Application of metabolomics for cosmetics

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Abstract

If genomic is well known now, metabolomic is a more recent science that focuses on giving the broadest characterisation of the metabolome of one cell, one organ or a whole organism. The goal is to give a comprehensive snapshot of physiological state and to identify differences between chemical profiles.

The human skin is an organ with a surface area reaching $2m^2$ that provides interface with our environment. It is composed of numerous molecules coming from host cells, microbiota and external molecules, applied deliberately or not. Its surface can be sampled to detect and quantify metabolites, using metabolomics approaches. It has been done in pathological context but until today, nobody has described metabolomics to assess cosmetics and/or active ingredient impact on skin metabolome. We demonstrated the interest and the great potential of metabolomics for our studies using HPLC hyphenated to high-resolution mass spectrometry (Orbitrap).

Panellists applied the formulated active on one cheek and the corresponding placebo formula on the other one, twice a day, during 28 days. Results of both were compared to the initial panellist's skin profiles. With this method, we have found nearly 300 statistically significant features (in positive and in negative modes) that are differentially expressed on the skin. They represent signature of formulas and active effects.

We described metabolomics study for the first time in a cosmetic context. It already seems to appear as a real powerful tool to go on in biological efficacy explanation and discovery.

Key words: Omics, Microbiota, HPLC, Mass spectrometry

Introduction

Omic sciences bring together several disciplines of biology that the name ends by omics such as genomics, transcriptomics, proteomics, metabolomics… The central dogma of biology

shows the flow of information from DNA to the phenotype. Associated with each stage is the corresponding systems biology tool, from genomics for DNA, transcriptomic for RNA, proteomic for proteins to metabolomics for biochemical. This is probably the last stage before the phenotype expression description. If genomic is well known now, metabolomic is a more recent science that focuses on giving the broadest characterisation of the metabolome of one cell, one organ or a whole organism. The metabolome represents the complete set of metabolites which are the end products of cellular processes. Conversely, metabolic profiling can give an instantaneous snapshot of the physiology of that cell, and thus, metabolomics provides a direct "functional readout of the physiological state" of an organism. Indeed, there are quantifiable correlations between the metabolome and the other cellular ensembles (genome, transcriptome, proteome and lipidome). The metabolome is composed of a large number of small metabolites (e.g from primary metabolites such as sugar, amino acids, nucleic acids, fatty acids, lipids…to secondary metabolites such as polyphenols, alkaloids, drugs, toxins, xenobiotics…). Metabolomics studies are currently done using combinations of liquid chromatography, mass spectrometry and/or nuclear magnetic resonance. One of their goals is to give a comprehensive signature of the physiological state of an event (treatment with active for example) and identify differences between the observed chemical profiles. For each study, it is mandatory to take into account that a metabolite profile results from many factors like the genetic background or the influence of environmental factors.

The human skin is an organ with a surface area reaching $2m^2$ that provides interface with our environment. It is composed of numerous molecules coming from host cells, microbiota and external molecules, applied deliberately or not. Functioning of skin structures (cells, sweat glands, sebaceous glands, interstitial fluids…) and of its microbiota influence the quantity and the heterogeneity of skin metabolites. But, lifestyle (diet, medicines, smoke…), environment (composition of the air from inside or outside, smoke, composition of clothes…) and everything touching our skin may interfere with its metabolites composition. It is the case for cosmetics.

The skin surface can be sampled to detect and quantify metabolites, using metabolomics approaches. It has been done in pathological context, to identify biomarkers signing diseases (fibrosis, psoriasis, Parkinson's disease or cancer) [1]. Until today, nobody has described metabolomics to assess cosmetics or active ingredient impact on skin metabolome. We interested in the great potential of metabolomics for our studies.

Diverse methodologies for specimen collection and identification using metabolomics are available. For that study, our aim was to collect skin metabolites following a non-invasive method [1]. In that goal, we avoided biopsy, suction blistering or other too invasive

methodologies, as we wanted to access to long treatment effect (few weeks). We have perfected the sampling using swabs, after selection of materials, their preparation, time of contact, etc. Concerning metabolomics analysis, the chosen technology was HPLC hyphenated to high-resolution mass spectrometry (Orbitrap). With that technology, each skin sample chemical profile is described by a list of features. Features are variable with at least three properties: intensity, retention time and mass to charge ratio. The intensities variations of each features were statistically analysed using principal component analysis and T-tests. In theory, every feature could be linked to a metabolite by using the chemical information present in the data. Despite the use of high-resolution mass-spectrometry that identification step is not trivial, we have added to our experiment one fragmentation step using Orbitrap system capabilities. With this additional step, we could access (for some features) to MS/MS spectra that can be compared to internal or external databases, gives structural information, and helps in the annotation step. We used those technics to screen the best way to sample the skin in a first step and secondly to characterise effects of active ingredients of interest on skin chemical profile.

Materials and Methods

- Volunteers & treatment

The area of interest was the cheek of several volunteers. Depending on the study, panellists applied the formulated active on one cheek (around 9 cm^2) and the corresponding placebo formula on the other one, twice a day, during 28 days.
The sampling was always realised by the same technician using swabs. After skin contact on a 2 cm^2 surface, the swabs were stored in 0.2 mL Eppendorf and kept frozen (-80°C).

- Chemicals and reagents

Acetonitrile LC/HRMS grade CHROMASOLV™ LC-MS (Riedel-de Haën), Ethanol LC/MS grade (Riedel-de Haën), Water LC/HRMS grade CHROMANORM® (VWR Chemicals).

- Sample preparation [2]

Frozen swab was removed from the 0.2 mL Eppendorf tube and put in a 2mL Eppendorf tube. To rinse the 0.2 mL tube, 200 µL of a Water/Ethanol mix (50/50 v/v) were added and mixed by vortex vibration for 20 seconds. With a Pasteur pipette, those 200 µL were retrieved and put in the 2 mL tube and 300 µL of the Water/Ethanol mix (50/50 v/v) were added. After 20 seconds of vortex vibration, samples were put on an agitator at 600 rpm for 30 minutes, at 4°C. Then,

swab was wrung out by pressing the swab on the tube's wall with a clamp and put back in the 0.2 mL tube for backup. Each 2mL tube was centrifuged at 10,000 rpm for 5 min at 4°C to eliminate the remaining residuals from the swab. 400 µL of the supernatant were retrieved and put in a new tube. Before injection, 60 µL were put in HPLC vial and were dried with a nitrogen evaporator at 25°C then dissolved in a Water/Acetonitrile (95/5 v/v) mix. A pooled sample (QC) was also prepared by mixing 10 µL of every sample. This pooled sample was used as quality control for column equilibration and as an analytical performance indicator of the ultra-performance liquid chromatography coupled with high-resolution mass spectrometer analysis (UHPLC-HRMS).

- UHPLC-MS/MS analysis

Samples were analysed using a Dionex Ultimate TM 3000 ultra-high performance liquid chromatography (UHPLC) system (Dionex Softron®, Germering, Germany) system coupled to a Q-Exactive Plus mass spectrometer (Thermo Scientific®, Bremen, Germany) equipped with a Heated Electro Spray Ionization (HESI) source operating in switching positive and negative electrospray ionization modes (ESI+ and ESI- respectively). The acquisition of the raw data was performed using a full scan mode within the m/z 65-1000 range at a resolving power of 70,000 at m/z 200. Da. Compound separation was performed using a Hypersil GOLD-C18 column (1.9 µm, 100 mm x 2.1 mm) from Thermo-Scientific (USA). The column temperature was set at 35°C. The mobile phases were composed of 0.1% of acetic acid in water (solvent A) and in acetonitrile (solvent B). The applied gradient (A:B, v/v) was as follows: 95:5 from 0 to 2.4 min, 75:25 at 4.5 min, 25:75 at 11 min, 0:100 from 14 at 16.5 min and 95:5 from 19 to 25 min. The flow rate was set to 0.40 mL/min. The injection volume was 5 µL. All samples were analysed in one batch without any stopping or recalibration step. The pool sample (QC) was injected regularly throughout the run after every ten samples approximately.

Data acquisition was settled with an automatic gain control of 5.105 and a C-Trap inject time of 20 ms. The acquisition spectrometric parameters were as follows: the spray voltage (+3 kV), the S-Lens RF level (50), the tube lens voltage (+100 V), the capillary temperature (350°C), the heater temperature (300°C), the sheath gas pressure (55 arbitrary units), the auxiliary gas flow rate (10 arbitrary units) and the sweep gas flow rate (0 arbitrary units). Full instrument calibration was performed using a MSCAL5 ProteoMassT LTQ/FT-Hybrid ESI Pos/Neg. Xcalibur V2.2 (Thermo Scientific®, Bremen, Germany) software was used for the generation of all chromatographic peaks acquired in full scan mode.

- Statistical analysis and annotation

Preprocessing of the data (automatic peak detection, integration, peak filtration, peak identification, peak grouping and smoothing, retention time correction, integration, annotation), quality control (metabolites correlation analysis) and statistical analysis (univariate testing and multivariate modeling) were conducted on the online and freely available Workflow4Metabolomics (W4M) platform [3] (https://workflow4metabololomics.org). Principal component analysis (PCA) was used for multivariate exploration of clusters and trends among the observations. Orthogonal supervised partial least-squares-discriminant analysis (OPLS-DA) were also built. For interest features identified, annotation was performed at "level 2". For level 2 annotations [4], data dependent MS/MS acquisitions (DDA) were run on samples. All the generated MS/MS were processed with msPurity module included in W4M [5] and compared to a MS/MS spectral database containing data from MoNA including Massbank, HMDB, LipidBlast and GNPS that can be downloaded at https://github.com/computational-metabolomics/msp2db/releases.

Results & Discussion

- Using of Swabs

Skin metabolome extraction tests were done on different types of swabs: cotton, foam and polyester swabs (figure 1). The selection for sampling was made regarding two parameters: the number of dissolved molecules from blank swabs and from swab after skin contact. Foam swabs do not desorb much molecules proper to its composition but do not desorb much molecules after skin contact, whereas polyester swabs desorb a lot of molecules proper to its composition and not much more after skin contact. Cotton swabs were the most suitable ones for metabolomics analysis: they desorb a few molecules proper to its composition, especially in negative mode, but they desorb much more molecules after sampling which allows to analyse specific metabolites collected from the skin.

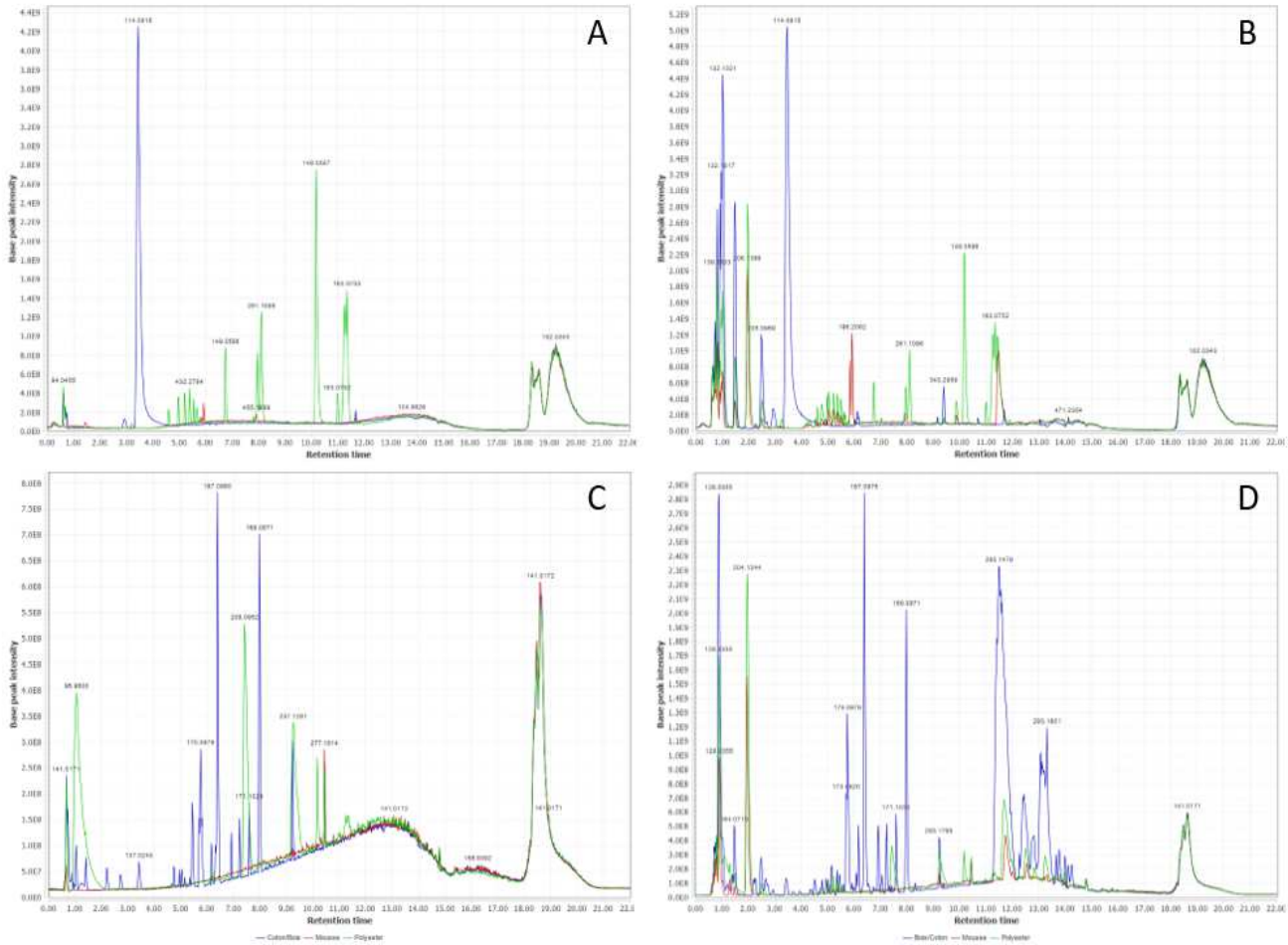

Figure 1: Chromatograms obtained from samples. In green: polyester swab, in red: foam swab, in blue: cotton swab. A- Samples from blank swabs in positive mode; B- Samples from swabs after skin contact in positive mode; C- Samples from blank swabs in negative mode; D- Sample from swabs after skin contact in negative mode.

In order to clean the cotton swabs chemical background, a cleaning step was added for the following experiments. The swabs were put into 3 successive ethanol/water (50/50 v/v) mix for 24h before sampling. This method allows to reduce desorbed impurities from blank cotton swabs as shown in the illustration below (figure2), and to have the as clear as possible swabs to analyse metabolites extracted from the skin only.

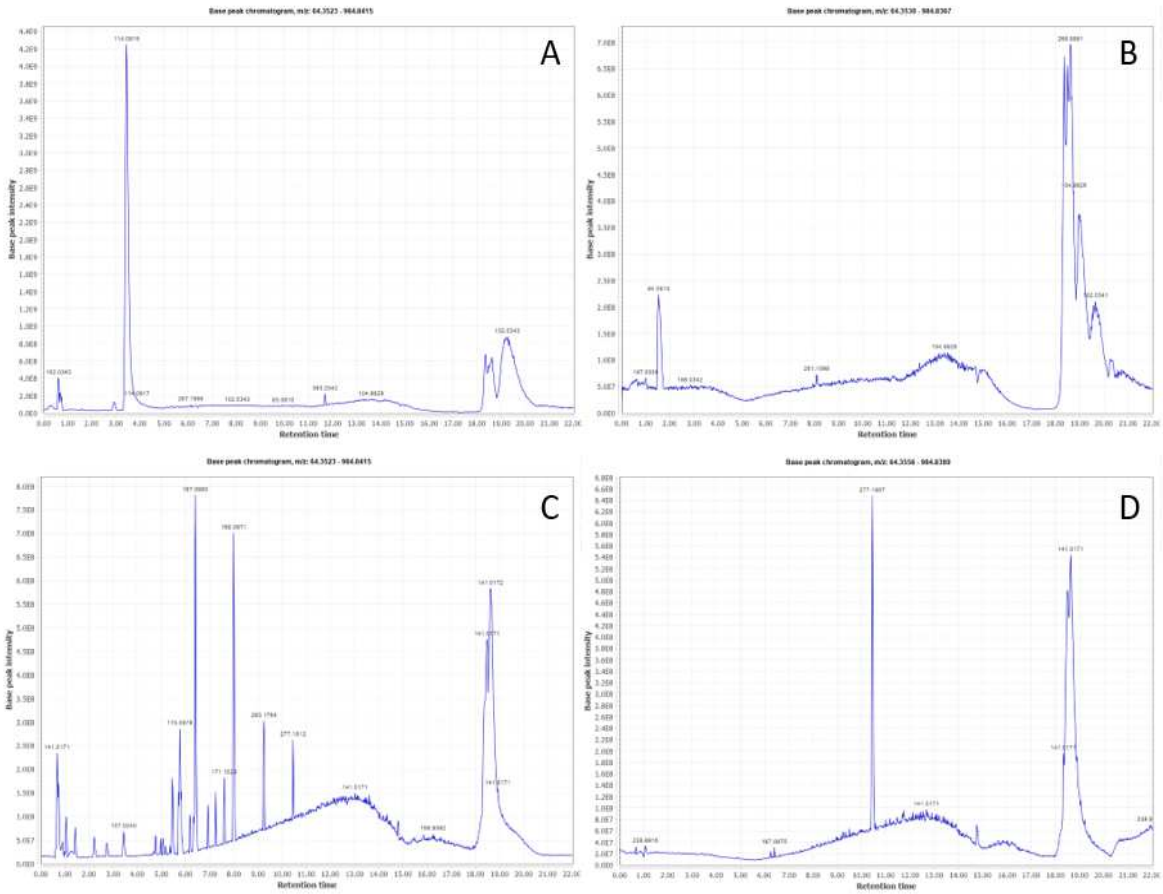

Figure 2: Total ion chromatograms obtained from blank cotton swab.
A- Before cleaning in positive mode; B- After cleaning in positive mode;
C- Before cleaning in negative mode; D- After cleaning in negative mode.

- Statistical analysis and annotation

In order to search for discriminant features, a statistical approach (OPLS-DA : Orthogonal Partial Least Square Discriminant Analysis) was made to attempt to distinguished groups between treated cheeks with an active ingredient treatment and non-treated cheeks (placebo treatment) after 28 days treatment. In positive mode, this analysis shows two distinguished groups with a p-value (pQ2) on the permutation test equal to 0.05 which validate this model. All the statistically significant features differentially expressed between the different conditions, 823 features in this case, are studied.

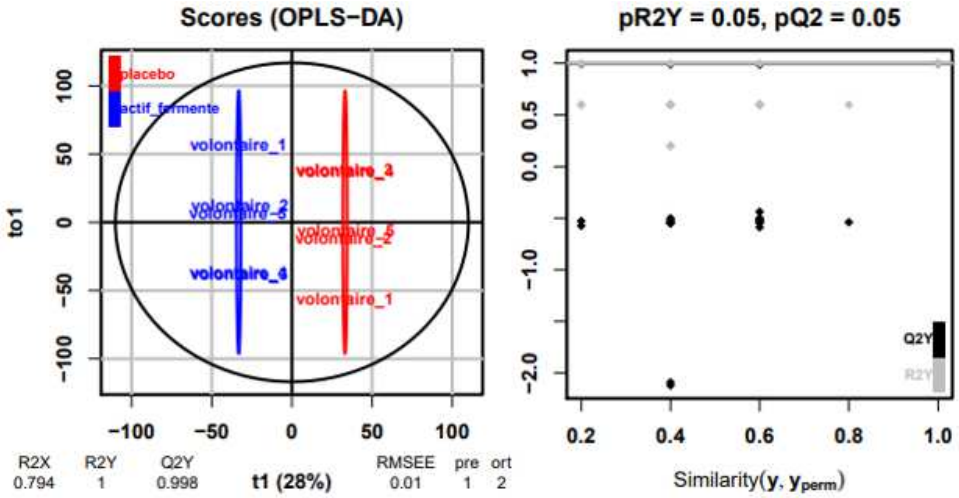

Figure 3: OPLS-DA using 823 positive mode features with a VIP (variable importance in projection) superior to 1 after a multilevel correction. On the left, score plot. On the right, permutation test. In blue, treated cheeks (active ingredient) after 28 days treatment. In red, non-treated cheeks (placebo treatment) after 28 days.

Thanks to the DDA method used on samples, MS/MS spectra are provided for the annotation step. 218 over the 823 statistically significant features have an MS/MS spectrum and only 104 have a putative annotation related after databases comparison. Those ones have an annotation level 2 based on Schymanski's rules [4], extracted in figure 4, that can be interesting for understanding active effects on skin.

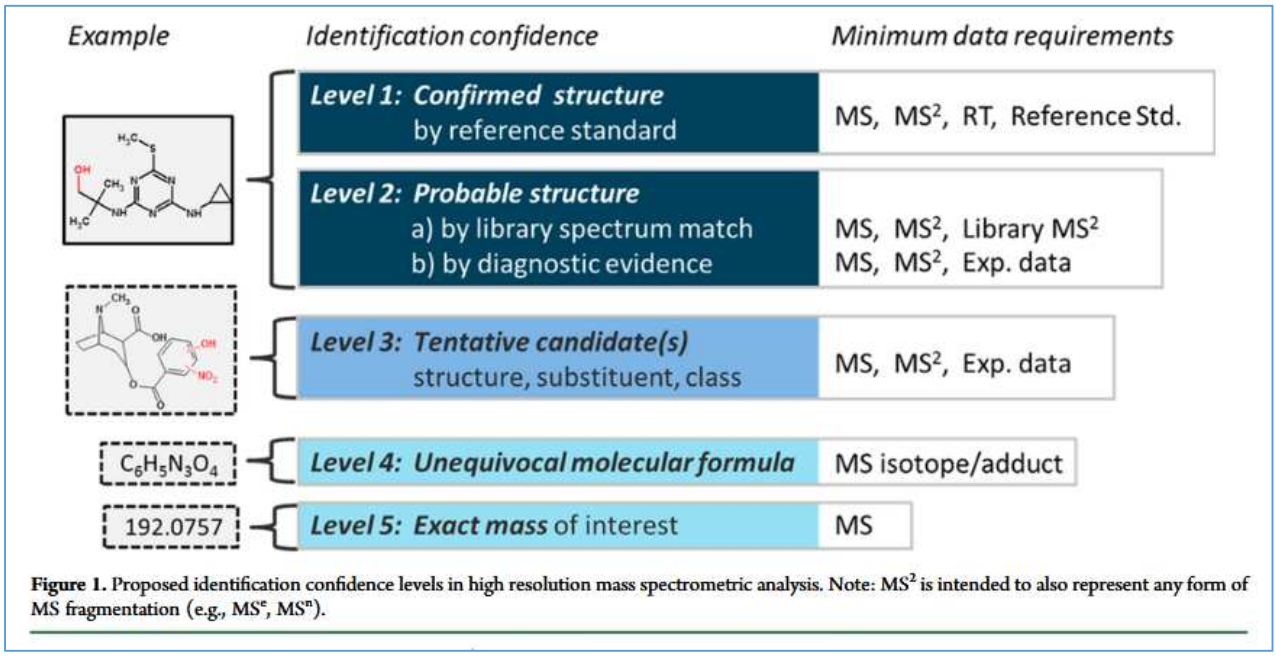

Figure 4: figure 1 of the fourth reference, Schymanski's rules [4].

Such untargeted metabolomics study generates a huge amount of data and in order to make easier data interpretation, molecular networks are one of the useful tools for data visualization and interpretation. Molecular networks for this project are based on all the MS/MS spectra provided by the DDA method. The illustration below shows the link between annotated molecule (sisomicin) with 13 others features. In that cluster, every node is a feature sharing some structural similarities with the others and the distance is proportional to the degree of similarity between features MS/MS spectra.

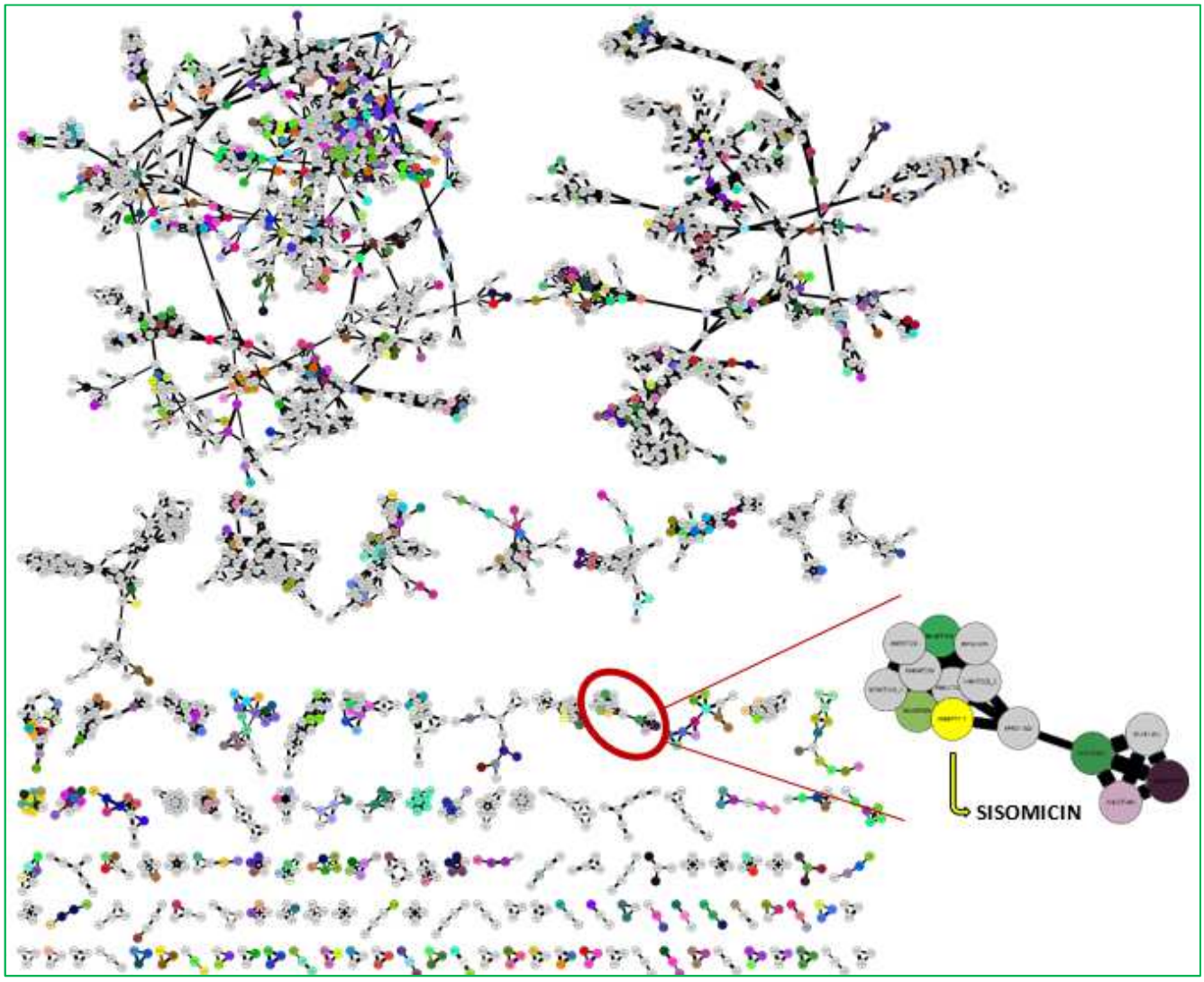

Figure 5: Molecular network built with MetGem [6] on the MS/MS spectra in positive mode. Each cluster represents ions with MS/MS spectra with similarity level above 0.7 (cosine score) and with at least 3 common fragmentation ions. The colored nodes represent annotated ions with Galaxy Spectral Matching tool. In grey, ions without putative annotation.

Conclusion

With this method, we have found numerous statistically significant features (in positive and in negative modes) that are differentially expressed on the skin. Some of these signals, can be linked to metabolites produced by the skin, the microbiota or both. They represent signature of formulas and active effects. All opened few ways of action and several mechanisms of interest, concerning host skin cells but also concerning skin microbiota.
Metabolomics study is described for the first time in a cosmetic context. It already seems to appear as a real powerful tool to go on in biological efficacy explanation and discovery.

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Conflict of interest: none

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