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Modern sample preparation approaches for small metabolite elucidation to support biomedical research☆

Sofia Malcangi[a], Monica Romagnoli[a], Marco Beccaria[a], Martina Catani[a], Tatiana Chenet[b], Chiara De Luca[a], Simona Felletti[a], Luisa Pasti[b], Alberto Cavazzini[a], Flavio Antonio Franchina[a,*]

[a] University of Ferrara, Department of Chemical, Pharmaceutical, and Agricultural Sciences, Via L. Borsari 46, 44121 Ferrara, Italy
[b] University of Ferrara, Department of Environmental and Prevention Sciences, Via L. Borsari 46, 44121 Ferrara, Italy

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ABSTRACT

The analysis of biological samples of clinical interest has certainly many analytical challenges. Among these, the preparation of samples for the determination of metabolites of interest is of paramount importance to avoid error propagation to the following separation/detection steps, but also to simplify the data elaboration. Sample preparation represents the initial key step for the acquisition of reliable and interpretable information. In this review, we showcase and discuss the most recent sample preparation techniques used for biological samples, limiting to those extracting the small (volatile or volatilizable) metabolites, thus those applications which enable the subsequent GC analysis. Essentially, these include the group of sorbent-based and liquid-based sampling techniques. Dedicated sections devoted to both assisted (i.e., ultrasound, microwave) extraction techniques and chemical derivatizations will also be presented. In the discussion, the importance and recent trends towards the development of green solutions in sample preparation, *via* automation and miniaturization, are highlighted.

1. Introduction

The study of metabolites in biomedical research is in constant development, making it one of the most active and promising fields of application. Studies are mainly driven by the quest for novel diagnostic molecules (or biomarkers) of health and altered states, and this rationale relies on the fact that metabolites (and their level) are affected at the early stage of the disease. Early diagnosis, together with an increased understanding of the pathological conditions, would improve patients' life quality and expectations.

However, there are several challenges associated with biomarker discovery that are related to the stability, variability, and complexity of the biological matrices (i.e., biological fluids, biopsies/tissues, cell cultures). Also, biological samples contain thousands of metabolites with a wide range of concentrations and physicochemical properties, which complicate further the analytical scenario. Indeed, a universal analytical platform, able to exhaustively cover and analyze such a large assortment of metabolites, does not exist, and multi-platform strategies should be pursued ideally [1].

No matter the instrumentation in use, it is important to get high-quality data with narrow confidence intervals to correlate and merge with other biological/clinical information for their interpretation. For this reason, devoting enough time to optimizing and tailoring each of the succeeding steps in the analysis workflow becomes essential. However, for practical limitations (e.g., time, resources, domain knowledge, etc.) this is not always bearable, and compromises are accepted. Definitively, a strong foundation of the early analytical steps assures a smaller propagation of errors and bias, before the final interpretation of the results. Quality control procedures can be implemented at each step of the entire workflow to monitor the performance and avoid misleading drift or bias.

Abbreviations: BTEX, Benzene, toluene, ethylbenzene and xylenes; DHS, Dynamic headspace; DI/HS-SPME, Direct immersion/Headspace solid-phase microextraction; DLLME, Dispersive liquid-liquid microextraction; DVB/CAR/PDMS, Divinylbenzene/Carboxen/Polydimethylsiloxane; EME, Electromembrane extraction; GC×GC-ToF MS, Comprehensive two-dimensional gas chromatography coupled to time-of-flight mass spectrometry; HF-LPME, Hollow-fiber liquid-phase microextraction; LLE, Liquid-liquid extraction; LLME, Liquid-liquid microextraction; LPME, Liquid-phase microextraction; MAE, Microwave-assisted extraction; MEPS, microextraction by packed sorbent; NTD, Needle trap device; NTE, Needle trap extraction; P&T, Purge and trap; POPs, Persistent organic pollutants; SBSE/HSSE, Stir bar sorptive extraction/Headspace sorptive extraction; SDME, Single-drop microextraction; SPE, Solid-phase extraction; SPME, Solid-phase microextraction; TD, Thermal desorption; TFME, Thin film microextraction; UAE, Ultrasound-assisted extraction; (s)VOC, (semi)Volatile organic compound.

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* Corresponding author.
E-mail address: frnfvn@unife.it (F.A. Franchina).

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Without questioning the importance of a proper study design to define appropriate sampling, control/case groups, sample number and replicates, etc., the actual processing of the sample is the most time-consuming and error-prone process.

The intention of this review is to feature the most used sample preparation techniques used for biological samples, especially considering those with a great premise in automation and in reducing the environmental impact. We focus the discussion on those techniques and applications aiming at extracting small molecules for the high-resolution analysis involving GC–MS for the following separation and detection.

2. Significance of small metabolites and associated analytical challenges

Metabolomics gathers several disciplines, from biochemistry to computer science with applications embracing many features of biological and biotech research (e.g., drug and biomarker discovery, toxicology, nutrition and food control, host interactions, etc.) [2]. The information on metabolites complements the upstream biochemical information obtained from genes, transcripts, and proteins, enlarging current genomic reconstructions of metabolism, and improves our understanding of cellular processes, physiology, and medicine by linking metabolic pathways to biological mechanisms.

A classic example of the indication of certain diseases using small molecules is the peculiar odor from the human body, an idea which was introduced 400 years BC by Hippocrates. He was the first using these odorants to gain insights into pathogenesis, and his essays describing the *fetor oris* and *fetor hepaticus* are notorious. Using these terms, he referred to the specific bad smell resulting from breath or from sputum and originating by the putrid humors in the stomach caused by liver failure [3]. Other body fluids odors were used in the past for the diagnosis of illness, like patients' urine: the smell of a rotten apple was considered as being a sign of diabetes condition [4]. Also naturally released microbial odors from bacterial cultures (*M. tuberculosis* and *P. aeruginosa*) were reported in the early 20th century as the result of their metabolism [5]. These antique olfactory assays provided clues leading to early diagnosis of disease or indication of microbial activity; nowadays instead, the availability of modern chemical instrumentation makes possible to analyze these odors more rationally and to chemically identify them.

These are just a few examples of the relevance of the more volatile metabolites. Many other small chemical entities are physiologically released from different parts of the human body, as products of metabolic processes, whose presence and amount characterize each individual. Identifying and understanding these metabolite patterns may lead to a new era for diagnosing various diseases and personalized medicine based on specific biomarkers.

Despite the wide interest, there are many analytical challenges along different points of the metabolomics workflow (from study design to data analysis), which make the progression and establishment of the discipline tedious and slow [6]. The analysis of metabolites can be carried out on a large variety of organisms and biological samples. Nowadays, the main investigated sources of metabolites are represented by urine, blood serum and plasma, breath, secretions, tissues/biopsies, and *in vitro* cell cultures. Each source of metabolites has its own peculiar challenges (e.g., the matrix heterogeneity and interferences, stability, physical state of the sample, etc.) generating a cascade of hurdles with the following analysis steps.

The host produces endogenous metabolites naturally or in response to a pathological condition, while exogenous metabolites are instead taken up by the organism because of interactions with the environment.

From an analytical standpoint, monitoring all these metabolites usually requires efforts to isolate them from the sample, to elucidate and identify their chemical structure, to quantify their real concentration level. When one (or a set of them) is characteristic of a specific condition, then it (or they) can be considered as biomarker(s). Indeed, a biomarker measures a given biological characteristic considered representative of a biological or a pathological status.

The metabolic profile over time, the chemical stability, and the sensitivity of the analytical method should be considered when selecting a biomarker as an unambiguous indicator of exposure or disease. Therefore, implementation of biomarker-related studies needs a sensitive and reliable methodology. The identification of biomarkers and progress in related bioanalytical methodologies for the diagnosis of several diseases in early stages is currently a hot topic in the clinical studies. In addition to biomarkers linked to disease, there is another group of biomarkers that might assess the individual exposure to specific external factors (e.g., the diet and environment) which can alter or influence the health status; these are referred as biomarkers of exposure [7].

The research studies with a more clinically thoughtful study design fall into two types of approaches, namely cross-sectional and longitudinal studies, depending on the research question(s). The main difference between the two approaches is that the first one compares different sample groups at a single time point, like a snapshot. Typical examples of this approach involve the differentiation of a particular atypical condition (e.g., infection, inflammation, etc.) versus a typical condition (e.g., healthy controls). Longitudinal studies instead involve several observations of the same sample-type (or subject) over time. Typical examples of this approach are the observation of a specific state progression, or metabolism kinetics.

3. Toolbox of sample preparation techniques for biomedical GC applications

Complex biological samples, such as biofluids and tissues, require efficient and high-throughput approaches to remove interferences from the sample matrix and to pre-concentrate the analytes of interest prior to analysis. This is particularly true when the analysis continues with GC separation, since non-volatile or solid residue can negatively affect the chromatographic apparatus. In addition, because sample preparation is the first step in the entire analytical procedure, selecting the optimal extraction technique and conditions is critical for further sample handling and data interpretation. This step can be the main source of errors, affecting method precision and accuracy, and may consume over 80% of analysis time [8]. Additionally, sample treatment is considered the most polluting step in an analytical workflow, being responsible for most of the generated waste.

Different sample preparation techniques have been developed based on the type of analytes and sample matrices [9]. Depending on the condition used, each extraction procedure differs in speed, selectivity, ease of use, and automation possibility. The most suitable method and conditions should be carefully selected and optimized, keeping in mind the objective and the sample matrix (e.g., high/low-fat content, liquid/solid/gaseous sample). Usually, the extraction process can be either solvent-based or sorbent-based, depending on whether the analytes of interest are partitioned into an extraction solvent or a sorptive material.

The recent and continuous attention to greener and sustainable concepts in general chemistry has driven towards the same direction also the sample preparation techniques. In this context, the main efforts were and are being focused on reducing (i.e., miniaturization) or eliminating the organic solvents, and in automatizing (i.e., automation) the procedures.

In general, this wave of developments in sample preparation includes the continuous shift from laborious, time-consuming, and multi-step methods to simple, more integrated, and automated protocols for fast and reliable analysis. In contrast to the classical liquid-based extraction (LLE), the shift towards liquid microextractions was accompanied by the development of the more recent sorbent-based techniques, in which the analyte is extracted and concentrated in a sorbent phase.

In fact, both miniaturization and automation of sample preparation procedures are among the most interesting ways to make analytical procedures more environmentally friendly [10]. Automation of analytical

methods has advantages such as reducing the analysis time and errors, faster sampling, and delivering higher sensitivity and greater reproducibility, in addition to facilitate the acceptance and diffusion of that analytical technique.

In the following sections, the most relevant techniques and their use for the GC determination of metabolites of biomedical relevance, will be reported and discussed. **Table S1** lists the applications and key information described in the following sections.

The necessity to have GC-amenable analytes makes the sample preparation tailored to a reduced number of metabolites. This implies the removal of these analytes of interest from the matrix bulk, or vice versa (extraction or purification). By nature, headspace (HS) techniques hold the capacity to extract easily the more accessible metabolites (VOC/sVOC) from the sample, in contrast to other immersion or liquid-based sampling techniques. However, because of the different penetration into sample matrix, the amount of information (thus, the extracted analytes) is totally different. Also, HS techniques find in GC–MS separation the most natural and powerful partner for the intrinsic nature of the analytes and the separation/identification capability of the hyphenation.

Another important consideration can be done for analyte extraction procedures in biomarker translational studies. Analyte extraction and conditions should be indeed carefully considered and ideally maintained identical when *in vitro* studies have to be compared to *in vivo* ones for biomarker discovery and translation. This is important to focus exclusively on biological differences and minimize the variability given by the differences in the sample preparation techniques. Each sample preparation technique is indeed characterized by peculiar properties (loading factors, exhaustiveness, sensitivity, selectivity). When they are not used consistently among *in vivo* and *in vitro* research, the translation of biomarkers might be troublesome.

3.1. Sorbent-based sampling techniques

This most recent group of sample preparation techniques makes possible the recovery and reuse of the sorbent material, which is instead more complicated in liquid-phase extractions [11].

Sorbent-based sampling techniques minimize, or even eliminate, the use of organic solvents in the entire procedure, thus reducing the environmental impact. In addition, these methods have high preconcentration capabilities (this latter advantage is also present in LPME), and a tunable selectivity due to the broad variety of solid materials available with specific affinity interactions towards the target compounds.

Furthermore, a wide variety of sorbent-based microextraction techniques have been developed over the last years due to the versatility of different sorbent materials for being: (i) packed in small devices, (ii) dispersed along the sample matrix, or (iii) coated on a solid support. The sorbent phases can be used either via a flow-through method, in direct immersion (DI), or in the HS mode [12].

The invention of SPME surely marked the new era of sorbent-based microextractions, in which low sorbent amounts and low (or none) solvent volumes are exploited for high analyte enrichment. A brief survey of SPME together with other relevant sampling techniques and variations (i.e., DI/HS-SPME, SBSE/HSSE, DHS, NTE, MEPS) for applications of clinical interest will follow, accompanied with the recent research works involving GC-based separations.

3.1.1. Solid-phase microextraction

Solid-phase microextraction (SPME) is the iconic modern sample preparation technique integrating sampling, preconcentration, and extraction into a single step. Furthermore, trapped analytes can be directly and easily introduced into the GC system. It is a non-exhaustive technique, and it probably represents the most automatable and GC-integrated sampling technique, which makes SPME very attractive and accessible nowadays.

The most used form of SPME consists of fused silica fibers coated with the extraction phase (single or in combination), which are exposed to the headspace of, immersed in, or put in direct contact with the sample [13]. Considering the chemical complexity of biological samples and metabolites GC-suitability, the most used approach in GC biomedical applications sees its use in the headspace [14].

SPME also paved the way for the development of novel sorbent-based microextractions and configurations. For example, different geometries and sorbent amounts have allowed the development of thin film (TF) and “arrow” SPME. Thin film microextraction (TF-SPME) initially emerged as an alternative to classical SPME, which provides a higher volume of extractive phase as well as a larger surface-to-volume ratio compared to SPME fibers, which results in an improved sensitivity with relatively shorter extraction times.

Several HS applications have been recently developed for the discovery and monitoring of both endogenous metabolites and exogenous compounds.

Regarding the exogenous compounds, Antonucci et al. developed and optimized a multiresidue HS-SPME method for the determination of BTEX, MTBE, ethyl tertiary-butyl ether, tert-amyl methyl ether, and diisopropyl ether in urine (14 mL) [15]. The method was validated by testing its linearity, precision, recovery, accuracy, and detection and quantitation limits. The authors collected urinary samples from 40 children exposed to different levels of the monitored compounds according to living environments and environmental tobacco smoke exposure and quantified the target compounds.

Vaníckova et al. applied HS-SPME on two analytical GC–MS platforms (an GC-orbitrap MS and a GC × GC-ToF MS) to study the volatile profiles produced by human peripheral blood monocytes [16]. In this *in vitro* study, the optimal fiber resulted in the triphasic DVB/CAR/PDMS in terms of VOCs coverage and chemical interference. The authors emphasized the differences in the volatile profile difference between the studied groups/conditions (the media and the blood monocytes, the different growth times, and the presence of inflammatory agents) and highlighted potential *in vitro* inflammatory biomarkers for further clinical studies. They also remarked on the advantage and effectiveness of the multidimensional technique for the separation and discovery of a consistently higher number of metabolites.

Also *in vivo* research can be carried out with SPME. For example, a study for the efficiency evaluation of non-invasive *in vivo* TF-SPME sampling of saliva, compared to *ex vivo* sampling, for the retrospective detection of substances consumed was reported [17]. The authors used both LC and GC platforms, and regarding the GC-amenable compounds, they detected alcohols, aldehydes, aromatic compounds, benzocaine, carboxylic acids, fatty acids, caffeine, etc. The sampling methodology involved the use of a pre-loaded calibrant on the fiber to correct for differences in sampling conditions and provide quantification of substances present in saliva. The results showed that short *in vivo* or *ex vivo* SPME sampling (thanks to the TF geometry) coupled to LC-MS and GC–MS analysis is sensitive enough to provide detection of hydrophilic compounds and acidic compounds, such as acetaminophen, caffeine, and benzocaine.

Another interesting study involving the use of TF-SPME was focused on the VOC profile of *in vitro* cancer cellular lines [18]. Notably, in addition to different TF coating evaluation, extraction time, and temperature optimization, the authors compared two different sampling protocols to avoid contact between the biological material (potentially infectious) and the sampling apparatus (**Fig. 1**). However, the extraction efficiency in this alternative sampling resulted in higher extraction rates for the conventional HS, probably because of deteriorated transfer through glass wool into the glass tube and the additional tubing.

An example of DI-SPME was conducted on saliva for the quantitative analysis of 13 cannabinoids of both natural and synthetic origin [19]. The half-life of drugs in saliva is short, thus this methodology is useful to reveal recent drug consumption (i.e., within hours from the sampling). Technically interesting for the DI mode, it was reported that a simple

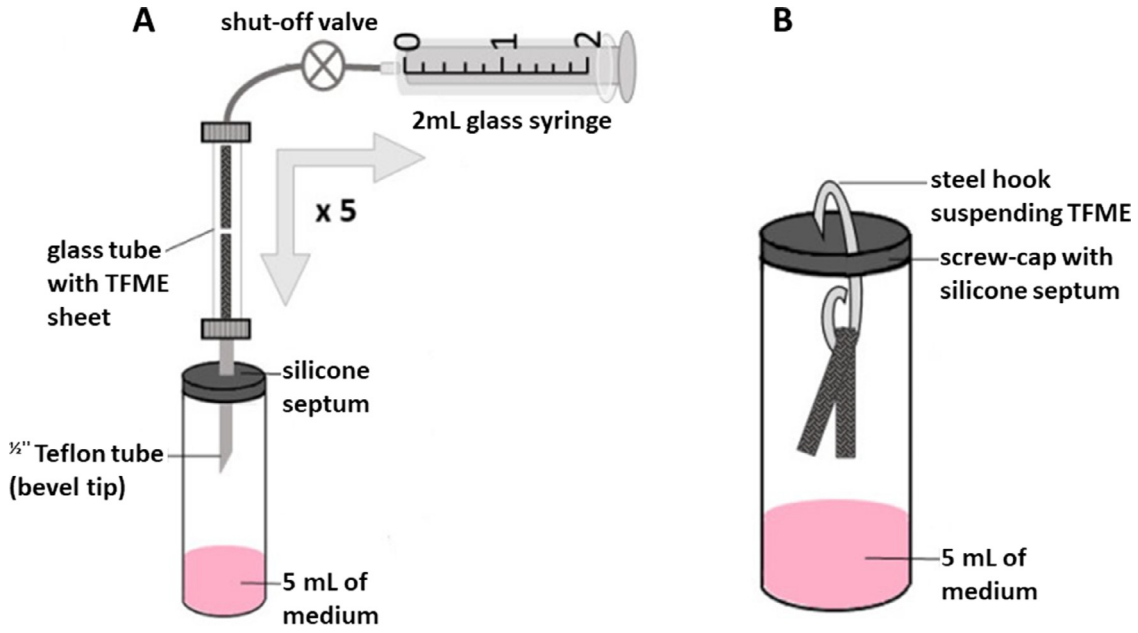

Fig. 1. Modes of HS sampling: (A) External headspace TFME mesh was placed inside a sorption glass tube exposed to the sample via a 1/4″ Teflon tube (flushed five times in both directions prior to extraction), (B) Internal headspace, where TFME was suspended on a steel hook inside the tightly closed 20 mL glass vial containing 5 mL of liquid sample. Adapted from [18].

centrifugation step was able to remove most of the proteins and other potentially interfering components, avoiding significant interference from the matrix and making the sample pretreatment fast and simple.

3.1.2. Stir-bar sorptive extraction

As a technique derived directly from SPME, the stir bar sorptive extraction (SBSE) is based on the same principles of distribution of the analytes between the sorbent and the sample. As SPME, the stir bar can be immersed (classical SBSE) or suspended in the headspace (HSSE) of the samples. This technique is based on the use of a small device consisting of a magnetic bar which is jacketed in a glass tube coated by the sorbent, that is PDMS in most of the cases. Generally, the sorbent amount is 50–300 times higher than the sorbent amount used in SPME, providing a significant increase in enrichment factors and giving at the same time higher extraction efficiency thanks to the larger volume and surface area [20,21]. Moreover, SBSE desorption can be directly obtained in a thermal desorption (TD) unit in many GC systems, so it maintains one of the main advantages derived from the use of SPME. However, the entire extraction procedure is more difficult to automatize fully, affecting the analysis throughput.

The SBSE coupled with GC–MS has been used to extract and analyze mainly hydrophobic organic compounds from aqueous samples. However, a variety of laboratory made coatings have been developed to extend the field of application of SBSE, especially to the extraction of polar compounds, as well as to improve its selectivity [22].

A recent study presented a modified HSSE collection system for metabolites from a human airway cell culture system [23]. Preliminary, the two volatile signatures from the liquid below cells (SBSE) and the headspace above cells (HSSE) in cell culture samples and in media controls were compared. From this evaluation, HSSE mode was chosen because it yielded better results regarding the distinction of the cell cultures from media controls. Also, a comparison with the HS-SPME was carried out, as shown in **Fig. 2**, in which the authors reported an increase in recovery of 52 times.

Similarly, Bean et al. used SBSE for *in vitro* experiments to study the antifungal activity of VOC produced by different isolates of *C. vaccinii* co-cultured with common plant pathogens (5 different strains of fungi) [24]. The authors placed 3 stir bars, coated with both PDMS and polyethylene, in the sandwich plate used for culture and analyzed both the single culture and the various co-culture combinations. The VOC analysis revealed that the volatile metabolomes of both the fungi and the bacteria are altered in co-culture compared to their respective monocultures. Indeed, as a protective behavior, also the fungi species release defensive VOCs to reduce the production of antifungals by *C. vaccinii*.

The SBSE is a very well-suited technique for contaminant analysis, also for samples of biological origin. For example, a quantitative method exploiting SBSE and using isotope dilution mass spectrometry was developed to quantify POPs in human serum and whole blood samples [25,26]. Because of the non-polar nature of the targeted contaminants, PDMS-coated stir bars were used. Traditional POPs classes were included (i.e., polychlorinated biphenyls, polybrominated diphenyl ethers) for the optimization of extraction conditions, along with compounds that have not traditionally been classified as POPs but show similar behavior in volatility, environmental persistence and ubiquity, and hydrophobicity. The authors highlighted the importance and robustness of the use of the direct isotope dilution for quantification purposes [25].

Similarly to TF-SPME, the use of a thin layer geometry of the sorbent demonstrated significant advantages due to its flat configuration and high surface-to-volume ratio. Indeed, a larger volume of the thin film results in higher sampling capacity, and thus higher sensitivity. This “sorptive tape” extraction finds application in the collection of small metabolites secreted from skin, which are typically present at very low concentrations.

Also, the flat geometry of the thin film provides great flexibility for *in vivo* sampling. Both headspace and direct contact sampling are easy to conduct, and air contamination is reduced. As for the SPME and the SBSE, sorptive tape sampling can be directly coupled with a GC instrument for thermal desorption, which simplifies the sample preparation steps and improves the sensitivity.

This approach has found interesting use in the passive *in vivo* skin VOCs analysis.

Pawliszyn and coworkers used a thin PDMS sorptive pad as the extraction phase for skin VOCs sampling. They evaluated the approach reproducibility and storage conditions to minimize sample loss and external contamination [27]. In this study, a piece of stainless-steel mesh

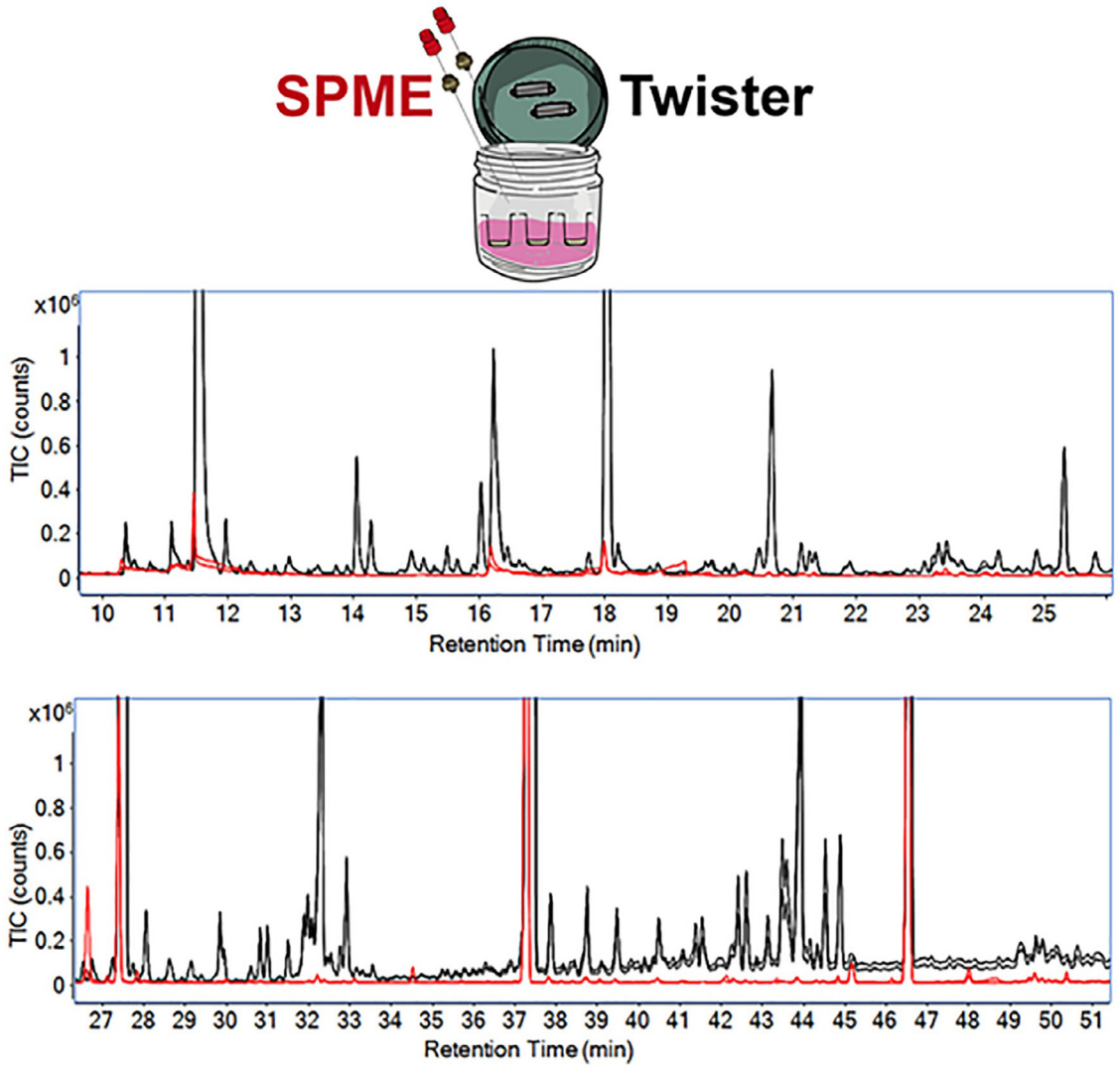

Fig. 2. GC chromatograms of HS-SPME (red trace) versus HSSE (black trace) sampling from the growth media control (at the top) and from the same cell culture (at the bottom). Reproduced from [23]. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

was used to avoid the pad from coming into direct contact with the skin. This "membrane sandwich" gave similar sampling efficiency for the most volatile compounds, while avoiding direct contact with lipids and dust, which are possible sources of issues for the GC apparatus. As for the storage conditions, being often impossible to analyze samples immediately after collection, the authors concluded that storing in dry ice is the best way to preserve volatiles on the membrane and prevent contamination. Similarly, Naudé et al. investigated the relation of specific skin VOCs with mosquitoes' bite [28–30]. The authors developed a PDMS minisampler in the form of a loop to be used non-invasively as bracelets or anklets (**Fig. 3**). This was placed in direct contact with the skin and covered with an aluminum sheet, everything secured with medical tape. After sampling (several wearing times were tested, from 1 to 9 h), the PDMS loop was thermally desorbed prior to GC separation. An internal standard was added to the PDMS samplers prior to sampling to alleviate possible variations. This approach proved to have great potential as a non-invasive sampling method for skin metabolite emissions/secretions.

3.1.3. Dynamic headspace extraction

The enrichment factor and the transportability have made the thermal desorption tubes a common device used for sampling, and especially for off-line breath analysis [31–34]. Indeed, numerous are the studies in these challenging clinical studies which relies on dynamic headspace (DHS) sampling, using TD tubes [35,36]. Many applications are focused on *in vitro* cultures to study the role of metabolites in a more controlled environment [37,38]. It is well known that antibiotic resistance in bacteria affects the production of small metabolites. Smart and coworkers highlighted the difference in the VOC production in sensitive and resistant urinary tract infections-causing bacteria [39]. In this study, the HS of 18 bacterial isolates (*E. coli, K. pneumoniae*, and *P. aeruginosa*) were sampled using TD tubes packed either with Tenax or a graphitized carbon black. Significant differences in volatile profiles were found between cephalexin resistant and sensitive isolates of *E. coli* and *K. pneumoniae*, and between ciprofloxacin sensitive and resistant isolates of the same bacterial species.

Analogously to SPME, also TD tubes can rely on several adsorbent materials designed to tune the selectivity toward specific classes of compounds. Small volatile metabolites bio-sampling in serum and exhaled breath using trap tubes was evaluated among different packing materials consisting of carbon molecular sieves, graphitized carbons and porous polymers, alone or in combination (Carboxen 1000, 1016, and 1003; Carboxen 1016 and 1003; Carbopack Y, X, and B; Carbograph 5TD and 1TD; Suficarb; Tenax TA) [37,40]. These studies highlighted the importance of the sorbent selection due to the different matrix contributions (e.g., the relative humidity effect on the sorbent type and complexity).

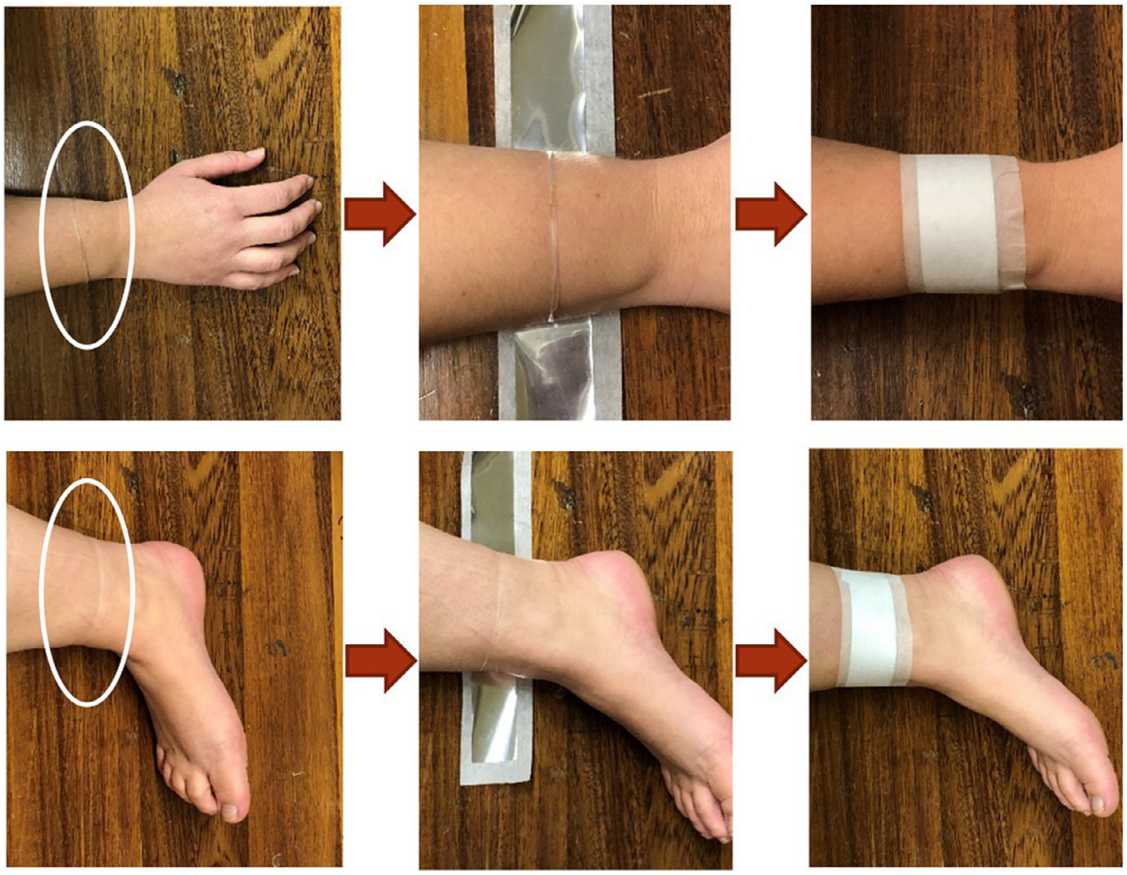

Fig. 3. Wrist (top) and ankle (bottom) skin sampling using PDMS samplers worn as bracelets (n = 3) and anklets (n = 3). The sampler is covered with a strip of aluminized sheeting and secured with surgical dressing tape. Reproduced with permission from [30].

Among the six tested sorbent traps, the tubes packed with Tenax TA showed the best sampling performance in terms of reproducibility and sensitivity, allowing longer metabolite tracking times and higher consistency of the measurements. The results reinforced the previous observations about the sampling for bacterial VOC [37].

DHS is definitively more suitable and used for analytes present at low concentration, for example, secondary metabolites in exhaled breath or blood [41,42]. Filipiak et al. investigated the effect of smoking habits as well as human exposure to indoor-air pollutants on the VOCs' profile in exhaled breath of more than 100 individuals. Common in this kind of analyses, exhaled breath (commonly 1–5 L) is first collected on container bags (different material are available, being Tedlar the most used), and then drawn into the TD tubes [43]. More recently, modern tools enabling to skip the Tedlar bag sampling, have been developed [33,41,44]. They are thought to minimize bias during the collection (volume, flow, portion of exhaled breath, sample loss, external contamination, etc.) and they are engineering devices which allow the patient to blow directly into the TD tube [33].

Kakuta et al. compared DHS and static HS-SPME for a multicomponent profiling of VOC in blood in two *in vivo* models, acute and chronic inflammation [42]. The DHS method showed higher sensitivity compared to classical SPME, being in addition able to extract the entire gas phase by purging the headspace of the vial. Additionally, the authors compared various sorbents for the extraction and evaluated the response of the target analytes generated on the GC–MS instrumentation, as shown in **Fig. 4**.

Purge-and-trap (P&T) is another dynamic extraction approach, classically used for water samples, which was recently explored in whole blood samples for the determination of BTEX [45]. Fresh blood was initially stored with heparin to minimize clotting. As for the P&T technique, blood samples were spiked with isotope IS and a defoamer before dilution in water. Authors optimized the purge time and temperature, as well as the desorption temperature and time on the analyte response.

3.1.4. Needle trap extraction

A needle trap device (NTD), also known as in-tube or needle trap extraction (NTE), is an apparatus utilized for VOC sample extraction for GC analysis. An extraction needle device can be used to perform the extraction of VOCs from either gas or aqueous samples. Since sample preparation using a needle extraction device is typically performed *via* active sampling, the sensitivity of the following analysis depends on the sample volume used and can be increased until the corresponding breakthrough amount of the analytes from the needle device is reached. This method can be seen as an upgrade of classical static HS extraction implementing a sorbent material, or, similarly, as a further miniaturization of DHS, or even as an approach in between them.

Analogously to DHS, when using an NTE device, an appropriate choice of the adsorbent needs to be made based on the volatility and/or polarity of the target analyte(s). Notably, the needle extraction device can be reusable, making the approach cost-effective for VOC analysis.

The needle extraction technique can be employed in combination with an autosampler, enabling high throughput in the routine analysis of VOCs.

A series of recent NTD applications can be recently found in biological fluids, such as in urine [46] and breath [47–49].

With this approach, smaller volumes can be sampled usually, for example, down to 2 mL for urine [46] and 20 mL for exhaled breath [47]. In the case of exhaled breath sampling, a thorough evaluation of parameters affecting the performance of NTE sampling can be found in [47].

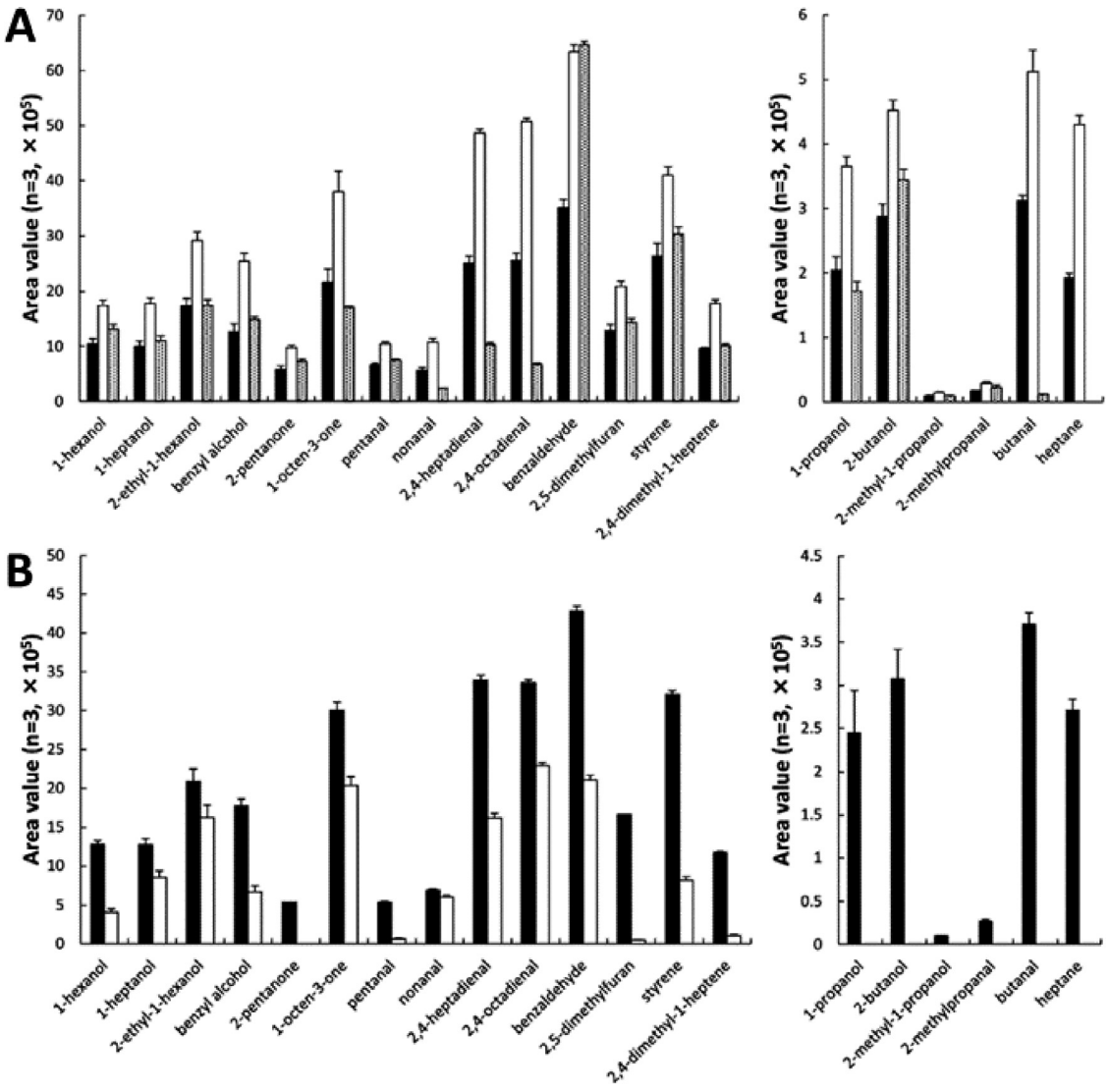

Fig. 4. A) Response comparison between different TD tubes packed with sorbents: Tenax TA (closed bars), Tenax GR (open bars), Carboxen100/Carbopack (dotted bars); B) Response comparison between DHS (closed bars, TD packed with Tenax TA) and HS-SPME (open bars). Reproduced from Kakuta et al. [42].

The importance of NTE or NTE-based approaches to gather relevant information from exhaled breath samples can be evidenced by a recent study showing that acetone concentration in breath increases by a factor of at least three in patients at home whose health conditions worsen following a heart failure relapse [48].

In another study, in order to provide an alternative source of information on gastric cancer-related biomarkers and their sources, a headspace needle trap extraction (HS-NTE) was developed [50]. Tissue samples were taken after gastric surgery (41 patients) and approx. 100 mg were used for the HS analysis (2-bed sorbent). The highly volatile species C_3–C_{12} were the target analytes. From this *ex vivo* analysis of tissue HS, the authors identified 32 VOCs, correlating them to the presence of cancer, smoking conditions, or bacterial infection.

3.1.5. Microextraction by packed sorbent

Microextraction by packed sorbent (MEPS) is a miniaturized form of SPE considered among the green sample pretreatment methodologies. MEPS needs only 1–2 mg of sorbent and is suitable for small sample volumes and can easily be interfaced with different chromatographic separation techniques without modification. The sorbent(s) can also be integrated into a liquid handling syringe that allows for low void volume and allows for sample manipulations, either manually or in combination with automated laboratory robotics. Nowadays, the MEPS is gaining acceptance in many fields, including bioanalytical applications.

With the MEPS, the extraction regards the less volatile sample fraction. Therefore, a derivatization step is often necessary for the fraction of interest before GC analysis and only rare examples exist in which biological samples are analyzed directly without additional pretreatments [51].

In a recent research, pyrethroid metabolites in urine were extracted and quantified using MEPS [52]. The authors had to deconjugate the glucuronide metabolites (*via* enzymatic reaction) before passing the sample in the MEPS sorbent. In this study, a deep investigation and optimization of the extracting sorbent, washing solvent(s), and draw-eject cycles was conducted on spiked pooled urine samples. The final condition used 100 µL of urine, C_{18} sorbent, methanol as conditioning solvent, while hexane was the final elution solvent for large volume GC injection. The method was validated for 5 target pesticides and compared to previously validated methods involving classical LLE.

In another example, metabolites from psychoactive substances were investigated using the MEPS followed by derivatization [53]. The starting matrix was whole blood (250 µL), on which a preliminary protein precipitation was performed. The diluted sample was then processed with the MEPS cation exchange sorbent (C_8 and SCX) and re-

tained/eluted using an acid (methanol added with formic acid)/basic (methanol added with ammonia hydrate) solution. Because of the pK_a of the target drugs (codeine, morphine, and 6-monoacetylmorphine), this allowed first the target to be positively charged and retained on the sorbent in the first wash, and then eluted (negatively charged) in the second one. After collecting the fraction of interest, the metabolites were derivatized as trimethyl-silyl ethers. The final extraction parameters were obtained using a 2-factorial design of experiments, considering wash solvent, formic acid and NH_4OH content on spiked blood samples. Even though low recoveries were obtained (< 20%), the method showed satisfactory figures of merit (sensitivity, linearity, and robustness) and the quantification of the target analytes was not affected in the real samples, also thanks to the sensitivity of the GC–MS/MS instrument.

Similarly, Rosado et al. extracted cannabinoid metabolites from plasma (250 μL) using MEPS [54]. They first optimized conditions with a design-of-experiment support and validated the method for tetrahydrocannabinol and 2 other metabolites for routine drug monitoring. Prior to analysis, a protein precipitation step with frozen acetonitrile (3:1) was performed, followed by a dilution with potassium phosphate (5 mL, 0.1 mM). After this, the samples were processed with a cation exchange sorbent (C_8 and SCX) and exploit the same ionic exchange properties of the sorbent and the differently ionized target molecules.

Few examples of direct GC injection of the fraction of interest without derivatization after the MEPS elution were reported for the analysis of methadone and monoterpenes and their metabolites in urine [55,56]. In the latter, the measurement of monoterpenes in biological fluid reflected the individual environmental uptake and thus the quality of indoor air (e.g., their presence in biofluids might be related to fungi presence in the environment that produce characteristic terpenes).

3.2. Liquid-based sampling techniques

The old fashioned solvent extraction can be considered the mother of all the extraction techniques [57]. It is based on the different partition/solubility of the analytes in between the sample and the extraction phase. This direct contact allows for a formidable penetration in the sample, and thus for a comprehensive accessibility to its components.

Its further advancement and use have been hindered by the apparent incompatibility with the recent principles of green chemistry, by the limited possibilities for automation and the growing and flourishing “competition” of modern solid-phase extraction techniques.

However, the trend towards simplification and miniaturization also touched these liquid-based techniques, thus reducing quantities of organic solvents needed, and making them contribute to the greening movement in chemistry, under the class of liquid-phase microextractions (LPME). These indeed, apart from reduced solvent consumption, are characterized by the small-scale and possibility of automation. In this sense, microextraction techniques have gained attention since the extraction might be carried out using amounts of extracting phase smaller than the sample amount (extraction of analytes is not always exhaustive).

In LPME, extraction normally takes place in a small amount of a water-immiscible solvent (acceptor phase) from an aqueous sample containing analytes (donor phase). It can be divided into three main categories: single-drop microextraction (SDME) dispersive liquid-liquid microextraction (DLLME), and hollow-fiber liquid-phase microextraction (HF-LPME).

Another great promise in increasing extraction yield and throughput is the use of external physical factors (e.g., ultrasound, microwave) which are discussed later in the dedicated section (Assisted techniques).

3.2.1. Microextractions (LPMEs)

Liquid-phase microextractions are the miniaturized forms of the liquid-liquid extractions, consequently consuming less volume of solvent(s). In 2006, Rezaee et al. developed a variation of LLME by combining the dispersion of fine droplets of extraction solvent in an aqueous sample, defined as dispersive liquid-liquid microextraction [58]. This technique has become one of the most widely used liquid-based extractions in biomedical area due to its simplicity, rapidity, low cost, and high recovery values. DLLME uses an extraction phase, formed by a water-immiscible organic solvent (extraction solvent) and a water-miscible organic solvent (dispersion solvent), that is injected into the aqueous sample solution. Through agitation, a cloudy solution of finely dispersed extraction phase droplets is formed. The organic phase is then separated and analyzed.

Šimek et al. developed a method for the determination of sterols and tocopherols in human serum and amniotic fluid [59]. This consisted of the acylation of cholesterol and related metabolites hydroxyl groups followed by liquid-liquid microextraction in which 500 μL of hexane were used to extract 100 μL of sample. Authors validated the method studying calibration curve, lower limit of quantification, precision and accuracy, recovery, and stability and tested it for GC–MS analysis.

The same research group also developed a sample preparation protocol to analyze the metabolomic profiling of protic metabolites in aqueous biological matrices with low protein content [60]. This method used simultaneous in situ derivatization and DLLME, followed by GC–MS analysis. The derivatization was performed by heptafluorobutyl chloroformate, while the solvents used for the extraction were isooctane as extraction solvent, and NaOH-pyridine (24:1, *v/v*) as dispersion solvent. In this case, only 150 μL total of organic solvent was used for the extraction of 25 μL of sample, reducing the solvent volume even more than the previous reports.

A further variation of DLLME was developed by Karami and Yamini [61]. They used an electromembrane extraction-dispersive liquid-liquid microextraction (EME-DLLME), performed using a lab-on-a-disk device (LOAD), for the determination of tricyclic antidepressants in biofluids. EME is a technique based on electrokinetic migration of ionized analytes from an aqueous donor phase to an acceptor solution across a liquid membrane. Since EME is a membrane-based extraction, it has become one of the preferred techniques for the cleaning-up of biological samples. LOAD is a microfluidic platform based on the centrifugal force for flow control. The centrifugal force is created by a rotary motor and there is no need for external pumps. Moreover, it has the advantages of handling bubble-free liquids and without dead volume, and the closed fluidic systems offer the capability of parallel sample operation on the same disk.

Single drop microextraction is another interesting and miniaturized alternative to traditional LLE, as it is a fast, inexpensive and virtually solvent-free sample pre-treatment technique. The concept of SDME is simple: a single drop of organic solvent is suspended by a syringe needle in the aqueous phase, while the system is stirred to drive the organic compounds towards the drop.

An efficient method for the determination of amphetamine and methylamphetamine was developed using electro-enhanced single-drop microextraction (EE-SDME) and GC [62]. The optimized EE-SDME-GC-FID method was applied to analyze a urine sample from suspected amphetamine users. Prior to extraction, 1 mL of urine was diluted 5 times with deionized water to reduce viscosity and ionic strength. The pH of the diluted urine sample was adjusted to 7.0. An advantage of the proposed method is that the extraction efficiency is greatly improved by the acceleration of the electric field of the mass transfer of the target analytes from the sample solution to the drop of organic solvent. In addition, the extracted analytes were derivatized in situ with isobutyl chloroformate (IBCF) in the droplet, which further improved the sensitivity.

In another study, pyrethroid metabolites in workers’ urine samples were monitored with a novel sample pretreatment process combining hollow-fiber liquid-phase microextraction and in-syringe derivatization, followed by gas chromatography-electron capture detector analysis [63]. The hollow-fiber liquid phase microextraction is a variant of the LPME technique; in this, the HF is immersed in an organic solvent, which is immobilized in the pores of the fiber, while another solvent in the fiber lumen is used as the acceptor phase. The acceptor phase is

usually a solvent with a different pH than the donor phase, which traps the analytes within the lumen, increasing extraction rates.

Pyrethroid metabolites were simultaneously extracted and enriched from urine samples by HF-LPME sampling and acid hydrolysis at 70 °C for 10 min. The derivatizing agents were pre-filled into the microsyringe barrel, and a segment of hollow fiber impregnated with extraction solvent was inserted into the syringe needle to be used as the LPME probe. After extraction, the LPME probe was extracted from the sample solution, and the in-syringe derivatization was achieved within the syringe barrel by mixing the extraction solution and derivatizing agents through plunger movements. To collect metabolites of synthetic pyrethroids, the derivatization step is also required to improve detection sensitivity since LPME does not allow total extraction [63].

3.2.2. Assisted techniques

Compared to the conventional procedures alone (e.g., LLE), the aid of ultrasound (UAE) and microwave (MAE) considerably support the extraction efficiency by reducing processing time, energy, and the amount of solvents, making the process more environmental-friendly [64].

Microwave heating is indeed more efficient and rapid than convection and conduction and enhances the migration rate of compounds into the extraction solvent. The ultrasound energy in UAE is used to increase the extraction rate by enhancing the contact area between the sample and solvent and by promoting efficient agitation.

The support of ultrasound/microwave can occur also on the miniaturized liquid-based extraction techniques.

Even though these assisted extractions find more use with food and environmental applications, few reports are present dealing with the analysis of biological samples. Lin and coworkers proposed for the first time an ultrasound-assisted-DLLME approach for the GC–MS determination of seven common recreational drugs (amphetamine, methamphetamine, 3,4-methylenedioxyamphetamine, 3,4-methylenedioxymethamphetamine, meperidine, methadone, and ketamine) in human whole blood without procedures of derivatization, vaporization, and reconstitution [65]. Authors compared several dispersive (methanol, ethanol, acetone, acetonitrile, and isopropanol) and extraction (cyclohexane, 1-chlorobutane, benzene, methylbenzene, methyl tert-butyl ether, dichloromethane, trichloromethane and tetrachloromethane) solvents, among which a combination of methanol/dichloromethane as dispersive/extraction solvent-mixture was selected, thanks to its best performance in terms of emulsification and extraction efficiency. The support of ultrasound shortened the DLLME time, achieving higher recoveries in only 2 min. The entire procedure was validated in terms of selectivity, linearity, LOD, LOQ, enrichment factor, extraction recovery, intra-day and inter-day precision and accuracy [65].

The use of low-density solvents (LDS) during UA-DLLME can facilitate the collection of the extract before injection. Meng and coworkers compared UA-LDS-DLLME with hollow-fiber liquid-phase microextraction for the GC–MS determination of drugs of abuse in biological samples, obtaining comparable results in terms of linearity, LOD, and repeatability. However, compared with HF-LPME, the UA-LDS-DLLME approach showed shorter extraction time and a better suitability for batches of sample pretreatment simultaneously [66].

3.3. Chemical transformations (derivatization)

Derivatization is used to chemically transform polar and/or non-volatile compounds to make them more compatible with GC analysis. It can be used for different purposes, such as for solubility improvement, for clean-up or fractionation purposes, and for separation or detection enhancement. The derivatization strongly affects the non-polar/polar property of the analyte, which modifies the interactions during the subsequent analytical separation.

The most essential requirements to make a derivatization process acceptable are the fullness of the reaction process and the stability of the products generated. The replacement of the active hydrogen (-H) in polar groups represents a large percentage of the derivatization process. Among the different derivatization reactions, i) alkylation, ii) acylation, and iii) trimethylsilylation are the most common and widely used [67,68].

According to the scientific goal, the derivatization process can be performed at different analytical stages (i.e., pre/post-extraction), involving a fraction of the sample, or the whole matrix.

Alkylation consists in the replacement of the active hydrogen into polar groups, such as -OH, -SH, and -NH with alkyl groups to give (thio/amino) ethers, (thio/amino) esters, etc. Particularly active on amines and carboxylic groups, it is mainly applied to derivatize esterified and/or not esterified fatty acids in alkyl esters. Among different derivatization reagents for fatty acid alkyl esters preparation, trimethylsulfonium hydroxide (TMSH) [69] has been widely used in clinical researches on different biological samples such as muscular biopsies [70], human plasma [71], and bacteria strains [72,73]. The TMSH methodology can be directly applied to the bio-sample and/or after isolation of the target analytes (i.e., extraction of phospholipids). Due to the simplicity, this approach can be useful for large batch analysis, but the limit of the TMSH method is the insufficient derivatization efficiency for the polyunsaturated fatty acids [74]. According to the needs, various direct trans-esterification approaches can be applied in biological samples, each one with advantages and disadvantages [75].

Thanks to the continuous progress in analytical technologies, it is possible to automate the entire derivatization process by using dedicated robotic sample preparation systems [76].

Another popular approach for alkylation in clinical samples involves the use of alkyl chloroformates , as recently applied for the GC–MS determination of sterols and tocopherols in human serum and amniotic fluid [59], as well as for the GC–MS investigation of more than 150 possible diagnostic amino-carboxylic metabolites in human urines [77].

In the acylation process, an acyl group is introduced to an organic compound where the active -H is converted into ester, thioester, or amide. If the organic compound is a carboxylic acid, there will be the loss of the hydroxyl group. Acylation, more suitable for the derivatization of amines, hydroxyls, and thiols, is a derivatization process that prefers an anhydrous environment, and it mainly occurs after an extraction procedure to isolate the target analytes. It has been successfully applied for the GC–MS determination of amino acids and their metabolites in different biological samples such as in urine, sputum, and blood plasma [78].

Silylation is the most common derivatization method applied in metabolomic studies due to its comprehensive coverage of compound classes and relative ease of use. Indeed, the wide choice of silyl-reagents available makes silylation suitable for most of the functional groups containing an active hydrogen. To avoid hydrolysis of reagent/derivatize products, care during this process must be taken, ensuring that both samples and solvents are in an anhydrous environment [79].

According to the chemical nature of target analytes, a two-step derivatization combining consecutive multiple reagents (e.g., for the acylation, the silylation, and the methoxylation) is also possible and automatable [80,81].

The derivatization of metabolite extracts can potentially require long incubation times before injection, possibly leading to hydrolysis of the derivatization reagent and derivatives. Thus, automation of the entire process, together with the use of dedicated analytical tools to improve the throughput, is highly desirable. An automated silyl-derivatization protocol for GC–MS non-target based metabolomic analysis of urine samples has been proposed by Abbis and coworkers, in which each sample was automatically ready for injection every 70 min. The automated protocols were compared with a manual two-step derivatization approach, obtaining comparable results [82].

All the derivatization processes can benefit from the presence of external physical phenomena (e.g., microwaves, ultrasounds) as discussed in the previous section. The use of microwave heating (microwave-

assisted derivatization) in combination with the common derivatization methods (alkylation, acylation, and silylanization) can dramatically reduce the time needed to derivatize samples compared to conventional "offline" approaches [82].

Also thanks to the advancement of specialized scientific microwave instruments with high throughput rotors capable of housing GC-ready vials, it is possible to undertake high throughput sample derivatization of multiple samples per reaction in large scale omics research [67]. Recently, Chou and coworkers developed and validated a protocol using direct ultrasound-assisted derivatization for the GC–MS determination of organic acids in human serum [83].

There are other variations to save time/reduce the number of sample processing steps before GC injection, such as performing the derivatization in-syringe, in which sample and derivatization reagent are drawn into the GC syringe creating a multi-layer [84]. Another approach is to combine the extraction with the derivatization process, such as for the on-fiber SPME derivatization. This can occur absorbing the derivatization reagent onto the SPME fiber before the sampling, allowing then a simultaneous sampling and derivatization step before the desorption into the GC system, as reported by Fuchs and coworkers [85]. They investigated several short-chain aldehydes in human breath as possible biomarkers in the context of lung cancer disease by on-fiber derivatization-HS-SPME-GC–MS analysis. In general, examples of on-fiber SPME derivatization and derivatization in-syringe are more frequent in environmental, food, and forensic sciences rather than in clinical studies.

Finally, it is also important to mention that an appropriate selection and regular replacement of the GC liner is necessary to limit unwanted reactions and surface adsorption phenomena, with a possible under/overestimation and degradation of the metabolites of interest [86]. The presence of active catalytic sites in the liner might indeed generate distorted peaks and contribute to carryover effects, both affecting the reliability of analytes detection and quantification.

4. Conclusions and perspectives

A portrait of the current approaches for the extraction of GC-amenable analytes in biomedical research has been outlined and they essentially are grouped in sorbent-based and liquid-based techniques.

The techniques based on sorbent(s) represent the current trend in sample preparation and a further consolidation is expected during the next years thanks to their intrinsic advantages also in terms of the green analytical chemistry principles. Besides the variety of available sorbents and the continuous discovery of novel ones, this type of extraction is more suitable for automation, which increases analysis throughput and robustness (by reducing analytical errors).

The intriguing idea of volatile biomarkers associated to the availability of the headspace sampling technology has induced and keeps a frenzy of research activities in this direction. The study of the volatilome has its biological rationale, even though the biochemical pathways and metabolic intermediates are yet far from being well known.

Regarding the less volatile molecules (including amino acids, fatty acids, etc.), which are intrinsically more bonded to the biological matrix, the chemical transformation is the unavoidable step to make them suitable for GC analysis, and often this is combined with additional purification/extraction steps. In this regard, more efforts are being focused on automation and miniaturization with dedicated robotic apparatus, both offline and online.

Among the three practical analytical steps (i.e., sample extraction, separation, and detection), the sample preparation is surely contributing greatly to the final reliability and robustness of the methodology. However, it is paying the cost of the different pace of the technological advancement of chromatography and detection techniques (especially mass spectrometry). Despite the fast growing of automation, robustness, selectivity, and sensitivity of modern instrumentation could apparently question the need for analyte enrichment and purification during the sample preparation, in the authors' opinion, not only the appropriateness but also the optimization of this initial step must not be undervalued, especially in addressing biomedical questions.

Finally, in this growing quest for greener, miniaturized, faster, selective/universal, and robust sampling strategies, it will be interesting to see how some recently developed extraction concepts and variations (vacuum-based, paper-based, freeze-concentration extractions) will contribute to study biological samples.

Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Supplementary materials

Supplementary material associated with this article can be found in the online version, at doi:10.1016/j.sampre.2022.100017.

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