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Volatile profiles of human skin cell cultures in different degrees of senescence

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ABSTRACT

It is known that skin releases volatile organic compounds to the environment, and also that its emission pattern changes with aging of the skin. It could be considered, that these compounds are intermediaries in cell metabolism, since many intermediaries of metabolic pathways have a volatile potential. In this work, a simple and non-destructive method consisting of SPME sampling and GC/MS analysis was developed to identify volatile organic emanations from cell cultures. This technique, applied to skin cells culture, indicates that the cells or cell metabolism produce several skin emissions. Chemometric analysis was performed in order to explore the relationship between a volatile profile and the senescence of cell cultures. Volatile profiles were different for cell cultures in different degrees of senescence, indicating that volatile compound patterns could be used to provide information about the age of skin cells.

1. Introduction

It is known that human metabolism synthesizes a range of volatile organic compounds (VOCs) but only recently, has it been noted that several are emanated by the skin [1]. Since the skin is the largest organ in the body, there is great interest in relation to VOCs and their functions. Hundreds of different kinds of organic substances appear in human skin emanations, which can be classified into several groups according to their functional groups such as carboxylic acids, alcohols, aldehydes, aliphatics, esters, ketones, amines, heterocyclics, and others [2].

Studies of body emanations, particularly those associated with perspiration, generate knowledge about the compounds present in skin, but not necessarily about the volatiles that are of importance to cell metabolism. Perspiration, being mainly formed of water (99%), is a dilute solution of compounds containing salts, non-volatile compounds, as well as small amounts of volatiles. Many amino acids (phenylalanine, leucine, valine, and alanine) have been identified in perspiration [3] together with ammonia, urea, lactate, uric acid, creatine polysaccharides, immunoglobulin A, epidermal growth factor, some hormones, vitamins and proteolytic enzymes [4].

It seems that VOC-profiles could be of importance in understanding the metabolism of the skin and other organs: skin emission patterns of VOCs have been found to be different in winter as they are in spring [1], plus it has been reported that alterations of the metabolic balance produced by pathologies, results in modifications in human VOC-profiles [5].

Several studies in humans have suggested that skin emanations are a source of chemical signals containing physiologically active components capable of altering the menstrual cycle in women [6], indicating that volatiles are part of a complex biological system. Several types of skin glands, including apocrine, eccrine, sebaceous and apoeccrine glands, contribute to volatile releases [7]. On the other hand, skin and skin emanations change with aging. The number and function of eccrine sweat glands decreases with age. In contrast, sebaceous glands increase with age, paradoxically accompanied by a marked decreased in sebum production [8], and an increase of senescent skin cells [9].

Senescence is an important characteristic of cells related mainly to the arrest of proliferation after a finite number of divisions. Since it is an irreversible process and the principal cause of tissue aging attracts much attention. The classic method used to identify senescence cells makes use of a biochemical reaction associated with the enzyme β-galactosidase present in the cell. Pre-senescent cells express enzymes with activity only at pH 4, but senescent cells also produce a β-galactosidase with activity at pH 6. This enzyme is called "Senescence-Associated/β-Galactosidase" (SA/β-Gal), and is considered a cellular marker used to identify senescent cells. SA/β-Gal expression can be detected with a stain which produced a blue colour after 16 h of chromogen addition into the cell culture [9]. Other methods have been proposed to measure SA/β-Gal activity [10], such as fluorescent assays [11,12] or differential-pH assays [13], looking after faster analytical methodologies. All of these methods are destructive in relationship to the cell sample. Our approach is based on the use of solid phase microextraction

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(SPME) a technology recently applied to study biological samples with fast and reliable protocols. This technique has been applied to measure VOCs in cell cultures [14,15], human blood [5], human skin [1] and other human samples [16] as their main characteristic is a non-destructive approach.

Aging of the skin is usually accompanied by marked changes in emanation releases [8] and dramatic changes in the synthesis capacity and gene expression of skin-fibroblasts [17,18]. For this reason, we hypothesized that the senescence modifies the VOC-profiles obtained from skin cells. In the present work, the monitoring of these changes by using GC/MS–SPME analysis was done.

2. Materials and methods

2.1. Cell culture

Cells were isolated using standard cell and tissue culture procedures [19,20], adapted to obtain human dermal fibroblasts [21]. Biopsies of human skin were obtained from foreskin of Chilean (mestizo-white) healthy young donors (between 3 and 6 years old), under informed consent from their parents, approval of the surgeon in charge and Ethic Committee, as required by the Helsinki Declaration. Since that circumcision of children is a routine medical procedure, the discarded piece of foreskin was used to obtain primary cell cultures (pre-senescent cells).

The biopsy was washed three times with phosphate buffer saline (PBS) (0.1 M pH 7.4) containing penicillin (100 U/mL), streptomycin (100 μg/mL) and amphotericin B (25 μg/mL). The visible fat was removed. The biopsy was incubated for 1 h at 37 °C in trypsin–EDTA (0.05%–0.53 mM) (Gibco-Invitrogen). The dermal layer was separated from the epidermis, cut in 1 mm^2 pieces and incubated for 1 h in trypsin–EDTA. Cells were recovered by centrifugation and cultured in DMEM/F12 (1:1) (Gibco-Invitrogen) with 10% FBS in 25 cm^2 section flask (Falcon).

The cell cultures were treated with commercial solutions to prevent mycoplasma contamination (BioMyc1/BioMyc2; Biological Industries) and tested for the presence of mycoplasma using a PCR-assay (EZ-PCR Mycoplasma Test Kit; Biological Industries).

Lactate concentration in the culture supernatant was measured with a commercial enzymatic kit (Lactate oxidase/Peroxidase; Sentinel Diagnostic).

2.2. Generation of senescent cell cultures

To evaluate the aging of the fibroblast cultures, the senescent cell assay was made using the conventional protocol for Senescence-Associated/Beta-Galactosidase (SA/β-Gal) staining [9] in non-confluent cultures [17].

Senescence in human skin cells was induced by serial passage. In each passage, the cells were grown up to approximately 80% of confluence and trypsinized (trypsin–EDTA 0.05%–0.53 mM; Gibco-Invitrogen). The senescence status was verified by SA/β-Gal staining in each passage of the cell cultures. When cells reached 10, 15, 20, 30, 50 and 70%SA/β-Gal, they were used in VOCs experiments.

2.3. Selection of the solid phase microextraction fiber

Three commercial solid phase microextraction (SPME) fibers were used together with a SPME manual holder: 100 μm polydimethylsiloxane (PDMS), 65 μm polydimethylsiloxane–divinylbenzene (PDMS-DVB) and 75 μm carboxen–polydimethylsiloxane (Car-PDMS). The SPME fibers were conditioned as recommended by the manufacturer (Supelco) at some degrees below each fiber's maximum temperature before they were used for the first time.

Conditioned media was used as a sample [22,23], prepared by mixing cell culture supernatants and fresh medium (DMEM/F12, 10% FBS), in a 3:1 ratio. Supernatants were obtained from five cell cultures, each one prepared from different donors.

The SPME fiber was introduced inside a headspace-vial of 20 ml, through a silicon septum (Supelco). Previously, the headspace-vial was loaded with 1 ml of sample (for 25 cm^2 culture flasks, no more than 5 ml conditioned media were available, obtaining a sample of 1 ml per quintuplicate). The loaded vial was stabilized for 15 min at extraction temperature (60 °C). Since the culture media contain blood serum, extraction was performed at the optimal extraction temperature to human blood (60 °C) for 60 min, therefore optimizing VOCs extraction and preventing protein denaturation [5].

2.4. Gas chromatography parameters

Targeted analytes loaded in the fiber were analyzed in a Hewlett-Packard (HP) 6890 gas chromatography (with a HP MD5973 quadrupole mass spectrometer) in splitless mode (5 min). Desorption was carried out at 250 °C for 5 min [5]. Helium at a constant flow (1.5 mL/min, 32 cm/s) served as a carrier gas. Separation was conducted in a 60 m length × 0.32 mm i.d. × 1.8 μm film thickness HP-VOC column (Agilent Scientific, USA). The oven was programmed as follows [1]: initial temperature of the column at 60 °C (2 min hold) followed by a ramp of 8 °C/min until 200 °C (15 min hold) and a second ramp of 5 °C/min to reach 260 °C, finally ramped to 270 °C with a post-run for 3 min. The parameters of detector were: Ion mass/charge ratio 20-550 m/z; quadrupole temperature 150 °C; electron impact ionization (EI); EI source temperature 230 °C; interface temperature 280 °C; electron multiplier voltage 1780 eV; scan mode.

2.5. Blanks

Two sorts of blanks were required: Blanks associated with the equipment (to discard contaminant related to the SPME fiber and chromatographic column, like siloxanes) and environmental blanks (to discard possible contaminants present in the laboratory environment). For an equipment blank, a SPME fiber was injected into the GC/MS without extraction samples. For an environment blank, a sterile PBS was used, in the same extracted condition of the respective sample.

The called "compensed-chromatogram", used to compare different volatile profiles, is the result of balancing the sample chromatogram to the related blank chromatogram.

2.6. VOCs identifications

Potential emanations were analyzed by matching sample mass spectrums with those of the National Institute of Standards and Technology (NIST) MS spectral library (98/02) for peaks presented in the chromatograms. Emanations were considered "unknown", when their mass spectral fit values were <90%, and discarded of this identification process. Putative emanations with mass spectral fit value ≥90% were compared with their respective chemical standard (Sigma–Aldrich), and informed as "identified", if retention times and mass spectra fitted those of standard compounds.

2.7. Comparative analysis of VOC-profiles of different cell cultures

Three cell cultures replicates of each senescence degrees evaluated by SA/β-Gal (10, 15, 20, 30, 50 and 70%SA/β-Gal) were seeded in six well plates at 2×10^4 cell/cm^2. The selected SPME fiber associated process was applied to detect the VOC-profiles. Supernatant of these 18 cell cultures after 72 h of seeded were analyzed per triplicate (generating 54 chromatograms). One ml of sample

Table 1
VOCs identified using GC/MS-SPME/HS.

Retention time (min)	Compound	Fresh medium	Medium incubated cell-free[a]	Primary culture 10%SA/β-Gal	Senescent culture 70%SA/β-Gal
14.41	1,4-Dimethylbenzene[b]			×	×
14.80	Cyclohexanol	×			
14.56	Nonane[b]			×	
15.05	Styrene[b]		×	×	×
16.93	Decane[b]				×
17.01	Benzaldehyde[c,b]	×	×		
17.22	β-Pinene			×	
17.52	1,2,4-Trimethylbenzene			×	
17.82	Ethylhexanol[c]	×	×	×	×
18.52	Benzyl alcohol[c]		×		
19.47	Acetophenone[c]		×	×	×
41.99	Heptadecane[b]				×

[a] Medium was incubated 72 h without cells in cell culture plates at 37 °C, in identical conditions of skin cells.
[b] Also identified in human skin emanations with other methods by Bernier et al. [2].
[c] Also identified in human skin using GC/MS–SPME by Zhang et al. [1].

(supernatant medium) was introduced into headspace glass vial. When necessary, the respective standards were used to quantify the cellular production or consumption (fg/cell day) of the VOCs.

2.8. Chemometrics and bioinformatics analysis

The chemometrics analysis based on PCA (Principal Component Analysis) was performed using SIMCA-P software (UMETRICS). PCA is a statistical technique that can be described as a reduction of multidimensional data sets. The principle of PCA is to find the linear combinations (called principal components) of the original-variables that contribute more to make the observations different from each other. To discriminate among different observations, a PCA-Class model was used with $p = 0.05$ [24]. Standardized variables were used and cross-validation was performed to determine the optimal number of principal components [25].

The multivariate calibration based on PLS (Partial Least Squares or Projection to Latent Structures) was made in SIMCA-P software. PLS is a multivariate method, where the X variables are projected onto a small number of latent variables (also called principal components) to simplify the relationship prediction between X and Y by the smallest number of variables. The retention time of the compensed-chromatograms (each 0.3 s), previously processed using a moving-averages smoothing-function [25], were set as independent variables (X), and the senescence (%SA/β-Gal) was set as the dependent variable or response (Y). The validation of the PLS model was made with the protocol proposed by Van der Voet [26], using about 100 permutations. This protocol (implemented in SIMCA-P software) estimate the degree of fit (R^2Y) and capac-

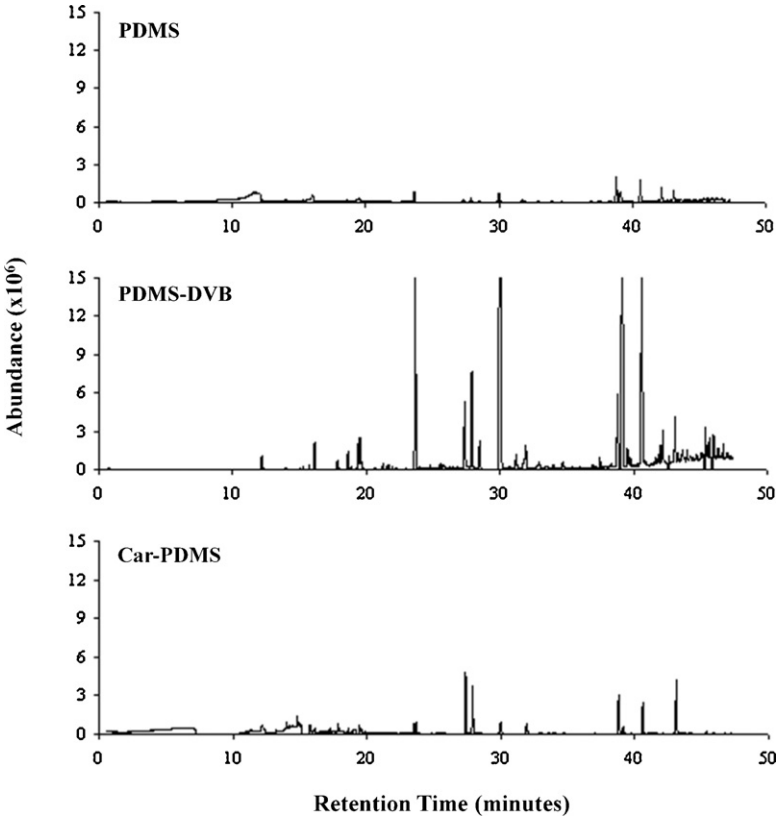

Fig. 1. Selection of the best SPME fiber.
Compensed-chromatograms were obtained by an extraction process of 60 min at 60 °C, on 1 ml of conditioned medium.

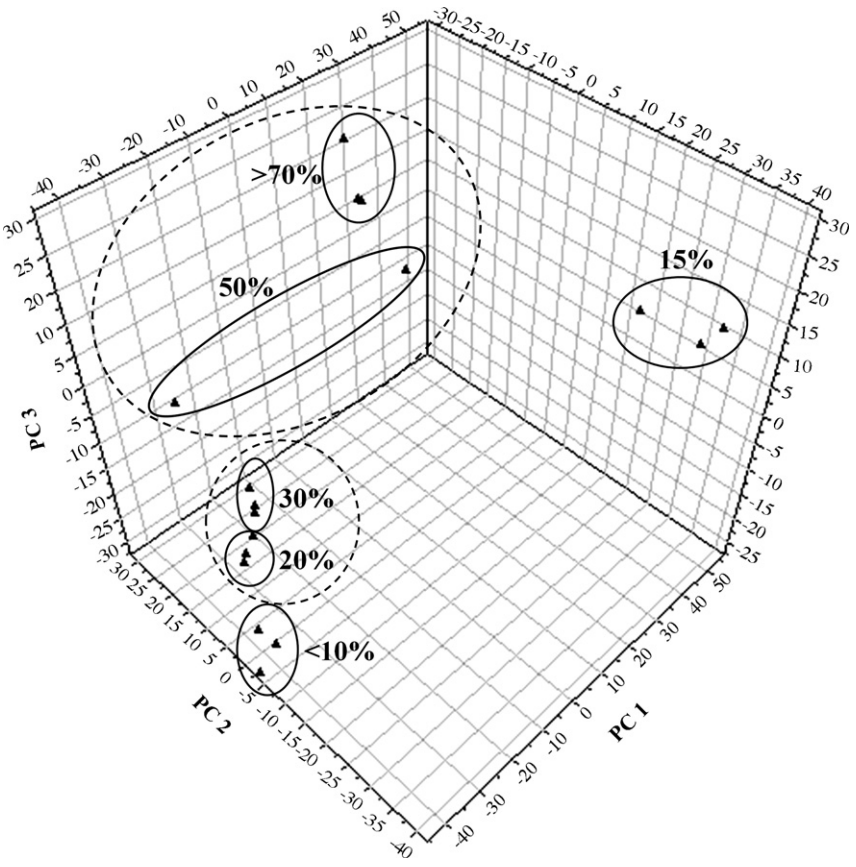

Fig. 2. PCA of VOC-profiles using cell cultures with different senescence state.
The principal component analysis was performed with SIMCA-P software (UMETRICS). Variables were abundance of the retention time between 14 and 47 min (taking one variable each for 0.3 s). Observations were the cell cultures with different expression of senescence. Percentage symbol next to the circles indicate senescent percentage measurement with SA/β-Gal.

ity of prediction (Q^2Y) using random permutations. As a criterion, when the parameters are $R^2Y < 0.3$ and $Q^2Y < 0.05$, is obtained a good model [24].

The bioinformatics analysis based on metabolic approach were made using the "Kyoto Encyclopedia of Genes and Genomes" (KEGG), available in http://www.genome.jp/kegg/. KEGG is a free database (Kanehisa Laboratory, Kyoto University). The identified VOCs by GC/MS–SPME were matched with the KEGG Pathway Database. Then, the cellular production or consumption (fg/cell day) of the matched VOCs, were associated at the senescence degree (%SA/β-Gal) using the Pearson's correlation coefficient and loadings of principal components.

3. Results and discussion

3.1. Selection of SPME fiber and VOCs identification

Examination of the compensed-chromatograms resulting from the three fibers (PDMS, PDMS-DVB and Car-PDMS), reveals the PDMS-DVB is the most suitable for extraction of volatile compounds for cell cultures, as shown in Fig. 1, which shows more and more distinctive peaks.

Application of the PDMS-DVB results in approximately 50 distinctive peaks in the chromatograms (see Fig. 1), 30 of which can be attributed to the blanks, being principally environmental contamination. When excluded from the analysis, the remaining 20 VOCs were assumed to occur in the cell culture media. Of these, 17 compounds were matched using the NIST Library – with reproducibility and low variation in quintuplicates – 12 of which were identified using standards (Table 1).

3.2. VOCs release from cell culture

Table 1 shows VOCs identified using the GC/MS-SPME/HS method. Nine compounds present in culture medium or cell culture has been reported in human emanations (1,4-dimethylbenzene,

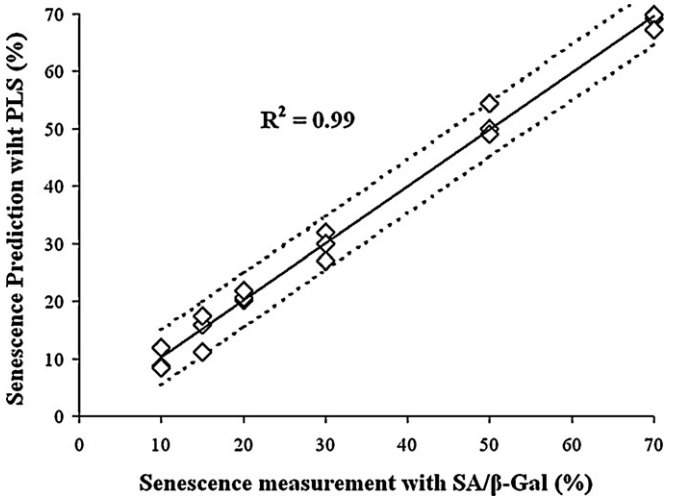

Fig. 3. Senescence correlation with VOC-profile using PLS.
The PLS regression method was performed with SIMCA-P software (UMETRICS). Dependent variable was Senescence (%SA/β-Gal) and independent variables were abundance of the retention time between 14 and 47 min (taking one variable each for 0.3 s). Observations were the cell cultures with different expression of senescence. Segmented lines represent the confidence interval (95%).

Table 2
VOCs linked at metabolic pathways and their productions (or consumptions) for primary (10%SA/β-Gal) and senescent cultures (70%SA/β-Gal).

VOC	Reference pathway linked	Production (+) or consumption (−) of VOCs (fg/cell day)	
		Primary culture 10%SA/β-Gal	Senescent culture 70%SA/β-Gal
1,4-Dimethylbenzene	Toluene and xylene degradation	+10.4	+6.7
Styrene	Styrene degradation Ethylbenzene degradation	+70.4	+10.6
Benzaldehyde	Toluene and xylene degradation	−75.5	−25.2
β-Pinene	Monoterpenoid biosynthesis	+100.6	0.0
Benzyl alcohol	Toluene and xylene degradation	−56.2	−18.7
Acetophenone	Ethylbenzene degradation	−15.2	−0.1

nonane, styrene, decane, benzaldehyde, ethylhexanol, benzyl alcohol, acetophenone, heptadecane) [1,2], showing that these VOCs could be cellular compounds released to the extracellular medium. However, many human skin emanations may have exogenous precedence [2]. VOCs could be absorbed or adsorbed by the cell when in the skin tissue, to be afterward released into the cell culture medium. Additionally, we identified β-pinene, but Zhang et al. [1] reported α-pinene in human skin. Our data was confirmed using both standards (16.14 min of retention time to α-pinene and 17.22 to β-pinene). It has been reported biological activity of β-pinene in mammalian cells, most likely interacting with cell receptors [27].

Six VOCs were found in the medium (fresh medium or medium incubated cell-free) (see Table 1), indicating that cells exposed to these VOCs, may alter the original metabolism of the tissue. Five of these six compounds were identified in human skin emanations (styrene, benzaldehyde, ethylhexanol, benzyl alcohol and acetophenone) [1,2]. Cyclohexanol, only present in fresh medium, has been reported in cell cultures as exogenous compound contained in the media [15].

Styrene is not present in fresh medium, but appears in the skin cell cultures and medium incubated cell-free (see Table 1). This suggests that styrene can be a degradation product from

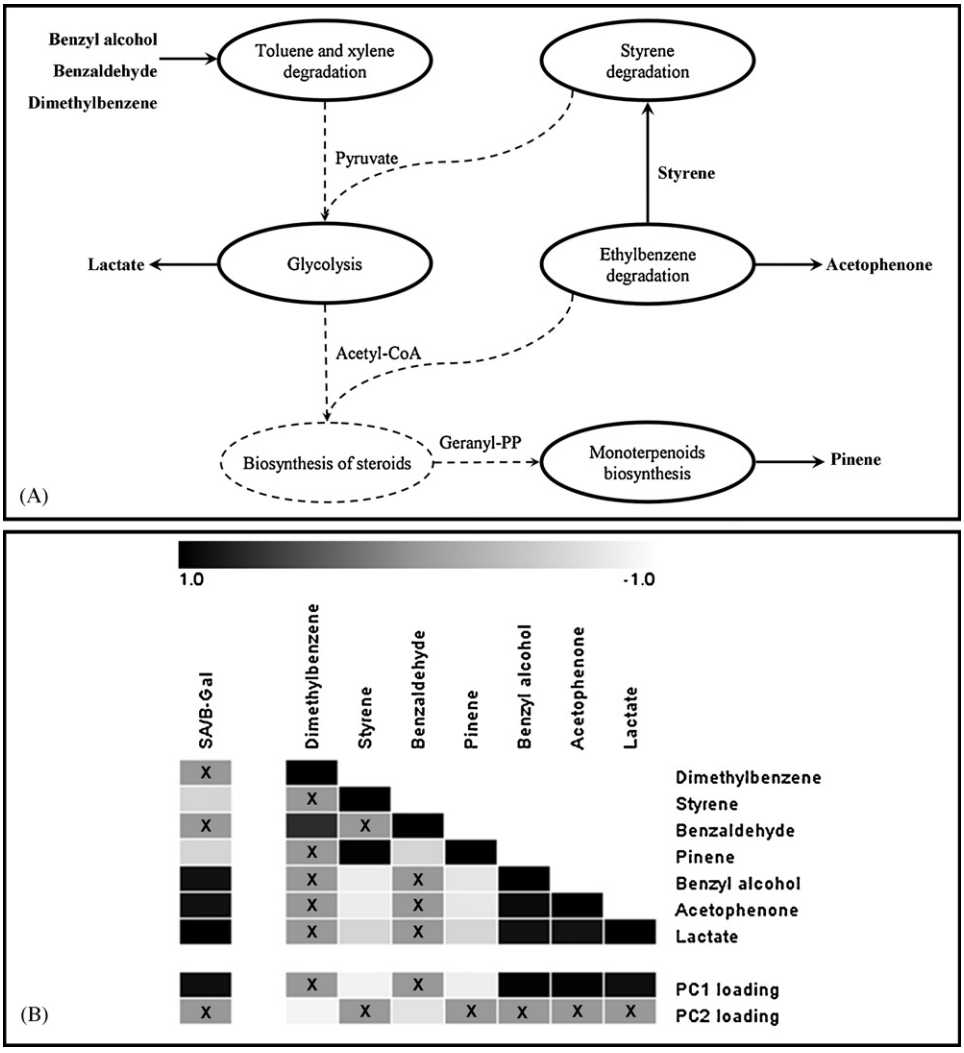

Fig. 4. Bioinformatic analysis of the VOC fluxes.
A: Reconstruction of metabolic pathways using KEGG database. Segmented lines represent putative pathways.
B: Matrix of Pearson's correlation and loadings of the principal component (PC). Variables were the fluxes (production or consumption, fg/cell day). Observations were the cell cultures with different expression of senescence. Bar of gray intensity represent the values between −1 and +1, and the symbol "×" shows no significant correlation ($p > 0.05$; $r = 0$).

the polystyrene flask (cell culture flask of 25 cm^2, Falcon). Our experiments seeding cells on glass (treated with poly-L-lysine for cell adherence) confirmed this hypothesis, because styrene was not been found in these cell cultures. In principle, polystyrene resists acid, alkalis and alcohol, but it can be attacked by some organic solvents. Styrene diffusion into foods has been reported for polystyrene packages [28] and is being reported as present in human blood samples [5], human skin [2] and cell cultures [15].

Trimethylbenzene was not found in the medium (fresh medium or medium incubated cell-free) (see Table 1), neither in the classic reports of human emanations [1,2]. Moreover it displays similarity with emanations reported in human skin as 1,4-dimethylbenzene and ethylbenzene [2].

3.3. Comparative analysis of VOC-profiles from different senescent cell cultures

Having proved that the proposed method permit obtain VOC-profiles from cultures with compounds associated to skin cells, we moved towards applying this method to cell cultures in different aging stages to see if it is possible to discern some characteristics through the analysis of the VOCs pattern. Such is the case of senescence achieved through growth over several consecutive passages. Supernatants of 18 cell cultures (per triplicate) were analyzed with different degrees of senescence (between 10% and 70% of senescence), evaluated by SA/β-Gal staining.

Principal component analysis (PCA) was performed in order to investigate the relationship between VOCs profiles and the state of the cell cultures. This chemometric technique has been used to study chromatographic VOC-profiles in human skin [1]. PCA was made using the compensed-chromatograms (retention time between 14 and 47 min, taking one variable each for 0.3 s) of the cell cultures. Thus, it was possible to have a complete vision of the VOC-profiles. The result in Fig. 2, clearly shows four clusters separated by the degree of senescence ($p < 0.05$; PCA-Class). The VOC-profiles were different in senescent and pre-senescent cultures. Pre-senescent and senescent cultures generally contained >70% and <10% proliferating cells and were $\leq$10% and $\geq$70% SA/β-Gal positive, respectively [9,29].

To investigate the possibility of determining the degree of senescence through VOC-profiles, a regression analysis was made with Partial Least Squares (PLS) methods, using the compensed-chromatograms of the 18 cell cultures (between 10% and 70% of senescence). A significant correlation coefficient ($p < 0.05$) was obtained between VOC-profile and the degree of cell senescence (Fig. 3). The PLS model was made with cross-validation protocol and validation using about 100 permutation of the data [26], obtaining good parameters to fit and prediction ($R^2Y < 0.3$ and $Q^2Y < 0.05$) [24]. The linear fit shown in Fig. 3 is similar at a bisector – zero offset ($p > 0.05$) and unitary slope ($p > 0.05$) – indicating that the predicted data are non-different to experimental data.

3.4. Compounds associated with senescence

In order to find possible association between some VOCs with the physiological state of cell senescence, a brief bioinformatic analysis was made. The emanations identified (see Table 1) were matched with KEGG bioinformatics database, obtaining six compounds associated to four reference metabolic pathways (Table 2), putatively interconnected such as is showed in Fig. 4A. These metabolic pathways were linked through a central branch mediated by glycolysis, it was then considered pertinent to study the glycolytic activity, finding an increase in lactate production with senescence: 2.8 (mg/cell day) to primary cells (10%SA/β-Gal) and 5.3 (mg/cell day) to senescent cells (70%SA/β-Gal). This is consistent with evidence which indicates that glycolysis increased with senescence [21].

The matrix of Pearson's correlation using cell cultures with different expression of SA/β-Gal as observations, showed significant correlations ($p < 0.05$) between metabolic-fluxes (production or consumption per cells, fg/cell day) (Fig. 4B). Four fluxes show significant correlations ($p < 0.05$) with SA/β-Gal expression (styrene, β-pinene, benzyl alcohol and acetophenone). Additionally, lactate production (glycolysis) shows significant correlation ($p < 0.05$) with VOCs and senescence (Fig. 4B). Principal component analysis shows that lactate, acetophenone and benzyl alcohol have strong positive loadings with first principal component (PC1), which has significant correlation ($p < 0.05$) with senescence (Fig. 4B).

We found evidence that some of the differences in VOCs release by skin cells are modified by senescence. It is likely that volatile changes in aged skin are indirectly influenced by cellular metabolism.

4. Conclusion

A simple and non-destructive method consisting of SPME sampling and GC/MS analysis was created to identify volatile organic emanations from human skin cell cultures. The best fiber for VOCs study in cell cultures was PDMS-DVB for the determination of whole profiles.

Twelve compounds were identified, nine of which have already been reported in human skin emanations.

Chemometrics and bioinformatics methods permitted to found that VOC-profiles change with different degrees of cell senescence.

We also concluded that skin cells release VOCs to the extracellular medium, many of these were already found in human skin.

Finally, VOC-profiles are statistically different for cell cultures in different states of senescence, indicating that VOCs could provide information on the physiological state of skin or skin cells, and this method could be used in recognizing the aging of skin or senescence in cells.

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