

**Modifications induced by chemical skin allergens on
the metabolome of reconstructed human epidermis:
A pilot HRMAS NMR study.**

François-Marie Moussallieh¹, Eric Moss¹, Karim Elbayed², Guillaume Lereaux³, Fleur
Tourneix³, Jean-Pierre Lepoittevin^{1,*}

¹ Institute of Chemistry, CNRS UMR 7177 and University of Strasbourg, Strasbourg, France

² Laboratoire des sciences de l'ingénieur, de l'informatique et de l'imagerie (ICube), CNRS
UMR 7357 and University of Strasbourg, Strasbourg, France

³ L'Oréal R&I, Aulnay-sous-Bois, France

* Corresponding author, jplepoit@unistra.fr

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Abstract

Background: High-resolution magic angle spinning (HRMAS) is a nuclear magnetic resonance (NMR) technique that enables the characterization of metabolic phenotypes/metabolite profiles of cells, tissues and organs, under both normal and pathological conditions, without resorting to time-consuming extraction techniques.

Objectives: To assess the impact of chemical skin sensitizers vs non-sensitizers on the metabolome of 3D reconstructed human epidermis (RHE) by HRMAS NMR.

Methods: Based on the SENS-IS assay, 12 skin sensitizers and 5 non-sensitizing chemicals were investigated and applied on EpiSkin RHE at the published maximal non irritating concentrations under the conditions of the test. The metabolome of RHE samples was then analyzed by HRMAS NMR.

Results: Thirty-two different metabolites were identified; 20 of these were quantified for all samples. Statistical univariate analysis showed first that the tissue content of most measured metabolites (with the exception of acetate and glucose) were different in the untreated, treated with non-sensitizers and treated with sensitizers. In RHE samples in contact with sensitizing chemicals, concentrations of all these 18 metabolites were significantly decreased. Alanine and tyrosine could not discriminate between sensitizer and non-sensitizer treated groups. A multivariate PLS-DA analysis was performed on the two treated groups discriminating sensitizing and non-sensitizing chemicals with very good R²Y value of 0.87 and a good Q² value of 0.70.

Conclusions: Data suggest that HRMAS NMR could be used to monitor the impact of chemicals, skin allergens vs non-sensitizers, on the metabolome of 3D RHE.

Keywords

Reconstructed Human Epidermis, HRMAS NMR, chemical skin allergens, metabolome.

1. INTRODUCTION

In addition to its physical barrier function,¹ the skin is a major immunocompetent² and xenobiotic-metabolizing organ.³ As a consequence, prolonged or repeated dermal exposure to chemicals can lead to a hypersensitivity immune response in some cases such as skin allergy/allergic contact dermatitis (ACD), a very common inflammatory skin disease.^{4,5} Currently, in the western world, 15-20% of the population are allergic to at least one chemical present in their environment.⁶ As there is no treatment for skin allergy other than symptomatic, the prevention of this disease relies on early identification of sensitizing chemicals prior to their introduction to the market. So far this has been done on the basis of animal methods such as the murine local lymph node assay (OECD TG 429) or the Magnusson-Kligman guinea pig based tests (OECD TG 406). However, since the implementation of EU regulations such as REACH⁷ and the Cosmetics Regulation,⁸ there considerable efforts have been made to develop non-animal methods to assess skin sensitization. The adverse outcome pathway (AOP) for skin sensitization, published by the OECD in 2012,^{9,10} has been the basis for new alternative methods addressing specific key events (KE) of this AOP. Up to now three methods are available as OECD test guidelines: the Direct Peptide Reactivity Assay (DPRA),¹¹ the KeratinoSens,¹² and the human Cell Line Activation Test (h-CLAT)¹³ addressing KE1 (protein reactivity), KE2 (keratinocytes activation) and KE3 (antigen presenting cells activation), respectively. While these methods are individually performing quite well to identify potential skin sensitizers, they only

address one specific key event. Moreover, they show some limitations in addressing bioavailability, metabolic activation and danger signal.

One alternative to these peptide/cell based assays would be to use a 3D reconstructed human epidermis (RHE) model that is very similar to the *in vivo* human epidermis^{14,15} in terms of morphology and metabolic activity, although some differences in terms of skin penetration¹⁶ and enzymatic activation^{17,18} should be pointed out. Advantages of using a 3D reconstructed skin model would be to test chemicals under conditions similar to human use (topical application), take advantage of the metabolizing enzymes present, and benefit from the tridimensional microenvironment. On the one hand, an alternative approach taking advantage of a 3D RHE model (EpiSkin), has recently been proposed.¹⁹ The SENS-IS assay is based on the expression analysis of a large panel of genes relevant to the sensitization process in RHE after exposure to a chemical.²⁰ This assay has been shown to have a very good predictive capacity, to be easily transferable and have a good reproducibility.²⁰ On the other hand, metabolomics is a rapidly growing branch of science that aims at analyzing a whole set of metabolites, the metabolome, existing in a biological environment. Metabolites are small molecules with a molecular weight of less than 1-1.5 kDa produced by the cells and taking part to the biochemical reactions essential for the cellular integrity. Their levels can be regarded as the ultimate responses of the biological systems to genetic and/or environmental challenges.²¹ The metabolome is therefore the resulting product of the genomic expression implied in the cellular metabolism.

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We have therefore been interested in the impact of chemical skin sensitizers on the metabolome of RHE. The techniques most commonly utilized in metabolomics are mass spectrometry (MS) coupled with gas or liquid chromatography (GC or LC) or liquid nuclear magnetic resonance (NMR) spectroscopy. However these techniques are time-consuming by requiring a step of extraction which can also introduce bias.²² An alternative could be achieved by using high resolution magic angle spinning (HRMAS) nuclear magnetic resonance (NMR) technique.²³⁻²⁵ Indeed, this technique offers, without resorting to time-consuming extractions, a detailed and accurate analysis of the metabolic composition of intact tissue specimen. This enables the characterization of metabolic phenotypes/metabolite profiles of cells, tissues and organs, under both normal and pathological conditions.²⁶⁻²⁸ Several gynecological, neurological and urological human malignancies have thus been studied by HRMAS with promising results and the technique has been applied to a variety of pathologies.²⁹ More recently, our studies have demonstrated HRMAS to be a promising tool in new domains such as organ transplantation by allowing the assessment of the quality of the graft,^{30,31} and surgical operations as an additional diagnostic tool providing real-time information.³² We have also been showing that HRMAS NMR was well adapted to study RHE models,³³⁻³⁶ more precisely the metabolism of different ¹³C-substituted sensitizers.

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In this work we investigated if this approach based on the HRMAS NMR analysis of RHE could be used to characterize the impact of chemical skin allergens. To this end, we have been testing a series of 12 skin allergens versus 5 non-sensitizing chemicals on EpiSkin following conditions used for the SENS-IS assay^{19,20} showing a modification of the metabolome allowing to discriminate between sensitizers and non-sensitizers.

2. MATERIALS AND METHODS

2.1 Chemicals and reagents

Chemicals used in this study were: Oxazolone (CAS no. 15646-46-5), diphenylcyclopropanone (CAS no. 886-38-4), *p*-benzoquinone (CAS no. 106-51-4), 2,4-dinitrochlorobenzene (CAS no. 97-00-7), 4-nitrobenzylbromide (CAS no. 10011-8), 3-methylcatechol (CAS no. 488-17-5), methyldibromo glutaronitrile (CAS no. 35691-65-7), *o*-aminophenol (CAS no. 95-55-6), formaldehyde (CAS no. 50-00-0), glutaraldehyde (CAS no. 111-30-8), propyl gallate (CAS no. 121-79-9), methylchloroisothiazolinone (CAS no. 26172-55-4), *N*-butyl alcohol (1-butanol; CAS no. 71-36-3), propylene glycol (CAS no. 57-55-6), sulfanilamide (CAS no. 63-74-1), vanillin (CAS no. 121-33-5), glycerol (CAS no. 56-81-5), and dimethylsulfoxide (CAS no. 67-68-5).

2.2 Reconstructed Human Epidermis

Cell Culture: The EpiSkin reconstructed human epidermises, large model (thirteen days old, 1.07 cm²), were obtained from Skinethic, Lyon, France. These models are differentiated from human keratinocytes isolated from healthy donors and include a human collagen (Type I) matrix, coated with a layer of Type IV human collagen, and a fully stratified and differentiated epidermis covered with a stratum corneum. The EpiSkin cultures are shipped onto a nutritive gel for transportation to maintain viability. Upon reception, RHE were kept under sterile conditions in a 12-well culture dishes containing 2 mL/well of supplied maintenance medium (DMEM/Ham's F12) pre-warmed at 37°C. The medium was changed (1 mL) each 24h.

Treatment protocol: At day 14 of culture, the EpiSkin tissues were exposed to the test chemicals. A solution of each chemical in PBS or DMSO (30 µL) at the maximum non-toxic concentration¹⁹ was applied with a micropipette onto the stratum corneum of the epidermis in duplicate. The tissues were then incubated with chemical for 15 min. at 37°C/5% CO₂ and washed with 25 mL PBS per epidermis. After washing, the tissues were further incubated at 37°C/5% CO₂ for 6 hours. Each epidermis was then lifted from the insert using forceps and transferred into a vial for snap freezing. The two RHE samples treated with the same chemical were pooled into the same vial prior to the snap freezing.

2.3 Rotor preparation and data acquisition by HRMAS NMR

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The rotor preparation and the data acquisition by HRMAS NMR were carried out according to the methodology already described. Briefly, each sample was prepared at -20 °C by introducing 15 to 20 mg of frozen RHE completed with D₂O into a disposable 30 µL Kelf inserts. Shortly before HRMAS analysis, the insert was placed into a standard 4 mm ZrO₂ rotor and closed with a cap. The HRMAS experiments were performed at 3 °C. Upon completion of the analysis, the insert was taken out of the rotor and stored back at -80 °C for further complementary NMR analysis at a later stage.

HRMAS spectra were recorded on a Bruker Avance III 500 spectrometer (Haute-pierre University Hospital, Strasbourg) operating at a proton frequency of 500.13 MHz, equipped with a 4 mm double resonance (¹H, ¹³C) gradient HRMAS probe. The conditions of acquisition and processing for the 1D ¹H experiments using standard one pulse and Carr-Purcell-Meiboom-Gill (CPMG) pulse sequences coupled with water presaturation, and for the 2D ¹H-¹³C g-HSQC (gradient Heteronuclear Single Quantum Coherence) experiments using echo-antiecho gradient selection, were those described in the detailed methodology. All spectra were referenced by setting the lactate doublet chemical shift to 1.33 ppm in ¹H and to 22.7 ppm in ¹³C.

2.4 Metabolite identification.

Spectral assignments were based on matching 1D NMR data to reference spectra in a home made reference database, as well as with other databases

(<http://www.bmrp.wisc.edu/metabolomics/>; <http://www.hmdb.ca/>), and reports in the literature. Some unknown signals (marked with an “X” on 1D and 2D spectra) were detected but they were not referenced, to our knowledge in any spectral database.

2.5 Discriminant analysis according to experimental groups.

A non-parametric Kruskal-Wallis test with a critical *P*-value of .05 was used to determine whether there was a significant difference between the three groups for each metabolite obtained from the partial least squares discriminant analysis (PLS-DA) models. *P*-values were adjusted for multiple testing corrections using the False Discovery Rate.³⁷ This test was conducted using Minitab software (version 18). Statistically significant changes between the metabolic content of RHE samples exposed either to sensitizers or non-sensitizers and control groups were also assessed using a non-parametric Kruskal-Wallis test followed by a Mann-Whitney test (*n*= 3 to 12 per group, *P*-value < .05).

A multivariate approach was also used. To this end, concentrations of the different metabolites were imported as an input matrix into Python 3 using Matplotlib (Atlanta, MA)³⁸ and Scikit-learn libraries.³⁹ Principal component analysis (PCA) was performed to separate the exposed and control groups and remove outliers, if necessary (data not shown). The PLS-DA method was then applied to identify potential metabolites corresponding to the buckets with variable importance in the projection (VIP) above 1. Cross-validation was used to determine the number of linear combinations to be included

in the PLS-DA model. The quality of the model was indicated by two parameters, R²Y (proportion of explained variance) and Q²Y (predictive ability). The Q² value was evaluated using seven-fold cross-validation.

3. RESULTS AND DISCUSSION

Based on the SENS-IS assay,^{19,20} 12 skin sensitizers and 5 non-sensitizing chemicals (Table 1) were investigated and applied on EpiSkin RHE at the published maximal non irritating concentrations under the conditions of the test (15 min. exposure, washing with PBS followed by 6h post-incubation).

3.1 HRMAS NMR analysis of untreated RHE

HRMAS NMR spectroscopy experiments were performed on several samples belonging to two different EpiSkin RHE batches. 1D and 2D NMR spectra were found to be quantitatively and qualitatively similar. A representative HRMAS Carr-Purcell-Meiboom-Gill (CPMG) spectrum of an untreated RHE sample is presented in Figure 1A. Metabolites were assigned using standard metabolite chemical shift tables available in the literature.⁴⁰ ¹H–¹³C g-HSQC standard and edited spectra as well as ¹H–¹H DIPSI spectra were also used to confirm some assignments. A representative g-HSQC standard spectrum is presented in Figure 1B, and Supplementary Table 1 presents detailed assignments of metabolites detected in EpiSkin RHE. A total of 32 different metabolites were thus identified.

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Since magnetic resonance is a quantitative technique, it was possible to obtain metabolite levels for such samples. According to the protocol described elsewhere,²⁹ metabolite concentrations were measured by integrating the areas under individual peaks of the metabolites. Note that these concentrations were obtained from about 15 mg of RHE within an experimental time of about 19 min. These concentrations are indicative since they were obtained on not fully relaxed systems. However, as all experiments were performed under the same conditions, the measured concentrations can be used for statistical analyses. A representative 1D ¹H spectrum of the control group is presented Figure 1A, and a representative 2D g-HSQC is presented in Figure 1B.

For some metabolites presenting at least one resonance with sufficient spectral resolution (20 among the 32 described by 2D experiments), the obtained values in (nmol/mg of RHE) were (on average) for an untreated sample alanine (10.3), aspartic acid (4.0), citrulline (2.8), free choline (1.4), phosphocholines (4.0), total choline (5.4), beta-glucose (5.3), glutamate (11.5), glutamine (23.9), glutathione (1.7), glycine (9.2), isoleucine (3.9), lactate (17.8), lysine (12.0), pyroglutamic acid (10.1), serine (16.0), threonine (8.1), tyrosine (3.6), and valine (3.6).

3.2 HRMAS analysis of treated RHE

The representative ¹H spectra of the two treated groups of samples are also presented on Figure 2 (B and C), along with the one of the control group. The NMR spectra of the skin

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samples showed signals from numerous small molecular weight metabolites and lipids. The 20 metabolites were for the majority of them detectable in all spectra, and were identified as cell building blocks (amino acids and choline compounds), metabolites involved in glutaminolysis (glutamate, glutamine), metabolites involved in detoxification (glutathione) and metabolites involved in energy consumption (glucose and lactate). Peak areas of the 20 selected metabolites could be calculated for all samples. Note that acetate, present and detectable in some RHE samples treated with sensitizers, was not detectable in control samples. The average concentrations of the different metabolites quantified are presented in Table 2, $\pm$ SD. The individual concentrations of each metabolite for all the samples analyzed are listed in Supplementary Table 2. A simple visual inspection of the three representative ^1H spectra presented in Figure 2 shows a global decrease of the metabolic content between the control group, the non-sensitizer treated group and the sensitizer treated group.

3.3 Modifications induced in RHE by sensitizers and non-sensitizers

Statistical univariate analysis showed first that the tissue content of most of the measured metabolites (with the exception of acetate and glucose) were significantly different in the three types of analyzed samples. This striking result is emphasized by a P -value $< .05$ in the Kruskal-Wallis test, as depicted in Table 2. The levels of 17 of the quantified metabolites

were lower in treated RHE compared to naive RHE samples. Note that glycine cannot discriminate between naive samples and samples treated by a non-sensitizing chemical.

We also found metabolic changes for 18 of the quantified metabolites ($P < .05$, Mann-Whitney test) (Table 2) between the two treated samples groups (sensitizers vs non-sensitizers). Indeed, all these 18 metabolites were significantly decreased in RHE samples exposed to a sensitizing compound even if alanine and tyrosine cannot discriminate between the sensitizer and non-sensitizer treated sample groups. We can hence confirm the global decreasing trend of the metabolic fingerprint of the RHE for distinguishing the samples treated either by a non-sensitizing or a sensitizing chemical. We have also checked that both treated groups (sensitizers and non-sensitizers) could be distinguished from the untreated control group.

Among affected metabolites, glutathione (GSH) attracted our attention, as this peptide is known to react readily with electrophiles and be involved in detoxication processes. Overall, the average concentration of GSH was found to be 1.1 ± 0.1 nmol/mg and 0.3 ± 0.4 nmol/mg in non-sensitizers and in sensitizers treated RHE samples, respectively (Fig 3A). For 7 over 12 sensitizers, GSH was even completely depleted, with no detectable signal. Very interestingly the depletion was not complete either with prohaptenes that need to be activated (propylgallate, *o*-aminophenol, 3-methylcatechol) or aldehydes that are known to be less reactive towards thiol nucleophiles (formaldehyde and glutaraldehyde).

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Interestingly, formaldehyde induced in parallel the second most important depletion for lysine which is in very good agreement with its known chemical reactivity. A threshold based on the measured concentration of GSH in RHE treated with formaldehyde at 0.97 nmol/mg could allow discriminating sensitizers from non-sensitizers (Fig. 3A). Similar observations could be made with glutamate which could also discriminate sensitizers from non-sensitizers with a threshold based on the concentration measured for *o*-aminophenol at 4.70 nmol/mg of RHE (Fig 3B). Looking at depletion levels associated with vanillin that are close to the threshold, the classification of this chemical as a non-sensitizer could be questioned. Indeed, it is classified as such based on LLNA data, but cases of skin allergy have been reported in humans and it could therefore be considered as a weak sensitizer. If vanillin was considered as a sensitizer, many more metabolites could discriminate between sensitizers and non-sensitizer such as serine, alanine or lysine. This very well illustrates the complexity of analyzing such a large dataset, and therefore in order to include all data without any “a priori”, more accurate statistical analysis based on multivariate tests were needed.

3.4 Multivariate Statistical analyses

Multivariate partial least square-discriminant analysis (PLS-DA) using our in-house built Python algorithm was performed on the two treated groups using all concentrations of the different analyzed metabolites as an input. The variable importance in the projection (VIP)

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values determined following this analysis showed that citrulline, glycine, tyrosine and aspartate had a low predictive value while all the remaining metabolites had the best predictive values characterized by a VIP value above 0.9. Among these 16 metabolites, the strongest discriminants were found to be acetate, free choline, glutathione, pyroglutamic acid, lysine and threonine. A partial least square-discriminant analysis (PLS-DA) restrained on these most discriminating metabolites has therefore been performed and a score plot is presented in Figure 4A. It is characterized by a very good R²Y value of 0.87 and a good Q² value of 0.70 obtained with the first two PCs of the model. A R²Y value and a Q² value superior respectively to 0.7 and to 0.5 are generally considered as very good indicators for a robust classification model.²⁹ Finally, we used this model to predict the control group. As we can see on the score plot presented in Figure 4B, this group is also well distinguished for the two others and is located close to the non-sensitizer group, reflecting the observation made previously by a visual inspection in the three average NMR spectra.

3.5 Pathophysiological considerations

If in the case of GSH and Lysine the depletion following contact with skin sensitizers could be explained by a direct reaction with the chemical, like in the DPRA, it is less clear for other metabolites even if some of them have been linked to some pathophysiological processes occurring in the skin. Thus, serine can be used by cells for energy supply being converted into lactate,⁴¹ even if the origin of the intracellular serine remains unclear: either

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taken up from the medium, de novo synthesized from 3-phosphoglycerate or finally resulting from the conversion of reduced serine. Some metabolites appear to be associated with hyperproliferative or hypermetabolic states: glutamic acid, choline, valine, and glucose. This parallels cancer metabolic profiles as both diseases are characterized by high cellular proliferation rates.^{28, 42} Similarly, choline is a building block needed for cell growth and division and was found to be elevated in psoriatic skin compared to symptom-free psoriatic skin and healthy controls in two studies using NMR and MS analysis.^{43,44} Moreover, both glutamic acid and choline were found to have a positive correlation with psoriasis severity based on the plaque severity scale, likely due to the increasing keratinocyte proliferation and inflammation.⁴³ Alternatively, decreased levels of glucose and valine-leucine/isoleucine ratios were found in psoriatic lesions compared to non-psoriatic skin, which may be due to accelerated turnover of these metabolites in rapidly proliferating cells. Increased levels of choline in inflammatory processes have also been observed.⁴²

4. CONCLUSION

The use of a metabolomic approach based on HRMAS NMR Spectroscopy could be a good alternative to assess the sensitizing capacity of chemicals. In this pilot study we have been using a still limited number of potent skin sensitizers (12) and non-sensitizers (5). Sensitizing chemicals were either classified as extreme (6) or strong (6) according to the

LLNA and were covering some chemical diversity including haptens and prohaptens. It is striking to note that even after a very short contact of 15 min, followed by an extensive washing and a post incubation of 6h, a deep impact was observed on the metabolome of RHE. It should also be noted that all chemicals were tested at non-toxic concentrations previously determined under the conditions of SENS-IS and that not all metabolites were affected by skin sensitizers. If the depletion of GSH and/or lysine could be explained by a reaction with electrophilic intermediates, the decrease of some other metabolites is still unexplained at this stage. To reinforce the model, experiments should be repeated and more sensitizers, particularly those classified as moderate and weak or with a known metabolic pathway should be included. Finally, the concentrations of the different metabolites used for building our model were determined by using the area under the corresponding peaks; we will reinforce thus our model by using some more accurate quantification procedures by using commonly accepted methods like deconvolution.

ABBREVIATIONS

ACD, allergic contact dermatitis; AOP, adverse outcome pathway; DPRA, direct peptide reactivity assay; ECVAM, European Center for Validation of Alternative Methods; HRMAS, high-resolution at magic angle spinning; g-HSQC, gradient Heteronuclear Single Quantum Coherence; LLNA, local lymph node assay; RHE, reconstructed human epidermis; PCA,

Principal Component Analysis; PLS-DA: Partial Least Square-Discriminant Analysis; NMR: Nuclear Magnetic Resonance. VIP: Variable Importance in the Projection.

References

1. Tobin DJ. Biochemistry of human skin - our brain on the outside. *Chem Soc Rev.* 2006;35(1):52–67.
2. Bos JD. *Skin immune system: cutaneous immunology and clinical immunodermatology 3rd edition*. Boca Raton: CRC Press; 2005. p 3-12.
3. Luu-The V, Duche D, Ferraris C, Meunier J-R, Leclaire J, Labrie F. Expression profiles of phase 1 and 2 metabolizing enzymes in human skin and the reconstructed skin models Episkin™ and full thickness model from Episkin™. *J Steroid Biochem Mol Biol.* 2009;116(3-5):178-186.
4. Karlberg A-T, Bergström MA, Börje A, Luthman K, Nilsson, JLG. Allergic contact dermatitis-formation, structural requirements and reactivity of skin sensitizers. *Chem Res Toxicol.* 2008;21(1):53–69.
5. Peiser M, Tralau T, Heidler J, et al. Allergic contact dermatitis: epidemiology, molecular mechanisms, in vitro methods and regulatory aspects. Current knowledge assembled at an international workshop at BfR, Germany. *Cell Mol Life Sci.* 2012;69(5):763–781.
6. Thyssen JP, Linneberg A, Menné T, Johansen JD. The epidemiology of contact allergy in the general population–prevalence and main findings. *Contact Dermatitis.* 2007; 57(5):287–299.
7. European Commission. Regulation (EC) No 1907/2006 of the European Parliament and of the Council of 18 December 2006 concerning the Registration, Evaluation, Authorisation

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and Restriction of Chemicals (REACH), establishing a European Chemicals Agency, amending Directive 1999/45/EC and repealing Council Regulation (EEC) No 793/93 and Commission Regulation (EC) No 1488/94 as well as Council Directive 76/769/EEC and Commission Directives 91/155/EEC, 93/67/EEC, 93/105/EC and 2000/21/EC. *Off J Eur Union*. 2006; L396/1 of 30.12.2006.

8. European Commission. Regulation (EC) No 1223/2009 of the European Parliament and the Council of 30 November 2009 on cosmetic products. *Off J Eur Union*. 2009;L342: 59-209.

9. OECD. The adverse outcome pathway for skin sensitisation initiated by covalent binding to proteins. Part 1: scientific evidence. *OECD Environment, Health and Safety Publications Series on Testing and Assessment*. 2012; 168: 1-59.

10. OECD. The adverse outcome pathway for skin sensitization initiated by covalent binding to proteins. Part 2: Use of the AOP to develop chemical categories and integrated assessment and testing approaches. *OECD Environment, Health and Safety Publications Series on Testing and Assessment*. 2012; 169: 1-46.

11. European Commission EURL-ECVAM. Recommendation on the Direct Peptide Reactivity Assay (DPRA) for skin sensitization testing.

http://ihcp.jrc.ec.europa.eu/our_labs/eurl-ecvam/eurlecvam-recommendations/eurl-ecvam-recommendation-on-the-direct-peptide-reactivity-assaydpra. 2013.

- Accepted Article
12. EC EURL-ECVAM. Recommendation on the KeratinoSens™ assay for skin sensitisation testing, 42 pp. http://ihcp.jrc.ec.europa.eu/our_labs/eurl-ecvam/eurl-ecvamrecommendations/recommendation-keratinosens-skin-sensitisation. 2014.
13. EC EURL-ECVAM (2015) Recommendation on the human Cell Line Activation Test (h-CLAT) for skin sensitising testing. https://eurl-ecvam.jrc.ec.europa.eu/news/news_docs/eurl-ecvam-recommendation-on-the-human-cell-line-activation-test-h-clat-for-skin-sensitisation-testing.
14. Rosdy M, Clauss LC. Terminal epidermal differentiation of human keratinocytes grown in chemically defined medium on inert filter substrates at the air-liquid interface. *J Invest Dermatol*. 1990;95(4):409-414.
15. Ponc M. Reconstructed human epidermis in vitro: an alternative to animal testing. *ATLA*. 1995;23:97-110.
16. Netzla F, Lehr C-M, Wertz PW, Schaefer UF. The human epidermis models EpiSkin®, SkinEthic® and EpiDerm®: An evaluation of morphology and their suitability for testing phototoxicity, irritancy, corrosivity, and substance transport. *Eur J Pharm Biopharm*. 2005;60:167-178.
17. Götz C, Pfeiffer R, Tigges J, et al. Xenobiotic metabolism capacities of human skin in comparison with 3D epidermis model and keratinocyte-based cell culture as *in vitro* alternatives for chemical testing: activating enzymes (Phase I). *Exp Dermatol*. 2012;21(5):358-363.

- Accepted Article
18. Eilstein J, Léreaux G, Budimir N, Hussler G, Wilkinson S, Duché D. Comparison of xenobiotic metabolizing enzyme activities in ex vivo human skin and reconstructed human skin models from SkinEthic. *Arch Toxicol*. 2014;88(9): 1681-1694.
 19. Cottrez F, Boitel E, Auriault C, Groux H. Genes specifically modulated in sensitized skins allow the detection of sensitizers in a reconstructed human skin model. Development of the SENS-IS assay. *Toxicol in Vitro*. 2015;29(4): 787-802.
 20. Cottrez F, Boitel E, Ourlin J-C, et al. SENS-IS, a 3D reconstructed epidermis based model for quantifying chemical sensitization potency: reproducibility and predictivity results from an inter-laboratory study. *Toxicol in Vitro*. 2016;32(4): 248-260.
 21. Fiehn O. Metabolomics--the link between genotypes and phenotypes. *Plant Mol Biol*. 2002;48(1-2): 155-71.
 22. Beckonert O, Coen M, Keun HC, et al. High-resolution magic-angle-spinning NMR spectroscopy for metabolic profiling of intact tissues. *Nat Protoc*. 2010;5(6): 1019-32.
 23. Lowe I J. Free induction decays of rotating solids. *Phys Rev Lett*. 1959;2: 285-287.
 24. Andrew E R, Bradbury A, Eades R G. Removal of dipolar broadening of nuclear magnetic resonance spectra of solids by specimen rotation. *Nature*. 1959;183: 1802-1803.
 25. Lippens G, Bourdonneau M, Dhalluin C, et al. Study of compounds attached to solid supports using high resolution magic angle spinning NMR. *Curr Org Chem*. 1999;3: 147-169.

26. Cheng L L, Lean C L, Bogdanova A, et al. Enhanced resolution of proton NMR spectra of malignant lymph nodes using magic-angle spinning. *Magn Reson Med*. 1996;36(5): 653-658.
27. Cheng L L, Ma M J, Becerra L, et al. Quantitative neuropathology by high resolution magic angle spinning proton magnetic resonance spectroscopy. *Proc Natl Acad Sci U.S.A.* 1997;94(12): 6408-13.
28. Bathen T F, Sitter B, Sjøbakk T E, Tessem M B, Gribbestad I S. Magnetic resonance metabolomics of intact tissue: a biotechnological tool in cancer diagnostics and treatment evaluation. *Cancer Res*. 2010;70(17): 6692-6.
29. Piotto M, Moussallieh F-M, Imperiale A, et al. (2010) Reproducible sample preparation and spectrum acquisition techniques for metabolic profiling of human tissues by ^1H HRMAS NMR. *Methodologies in metabolomics: Experimental strategies and techniques for metabolomics research*. P. Lutz (Eds.), Cambridge University Press; 2010.
30. Benahmed MA, Santelmo N, Elbayed, et al. The assessment of the quality of the graft in an animal model for lung transplantation using the metabolomics (^1H) high-resolution magic angle spinning NMR spectroscopy. *Magn Reson Med*. 2012;68:2026-38.

31. Faitot F, Besch C, Battini S, et al. Impact of real-time metabolomics in liver transplantation: Graft evaluation and donor-recipient matching. *J Hepatol*. 2018;68(4): 699-706.
32. Piotto M, Moussallieh F-M, Neuville A, Bellocq J-P, Elbayed K, Namer IJ. Towards real-time metabolic profiling of a biopsy specimen during a surgical operation by ^1H high resolution magic angle spinning nuclear magnetic resonance: a case report. *J Med Case Reports*. 2012;6(1): 22.
33. Elbayed K, Berl V, Debeuckelaere C, et al. HR-MAS NMR spectroscopy of reconstructed human epidermis: potential for the *in situ* investigation of the chemical interactions between skin allergens and nucleophilic amino acids. *Chem Res Toxicol*. 2013;26(1): 136-145.
34. Debeuckelaere C, Berl V, Elbayed K, Moussallieh F-M, Namer I-J, Lepoittevin J-P. Matrix Effect of Human Reconstructed Epidermis on the Chemoselectivity of a Skin Sensitizing α -Methylene- γ -Butyrolactone: Consequences for the Development of in Chemico Alternative Methods. *Chem Res Toxicol*. 2015;28(11): 2192-2198.
35. Debeuckelaere C, Moussallieh F-M, Elbayed K, et al. In situ chemical behaviour of methylisothiazolinone (MI) and methylchloroisothiazolinone (MCI) in reconstructed human epidermis: a new approach to the cross-reactivity issue. *Contact Dermatitis*. 2016;74(3): 159-167.

36. Moss E, Debeuckelaere C, Berl V, et al. In situ Metabolism of Cinnamyl Alcohol in Reconstructed Human Epidermis: New Insights into the Activation of this Fragrance Skin Sensitizer. *Chem Res Toxicol*. 2016;29(7): 1172-1178.
37. Benjamini Y, Hochberg Y. Controlling the false discovery rate: a practical and powerful approach to multiple testing. *Journal of the Royal Statistical Society, Series B*. 1995;57: 289-300.
38. Hunter JD. Matplotlib: a 2D graphics environment. *Comput Sci Eng*. 2007;9: 90–95.
39. Pedregosa F, Varoquaux G, Gramfort A, et al. Scikit-learn: machine learning in python. *J. Machine Learn Res*. 2011;12: 2825–2830.
40. Martínez-Granados B, Monleón D, Martínez-Bisbal MC, et al. Metabolite identification in human liver needle biopsies by high-resolution magic angle spinning ¹H NMR spectroscopy. *NMR Biomed*. 2006;19(1): 90–100.
41. Mazurek S, Zwerschke W, Jansen-Dürr P, Eigenbrodt E. Effects of the human papilloma virus HPV-16 E7 oncoprotein on glycolysis and glutaminolysis: role of pyruvate kinase type M2 and the glycolytic-enzyme complex. *Biochem J*. 2001;356 (Pt 1): 247–256.
42. van Waarde A, Elsinga PH. Proliferation markers for the differential diagnosis of tumor and inflammation. *Curr Pharm Design*. 2008;14(31): 3326–3339.
43. Dutkiewicz EP, Hsieh KT, Wang YS, Chiu HY, Urban PL. Hydrogel micropatch and mass spectrometry-assisted screening for psoriasis related skin metabolites. *Clin Chem*. 2016;62(8): 1120–1128.

44. Sitter B, Johnsson MK, Halgunset J, Bathen TF. Metabolic changes in psoriatic skin under topical corticosteroid treatment. *BMC Dermatol.* 2013;13: 8.

Table 1: Test chemicals used in the study

Chemical name	CAS number	Dose used (%)	Solvent	Sensitizer
Oxazolone	15646-46-5	1	DMSO	Y
Diphenylcyclopropenone	886-38-4	1	DMSO	Y
<i>p</i> -Benzoquinone	106-51-4	10	DMSO	Y
2,4-Dinitrochlorobenzene	97-00-7	1	DMSO	Y
4-Nitrobenzyl bromide	100-11-8	1	DMSO	Y
3-Methylcatechol	488-17-5	10	DMSO	Y
Methyldibromo glutaronitrile	35691-65-7	10	DMSO	Y
<i>o</i> -Aminophenol	95-55-6	10	DMSO	Y
Formaldehyde	50-00-0	10	PBS	Y
Glutaraldehyde	111-30-8	10	PBS	Y
Propyl gallate	121-79-9	10	DMSO	Y
Methylchloroisothiazolinone	26172-55-4	50	DMSO	Y
<i>N</i> -Butyl alcohol (1-butanol)	71-36-3	10	PBS	N
Propylene glycol	57-55-6	50	PBS	N
Sulfanilamide	63-74-1	50	DMSO	N
Vanillin	71-36-3	50	DMSO	N
Glycerol	56-81-5	50	PBS	N

TABLE 2: Average concentrations of the different metabolites in control (untreated RHE) and in RHE after treatment by non-sensitizers or sensitizers.

Metabolite	Control (n=3)	Non Sensitizers (n=5)	Sensitizers (n=12)	<i>P</i> -Value*
Acetate	0	0	0.22±0.20	-
Alanine	14.4±3.6	6.6±1.7 [#]	2.8±1.2 ^{##} ††	.003
Aspartate	5.0±0.8	2.8±1.7	0.3±0.7 ^{##} †(††)	.006
Citrulline	4.0±1.2	2.2±0.6	1.2±0.6 ^{##} †	.005
Free Choline	1.1±0.3	0.6±0.1 [#]	0.3±0.1 ^{##} ††	.001
Phosphocholines	3.7±0.3	2.6±0.5 [#]	1.3±0.6 ^{##} ††	.001
Total Choline	4.8±0.5	3.2±0.5 [#]	1.6±0.7 ^{##} ††	.001
Beta Glucose	4.5±1.2	2.6±0.5	3.3±1.7	.18
Glutamate	14.0±2.1	6.7±1.2 [#]	2.9±1.2 ^{##} ††	.001
Glutamine	26.5±2.2	14.3±3.4 [#]	6.7±3.0 ^{##} ††	.001
Glutathione	1.5±0.3	1.1±0.1 [#]	0.3±0.4 ^{##} ††	.001

TABLE 2 (continued)

Metabolite	Control (n=3)	Non Sensitizers (n=5)	Sensitizers (n=12)	P-Value [†]
Glycine	13.1±3.4	8.1±4.2	2.7±1.2 ^{##} ^{††}	.002
Isoleucine	3.3±0.6	2.3±0.4 [#]	1.1±0.4 ^{##} ^{††}	.002
Lactate	18.0±0.8	10.3±0.9 [#]	8.6±3.9 ^{##}	.009
Lysine	13.5±1.4	6.9±1.4 [#]	3.0±1.2 ^{##} ^{††}	.001
Pyroglutamate	12.7±2.4	7.1±2.2 [#]	3.4±1.9 [†]	.005
Serine	24.7±7.7	12.4±2.7 [#]	6.4±1.7 ^{##} ^{††}	.001
Threonine	9.5±1.3	5.4±1.1 [#]	2.4±1.0 ^{##} ^{††}	.001
Tyrosine	1.7±0.1	1.0±0.2 [#]	0.6±0.2 ^{##} ^{††}	.003
Valine	6.5±2.6	2.4±0.2 [#]	1.2±0.5 ^{##} ^{††}	.001

[†] A non-parametric Mann-Whitney U test has followed a non-parametric Kruskal-Wallis test, both based on average concentrations of the different metabolites quantified.

[#] ($P<.05$), ^{##} ($P<.02$) indicate a significant change with respect to the “control” group; [†] ($P<.05$), ^{††} ($P<.02$) indicate significant change with respect to the “non-sensitizer” group. Between brackets: when adjusted for numbers with identical ranges.

Figure captions

Figure 1

Representative NMR spectra of the control group: 1A - Representative 1D ^1H Carr-Purcell-Meiboom-Gill (CPMG) spectrum of an untreated RHE sample. 1B (up and down) - Representative 2D heteronuclear g-HSQC standard spectrum of an untreated RHE sample. Characteristic metabolites, fatty acids and macromolecules are annotated. Numbers refer to metabolites listed in Supplementary Table 1.

Figure 2

Representative 1D ^1H HRMAS Carr-Purcell-Meiboom-Gill (CPMG) spectra of RHE samples – untreated (**A**), treated with a non-sensitizer (**B**), treated with a sensitizer (**C**). The violet arrow indicates a global decrease of the metabolic profile in NS treated samples compared to blank samples and the red one indicates a more accentuated global decrease in S treated samples compared to the control group. Characteristic metabolites, fatty acids and macromolecules are annotated. Numbers refer to metabolites listed in Supplementary Table 1.

Figure 3

Box-plots of GSH (1A) and Glutamate (1B) concentrations (nmol/mg of RHE) in untreated samples (Blank), treated with non-sensitizers (NS) and with sensitizers (S). The bottom line

of each box represents the first quartile, the intermediate line the median and the top line the third quartile

Figure 4

Partial Least Squares Discriminant Analysis (PLS-DA) Score plots of the three analyzed groups: 4A - PLS-DA Score plot between RHE samples treated by a non-sensitizing chemical (triangles) and RHE samples treated by a sensitizing chemical (circles). 4B - Predictive Model based on the previous one with the two treated groups represented by triangles and circles and untreated control RHE represented by diamonds.

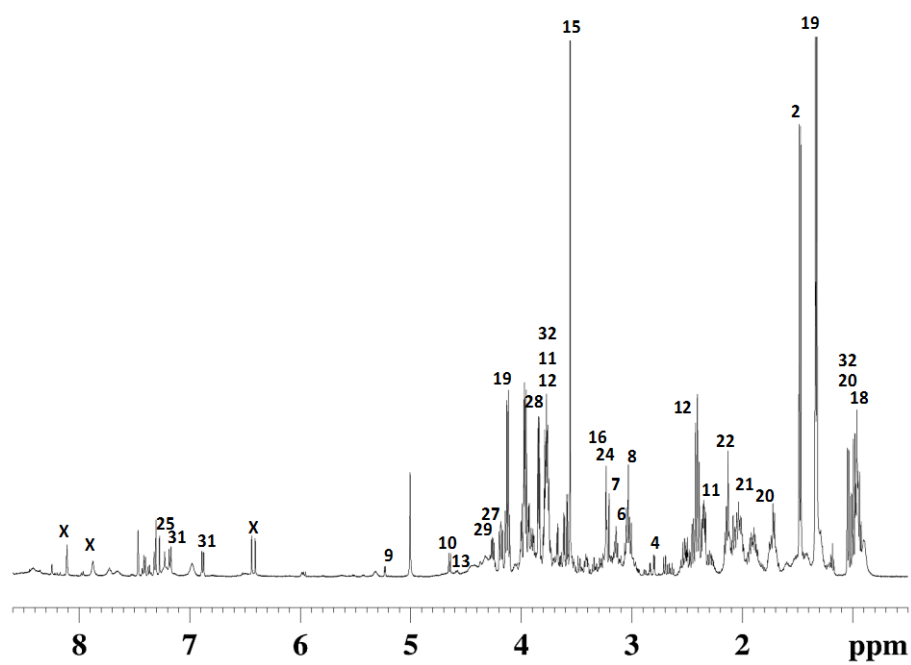
Fig 1A:

Fig 1B:

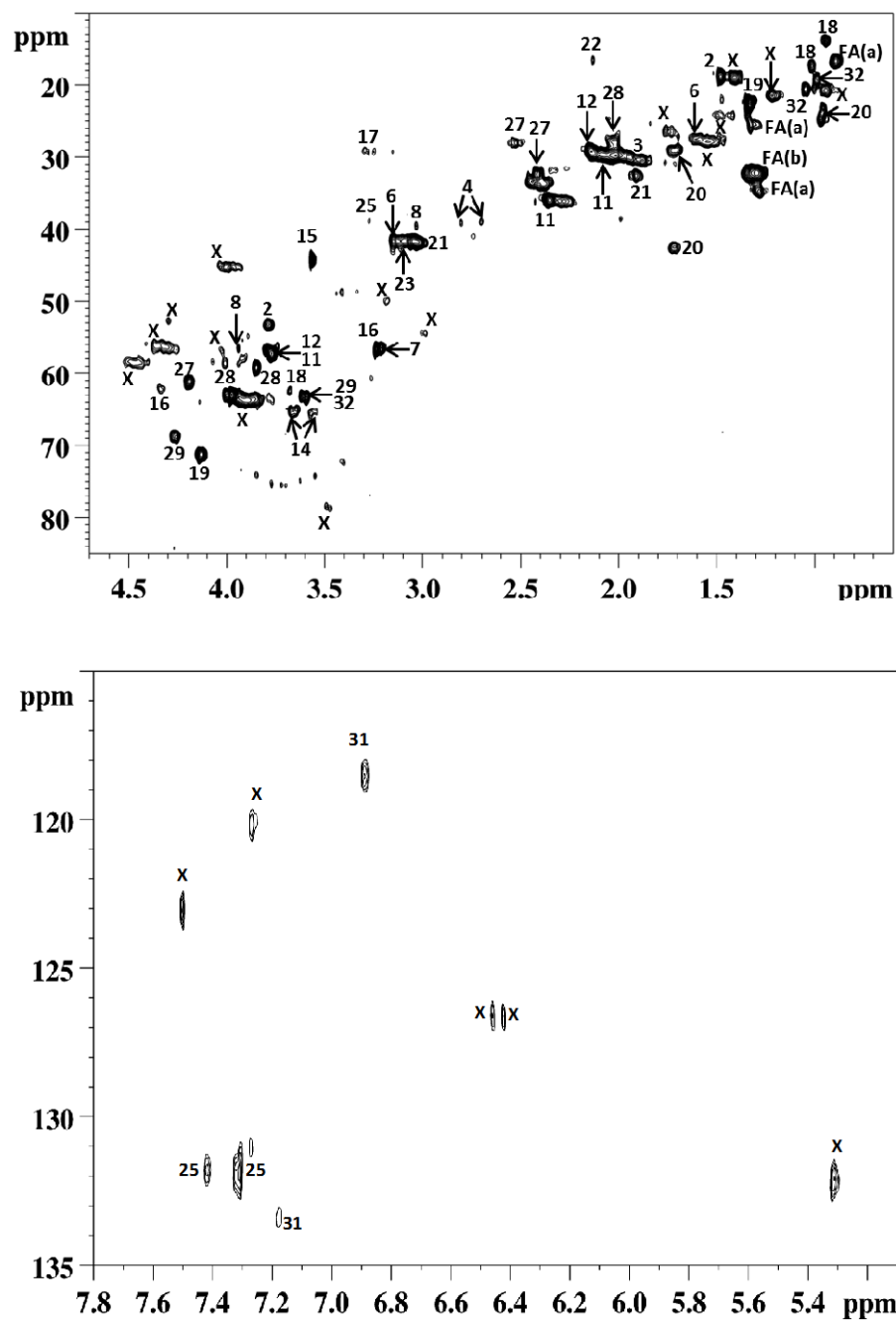


Fig 2:

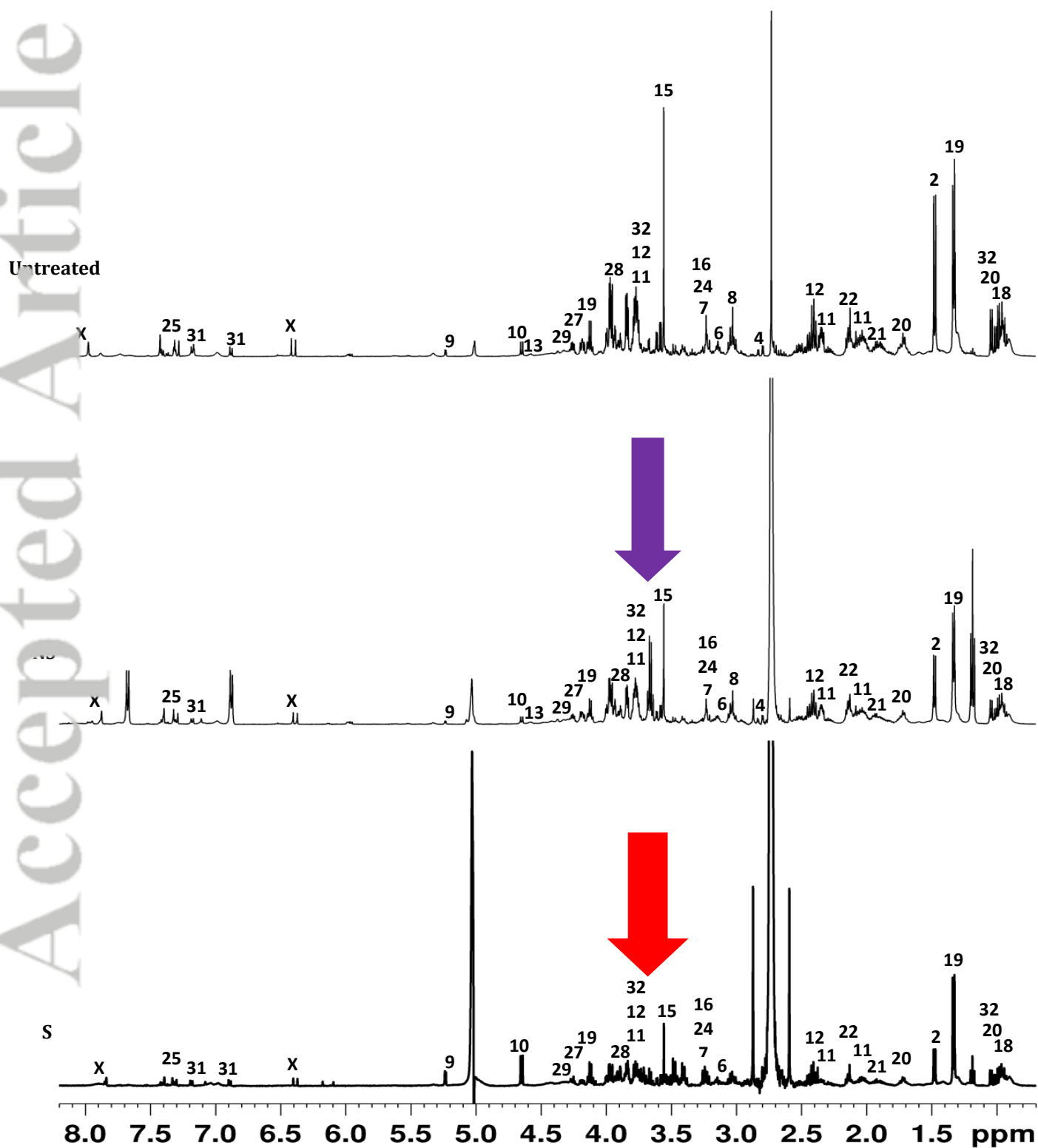
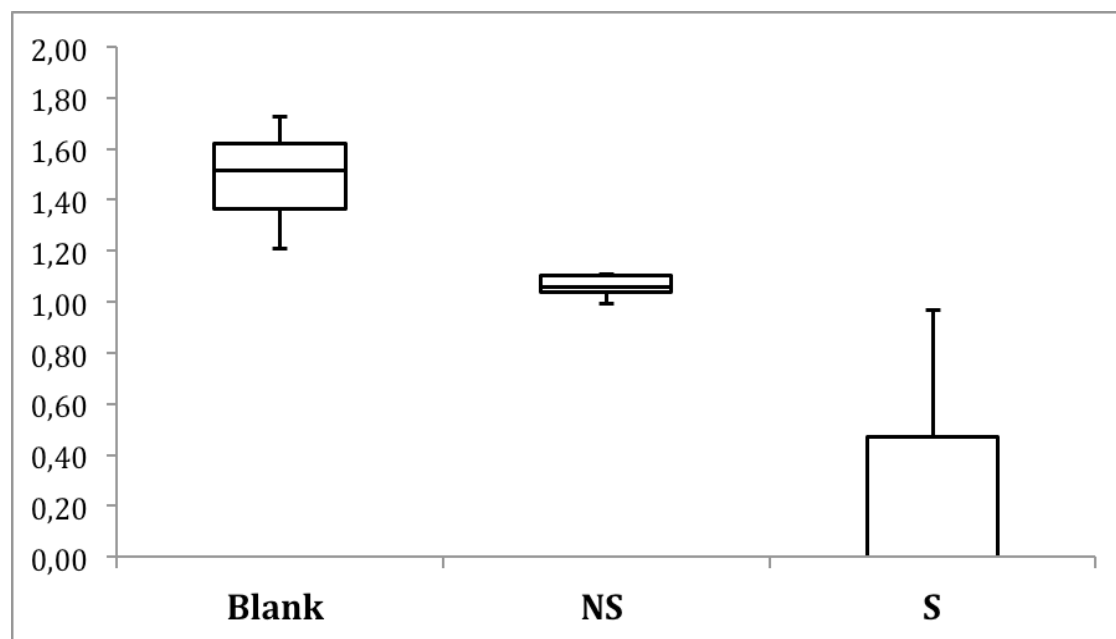


Figure 3



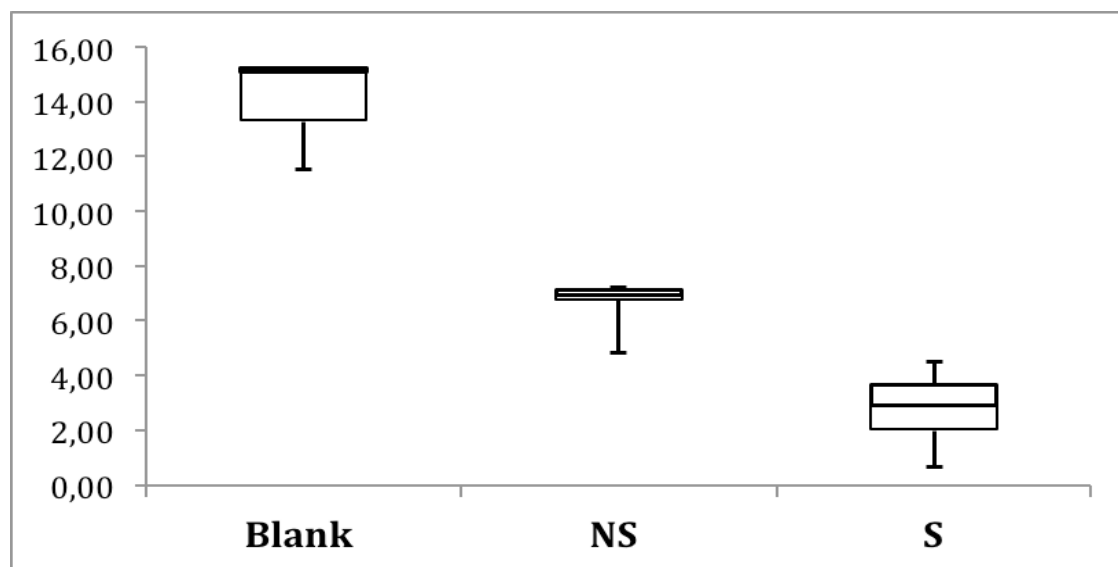
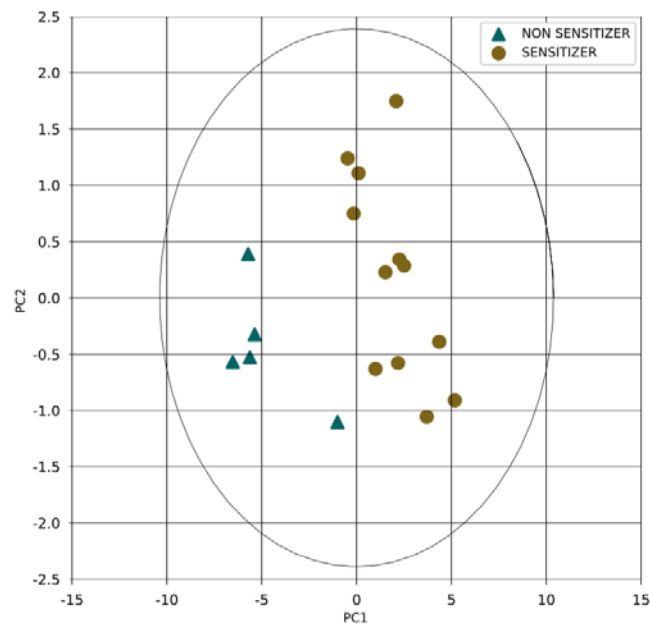
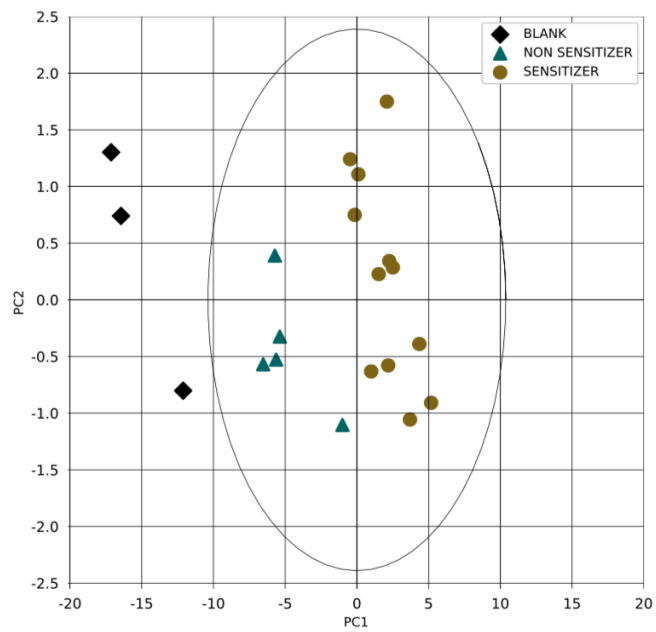
B

Figure 4



B

Supplementary Table 1: ^1H and ^{13}C resonance assignments for metabolites, fatty acids, and macromolecules present in EpiSkinTM RHE samples.

	Metabolite	Group	^1H	^{13}C
1	Acetate	β -CH ₃	1.92	
2	Alanine	β -CH ₃	1.48	18.71
		α -CH	3.78	53.13
3	Arginine	β -CH ₂	1.88	30.43
		α -CH	3.91	57.80
4	Asparagine	β -CH ₂ (u)	2.87	
		β -CH ₂ (d)	2.93	
		α -CH	4.00	
5	Aspartic acid	β -CH ₂ (u)	2.70	38.92
		β -CH ₂ (d)	2.80	39.05
		α -CH	3.89	27.83
6	Citrulline	γ -CH ₂	1.58	27.50
		β -CH ₂	1.88	41.54
		δ -CH ₂	3.14	41.62
		α -CH	3.75	13.87
7	Choline (Cho)	N+(CH ₃) ₃	3.20	56.33
		β -CH ₂	3.52	
		α -CH ₂	4.07	58.16
8	Creatine	CH ₂	3.93	56.38
		CH ₃	3.03	39.41
9	α -Glucose	C ₂ H	3.54	74.00
		C ₃ H	3.69-3.71	75.40
		C ₅ H	3.84	73.98
		C ₆ H or C ₆ H (d)	3.89	63.67
		C ₁ H	5.23	94.83
10	β -Glucose	C ₂ H	3.24	
		C ₄ H	3.40	72.08
		C ₃ H, C ₅ H	3.47	78.6
		C ₃ H, C ₅ H	3.48	78.3
		C ₆ H or C ₆ H (u)	3.77	63.27
		C ₁ H	4.65	98.65
11	Glutamate	β -CH ₂	2.04-2.11	29.60
		γ -CH ₂	2.35	35.91
		α -CH	3.76	57.20
12	Glutamine	β -CH ₂	2.14	29.18
		γ -CH ₂	2.45	33.38
		α -CH	3.77	57.20
13	Glutathione, reduced	β -CH ₂ , Glu	2.17	
		α -CH ₂ , Cys	2.56	
		α -CH ₂ , Gly	2.96	
		α -CH	3.78	
		β -CH ₂	4.57	
		NH, Gly	8.37	
		NH, Gly	8.56	
14	Glycerol	1,3 CH ₂ OH	3.56	65.30
		1,3 CH ₂ OH	3.66	65.30
		CH(OH)	3.76	75.08
15	Glycine	α -CH	3.56	44.08
16	GPCho	N+(CH ₃) ₃	3.23	56.45
		β -CH ₂	3.69	68.38
17	Histidine	CH ₂	3.15-3.29	
		C ₄ H(ring)	7.11	
		C ₂ H(ring)	7.92	

Supplementary Table 1 (cont.)

	Metabolite	Group	¹ H	¹³ C
18	Isoleucine	δ-CH3	0.93	13.87
		γ-CH3	1.01	17.36
		γ-CH2 (u)	1.25	
		γ-CH2 (d)	1.46	27.74
		β-CH	1.98	38.57
		α-CH	3.67	62.29
19	Lactate	CH3	1.33	22.70
		CH	4.12	71.17
20	Leucine	δ-CH3	0.95	23.44
		δ'-CH3	0.96	24.68
		γ-CH	1.70	29.12
		β-CH2	1.71	42.54
		α-CH	3.73	
21	Lysine	γ-CH2	1.47	24.28
		δ-CH2	1.72	
		β-CH2	1.91	32.54
		ε-CH2	3.03	41.63
		α-CH	3.79	
22	Methionine	ε-CH3	2.12	16.51
		γ-CH2	2.63	31.00
		α-CH	3.87	
23	Ornithine	β-CH2	1.94	30.07
		δ-CH2	3.08	41.55
24	PCho	N+(CH3)3	3.22	56.51
25	Phenylalanine	β-CH2 (u)	3.12	
		β-CH2 (d)	3.28	38.80
		α-CH	4.00	58.81
		CH 2,6	7.31	
		CH 4	7.37	
26	Proline	CH 3,5	7.41	
		γ-CH2	2.00	
		β-CH2 (d)	2.34	31.89
		δ-CH2 (u)	3.33	48.37
		δ-CH2 (d)	3.33	48.37
27	Pyroglutamic acid	α-CH	4.13	63.75
		β-CH2 (u)	2.02	29.70
		γ-CH2	2.40	32.31
		β-CH2 (d)	2.51	28.03
		α-CH	4.19	61.14
28	Serine	α-CH	3.84	58.98
		β-CH2	3.97	62.86
29	Threonine	γ-CH3	1.32	
		α-CH	3.59	63.15
		β-CH	4.25	68.65
30	Tryptophan	CH-arom	7.26	130.97
		CH-arom	7.71	
31	Tyrosine	α-CH	3.93	58.59
		CH 3,5	6.89	118.47
		CH 2,6	7.17	133.40
32	Valine	γ-CH3	0.99	19.23
		γ'-CH3	1.04	20.62
		β-CH	2.28	36.14
		α-CH	3.61	62.92

Supplementary Table 1 (cont.)

Metabolite		Group	¹ H	¹³ C
FA	Fatty acids (a)	CH3	0.90	16.65
		CH2	1.28	34.62
FA	Fatty acids (b)	CH2	1.29	32.16
FA	Fatty acids (c)	CH2	1.30	
		CH2	2.01	28.01
			5.32	
FA	Fatty acids (b) (c)	CH2	1.58	27.54
		CH2	2.24	
MM	Macromolecules	CH3	1.40	18.94
		CH	4.29	56.39
		CH	4.47	58.39
EtOH	Ethanol	CH3	1.18	
		CH2	3.65	
X	—	CH?	0.94	20.85
X	—	CH?	1.22	21.54
X	—	CH?	1.41	19.05
FA	Fatty acids (a)	CH3	0.90	16.65
		CH2	1.28	34.62
FA	Fatty acids (b)	CH2	1.29	32.16
FA	Fatty acids (c)	CH2	1.30	
		CH2	2.01	28.01
			5.32	
FA	Fatty acids (b) (c)	CH2	1.58	27.54
		CH2	2.24	
MM	Macromolecules	CH3	1.40	18.94
		CH	4.29	56.39
		CH	4.47	58.39
EtOH	Ethanol*	CH3	1.18	
		CH2	3.65	
X	—	CH?	0.94	20.85
X	—	CH?	1.22	21.54
X	—	CH?	1.41	19.05
X	—	CH?	1.42	24.33
X	—	CH?	1.50	24.37
X	—	CH?	2.98	54.50
X	—	CH?	3.18	49.99
X	—	CH?	3.49	78.45
X	—	CH?	3.89	63.71
X	—	CH?	4.00	58.53
X	—	CH?	4.29	52.76
X	—	CH?	4.32	56.08
X	—	CH?	4.48	58.53
X	—	CH?	5.31	132.05
X	—	CH?	6.42	126.56
X	—	CH?	6.46	126.54
X	—	CH?	7.27	120.11
X	—	CH?	7.50	122.98
X	—	CH?	1.42	24.33
X	—	CH?	1.50	24.37
X	—	CH?	2.98	54.50
X	—	CH?	3.18	49.99
X	—	CH?	3.49	78.45
X	—	CH?	3.89	63.71
X	—	CH?	4.00	58.53
X	—	CH?	4.29	52.76

Supplementary Table 1 (cont.)

Metabolite		Group	¹ H	¹³ C
x	–	CH?	4.29	52.76
x	–	CH?	4.32	56.08
x	–	CH?	4.48	58.53
x	–	CH?	5.31	132.05
x	–	CH?	6.42	126.56
x	–	CH?	6.46	126.54
x	–	CH?	7.27	120.11
x	–	CH?	7.50	122.98

* EtOH is not a metabolite but a technical residue.

(d): down field

(u): up field

arom: aromatic

x: unidentified metabolites or with ambiguous assignments given by available databases.

Supplementary Table 2: Concentration of metabolites in EpiSkin™ RHE samples (untreated or treated) expressed in nmol/mg of RHE

Metabolites	Acetate	Alanine	Aspartate	Citrulline	free Choline	Phosphocholines	total choline
RHE samples							
Untreated	0,00	10,31	4,04	2,77	1,42	3,95	5,37
Untreated	0,00	16,48	5,38	4,12	0,96	3,79	4,75
Untreated	0,00	16,43	5,48	5,16	0,92	3,38	4,30
DMSO	0,00	3,13	0,00	1,30	0,52	1,66	2,17
PBS	0,00	9,04	3,95	3,31	1,00	3,12	4,11
Vanilin	0,00	3,61	0,00	1,44	0,58	2,05	2,63
Sulfanilamide	0,00	7,51	3,97	3,01	0,51	2,17	2,68
1-Butanol	0,00	7,55	2,84	2,58	0,60	2,91	3,51
Propylene glycol	0,00	6,92	2,83	2,09	0,67	3,01	3,67
Glycerol	0,00	7,22	4,13	1,97	0,72	2,83	3,55
Propylgallate	0,38	4,16	0,00	1,79	0,31	1,49	1,80
2-Aminophenol	0,31	3,79	0,00	1,34	0,46	1,66	2,12
Methylchloroisoithiazolinone	0,63	3,68	0,00	2,16	0,21	0,55	0,76
Methyldibromo glutaronitrile	0,00	1,65	0,00	1,53	0,26	0,53	0,79
Formaldehyde	0,43	2,03	1,65	0,24	0,54	2,00	2,55
Glutaraldehyde	0,23	3,76	1,77	1,00	0,48	2,34	2,82
2,4-dinitrochlorobenzene	0,00	2,98	0,00	2,06	0,25	0,68	0,93
<i>p</i> -Benzoquinone	0,28	0,60	0,00	0,44	0,42	0,98	1,41
3-Methylcatechol	0,19	2,74	0,00	1,09	0,27	1,19	1,45
4-Nitrobenzyl bromide	0,00	3,76	0,00	1,10	0,20	1,30	1,50
Oxazolone	0,00	3,02	0,00	1,02	0,50	1,75	2,25
Diphenylcyclopropenone	0,15	1,32	0,00	1,05	0,19	0,57	0,76

Supplementary Table 2 (cont.)

Metabolites	-Glucose	Glutamate	Glutamine	Glutathione	Glycine	Isoleucine	Lactate	Lysine
RHE samples								
Untreated	5,31	11,53	23,94	1,73	9,23	3,93	17,82	11,95
Untreated	5,07	15,34	27,75	1,51	14,14	3,11	17,24	14,72
Untreated	3,05	15,08	27,65	1,21	15,84	2,78	18,84	13,92
DMSO	2,58	4,13	8,02	0,66	3,26	1,17	10,51	4,05
PBS	4,58	7,92	18,58	0,56	9,03	3,00	9,70	8,90
Vanilin	2,82	4,81	8,47	1,06	3,58	1,73	10,84	4,56
Sulfanilamide	2,50	6,92	16,23	1,04	8,00	2,53	10,71	7,07
1-Butanol	2,45	7,12	14,37	1,11	7,18	2,70	9,36	7,84
Propylene glycol	2,10	6,77	15,27	0,99	6,98	2,21	11,19	8,03
Glycerol	3,33	7,97	17,20	1,10	14,96	2,37	9,44	7,22
Propylgallate	2,52	4,20	10,09	0,77	4,65	1,75	10,41	4,72
2-Aminophenol	1,66	4,70	7,85	0,80	3,40	1,30	13,79	4,07
Methylchloroisothiazolinone	2,39	3,07	6,98	0,00	4,13	1,36	4,21	3,51
Methyldibromo glutaronitrile	4,11	1,70	5,01	0,00	1,71	0,75	4,74	2,63
Formaldehyde	6,62	1,96	5,84	0,97	1,56	1,00	17,45	1,18
Glutaraldehyde	4,73	4,17	13,70	0,29	2,61	1,57	9,95	3,14
2,4-dinitrochlorobenzene	2,95	2,96	5,23	0,00	2,86	1,18	6,48	3,29
<i>p</i>-Benzoquinone	5,46	0,68	3,11	0,00	0,48	0,35	5,69	0,93
3-Methylcatechol	0,91	2,67	5,19	0,37	2,79	0,96	8,26	2,40
4-Nitrobenzyl bromide	2,05	2,85	6,88	0,00	3,33	1,39	7,70	4,26
Oxazolone	3,35	3,51	7,26	0,00	2,79	1,43	9,22	3,97
Diphenylcyclopropenone	2,63	2,04	3,15	0,00	1,56	0,66	5,49	1,91

Supplementary Table 2 (cont.)

Metabolites	Pyroglutamate	Serine	Threonine	Tyrosine	Valine
RHE samples					
Untreated	10,12	15,96	8,05	1,59	3,62
Untreated	13,25	27,66	9,96	1,83	7,28
Untreated	14,72	30,60	10,45	1,75	8,60
DMSO	2,70	6,96	3,08	0,64	1,30
PBS	8,96	18,20	6,63	1,27	2,92
Vanilin	3,45	7,77	3,63	0,72	2,07
Sulfanilamide	9,15	14,45	6,11	1,25	2,15
1-Butanol	7,15	12,97	5,75	1,00	2,49
Propylene glycol	7,22	12,55	5,33	1,03	2,61
Glycerol	8,65	14,24	6,18	0,94	2,50
Propylgallate	5,11	8,48	3,95	0,84	2,19
2-Aminophenol	4,48	5,99	3,39	1,01	1,25
Methylchloroisothiazolinone	4,12	9,55	2,76	0,67	1,31
Methyldibromo glutaronitrile	2,00	4,54	2,39	0,44	0,76
Formaldehyde	2,69	6,75	1,07	0,43	0,77
Glutaraldehyde	8,12	7,71	1,84	0,47	1,63
2,4-dinitrochlorobenzene	2,65	6,30	3,24	0,58	1,21
<i>p</i>-Benzoquinone	0,84	4,35	0,74	0,21	0,47
3-Methylcatechol	3,33	5,18	1,92	0,51	1,28
4-Nitrobenzyl bromide	3,16	7,34	2,60	0,84	1,28
Oxazolone	2,84	6,75	2,73	0,65	1,55
Diphenylcyclopropenone	1,42	3,93	1,62	0,36	0,99