Current Developments in Analyzing Food Volatiles by Multidimensional Gas Chromatographic Techniques

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Abstract

This paper presents current developments and future perspectives on the spreading of advanced analytical tasks in the field of food volatile analysis. The topics outlined comprise: (a) recent advances on miniaturized sampling techniques; (b) the potential and challenges of multidimensional gas chromatography coupled with mass spectrometric detection for volatile identification and quantitation in samples with complex matrices; (c) the potential of comprehensive two-dimensional gas chromatography in fingerprinting studies, in particular for classifying complex samples in routine analysis; and (d) the key role of dedicated software tools for data elaboration with comprehensive two-dimensional separations.

Key-words:

Analysis of food volatiles; sample preparation; multidimensional gas chromatography; comprehensive two-dimensional gas chromatography; fingerprinting

Introduction

Although understanding of food aroma comprises the knowledge about its volatile and non-volatile fractions, in this work, we focus our discussion on the analysis of volatile compounds. Concepts for the analyses and understanding of the contributions of non-volatiles to food aroma can be found elsewhere.[1]

Basically, food volatile analyses can be differentiated into targeted analysis of key odorants and profiling or fingerprinting analyses, targeting at best the sum of volatile compounds amenable to the chosen analytical technique. Fingerprinting analyses often involve *-omics* strategies tackling, for example, food sensory quality characterization (*sensomics*, *flavor metabolomics* and *flavoromics*).[2,3] A focus usually is on sensory-active compounds to correlate stimuli of multimodal perceptions of food (i.e., aroma, taste, texture, etc.) with specific and peculiar chemical signatures, i.e., the chemical pattern associated with the perceived property. In this perspective, comprehensive investigations are required to combine chemical information on sample components (analyte identity and concentration in the matrix) with their sensory quality.

Well established investigation protocols, such as the molecular sensory science approach,[4] focus on isolation, identification, and quantitation of key-aroma compounds by combining extraction (e.g., liquid-liquid extraction (LLE), solvent assisted flavor evaporation (SAFE), simultaneous distillation extraction (SDE), solid phase extraction (SPE), or supercritical fluid extraction (SFE)) and analysis by gas chromatographic separation and mass spectrometric detection (GC-MS), odorant detection by GC-Olfactometry (GC-O), and structure elucidation. An important part of the characterization of key-aroma compounds is their accurate quantitative measurement, together with the knowledge of their sensory threshold in the respective food. Each of these discrete and often manifold analytical operations can be difficult, e.g., due to the very low concentration of potent odorants in complex matrices, but are fundamental to detailing

flavor-chemical signatures. The option and possibility of extending such detailed protocols to high-throughput screening and/or fingerprinting would be very useful and attractive.[5]

Based on their experience, the authors here present their viewpoint and outline perspectives on the future of advanced approaches in the field of volatile aroma analysis. The following steps within the analytical work-flow are addressed: (a) recent advances on miniaturized sampling techniques; (b) the potential and challenge of multidimensional (MD) GC-MS for volatile identification and quantitation in samples with complex matrices; (c) the potential of fingerprinting studies with comprehensive two-dimensional gas chromatography (GC×GC), in particular for routine analyses to classify complex samples; and (d) the key role of dedicated software tools for data elaboration with comprehensive two-dimensional separations.

Recent advances in sample preparation

Sample preparation usually is the beginning of an analytical workflow and thus may be considered as the first dimension of the total analysis system. In multi-dimensional separations, with the first separation usually assigned to the first dimension, so sample preparation could be designated the zeroth dimension, as it presents an initial discrimination of the analytes in function of one specific characteristic (polarity, volatility, etc.). Sample preparation should match with the sample's chemical dimensions[6] to deliver a meaningful picture of its constituents, allowing access to the multiple levels of information.

The so-called "aroma-active compounds" are part of the volatile and semi-volatile fraction of a sample and their distribution is highly informative for its sensory quality. Extraction and sampling conditions should be carefully considered to meet fundamental requirements. A sampling system capable of mapping odor-active compounds should have: (a) an appropriate, and possibly tunable, extraction selectivity; (b) high extraction efficiency/capability to capture trace

and ultra-trace analytes (with high odor impact); (c) extraction mechanisms based on mild interactions (sorption/partition is preferable to adsorption) to limit artifact formation (e.g., as often seen in thermo-desorption at higher temperatures); and (d) a good integration into the instrumental analytical system, including the software-assisted automation of all operation steps.[5,7,8] The choice and optimization of sample preparation depends on the fundamental question(s) driving the analysis. In some cases, the goal is a more or less holistic understanding of the sample's nature; then, a comprehensive extraction comprising as many compounds as possible is required. In other cases, the goal may be a target analysis, e.g., the common problem in off-flavor analysis. Then, a more specific extraction is preferred to reduce the concentration of unwanted matrix compounds and possibly enrich the target analyte(s). Affinity chromatography, e.g., for the selective enrichment of thiols (sulfanyls), is an interesting example in this respect.[9]

Headspace sampling approaches, particularly those with a concentration step (high concentration capacity headspace techniques, HCC-HS), often are preferred for satisfactory throughput in volatile aroma analysis. Headspace solid phase microextraction (HS-SPME) is the most popular HCC techniques and its hyphenation with gas chromatography is extensively documented in literature.[7,8,10–12] A recent advancement of this technique in flavor analysis is the so-called SPME Arrow,[13,14] which has increased sorption phase volumes (from 0.5-1 µL of standard SPME fibers to about 15 µL of SPME Arrows) and overall mechanical stability. A schematic of the commercial device is illustrated in Figure 1. The higher amount of sorption phase(s) provide(s) an improved sensitivity and polymer chemistry (polydimethylsiloxane/carboxen 1000 - PDMS/CAR 1000; PDMS/Carbon WR -PDMS/CAR WR; polyacrylate -PA; polydimethylsiloxane - PDMS) thereby enabling better tuning of extraction selectivity for analytes of interest.

Preliminary studies with the SPME Arrow for profiling volatile amines in water samples and in the atmosphere[15] achieved better sensitivity and robustness compared to conventional SPME.

Limited effects of competitive adsorption with complex samples were observed. This aspect is of particular interest for profiling volatiles because thermally transformed food (coffee, cocoa, roasted fruits, etc.) exhibit very complex patterns that also exert strong matrix effects on headspace composition.

Another interesting study by Kremser et al.[14] systematically compared the effectiveness of the SPME Arrow with established static and dynamic headspace techniques. Results on a model mixture of volatiles covering a wide range of polarity and volatility (from C2 to C10), confirmed its efficiency in recovering a wide range of volatiles from the sample HS and showed relative recoveries comparable or even better than SPME.

Conventional SPME, combined with multidimensional GC separations is very common [16–22] although its extraction capability is sometimes limiting for ultra-trace odorant analysis. In an interesting experiment to enhance method sensitivity for GC-O in wine aroma assessment, Chin et al.[23] proposed cumulative multiple HS-SPME samplings with two different fiber coatings, followed by successive GC injections delayed over time. Desorbed volatiles were collected at the first section of the separation column using cryo-trapping (CT) to guarantee short initial band widths for analysis. This experimental design aimed at matching sensitivity requirements for GC-O screening, although it presented challenging aspects: the difficulty of automation and of performing replicate assays or dilution experiments with GC-O. With the complex wine matrix and a one-dimensional separation, the authors also addressed the necessity for an improved separation strategy to better separate individual compounds. New developments in this field of aroma analysis are discussed in more detail in the next paragraph.

Higher HS extraction efficiency also can be achieved by dynamic headspace (D-HS) or by adopting sampling approaches with a higher amount of extraction phase. In a study aimed at characterizing the volatile fraction from dried milk samples, Cordero et al.[24] reported a systematic

investigation on different but complementary sampling techniques, based on either sorption or adsorption, or on their combination. The approaches investigated show a high degree of automation, and included SPME, stir bar sorptive extraction (SBSE) and headspace sorptive extraction (HSSE) with PDMS and dual phase SBSE, and D-HS with silicone sorbents or polar adsorbents (e.g., Tenax TA™). Information for analytes, including key-odorants and off-odors, extracted by headspace and in-solution sampling were compared to evaluate whether a given orthogonal approach was advantageous to describe samples' sensory properties. Within headspace approaches, HCC techniques with higher amounts of polymeric accumulation phase (i.e., HSSE and D-HS) gave better results in terms of concentration capacity. Single-polymer SPME fibers, including polar and selective polymers (i.e., polyacrylate-PA and polyethylene glycol - PEG), were less effective than multi-polymer devices (e.g., DVB/CAR/PDMS SPME). With in-solution sampling, the concentration capacity of SBSE was superior for both sampling systems (100% PDMS and dual-phase PDMS Carbopack BTM), achieving concentration factors of 6 to 7 times compared to HCC-HS techniques. This aspect is crucial for an integrated analytical platform for sensomics, where GC-O should be carried out contemporarily to the identification and quantitation of odor-active compounds. However, in-solution sampling by SBSE or SPME has to be carefully evaluated for aroma analysis being prone to artifacts formation, during thermal-desorption of analytes, due to the presence of aroma precursors in the sample, and to possible recovery discrimination due to the different analyte/accumulating phase interaction.

Exploiting multidimensional separation in targeted aroma analysis

Multidimensional gas chromatography (MDGC) plays a crucial role in flavor research either in the conventional heart-cut (H/C MDGC) or in comprehensive (GC×GC) mode.[25] H/C MDGC has long been used, although its widespread application still remains unfulfilled. Behind the

development of MDGC, in the early days of capillary GC, there was the need for well separated chromatographic peaks for accurate quantitative analyses or for the characterization of complex fractions, including enantiomeric recognition for authentication purposes. Despite instrumental advancements and full automation of heart-cut (H/C) procedures, H/C MDGC is still considered a niche technique for applications in which 1D-GC does not offer sufficient separation power or when the hyphenation with MS (including tandem MS) does not provide the required level of information (e.g. enantiomer recognition).

In a study aimed at investigating the enantiomeric distribution of a potent aroma compound, 3-*sec*-butyl-2-methoxypyrazine (SBMP), in a number of vegetal and fruit species, H/C MDGC was used for chiral recognition with heptakis-(2,3-di-*O*-methyl-6-*O*-tert-butyldimethylsilyl)-β-cyclodextrin as chiral selector in the second dimension (^{2}D). Sensory evaluation of the individual (*R*)-and (*S*)-SBMP did not show differences in their odor quality, but their odor thresholds differ by an order of magnitude, 0.01 and 0.1 ppb in water for (*R*)- and (*S*)-SBMP, respectively.

Legrum et al.[26] described the chiral recognition of SBMP with different GC-MS systems. Trace-level analysis (ng/L or ng/kg) of SBMP in lady beetles and *Vitis vinifera* species was successfully performed with a single-oven H/C MDGC system with a cryo modulator (as cold trap) for trapping analytes transferred from the achiral first-dimension (^{1}D) column to the chiral second-dimension (^{2}D) column before starting the ^{2}D enantioseparation with an independent temperature ramp. Highly selective detection was achieved with a triple quadrupole MS (QqQ-MS) in MS/MS mode by selecting suitable mass transitions. Figure 2 illustrates the system proposed by Legrum et al.[26] for this study. Experimental results confirmed discrimination of the *(R)*- and (*S*)- enantiomers of SBMP and revealed that only (*S*)-SBMP was detected. This result supports the hypothesis of natural amino acids serving as starting material for the biosynthesis of alkyl-methoxypyrazines. For higher concentration levels (μg/Kg) such as those found in matrices from peppers, carrots, sugar

peas, and potatoes, a classical H/C MDGC system with two independent GC ovens and selected ion monitoring (SIM) with a single quadrupole MS (qMS) usually was sufficient to achieve reliable quantitative data. However, the authors mention co-elution problems within some matrices (e.g., parsnips), that would demand either a more selective detection mode (MS/MS) or a better separation (or both). At trace-level alkyl-methoxypyrazine analysis, selective MS detection was necessary for complex samples and/or for accurate quantitation below 1 µg/Kg. In these cases, selective reaction monitoring (SRM) by QqQ-MS was succesful.[26,27]

GC×GC can today be considered as the state-of-the-art technique in terms of separation efficiency. Legrum et al. achieved the enantiomer separation of SBMP with a GC×GC system that automatically trapped analytes eluting from the chiral ^{1}D column and re-injected them into the achiral ^{2}D column with a dual-jet cryo modulator and monitored characteristic fragment ions with a time-of-flight mass spectrometer (ToF-MS). They also discussed the influence of the modulation period that must be short for such enantioseparations with limited chiral resolution and the alternative of using a GC×enantioGC system. Besides the separation efficiency of GC×GC in general, trace-level analysis can further benefit from spectral deconvolution algorithms more effective with ToF-MS data.[28] In general, data quality obtained in such trace analysis benefits from additional analytical refinements and data obtained with less analytical effort in some cases may be wrong with critical matrices.[29,30]

Several challenging aspects are noteworthy in order to optimize H/C MDGC analyses. The most important aspect of H/C MDGC is probably to increase the separation selectivity for target compounds within a complex matrix. Defining the H/C temporal window then should minimize the risk for co-transferring eventually disturbing matrix compounds. A careful definition of such H/C temporal windows for a quantitative transfer of both analyte and isotopologue is mandatory in particularly when quantification via a stable isotope dilution assay (SIDA) is intended, and ^{1}D

separation conditions as well as isotopic labeling should be optimized. The reason lies in the chromatographic separation of isotopologues that often shows an *isotope* effect,[31] thus a retention shift along the ^{1}D separation that demands an elongation of the temporal H/C window to avoid losses of analyte and/or the isotopic internal standard. Then, a partial transfer of either ones results in inaccurate quantifications. This should be avoided because an increase of the temporal transfer windows enhances the risk for transferring disturbing matrix compounds. Selecting an appropriate isotopic labeling strategy and optimized conditions for the chromatographic separation, notably choosing an appropriate polarity for the separation column stationary phase, then affords a complete or almost complete co-elution situation on the ^{1}D. Such optimization thus allows for a minimized H/C temporal window and a reliable SIDA-based quantification.[32]

In the field of food adulteration, Langen et al.[33] proposed an interesting application of H/C MDGC for quantitative determinations of α- and β-ionone and β-damascenone and enantiomeric separation of α-ionone in wine samples. These potent odorants, deriving from carotenoids cleavage, are reminiscent of violet, raspberry, and floral flavor attributes, and generally are present as key-odorants in raspberries, tea, and tobacco. In wine, when present, their concentrations vary between a few micrograms and 60 μg/L.[33] Their impact on wine aroma is favored by an interesting synergism with other odorants (e.g., ethyl cinnamate and hexanoate) also inducing a masking effect against the herbaceous, bell-pepper like aroma of isobutyl methoxypyrazine.[34] The control authorities consider the assessment of the enantiomeric distribution of chiral aroma compounds as a point of interest to reveal adulterations by artificial aroma. In order to enhance the distinctive raspberry note often found in rosé wines, α-ionone might be added with a fraudulent purpose. In natural raspberry, α-ionone occurs primarily as the (*R*)-enantiomer. This also is found in wines made from *Vitis vinifera* varieties, together with low

amounts of β-ionone and β-damascenone. In this respect, concentration levels and enantiomeric distribution of chiral compounds are excellent markers for authenticity studies. In order to achieve low detection limits, Langen et al.[33] successfully applied an optimized H/C MDGC with an enantioselective GC column in the ^{2}D and a highly selective QqQ-MS detector (Limit of Quantitation (LOQ) were 0.007 µg/L for (*S*)- and (*R*)-α-ionone, 0.016 µg/L for β-ionone and 0.026 µg/L for β-damascenone). Accurate quantitation was achieved by SIDA in a set of 95 red, 75 rosé, and 64 white wines and revealed the presence of most of these key odorants in the range between method LOQ and 10 µg/L. The (*R*)-α-ionone clearly dominates in authentic wines; the *R*/*S* ratio could be adopted as a good indicator of wine (aroma) authenticity.

During their method development, Langen et al.[33] showed that a careful selection of a suitable MS/MS transition is also necessary for unambiguous identification of (*S*)- and (*R*)-α-ionone – even after H/C MDGC as an efficient method for matrix reduction. The reliability of the quantitation accuracy is affected by an interfering peak eluting between the two enantiomers on the enantioselective (ES) ^{2}D column (here heptakis-(2,3-di-*O*-methyl-6-*O*-tert-butyldimethylsilyl)-β-cyclodextrin). Figure 3 shows the different ^{2}D profiles corresponding to three specific transitions (SRM) in the elution region of (*S*)- and (*R*)-α-ionone.

The authors discussed the critical selection of precursor ions for further fragmentation in the context of the low molecular weight and often highly fragmented spectra of aroma compounds with classical electron impact ionization (EI). They propose favoring the highest possible mass for MS-MS aroma analyses, as otherwise ubiquitous small mass fragments may lead to nonselective SRM transitions that could yield erroneous results. In volatile aroma analysis from complex matrices, this should be considered and often calls for a selective sample preparation, increased separation efficiency (e.g. by H/C MDGC), and a very selective detection. In a recent work, the authors thus proposed for a relatively simple determination of 2-aminoacetophenon in

wine on μg/L an application of H/C MDGC-MS-MS, as otherwise erroneous results may occur in some wines when the separation efficiency or detection selectivity is lowered to a 1D approach. [35]

The fundamental drawback of small fragment ions encountered with volatile aroma compounds might be overcome by soft ionization techniques such as classical chemical ionization (CI) or new developments, e.g., the "Cold-EI" technique that is based on a supersonic molecular beam (SMB)[36] (Aviv Analytical, Tel Aviv, Israel) or the "Select-eV" ion source that uses a novel electron lens for maintaining high ionization efficiency at low eV levels (Markes International, Llantrisant, UK). However, at present, Select-eV is not yet offered with MS/MS detection. On the other hand, Select-eV already can be used in a fast switching ionization mode, named tandem ionization, which affords two sets of MS data to be acquired and further processed. Applications for Select-eV have been described in the petrochemical field[37] but also in aroma analysis[38]. An example of mass spectral signatures with enhanced molecular ions for ethylphenols in wine is provided as supplementary material in Supplementary Figure 1 (SF1).

High resolution MS (HR-MS) represents an ultimate option for selective detection leading to unambiguous structure elucidation or confirmation.Marketed solutions for GC hyphenation are available from Agilent, Waters, LECO and ThermoFisher). A clear example of the potentials is given by recent experiments on aroma active compounds by GC-HR-MS and Orbitrap. The Orbitrap technology had earlier been established for LC-HR-MS and matured over the past years. Technical specifications for the GC-Orbitrap claim a mass resolution of 12.500 to 100.000 (18 Hz to 3 Hz) at *m/z* = 272 (ThermoFisher Scientific, Dreieich, Germany). These systems are especially suitable for structure elucidation, as shown for β-damascenone and α-ionone with the mass fragment of *m/z* = 121 common to both. The exact mass reveals that *m/z* = 121.06480 in the case of α-ionone contains an oxygen atom that is compatible with the structure shown in the supplementary material (Supplementary Figure SF2). At present, no application in the aroma field has been

published, but one can expect the beneficial use of HR-MS in the near future – although, at the high costs for such detection systems.

Exploiting multidimensional separation in non-targeted aroma analysis

Highly specific and targeted H/C MDGC methodologies only partially answer the analytical needs of detailed analysis of volatiles fractions in aroma chemistry. A number of studies based on multidimensional separations followed by non-targeted data interpretation methodologies have demonstrated the informative potentials of such detailed chemical patterns.[5] Since its introduction by Liu and Phillips in 1991,[39] it was immediately evident that GC×GC would provide substantial advantages for detailed characterizations of complex mixtures such as some food-derived volatile fractions, containing odor-active compounds responsible for sensory attributes. These advantages derive from the adoption of (almost) orthogonal separation mechanisms in the two dimensions of the separation system. Resulting patterns show ordered structures for chemically related analytes and are helpful for group-type analysis or for identification purposes.

Figure 4 shows the 2D pattern of volatiles from an extra-virgin olive (EVO) oil sample. Figure 4A reports the full pattern of the volatile fraction separated on a GC×GC system with a poly ethylene glycol ^{1}D column (PEG) and a medium polarity ^{2}D column (OV1701). Figure 4B localizes the group of target analytes (identified through their EI-MS fragmentation pattern and linear retention index I^{T}_{S}). Figure 4C illustrates the ordered spatial distribution of homologues series and groups of analytes (esters, saturated and unsaturated aldehydes, hydrocarbons, and alcohols).

Adahchour et al.[40,41] were early investigators of the potential of GC×GC in the field of aroma characterization. They investigated aroma extracts from milk-derived products (dairy and non-dairy sour cream and dairy spread) obtained by SAFE and CF (cold finger) distillation. The analytical platform was equipped with a thermal modulator (a longitudinally modulated cryogenic

system - LMCS). The authors convincingly showed the merits of the technique in providing valuable information about volatiles distribution (profiling) at the same time informing on the presence of key-flavor components in milk-derived samples. The enhanced overall chromatographic resolution facilitated accurate quantitation of a selection of target compounds, such as methional and sotolon, found in the milk-product extracts at mg/kg concentration. The need for further improvements of the technique by designing alternative separation strategies were the seeds of subsequent instrumental developments that made the technique so successful in food investigations.[16,42]

The so called "multi-multidimensional" platforms, introduced by Marriott and co-workers, [43–46] effectively combine most of well established GC related techniques adopted in flavor analysis. An example of a system implementing H/C MDGC, GC×GC and GC-O is reported in the supplementary material as Supplementary Figure SF3.

Maikhunthod et al.[46] presented a platform implementing a switchable device between comprehensive two-dimensional gas chromatography and targeted multidimensional gas chromatography system (i.e., switchable GC×GC/targeted MDGC). The system enabled independent analyses by 1D-GC, GC×GC, and targeted MDGC with the possibility of switching from GC×GC to targeted MDGC at any times throughout a single analysis. A Deans switch microfluidic transfer module and a cryotrapping device (CT) are core components enabling classical H/C operations, GC×GC, or targeted MDGC. System operational performances were evaluated by analyzing volatiles of interest in the flavor and fragrance field and on medium complexity essential oils. Experiments were mainly focused on obtaining better resolved peaks by a targeted separation on a longer 2D column by diverging specific regions of a GC×GC separation in which co-elutions occurred, thus allowing reliable identification and quantitation of target analytes.

The potentials of hyphenated and multi-multidimensional systems to study aroma-impact

304 compounds were extensively illustrated by Cordero et al.[5] in an article reviewing the existing
305 literature with an eye to the future. The authors touted the still unexplored potential of
306 comprehensive GC in food fingerprinting investigations. The dense and multidimensional data sets
307 produced by each single analytical run necessitate suitable data mining approaches that expand
308 the simple targeted investigation methodology. Clear benefits of combined targeted and
309 untargeted fingerprinting are obtained in the area of complex samples classification and
310 discrimination. Some examples will be dealt with in the following discussion on data elaboration.

311 Another area of active research in the field of GC×GC is to develop effective modulation
312 devices that are cheaper and consumable-free and so suitable for adoption of GC×GC in routine
313 applications and quality control (QC) procedures. The characterization of key odorants requires
314 devices for efficient trapping and release of (highly) volatile components, most of them
315 responsible for distinct odor notes. Dual-stage thermal modulators (TM) with cooling media (CO_2,
316 liquid N_2 or closed cycle refrigerator/heat exchangers) prevail because of their flexibility and ability
317 to produce narrow pulses. However, over the last five years, flavor and fragrance applications of
318 GC×GC with cryogenic-free thermal modulators based on differential-flow modulation (FM)
319 dynamics have been described.[47–49]

320 FM devices, based on the original design by Seeley *et al.,*[50] have a simple and effective
321 design, low operational and hardware costs, and high robustness. Interesting solutions in this field
322 have been introduced by Tranchida et al. [49,51,52] and commercial products are nowadays available
323 from different manufacturers (e.g., Agilent Technologies, Sep Solve Analytical, and Chromaleont).
324 Cordero et al.[53] recently discussed the advantages of a new generation of FM devices
325 implemented with a Capillary Flow Technology (CFT) microfluidic plate and reverse fill/flush (RFF)
326 injection. The prototype, made available by Agilent is shown in the supplementary material
327 (Supplementary Figure SF4), has several advantages: (*a*) efficient band re-injection, (*b*) improved

^{2}D peak widths and symmetry, and (*c*) effective handling of collection-channel overloading.

Results on system performances (separation power, peak-widths, and separation space used) were assessed for a model mixture of volatiles while medium complexity essential oils [53,54] (e.g., mint and lavender) were used to test profiling and quantitation accuracy with both external calibration on the MS signals and predicted relative response factors (RRF) on the flame ionization detector (FID) signals. Figure 5 shows the contour plots of peppermint and spearmint essential oils. The enlarged areas show details of the elution regions of (A) menthols and (B) carvone derivatives. For details on analysis conditions see the figure caption.

The system potentials for quantitative profiling were confirmed by accuracy results obtained by cross-validating quantitation data from dual-parallel secondary columns and detection. Identity confirmation and quantitation by MS signals completed the information provided by FID that, in its turn, enabled extending quantitation to all identified components by using FID-Predicted Retention Factors (PRFs)[55]. Experimental results presented in this study, together with the acceptable operational costs and relative ease of use and simple maintenance, indicate that CFT reverse-inject differential flow modulation can contribute to promoting the use of GC×GC for routine analysis in the flavor and fragrance field.

Multidimensional data set elaboration challenges

Multidimensional separation techniques enable effective insights into the composition of complex samples. In particular, comprehensive GC offers unequaled information on samples dimensionality by producing resolved and informative separation patterns, i.e., chemical fingerprints with a great potential for comparative purposes. However, the data size and complexity is challenging. Cross-sample studies in food aroma characterization have several purposes: (*a*) sample classification *versus* sensory profile; (*b*) chemical fingerprinting to

characterize samples against reference standards; (*c*) progressive and time resolved monitoring of chemical changes a function of technological/enzymatic treatments; and (*d*) discovering informative markers for botanical/geographical assessment.

The most relevant *features* (i.e., chemical constituents characterized by relative position in the chromatographic space and detector or mass spectral intensities) of a cross-sample analysis are generally not known a priori and sometimes correspond to trace analytes. Informative data elaboration therefore should combine non-targeted and targeted approaches to achieve the most inclusive fingerprinting investigation.[56]

Most of the studies in the field of aroma characterization by H/C MDGC and GC×GC adopt targeted approaches: relevant analytes are first identified on the basis of their EI-MS spectrum and relative retention (^{1}D I^T*s*) then their relative amounts are compared across samples for classification and characterization. Interestingly, a bio-guided assay (e.g. GC-O) could preliminarily target/tag odor informative retention regions driving targeted data elaboration in specific regions of the chromatographic space.

In the authors' opinion, the most challenging aspect involves untargeted analysis by GC×GC because it requires dedicated software(s) and skillful analysts and is in general under-exploited in the field of aroma investigations.

In a study aimed at profiling volatiles from apple, pear, and quince fruits, Schmarr and Bernhardt[57] analyzed 2D patterns by adapting a *peak-region feature* approach commonly used for 2D gel electrophoresis. The volatile fraction, sampled by HS-SPME, was analyzed with GC×GC-MS to generate a 3D data matrix for each single analysis. The raw data then was converted to a grayscale jpeg image by an open-source software (ImageJ™, Wayne Rasband, National Institute of Health, USA) and processed with algorithms commonly used for 2D gel electrophoresis. The approach included pre-processing operations (image alignment by warping and summation) that

produced a representative cumulative chromatogram (fusion image) of all of the constituents in all samples. Figure 6 shows the complete workflow of the proposed method.

Boundaries around single 2D peaks were treated as regions in a template. The template geometrically mapped back to each chromatogram and used to extract detector responses from each chromatographic region, thus generating a data matrix of aligned regions for all samples. Feature matching was, at that time, performed by retention-times mapping and visual interpretation. As a constraint, at that time, MS data were not included for a direct verification of alignments or for peak identification. Nowadays, an improved release of the original software is available offering additional tools for spectra matching, visual comparison of chromatogram pairs, and various post-processing possibilities including multivariate analysis (MVA) and chemometrics (Decodon, Greifswald, Germany).

Smart Templates™ with peak-region features were developed by Reichenbach and co-workers,[58] then implemented as a basic tool for comparative and explorative analysis in a commercial software package (GC Image, Lincoln NE, USA) and used for targeted and untargeted fingerprinting in different application fields. Very recently, a straightforward concept has been exploited by combining targeted and untargeted approaches to obtain most comprehensive results. The procedure, defined *UT fingerprinting* (i.e. *untargeted* and *targeted* fingerprinting), was tested on extra-virgin olive oil (EVO oil) volatiles[56] and provided results for characterizing the degree of olive ripening and EVO oil quality. Moreover, thanks to effective global transformation algorithms[59,60] for pattern re-alignment and template matching, a retrospective analysis was also possible. This last option allowed retrospective re-evaluation of samples in light of new informative features. Figures 7 show pseudocolor images that visualize the volatiles pattern from EVO oils from two analytical campaigns within the three years of analysis. Figure 7A shows the 2D chromatogram of a Spanish sample from 2015 harvest with an overlay of the template of peak-

regions adopted for cross-comparisons; Figure 7B shows the 2D chromatogram of an Italian EVO oils sample (PDO Monti Iblei e Sicily Italy) analyzed in 2013. In the 2D patterns of these sample, the peak-regions templates (light blue graphics and green circles) were matched after global transformation of the original template to adapt the relative position of all peak-regions over the 2D pattern.[60,61] This operation requires inspection by the analyst to check for relative misalignments due to the column combination differences after two years of system operations, but enables retrospective analysis of samples and re-consideration of analytical results.

EVO oils were the topic of an interesting study focused on sensory defects and their blue-print within the volatiles mapped by GC×GC-MS. Purcaro et al.[62] adopted the Smart Templates™ approach for an advanced fingerprinting investigation on olive oil samples, including reference standards obtained from the International Olive Oil Council and commercial EVO oils. Samples, submitted to sensory evaluation by an official panel prior to GC×GC analysis, were characterized by targeted and untargeted approaches. A list of 261 reliably identified compounds was adopted for the template and used to reveal the informative fingerprints related to the sensory characteristics defined for each sample. These most informative compounds were collected in a *blueprint* of specific defects (or combination of defects) adopted to discriminate *extra-virgin* from defected oils (i.e. *lampante* oil) with the aid of a supervised approach, i.e. Partial Least Squares-Discriminant Analysis (PLS-DA). The principle of sensomics, assigning higher information potential to analytes with lower odor threshold, proved to be successful, and a much more powerful discrimination of samples was obtained in view of a sensory quality assessment.

In the aroma analysis, multidimensional gas chromatographic techniques assumes a valuable, sometimes indispensable, role (a) to study the composition of complex volatile fractions very often consisting of hundreds of components (e.g., coffee, tea or cocoa) by profiling analysis, (b) to detect key-odorants and explain their formation from precursors, (c) to discriminate

between enantiomer or coeluting (trace) components with different odor characteristics, and (d) to understand the interaction/relationship with flavor perception, personal behavior, and health. As extensively illustrated by the selected examples, MDGC is crucial to study the chemistry behind sensory perception(s) since it offers high separation power and sensitivity that are fundamental for accurate quantitation and to define informative fingerprints of complex samples to be correlated with sensory qualities.

Crucial to MDGC advancement, in particular for GC×GC, is its link with sample preparation and data elaboration, also in view of the development of a true “total analysis system”. The present research trend goes towards the full on-line integration of sample preparation through the adaption or introduction of new and/or dedicated techniques to make sampling a “true” additional dimension of the analytical platform. At the same time, data elaboration is expected to become the object of a radical evolution in the next years, as concurrently has happened in metabolomics, again with an ever full integration with the analytical process. Effective and/but operator-friendly processing tools enabling combined targeted and untargeted (fingerprinting) investigations are desirable and expected especially when analytical data directly define peculiar characteristic of the matrix.

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Figures captions

Figure 1: The Arrow SPME system with sorbent exposed (left) and with sorbent covered by a steel tube (right).[From Helin *et al*[15].]

Figure 2: Configuration for the H/C MDGC-MS/MS system, as reported by Legrum *et al*.[18], with AC (analytical column), RC (restriction capillary), EPC (electronic pressure control), and QqQ (triple quadrupole) MS.

Figure 3: H/C MDGC chromatograms (^{2}D) showing co-elution in a real wine sample (Pinot Noir) during the analysis of α-ionone depending on selection of SRM transitions (from Langen *et al.* [33]). Analytical columns: ^{1}D column (30m×0.25 mm i.d. ×0.25 μm of polyethylene glycol (StabilWax-MS, Restek, Bad Homburg, Germany); ^{2}D column (25 m×0.25 mm i.d. Lipodex G® - octakis(2,3-di-*O*-pentyl-6-*O*-methyl)-γ-cyclodextrin, Macherey-Nagel). Carrier gas: helium in constant pressure. H/C and Oven temperature conditions are detailed in the reference paper by Langen *et al.*[33].

Figure 4: (**4A**) Pseudocolorized GC×GC chromatogram of the volatile fraction of an Extra Virgin Olive oil from the Granada region (Spain). (**4B**) Positions of 119 known target peaks (empty light green circles) linked by red lines to the ISTD α-tujone (black circle). (**4C**) Retention area of highly volatile compounds in the white rectangle of Figure 6A. [From Magagna *et al*. [56]]

GC×GC-MS analyses used: ^{1}D column (30 m×0.25 mm i.d. ×0.25 μm of polyethylene glycol (Solgel-Wax; SGE, Ringwood, Austalia); ^{2}D column (1 m×0.10 mm i.d. × 0.1 μm of 86% polydimethylsiloxane, 7% phenyl, 7% cyanopropyl (OV1701, Mega, Legnano, Italy). MS was a fast scanning single quadrupole operating at 12,500 amu/s; scan range 40-240 *m/z*, acquisition frequency 28 Hz. Carrier gas: helium in constant flow. Modulation parameters and oven temperature conditions are detailed in the reference paper. Volatiles were extracted by HS-SPME from 1.500 g of EVO oil in a 20 mL glass vial; sampling time 40 min at 50°C. SPME fiber was a divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS) 50/30 μm, 2 cm length

stableflex fiber from Supelco (Bellefonte, PA, USA).

Figure 5: Pseudocolor images of a peppermint and of a spearmint essential oil. Enlarged areas show details of the elution of (A) menthols and (B) carvone derivatives. [From Cordero *et al.* [53]] GC×GC-MS analysis with reverse-inject differential flow modulation used: ^{1}D column (10 m×0.10 mm i.d. ×0.10 µm of polyethylene glycol (Solgel-Wax; SGE, Ringwood, Austalia); two parallel ^{2}D columns (1.5 m×0.10 mm i.d. × 0.1 µm of 86% polydimethylsiloxane, 7% phenyl, 7% cyanopropyl (OV1701, Mega, Legnano, Italy). Detection was by parallel FID/MS; MS was a fast scanning single quadrupole operating at 12,500 amu/s; scan range 40-240 *m/z*, acquisition frequency 28 Hz. Carrier gas: helium. Modulation parameters and oven temperature conditions are detailed in the reference paper.

Figure 6: Workflow from Schmarr and Bernhardt [57]: (1) Samples prepared and analyzed by HS-SPME-GC×GC-qMS; (2) 2D GC chromatograms transformed into 32-bit images; (3) 2D GC images stored in Delta2D™ software; (4) Positional correction (warp vectors) for image congruency (dual channel overlay color code: blue = image1, orange = image2, and black = overlap); (5) Volatiles map from project-wide 2D GC image fusion; (6) Detected spot consensus; (7) Spot consensus boundaries applied to all 2D GC images for gray level integration; (8) Gray level integration results in quantitative data which can be summarized in volatile profiles (blue – low amount, black – average amount, orange – large amount of volatile).

Figure 7: Contour plots of the volatiles pattern from EVO oils from two analytical campaigns within a three years of analysis. [From Magagna *et al*.[56]] **(7A)** Spanish sample from 2015 harvest with an overlay of the template of *peak-regions* (light blue graphics) adopted for cross-comparisons; **(7B)** Italian EVO oils sample (PDO Monti Iblei e Sicily Italy) analyzed in 2013 with an overlay of the *peak-regions* template from **7A** after matching and global transformation. For analytical conditions, see the caption of Figure 4.

Associated content

Supplementary Figure SF1: *Brettanomyces* off-flavor compounds in a red wine after HS-SPME and GC×GC-TofMS analysis. The enlarged area shows the increased selectivity when using molecular ions for generating an extracted ion 3D plot facilitating identification of (A) 4-ethylguaiacol and (B) 4-ethylphenol. [From Schmarr *et al*. [38]]

Supplementary Figure SF2: Hi-res MS as a powerful tool for structure elucidation, here for identifying the structure of a common fragment ion (*m/z* = 121) in the spectra of β -damascenone and α –ionone (these can be retrieved at http://webbook.nist.gov/chemistry).

Supplementary Figure SF3: Instrument schematic of the integrated GC×GC/GC–GC system with flame ionisation, olfactory and mass spectral detections. [From Chin *et al.*[43]]

Supplementary Figure SF4: Schematic diagram of the reverse-inject differential flow modulator prototyped by Agilent in loading state (A) and injection state (B). [From Cordero *et al.*[53]]

Figure 1

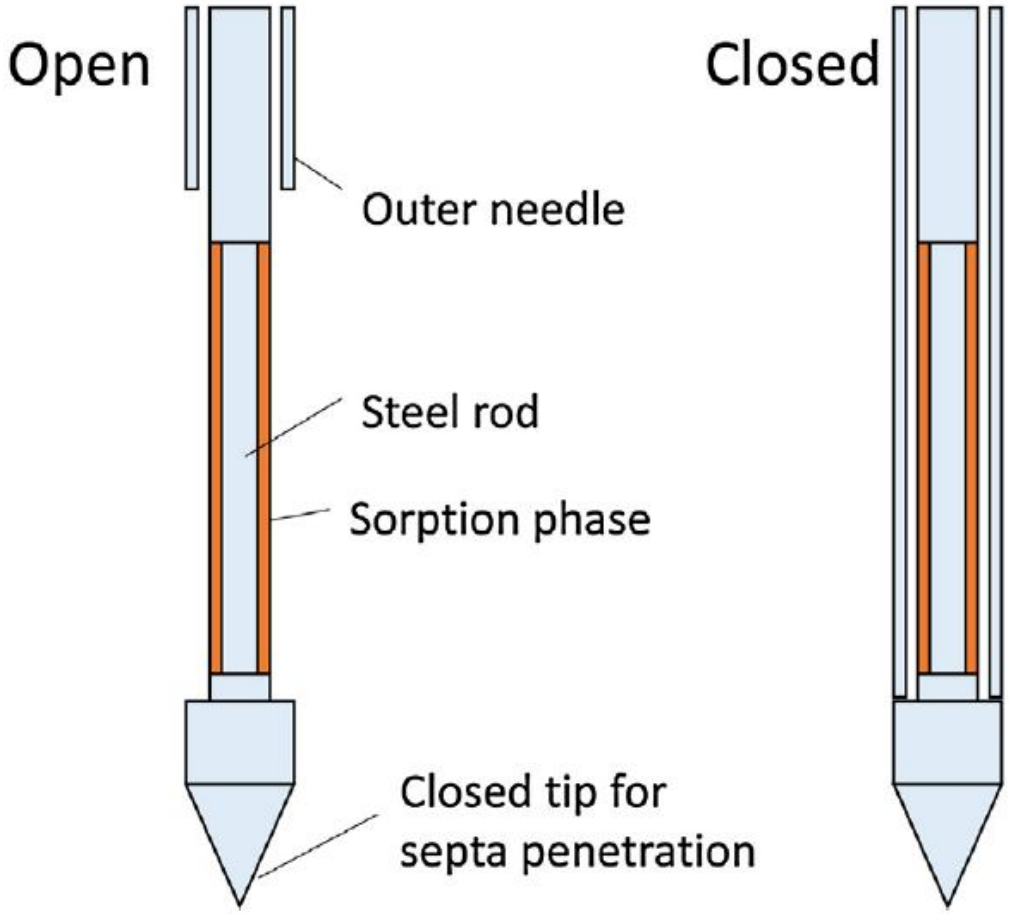

Open
Closed
Outer needle
Steel rod
Sorption phase
Closed tip for
septa penetration

Figure 2

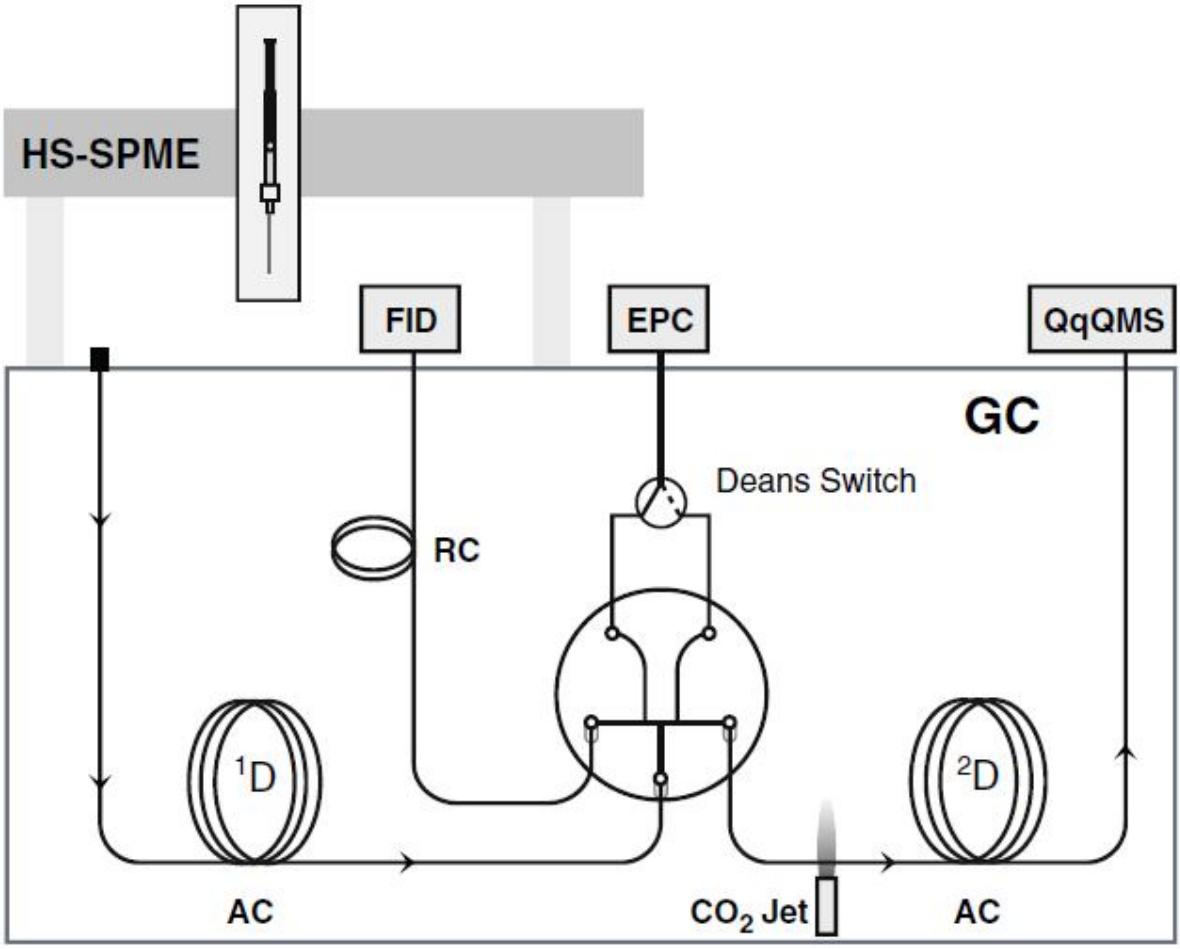


Figure 3

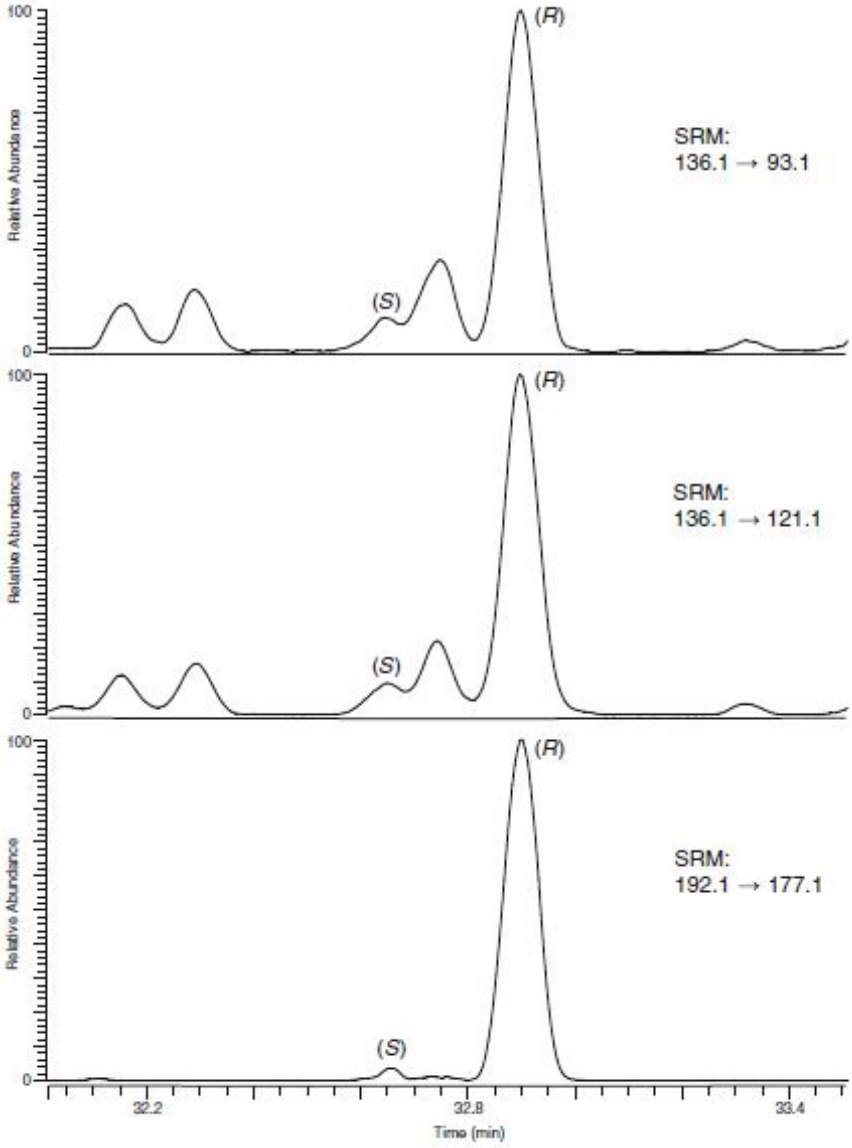

100
Relative Abundance
0
(R)
(S)
SRM:
136.1 → 93.1
100
Relative Abundance
0
(R)
(S)
SRM:
136.1 → 121.1
100
Relative Abundance
0
(R)
(S)
SRM:
192.1 → 177.1
32.2
32.8
33.4
Time (min)

Figure 4

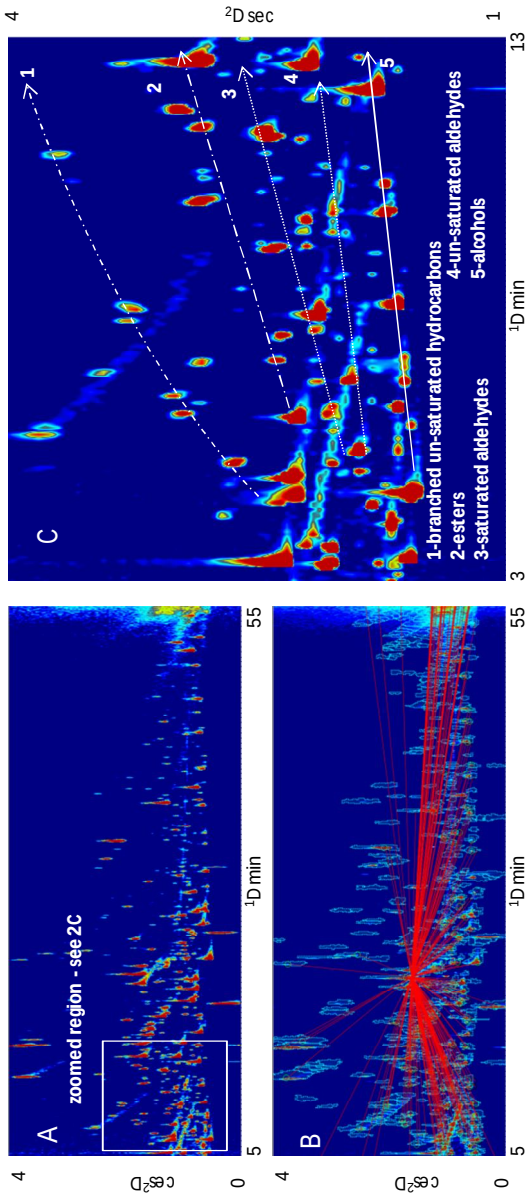

A
zoomed region - see 2C
B
C
1-branched un-saturated hydrocarbons
2-esters
3-saturated aldehydes
4-un-saturated aldehydes
5-alcohols
1
2
3
4
5
^{1}D min
^{2}D sec

Figure 5

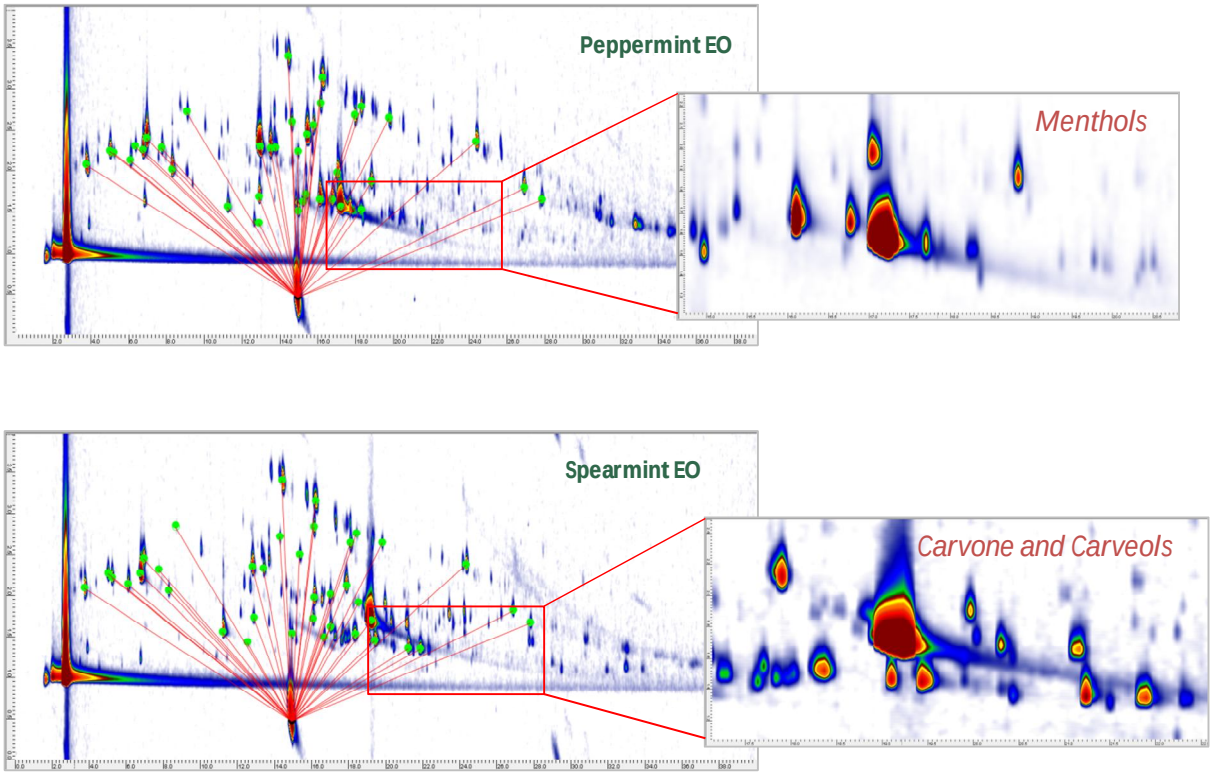


Figure 6

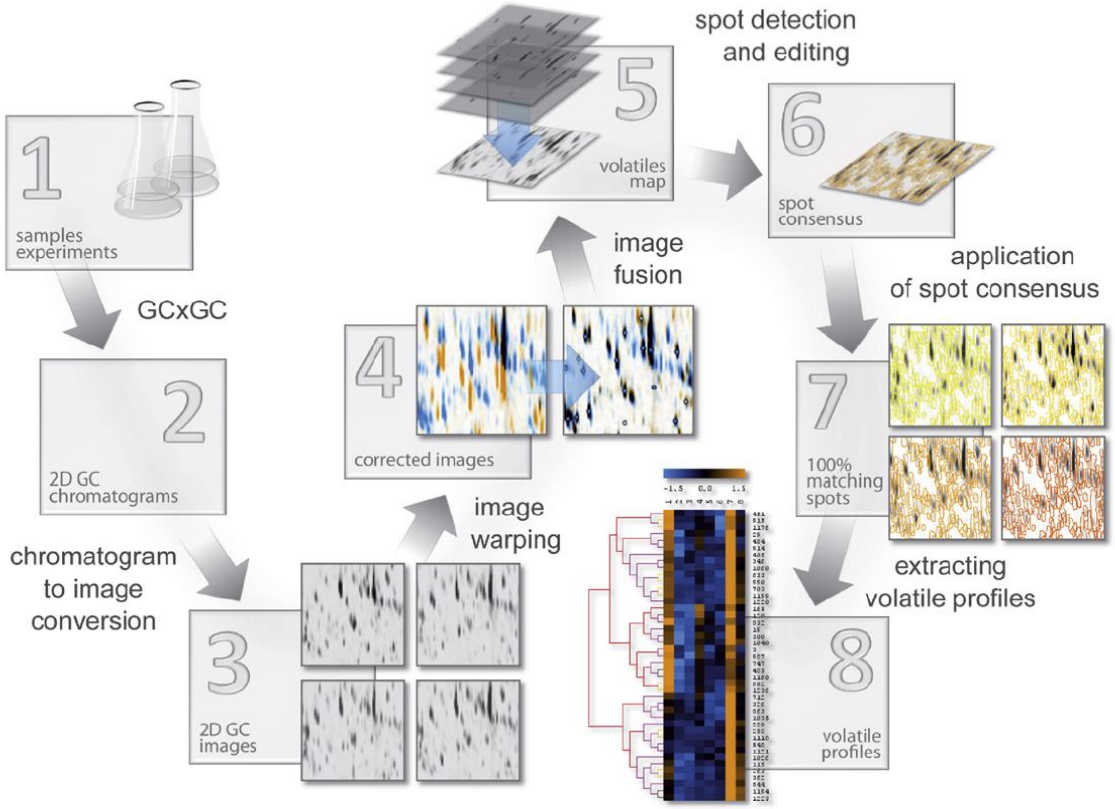
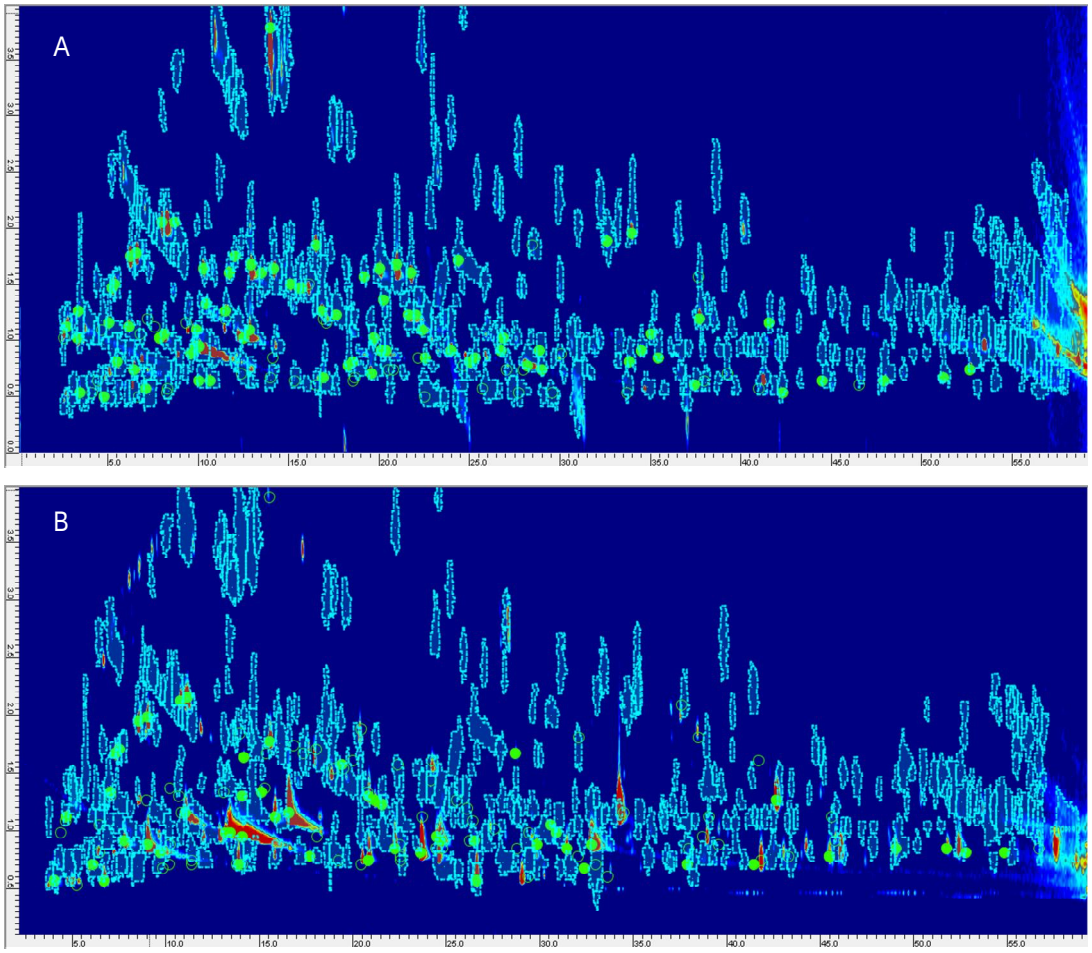

1
samples
experiments
GCxGC
2
2D GC
chromatograms
chromatogram
to image
conversion
3
2D GC
images
image
warping
4
corrected images
image
fusion
5
volatiles
map
spot detection
and editing
6
spot
consensus
application
of spot consensus
7
100%
matching
spots
extracting
volatile profiles
8
volatile
profiles

Figure 7



TOC graphic