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Review

Strategies for Metabolomic Analysis of Damaged Skin from Cell and Tissue Samples Using Gas Chromatography-Mass Spectrometry

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Metabolomics can provide a readout of the biochemical and physiological state of a biological system. Gas chromatography coupled to mass spectrometry (GC-MS) has been widely applied for metabolomic analysis due to its numerous advantages, such as good sensitivity, high resolution, reproducibility, extensive database, lower acquisition cost and greater coverage. In addition, combined with efficient methods of sampling and sample preparation, the metabolomic analysis of damaged skin based on GC-MS can provide an important step toward elucidating several skin diseases. Based on this, this review presents a comprehensive overview of sampling, sample preparation, data processing and GC-MS analysis of metabolomic studies of damaged skin. Also, part of the biological interpretation of metabolites such as *cis*- and *trans*-urocanic acid (UCA) altered in photoexposed skin and lauric acid (C12:0) and palmitic acid (C16:0) in melanoma is discussed. Finally, to improve decision-making, a part of the integration of skin metabolomics with other omics sciences for the advancement of diagnosis is presented.

Keywords: metabolomics, GC-MS, human skin, sample preparation, data analysis

1. Introduction

The skin endogenously declines functionality due to the progressive damage caused to its biochemical, physiological, and morphological functions. This deterioration is aggravated by several environmental exposures, physical, chemical, or biological, occurring over the years. This environmental exposure results in early damage evidenced mainly in the most exposed areas of the body, such as the face, neck, and hands.[1-3] Exposure to ultraviolet (UV) radiation, tobacco abuse, and atmospheric environmental pollution are some factors that damage skin.[4-11] The consequences of skin degradation have already been reported at both genomic and proteomic levels.[12-16] However, studies on biochemical alterations that result in metabolome changes in the skin are limited.[17] Since the skin is an active organ, studying the changes in its metabolomic profile is fundamentally important to understand the negative effects, such as oxidative stress, and improve repair mechanisms.

Metabolomics can be applied to understand the changes that occur in the skin. It provides a comprehensive view of primary and intermediate metabolites and exogenous compounds, such as drugs and other chemical compounds that constitute cells, tissues, or organisms. Thus, metabolomic studies involve the identification and quantification of biomolecules that participate in metabolic reactions and correlate their changes with pathological states or the effect of external factors.[18-20]

This research field can employ two analytical approaches: target and non-target analysis. A targeted approach is driven by known biological issues and allows for more accurate detection and quantification but requires that the compounds of interest be known *a priori* and are available in their purified form. The non-targeted approach is driven by a hypothesis, and usually its analyzes are carried out qualitatively by observing total changes in chromatographic patterns.[21,22]

Nuclear magnetic resonance (NMR), gas chromatography coupled to mass spectrometry (GC-MS), and liquid chromatography coupled to mass spectrometry (LC-MS) are some of the analytical techniques that have

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been applied in metabolomic studies.[23-26] Due to the greater sensitivity achieved by mass spectrometry (MS), hyphenated techniques such as GC-MS and LC-MS have become more acceptable in this field of research. GC-MS has high sensitivity and resolution, good reproducibility, an extensive database, and greater ease of operation. Furthermore, together with derivatization, a wide range of compounds of different polarities can be investigated with GC-MS.[27-29] In addition, comprehensive two-dimensional gas chromatography (GC×GC), which comprises the use of two columns connected in series using a modulator, also presents itself as a very effective technique for skin metabolomic studies, as it allows a detailed profile of known compounds and is highly accurate, allowing the acquisition of a unique fingerprint of a given sample.[30] This review summarizes the current methods used for sampling, sample preparation, GC-MS analysis of cells and tissues in the metabolomic studies of skin, and the challenges and future perspectives of this field.

2. Sampling

Sample extraction and collection is an important step in metabolomics study because it directly affects data quality. Because the skin is the largest and most accessible organ of the human body, many sampling methods have been applied to identify skin biomarkers and understand the details of its molecular composition. In addition, skin sampling can be performed on conventional matrices such as layers of the organ (dermis, epidermis or superficial fat), as well as analysis of sweat, sebum, and volatile compounds emanating from the skin.[31,32]

The common collection techniques applied to obtain skin samples include invasive methods such as stripping, iontophoresis, microneedle, micro-dialysis, suction-blister and biopsy (Table 1). A biopsy is commonly employed because of its advantages of simple operation, morphological preservation and the collection of complete targets. However, invasive methods are considered patient unfriendly because they are time-consuming, inflict pain,

Table 1. Sampling techniques for skin studies

Sampling technique	Collection	Method	Advantages	Limitation	Reference
Biopsy	epidermis dermis superficial fat	invasive	simple operation, morphological preservation and collection of complete targets	potential tissue degradation during collection and variable handling of samples can lead to significant loss and distortion of molecular information complex sample preparation	33
Ultrasound	interstitial fluid	minimally invasive	minimally invasive, accessibility to deeper skin layers and improved temporal sampling sensitivity	potential denaturation of biomarkers, complex device	33
Tape strip	stratum corneum and skin excretions	minimally invasive	fast, convenient and patient-friendly	lack of standardized protocol and heterogeneous sampling variation in procedure details such as skin hydration, corneocyte cohesiveness, body site, and inter-individual differences	33
Reverse iontophoresis	interstitial fluid	minimally invasive	simple and possible to follow a variety of analytes simultaneously	time spent, interruption by sweating, need calibration; some factors affect the transport process; namely the thickness of the skin, transport number of the drug, and charge of the skin, among others	34,35
Microdialysis	interstitial fluid	invasive	samples are protein-free and readily analyzable without the need for additional analytical purification	low relative recovery mainly of very lipophilic substances	36
Suction-blister	epidermis interstitial fluid	invasive	simple	long sampling time that can change the biomarker structure	37
Microneedle	interstitial fluid	minimally invasive	simple to use and low consumption of reagents	high sampling variation due to a variety of factors that can affect needle penetration and the amount of sample absorption, including skin thickness and microneedle size	37

and incur a potential risk of complications. As a result, non-invasive methods, such as skin cells and tissues transplanted in culture, reconstructed human skin, skin microsomal and cytoplasmic homogenates and skin tissue fluid can be applied such as patient-friendly sampling.[31,32]

Non-invasive *in vitro* analysis, such as cells and tissues transplanted in culture, has been gaining attention from researchers, as this type of sampling offers several advantages over other models of subjects or animals. Some of these advantages include easy-to-control experiments, greater reproducibility, lower cost, and ease of interpretation of results. Furthermore, variables such as age, sex, variation between individuals and population control are not problems for *in vitro* studies involving skin cells. In this sense, this review focuses on sampling and sample preparation cells and tissues of skin.[38]

Before starting all the steps of the analytical procedure, determining the sample size for metabolomic experiments is of paramount importance to obtain robust and transferable results to establish comparisons between organisms. Generally, a large number of samples are used in order to minimize technical variability and find biological variations linked to biochemical processes. However, due to the complexity of these experiments, there are currently no standard methods for sample size estimation in metabolomics.[39]

In this sense, although tissue metabolomics offers a spatial description of the distribution of metabolites in which it provides information about their mechanisms of action and effects, some disadvantages must be taken into account, such as sample heterogeneity and low tissue availability.[40] The study of mammalian cell cultures, in turn, is one of the most applicable approaches, as a large number of replicates can be used, for example from 3 to 15 replicates or more depending on the type of analyte and the metabolomic approach.[41] Furthermore, the analysis of the cell metabolome can be divided into two investigative fractions between extracellular and intracellular metabolites, which constitute the endometabolome and the exometabolome, respectively.

2.1. Quenching

A rapid and efficient interruption of metabolism during cell and tissue sampling must be achieved by inhibiting endogenous enzymes in order to suppress changes in the metabolic profile during the analysis process without modifying the cellular environment.[42] This process must also be fast enough to stop metabolic changes instantly. Furthermore, the process must not induce any variation in the metabolic physical, chemical, and quantitative properties concerning subsequent steps in the analytical procedure.[43]

Conventionally, cells are extinguished by trypsin solutions; however, the influence on intracellular concentrations of metabolites is considerable since this enzyme can modify the physiological state of cells due to its interaction with membrane proteins. In addition, the conventional method requires several time-consuming steps that can lead to considerable losses in the number of metabolites.[44]

Other strategies that consider the sample conditions, such as changing pH and temperature, can be applied. The pH can be changed by subjecting the samples to extreme pH values, adding potassium hydroxide or sodium hydroxide for basic solutions, or perchloric acid and hydrochloric acid for acid solutions.[45] Temperature quenching is applied when the sample is cooled using liquid nitrogen or a cold methanol mixture.[46]

Wilkins *et al*.[47] studied four different methods to extinguish the metabolism of fibroblast cells derived from human skin: (*i*) incubation with trypsin, (*ii*) mechanical collection in phosphate buffered saline (PBS) solution, (*iii*) addition of 80% methanol pre-cooled, on dry ice, and (*iv*) addition of cold 80% methanol with subsequent evaporation solvent. The authors observed that mechanical collection in PBS solution obtained the least recovered metabolites due to possible errors in membrane rupture and extracellular contamination. The addition of 80% cold methanol achieved better recovery than incubation with trypsin or the addition and subsequent evaporation of 80% methanol. Similarly, the study by Bennett *et al*.[48] shows satisfactory results in the recovery of metabolites from human fibroblasts in adherent growth when 80% methanol was added cold directly to cells after removing the medium.

Due to the wide range of physicochemical properties of the metabolites, it is possible that there is variation in the detection of these analytes after storage, especially those that are more unstable. Wilkinks *et al*.[47] also studied the effect of sample storage on the concentration of metabolites. Using (*i-iv*) collection methods, the authors investigated the composition of the samples with respect to storage time of 48 h, 2 weeks and 1 month at −80 ºC. After 4 weeks of storage, samples collected by methods (*iii*) and (*iv*) had better results as they showed significant changes in two metabolites (method (*iii*)) and one (method (*iv*)) when compared to methods (*i*) and (*ii*) which showed significant changes in 3 and 19 metabolites, respectively. The authors then concluded that overall, the results demonstrate that storage for up to 1 month minimally impacted the concentration of metabolites when collected using methods (*iii*) and (*iv*). Biological samples have a

very complex matrix due to the presence of proteins and a wide variety of cellular and extracellular components; therefore, they need to be prepared for subsequent data acquisition and decryption. The sample preparation, in target analysis, generally aims to enrich the metabolites of interest and remove interfering substances. In addition, this step must be simple and quick to avoid metabolite loss and/or degradation, reproducible, robust, and incorporate an extinction step to obtain representative metabolomic profiles and comprehensive coverage of the metabolites.[49,50]

Sample preparation is a critical step in a metabolomic analysis, as together with sample collection and storage, it has the most common set of errors in this type of study. In addition, bacterial contamination and metabolism can cause degradation or the appearance of metabolites. Therefore, to avoid misinterpretation, samples must be free of enzymatic reactions during sample preparation stage.[28,51]

3. Sample Preparation

3.1. Extraction procedures

The sample preparation step in the GC-MS target metabolomic analysis consists of two main parts: extraction and derivatization. The extraction procedure aims to isolate analytes and can be achieved through techniques such as solid-phase extraction (SPE), liquid-liquid extraction (LLE), and protein precipitation (PPT). The derivatization process avoids the degradation of the metabolites of interest and increases the volatility of metabolites containing polar functional groups and increases the sensitivity of the method.[52]

The extraction methods conditions result in different metabolite recoveries. Simple changes, such as varying the temperature, the solvent extraction composition, or storage time, will cause detectable effects on the metabolites. Thus, optimizing metabolite extraction protocols is extremely important to obtain a more robust metabolomics study that enables the greatest number of analytes to be recovered.[53]

Proteins, for example, can be present in large amounts, which produces signal suppression of less abundant compounds. Therefore, the applicability of an extraction method can be achieved by the precipitation of proteins using organic solvents, such as methanol and acetonitrile, by denaturing them using reactions with acids or changing the temperature.[54,55]

The LLE can be applied to obtain and/or separate metabolites of different polarities through partially miscible or immiscible solvents. Compounds such as isopropanol, ethanol, methanol, or a mixture of these solvents can be applied to extract polar metabolites. In contrast, chloroform or ethyl acetate can be applied to extract nonpolar metabolites.[56]

Several successful methods have been reported to extract intracellular metabolites using solvents, such as methanol, water/chloroform in different proportions, cold methanol and cold acetonitrile for mammalian cells.[57-59] However, the methanol/water mixture was the solvent that showed the greatest recovery for metabolite extraction in skin fibroblast cells, and skin tissue samples.[17,47,48,57,60]

SPE is widely used to sufficiently separate the analytes from the interfering matrix or to concentrate the analytes, increase sensitivity, and improve the limits of detection. SPE involves adsorption on an appropriate solid material and desorption with a well-selected solvent. Therefore, SPE achieves a cleaning step through selective retention of analytes while limiting interference due to the careful selection of an adsorbent with a strong affinity for the analyte.[61] SPE was used to explore alterations in the ceramide profile of fibroblasts and keratinocytes derived from the dermis and epidermis of patients with psoriasis vulgaris and healthy subjects. Using a liquid chromatography platform in conjunction with sample preparation methods, the authors identified differences in ceramide levels in patients with psoriasis both in the dermis and epidermis, demonstrating the efficiency of the developed method.[62] Another method is solid-phase microextraction (SPME) which can be an alternative to LLE and SPE, because in both techniques a large amount of sample volume is used, with high consumption of organic solvents that generate a high volume of waste and limited capacity of sorbents that can induce the matrix effect.[63] SPME is easy to use and allows sampling, pretreatment, enrichment, and sample introduction in a single step without using a solvent. In addition, it is known as a non-invasive and easy sampling method in metabolomic studies.[64]

SPME consists of the equilibrium distribution of analytes between the matrix and a fiber sorbent that can be liquid or solid. Sorbents with different compositions and polarities are used to coat the silica fiber to obtain the greatest selectivity of the analytes. SPME fiber can be immersed in the sample or can be applied in the headspace above the sample, allowing easy fractionation of volatile analytes in complex matrices and preventing damage to the fiber coating, signal suppression, and instrument contamination.[65] Abaffy *et al.*,[66] for example, used head space solid phase micro-extraction (HS-SPME) and GC-MS to identify volatile signatures from fresh and frozen biopsy specimens of melanoma, nevus, and skin. The authors compared the difference in frequency distribution and their level of expression and found possible candidates for biomarkers such as volatile 4-methyl decane, dodecane,

and undecane, which were preferentially expressed in fresh and frozen melanoma. Other variations of micro-extraction methods can also be applied for metabolomic analysis, such as thin film microextraction (TFME), stirbar sorptive extraction (SBSE), and dispersive-magnetic solid-phase extraction (d-MSPE).[67,68]

Finally, ultrafiltration can be applied to *in vivo* samples and can also be described as a sampling technique that can physically extract biological samples from the target tissue. Ultrafiltration employs the extraction of biological samples through a semipermeable membrane, which filters molecules according to their molecular weight. Due to the filtration property of the membrane, all large molecule impurities are blocked on the tissue side of the membrane, while small molecule analytes pass through the membrane to the collector side.[69] Table 2 describes the extraction methods normally used in metabolomic studies, highlighting their advantages and disadvantages.

For target analysis or quantitative metabolic profile, in which known compounds are analyzed, sample preparation may be accompanied by the addition of isotope surrogate standards to optimize the extraction and determination process. Furthermore, for samples containing low concentration of metabolites, the extraction procedure may include an additional pre-concentration step to reach the limits of detection of the determination method.[65]

3.2. Derivatization

The metabolomic study of the skin produces an infinite number of classes of metabolites such as amino acids, organic compounds, fatty acids, lipids, nucleotides, carbohydrates, among others. For this reason, due to the limitation of the GC-MS technique based on the analysis of non-polar, volatile and thermally stable compounds, a derivatization step must take place to cover most of the class of skin-derived metabolites. For example, amino acids that are considered key metabolites for wound healing, acid-base balance and water retention, protection against sunlight damage, and maintenance of the microbiome of the skin.[72] Thus, derivatization step is performed to reduce the polarity and increase the thermal stability and volatility of the analyte. Molecules that have functional groups with active hydrogens, such as carboxylic acids, alcohols, amines, and thiols are derivatized before chromatography analysis.[73]

The chemical derivatization process can be performed through alkylation, acylation, or silylation using physical processes such as agitation, heating blocks, microwaves, or automated instrumentation that allow samples to be derivatized over time. These steps can improve the performance of sample preparation by reducing time and facilitating reactions.[73]

Trimethylsilylation (TMS) is the most frequently used derivatization process due to its wide coverage, relative simplicity, and comparable silylation strength. TMS uses reagents such as *N*-methyl-*N*-(trimethylsilyl) trifluoroacetamide (MSTFA), *N*-*tert*-butyldimethylsilyl-*N*-methyltrifluoroacetamide (MTBSTFA), and *N*,*O*-bistrifluoroacetamide (BSTFA). However, the performance of the silylation reactions can be improved by the addition of a catalyst. Therefore, substances such as trimethylchlorosilane

Table 2. Summary of the extraction techniques most widely used in sample preparation for metabolomic studies

Extraction method	Description	Advantages	Disadvantages	Reference
Protein precipitation (PPT)	addition of an organic solvent to the biological matrix for protein precipitation, followed by centrifugation and filtration	simple and easy to use; high recovery, high precision and accuracy	low selectivity, coelution caused by remaining impurities may arise	56,69
Liquid-liquid extraction (LLE)	migration of solute from one solvent phase to another; the two phases are immiscible or slightly miscible	low cost and easy to implement; high selectivity over protein precipitation	time-consuming, often involving toxic solvents; low recovery for polar compounds, matrix effect due to remaining impurities	56,69
Solid-phase extraction (SPE)	enrichment of analytes by adsorption on appropriate solid material followed by desorption with a well-selected solvent	a small amount of solvent, high specificity wide coverage of analytes	low recovery, high cost, labor intensive	70
Solid-phase microextraction (SPME)	a fiber coated with an extraction phase can extract the analyte without prior need for protein precipitation; just as SPE is composed of two stages (adsorption/ desorption)	little sample preparation, direct sampling, automation capability	fiber fragility, low reproducibility	71
Ultrafiltration	filtration through a semipermeable membrane allows the passage of molecules with a specific molecular weight range, under negative pressure	simple, high reproducibility, direct sampling	semi-quantitative technique, high cost, limited metabolite extraction capacity	69

(TMCS), *tert*-butyldimethylchlorosilane (TBDMCS), trifluoroacetic acid (TFA), and pyridine are widely used as catalysts for the silylation derivatization reaction. These substances help in the hydrolytic stability of the mixture and increase the reactivity.[74,75]

For the silylation derivatization reaction, the extracts must be free of residual water to avoid unwanted hydrolysis, which interferes with the result. Thus, to ensure complete sample derivatization, an excessive amount of silylation reagents is used to react with all water molecules in the reaction pool.[74]

To avoid the hydrolysis issue, alkyl chloroformates such as methyl chloroformate (MCF), ethyl chloroformate (ECF), propyl chloroformate (PCF), and isobutyl chloroformate (IBCF), can be applied as an alternative reagent for derivatization in aqueous samples. These compounds interact with the target molecules, replacing the active hydrogen atom in the carboxyl, amino or phenolic hydroxyl groups with an alkyl group. One of the main advantages of alkyl chloroformates is that the reaction can be done in an aqueous medium within a few minutes without heating. Other benefits are the low cost of the reagents and the clear separation of their derivatives using an organic solvent.[74,76]

The derivatization process for GC-MS metabolomics analysis usually comes with an oximation step. The chromatogram is simplified by reducing carbohydrate peaks and protecting α-keto acids and aldehydes from decarboxylation and tautomerism of keto-enol. Hydroxylamine and alkoxamine reagents are often used in this process.[77-79]

Finally, physical processes are fundamentally important to complete the derivatization step, as they can facilitate, accelerate and/or promote reactions. The strategies for this step include off-line derivatization, microwave-assisted derivatization (MAD) or ultrasound, and those that employ automated systems.

Off-line derivatization generally refers to the process made from dry extracts that are reconstituted in the derivatization reagent, heated for a while, with or without stirring. After derivatization, the samples are transferred to clean flasks before analysis. This method is by far the most applied in metabolomic studies; however, the time when the samples are stagnant before analysis and the long incubation times can vary.[74]

A strategy to mitigate these errors is to use microwaves or ultrasound that can drastically reduce the time needed to derivatize the samples.[80] Liu *et al*.[81] used ultrasound after off-line derivatization of endogenous metabolites from common fibroblast cells. They observed that this method significantly increased the derivatization efficiency of metabolites and dramatically reduced the time.

The derivatization in the liner occurs within a few seconds inside the heated GC inlet. The sample and the derivatization reagent can be injected separately or simultaneously through a multi-layer "sandwich" injection with air space between them, which is more repeatable and automates the analytical sequence.[82]

4. GC-MS in Damaged Skin Target Metabolomics

In the injection system, two parameters are focused on: the inlet and the injection mode. The GC inlet liner is where the samples are introduced into the instrument to avoid contact with the metal walls of the GC. Choosing the right liner is crucial to obtain correct, reproducible, and accurate results in any type of analysis based on GC metabolomics. Thus, liners with internal strangulation are the most suitable for target metabolomic analysis in the split mode because they enable better discrimination of the molar mass. Inlet liners filled with glass wool are also indicated in this study because the glass wool acts as a filter for non-volatile compounds, protecting the injector and the column.[28]

The split and splitless injection modes were well used in metabolomic studies of damaged skin. However, the split mode is generally preferred, as the metabolites present in these samples have a variety of concentrations. When the splitless mode is chosen, some overload on the column may occur (Table 2).

The column choice must consider the types of stationary phases to obtain greater selectivity of the analytes, the temperature range to enable good resolution in a short time, and the sample quantity to avoid overload conditions. Thus, the columns widely used in damaged skin metabolomics contain the stationary phase of 5% diphenyl crosslinked with 95% dimethylpolysiloxane with a dimension of 30 m × 0.25 mm × 0.25 μm (length × internal diameter × film thickness). However, columns with smaller dimensions, for example, 20 m × 0.18 × 0.18 μm, have also been used. This type of column, while offering a higher peak resolution, also limits the sampling capacity, thereby compromising the sensitivity.[28]

Table 3 shows the analytical instrumentation used to study damaged skin by intrinsic means or exogenous factors such as exposure to ultraviolet radiation. The parameters verified are column, injection mode, temperature program, and the types of analytes identified.

Mass spectrometry coupled to GC are the most indicated for metabolomic studies to increase the specificity of the chromatographic technique. In GC-MS, the most widely applied mass spectrometer conditions are electron

Table 3. Analytical instrumentation, such as column, injection mode, temperature program, as well as the types of analytes identified used during metabolomic studies of damaged skin

Matrix	Quenching	GC column	Injection mode	Oven temperature program	Mass analyzer	Compounds	Reference
Tissue	liquid nitrogen	HP-5 (20 m × 0.18 × 0.18 µm)	splitless	the initial temperature was 60 °C raised to 340 °C at a rate of 18 °C min^{-1}, approximately	single-quadrupole	glucose, lactate, 3 phosphoglycerate, carnitine, acetylcarnitine, butyrylcarnitine, octanoylcarnitine, glycerol, glutatione metabolism, and others	60
Tissue	MeOH 80%	HP-5 (20 m × 0.18 × 0.18 µm)	splitless	the initial temperature was 60 °C raised to 340 °C at a rate of 18 °C min^{-1}, approximately	single-quadrupole	amino acids, nucleotides, sugars, peptides, cofactors, lipid metabolism, and others	17
Tissue	MeOH	Rtx-5MS (29.8 m × 0.25 mm × 0.25 µm)	split 10:1	the initial oven temperature was 75 °C for 2 min, increased to 300 °C at a rate of 15 °C min^{-1} and then maintained at 300 °C for 3 min	Q-TOF	GC: primary metabolites, amino acids, fatty acids, and saccharides LC: phospholipids MS: ceramide	83
Tissue	cold MeOH 80%	Rtx-5MS (29.8 m × 0.25 mm × 0.25 µm)	split 5:1	75 °C for 2 min, raised to 300 °C at a rate of 15 °C min^{-1}, and then maintained at 300 °C for 3 min	TOF	metabolites of TCA (tricarboxylic acid) cycle: pyruvate, citric acid, malic acid, and fumaric acid	15
Cell	cold MeOH 80%	DB-5MS (30 m × 0.25 mm × 0.25 µm)	splitless	the initial oven temperature was set at 120 ° C with the following rates: increased to 180 °C at 25 °C min^{-1}; raised to 270 °C at 6 °C min^{-1}; raised to 325 °C at 30 °C min^{-1}	single quadrupole	metabolites of the TCA cycle: 2-hydroxyglutaric acid, alpha-ketoglutaric acid, *cis*-aconitic acid, citric acid, fumaric acid, isocitric acid, lactic acid, malic acid, oxaloacetic acid, and succinic acid	47

GC: gas chromatography; MeOH: methanol; HP-5: 5%-phenyl 95% methylpolysiloxane, nonpolar column; Rtx-5MS: 5% difenil/95% dimethylpolysiloxan, low-polarity column (Restek); DB-5MS: 5% diphenyl/95% dimethylpolysiloxan, low-polarity column (Agilent); Q-TOF: quadrupole- time of light; LC: liquid chromatography; MS: mass spectrometry.

ionization (EI) at high energy (70 eV) and low pressures because EI generates more stable spectra that are available in many reference libraries.[84,85] However, due to the high applied energy, fragmentation of many organic compounds results in low mass ions that decrease sensitivity and significantly interfere with identifying unknown ions.[86]

On the other hand, chemical ionization (CI) leads to fewer molecular ion fragmentations, producing larger abundant mass ions. CI requires electron bombardment of a reagent gas at high ion source pressures. Therefore, CI is less suitable for database research, and its use is limited only to studies of targeted metabolomics.[87,88]

The mass spectrometer used in metabolomic investigations are based on single quadrupole (Q) and time of flight (TOF) analyzers. The high mass resolution and the fast scan rate make TOF one of the most advantageous technologies for the deconvolution of overlapping peaks and are an important tool for identifying strangers. However, quadrupole analyzers are the most used in metabolomic studies because they are easy to use, provide fast scanning, and are suitable for determining masses and compound fragment ions; moreover, their results are easy to interpret.[52,89] Serial analyzers (GC-MS/MS), such as triple quadrupole, are alternatives to the targeted metabolomic study of damaged skin, as this technique allows greater sensitivity and precision for quantitative analysis.[90]

Recent high-resolution technologies, such as the orbitrap type analyzers, have also been applied in metabolomic studies. Although orbitrap analyzers have gained interest due to the high acquisition speed, high

sensitivity and/or high spectral resolution, and greater mass accuracy, they are still rarely applied due to a poorly developed computational support that makes the absolute gain minimally explored.[84,91]

4.1. Advances of chromatography analysis-comprehensive two-dimensional gas chromatography as a powerful tool for metabolomics studies

The standard one-dimension capillary columns show inefficient separation when applied to the analysis of very complex biological samples. Liu and Phillips[92] developed a comprehensive two-dimensional gas chromatography (GC×GC) method to increase column separation by preventing coelutions in complex matrix analyzes. In GC×GC, two columns are coupled in line through a modulator. The eluate emerging from the first dimension (^{1}D), is concentrated, preserving the separation obtained, and is quickly sent to the second-dimension (^{2}D). These independent separation processes allow for complete transfer of the sample from ^{1}D to ^{2}D. GC×GC has several advantages over the one-dimensional gas chromatographic technique.[92]

In GC×GC, the first column has conventional dimensions (generally 30 m long, 0.20 mm internal diameter and approximately 0.2 or 0.3 μm of stationary phase thickness). Still, the second column is short and narrow, such as the columns used in fast gas chromatography, with a length of up to 2 m, an internal diameter of approximately 0.10 mm, and approximately 0.1 μm of stationary phase thickness. Thus, the elution in ^{2}D takes place in a few seconds before the injection of the next fraction by the modulator without the extension of the total run time. The columns should ideally be orthogonal with separation principles independent and different, such as using a low polarity column in ^{1}D and a high polarity column in ^{2}D.[93]

The modulator is considered the heart of the GC×GC system, allowing the effluent from the primary column to be focused and periodically injected into the second column. Furthermore, due to this effluent focus, the peaks obtained at the end of the second dimension are narrower, improving the sensitivity of the technique.[94] Several modulators are commercially available; those based on temperature, with heating or cryogenic interfaces, and valve-based modulators that use pneumatic means to perform the modulation. However, cryogenic modulators are the most widely used due to their successful application for many types of compounds and their ability to produce very small peak widths.[94]

The time of a complete modulation cycle corresponds to the modulation period, the same separation period in the second dimension. As all effluent from the first column passes into the second simultaneously, the total analysis time is equivalent to the time used for separation in the first column, as described in a one-dimensional analysis.[95]

GC×GC presents itself as a well-established analytical technique to elucidate complex matrices such as those used in metabolomic studies.[96,97] This technique allows a detailed profile of known compounds (targeted analytes), two to five times larger than those for one-dimensional chromatography. Furthermore, two-dimensional chromatograms are highly accurate and allow the acquisition of a unique fingerprint of a given sample. The fingerprint of a sample is essential when you want to discover changes due to metabolism over the years or by exogenous means. It can be a very effective alternative in the metabolomic study of damaged skin.[96,98]

Welthagen *et al.*[99] demonstrated the power of a comprehensive two-dimensional gas chromatography analysis by studying metabolite profiles of mouse spleen tissue. A GC×GC-MS method showed that the quality and sensitivity of the mass spectrum were improved by the better resolving power of the GC×GC. This work identified 1200 metabolites instead of 500 compounds identified in one-dimensional chromatography. Furthermore, the results showed that the identified compounds were very similar to those previously reported in NMR studies and other methods in mammalian tissues.

4.2. Some limitations based on metabolomics by GC-MS

Although GC-MS is an efficient technique for the analysis of several compounds, it has some limitations that must be taken into account in metabolomics studies, especially when the metabolomics approach is untarget. The main limitation is due to its nature based on the analysis of volatile and thermally stable small molecules. As a consequence, other problems can arise due to the derivatization step, which ranges from the time-consuming preparation of the sample to inaccurate quantifications due to incomplete derivatization of the analytes. Another factor is due to the conversion of different forms of metabolite derivatives during the derivatization reaction, which produces a sample where there are different forms of the same original metabolite. One factor that can reduce these inconveniences is through the use of standard derivatized compounds and data correction strategies to normalize this bias.[100]

5. Data Interpretation

Metabolomic measurements produce an enormous amount of data that require a high capacity of investigation

and interpretation to provide a wealth of information on the biochemical status of cells, tissues, or organisms. Data processing can occur in two distinct stages. The raw data are filtered, detected, aligned, and normalized in the preprocessing to simplify the subsequent analysis. Then, the analysis stage uses univariate or multivariate artifacts to interpret the processed data.[51]

For MS-based chromatographic information, the preprocessing step primarily aims to improve peak visualization by removing noise. In detection, all signals that present relative intensities above noise are identified, and the alignment aims to correct differences in retention times between runs and combine data from different samples.[51,101-103]

The data, after pre-processing, must be analyzed through statistical analysis to show relevant biological information. The statistical method applied depends on the type of information to obtain. Unsupervised methods can be used if the objective is to summarize, explore, and discover when previous information about the identity of the sample is unknown. Hierarchical cluster analysis (HCA), principal component analysis (PCA), clustering, independent component analysis (ICA), or a type of neural network such as the self-organization map (SOM) are the most widely used.[101,103]

For example, PCA was used for Randhawa *et al*.[17] to compare the global pattern of metabolites in skin biopsies obtained from sun-exposed and protected sites. The results clearly demonstrated that sun exposure altered the metabolic profile in skin biopsies exposed to the sun. A subset of 122 metabolites was significantly different (p-value < 0.05) with a false discovery rate threshold of less than or equal to 5% between the two sets of samples. Of the 122 metabolites, 46 were minor and 76 were major in biopsies extracted from sun-exposed sites compared to biopsies from sun-protected regions. The identified metabolites belonged to a total of 52 biological pathways and a subset of 42 pathways had one or more significantly different metabolites. These pathways encompass amino acids, nucleotides, sugars, peptides, cofactors, lipid metabolism, and others. On the other hand, supervised methods are applied when the identity of the sample is known and these methods aim to classify the biomarkers following their characteristics. Analysis of variance (ANOVA), partial least square (PLS), discriminant analysis of partial least square (PLS-DA), discriminant analysis of partial orthogonal least squares (OPLS-DA), and support vector regression (SVR), among others, have been used in metabolomic studies.[21,101,103] The profiles of mouse skin primary metabolites in response to UVB irradiation, for example, were evaluated by the OPLS-DA method to identify discriminable variables between the experimental groups. Amino acids, organic compounds, fatty acids, lipids, carbohydrates, and *cis*- and *trans*-urocanic acid (UCA) were identified as discriminators that characterize the differences between the groups. Additionally, radiation exposure time was investigated and analyzed by the same method. The authors concluded that chronic UVB exposure for 12 weeks had a greater impact on a greater number of primary metabolites than UVB exposure for 6 weeks. However, the *cis*-UCA metabolite and cholesterol showed the most dramatic changes at 6 and 12 weeks, respectively.[83]

Machine learning (ML) algorithms can also be applied to analyze metabolomics data sets to provide varied perspectives on cellular metabolic processes. ML can elucidate complex cellular mechanisms, identify molecular signatures, and predict clinical results of large biomedical data sets. In addition, ML can be used in multi-omic approaches to integrate more than one method such as genomics, proteomics and metabolomics, where the amount of data is much larger and the integration more complex.[104] Additionally, this artifact can facilitate integrative analysis by effectively addressing data heterogeneity, missing data, class imbalance, scalability of problems, and the curse of dimensionality. The number of variables is different from the number of samples.[104]

ML provides a wide spectrum of developed algorithms with easy-to-use interfaces. Data analysis based on artificial neural networks (ANN), convolutional neural networks (CNN), random forest (RF), support vector machine (SVM), genetic algorithm (GA), among others, are recent learning tools for supervised machine metabolomic analysis.[105]

The random forest, for example, was also used by Randhawa *et al*.[17] to discover a metabolic signature that would determine the state of photoexposed skin. The authors performed this classification on the entire dataset, significant and non-significant. From these data, 30 metabolites were ranked in order of importance between exposed and unexposed skin samples. As a result, the authors found *cis*-urocanate to be the third largest reduction in misclassification, corroborating other works that indicated this compound as a possible biomarker of UV damage.[17] The new ML subdomain that incorporates complex artificial neural network architectures, known as deep learning (DL), presents a powerful approach to omic data integration and precision medicine. This tool is efficient because it is capable of encoding and modeling many forms of complex data, such as numeric, text, audio and image data in both supervised mode (biomarker identification) and unsupervised configurations (anomaly detection).[106] The last step is to interpret and correlate the data with the biological context of the study. An extensive

number of databases containing detailed information on various metabolites are employed for data interpretation. Databases include the Human Metabolome Database (HMDB), MassBank, METLIN, and lipid metabolites and pathway strategy (LIPID MAPS), and PubChem.[20,103,107,108]

Metabolic data can also be integrated with other omic sciences to interpret all biological processes involved. Network-based visualization tools can be widely applied in addition to integration with biological knowledge derived from previous literature and/or experimental data. Tools like MetaboAnalyst, MetaCore TM, 3Omics, and InCroMAP can be used. Among them, MetaboAnalyst is a complete platform based on the integrated free access Web tool.[20]

Finally, the processing of data in general and the characterization of metabolites must be compatible with the experimental procedure and research purpose. However, due to the diversity of data generated, the choice of a treatment model is still very complex. It requires more knowledge and studies to be applied and discussed, which is beyond the scope of this review.

6. Biomarker Identification and Biological Interpretation

The chemical composition of the skin matrix contains an enormous amount of information about metabolic processes. As previously presented, recent studies[17,83] using GC-MS showed that several metabolic pathways were affected by exposure to solar radiation; most of these pathways indicate an increase in the production of reactive oxygen species (ROS), which resulted in increased oxidative stress that may be responsible for changes in the phenotypic appearance of sun-exposed skin. Metabolites such as amino acids, organic compounds, fatty acids, lipids, nucleotides, carbohydrates, and *cis*- and *trans*-UCA were identified as discriminators that characterized the differences between the groups. However, it is observed that in most of these studies, the UCA metabolite was the one that showed the greatest significant difference between photoexposed skin and unexposed skin.[17,83]

UCA is found largely in the stratum corneum in its *trans*-UCA form. This isomer plays an important role in sun protection[109] and has been proposed as the main acid-base regulator of the epidermis.[110] Interest in UCA has increased in recent years due not only to its beneficial properties but also to its harmful effects to the skin caused by the photoisomerization of the *cis*-UCA isomer by UV exposure. Unlike the *trans* isomer, *cis*-UCA produces pro-apoptotic intracellular acidification, oxidative deoxyribonucleic acid (DNA) damage and triggers specific immune responses, which can nullify any protective effect.[110,111] This fact indicates that UCA may be a signaling pathway for discovering a biomarker that indicates damage caused by UV exposure. However, it should be noted that their roles in skin homeostasis are still complex and require further investigation.

Metabolite changes caused by skin photoaging were also associated with the tricarboxylic acid (TCA) cycle. Moon *et al.*[15] found that dihydrolipoyl dehydrogenase (DLD) was the only TCA cycle protein that showed a decrease in expression after exposure to ultraviolet radiation in mice. And to confirm their results, using GC-TOF/MS, they identified 2 metabolites, malic acid and fumaric acid, that were downregulated after UVB induction. With the results, the authors correlated that the decrease of the DLD enzyme can contribute to the increase of oxidative damage, reduction of energy metabolism, regulation of Fe metabolism and metabolic acidosis. Therefore, DLD regulation may be an important target for skin photodamage.[15]

GC-MS analyses were also applied to the metabolomic study of volatile organic compounds to discover skin cancer biomarkers. The authors identified elevated levels of lauric acid (C12:0) and palmitic acid (C16:0) in melanoma which correlated with increased oxidative stress and also as a consequence of deregulated lipid synthesis, a known feature of cancer.[112]

Masutin *et al.*,[113] in a review study, obtained an overview of the metabolic changes of the skin under conditions altered by either internal or external factors and identified by several types of analytical platforms, including GC-MS. The authors identified 364 metabolites from a cross-comparison of studies extracted from different databases and assigned the corresponding metabolic pathways and the most strongly affected signaling pathways were identified. Lipid metabolism, the Krebs cycle, purine metabolism, and pentose phosphate pathway were some of the pathways affected by these changes. In summary, it can be seen that mainly energy metabolism, lipid metabolism, antioxidant defense, and DNA repair systems were affected by the factors studied in this work. In this sense, it is possible to observe that several types of research have been done to identify possible markers of damaged skin based on metabolomics. However, they are still in the early stages and are still very focused on photoaging and cancer. Therefore, greater efforts must be made to obtain more concrete answers for the metabolomic analysis of damaged skin, not only from UV radiation or cancer, but also for other areas of clinical experience such as dermatitis, psoriasis, eczema, changes caused by environmental pollutants, chemical agents, among others.

7. Multi-Omics Integration toward Systems Biology

Multi-omics integration can be an immensely powerful resource for studies on damaged skin as it takes an integral view of the molecules that make up a cell, tissue, or organism. The first applied omic approach was genomics, which is intended for the universal detection of genes; followed by transcriptomics, which is intended for the study of ribonucleic acid (RNA) transcripts; then proteomics, which is the study of proteins that are produced by cells or organisms; and more recently metabolomics, which adopts a comprehensive analysis of metabolites.[18]

Genomics, transcriptomics, and proteomics have already been widely applied to the study of damaged skin.[12-14,114-117] However, skin research based on metabolomics data is still in its early stages. Therefore, it is often necessary to integrate these different omic methodologies to find reliable biomarkers to be applied in clinical practice.

Kuehne *et al.*,[118] for example, identified that the combination of transcriptome and metabolome data was essential to understand the metabolism of intrinsic skin aging. From the transcription data, it was possible to analyze changes in metabolism during aging, such as changes in glucose, glycolipid biosynthesis and decreased protein and polyamine biosynthesis.

Finally, metabolomics can be a fundamental complement to the broader understanding of biological systems when integrated with these other omic sciences because the metabolome represents the sum of all metabolites that can be generated or decomposed by cells as the final products of complex interactions at the genomic, transcriptomic, and proteomic levels.

8. Conclusion

This review demonstrated that applying GC-MS has been a very useful and effective tool in the metabolomic study of damaged skin. Furthermore, according to the works presented, using two columns coupled in series (GC×GC) together with high-resolution mass spectrometers improves separation efficiency, offers greater selectivity, mass resolution, and sensitivity when applied to complex biological samples.

In general, changes can be observed in metabolites derived from human skin tissue or cell samples, for example, *cis*- and *trans*- UCA in photoexposed skin or lauric acid (C12:0) and palmitic acid (C16:0) in melanoma, in addition to some metabolic pathways affected by sun exposure, such as the tricarboxylic acid cycle, which has been correlated with oxidative stress. These studies[17,83,112,113] advance greatly in understanding the processes of deterioration and in assessing repair mechanisms. However, there is still nothing conclusive about discovering biomarkers, as the metabolomic study of damaged skin is still in its early stages. Thus, understanding metabolomics along with other omics science and clinical data is fundamentally important in determining predisposition, diagnosis, prognosis, and predictive biomarkers. Finally, the continued advancement in the implementation of precision medicine aims to improve the traditional practice of medicine based on symptoms. These advances lead us to propose new studies on integrating biomedical sciences and technologies applied to data processing, to apply safer early interventions using predictive diagnoses and personalized treatments for each type of patient.

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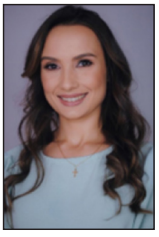

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