The role of sample preparation in multidimensional gas chromatographic separations for non-targeted analysis with the focus on recent biomedical, food, and plant applications

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Abbreviations: CAR, carboxen; CBD, cannabidiol; CBN, cannabinol; CSC, coconut shell charcoal; DHS, dynamic headspace; DI, direct immersion; DVB, divinylbenzene; EG, ethyleneglycol; FID, flame ionization detector; FM, flow modulation; GC-GC, heartcutting multidimensional gas chromatography; GPe, gas-phase extractions; HR, high-resolution; HS, headspace; HSSE, headpace sorptive extraction; LPe, liquid-phase extractions; MDE, magnetic dispersive extraction; MSTFA, N-Trimethylsilyl-N-methyl trifluoroacetamide; NTME, needle trap microextraction; ODS/GC, octa-decyl silica and graphite carbon; P&T, purge and trap; PBS, phosphate-buffered saline; PDMS, polydimethylsiloxane; PIL, poly ionic-liquid; PLE, pressurized liquid extraction; QTOF, quadrupole-time of flight mass analyzer; SBSE, stir bar sorptive extraction; SDE, simultaneous distillation extraction; SFE, supercritical fluid extraction; SHS, static headspace; SPe,solid-phase extractions; SVOC, semi volatile organic compounds; Tenax TA, 2,6-diphenyl-p-phenylene oxide; THC, tetrahydrocannabinol; VOC, volatile organic compounds; XAD-2, styrene-divinylbenzene resin.

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Abstract

In this review, we consider and discuss the affinity and complementarity between a generic sample preparation technique and the comprehensive two-dimensional gas chromatography process. From the initial technical development focus (*e.g.*, on the GC×GC and solid-phase microextraction techniques), the trend is inevitably shifting toward more applied challenges, and therefore the preparation of the sample should be carefully considered in any GC×GC separation for an overreaching research.

We highlight recent biomedical, food and plant applications (2016-July 2020), and specifically those in which the combination of tailored sample preparation methods and GC×GC-MS has proven to be beneficial in the challenging aspects of non-targeted analysis. Specifically on the sample preparation, we report on gas-phase, solid-phase, liquid-phase extractions, and derivatization procedures that have been used to extract and prepare volatile and semi-volatile metabolites for the successive GC×GC analysis.

Moreover, we also present a milestone section reporting the early works which pioneered the combination of sample preparation techniques with GC×GC for non-targeted analysis.

1. Introduction

The first decade after the seminal paper from Phillips [1] was especially abundant in the research for a better understanding of the GC×GC process and orthogonality, the visualization of the data, and the surprising “re-discovery” of samples’ composition. These, in addition to the advancements of the transfer process, which also led to the development of the different modulation forms available nowadays, represented the main playground for researchers in the early stage of GC×GC [2].

The sample preparation initially retained little attention within GC×GC studies, with extremely limited or basic procedures, as already pointed out [3], and with the main attention focused around the novelty of the multidimensional separation concept. The minor consideration for the sample preparation when combined with GC×GC was also partially due to the additional features of the multidimensional technique (increased selectivity, sensitivity, speed, separation power, structure), compared to the conventional (1D) GC separation. Nevertheless, even if the additional 2D separation helps with analytes of interest enrichment (*i.e.*, the focusing effect especially for thermal modulators), cleanup,

and interference removal (fewer coelutions, and separation from the chemical noise, *i.e.*, columns bleed), it does not justify overlooking a proper and optimized sample preparation method. The preparation and extraction of the analytes of interest indeed represent the most crucial step in the analytical workflow as it should provide an authentic representation of the sample, setting a solid basis for the following analytical stages [4].

The introduction of solid-phase microextraction (SPME) by Pawliszyn represented the other groundbreaking advance which contributed strongly to the increase of the consideration and use of novel, green, and miniaturized sample preparation techniques with GC×GC [5,6]. The most used form of SPME consists in fused silica fibers coated with the extraction phase(s), which are exposed to the headspace of, immerged in, or put in direct contact with the sample [4].

It is worthy to highlight the fact that GC×GC and SPME originated (1) in the same period [1,7], (2) from two researchers who shared the same research laboratory for some time, and (3) from similar fundamental concepts; these facts are signs of the intimate relation of the two techniques. **Figure 1** resumes some of the key features of a generic sample preparation and the GC×GC separation, highlighting the commonalities between the two processes. Shared features and attributes can be identified for both the sample preparation and the standalone GC×GC process. Among these, the enrichment and clean-up of analytes of interest, the interference removal, the tuning of the selectivity, and the versatility of the approaches make them the most powerful analytical alliance for complex samples analysis. This becomes exceptionally true when the detection is represented by mass spectrometry, thanks to the unique identification capabilities to handle a multitude of the extracted and separated analytes. In this case, the data generated intrinsically contain all the information for applying either a fingerprinting, a profiling, and/or a targeted approach, based on the research questions.

An additional and common point regards the trend toward greener approaches and miniaturization which is true for both sample preparation and the GC×GC separation [3,5].

All the new evolutions after the introduction of SPME and GC×GC, together with the rapid development of MS and software-data handling tools since early 2000, surely ignited the growing and valued the use of non-targeted analysis in all applications fields. Nowadays, it is possible to find demonstrations of the usefulness of non-targeted analysis combining the many forms of sample preparation with the high-resolution GC×GC-MS technique. In this review, we report on gas-phase (GPe), solid-phase (SPe), liquid-phase (LPe) extractions, and derivatization procedures which have been used to extract and analyze volatile (boiling point range between 50-260 °C) and semi-volatile (boiling point range between 240-400 °C) metabolites from biological, food and plant samples, with the focus on non-targeted applications in last 5-years period (2016-July2020). The description of the underlying mechanism of the extraction types (*i.e.*, GPe, SPe, LPe) is reported in the following paragraph 3.

2. Milestones in combining sample preparation techniques and GC×GC

Thirthy years have passed since the first report of the modern sample preparation SPME and comprehensive two-dimensional gas chromatography techniques. The intent of this paragraph is to recognize the early contributions which pioneered the combination of sample preparation with high-resolution separation and detection for non-targeted analysis in biological, food and plant applications. It must be said that these early works that initiated the use of innovative sample preparation techniques as up-front tools for GC×GC, yet focused mainly on the increase of selectivity and sensitivity of the multidimensional separation technique.

SPe techniques were the earliest sample preparation procedures used with GC×GC. In 2002, Adahchour *et al.* evaluated the general practicability of combining headspace (HS)-SPME with GC×GC for the analysis of flavors in garlic cloves [8]. Compared to conventional GC, a 10 to 50-fold increase in sensitivity together with a 10-times higher peak capacity were reported. In addition, the comparison of the PDMS and DVB/CAR/PDMS fibers highlighted the improved extraction efficiency (up to 20-fold) when using the latter. Their study opened the way for HS-SPME-GC×GC for detailed flavor analysis and identification of aroma active compounds. In the same year, Perera *et al.* reported increased sensitivity and improved peak capacity using HS-SPME and GC×GC for the analysis of wounded plants [9]. Furthermore, they highlighted the usefulness of the fingerprinting approach to define chromatographic regions of interest enabling direct and easy sample comparison. Worth mentioning, even if not relevant with the applications' fields of this review, is the very first HS-SPME-GC×GC application carried out by Frisinger *et al.* in 1998 for environmental volatile organic compound (VOC) determination. The authors were still using the first thermal sweeper modulator and they discussed about the special care and the details for the (manual) SPME sampling and injection procedures since no automation was yet available [10].

In 2003, GC×GC followed mixed-bed SPE cartridges to analyze drugs in horse and dog urine [11]. These cartridges represent the most classical SPe, and are short disposable columns containing a bed of porous particles, in which the analytes or the fractions of interest are retained after a first solvent elution, and then re-mobilized by elution using a different solvent [12]. The authors performed the derivatization of the amino groups of the drug metabolites to produce thermally-stable analytes. This study demonstrated the potential of the overall methodology in drug analysis by providing lower detection limits of 0.04 mg/L against 0.1 mg/L using classical 1D GC, good reproducibility, and the possibility of high-throughput sample screening using the 2D chromatographic space.

In 2002, Seeley *et al.* employed sorbent traps with GC×GC, equipped with a differential flow modulator and dual secondary columns, to characterize exhaled breath from healthy humans [13]. Multi-bed sorbent trap tubes (Carbotrap C/Carbotrap/Carbosieve SIII) were used to collect 1.5 L of breath. The system enabled the characterization of 100 compounds over a 10 min analysis. The unique selectivity of the two secondary columns allowed increasing further the resolution and the qualitative information provided by GC×GC analysis. Even though this contribution was mainly focused on the characteristics of the flow modulated system, it demonstrated the effectiveness of GC×GC for breath analysis, which was further investigated in the following years [14]. An alternative and interesting approach for breath analysis was highlighted in 2010 by Mieth *et al.*, with the use of a needle trap microextraction (NTME) device [15]. Alveolar breath samples were taken from patients undergoing cardiac surgery showing the potential of the device for on-site sampling and pre-concentration, for biomarkers discovery. Good storage capabilities, up to one day according to the physico-chemical properties of the analytes, and high throughput analysis using an optimized autosampler were described.

In 2012, Risticevic *et al.* investigated for the first time the potential of direct immersion (DI)-SPME and GC×GC for metabolite profiling of apples [16]. An increased (351 supplementary metabolites) and more balanced metabolite coverage were obtained when using DI-SPME, avoiding loss of polar and high molecular weight analytes. However, it was highlighted that a comprehensive characterization of the metabolome was only achievable under optimized conditions for both the sample preparation and the separation. The authors fully exploited GC×GC attributes, *i.e.*, the 2D separation space occupation and the structured separation, to optimize the SPME method.

Cordero *et al.* also took advantage of multiple sampling techniques with the GC×GC attributes [17]. The complementarity of gas-phase (*i.e.*, dynamic headspace (DHS)) and solid-phase (*i.e.*, headspace sorptive extraction (HSSE), HS-SPME, DI-SPME, and stir bar sorptive extraction (SBSE)) sampling approaches was demonstrated in the characterization of VOCs and semi volatile organic compounds (SVOCs) in dry milk powder. In DHS, a continuous removal of volatiles from the matrix is obtained with a constant inert gas flow to increase the efficiency of the extraction. Regarding SBSE, it has the same working principles of SPME: in this case a magnetic stir bar is coated with the sorbent and can be immersed (classical SBSE), or suspended in the headspace (HSSE) of the samples. The authors highlighted for the first time the effectiveness of the combination of HSSE and SBSE with GC×GC for a sensomics investigation in food analysis thanks to their high concentration factors enabling broad analyte coverage. These results pointed-out the importance of the choice of the sample preparation since this latter can greatly influence the obtained fingerprints of complex volatile fractions.

Shortly after its introduction, GC×GC-MS also served the emerging metabolomics field. The great potential of structured 2D chromatograms has been initially shown for lipids [18,19].

In these early reports, a derivatization procedure (*i.e.*, transesterification) was necessary to isolate the fatty acids from the rest of the matrix and make them more suitable for GC×GC analysis, as methyl ester derivatives. The modulator band compression allowed the concurrent determination of the major as well as minor components of milk and various vegetable and fish oils, revealing a novel chemical complexity of those "well-known" samples.

Following such a demonstration of the increased capacities of GC×GC over 1D GC, in 2005 GC×GC was used to identify biomarkers of obesity extracted from mouse spleen [20]. The extracted metabolites were derivatized with commonly used silylation agents, prior to GC×GC analysis. Amino- and hydroxyl acids together with few carbohydrates' derivatives were identified in two populations, with sugar alcohols classified as potential biomarkers of obesity. This was the first claimed non-targeted metabolomics study using GC×GC-MS, demonstrating that direct translation from 1D GC-MS to 2D GC-MS was possible, and set the basis for the use of GC×GC in biomedical research.

3. Non-targeted analysis applications

With the refinement and advancement of the hardware, which improves the technique robustness and reliability, and software tools, which help with the high-dimensional data handling and processing, recent applications are more and more focused on applied challenges. The workflow used for the literature survey in the period covering 2016 until mid-2020 is provided in the supporting information (**Figure S1**). These references are listed in **Table S1**, which also contains brief information on the sample type, the sample preparation, and the GC×GC-MS methodology. Selected biomedical-, food- and plant-related applications which reported more emphasis on the sampling step are instead described within each of the following section to summarize the recent trends and challenges of non-targeted analysis combining innovative sample preparation techniques and GC×GC. Unless otherwise stated, all the applications described in this review involved GC×GC-MS with cryogenic modulation. Some remarkable applications during the covered period are summarized in **Table 1**, in which more technical details on the sample preparation conditions are reported.

Along the review, the various sample preparation techniques which preceded the GC×GC-MS analysis were grouped into the principal classifications of GPe, SPe, LPe, and derivatization protocols (**Figure 2**) [21].

In GPe, a gas is used to strip the volatile compounds off the sample, which can be injected directly (traditional static HS) or trapped into a sorbent trap. SPe are based on the transfer of the compounds of interest from a gas, liquid, or supercritical fluid matrix to a solid

sorbent. In LPe, the extracting agent is a liquid fluid, as in the common case of liquid-liquid extractions [22].

SPe and most of the GPe techniques rely on sorbent-based interactions with the analytes to extract. Among these techniques, surely SPE, SPME, SBSE, NTME, and DHS through trap tubes are the most promising and the most used with GC×GC. For a more detailed description of the techniques reported in this review, please refer to the literature [12,23,24].

Generally, GPe methods are exclusively dedicated to VOC sampling, while SPe, LPe, and the derivatization approaches are suitable also for the heavier SVOCs. Among SPe, some techniques can be used both in headspace or direct immersion approaches (*e.g.*, HS/DI-SPME and HSSE/SBSE) [25]. In the case of multistep sample preparation protocols, for example, a derivatization step followed by multiple extractions, the sample preparation techniques were counted separately for the generation of **Figure 2**.

As already highlighted, each analytical step and especially the sample preparation could introduce a bias (in the selectivity) in the final result, which makes the realization of a true non-targeted and unbiased analysis very challenging and arguable. For example, any extraction strategy sets at the inception of the whole methodology a certain degree of selectivity towards a defined class of analytes, depending on their chemical or physical properties. Keeping in mind this unavoidable bias-introduction, non-targeted strategies generally tend to use wide-selectivity analytical steps to characterize in-depth the samples, track and/or compare hundreds of analytes between groups of samples to address specific research questions. Like for the GC column chemistries, different materials with variable selectivity are available for the sorbent-based extractions. The discussion on the different sorbent materials and their selectivity will not be herein described, and a series of reviews and books are provided for more detailed information [12,23,24,26].

Over this period of 5 years (2016-2020), we observed that the characterization of volatile metabolites from biological, food and plant samples through headspace sampling represents the most common approach (57 %) used in combination with GC×GC. A dominant position for sampling is held by SPME, which appears to be used preferably in the HS mode (39 %) rather than in DI mode (1 %).

3.1. Biomedical-related

The interest for non-targeted metabolomic analysis 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 search for novel diagnostic and prognostic biomarkers of heath-altered states, and their basis relies on the fact that metabolite levels vary at the early stage

of the diseases. Early-diagnosis together with an increased understanding of human diseases would allow improving the quality of life and expectations of the patients. However, numerous are the challenges associated with biomarker discovery in this field. In particular, the variability and complexity of the biological matrices (*i.e.*, biological fluids, biopsies, tissues, cell cultures), comprising thousands of metabolites characterized by a wide range of concentrations and physico-chemical properties, are posing the major challenges. In addition, a single analytical platform is not sufficient to exhaustively cover and analyze such a broad range of metabolites, and thus multiplatform strategies should ideally be sought [27]. The diversity of the metabolome and the related difficulties to identify *de novo* metabolites delay the direct correlation or mapping of (novel) biochemical pathways [28].

In view of the sample preparation techniques used over the 5 past years, HS-SPME together with sorbent traps and derivatization procedures have been the most used sampling techniques, representing together ~70 % of the methods reported in the 2016-2020 literature (**Figure 2**).

3.1.1. Biological fluids

Blood, urine, and exhaled breath have been thoroughly studied matrices to identify potential biomarkers of metabolism perturbations [29,30].

The growing interest for exhaled breath analysis can be explained by its non-invasive nature, allowing its use for a broad range of patients. Exhaled breath sampling for GC×GC analysis is a two-stage process since the direct injection into the separation system has not yet been demonstrated. In most of the studies, breath collection was performed using Tedlar sampling bags before the pre-concentration on sorbent traps. In this case, the pre-concentration step which enables sample enrichment is particularly important, as analytes are present in trace-levels. Over the past 5 years, trap tubes have been the most commonly used sampling technique for breath analysis [31–36], although a more recent trapping technique, the NTME has demonstrated great potential for breath analysis [37]. Ideally, NTME devices and trap tubes could be used for simultaneous collection and pre-concentration avoiding the intermediate use of Tedlar bags, but until today, only few specific devices exist [38–40]. In a recent study, Wilde *et al.* optimized the sampling parameters of a device enabling the direct collection and pre-concentration of breath, with no need for a derivatization step, onto trap tubes to identify potential markers of acute breathlessness [35]. In their study, the trap tubes were desorbed in a flow-modulated (FM)-GC×GC system with dual MS and flame ionization detection (FID), combining high-separation power, more uniform response factors, and identification capabilities.

In another work, VOC bio-sampling in serum and exhaled breath using trap tubes was evaluated among different packing materials, highlighting the importance of the sorbent selection due to the different matrix contributions (*e.g.,* relative humidity effect on the

sorbent type and complexity) [41]. In **Figure 3** is shown the longitudinal sampling profile of some endogenous metabolites released in breath after coffee ingestion using differently-packed tubes. 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 (see paragraph 3.1.3) [42].

In the context of biofluids, SPME in HS mode is the second most popular technique for volatile analytes characterization by GC×GC [43–46]. Mack *et al.* evaluated the efficiency of different HS-SPME fibers to measure urinary volatile metabolites linked to dietary intake [43]. As can be seen in **Figure 4**, a richer chromatogram and higher extraction yields were obtained when using the triphasic DVB/CAR/PDMS fiber, with the authors pointing out the importance of careful evaluation and optimization of the materials/conditions for analyte extraction. In addition, they highlighted the impact of data treatment and especially the normalization. They compared different normalization approaches relying on physiologic properties (*i.e.*, osmolality, creatinine, and urine volume), and data-driven normalizations (*i.e.*, mass spectral total useful signal and probabilistic quotient normalization), and among six compounds identified as potential markers of coffee consumption, only one was confirmed independently of the normalization used.

The triphasic fiber is common and appears to be an optimal fiber for biofluid analysis, and has been used to extract VOCs from urine and blood for different objectives such as asthma phenotyping [44], colorectal cancer diagnosis [45], and fertility monitoring [46].

When seeking for increased metabolite coverage, for example, high molecular weight and/or polar metabolites, a derivatization procedure is widely used to make sugars, amino acids, fatty acids, and steroids amenable for GC analysis and to characterize them. Such metabolites are essential to living organisms and their levels are most of the time correlated to metabolism perturbations or disease states. Even though the derivatization protocols make the sample preparation more extensive, their utilization can be maximized in combination with GC×GC [47–55].

Bileck *et al.* described a non-targeted method for the comprehensive analysis of steroid metabolites in urine [47]. Using a previously optimized derivatization procedure, they highlighted the complexity of the matrix and the numerous co-elutions between urinary steroids and other metabolites (**Figure 5A-B**). The increased separation power of GC×GC enabled the characterization of up to 70 steroids in urine from different sources (infants, adults, and rodents) against 26 using classical GC- and LC-MS methods.

Additional studies reported the increased characterization of the urine metabolome combining a well-established derivatization process and GC×GC separation, which enabled

to discover new tuberculosis markers reflecting adaptations from the host-pathogen interaction and treatment failure [48,49].

In some cases, the increased resolution provided by GC×GC was not sufficient, and specific sample preparation was necessary for the determination of specific classes of metabolites. For example, the derivatization of serum and urine samples using metoxyamine and MSTFA (1% TMSCI) yielded in the trimethylsilane adducts of lactate and pyruvate that were not sufficiently different to be separated. Instead, the use of one-step derivatization protocol using MSTFA (1 % TMSCI) enabled the separation of the derivatized forms of lactate and pyruvate, suggesting that variations in derivatization strategies may further increase molecular feature detection in complex biomedical samples [50].

At the cost of making the global sample preparation more extensive, Di Giovani *et al.* insisted on the need for careful optimization of each step of the extraction and derivatization process together with a robust experimental design to identify serological biomarkers of Crohn's diseases, and long-term quality controls [52]. Here, the optimized sample preparation procedure allowed identifying 33 potential biomarkers in serum to discriminate healthy volunteers from three subgroups of patients having Crohn's disease, namely with high, low, and quiescent endoscopic activity.

3.1.2. Tissue or biopsies

When the starting material is a tissue, time-consuming multistep sample preparation is required, especially for hypothesis-generating, non-targeted metabolomics studies. Sample homogenization, metabolites extraction, and derivatization are some of the steps usually involved in the sample preparation [50,56]. Joseph *et al.* identified some metabolites related to lipid metabolism in rat muscle which could be used in therapeutic approaches for monitoring type 2 diabetes and obesity [56]. However, the sample preparation required approximately 14 h of labor. Yu *et al.* developed and validated a sampling protocol requiring only 2 h for metabolite extraction and derivatization from tissue, demonstrating the potential for further improvement towards high-throughput sample preparation protocols [50].

The *in vivo* sampling and investigation of VOCs emitted from human skin has been recently exploited and investigated with GC×GC [57,58]. Roodt *et al.* described a PDMS silicone rubber in the form of bracelets and anklets to characterize the human skin microbiome [57]. After the passive sampling for 4 h, the bracelets were thermally desorbed into the GC×GC inlet, enabling the characterization of around 300 volatiles with acceptable reproducibility (< 40 % RSD for 75 % of the detected compounds). To analyze the heavier SVOCs from human skin, Dolezal *et al.* reported instead the use of glass beads. Following a cleaning step, the glass beads were handled and rolled in the palms of the volunteers for 10 min. Hexane was then used to extract the scent molecules [58].

As for breath analysis, such a straightforward and non-invasive *in vivo* skin sampling holds high interest and potential in the medical field. However, so far, not many reports have made use of this alternative *in vivo* sampling techniques with GC×GC, and further studies are required to understand their full capabilities.

3.1.3. In vitro or cell culture-based

In vitro research is of particular interest since it enables the study of biological processes leading to the production of VOC and SVOC in an "easy-to-manipulate" environment compared to *in vivo* systems.

HS-SPME is a commonly used approach in *in vitro* studies, mainly due to its simplicity, reproducibility, and possible automation [41,59–65]. Recently, this approach was employed to study the volatile metabolites from lung epithelial cells subjected to chemical and biological inflammation using, hydrogen peroxide and sputum from asthmatic patients, respectively [65]. A DVB/CAR/PDMS fiber was selected to extract the widest range of VOC emitted from the cell culture subjected to the different stressors. According to the type of inflammation induced and the associated status of the cells, different volatile metabolites have been characterized. Increased production of volatile compounds was reported following the co-incubation of epithelial cells with inflammatory sputum when compared to cells treated with non-inflammatory sputum and non-treated cells. The principal component analysis of **Figure 6** is demonstrating the differentiation between chemically and biologically stressed epithelial cells and three different cellular controls consisting of non-treated cells (green), cells diluted in phosphate-buffered saline (PBS) which is used to treat the sputum samples (yellow), and cells treated with sputum from healthy volunteers (pink). Such clear differentiation is confirming the potential of volatile metabolites to reflect the status of lung epithelial cells and therefore, alterations in their metabolism.

HS-SPME has also been used to characterize virally-infected respiratory cells [60] and to study the volatile profile of numerous bacteria and fungi [66,67]. Martins *et al.* have evidenced differences in the extracellular volatile metabolites of two different yeasts found in beer and wine over 36-h [66]. *S.cerevisiae* and *S. pastorianus*, at different sampling points (HS-SPME) corresponding to different growth phases (lag, exponential, and logarithmic), revealed a high number of compounds which were correlated with specific metabolic pathways. A similar approach was used to study the extracellular and intracellular metabolites on bacteria exposed to heavy metals [67]. In this case, the rapid extraction of bacterial volatiles (the extracellular and intracellular fractions were obtained in only 15 min and a 60 min SPME fiber exposure was necessary to extract the volatile compounds) allowed identifying the evolution in the volatile metabolome to further understand bacterial survival in a contaminated environment. Mousavi *et al.* reported the use of HS-SPME to successfully follow metabolic changes of *E. coli* induced by natural antibacterial agents such as clove oil and eugenol in real-time and *in vivo* [68]. The treatment of *E. coli* with clove oil

induced a dysregulation of 125 volatile metabolites, among which derivatives formed along with the fatty acid biosynthetic pathways, such as alcohols, ketones, and esters, were the most affected. This exposition of SPME fiber during the bacterial growth (*in vivo*) enhanced the amount of chemical information that can be obtained from the biological system under study while avoiding any *a priori* sample manipulation. Similarly, an HSSE method has been exploited to characterize antifungal VOC activity of bacteria and enabled to highlight new volatile metabolome properties from the co-culture of bacteria and fungi [69].

Thermal desorption tubes represent a valid alternative also for *in vitro* research sampling. The need for consistent analyte extraction between *in vitro* and *in vivo* studies for biomarker discovery and translation (*e.g.*, breath or urine and cell cultures) has been recently reported [42]. The authors highlighted the importance of the sorbent material selection for the trap tubes in bacterial VOC sampling from cultures. Indeed, the physico-chemical characteristics of the numerous adsorbent materials available are influenced differently by the sampling conditions (*e.g.*, humidity) and sample complexity, affecting the final results. **Figure 7** shows the heatmap of three bacterial strains (*P. aeruginosa, S. aureus, E. coli*) involved in lung infections and obtained with two differently-packed traps. The better separation of the three bacterial strains was highlighted when using Tenax TA, thanks to its higher reproducibility and metabolites coverage during the sampling.

Despite the advantages of HS-based sampling, the study of cell and bacteria metabolism often requires the characterization of SVOCs, which are intrinsically more interrelated with the matrix and can be more challenging to extract. In these cases, conventional extractions followed by derivatization techniques are the approaches generally used. As an example, Miguez *et al.* used silylation agents to study small polar metabolites from the lycopene and mevalonate pathways of *E. coli* [70]. Among these, homocysteine and homoserine have been identified as possible growth inhibitors associated with the overnight induction of the mevalonate pathway of *E. coli*. Their optimized quenching protocol allowed the measurement of the metabolites over 6 h with a high turnover rate. The protocol provided an increased understanding of the central carbon metabolism of *E. coli* that can be used to drive pathway and strain engineering.

3.2. Food and plant-related

The non-targeted analysis of metabolites in food or plant-related products commonly aims for obtaining a chemical snapshot of the sample composition. Regarding food-related products, the detection of small molecules such as acids, alcohols, aldehydes, carbonyl compounds, esters, ketones, phenols, or terpenoids is of high importance as this information can be used for a variety of applications. For example, it allows controlling food production processes, maintaining or improving food quality, or developing a particular organoleptic property. In plants instead, the chemical signature is related to a particular metabolic setting of a physiological/altered state. The study of small metabolites as a picture of the metabolic state using high-resolution techniques appeared in early 2000 and

progressed until nowadays with the continuous implementation of cutting-edge analytical technologies [71]. In the context of GC×GC, the recent years have seen the majority of the non-targeted analysis in food and plant-related samples focusing on the more volatile metabolites, thus exploiting HS techniques (94 %) involving GPe (DHS, purge-and-trap (P&T), static headspace (SHS)), and SPe (HS-SPME, HSSE).

Especially miniaturized sampling techniques allowing for preconcentration such as SPME, HSSE, and DHS have shown to be useful to limit artifact formation and match the analytical requirements of automation [72]. It has become more and more evident that the hyphenation of a robust sample preparation technique with advanced GC×GC separation and MS detection is important and that suitable software and data-handling tools with statistical analysis are necessary to make proper use of the high-dense acquired information.

Regarding food and plant-related products, 82 % of the revised literature of the last 5 years used non-targeted approaches (metabolite profiling and/or fingerprinting), 18 % aimed for a targeted approach, and ~80 % involved the use of HS-SPME.

3.2.1. Beverages

The understanding of the aroma composition, for example, to improve sensory properties, is one of the main focus in the analysis of beverages. The aroma, which can be considered as a major consideration for consumers' choice, derives primarily from its ingredients; however, it is also strongly influenced by its production process. Concerning the aroma and sensory studies of any kind of beverage, surely the headspace sampling is the primary and most used technique exploited with GC×GC [72].

The majority of the revised literature investigating alcoholic beverages used SPME in HS-mode for aroma analysis. HS-SPME in combination with FM-GC×GC-MS has been used to distinguish beers fermented with two different yeast strains. Multiway principal components analysis revealed 46 compounds, among the approximately 210 detected, which were relevant for the classification [73]. Paiva *et al.* chose the commercially available DVB/CAR/PDMS fiber to obtain the widest analyte coverage for their non-targeted analysis.

Static (classical SHS, HS-SPME) and dynamic HS extraction techniques (DHS) have been compared for the aroma analysis of Belgian Trappist beers [74]. The authors also tested the extraction efficiency of classical and multiple SBSE, concluding that DHS exhibited a satisfying trapping efficiency for aroma VOCs while producing limited chromatographic artifacts. An alternative dynamic sampling, involving the use of P&T (Tenax TA) was explored with an FM-GC×GC-MS system, discussing a straightforward and informative workflow for aroma analysis on different fruity beers [75]. As for the DHS, purge-and-trap represents a long-established dynamic technique, which consists of pushing the VOCs continuously from the liquid sample (bubbling an inert gas through the sample) into the HS, resulting in a more

efficient extraction of the analytes. Also, this study is the first published paper reporting on the combination of purge-and-trap with GC×GC.

In the quest for unique selectivity, ionic liquid coatings have been recently developed for sampling methods [76,77], in addition to their use as stationary phase for GC columns [78,79]. Few recent pieces of research reported on the use of poly ionic-liquid (PIL)-based sorbent coatings for HS-SPME in GC×GC [80,81]. Crucello *et al.* investigated several non-ionic and PIL-based sorbent coatings for HS-SPME, analyzing VOCs in Brazilian wine [80]. About 350 peaks were detected in four wine samples. Among the five different fibers tested, the DVB/CAR/PDMS and one of the PIL coated fibers exhibited improved selectivity toward a broad range of aroma VOCs present in wine. That particular PIL-coated fiber extracted more effectively polar analytes found in wine compared to the popular DVB/CAR/PDMS fiber because significantly higher distribution coefficients can be obtained with ionic liquid coatings. Noteworthy is that some compounds were solely detected employing the aforementioned PIL fiber. For example, two of the four potential markers to distinguish “Isabella” and “BRS Magna” wine cultivars, namely ethyl butanoate and ethyl methyl succinate, were only extracted when using the PIL-based SPME fiber.

Similarly, for non-alcoholic beverages such as tea and coffee, HS-SPME has been extensively applied for the analysis of infusions or dried plant material [82–84]. Nevertheless, also the direct immersion approach (DI-SPME) [82], simultaneous distillation extraction (SDE) [85], and derivatization [86] have been reported.

Aroma components of several green tea and chestnut were analyzed by Zhu *et al.*, using two different sample preparation techniques (SDE and HS-SPME) [87]. The authors considered SDE as the most suitable sample preparation technique for green tea aroma; however, due to the poor solubility and volatility of chestnut aroma compounds in the SDE solvents, HS-SPME was applied as an alternative extraction technique. Eventually, (1) the combination of SDE with GC×GC-MS used to determine the odor activity value, and (2) the HS-SPME/GC-MS/GC-O used for the identification of key odorants, revealed eight VOCs as the most definite odorants to promote the formation of the chestnut-like aroma of green tea.

Magagna *et al.* sampled VOCs from the HS of dried black tea leaves in different conditions (*i.e.*, untreated, suspended, and infused in water) [82]. The dry plant material was sampled *via* HS-SPME, SBSE, and DHS and the infusion *via* DI-SPME, and SBSE. The efficiency of each sampling method was compared in terms of numbers of analytes extracted and their response. Significant differences in extraction capabilities were shown, depending on the technique used. On one hand, DHS and HSSE (both using PDMS) revealed good recovery of less polar and low volatility analytes such as saturated aldehydes (C_{10} to C_{18}), methyl ketones (C_{11} to C_{15}), and some medium-chain alcohols. On the other hand, HS-SPME (DVB/CAR/PDMS) showed the highest suitability for highly-volatile aldehydes (C_5 to C_7) and was therefore considered by the authors as the most suitable sampling technique to establish aroma fingerprinting. Nevertheless, the authors suggested that the most

comprehensive chemical signature would be provided by the combination of the complementary information offered by the different sampling approaches.

Similarly, the chemical composition of raw coffee has been correlated to the end products' quality, authenticity, and sensory properties [88]. Novaes *et al.* reported a method for the analysis of low volatility compounds in green Arabica coffee extracts without the need for prior fractionation or another pre-treatment (*e.g.,* derivatization) [89]. After liquid-liquid extraction (at circa 77°C), samples were subjected to high-temperature GC and GC×GC. The GC×GC set-up, consisting of a relatively short 1D column (11 m), therefore requiring the optimization of some separation conditions such as trap temperature, oven temperature program, and flow rate allowed for the elution also of thermo-labile and low volatility compounds. Despite minimal sample preparation, superior performances of GC×GC in comparison to GC resulted in improved compound identification and group-type analysis of the approximately 200 detected compounds. The same authors developed a sampling protocol for the trapping of VOCs and SVOCs during the roasting process of coffee beans [88]. For the dynamic sampling, the authors tested two different sorbents (XAD-2 and CSC). **Figure 8** displays the GC×GC results for VOCs and SVOCs extraction of the coffee roasting process using XAD-2 (left side) and CSC (right side) adsorbents for different time-intervals. The boxed zones highlight SVOCs elution regions. **Figure 8** readily demonstrates that the use of XAD-2 for the sample preparation, in combination with a high-temperature column set for the two-dimensional separation allowed the specific identification of SVOCs for the chemical profiling of the roasting process.

3.2.2. Plant-based edibles

The assessment of quality represents often a central aim in the research in the field of edibles, and the combination of robust sample preparation and high-resolution separation techniques contribute to providing dense information about sample composition. Among the most common food products derived from plants, cocoa and hazelnut composition have been studied deeply using HS techniques [90–94]. For example, the volatile metabolome of cocoa from different regions was analyzed using HS-SPME and GC×GC-MS [90]. The SPME method was standardized, preloading an internal standard (α-thujone) onto the fiber before sampling for the normalization of analyte response. In their study, they exploited the complexity of the cocoa headspace to develop new workflows for non-targeted and targeted analysis. Also, they highlighted the value of the combination of softer ionization with conventional electronic ionization to increase the level of confidence for the identification of VOCs.

The HS of vegetable oils generally carries sensory-relevant information which can be used not only to define the quality but also to discriminate different varieties and geographical origins. HS-SPME was used by Lukić *et al.* for the characterization and differentiation of virgin olive oils made from different varieties grown in different regions [95].

Multiple headspace sampling techniques have been applied to study the complex volatilome of olive oils [96]. The extraction capabilities of static (HSSE, HS-SPME), monolithic material sorptive extraction, and dynamic headspace extraction (DHS), were compared as well as several sorbent materials (DVB/CAR/PDMS, PDMS, PDMS/Carbopack B, PDMS/EG, ODS, ODS/GC, and Tenax TA). Interestingly, the authors pointed out that the sampling techniques with higher amounts of sorbent, such as HSSE and DHS (Tenax TA), gave better results in terms of concentration capacity, resulting in a higher extraction yield. However, they focused the attention principally on the use of the HS-SPME approach and discussed how the combination of such a technique can successfully be integrated with GC×GC-MS.

On the less volatile fraction of vegetable oils, derivatization approaches for the characterization of the minor components (unsaponifiable fraction) were also proposed, combining a silylation method with GC×GC-high resolution (HR) MS [97] and a miniaturized-SPE with dual detection GC×GC-MS/FID [98].

After the relevant contribution from Risticevic *et al.* showing the potential of DI-SPME [16], further *in vivo* and *in situ* apple metabolome studies have been performed [99,100]. In these studies, the authors confirmed the feasibility of the approach to provide unbiased metabolite coverage, compared to traditional approaches requiring metabolism quenching and laborious sample preparation. Apple was used as a living plant sample to detect changes in metabolic fingerprints in response to fruit maturation. An overcoated triphasic SPME fiber was used, showing more effective cleanup and reduced decomposition products. In these works, esters were the most important contributors to the aroma profile of apples, both in quantitative and qualitative terms. Also, the comprehensive metabolome coverage obtained with *in vivo* DI-SPME was reflected by the extraction of aldehydes, ketones, alcohols, aromatic compounds, carboxylic acids, aromatic aldehydes, aromatic ketones, benzyl alcohols, alkanes, benzyl acetates, and glycol ethers, as well as metabolites originating from the lipoxygenase pathway. For the esters profiling, the structurally ordered GC×GC chromatograms supported the identification of 13 acetates, 5 propanoates, 10 butanoates, and ethyl hexanoates. Interestingly, a comparison of the *in vivo* sampling on the intact apple with the *ex vivo* sampling following homogenization and metabolism quenching, revealed differences in metabolites, corroborating the fact that the metabolome is influenced by the sample preparation procedure and that many metabolites can be produced or altered during this step (**Figure 9**).

The analysis of fatty acids in foodstuff is another common area of research, which typically involves the derivatization and isolation of targeted classes before the GC×GC separation. The determination of free fatty acids in edible oils is particularly important for quality and authenticity control, and it was recently investigated by performing magnetic dispersive extraction (MDE) followed by derivatization using silylation agents [101]. The extraction method combined the advantages of magnetic solid-phase extraction and dispersive liquid-liquid microextraction. However, the multiple steps and the emulsification issues can

impede its widespread use. After the extraction, free fatty acids were derivatized using silylation agents, concentrated and then injected in a GC×GC-MS system. The authors highlighted the help given by the regular and predictable patterns of the homolog fatty acids, which facilitated the qualitative analysis of fatty acids without the use of analytical standards (**Figure 10**).

3.2.3. Plant parts and extracts

The non-targeted analysis of plant parts and extracts usually aims at the better characterization of the samples and understanding of biochemical processes, and the acquired knowledge could support in correlating the health-beneficial properties of the natural products.

The composition of a variety of plant extracts or resins used in traditional oriental medicine has been characterized by GC×GC [102,103]. The analysis of these typically complex natural products benefits substantially from the increased separation power allowing for the precise determination of the compounds with potential biological activity [102]. In a comparative study of the volatile metabolite profiles of resin from five *Dracaena* species, HS-SPME-GC×GC-MS allowed identifying 20 terpenoid components. It was found that the terpenoid compounds are species-specific and that the monoterpene fraction could serve to determine chemotaxonomic markers [103].

The VOCs from Frankincense (the gum resin of *Boswellia papyrifera*), which also present potent antimicrobial activities, were studied using a PDMS/DVB SPME fiber allowing the detection of about 220 compounds in a single analysis [104]. Group-type separation allowed for the classification of monoterpenes, sesquiterpenes and diterpenes, and unidentified compounds could be further classified based on the elution pattern and mass spectral information.

A continuously emerging field is the analysis of cannabis and its related products, which are mainly used for their psychoactive and medicinal activities. About 500 compounds have been identified and, among them, cannabinoids and terpenoids currently hold the highest interest for their biological and synergistic activity. Conventional studies have led to protocols optimized for the analysis of a few targeted analytes or chemical classes. Instead, GC×GC has shown its advantages in the multiclass and high-resolution analysis of a variety of analytes [105,106]. In a recent approach, a methodology involving the use of SBSE was employed for the multiclass metabolite profiling of cannabis inflorescences [107]. The SBSE was selected to cover the high molecular weight metabolites, and particular attention was dedicated to the optimization of the extraction conditions in order to extend the analytes class coverage (**Figure 11**). Using a fractional factorial design, five chemical classes were considered to optimize the extraction conditions in a non-targeted fashion. **Figure 11A** shows the response trends under the different extraction variables and levels considering

the investigated classes, which were group-type separated in the GC×GC space and tracked using characteristics ions (**Figure 11B**). Under optimized conditions, different cannabis chemovars belonging to sativa, indica, and hybrid subspecies were analyzed combining SBSE with an FM-GC×GC-MS system. In addition to chemotype classification, quantitative analysis was performed on three common cannabinoids (THC, CBD, and CBN). Noteworthy, the non-targeted sampling strategy found also confirmation with the detection of exogenous compounds like pesticides, plasticizers, and cannabinoids degradation products, which were identified with high confidence by using high-resolution MS (SBSE-GC×GC-HR MS).

Some high molecular weight molecules, such as small carbohydrates and phenylalkanoid glycosides play an important role in the biological activity of plants. Even though these molecules are typically analyzed via HPLC, Carrero-Carralero *et al.* reported a derivatization protocol employed in combination with GC×GC for the analysis of *Sedum roseum* root dietary supplement [108]. In this uncommon choice of analyzing sugar and glycosides, the authors highlighted the importance of the optimization of the derivatization step (silylation) for the class of phenylpropanoid glycosides. The final derivatization procedure allowed for the complete and reproducible identification and quantification of targeted compounds with a GC-based system. Also, the non-targeted analysis revealed the presence of a variety of other important compounds such as polyphenols, terpenes, phenylalkanoids, carbohydrates, and glycosides which were co-extracted and separated in the high-resolution chromatographic system.

Essential oils and other plant extracts from aromatic plants are widely appreciated in the food and flavor industry due to their olfactory properties. Essential oils, typically obtained by hydrodistillation, are mainly injected directly, although, a sample preparation beforehand can be beneficial to increase selectivity. For example, an additional liquid-liquid extraction combined with high-resolution GC×GC-MS (QTOF) was used for the detailed chemotypic profiling of essential oils of different hop genotypes [109]. Bendif *et al.* compared the extract of *T. munbyanus* obtained *via* hydrodistillation, pressurized liquid extraction (PLE), and supercritical fluid extraction (SFE) [110]. The authors discussed the impact of solvent polarity using PLE, and how the different ratios resulted in various total phenolic content which was related to the antioxidant activities. However, major differences were observed in the metabolic profile of essential oils obtained *via* hydrodistillation, mostly characterized by the presence of typical terpenoids, whereas the more lipophilic SFE extracts contained higher amounts of long-chain hydrocarbons and tocopherols.

Other pre-separation procedures, especially those based on chromatographic techniques have been proposed to select specific fractions of interest. Tissandie *et al.* reported a detailed investigation of vetiveryl acetates and vetiver oils and related it to the final olfactory properties [111]. A series of SPE cartridges (*i.e.,* silica and silver) were used to isolate the different chemical classes before GC×GC-MS/FID for qualitative and quantitative analysis. The authors exploited the different SPE characteristics and elution solvents polarity

to obtain a selective fractioning of the samples (*i.e.,* hydrocarbons, esters, aldehydes and ketones, tertiary alcohols, and vetivones) for detailed characterization. The authors finally concluded that the GC×GC analysis of vetiveryl acetates allowed more reliable quantification of their constituents, superior to what the conventional GC-FID could offer.

Another powerful approach that also holds the possibility for automation and on-line configuration, is the use of liquid chromatography as a pre-fractionation step before the GC×GC separation. A detailed qualitative analysis of the volatile fractions of mandarin and lime essential oils was achieved by combining HPLC and GC×GC-MS [112,113], although in an off-line configuration in these examples. The pre-separation using a normal phase HPLC process aimed at the isolation of the oxygenated compounds contributing to the composition of the essential oils.

Hydrodistillation followed by a hybrid heart-cut MDGC and GC×GC-MS system was combined for the analysis of hop (*Humulus lupulus L.*) essential oil and agarwood (*Aquilaria malaccensis*) oleoresin [114]. This represents a neat example to show the close relation of sample preparation and multidimensional chromatography in contributing to the removal of interferences and the increase of sensitivity. In this configuration, the initial heart-cut MDGC acted as the sample preparation step and replaced a conventional fractionation step, allowing to isolate the fraction of interest which was then further subjected to the high-resolution GC×GC separation. **Figure 12** shows the analysis of oxygenated sesquiterpenes in agarwood oleoresin using GC-FID (Figure 12A), heart-cut GC-GC (Figure 12B), and GC-GC×GC-MS (Figure 12C). An extension of compound coverage concerning oxygenated sesquiterpenes was apparent when samples were subjected to the high-dimensional separation system (GC-GC×GC-MS).

Generally, the use of DI-SPME appears not common, especially in the case of matrices with high-fat content, and due to issues related to the direct exposure of the coating with the matrix (lack of reproducibility, poor sensitivity, or insufficient ruggedness).

Noteworthy, DeGrazia *et al.* [115] presented an approach for DI-SPME analysis of high-fat content matrices (in avocado puree). A commercially available PDMS/DVB fiber was over-coated with an additional layer of PDMS to enhance inertness and prevent fouling of the fiber surface. Critical parameters for the sample extraction such as pre-desorption rinsing and post-desorption washing were optimized for time, solvent mixture, and agitation conditions, to assure satisfactory sensitivity and reproducibility of the method [4]. In their study, the utilization of GC×GC provided sufficient resolution for the evaluation of the fiber coatings. Indeed, the multidimensional separation allowed the assessment of the degree of matrix accumulation in the fiber and/or the formation of artifacts at the injector port, for example, resulting in high background signals and very complex 2D chromatograms. The modified PDMS/DVB/PDMS coating showed superior performance regarding its robustness which permitted more than 100 consecutive extraction cycles. Finally, the method was

automated, resulting in a high-throughput approach with minimal sample preparation for food analysis.

Conclusions and perspectives

The affinity and complementarity between a generic sample preparation technique and the GC×GC separation was herein considered and commented. Recent biomedical, food, and plant applications (2016-2020) were also discussed, specifically those in which non-targeted analysis was sought, and the combination of tailored sample preparation methods and GC×GC-MS has proven to be beneficial.

Even though common features between a typical sample preparation and the GC×GC separation can be identified (**Figure 1**), the full optimization of the two processes remains fundamental for reliable and reproducible results, especially in the challenging aspects of non-targeted analysis in biomedical, food and plant applications. Moreover, their combination with the identification capability of mass spectrometry generates the most powerful and flexible analytical platform for complex sample analyses. Indeed, a complete analytical methodology that exploits (1) a wide and an unbiased analyte extraction from the sampling step, (2) the sensitivity, the separation, and identification power from the GC×GC separation and the MS detection, represents a solid advantage for non-targeted analysis.

In the quest for greener, miniaturized, faster, selective/universal, and robust sampling strategies, it will be interesting to see how some recently developed sampling alternatives (vacuum-based, thin-film, paper-based, freeze-concentration, or solvent-assisted SPe techniques [116–119]) will perform with GC×GC.

An additional aspect to consider indeed is the use of GC×GC to discover new sample preparation techniques and to fully exploit, evaluate, and refine the existing ones. In fact, the GC×GC increased separation power provides a more comprehensive overview of the sampling performance, and finally of the sample composition.

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Conflict of interest statement

The authors declare no competing financial interest.

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Figure Legends:

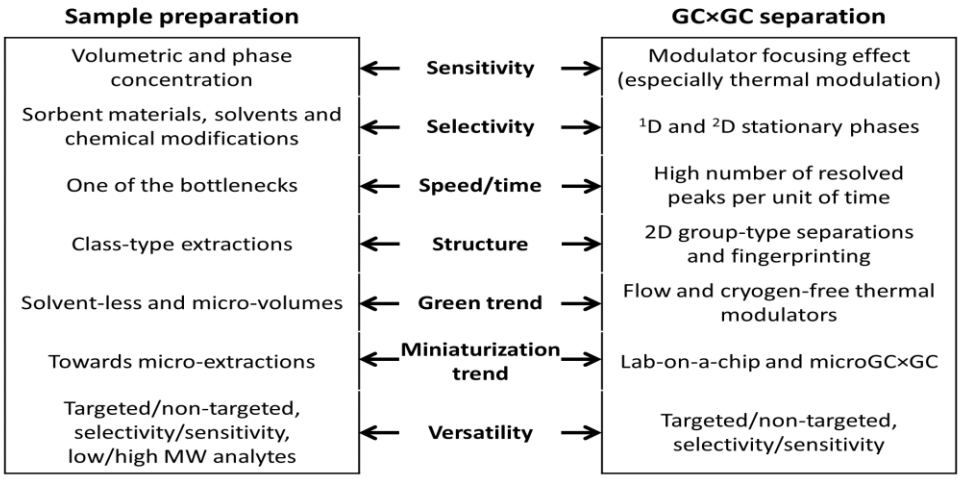

Figure 1. Commonalities and characteristics of a generic sample preparation and the GC×GC separation steps.

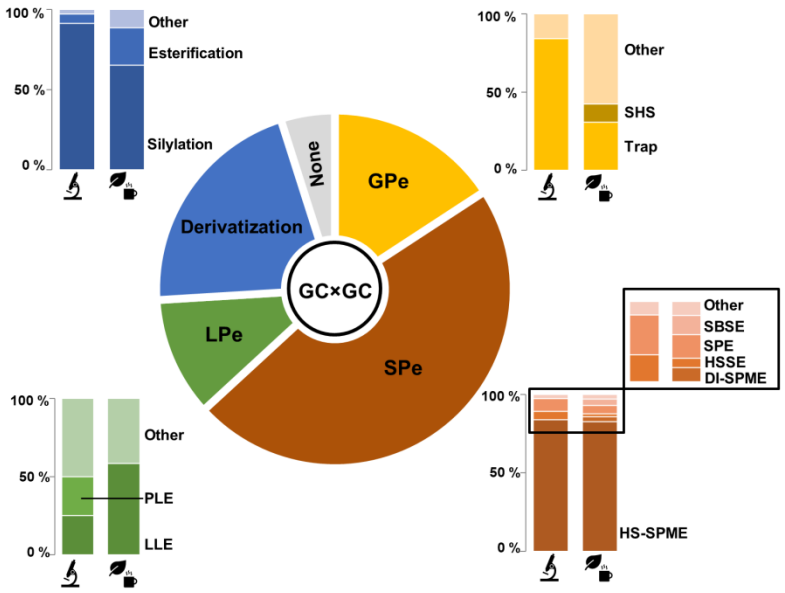

Figure 2. Distribution of the sample preparation types used in combination with GC×GC in the period 2016-2020. In the bar charts, the bar on the left groups biomedical-related applications, and the one on the right groups the food- and plant-related applications.

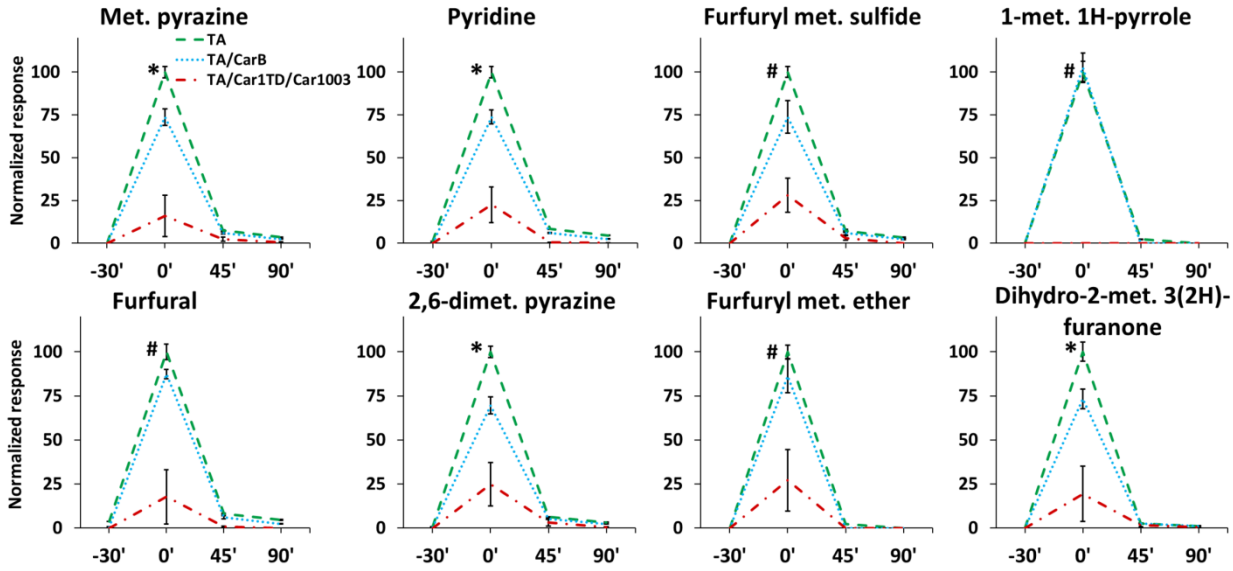

Figure 3. Longitudinal tracking of breath metabolites from coffee (-30, 0, 45, and 90 min with respect to its intake) using different sorbent traps Reproduced from [41] with permission from Elsevier.

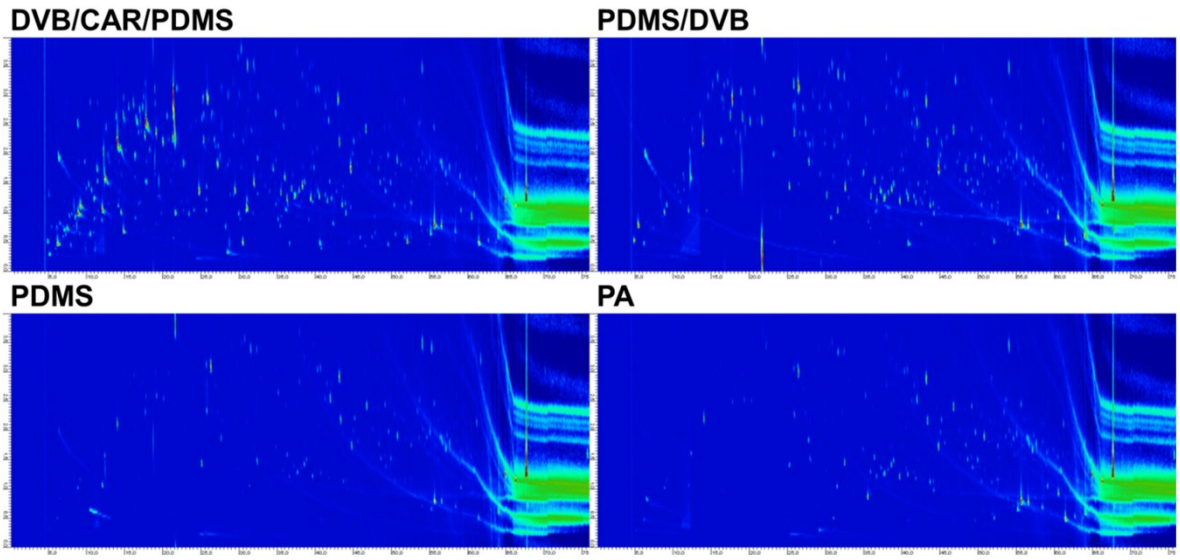

Figure 4. Different HS-SPME-GC×GC-MS chromatograms of a urine sample using different fiber sorbent phases. Reproduced with permission from [43].

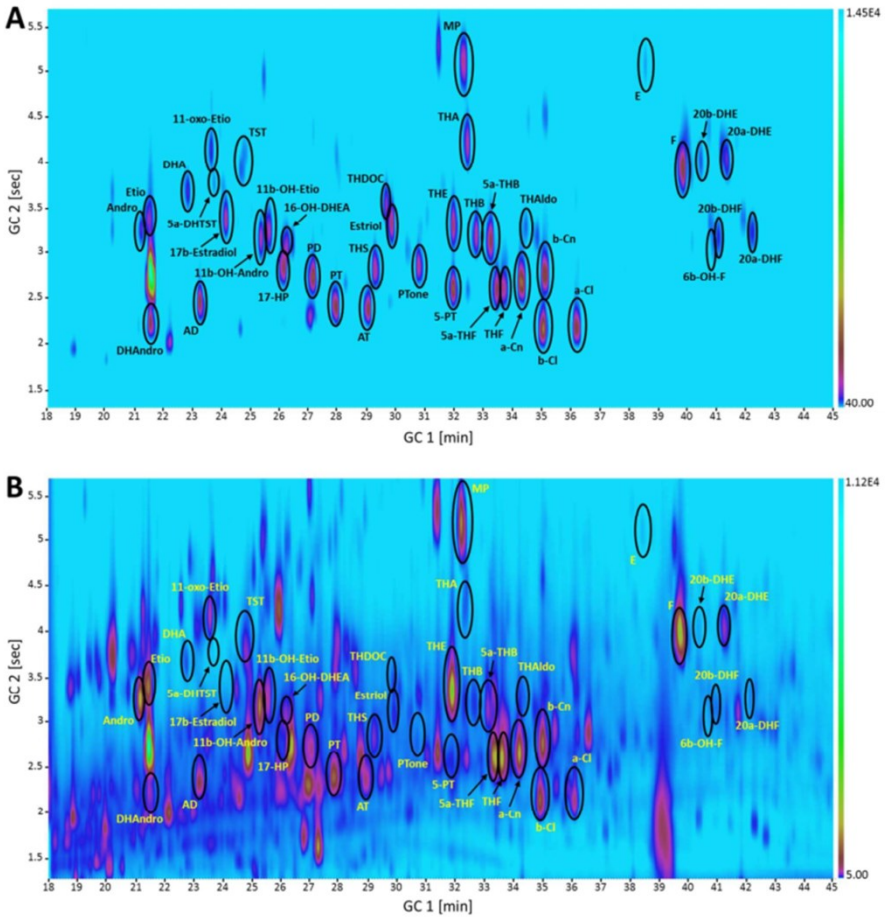

Figure 5. GC×GC-MS chromatograms of derivatized (A) 40 standard steroids and two internal standards and (B) a human urine sample. Reproduced from [47] with permission from the Royal Society of Chemistry.

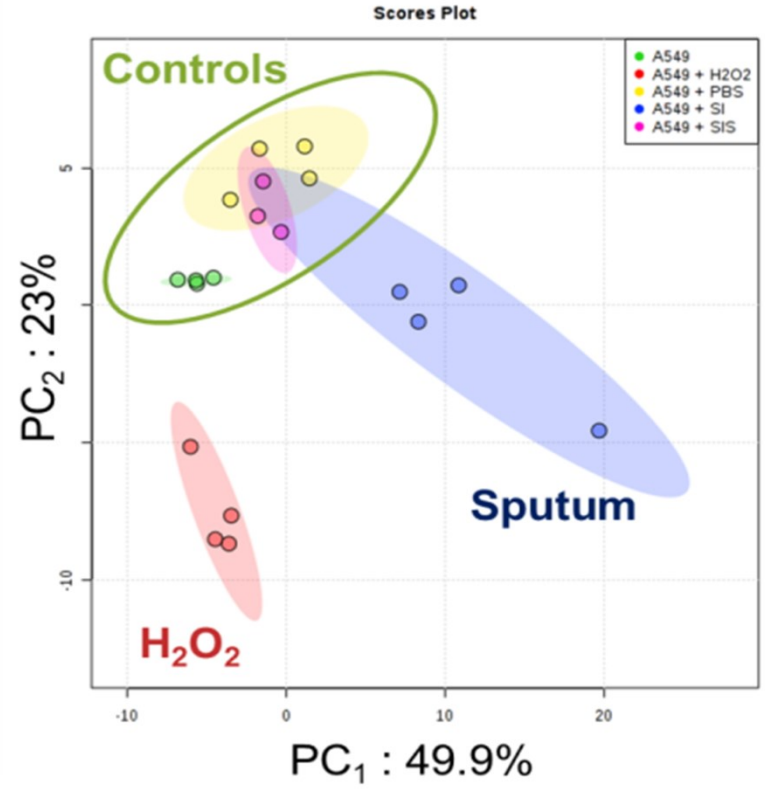

Figure 6. Principal component analysis of the chemical and biological induced inflammation on A549 lung epithelial cells using selected features. Adapted and reproduced from [65], with permission from the Royal Society of Chemistry.

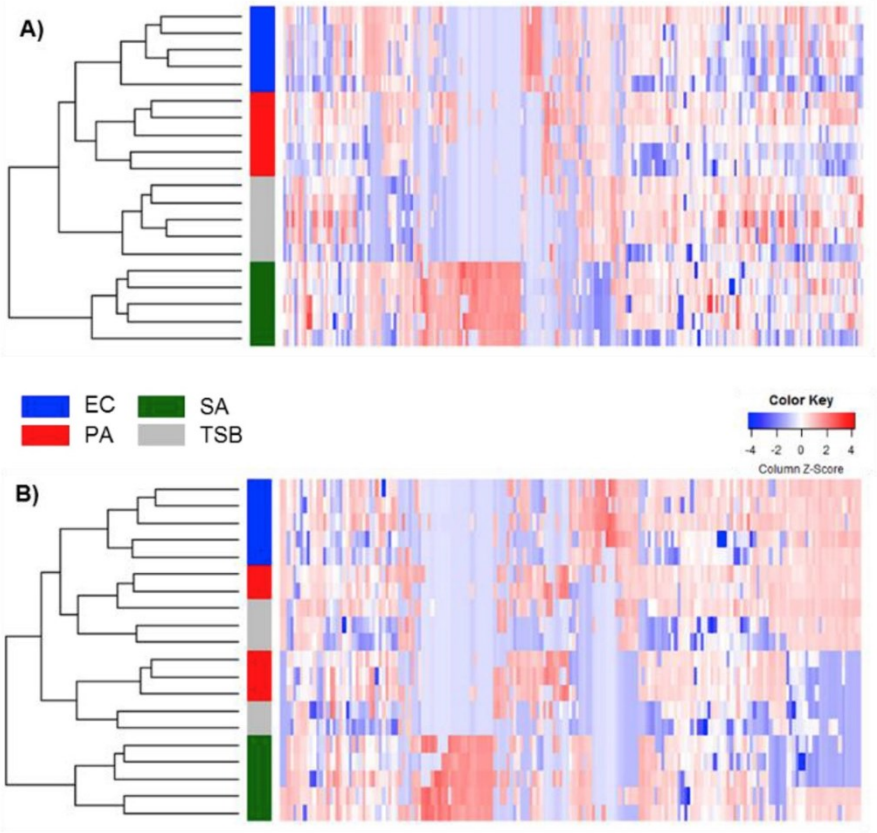

Figure 7. Heatmap and hierarchical showing the clustering of the control, and SA, PA and EC cultures using (A) Tenax TA and (B) CarbopackY/CarbopackX/Carboxen1000 as sorbent materials. Adapted and reproduced from [42] with permission from Elsevier.

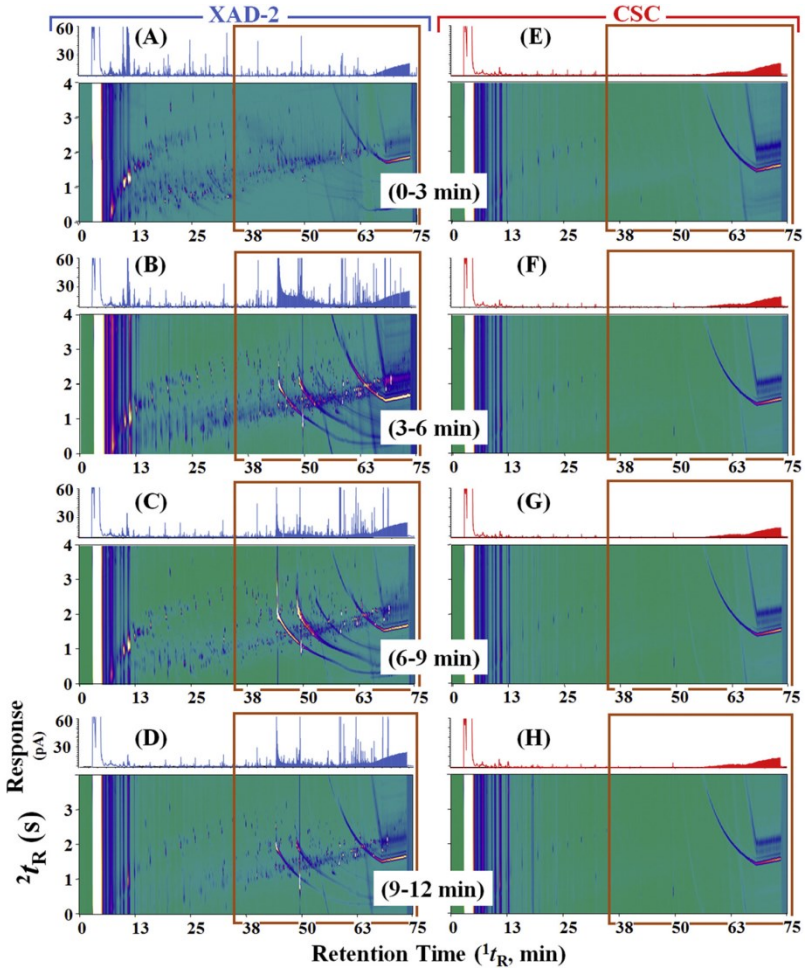

Figure 8. GC×GC results for VOCs and SVOCs extraction (DHS) of the coffee roasting process using flow-through/active sampling with two different adsorbents (XAD-2 and CSC, on the right and on the left, respectively) at different roasting time intervals. The boxed zones highlight SVOC elution regions. Adapted and reproduced from [88] with permission from Elsevier.

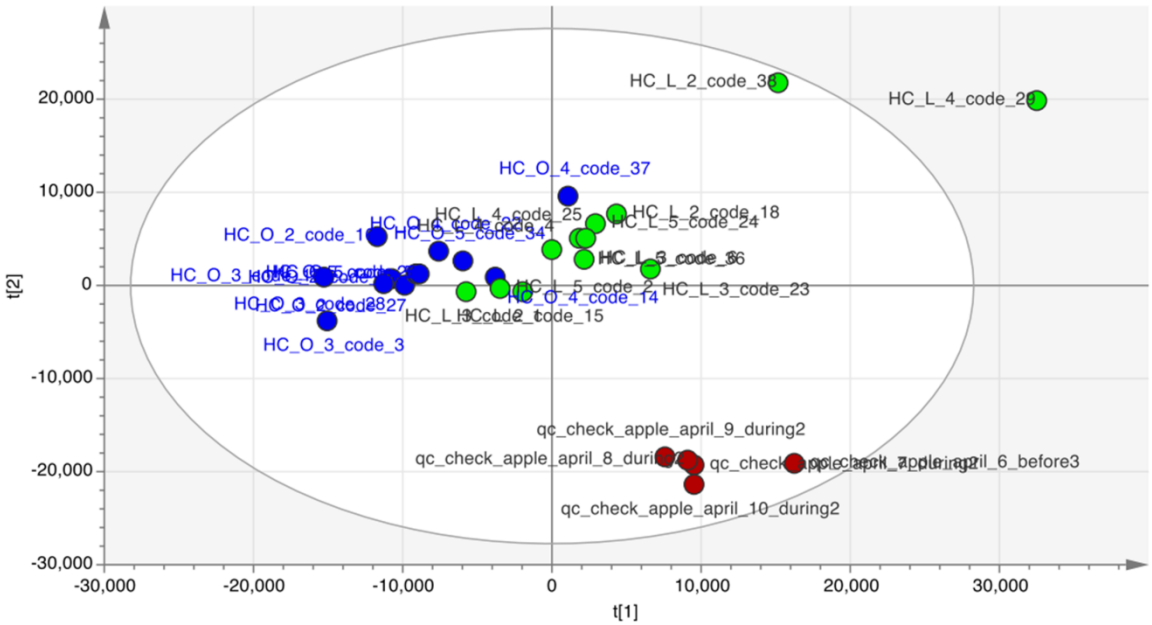

Figure 9. PCA scores plot of in vivo DI-SPME data for apples with higher maturity index (green circles), apples with lower maturity index (blue circles), and ex vivo HS-SPME data obtained for control samples (red circles). Reproduced from [100].

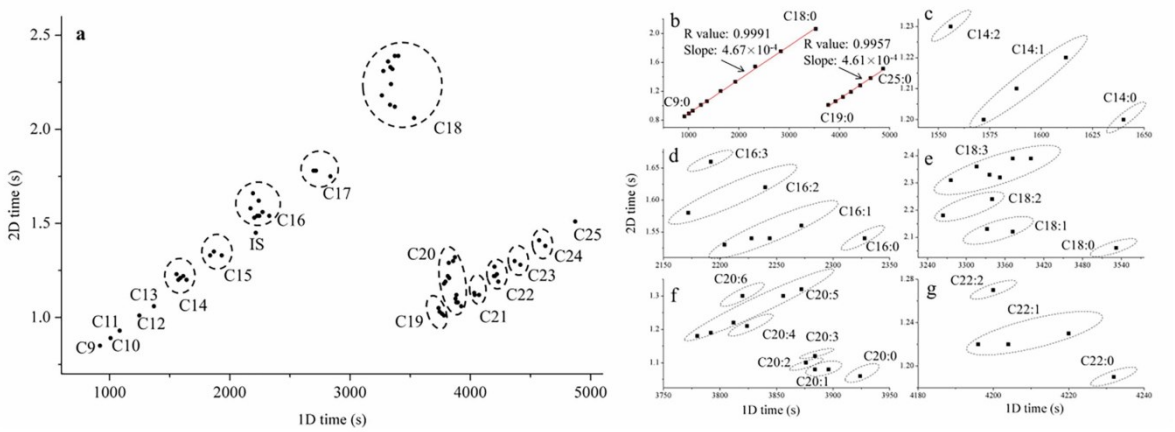

Figure 10. Apex/dot plot with the identified FFAs in edible oils using the MDE followed by GC×GC-MS (a). Saturated FFAs (b), $C_{14:N}$ (c), $C_{16:N}$ (d), $C_{18:N}$ (e), $C_{20:N}$ (f), $C_{22:N}$ (g), in edible oils via using the magnetic dispersive extraction followed by GC×GC-MS analysis. $C_{x:y}$ represent the carbon number and the unsaturation degree of the fatty acids. Reproduced from [101] with permission from Elsevier.

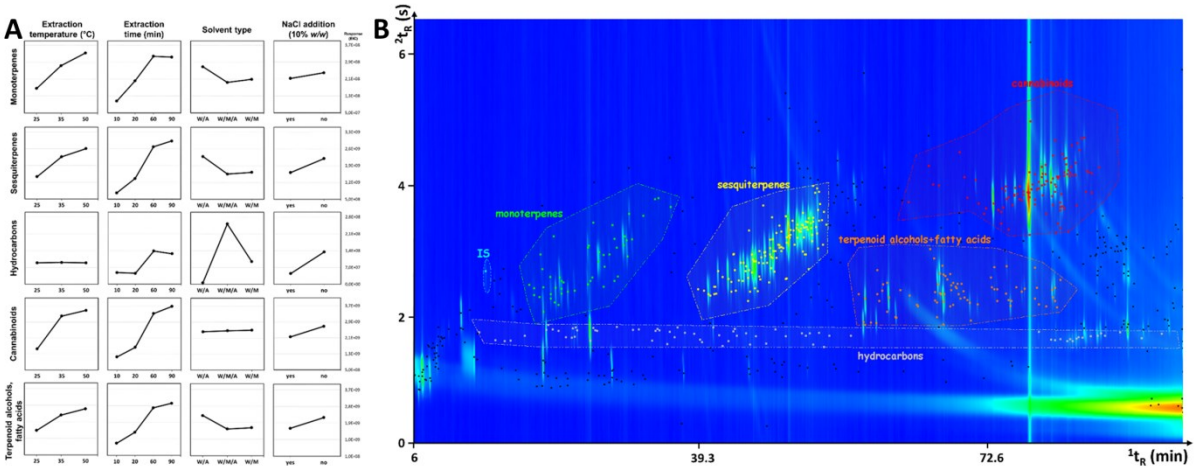

Figure 11. Response plots under different extraction conditions of monoterpenes, sesquiterpenes, hydrocarbons, cannabinoids, and terpenoid alcohols and fatty acids. B) SBSE FM-GC×GC-MS chromatogram of a cannabis inflorescence sample. Adapted and reprinted with permission from [107]. Copyright 2020 American Chemical Society.

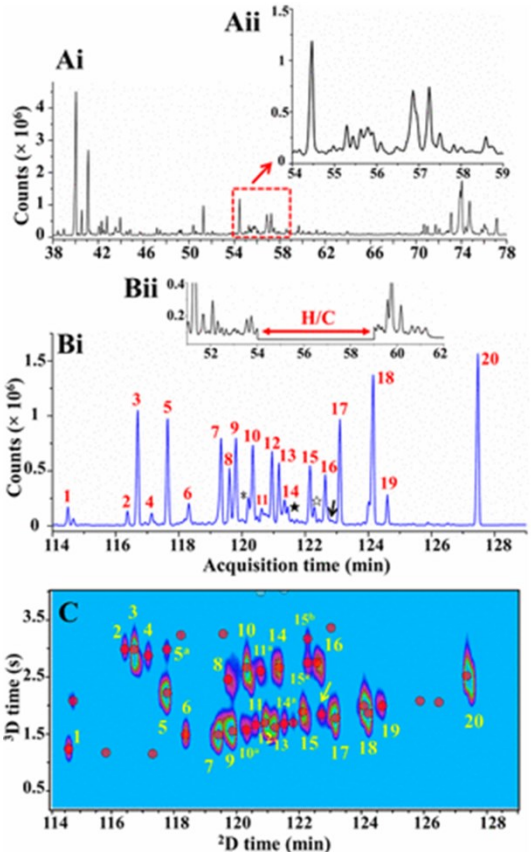

Figure 12. Sequential GC-GC×GC-MS analysis of the oxygenated sesquiterpenes in *A. malaccensis* oleoresin. (A_i) 1D FID response, the region to be heart-cut (H/C) is denoted by the dotted rectangle, with an inset (A_{ii}) of the expansion of the target region. (B_i) H/C GC-GC-MS analysis of the target region and with an inset (B_{ii}) of the 1D FID response. (C) GC-GC×GC-MS analysis of the target region. Reprinted with permission from [114]. Copyright 2020 American Chemical Society.

Table 1: Selected applications in the order as they appear in the manuscript. All biofluids and tissues are from human unless otherwise stated. For SPME, the coating was DVB/CAR/PDMS if not specified further. For HSSE and SBSE, the coating was PDMS if not specified further. TM and FM are thermal and flow modulator, respectively.

Field	Sample type	Highlights	Sample preparation	GC×GC	Detector
Biomedical applications	Breath [30]	Standardization of exhaled breath sampling *via* direct collection and pre-concentration onto TD tubes	Trap (Tenax TA/Carbograph 1TD)	FM	Q/FID
	Urine (human and animal) [42]	Increased detection and characterization of trace-level steroids	SPE (C_{18}), derivatization (silylation, MOX + TMSI)	TM	HR TOF

	Urine [43]	Identification of new tuberculosis metabolites	Derivatization (silylation, BSTFA + TMCS)	TM	TOF
	Urine, tissue and cell lines (animal) [45]	Fast sample extraction protocol (2-h) for multiple matrices untargeted profiling	Derivatization (MSTFA + TMCS)	TM	Q
	Sputum [46]	Detection of markers of mycobacteria species and *M. tuberculosis*	Derivatization (alkylation, TMAH)	TM	FID
	Serum [47]	Novel biomarker identification of Crohn's diseases	Derivatization (silylation, MOX + MSTFA)	TM	HR TOF
	Skin VOCs [52]	Straightforward *in vivo* passive collection of skin VOCs using rubber bands	PDMS bracelets and anklets	TM	TOF
	Bacterial culture [62]	Monitoring volatile metabolome changes of bacterial cultures (*Rhizobium* sp.)	HS-SPME	TM	TOF
	Bacterial culture [63]	*In vivo* study of antibacterial agents' effect on the volatile profile and metabolomic pathways (*E. coli*)	HS-SPME	TM	TOF
	Bacterial and fungi co-cultures [64]	*In vivo* characterization of bacteria antifungal VOC activity and co-culture VOC profile changes (*C. vaccinii*)	HSSE (PDMS/EG)	TM	TOF
	Bacterial culture [37]	Sampling performance evaluation of different sorbent materials for untargeted and targeted bacterial VOCs analysis (*E. coli*, *S. aureus*, *P. aeruginosa*)	Trap (DHS, Tenax TA, CarbopackY/X/Carboxen1000, CarbopackB/X, CarbopackY)	TM	TOF
Food and plant applications	Trappist beer [69]	Comparison of different extraction techniques for volatile aroma profiling	HS-SPME (CAR/PDMS), classical and multiple SBSE (PDMS/EG), trap (SHS and DHS, Tenax TA)	TM	TOF
	Fruity beer [70]	Development of a purge-and-trap extraction method for aroma characterization	Trap (P&T, Tenax TA)	FM	TOF
	Wine [72]	PIL coating for SPME, increasing recovery for polar compounds	HS-SPME (PA, DVB/CAR/PDMS, three PIL coatings)	FM	Q/FID
	Coffee [81]	Chemical profiling (with focus on SVOCs) of coffee roasting process	Trap (DHS, CSC + XAD-2), liquid-extraction (DCM)	TM	Q, FID

	Cacoa [83]	Targeted and untargeted sampling approach; combined with low eV MS ionization for improved identification capacity	HS-SPME	TM	TOF
	Olive oil [90]	Comparison of four extraction methods and sorbents for volatile fingerprinting	HS-SPME, HSSE (PDMS, PDMS/Carbopack B, PDMS/EG), MMSE (ODS/CB), HSSE, trap (DHS, Tenax TA)	TM	TOF
	Apple [93-94]	*In vivo* and *ex vivo* metabolome profiling using over-coated fiber for improved performance	HS- and DI-SPME	TM	TOF
	Cannabis [101]	Targeted and untargeted multiclass metabolite profiling using a single extraction	SBSE	FM, TM	TOF, HR TOF
	Sedum roseum root food supplement [102]	Extraction and characterization of low molecular weight carbohydrates and phenylalkanoid-glycosides	Derivatization (silylation, TMSI + TMSC)	TM	TOF
	Essential oil [106]	LC-prefractionation for the analysis of oxygenated species	HPLC	TM	Q
	Avocado [109]	Protocol for direct determination of contaminants in fatty matrices based on improved fiber coating	DI-SPME	TM	TOF

MOX = methoxamine

TMSI = N-trimethylsilylimidazole

BSTFA = N,O-Bis(trimethylsilyl)trifluoroacetamide

TMCS = chlorotrimethylsilane

MSTFA = N-trimethylsilyltrifluoroacetamide

TMAH = Trimethylanilinium hydroxide