated with lowering the threshold dose for skin sensitization induction. On the other hand it has to be noted that CA, IE, OHC and HCA are skin irritants whereas DHC and Glyoxal are not irritants and therefore do not need an irritation signal to induce sensitization. Different combination of irritants and sensitizers should be tested to better understand the influence of irritation/inflammation on skin sensitization potency. Nevertheless, the SENS-IS assay provides a tool to investigate that relationship.

Aggregative effects of different sensitizers can also influence the skin sensitization potential of a mixture. To evaluate the ability of the SENS-IS assay to measure these effects, we replicated *in vitro* some analysis performed *in vivo* using the LLNA. Bonefeld et al. (Bonefeld et al., 2011) used a mixture of cinnamaldehyde, isoeugenol and HICC in order to show that a mixture of fragrance allergens had an increased sensitization potency as compared with the isolated fragrance allergens. Using similar high, medium and low-potency mixtures of the same ingredients we showed here that the SENS-IS assay is able to demonstrate also that a mixture of ingredients at a dose where they are individually negative can be detected as positive (Table 3). This suggests that at least an addition of signals can be detected in mixtures. To confirm that, we also used the mixture of PPD and BBMG described by Lowsey et al. (Jowsey et al., 2008a). Again, the SENS-IS assay was able to measure the difference between the mixture and the ingredients tested alone, even at doses where the two ingredients failed to induce a positive signal (Table 3).

Although the SENS-IS assay can detect a mixture component effect it is very difficult, like for the LLNA, to distinguish between additive and synergistic effects.

Finally we have analyzed the ability of the SENS-IS assay to evaluate the skin sensitization and irritation hazards, of finished cosmetic products. We tested 25 different cosmetic products from 18 different brands from different major or smaller providers of cosmetic products. The tested products were selected for being directly used onto skin with or without rinse off use and were tested without any dilution. None of them had a “hypoallergenic claim” and all of them contained at least one sensitizing ingredient. As shown in Table 2 most of the tested product were negative both for irritation and sensitization (21/25). One soap from brand N was positive for both irritation and sensitization but since it is a typical rinse off product it may not be problematic. In addition this product was tested pure. The 4 other positive sensitizing products (1 from brand A and C) were positive only after 6 hrs long application which is a maximizing situation that may not completely reflects the use of the product. Moreover two of them were rinse off products.

Consumer products are usually composed of cocktail of irritant and allergens. The current study indicates that the SENS-IS assay could be used to assess the sensitizing hazard and could help in the management of risk assessment of complex mixtures and finished products.

Legend to the figures

Figure 1 : Dose-range analysis of 6 different sensitizers using the SENS-IS assay

The number of over-expressed genes (1.25 x control vehicle value) was measured after application at each indicated doses of the 6 different skin sensitizers onto the epidermis. For each dose, the number of overexpressed genes in the SENS-IS (left bar) or REDOX (right bars) group of genes was measured. The line across shows the number of overexpressed genes above the cut off value of 6 genes (7 and over) showing a positive sensitizer dose. Results display the mean $\pm$ SD of three experiments.

Figure 2 : Influence of the vehicle on the potency of skin sensitizers

Cinnamaldehyde, Isoeugenol and Hydroxycitronellal were diluted into 5 different cosmetic bases, respectively oil, water-in-oil, cleansing water, oil-in-water and microemulsion. The result obtained using the SENS-IS test with the cosmetic formulations as vehicle (solid bars) was compared to the one obtained with Olive Oil (CinA, ISE) or DMSO (OHC) as vehicle (dashed bars). The number of overexpressed gene in the Redox group is displayed for CinA and ISE. For OHC, we analyzed the number of genes overexpressed from the SENS-IS group of genes. The line across shows the number of overexpressed genes above the cut off value of 6 genes (7 and over) showing a positive sensitizer dose. Results display the mean $\pm$ SD of two experiments.

Figure 3 : Influence of the vehicle on the potency of complex perfume mixtures

Fragrance blends coded RN and FM were diluted into either Olive oil (dashed bars) or the indicated cosmetic formulations (solid bars) and tested at different indicated concentrations using the SENS-IS test. The number of overexpressed genes in the Redox group is displayed for both RN and FM. The line across shows the number of overexpressed genes above the cut off value of 6 genes (7 and over) showing a positive sensitizer dose. Results display the mean $\pm$ SD of two experiments.

Figure 4 : Influence of irritants on the potency of skin sensitizers

Cinnamaldehyde, Isoeugenol Hydroxycitronellal, Hexyl cinnamaldehyde, Glyoxal and Dihydrocoumarin were tested diluted in DMSO at sensitizing and non-sensitizing doses as indicated. The non-sensitizing doses were also tested mixed with non-irritating doses of SLS (1%) or lactic acid (1%). The number of overexpressed genes in the Redox group is displayed for CinA, ISE, Glyoxal and DHC. For OHC and HCA, we analyzed the number of genes overexpressed from the SENS-IS group of genes. The line across shows the number of overexpressed genes above the cut off value of 6 genes (7 and over) showing a positive sensitizer dose. Results display the mean $\pm$ SD of two experiments.

Figure 5 : Influence of irritants on the potency of complex perfume blends

Fragrance mixes coded RN (A) and FM (B) were tested diluted in DMSO at sensitizing (10%) and non-sensitizing (1%) doses. The non-sensitizing doses were also tested mixed with non-irritating doses of SLS or lactic acid. The number of

overexpressed genes in the Redox group is displayed for both RN and FM. According to the color scheme, the green color represents negative values, whereas the red color represents the positive values. The line across shows the number of overexpressed genes above the cut off value of 6 genes (7 and over) showing a positive sensitizer dose. Results display the mean $\pm$ SD of two experiments.

Conflict of interest statement

HG and FC have filled on patent for the SENS-IS assay, all the other authors declare no conflict of interest

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Declaration of interests

☒ The authors, Elodie Boitel, Marie-Pierre Berrada-Gomez, Hanna Dalhuchyts, Catherine Bidan, Sandy Rattier, and Pierre-Jacques Ferret declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☒ The authors, Françoise Cottrez and Hervé Groux declare the following financial interests/personal relationships which may be considered as potential competing interests:

Patent that covers some aspects of the SENS-IS assay
Financial interest in the company ImmunoSearch

Table 1 : Definition and composition of the five different cosmetic preparations tested

	Microemulsion	Oil in water emulsion(O/W)	Water in oil emulsion (W/O)	Oil
Definition	Dispersion made of water, oil, and surfactant(s) that is anisotropic and thermodynamically stable system with dispersed domain diameter varying approximately from 1 to 100 nm, usually 10 to 50 nm.	An emulsion is a mixture of two or more liquids that are normally immiscible. It is called an oil/water (o/w) emulsion if the dispersed phase is an organic material and the <i>continuous phase</i> is water or an aqueous solution	An emulsion is a mixture of two or more liquids that are normally immiscible. It is called water/oil (w/o) if the dispersed phase is water or an aqueous solution and the continuous phase is an organic liquid (an "oil").	An oil is any chemical substance that is a viscous liquid at ambient temperature and is both hydrophobic and lipophilic
Composition	20 %emulsifying agent O/W 50% oil 20%emollient (+ antioxidant)	20% oil 77% water (+ preservative/ pH regulating agent/ emulsifying O/W agent)	67% water 30% oil (+Preservative/ pH regulating agent/ emulsifying agent W/O)	Mix of 5 oils (+ antioxidant)

Table 2 : Analysis of finished cosmetics products irritation and sensitization hazard

BRANDS	TYPE	SENSITIZING CONSTITUANTS	IRRITATION	SENSITIZATION
A	eye make up remover	Fragrance triethanol- amine	N	N
A	skin make-up remover	Fragrance preservative	N	N
A	skin make-up remover	Fragrance	P	P
A	shave cream	Fragrance triethanol- amine	N	P*
B	body milk	Benzyl alcohol Fragrance	N	N
C	skin care	Fragrance	N	N
C	skin care	Active ingredient Fragrance	N	P
C	skin care	Active ingredient Fragrance	N	P
D	skin care	Fragrance	N	N
E	skin care	Siloxane Fragrance	N	N
F	skin care	Fragrance	N	N
G	body milk	Fragrance	N	N
H	Soap for babies	Fragrance	N	N
I	skin care	Fragrance	N	N
I	skin care	Fragrance	N	N
J	Soap	Benzyl alcohol	P	N
K	skin care	preservative	N	N
L	skin care	Fragrance preservative	N	N
M	Soap for babies	Fragrance	N	N
N	Soap	Fragrance	P	P
O	skin care	Siloxane Fragrance	N	N
O	skin care	Fragrance	N	N
P	skin care	Preservative Fragrance	P	N
Q	skin care	Benzyl alcohol	N	N
R	skin care	Fragrance	N	N

25 commercially available cosmetic finished products from 13 different brands coded A to R were tested using the SENS-IS assay. All products were apply to the Episkin® large model for a pre incubation time of 15 mn or 6 hrs as described in the Material and Method section. Irritation was measured positive when more than 15 genes were over-induced. Sensitization was measured positive when more than 7 genes in the SENS-IS or ARE groups of genes were over-induced (see Material and Method section). P* means that the product was positive with the short time (15 mn) application protocole.

Table 3 : Analysis of the minimum positive concentration of sensitizing chemicals and mixtures using the SENS-IS assay.

Products	High	Medium	Low
Isoeugenol	11	9	4
Cinamaldehyde	12	9	5
HICC	9	8	3
mixture IE+CA	10	9	7
mixture IE+CA+HICC	15	14	11
methyldibromo glutaronitrile	12	8	3
1,4-phenylenediamine	15	9	4
Mixture MDG + PPD	14	10	9

The different sensitizers, Isoeugenol, cinnamaldehyde, HICC, methyldibromo glutaronitrile and 1,4-phenylenediamine were tested alone or incorporated into the indicated mixtures at high-potency (25% for HICC and 10% for the other sensitizers), medium-potency (10% for HICC and 1% for the other sensitizers) or low-potency concentrations (2% for HICC and 0.2% for the other sensitizers). After application onto the epidermises, the number of genes over-expressed in the ARE group of genes of the SENS-IS assay were measured. A positive signal, in grey, is measured were more that 7 genes out of 17 are overexpressed.

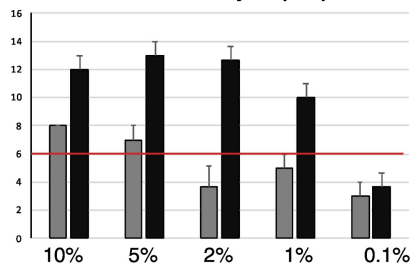
HIGHLIGHTS

- Cosmetic formulations can impact skin sensitization potency
- Irritant and mixtures of sensitizers can have the potential to influence skin sensitization potency
- Mixture component effect can be gauged by the SENS-IS assay
- Irritation and sensitization hazard of cosmetic finished products can be assessed by the SENS-IS assay

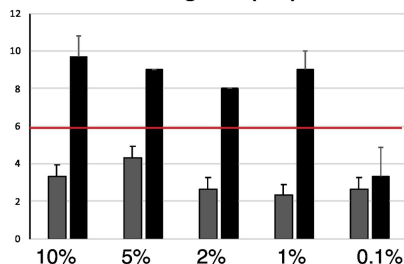
Journal Pre-proof

Nb over-expressed genes

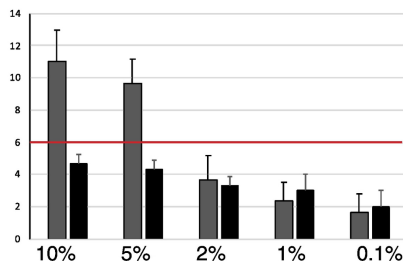
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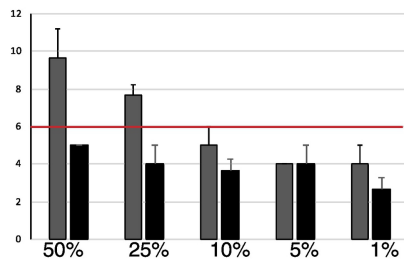
Isoeugenol (OO)



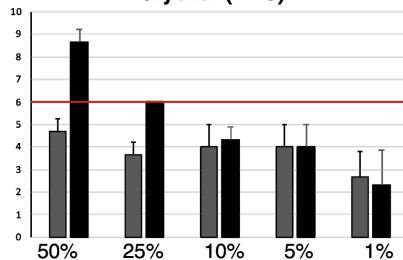
Hydroxycitronellal (DMSO)



alphahexyl cinnamaldehyde (OO)



Glyoxal (PBS)



Dihydrocoumarin (DMSO)

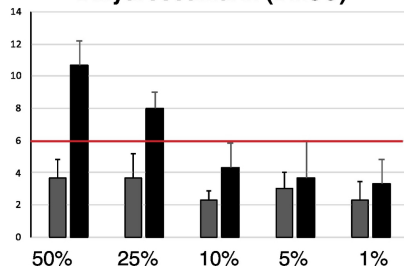


Figure 1

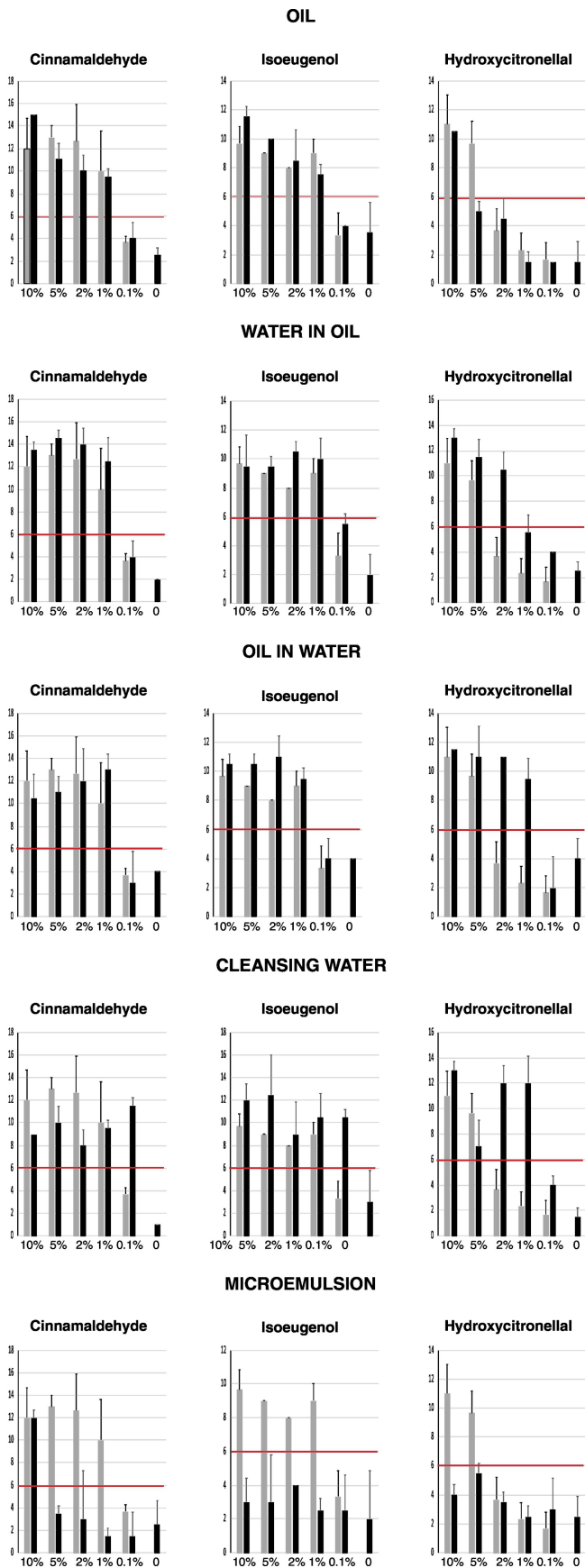
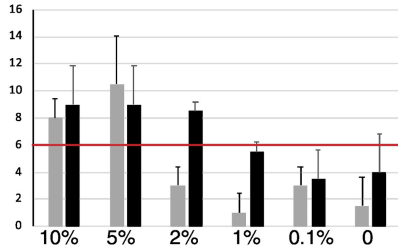


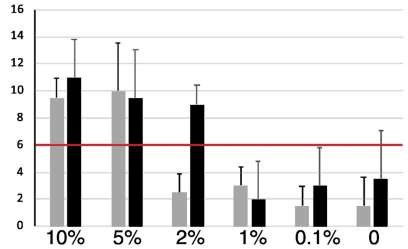
Figure 2

CLEANSING WATER

RN Fragrance blend

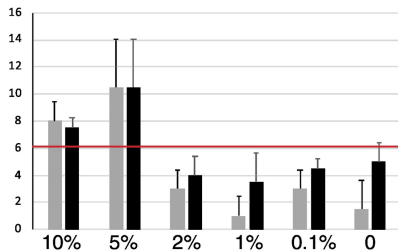


FM fragrance blend

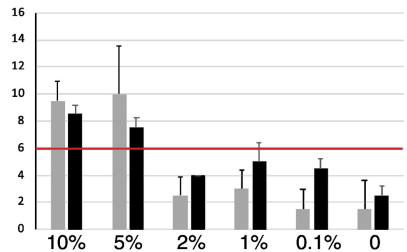


OIL

RN Fragrance blend

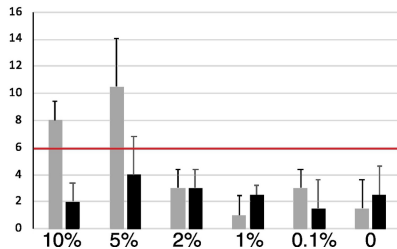


FM fragrance blend



MICROEMULSION

RN Fragrance blend



FM fragrance blend

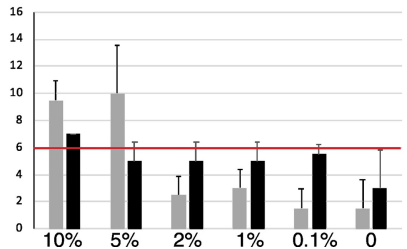


Figure 3

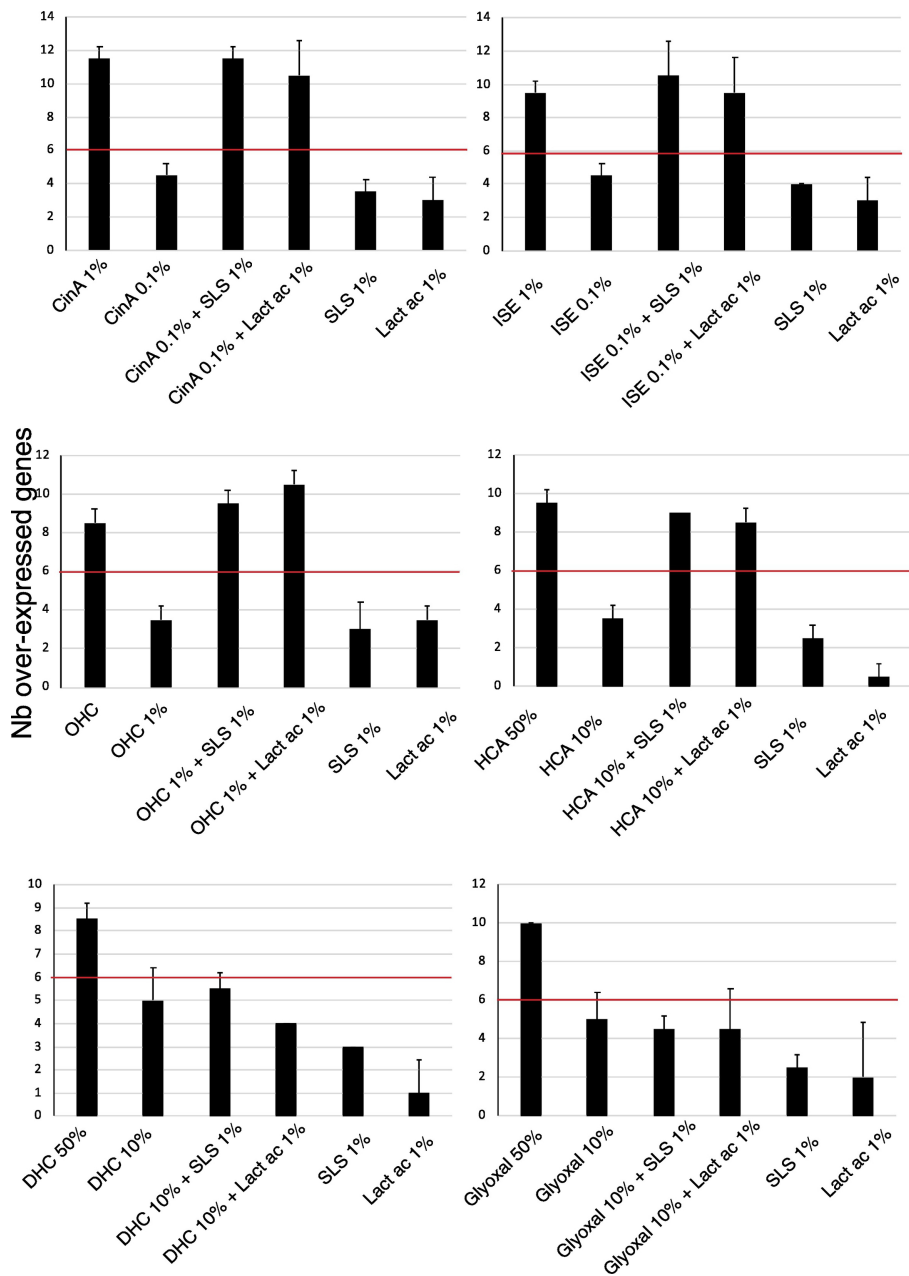


Figure 4

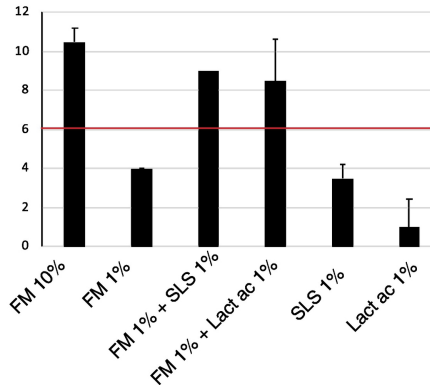
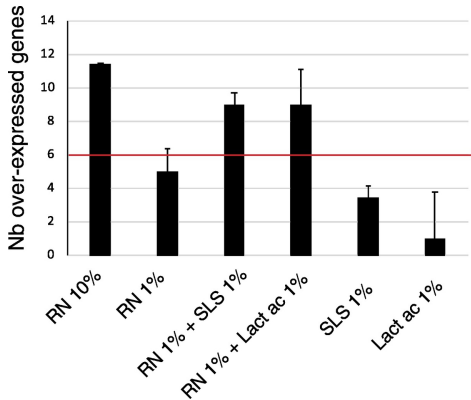


Figure 5