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Emerging Opportunities for Flavor Analysis through Hyphenated Gas Chromatography†

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Important advances in chemical and flavor knowledge are possible through improved analytical separation and identification. Along with greater separation power, both a more robust analysis and an improved identified sample composition result. In this paper, a number of new integrated methods are explored that permit improved resolution and superior analyses for a range of studies. These methods focus on multidimensional gas chromatography (MDGC), comprehensive two-dimensional gas chromatography (GC×GC), olfactometry, mass spectrometry, and nuclear magnetic resonance (NMR) spectroscopy. The overriding objective is to provide technical solutions that employ the best possible separation of compounds, allowing tools such as olfactometry, mass spectrometry, NMR spectroscopy, and other detectors to provide much better characterization of separated chemical species. Various novel strategies are demonstrated that provide the necessary increased separation power, integrated with specific detection steps. Case studies presented include the sensory-directed identification of a woody odorant in hop essential oil, correlation of compound identifications in coriander leaf, and development of new preparative isolation capabilities using MDGC with NMR spectroscopy.

KEYWORDS: Multidimensional gas chromatography; comprehensive two-dimensional GC (GC×GC); hyphenation; spectroscopic detection; olfactometry; preparative gas chromatography

INTRODUCTION

A trivial definition of hyphenated gas chromatography (GC) simply defines the type of detector or "system" that is coupled to the GC analysis. Thus, we can discuss GC hyphenated with flame ionization detection (FID) or with nitrogen−phosphorus detection (NPD) or hyphenation with a spectroscopic/spectrometric "analysis" system, such as mass spectrometry (MS) or Fourier transform infrared (FTIR) spectroscopy. A further hyphenation type can include GC coupled with another GC analysis system. This defines the so-called multidimensional gas chromatography (MDGC) analysis methods, which are presently enjoying a resurgence in interest among both instrument companies and, importantly, the analytical GC community.

Multidimensional analysis (MDA) constitutes two (or more) different, bone fide analytical techniques that are hyphenated in the one analytical process. This means that neither GC-FID nor GC-NPD is defined as a MDA, whereas GC-MS is included under MDA. We can propose that GC-MS in selected ion monitoring mode does not constitute MDA, because the MS stage does not function as a full spectroscopic analytical method, and in this manner is more like a specific detector in GC, akin to, for example, flame photometric detection (FPD) or electron capture detection (ECD).

GC−olfactometry (GC-O) is an important hyphenated technique for flavor analysis, wherein human assessors are used to detect odor-active compounds as they elute from a GC separation (*1*). However, the definition of the olfactometric detection system according to the MDA classification system is unclear. Is the olfactometry step just a specific detector? Or is it a "spectroscopic" detector, which therefore classifies GC-O as MDA? Regardless of this question, GC-O is an indispensible tool for determining odor-active compounds in flavor analysis.

The role, implementation, and use of MDGC is now well established (*2*, *3*). MDGC has a long history, although the promise and widespread application of MDGC remained unfulfilled for many years (*4*). The driving force that catalyzed the development of MDGC in the early days of capillary GC can be considered as twofold. First, the elegance of linking two or more columns in a hyphenated arrangement demanded investigation, just to evaluate whether it might be possible and then to decide if it could benefit the technology. The other, more critical, factor was

†This paper is dedicated to the memory of Prof. Jean-Pierre Dufour, who tragically passed away on the 26th February 2007, and is included as a posthumous coauthor. The present work represents the culmination of a collaborative research program undertaken by GTE under the supervision of J.-P.D. and P.J.M during Ph.D. and postdoctoral research. Jean-Pierre had an abiding passion for science and believed deeply in the value of developing new technologies to improve separations for flavor analysis. His enthusiasm, knowledge, and friendship are greatly missed.

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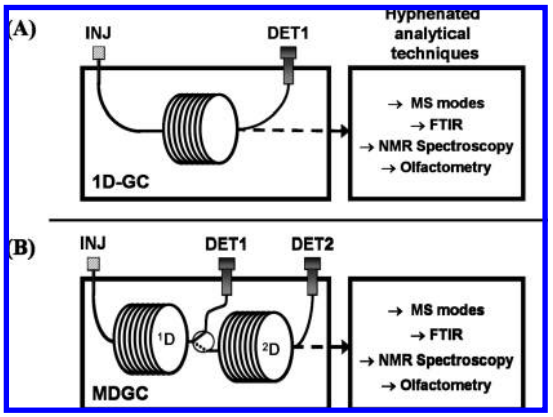

Figure 1. Schematic diagram of hyphenated detection systems for (**A**) single-dimension GC (1D-GC) and (**B**) multidimensional GC (MDGC). Spectroscopic techniques are hyphenated either offline or online (see text for details).

the recognition that in many cases single-column GC was inadequate to provide the analytical answers that an analyst seeks for complex samples. The demand for resolved chromatographic peaks was the force behind this search. In this context, we have little solution other than to resort to multiple separation dimensions.

The present paper will focus attention on hyphenated systems that come under the realm of MDA, specifically on multidimensional separation technologies that have been recently making significant inroads in flavor analysis, and include case studies of various methods that incorporate olfactometry. Thus, it will cover the classical coupled gas chromatographic method of MDGC but include some recently reported innovations and also will present example applications using the comprehensive two-dimensional gas chromatography technique (GC×GC). Methods that incorporate liquid chromatography analysis steps (including LC-GC) will not be included.

Figure 1 summarizes the basic approach to the present paper and defines the scope of hyphenated multidimensional analysis with either a one-dimensional GC method (1D-GC) with spectroscopic (and olfactometry) analysis shown in **Figure 1A** or the corresponding hyphenated MDGC technique (**Figure 1B**).

ANALYTICAL TECHNIQUES

Fetterolf and Yost (*5*), followed by Kidwell and Riggs (*6*), presented discussions on the informing power of hyphenated analytical separations combined with mass spectrometry (MS). Because GC-MS has been the mainstay analytical tool for volatile flavor analysis, it is important that the role of MS be recognized here. A single GC column has an informing power or peak capacity of about 500−600 (i.e., it can separate 500−600 evenly distributed peaks (compounds) in a single analysis) (*6*). However, peaks are neither evenly nor randomly distributed in a chromatogram because related compounds demonstrate related chemical properties, particularly in essential oil samples (*7*). Consequently, to achieve complete resolution, the peak capacity required for the system needs to be much greater than the actual number of compounds in the sample. The result of these factors is that complex samples have a high likelihood of multiple peak coelutions and, according to Davis and Giddings (*8*−*10*), may demand multidimensional separations. Electron ionization (EI) GC-MS increases informing power to about 6.6×10^6 due to the discrimination of the mass spectral dimension. MS is able to provide considerable identification power due to the use of library matching to established databases. GC-MS/MS further extends

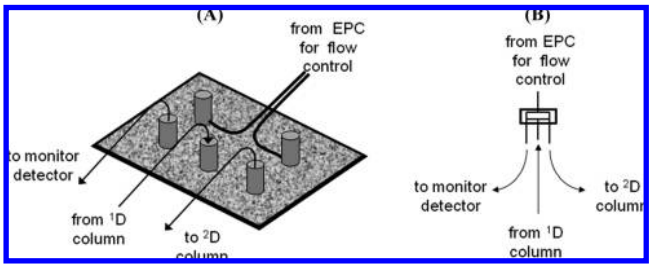

Figure 2. (**A**) Schematic diagram of the physical design of a Deans switch system. (**B**) Diagrammatic representation of the Deans switch used in **Figure 8**.

informing power to an amazing 6.6×10^9, due to the further selectivity of the second MS dimension (*6*).

Similarly, MDGC can provide significantly more resolving power by selecting small zones of eluting peaks from a first column and separating them on a second column. The separation mechanism of the second dimension can be made "somewhat orthogonal" to the first by selection of different stationary phases. The least sophisticated technique to perform MDGC analysis employs an offline system, wherein a particular region eluting from the column is collected using, for example, an adsorbent trap and then reintroduced into a second column (*4*, *11*). Online systems require a controllable interface to selectively transfer regions of interest from the first column to the second column, both accurately and reproducibly, in a process known as "heart-cutting". Mechanical rotary valves were the original type of interface used to perform online heart-cutting. However, it has been demonstrated that labile compounds (e.g., sulfur compounds, acids, alcohols, and aldehydes) can degrade or adsorb to active surfaces in mechanical valves, or peaks can be lost through incomplete collection or leakage of valves (*12*, *13*). Early valve systems also suffered from large dead volumes, causing peak broadening, peak tailing, and carry-over effects. Microswitching valves have since been greatly improved to reduce the internal volumes and levels of activity, and subsequently many successful valve-based analyses have been reported (*3*, *12*, *14*).

An alternative approach to implement MDGC is using pneumatic flow switching with a valveless Deans switch system (**Figure 2**) (*15*). In this case, flow switching is effected by pressure-directed changes in flow from an auxiliary electronic pressure control (EPC) module. The direction of the flow is controlled by a solenoid valve to determine whether flow eluting from the first column is directed to either a monitor detector (to record the ^{1}D chromatogram) or the ^{2}D column, upon elution of the target region (*16*). The main advantage of the Deans switch system is that it uses fixed flow channels with no valves or rotor faces. Thus, the sample is not in contact with any moving parts, making inactivation of the interface surfaces much more effective and providing greater inertness (*17*). A cryotrapping device incorporated into the system, to cryofocus the heart-cut analyte band at the head of the second column, would be a preferred option, particularly to maximize performance for short, thin-film columns. The thermal mass of the device is typically much lower than a valve system and so provides good temperature tracking (fast equilibration) with the oven. Pneumatic flow switching with a microfluidic device should also reduce peak broadening during the transfer process compared to valve switching (*18*).

A simplified MDGC method has also been suggested, using a moving cryotrap device known as a longitudinally modulated cryogenic system (LMCS) developed for MDGC and GC×GC, in what is described as the "target mode" (*19*). Begnaud and Chaintreau (*13*, *20*) described a further development to utilize the

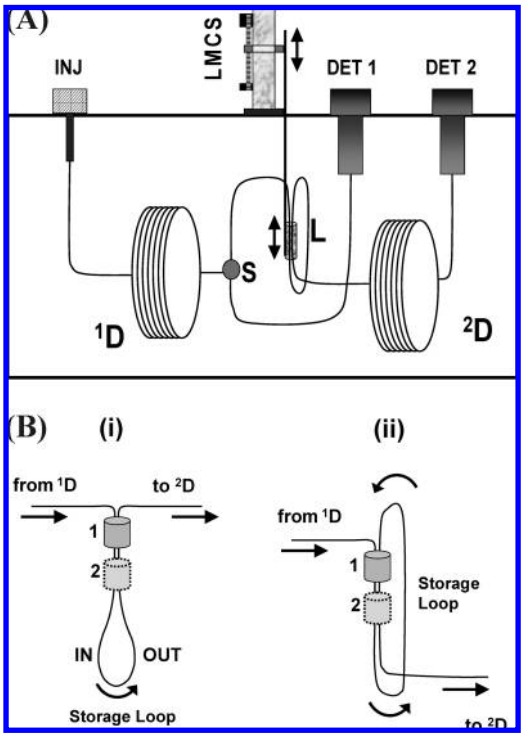

Figure 3. (**A**) Schematic diagram of a "double cool-strand loop" (DCSI) modulator incorporating a looped column (L) through a longitudinally modulated cryogenic system (LMCS). Target regions are selected, sampled, and passed to the second dimension (^{2}D) by action of the LMCS cryotrap. The splitter union (S) allows a small flow to pass to the first detector (DET 1) to allow precise selection of the target region. (**B**) Two different arrangements for the looped column modulator.

LMCS cryotrap as a new transfer mechanism for MDGC. The authors termed this the double cool-strand interface (DCSI) (**Figure 3A**). In this arrangement, the column is looped through the LMCS cryotrap to create two trapping zones, allowing compounds to be quantitatively trapped in this storage loop, isolating them online from preceding and following peaks, and then introducing the target region into the ^{2}D column for effective resolution from interfering matrix compounds. By use of a ^{2}D column of adequate length, this technique produces peak widths and sufficient separation to allow both MS and olfactory analysis. A splitter (S) prior to the interface directs a small split flow (~10%) to a monitor detector to determine correct event timing to ensure target trapping in the loop, which must be sufficiently cool to retain the trapped compounds of the target region. The storage loop may operate in one of two configurations (**Figure 3B**). The first is a reverse direction loop through the cryotrap (**Figure 3B**(i)), whereas the second (**Figure 3B**(ii)), as originally proposed, passes the loop through the same direction as the incoming strand. The former arrangement allows a smaller (and more flexible) loop size and achieves the transfer operation from ^{1}D to ^{2}D with fewer movements of the cryotrap.

FAST TWO-DIMENSIONAL GC TECHNIQUES

Today there is much interest in developing faster methods of GC analysis, primarily to provide higher throughput of analysis while maintaining comparable separation power with the conventional analysis. Approaches such as "method translation" allow development of significantly faster methods employing shorter, narrow-bore columns, faster temperature program rates, and higher carrier gas velocities. These approaches do not necessarily provide enhanced resolution power, and so for a complex mixture, faster analysis will not generate better component separation.

Within the competing (and conflicting) demands for greater peak separation and higher sample throughput, the challenge for productivity of analysis might be considerable. However, there can be ways to achieve both objectives. Consider selection of a complex group of peaks eluting within a zone equivalent to the width of 10 peaks (constituting approximately 1 min of effluent) but possibly comprising 50 overlapping peaks for a complex sample. Transferring this region to a ^{2}D column with a peak capacity of 500 should statistically allow resolution of each sampled peak. This situation describes a system incorporating a long ^{2}D column in a traditional MDGC experiment. However, can we develop a method that can separate all such sections in a chromatogram with one injection, in real time? Unfortunately, the ^{2}D column cannot provide a capacity of 500, within a 1 min analysis time, in a manner that could permit separation of the next fraction sampled from the ^{1}D column and every subsequent fraction thereafter.

Two hyphenated GC techniques achieve the rapid sequential 2D analysis for all compounds in a sample. The first approach is based on a method called rapid targeted MDGC, wherein a moving LMCS cryotrap repetitively selects contiguous zones of effluent from the ^{1}D column and sequentially transfers each zone to the ^{2}D column (*14*, *21*). As the cryotrap is moved away from the cooled zone in the direction of the incoming carrier flow, the trapped components are remobilized onto the ^{2}D column in a narrow re-injection band. A peak capacity of 40+ peaks in a 1 min sampled zone is realized by using a fast 0.1 mm i.d. ^{2}D column with a length of 5−10 m, which generates a very fast ^{2}D analysis. The number of applications using targeted MDGC to date remains small, but an example has been reported for separation of suspected allergens in personal products (*22*), wherein the analytical challenge involves isolation of small amounts of allergen in the presence of large amounts of matrix materials. For terpenes and related compounds, the similarity of MS library matches makes positive identification difficult, but better resolution provides improved analysis certainty.

The second technique able to separate each sample component better is comprehensive two-dimensional GC (GC×GC) (*23*, *24*). Here, a much shorter, narrow-bore ^{2}D column (ca. 1 m long; 0.1 mm i.d.) is used and a modulator (e.g., the LMCS or other cryogenic devices developed subsequently) controls the transfer of eluting compounds from the first column to the second column. The modulator repetitively traps regions from the ^{1}D separation and then re-injects them as narrow pulses onto the ^{2}D column according to the modulation period (P_M), for example, every 4 s. The modulation process effectively samples the eluate from the ^{1}D column, creating a series of multiple ^{2}D peaks for each original peak according to the modulation ratio (M_R) selected (*25*). Ideally, the separation of the peaks on the second column is completed within the same time frame as the modulation period, thereby preventing any potential overlap with peaks from a subsequent modulation event, a phenomenon called wrap-around. This is achieved by the fast separation facilitated by the short, narrow-bore ^{2}D column. A further technique describes the use of multiple trapping elements at the end of the first column (*26*), but because each trap needs to be separately eluted through a second conventional column (or to different detectors), it cannot be deemed to be a rapid method.

Another technology that is rapidly gaining a foothold in advanced GC methodology is the "capillary flow technology" approach based on Deans switching, which can be used for both GC×GC and MDGC (*27*). This has recently been reported for alkyl mercaptans in natural gas, and this application is typical of the basic approach used in the MDGC mode.

Junge and co-workers (*28*) examined the capabilities for GC×GC to be extended into a faster analytical domain using the example of a standard mixture of suspected allergens that might be present in perfumery-type samples. The researchers determined that a 0.1 mm i.d. ^{1}D column coupled with a ^{2}D column of 0.05 mm i.d. would provide faster overall analysis with a faster modulation period (P_M) that maintains an acceptable modulation ratio (M_R). The column lengths were about 5 and 0.35 m for ^{1}D and ^{2}D, respectively, and a temperature programming rate of about 35 °C min^{-1} was employed. Given that the two column systems had slightly different phase combinations in the classical and fast methods, reasonably good correlation of peak positions was observed, but with the fast method giving analysis in about 5 min.

A current research direction in gas chromatography is miniaturization using microfabricated columns. Lambertus and Sacks (*29*) reported the use of 3 m × 150 μm × 240 μm columns etched onto a 3.2 cm × 3.2 cm silicon chip, with each column capable of generating between 5000 and 6000 theoretical plates. In the cited paper, two such columns coated with different stationary phases were connected in series with a stop-flow valve at the interface, which further improved the resolution of coeluting peaks.

SELECTED APPLICATIONS OF TWO-DIMENSIONAL GC

An elegant analysis that almost demands MDGC is the separation of enantiomeric compounds. The most logical approach is to use an achiral ^{1}D column, followed by an enantioselective ^{2}D (e-^{2}D) column. A narrow zone of effluent from ^{1}D containing the target enantiomers is passed to the second chiral (e-^{2}D) column for complete enantiomer separation. Ideally, the two fully resolved enantiomers will be completely resolved from any matrix compounds transferred in the target region with the enantiomeric compounds (*30*). Use of an e-^{1}D column is not appropriate because a wide transfer zone would be necessary to encompass both resolved enantiomers and would thereby capture a greater number of matrix compounds, which would have to be resolved from the enantiomers on the second column. Additionally, if we use a cryofocusing step after the e-^{1}D, then we lose enantioseparation if an achiral ^{2}D column is used.

The double cool-strand interface MDGC system described by Begnaud and Chaintreau was applied to resolve α-isomethylionone (a suspected allergen) from fragrance samples (*20*). MDGC was required because α-isomethylionone coelutes either with α-ionone on a polar column or with β-ionone on a nonpolar column. The system was also used with a chiral ^{2}D column to determine enantiomer ratios of linalool and linalyl acetate in essential oil samples to detect any adulteration (*20*). A second application was to resolve and quantify the enantiomers of a sweat malodor compound, 3-methyl-3-sulfanylhexan-1-ol (*13*). In both applications, the LMCS cryotrap interface allowed the target region to be retained in the storage loop while the oven temperature was cooled to optimize the ^{2}D chiral separation.

GC×GC has been used for analysis of a broad range of essential oils (*31*). In a typical application, GC×GC/TOFMS was used to compare volatile compounds in the headspace of 13 different varieties of true pepper (*Piper* sp.) and other spices typically described as "pepper" (*32*). Solid-phase microextraction (SPME) sampling, with a fiber-drying step and on-fiber derivatization prior to GC×GC-FID and GC×GC/TOFMS, allowed direct analysis of resveratrol in wine (*33*). A further study of methoxypyrazines in Semillon wine with deuterated internal standard addition and SPME (*34*) permitted quantitative analysis of the target pyrazine in the GC×GC experiment; a multidimensional GC approach was also reported for a similar application (*35*). A recent example of fish oil analysis for trace contaminants was conducted by using a direct sample introduction interface followed by GC×GC/TOFMS analysis (*36*). Further applications have been reported in the review series by Adahchour and coauthors (*37*, *38*), including examples such as ginger, hops, sandalwood, and wine. This series of reviews is an excellent overview of the technology of GC×GC.

Despite the obvious advantages of the rapid methods presented above, there are some applications in which traditional MDGC with a long ^{2}D column is more appropriate. Such an example applies to olfactometry, wherein human assessors evaluate the odor activity of compounds as they elute after completion of the GC separation. In this situation, broader peak widths and greater separation between compounds allow for easier odor detection (*20*, *39*).

INCORPORATION OF OLFACTOMETRY WITH GC×GC AND MDGC

Hyphenation of a GC separation with detection by human assessors (GC−olfactometry; GC-O) is a common and important technique to determine odor activity of individual compounds in a mixture and to identify character-impact odorants. Various GC-O protocols, based on different principles (e.g., odor intensity, dilution-to-threshold, or detection frequency), may be applied to assign a relative importance to odor-active compounds eluting from the GC (*1*). The chromatography objective in GC-O is for each compound to be completely resolved to enable their odor quality (such as character or intensity) to be assessed individually. However, the overall odor perception of a sample is the result of a complex combination of many interacting odorants and their proportional release from the original sample matrix. It is difficult to determine the recombined odor simply from individual separated compound(s), due to complex interactions and physiological effects (i.e., threshold, volatility, receptor biology) (*40*, *41*). Nevertheless, the technology of achieving individual component separation and odor assessment is still important.

The characterization of an unknown odor is more complex than might be acknowledged by the above commentary. Combining retention indices, mass spectrometry, and olfactory analysis is a powerful multidimensional identification strategy. However, often peak overlaps arise due to insufficient resolution in a complex sample, and so precisely assigning a chemical compound to the odor perception is challenging, particularly when a trace odor-active compound coelutes with an abundant odor-inactive peak. This uncertainty in identifying the compound responsible is an opportunity for application of multidimensional separation technologies, to provide a superior analytical confirmation.

The number of publications in which MDGC has been combined with olfactory analysis remains small but is now increasing. Reported applications include analysis of an orange extract (*42*), spearmint oil (*43*), ginger (*44*), off-flavor compounds in beer (*45*), kiwifruit puree (*46*), malt whiskey (*47*), orange oil (*48*), malodors in animal feed (*49*), fragrance models (*20*), sweat malodors (*13*), human milk (*50*), prune-like off-aroma in wine (*51*), and hop essential oils (*52*). Recent research serves to demonstrate the capabilities of some new hyphenated MDGC techniques available to chromatographers for flavor analysis.

Begnaud and coworkers (*13*) utilized the DCSI system described above for chiral GC-O to assess the odor character of the pure enantiomers and the racemic mixtures of linalool and 3-methyl-3-sulfanylhexan-1-ol. Both (*S*)- and (*R*)-enantiomers of each compound were resolved on a chiral ^{1}D column, isolated in

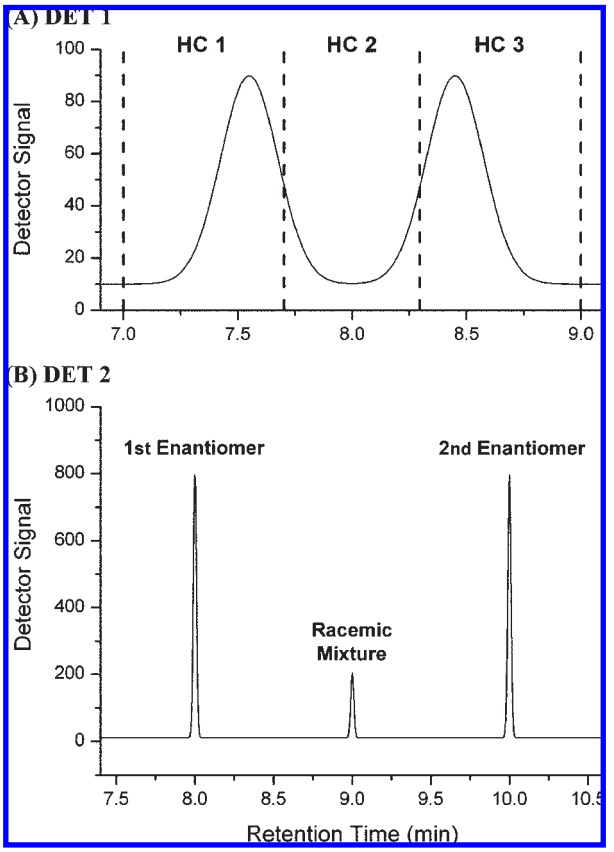

Figure 4. Chiral GC−olfactometry separation using the DCSI system to allow olfactory evaluation of pure enantiomers and the racemic mixture. (**A**) DET 1 response demonstrating resolution of the enantiomers on a chiral ^{1}D column. Three heart-cuts (HC) are made across the two peaks for transfer to the olfactory port/DET 2 via the ^{2}D transfer line. (**B**) DET 2 response following isolation of each HC with the DCSI. Olfactory evaluation is performed simultaneously. Schematic diagram was adapted and redrawn from ref *13*.

the DCSI storage loop, and then released to an odor port for characterization. Under normal chiral 1DGC-O conditions, enantiomers elute too closely to evaluate the compounds individually because the odor perception persists and sensory adaptation occurs. By making three heart-cuts across the two resolved peaks, the odor character and intensity of each pure enantiomer and the racemic mixture could be assessed in a single run, as shown schematically in **Figure 4** (*13*). Maintaining a time interval of 1 min between elution of the odorants ensured that assessors could sufficiently recover and eliminate any impact of adaptation on the odor assessment. A great advantage of this system is that sample components are only ever in contact with inert (deactivated) column walls and column phase, thus avoiding any activity or adsorption problems from contact with the hot metal surfaces found in valves and similar interfaces. This feature was a necessity for the analysis and characterization of the labile sulfur compound, 3-methyl-3-sulfanylhexan-1-ol (*13*).

A second case study incorporated both GC×GC with GC-O for characterization of coriander leaf volatiles (*53*). Character-impact odorants were located by GC-O and assigned a relative importance using the CharmAnalysis methodology. Compounds responsible for the odor perceptions were subsequently identified using GC×GC/TOFMS. The GC×GC distribution of components permitted identification of several homologous series of related compounds. Each series (*E*-2-alken-1-ols; alkanols; *E*-2-alkenals; *Z*-2-alkenals; *Z*-4-alkenals; alkanals; alkanes)

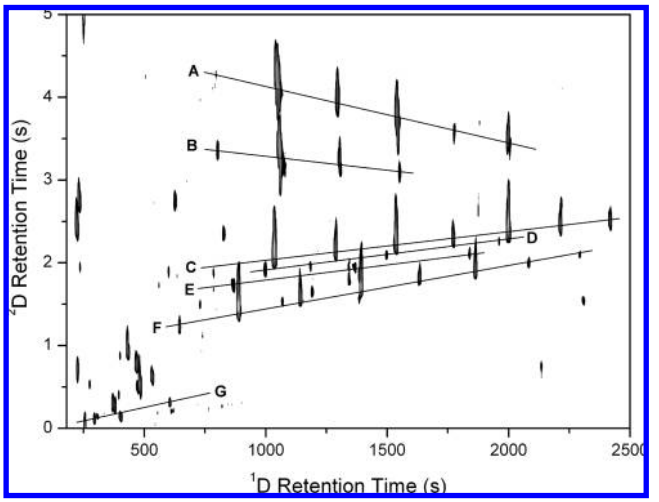

Figure 5. GC×GC analysis of coriander leaf essential oil. The series of compounds identified were (using GC×GC/TOFMS analysis) (A) *E*-2-alken-1-ols, (B) alkanols, (C) *E*-2-alkenals, (D) *Z*-2-alkenals, (E) *Z*-4-alkenals, (F) alkanals, and (G) alkanes. Adapted from ref *53*.

forms an approximate linear relationship within the 2D separation space, and peak identities were confirmed by using GC×GC/TOFMS (**Figure 5**). Such plots allow prediction of individual compound locations and allow rapid confirmation of their presence. Compound identification was supported using various combinations of retention index values, MS, and injection of reference compounds.

Note that this study did not employ GC×GC directly hyphenated with olfactometry to evaluate odor activity, although this group has briefly investigated this approach (unpublished results). It was found that the "modulation" process and the resultant peak intensity enhancement could promote the perception of volatile aroma compounds concentrated by the cryotrapping step. Although odors were successfully detected for modulated peaks of a reference standard, this technique was deemed to be too difficult for the assessors to maintain their focus and unreliable because the chance of missing an odor was high. We concluded that this was not the best approach for resolving and evaluating coeluting odorants, and so a heart-cut MDGC-O instrument was subsequently developed (see below).

Other researchers (*54*) have claimed olfactory detection can reliably be used with GC×GC, demonstrating identification of many compounds, but this is yet to be replicated by any other research groups. The results presented are impressive, reporting the detection of 818 odorants and identifying 481 of these. However, the main objective of GC-O is to determine the character-impact odorants that contribute most to the odor character of a sample, and so locating 818 odor-active compounds may not effectively meet this objective. The paper also illustrates the inherent drawbacks of GC×GC-O. Slow temperature programs are required (1−4 °C min^{-1}) for GC×GC, meaning that each analytical run is 30−60 min long. For example, d'Acampora Zellner et al. (*54*) used an oven rate of 1.5 °C min^{-1} with peaks of interest eluting with retention times up to 100 min and resulting in a total run time of over 2 h. Because of the intense concentration required for the task, sniffing was performed in stages of only 5 min interspersed by 10 min breaks. Therefore, it took each assessor three runs to assess the whole sample. Because the chance of missing odors is high, each assessor repeated the analysis six times to ensure results were valid, resulting in a very time-consuming investigation. To facilitate the GC×GC sniffing, a long modulation period of 8 s was also used, which could

possibly result in recombination of peaks already resolved on the ^{1}D column and risks reducing overall resolution.

d'Acampora Zellner et al. (*54*) cite a perception transduction time of 500 ms and use this value to justify the potential to detect odorants eluting within the 8 s modulation period. However, this does not consider the time required for the brain to react to the perception, that is, recognize the odor. The time required for this cognitive treatment has actually been reported to be > 700 ms (*55*). Therefore, the olfactory peak detection capacity is very low, particularly when the impact of the assessor's breathing rate and the extra time required to record the odor description are considered. Finally, performing any olfactory task more complicated than simple detection or description, such as measuring odor intensity, would certainly be extremely difficult in the short time frame required of a GC×GC experiment.

A further case study by the present researchers utilized GC-O, GC×GC, and MDGC-O to investigate odor-active compounds in a specific fraction of the essential oil from four hop varieties (*52*). The following summarizes the progression of the methodology to identify the compound responsible for a potent "woody, cedarwood" odor using various hyphenated techniques:

(i) GC-O located the odor-active region of interest, but the chromatogram demonstrated considerable complexity in this region (oxygenated sesquiterpenes). GC-MS analysis could not adequately identify a single component responsible for the odor perception. Peak overlap prevented correlation of the olfactory response with particular peak abundances (i.e., peaks in the GC did not correlate with relative odor perception, due to interfering peak overlaps).

(ii) GC×GC analysis vastly improved the separation of the target region, and TOFMS detection provided further resolution by deconvolution of coeluting isomers. Between 8 and 13 components were found to coincide with the odor-active region in each of the four samples. A number of peaks in this region could not be unambiguously identified from their mass spectra alone, so the odor quality of reference standards could not be assessed. This GC×GC result is not shown here.

Due to the improved separation, it was possible to compare relative peak abundances from GC×GC analysis of eight samples of hop oils (four additional whole oil samples were analyzed), with the odor potency values generated by CharmAnalysis for each sample. In this case, a greater log Charm value could be correlated with the log peak area of only one peak in the region of interest ($R^2 = 0.95$). However, this remains inadequate to provide confirmatory evidence that the suspected peak is responsible for the odor perception. In addition, this correlation approach cannot be applied to the analysis of a single sample, necessitating a third hyphenated approach.

(iii) Subsequently, MDGC separation based upon a Deans switching system allowed selective transfer of the target odor region to the ^{2}D column for resolution of coeluting peaks (**Figure 6**). The ^{2}D column separation for the sample is presented in **Figure 6B**, with the heart-cut region showing in excess of 15 peaks of various intensities. Two assessors could therefore easily assess the odor character of each peak eluting from the high-resolution ^{2}D separation over a 4 min period. The peak responsible for the odor perception is marked with an asterisk, which also matched the peak determined by the GC×GC correlation approach. The MDGC result for each of the four hop samples is shown in **Figure 7** (note that **Figure 7A** is a repeat of **Figure 6B**), demonstrating the presence of the same odor-active peak in each sample. Only one other odor was detected in all of the samples, which was a weak woody odor found in the HHE sample coinciding with coeluting peaks marked with a dagger (†). These peaks were identified by GC×GC/TOFMS as α-eudesmol

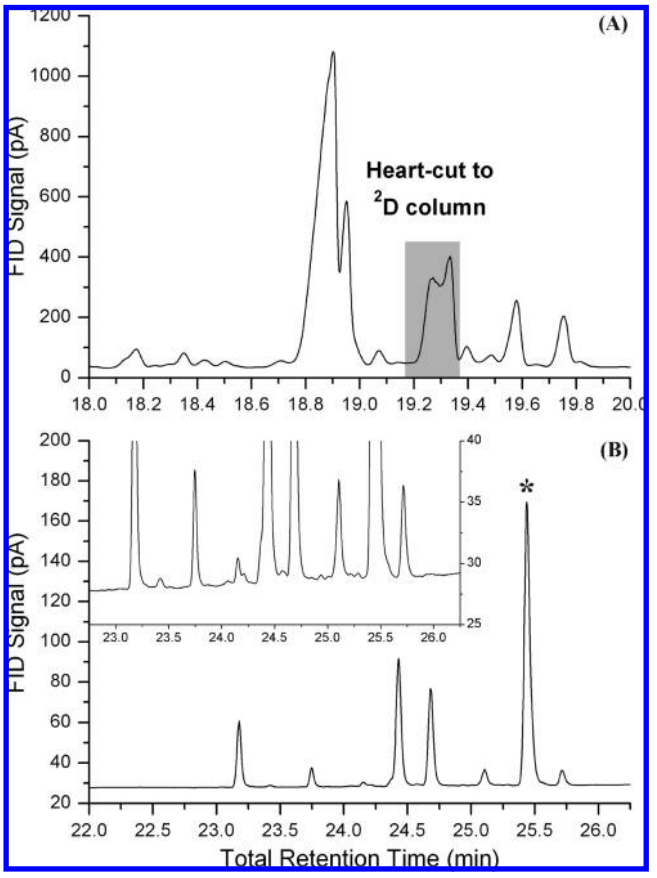

Figure 6. MDGC separation of a target region in the spicy fraction of Cascade hops: (**A**) partial ^{1}D chromatogram showing the target region where a woody odor was detected using 1DGC-O analysis; (**B**) separation of the isolated target region on the ^{2}D column over a 4 min period. The peak responsible for the woody, cedarwood odor is marked with an asterisk (∗) determined by parallel olfactometry. The inset illustrates that about 15 components are present in the heart-cut region. Reprinted from ref *52*. Copyright 2007 American Chemical Society.

and α-cadinol. These compounds are also present in the other spicy fraction samples but at concentrations below their detection thresholds. This lead to the conclusion that these compounds do not significantly contribute to the odor perceived.

The compound responsible for the woody odor was tentatively identified as 14-hydroxy-β-caryophyllene by comparison of its mass spectrum acquired with GC×GC/TOFMS with the MassFinder 3.58 library of terpenoids and related constituents of essential oils (Hochmuth Scientific Consulting, Hamburg, Germany). The identification was supported by matching the retention indices of the peak on two different stationary phase columns with past literature (*52*).

Difficulties were encountered when it came to confirming this identification, as a pure reference standard could not be acquired. Confirmation would then typically demand the compound be synthesized, which may be a time-consuming task, particularly when the identification is not certain. Therefore, the need for a preparative MDGC (prep-MDGC) system was recognized to be able to isolate pure compounds for characterization by NMR spectroscopy and/or other structure elucidation techniques.

DEVELOPMENT OF MICROSCALE PREPARATIVE CAPILLARY MDGC

The most information provided by MDGC is when it is hyphenated with MS detection, which in turn works best with

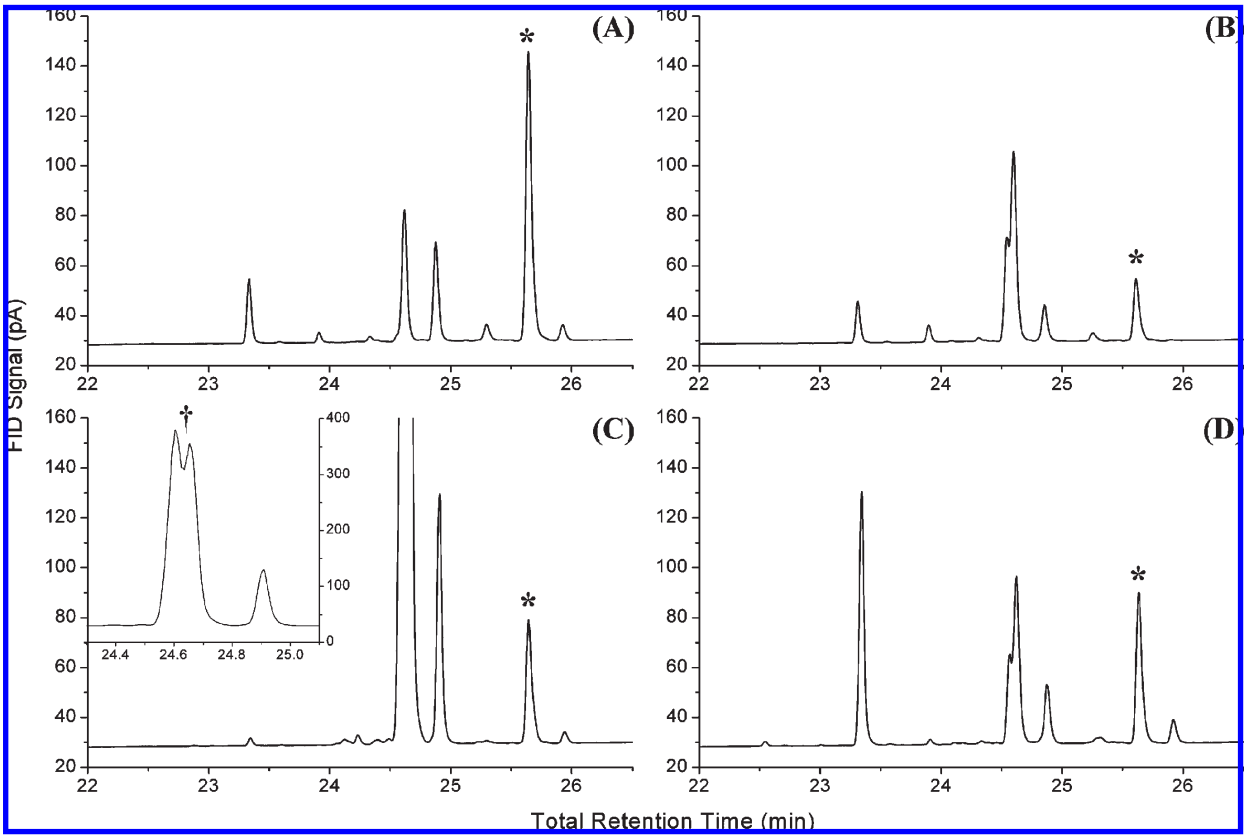

Figure 7. Comparison of the ^{2}D separations of the target regions in the spicy fractions of (**A**) Cascade hops, (**B**) Target hops, (**C**) HHE hops, and (**D**) Saaz hops. The odor-active peak is marked with an asterisk (∗).

well-resolved peaks, that is, with only a single peak entering the detector at a time. Overlapping peaks can still be successfully analyzed using data processing algorithms such as background subtraction, spectral deconvolution, and/or quantification by selected ion monitoring using identified unique ions. However, none of these techniques is necessary if baseline resolved, pure GC peaks are achieved using MDGC. With pure peaks, there should be no limitation to the detection tools that can be combined with GC, either hyphenated directly online or in an offline arrangement (provided each peak can be collected). The requirements that must be considered are as follows: (i) Can a suitable detector be directly hyphenated with MDGC? (ii) What procedure can deliver the separated analyte to the spectroscopic system? (iii) What is the relative sensitivity needed to provide adequate detection of target molecules?

For requirement i, online hyphenation requires compatibility of the detector with the separation step. The detector must be capable of real-time data acquisition in the time frame of the GC separation. Some methods cannot accomplish this, whereas others may have suitable sensitivity for GC hyphenation, but the interface may be unwieldy. Therefore, we must resort to situation ii using offline hyphenation with analyte collection in a preparative system. Point iii is a critical consideration if there is a significant mismatch between the amount of analyte injected into the MDGC system and the sensitivity of detection. The latter is normally the limiting step (of lower sensitivity), so multiple injections/trappings of analyte and/or overloading of the MDGC system is required.

Obtaining a pure isolated compound from a prep-MDGC system then allows the application of any combination of spectroscopic methods to identify the compound or to provide complete structure elucidation for an unknown. These methods may include NMR spectroscopy, high-resolution MS, Fourier transform infrared (FTIR) spectroscopy, or other suitable optical methods. Once the compound is unambiguously identified, the pure mass spectrum can be added to a database to facilitate its identification in other samples. Further analysis of the collected compound with GC-FID or GC-MS on several columns with different stationary phases would generate retention index data to assist identification. Another important assessment of the isolated compound would be its odor activity, in terms of its odor character, intensity, and detection threshold. In addition, the impact of the identified compound on the overall aroma perception of a mixture may be investigated using recombined odor models of an original sample (e.g., a food or beverage, a natural product, or an essential oil) (*41*). Obtaining the pure compound circumvents the requirement for organic synthesis for this purpose, although absolute structure elucidation would greatly facilitate the organic synthesis process.

Recent work of this group (*56*, *57*) developed a microscale preparative MDGC system, incorporating an LMCS cryotrap loop as the transfer interface and a Deans switch device at the outlet of the system to direct the target analyte to an external cryotrap (trapping capillary; xTC) for collection (**Figure 8**). Cryotrapping works well for analytes that may be suitably trapped at reasonable trapping temperatures (−30 to 0 °C) to allow good recovery rates. Other devices at the outlet of the prep-MDGC system might include a gas-phase trapping cell, or a solid-phase sorbent, the latter requiring elution of the analyte from the sorbent, which may affect the recovery and purity of the recovered compound. The system operates automatically, including making multiple injections, back flushing if required, and all event functions apart from manual elution of the trapping capillary into an NMR tube. Demonstration of this technique was presented for the isolation of geraniol from an essential oil matrix (*56*) where many compounds coeluted on the ^{1}D column

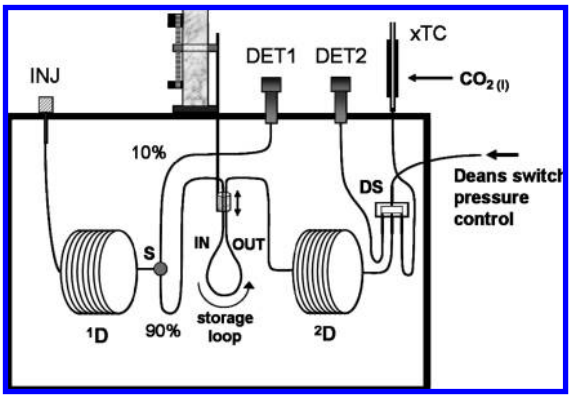

Figure 8. System developed for preparative MDGC for isolation of pure compounds for offline NMR spectroscopy. DET 1 provides a monitor signal for the ^{1}D separation using 10% of the column flow from a splitter (S). The column looped through an LMCS cryotrap (storage loop) isolates specific target regions to facilitate separation of the target zone on the ^{2}D column. The Deans switch (DS) diverts the target compound (geraniol) to an external trapping capillary (xTC) cooled with liquid CO_2 (l), to collect the trapped geraniol for subsequent NMR analysis.

under preparative-scale conditions. The major objective was to collect sufficient quantities of geraniol to perform offline NMR spectroscopy for structural confirmation.

Figure 9 demonstrates the method for isolation of geraniol from a complex mixture of essential oils. A narrow region from the ^{1}D column (ca. 25 s wide), which comprised the chosen target geraniol and interfering peaks carvone, pulegone, and linalyl acetate, was isolated in the storage loop (**Figure 8**). Briefly, process operations are as follows: Peaks eluting before the target region are initially collected at the incoming column segment cooled by the cryotrap (first trapping zone). At the start of the target region, simple movement of the cold region then passes the "before" fraction into the storage loop (second trapping zone) as the target peaks are trapped in the first zone. The complete target region is then isolated in the storage loop as the "before" region is simultaneously expelled onto the ^{2}D column. Any peaks after the target region are retained in the incoming cooled column segment and cannot proceed into the storage loop. Finally, downward movement of the cryotrap pulses the target region into the ^{2}D column for further separation. Thus, the result is that geraniol is completely resolved from the matrix components (**Figure 9B**), allowing collection of the pure component from a complex sample. Switching of the Deans switch (**Figure 8**) directs the ^{2}D chromatographic result from DET2, to the xTC (external trapping capillary), where the single pure geraniol peak is collected cryogenically and so it is missing from **Figure 9C**.

One hundred automated injections accumulated sufficient analyte (~78 μg) to perform ^{1}H NMR spectroscopy in both 1-D and various 2-D modes, but any desired spectroscopic technique may be used according to specific needs. To our knowledge, this constituted the first demonstration of 2-D NMR with a sample isolated from an automated preparative-scale multidimensional capillary GC separation. NMR identification, supported by MS information, makes improved structural confirmation possible. In a related research application, König and co-workers (*58*) described the occurrence of enantiomers of cadina-3,5-diene and δ-amorphene and employed a packed-column preparative 1D-GC system to separately isolate the target compounds. Subsequent chiral separation of the target analytes by re-injection on selected chiral columns afforded isolation of the individual enantiomers. Structural assignments were reported, based on 1-D and 2-D NMR methods, with ^{13}C NMR data also generated.

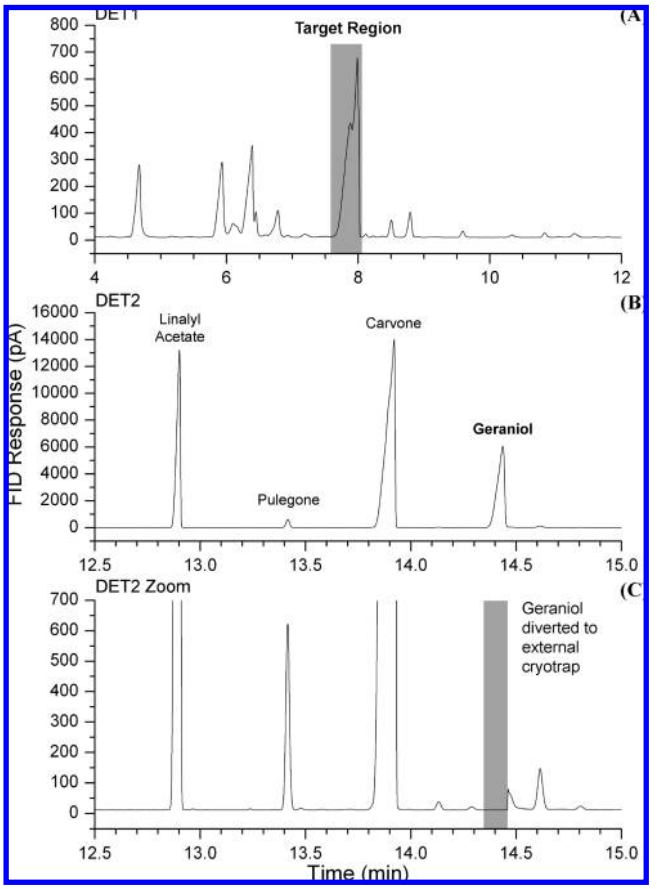

Figure 9. Resolution and isolation of geraniol using the prep-MDGC system: (**A**) DET 1 response monitoring the ^{1}D separation used to transfer the target region (shaded); (**B**) DET 2 response recording the ^{2}D separation of the target region and resolution of geraniol; (**C**) zoom of DET 2 response demonstrating diversion of geraniol to the external trapping capillary. Replotted original data from ref *56*.

A multidimensional capillary gas chromatography method using a nonpolar (^{1}D)/chiral (^{2}D) column system was used to quantify the relative enantiomers present in selected essential oils.

Although geraniol is a well-characterized compound, its isolation and identification served to demonstrate the potential of the technique (*56*). The next step is to apply the approach to a "blind" study of a randomly selected compound or to a "real" problem for which high-resolution separation is required to identify an unknown compound. For example, the prep-MDGC approach would have been effective as an alternative technique to that reported in a recent publication regarding the isolation and identification of a cockroach sex pheromone (*59*, *60*). This paper involved many liquid partitioning and chromatographic steps, with final gas chromatographic elution to produce a presumably pure peak, with collection of enough analyte (~5 μg) to perform ^{1}H NMR analysis with a microvolume NMR tube. Synthesis of the final suspected compound confirmed the structure and biological activity of the compound toward the male cockroach.

In a further recent study (*61*) it was demonstrated that multiple selected components could be diverted to the xTC, via the Deans switch, to effectively produce a new submixture of the original sample. With clean isolation of each heart-cut compound from the matrix, a unique mixture of (any) desired composition could be prepared in microscale. Such a mixture could then be available for any subsequent analysis by using, for example, GC-MS or GC×GC.

FINAL REMARKS

Hyphenated analysis for flavor monitoring, detection, and discovery is a very robust, but continually advancing, topic. For volatile compounds, GC-MS is used reliably, often providing the answer demanded by the analyst. However, it is increasingly recognized that complex samples require more than a single-column separation to provide sufficient resolution of target peaks. The power of MS is often insufficient to provide unambiguous measurement for grossly overlapping compounds. We must resort to higher peak capacity or multidimensional GC methods. Various new approaches have been introduced in our laboratory to address the above limitations by maximizing resolution to improve capabilities for detection. Case studies include analysis of coriander leaf oil showing trends in chemical class retention in GC×GC, identification of the compound responsible for a woody odor by using GC-O correlated with GC×GC/TOFMS data, and finally MDGC-O. A new study demonstrated the power of a microscale preparative MDGC separation method, with a loop modulator and a microfluidic Deans switch to isolate a pure geraniol component, followed by NMR analysis. Truly, the methods utilizing multidimensional separations and hyphenation with advanced spectroscopic procedures will permit many new and exciting future studies for flavor analysis.

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