Ion mobility spectrometry and mass spectrometry coupled to gas chromatography for analysis of microbial contaminated cosmetic creams

María García-Nicolás[a], Natalia Arroyo-Manzanares[a*], Juan de Dios Hernández[b], Isidro Guillén[b], Pascuala Vizcaíno[b], Marta Sánchez-Rubio[b], Ignacio López-García[a], Manuel Hernández-Córdoba[a], Pilar Viñas[a*]

[a]*Department of Analytical Chemistry, Faculty of Chemistry, Regional Campus of International Excellence "Campus Mare-Nostrum", University of Murcia, Murcia, Spain*

[b]*Productos Sur S.A. (Prosur) Av. Francisco Salzillo, P/27-2, San Ginés, 30169 San Ginés, Murcia, Spain*

Corresponding author:

Prof. Pilar Viñas / Prof. Natalia Arroyo-Manzanares

Department of Analytical Chemistry

Faculty of Chemistry

University of Murcia

E-30100 Murcia

SPAIN

Tel.: +34 868887415

FAX: +34 868887682

e-mail: pilarvi@um.es

Abstract

The most commonly used technique for monitoring microbial contamination in cosmetic products is plate counting. In this contribution, headspace - gas chromatography (HS-GC) coupled to mass spectrometry (MS) or ion mobility spectrometry (IMS) is proposed as a technique to evaluate rapidly and accurately the state of microbial colonies in cosmetic creams using the volatile organic compounds produced by microorganisms (MVOC). The work focuses on monitoring two of the microorganisms that most frequently occur in such creams, *Candida albicans* and *Staphylococcus aureus*. In addition, two different types of ingredient with antimicrobial properties (a chemical preservative and a natural preservative) were added to study the behaviour of these microorganisms under different conditions. The facial creams were elaborated and inoculated with the two above microorganisms, and then sampled weekly for 4 weeks, analysing the evolution of the MVOCs by HS-GC-MS and HS-GC-IMS. In addition, microbial contamination was determined by the classical plate counting method. The pH, colour, viscosity and water activity parameters were also measured. The use of chemometric tools is essential because of the large amount of data generated, and different models based on discriminant analysis with an orthogonal projection on latent structures (OPLS-DA) were constructed. The optimal models obtained by both analytical techniques allowed differentiation between contaminated and non-contaminated creams, with a validation success rate of 94.4%. In addition, MVOC monitoring also allowed assessment of the microbial concentration.

Keywords: ion mobility spectrometry; mass spectrometry; gas chromatography; cosmetic creams; microbial contamination.

1. Introduction

The microbial contamination of cosmetic products has been extensively studied [1, 2]. Cosmetic products can be contaminated with filamentous fungi, yeasts and bacteria from many different sources, including the natural raw materials, equipment, water, operators or even air. Research has shown that the most frequently microorganisms found in cosmetics are *Pseudomonas aeruginosa*, *Klebsiella oxytoca*, *Burkholderia cepacia*, *Staphylococcus aureus*, *Escherichia coli*, *Candida albicans*, *Enterobacter gergoviae* and *Serratia marcescens*, although other bacteria, fungi and yeasts may also be found [3]. In this work, two of these microorganisms, *S. aureus* and *C. albicans*, were investigated. *S. aureus* is an opportunistic human pathogen and it is one of the principal causes of skin infections, many of which involve episodes of cellulite and post-operative infections. In addition, skin lesions, such as atopic dermatitis and eczema, are often caused by colonization by high densities of this genus [4,5]. The presence of *S. aureus* in the cosmetic product or its feedstock indicates that contamination may occur as a result of human action, as it can be carried by dust, skin, clothing and water micro-droplets generated by moving, talking and sneezing [6]. In turn, *C. albicans* can produce secondary allergies and mycosis, behaving as a pathogen. Since it is a normal host of the human intestinal flora, it produces superficial or systemic candidiasis in weakened, new-born children and elderly people with a deficient immune system. Because of its propensity to grow in humid environments, *C. albicans* is commonly found in cosmetic creams [3,7].

The monitoring of microbial contamination in cosmetic creams is of great importance to assess the useful life of these products. The most commonly used technique to detect the presence of microbial contamination of both *S. aureus* and *C. albicans* is the plate counting of colony forming units (CFU). The main disadvantage of this methodology is the length of time needed to obtain results, and, consequently, the increase in the cost of analysis. It is for this reason that recent studies have focused on developing alternative methodologies to classical microbiological counting in order to save materials and time, while providing sufficient viability for the detection of microorganisms in cosmetics.

One of the alternative methods used for microbial determination in cosmetics is direct amplification of the DNA of the microorganism by polymerase chain reaction (PCR), enabling the identification of bacteria such as *S. aureus* in the amplified DNA [8]. In addition, for the detection of *S. aureus* and *C. albicans* in cosmetic products, rapid microbiological methods such as impedance, direct epifluorescent filter techniques

(DEFT) and bioluminescence ATP have been used [9, 10]. Matrix-assisted laser desorption ionization-time-of-flight (MALDI-TOF) MS has also been proposed for identification of microorganism isolated in cosmetic samples [11]. This technique allows the identification of microorganisms through an analysis of proteins, associating a specific mass spectrum to a given species. However, it presents drawbacks when used in direct samples due to its low sensitivity, and the need for a considerable amount of protein in order to obtain reliable profiles.

In recent years, the determination of microbial volatile organic compounds (MVOC) has proved to be very useful to assess the status of microbial communities quickly and reliably, as they represent different MVOC profiles depending on the environment in which they are found [12]. The difficulty of these experiments lies in the large number of metabolites produced, as well as the diversity of the chemical and physical properties of these compounds, which makes the simultaneous quantification of all the metabolites very complicated with current instrumental methods [13].

Volatile compounds in cosmetic creams have been determined using gas chromatography (GC) coupled to a flame ionization detector (FID) or mass spectrometry (MS) [14]. The coupling of GC-MS is the most widely used technique and it has been applied for the determination of suspicious volatile allergens [15], by placing the sample on a polydimethylsiloxane (PDMS) cylinder and using direct contact sorptive tape extraction (DC-STE-GC-MS) [16] or with a combination of full evaporation dynamic headspace (FEDHS-GC-MS) [17]. It has also been used to identify and quantify nitrosamines following solid-phase microextraction in the headspace (HS-SPME-GC-MS) [18] and for the determination of volatile methylsiloxane compounds with QuEChERS ("Quick, Easy, Cheap, Effective, Rugged, and Safe") sample treatment [19]. However, to date, the volatile profile has not been linked to microbial contamination of cosmetic products.

The principle of ion mobility spectrometry (IMS) separation is based on the different mobility of the gas phase ions inside a drift tube under the effect of a constant electric field at atmospheric pressure [20]. IMS is a rapid and very sensitive analytical technique, which is also characterized by minimal requirements for sample treatment and the low cost of the analysis compared with other analytical techniques. Its effectiveness has been demonstrated for the determination of VOCs in samples of a diverse nature, such as food [21,22], clinical [23] and environmental [24] samples. IMS has also been applied for the determination of trace impurities in cosmetic intermediates [25]. Coupling to GC

combines the high selectivity of GC separation and the good sensitivity of IMS, resulting in a two-dimensional separation. Therefore, each VOC is characterized by two parameters, GC retention time and IMS drift time, which is defined as the time required for ions to cross the distance between the ion shutter and detector. As far as we know, there are no procedures that have analysed cosmetic creams using GC-IMS.

Therefore, in this work, an alternative method to traditional microbial plate counting was developed based on the study of the MVOCs produced by two of the most important microorganisms found in cosmetic creams, *S. aureus* and C. *albicans*. Specifically, the study of volatile profiles using two different techniques HS-GC-MS and HS-GC-IMS, working in their respective optimal conditions, and chemometric approaches were proposed.

2. Materials and methods

2.1. Reagents and samples

All reagents used were of analytical reagent grade and the solvents were of HPLC grade. Chlorobenzene was supplied by Sigma Aldrich (St. Louis, MO, USA) and dimethyl sulfoxide by ApplicChem GmbH (Darmstadt, Germany). Chlorobenzene was used as internal standard (IS) in the GC–MS. The solution was prepared by diluting 2 μL of chlorobenzene in 25 mL of dimethyl sulfoxide to obtain a concentration of 80 μL L^{-1} and stored at -4 °C until use.

The ingredients for cosmetic cream preparation were obtained from a local dealer. Two different type of preservatives were used: a chemical preservative (a sorbate/benzoate mixture) and a natural preservative developed by Productos Sur S.A and obtained from vegetable material.

Tryptic soy broth (TSB), sabouraud dextrose agar with chloramphenicol, baird-parker agar with tellurite egg yolk and peptone water, all from Pronadisa Conda (Madrid, Spain), were used in the preparation of the culture media.

2.2. Instrumentation and software

HS-GC-MS analyses were carried out on a 7890A GC-System gas chromatograph from Agilent Technologies (California, USA), equipped with a temperature-controlled vaporizer (PTV) model CIS4-C506 and an automatic injector (Headspace model Multipurpose Sampler MPS), both from Gerstel (Mülheim an der Ruhr, Germany). The

GC system was coupled to a mass spectrometer (5975C inert MSD-triple axis detector from Agilent Technologies). The chromatographic separation was carried out on a DB-624 column with an internal diameter of 0.25 mm, a length of 60 m and a film thickness of 1.40 μm, which consisted of 94% dimethylpolysiloxane and 6% cyanopropylphenyl, also from Agilent Technologies. The injection was made in split mode with a ratio of 1:25. The GC temperature programme was: start temperature 40 °C, hold for 5 min, increase to 150 °C at 10 °C min^{-1} and maintain for 2 min; next, the temperature of 220 °C was reached at 25 °C min^{-1} and held for 10 min. The mass spectrometer was operated using electron ionization (EI) mode (70 V) and analyses were carried out using scan mode at *m/z* from 29 to 150, since this range was effective for the assessment of microbial contamination in mayonnaise [31]. The temperature of the GC-MS transfer line was set at 110 °C and temperatures of the ion source and the quadrupole used were 230 °C and 150 °C, respectively.

HS-GC-IMS analyses were performed using a Gerstel MPS headspace unit with a 2.5 mL syringe for gas injection. In this case, a gas chromatograph from Agilent 6890N (Agilent, Waldbronn, Germany) was used coupled to a commercial IMS from Gesellschaft für analytische Sensorsysteme mbH (G.A.S., Dortmund, Germany), equipped with a tritium (^3H) ionization source. Separation at the GC was carried out using an HP-5MS-UI column (Agilent J&W GC Column) with an internal diameter of 0.25 mm, a length of 30 m and a film thickness of 0.25 μm, which was composed of 95% dimethylpolysiloxane and 5% diphenyl. The oven programme was set as follows: initial temperature of 50 °C held 3 min, which was increased from 50 °C to 120 °C at 5 °C min^{-1} and held 120 °C for 3 min (total run 20 min). The analytes were introduced into the IMS module and ionized by a tritium source at atmospheric pressure in a positive ion mode. Nitrogen was used as drift gas at a constant flow of 150 mL min^{-1}. Once the ions had been formed in the ionisation chamber, they were placed in a 98 mm long drift tube operated with a constant field strength (500 V cm^{-1}) at 80 °C. Each spectrum had an average of 32 scans, which were obtained using a repetition rate of 30 ms, a grid pulse width of 150 μs and drift and blocking voltages of 241 and 80 V, respectively.

In both instruments, the samples were analysed in 20 mL vials with 18 mm aluminium magnetic screw cap and silicone septum.

A SensIONTM pHmeter (Hach, Colorado, USA) was used for pH measurements. Colour determination was carried out using a 962 colorimeter from X-Rite (Michigan, USA), a Visco Basic Plus viscometer was used to determine viscosity (Laboquimia, La

Rioja, Spain) and a Nicolet Evolution 300 spectrophotometer from Thermo Electron Corporation (Massachusetts, USA) was used to prepare the inoculum of *C. albicans* and *S. aureus*.

MS data were acquired using Maestro 2 Version 1.4.25.8 /3.5 software (GERSTEL) and MSD ChemStation D.02.00.275 (Agilent Technologies). The IMS data were acquired in positive polarity using the LAV (Laboratory Analytical Viewer) software version 2.1.1 (G.A.S.). Data were processed using Microsoft Office Excel (Microsoft, Washington, USA), Simca-P (Umetrics, Malmö, Sweden), Sigmaplot 13.1 (Systat, Software Inc., San Jose, CA).

2.3. Elaboration and inoculation of cosmetic creams

The experiments were carried out using face cosmetic creams prepared using a food processor under sterile conditions. The formulation of each cream consisted of 77.65% water phase (72.5% pure water; 0.15% rodicare S; 4% glycerin and 1% pantenol USP) along with 22.35% oil phase (5% emulium delta tablets; 2.5% rofetant GTCC/bergabest MCT-OIL; 2.5% isostearyl isostearate; 0.2% dragosantol/bisabolol; 2.70% mirasil DM-350; 0.5% vitamin E acetate; 2.95% jojoba oil; 2% apifil CG 2% massocare SQV; 2% cocoate BG). Two different preservatives were tested to increase the variability of samples and the profile of the VOCs generated. In this way, 1.5 kg of cosmetic cream was prepared, which was divided into 3 batches (0.5 kg each). A natural additive, a chemical additive or no additive was added to each batch in a 0.1% proportion.

Each batch was aseptically divided into 50 g aliquots, which were stored in polypropylene flasks with polyethylene caps. One third of the flasks were inoculated with *C. albicans* and another third with *S. aureus*, both at a concentration of 10^5 CFU/g. The flasks were heat sealed and incubated at 25 and 37 °C, respectively, until sampling. The remaining flasks were not inoculated in order to monitor the evolution of the cosmetic cream with no microbial contamination and were incubated in the same conditions (25 ºC). One gram from each flask was sampled (three times) weekly over four weeks, obtaining a sample for each temperature, each strain and each preservative.

2.4. Sample analysis by HS-GC-MS and HS-GC-IMS

For the analysis of VOCs by HS-GC-MS, 1 g of sample with 50 µL of IS chlorobenzene was incubated for 20 minutes at 80 °C at a stirring rate of 250 rpm. Using

228 a syringe at 80 °C, a volume of 2000 μL was then automatically injected from the
229 headspace into the CIS at 0 °C in Split mode with a 1:25 ratio. Helium with a constant
230 flow rate of 1 mL min^{-1} was used as the carrier gas.
231 For the analysis by HS-GC-IMS, 1 g of sample was also used. The sample was
232 incubated at 100 °C for 5 minutes at a speed of 750 rpm. Then, a volume of 750 μL taken
233 from the headspace was injected automatically by a syringe at 100 °C into the injector
234 (100 °C) in splitless mode. Nitrogen (99.9% purity) with a flow rate of 1 mL min^{-1} was
235 used as the carrier gas.
236
237 *2.5. Measurement of pH, water activity, colour and viscosity.*

238 The pH, water activity, colour and viscosity were measured in each sample. For
239 colour determination, three parameters (L*, a* and b*) were quantified with an X-Rite
240 962 spectrophotometer using the D65/10° illuminant/observer method, but only the L*
241 parameter was considered for data processing as a* and b* remained constant throughout
242 the experiment.
243
244 *2.6. Strain and culture conditions*

245 Two different microorganism species were used: *C. albicans* and *S. aureus*. The first
246 was obtained from the Spanish Collection of Valencia Type Crops, which was isolated
247 by Berkhout in 1923. *S. aureus* was isolated by Rosenbach in 1884 and also obtained
248 from the Spanish Collection of Valencia Type Crops. The *C. albicans* inoculum was
249 prepared by transferring a colony obtained on Sabouraud dextrose with chloramphenicol
250 agar plate to a soybean digested medium (TSB), which was incubated for 24 h at 25 °C.
251 The *S. aureus* inoculum was prepared by transferring a colony obtained on tellurite egg
252 yolk in baird-parker agar plate to a TSB medium, which was incubated for 24 h at 37 °C.
253 Both inoculums were standardized by dilution in TSB to a concentration of 10^5 CFU/mL.
254
255 *2.7. Statistical analysis*

256 The discriminant analysis carried out consisted of an orthogonal projection on latent
257 structures (OPLS-DA). OPLS-DA was introduced as an improvement of partial least
258 squares regression discriminant analysis (PLS-DA) to discriminate two or more groups
259 (classes) using multivariate data [26,27]. At first, each data matrix was divided into two

groups, a classification set (80% samples) was used to train the models, and the remaining 20% of the data was used for model validation. The samples selected to compile the calibration and validation matrices for the HS-GC-MS and HS-GC-IMS data sets were exactly the same. In order to choose the optimal models, the need to transform the data logarithmically was investigated and six different scales were tested: unit variance (UV), unit variance none (UVN), Pareto (Par), Pareto none (ParN), centering (Ctr) and freeze [28]. To ensure fit accuracy and predictive capability, the models were evaluated in R2X (cum), R2Y (cum) and Q2 (cum) terms. R2X is the cumulative fraction of the variation of X, R2Y is the percentage of the variation of the model-dependent variable and Q2 is a measure of the predictive capability of the cross-validation model [28,29]. These parameters have a range between 0 and 1, with values close to 1 representing the highest fitting to the model. At 50% or above of the value of parameter Q2 (cum), the method is considered valuable [29].

3. Results and discussion

3.1. Optimization of HS-GC-MS parameters

The sample amount, sample incubation time, incubation temperature, injection volume, CIS temperature and split injection ratio were investigated in order to optimize the HS-GC-MS method. A cosmetic cream sample without microbial contamination and without preservative was used as reference matrix. During data acquisition, retention time ranges between 8-9 min and 20-21 min were not collected since, at these retention times, dimethyl sulfoxide and ethanol signals were obtained, respectively. Dimethyl sulfoxide was used to prepare the IS solution and ethanol is present in the cosmetic cream preparations. The concentration of these two compounds is very high compared to other ingredients and consequently, their high intensity inhibited visualization of the rest of the signals.

Firstly, the sample amount was studied in the 0.1-1 g range. As the sample amount increased the signals improved, so 1 g of sample was selected as optimum. The effect of sample incubation temperature was studied between 60 °C and 80 °C. As the temperature increased, both the number and intensity of the signals increased (Supplemental Figure S1). This was because high temperatures facilitated the release of volatile organic compounds with high boiling points; hence 80 °C was chosen as the optimum temperature, which is the maximum suggested for the injector syringe. Moreover, the

sample incubation time was studied between 10 and 20 min. Best results were obtained using 20 min, so this temperature was selected. Longer times were not assayed in order to decrease the total analysis time (Supplemental Figure S2).

The injection volume was optimized between 500 and 2000 µL, the best results being obtained using the 2000 µL injection volume, when new peaks with increased intensity were observed. Then, the CIS temperature was optimized between -20 and 20 °C. Although working at -20 °C gave the best results, it was decided to set the temperature at 0 °C for economic and practical reasons, since the use of CO_2 for cooling is expensive and any differences between -20 °C and 0 °C were not significant.

Finally, the split injection ratio was studied between 1:25 and 1:100. As expected, the best intensities and number of peaks were obtained with the lower dilution rate. Therefore, the split ratio was set at 1:25 (Figure 1).

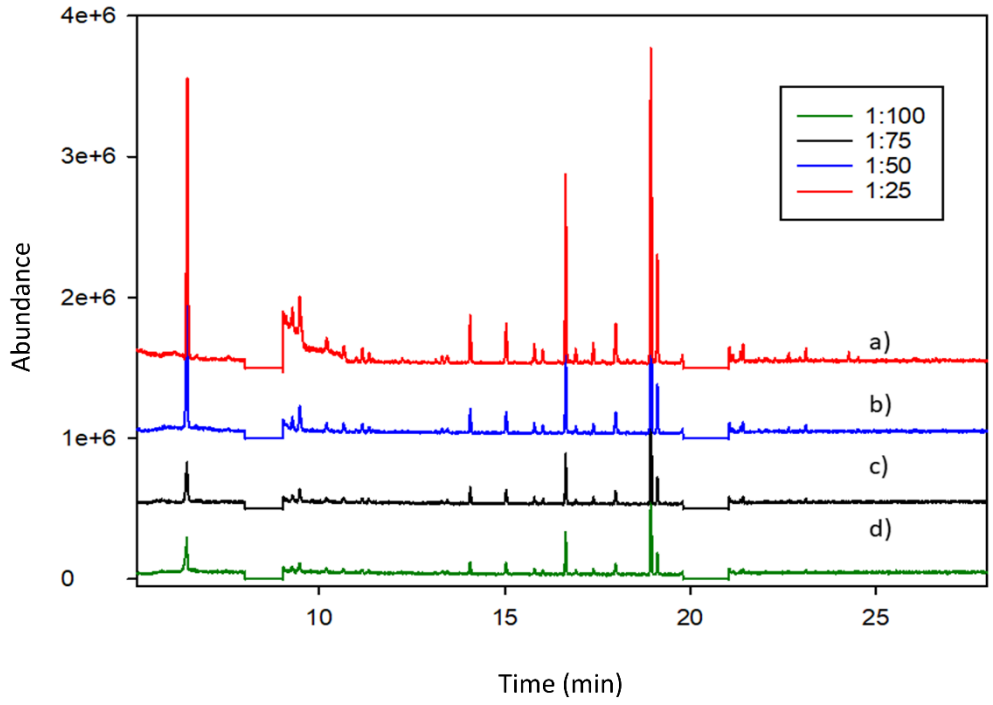

Figure 1. HS-GC-MS total ion chromatograms obtained for split ratios of a) 1:25, b) 1:50, c) 1:75 and d) 1:100.

3.2. Optimization of HS-GC-IMS parameters

In order to optimize the HS-GC-IMS method, the following parameters were investigated: sample amount, sample incubation temperature and time, and drift tube temperature. Optimum values were also selected based on the highest intensity obtained for the compounds and the best separation of the compounds on the topographic map. The effect of the sample incubation temperature was studied in the 80–120 °C range. It was found that the higher the incubation temperature, the greater both the intensity and the number of signals (Supplemental Figure S3). At temperatures above 100 °C, the IMS equipment became contaminated and, therefore, 100 °C was set as the optimum temperature. Then, the sample incubation time was studied between 1 and 20 min (Supplemental Figure S4). It was observed that at incubation times lower than 5 min the intensity of the signals decreased, and above this value no significant differences were found, therefore 5 min was selected as optimum. Finally, the drift tube temperature was studied between 60 and 100 °C (Figure 2). As the temperature rose, an increase in the intensity of the signals was clearly observed; however, 80 °C was selected as the drift tube optimum temperature since 100 °C was the limit value of this parameter according to the manufacturer.

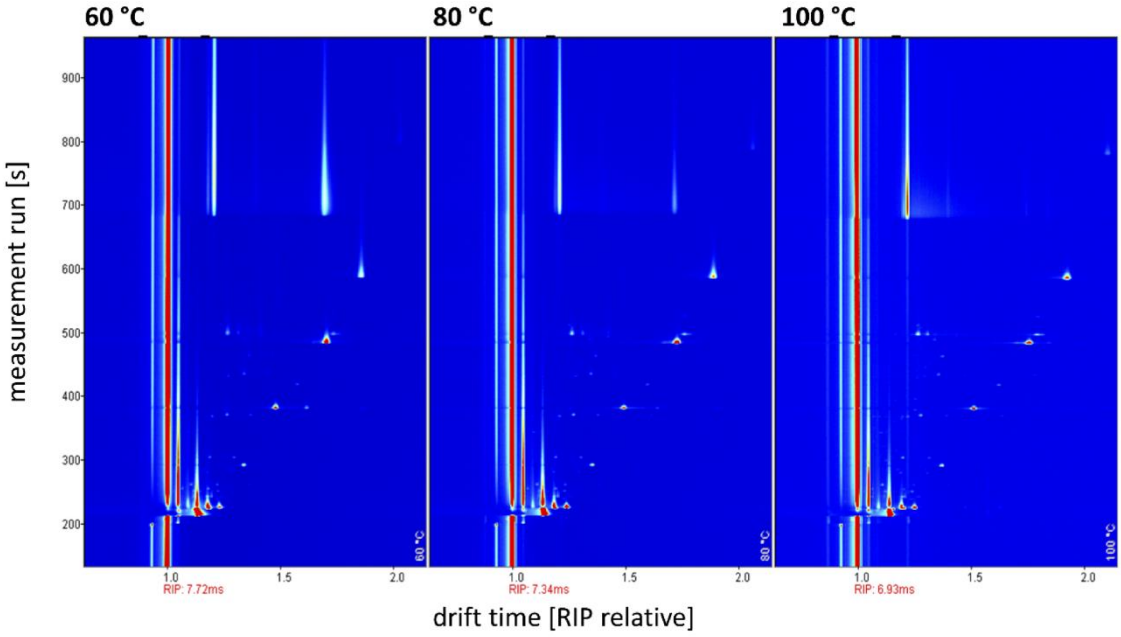

Figure 2. HS-GC-IMS topographic maps obtained for drift temperatures of 60, 80 and 100 °C.

3.3. Microbial contamination detection in cosmetic cream samples

Initially, all the cosmetic cream samples were analysed using plate counting to assess the microbial contamination level, the result was expressed as log10, the logarithmic-scale (base 10) for measuring colony-forming unit (CFU)/g. Based on these results, samples were divided into two groups: contaminated (55 samples) and non-contaminated (35 samples) and were analysed by both techniques, HS-GC–MS and HS-GC–IMS. The parameters pH, water activity, colour and viscosity were also measured and their evolution over four weeks is shown as supplementary material (Supplemental Figure S5). To study the evolution of these parameters over time, a statistical study was carried out. The data are included in Tables S2 and S3. Initially, the normality of these parameters was studied through the Shapiro-Wilk test. In all the cases, except the viscosity parameter, data were not adjusted to a normal distribution, therefore a Kruskal-Wallis one way-analysis of variance on ranks was carried out. No significant differences were found for contaminated and non-contaminated samples over four weeks for pH (p-value = 0.5606 for contaminated samples, p-value = 0.0809 for contaminated and non-contaminated samples), average pH remaining 5.5. However, water activity values increased in the first week in both group of samples (contaminated (p-value = 0.0003), non-contaminated p-value = 0.0126), and colour values suffered small fluctuation in weeks 2 and 3 for contaminated samples (p-value < 0.0001) and in week 3 for non-contaminated samples (p-value = 0.0032). Great differences were found in the viscosity values when chemical preservative was used. Therefore, the evolution of viscosity in the different batches was studied separately. Since data fit a normal distribution, one way-analysis of variance was carried out. In all the cases, no significant differences were found (p-value > 0.05).

In order to investigate the possibility of differentiating contaminated and non-contaminated samples in terms of viscosity, water activity, pH and colour, statistical tests were also carried out, grouping all contaminated or non-contaminated samples. For all parameter except for viscosity, a Mann-Whitney rank sum test was carried out since data were not normally distributed, and no significant differences were found (p-value of 0.0654, 0.4102 and 0.1129 for pH, water activity and colour, respectively). A t-test was performance for viscosity and also no significant differences were found (p-value = 0.7494 for samples with chemical preservative and p-value = 0.6454 for samples with natural preservative and with no preservative). In conclusion, these parameters did not

point to differences in the behaviour of creams depending on the degree of microbial contamination.

On the other hand, a new study has been carried out considering the evolution of the samples over time. Specifically, the evolution of microbial contamination by means of plate counting results and the evolution of the total VOCs content detected by HS-GC-IMS and HS-GC-MS were studied. The results are shown in Figure 3, demonstrating that microbial contamination and VOCs content increase with the time, and therefore the information of the four weeks had to be taken in account in the chemometric models for discriminating between contaminated and non-contaminated samples.

Chemometric models were constructed using the data obtained by HS-GC-MS and HS-GC-IMS in order to obtain a classification model that allowed differentiation of the contaminated and non-contaminated samples as an alternative to plate counting. Two different chemometric models were constructed using: a) the entire chromatographic profile (HS-GC-MS) [28] and b) a selection of the main markers of the topographic maps (HS-GC-IMS) [30].

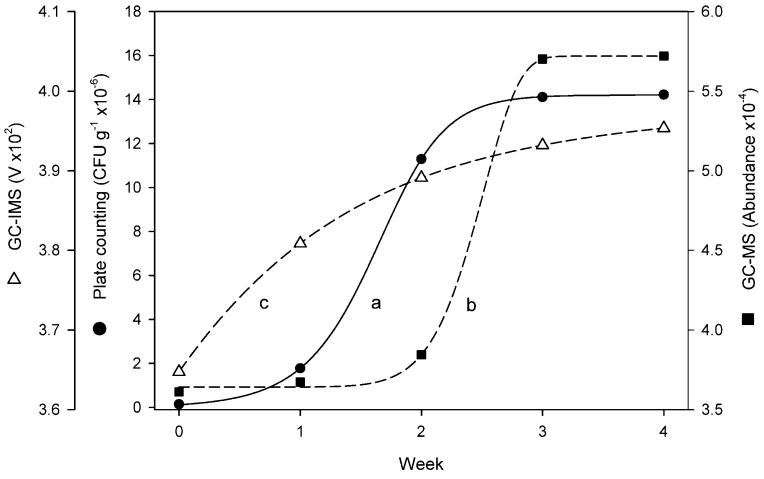

Figure 3. Evolution of microbial contamination over time by means of plate counting results (a) and evolution of VOCs detected by HS-GC-MS (b) and HS-GC-IMS (c).

3.3.1. Classification of samples using the total ion chromatogram obtained by HS-GC-MS

The total ion chromatogram (TIC) obtained by HS-GC-MS is the sum of the intensities of all the ions as a function of the retention time of the analytes in the column.

385 The use of TIC instead of the selection of specific markers for the assessment of microbial
386 contamination has already been demonstrated [31].
387 Data were processed following the previously described methodology [31]. Before
388 constructing the models, the need to correct the variations in retention times and the
389 baseline of the chromatograms was investigated. To correct possible variations in
390 retention time, the IS chlorobenzene was added. The baseline remained constant over
391 time and, therefore, it was not necessary to be corrected.
392 The dimensions of the data matrix obtained by HS-GC-MS were 90 rows (samples)
393 x 8779 columns (MS features). This matrix was divided into two: calibration or training
394 matrix and validation matrix. The calibration matrix was formed by 80% of samples, a
395 total of 72, of which 44 were contaminated samples and 28 were non-contaminated
396 samples. The validation matrix was formed by the remaining 20% of samples, a total of
397 18 (11 contaminated and 7 uncontaminated).
398 Using the calibration matrix, the OPLS-DA models were constructed. To obtain the
399 best model, different scales were used - UV, UVN, Par, ParN, Ctr and freeze - and also
400 raw data and the data normalized with the IS, and using logarithmic transformation. A
401 total of 24 different models were built and the best results were obtained with the
402 logarithmic transformation of the raw data and adjustment to the UV scale (Supplemental
403 Table S1). The chemometric model obtained is shown in Figure 4a. The results of
404 applying the classification model to the validation samples are shown in Table 1. As can
405 be seen, no false negatives were obtained, as all the contaminated samples were correctly
406 classified. Only one non-contaminated sample was misclassified as contaminated, giving
407 a total validation success rate of 94.4%.

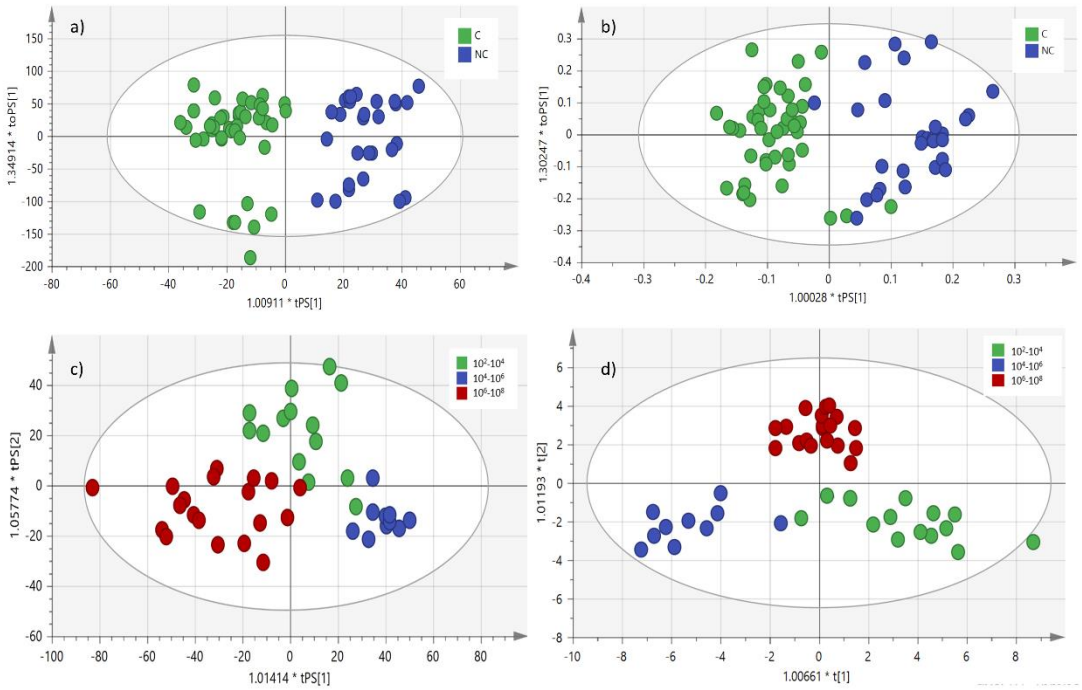

Figure 4. OPLS-DA models obtained using: a) total ion chromatogram raw data, UV scale and logarithmic transformation; b) topographic map data normalized with respect to the RIP intensity and Ctr scale; c) TIC raw data and freeze scale used to classify contaminated samples into three groups of microbial concentration; d) topographic raw data and freeze scale to classify contaminated samples into three groups of microbial concentration. NC: non-contaminated cosmetic cream samples; C: contaminated cosmetic cream samples (10^2-10^4: between 10^2 and 10^4 CFU/g; 10^4-10^6: between 10^4 and 10^6 CFU/g; 10^6-10^8: between 10^6 and 10^8 CFU/g).

3.3.2. Classification of samples using the topographic map obtained by HS-GC-IMS

The data obtained by HS-GC-IMS were treated following the methodology described by Arroyo-Manzanares et al. [32]. In this case, variations in retention time between samples were observed, so the first processing step was the manual alignment of the topographic maps. This alignment was performed with the LAV software, and all samples were aligned with respect to a sample used as reference. No significant variation in drift times were observed (tolerance of 0.001 ms).

Once all the samples were aligned, the topographic maps of all of them were studied visually and a total of 101 markers were selected. Hence, the dimensions of this data matrix were 90 rows (samples) x 101 columns (IMS features). As in the previous section, the OPLS-DA models were constructed and trained using 80% of the samples (calibration set) and validated with the remaining 20% (validation set). More specifically, the calibration set consisted of 72 samples (44 contaminated and 28 non-contaminated) and the validation set of 18 samples (11 contaminated and 7 non-contaminated). Six different scales (UV, UVN, Par, ParN, Ctr and freeze) were also tested working with raw data and data normalized with respect to the RIP intensity, and both were also tested with logarithmic transformation. The best OPLS-DA model constructed was obtained when the raw data adjusted to the Ctr scale and the intensity of the markers was corrected with the intensity of the RIP (Figure 4b, Supplemental Table S1). After applying the optimal model to the validation set, the success rate was 94.4% and only one non-contaminated sample was classified as contaminated (Table 2).

The spectra and chromatograms obtained by HS-GC-IMS and HS-GC-MS, respectively, throughout the experiment, showed clearly the evolution of VOCs over time (Supplemental Figure S6). In the case of HS-GC-IMS ten markers contributed most to the distinction of contaminated and uncontaminated samples. From these maps, it can be clearly appreciated the evolution of the MVOCs with time. These markers were: M1 (tr: 228.69 s, td: 9.19 ms), M2 (tr: 228.69 s, td: 8.81 ms), M3 (tr: 237.60 s, td: 8.40 ms), M4 (tr: 383.13 s, td: 11.07 ms), M5 (tr: 486.09 s, td: 12.77 ms), M6 (tr: 590.04 s, td: 13.96 ms), M7 (tr: 264.33 s, td: 9.14 ms), M8 (tr: 499.95 s, td: 9.36 ms), M9 (tr: 712.80 s, td: 8.93 ms), M10 (tr: 398.97 s, td: 9.19 ms). The evolution of these markers is shown in Figure S7, where the mean intensity normalized (V) of each marker along time within contaminated and uncontaminated samples is represented. Standard deviation error bars are also represented including all the replicates, which was different for each group of samples and for each week (n varied between 6 and 12 for the error bars). Statistical analysis was performed to these markers in order to evaluate their evolution through the experiment. Since data were not normally distributed Kruskal-Wallis one-way analysis of variance on ranks was carried out for all markers except for M6 (Tables S4 and S5). In the case of M8 marker, data was normally distributed and therefore a one-way analysis of variance was carried out. The data and results of this statistical analysis are a key aspect for building of a classification model, which is finally the major outcome of this study in demonstrating the potential of GC-IMS.

The intensity of M1 decreased over time for contaminated samples (p-value < 0.001) while remained practically constant for uncontaminated samples and no significant differences were found (p-value = 0.843). The M2 marker decreased over time for contaminated samples (p-value < 0.001), and had a maximum of intensity in the second week for non-contaminated samples (p-value = 0.021). The M3 marker (p-value = 0.019) had a minimum of intensity in the third week for non-contaminated samples, while no significant differences were found for contaminated (p-value = 0.846). Whereas, the intensity of M4 (p-value < 0.001), M5 (p-value < 0.001) and M6 (p-value < 0.001) decreased in both set of samples (contaminated and non-contaminated). The marker M7 had the opposite behaviour, increased over time for contaminated samples (p-value = 0.023), while remained almost constant for uncontaminated (p-value = 0.086). On the other hand, the intensity of M8 (p-value=0.019) was higher at the beginning of the experiment and had a drop in the first week for contaminated samples, being the intensity of M8 decreased over time for non-contaminated samples (p-value = 0.004). In a similar way, the intensity of M9 (p-value=0.032) was higher at the beginning of the experiment and had a drop in the first week for contaminated samples; while, for non-contaminated samples, no significant differences were found for M9 (p-value = 0.130). The marker M10 had the opposite behaviour, increased over time for contaminated and non-contaminated samples (p-value < 0.001).

Consequently, from these Figures S6 and S7 it was clear the evolution of the marker concentrations over the time.

3.4. Classification of contaminated samples according to microbial concentration

The chemometric models constructed using the TIC data obtained by HS-GC–MS and the main markers of the topographic maps obtained by HS-GC-IMS demonstrated a high success rate in classifying samples contaminated and non-contaminated by microorganisms. Therefore, the potential of both analytical techniques was investigated in order to quantify the microbial level of contamination.

For this reason, the 55 contaminated samples were divided into three groups with different levels of microbial contamination: group 1 (between 10^2 and 10^4 CFU/g) consisted of 17 samples and group 2 (between 10^4 and 10^6 CFU/g) of 14 samples while group 3 (between 10^6 and 10^8 CFU/g) was formed by 24 samples. OPLS-DA models were also constructed with 80% of samples (calibration set), using the data matrix obtained by HS-GC-MS with raw data and the data matrix normalized with respect to the IS using

different scales and logarithmic transformation. The best model was obtained using the raw data matrix adjusted to a freeze scale, with a success rate of 90.9% when the calibration model was applied to classifying the validation set (the 20% of remaining samples) (Figure 4c, Table 3). Although there was good separation between groups, the parameter Q2 was below 0.5, which indicates that the model fit was not satisfactory. Then, the same concentration groups were established to construct OPLS-DA models with HS-GC-IMS data. As in the previous case, 80% of the samples were used to construct the models (44 samples, of which 14 belonged to group 1, 11 to group 2 and 19 to group 3) and the remaining 20% was used to validate them (11 samples, of which 3 belonged to group 1, 3 to group 2 and 5 to group 3). Raw data obtained by HS-GC-IMS and the data normalized with respect to the reactant peak intensity was used to construct the models, testing different scaling and logarithmic transformation. As in the case of the MS detector, the best results were obtained using raw data fitted to the freeze scale. The final model obtained is shown in Figure 3d. When this model was applied to the validation set (Table 4), all the samples were classified correctly (100% validation success). With the HS-GC-IMS data, besides improving the validation success rate, the OPLS-DA model obtained fitted the behaviour of the samples, sincethe Q2 was 0.634 (>0.5).

3.5. Identification of compounds by HS-GC-IMS and HS-GC-MS

The identification of some compounds presents in the chromatographic profile of HS-GC-MS and the topographic plots of HS-GC-IMS was carried out.

Using the HS-GC-MS technique, the identification was enabled using available mass spectra libraries and several standards. A total of 17 compounds were identified, named acetaldehyde (RT=6.43 min), isopropyl alcohol (RT=9.49 min), 2-methyl-pentane (RT=10.20 min), 2-methyl-1-pentene (RT=10.99 min), hexane (RT=11.16 min), heptane (RT=14.07 min), methyl-cyclohexane (RT=15.04 min), 2-methyl-heptane (RT= 15.80 min), 3-methyl-heptane (RT=16.02 min), octane (RT=16.64 min), (Z)-2-octene (RT= 17.13 min), 2,4-dimethyl-heptane (RT= 17.25 min), ethyl-cyclohexane (RT= 17.98 min), 4-methyl-octane (RT= 18.30 min), chlorobenzene (RT= 18.94 min), nonane (RT= 19.11 min) and 2-phenoxy-ethanol (RT= 26.62 min). Acetaldehyde enable the differentiation between contaminated and uncontaminated samples since it is only identified in contaminated samples. Conversely, isopropyl alcohol, 3-methyl-heptane and 2-phenoxy-ethanol were identified exclusively in uncontaminated samples.

In order to identify the most important markers of HS-GC-IMS, formulation face cosmetic creams compounds that have been previously cited in Section 2.3, were prepared at its corresponding concentration in 1 g of sample and analysed. Thus, 3 of 10 markers, M4, M5 and M6, were identified with the component mirasil DM-350. This identification confirmed that the levels of these three markers were very similar for contaminated and non-contaminated samples because they correspond to an essential component of creams, which was not affected by microbial contamination.

Consequently, the development of this platform with HS-GC, MS and IMS could be strongly justified.

4. Conclusions

The usefulness of monitoring the volatile profile to detect creams contaminated by microorganisms is demonstrated. Two analytical methods, HS-GC-MS and HS-GC-IMS, were optimized and both are good alternatives discriminating between contaminated and uncontaminated samples (validation success of 94.4% in both cases). The IMS detector provided good results when classifying the samples according to microbial concentration. The OPLS-DA model obtained using the IMS detector showed a high validation success rate, as all samples were classified correctly (100% success), while the MS detector classified only 90.9% of the samples correctly. In addition, the IMS model showed a good fit to the behaviour of the data.

The HS-GC-IMS method can therefore be considered a good alternative to the classical microbial counting method, allowing saving in materials, time and money.

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Figure Captions

Figure 1. HS-GC-MS total ion chromatograms obtained for split ratios of a) 1:25, b) 1:50, c) 1:75 and d) 1:100.

Figure 2. HS-GC-IMS topographic maps obtained for drift temperatures of 60, 80 and 100 °C.

Figure 3. Evolution of microbial contamination over time by means of plate counting results (a) and evolution of VOCs detected by HS-GC-MS (b) and HS-GC-IMS (c).

Figure 4. OPLS-DA models obtained using: a) total ion chromatogram raw data, UV scale and logarithmic transformation; b) topographic map data normalized with respect to the RIP intensity and Ctr scale; c) TIC raw data and freeze scale used to classify contaminated samples into three groups of microbial concentration; d) topographic raw data and freeze scale to classify contaminated samples into three groups of microbial concentration. NC: non-contaminated cosmetic cream samples; C: contaminated cosmetic cream samples (10^2-10^4: between 10^2 and 10^4 CFU/g; 10^4-10^6: between 10^4 and 10^6 CFU/g; 10^6-10^8: between 10^6 and 10^8 CFU/g).

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Table 1. Validation matrix for non-contaminated/contaminated cosmetic cream samples of the OPLS-DA model built using the TIC raw data and UV scale with logarithmic transformation.

Actual/Prediction	Samples	% Correct	Contaminated	Non-contaminated
Contaminated	11	100	11	0
Non-contaminated	7	85.7	1	6
Total	18	**94.4**	12	6

Table 2. Validation matrix for non-contaminated/contaminated cosmetic cream samples of the OPLS-DA model built using the topographic map data normalized respect to the RIP intensity and Ctr scale.

Actual/Prediction	Samples	% Correct	Contaminated	Non-contaminated
Contaminated	11	100	11	0
Non-contaminated	7	85.7	1	6
Total	18	**94.4**	12	6

Table 3. Validation matrix for the classification of contaminated cosmetic cream samples according to microbial concentration of the OPLS-DA model built using the TIC raw data and freeze scale.

Actual/Prediction	Samples	% Correct	10^2 to 10^4 CFU/g	10^4 to 10^6 CFU/g	10^6 to 10^8 CFU/g
10^2 to 10^4 CFU/g	3	100	3	0	0
10^4 to 10^6 CFU/g	3	100	0	3	0
10^6 to 10^8 CFU/g	5	80	0	1	4
Total	11	**90.9**	3	4	4

Table 4. Validation matrix for the classification of contaminated cosmetic cream samples according to microbial concentration of the OPLS-DA model built using the topographic map raw data and freeze scale.

Actual/Prediction	Samples	% Correct	10^2 to 10^4 CFU/g	10^4 to 10^6 CFU/g	10^6 to 10^8 CFU/g
10^2 to 10^4 CFU/g	3	100	3	0	0
10^4 to 10^6 CFU/g	3	100	0	3	0
10^6 to 10^8 CFU/g	5	100	0	0	5
Total	11	**100**	3	3	5