OPPORTUNITIES FOR FLAVOUR ANALYSIS THROUGH HYPHENATION

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Abstract

The advances in chemical and flavour knowledge that can be made through improved separation and identification capabilities cannot be underestimated. In this paper, we explore a number of new integrated methods that we have recently introduced that permit improved resolution and separation power for a range of analytical studies. These focus on multidimensional gas chromatography, comprehensive two-dimensional gas chromatography (GC), olfactometry, mass spectrometry and nuclear magnetic resonance spectroscopy. The overriding aim is to provide technical solutions that employ the best possible separation of compounds. Better separation of compounds allows tools such as olfactometry, mass spectrometry, nuclear magnetic resonance spectroscopy, and other detectors to provide much better characterisation of separated chemical species. We demonstrate various novel strategies that provide the necessary separation power, integrated with specific detection steps. Case studies in the areas such as sensory-directed identification of a woody odorant in hop essential oil, correlation of compound identifications in coriander leaf, and development of new preparative capabilities in multidimensional GC with nuclear magnetic resonance spectroscopy.

Introduction

The role, implementation and use of multidimensional gas chromatography (MDGC) is now well established (1,2). MDGC has a long history although the promise and widespread application of MDGC remains unfulfilled (3). What driving force developed MDGC, even in the early days of capillary GC? The elegance of linking two or more columns in a hyphenated arrangement demanded investigation, just to see how it might benefit the technology. At the other extreme is the recognition that a single column GC solution is simply inadequate to provide the analytical answers that an analyst seeks. In this context, we have little solution other than to resort to multiple separation dimensions. Fetterolf and Yost (4) then Kidwell and Riggs (5) presented discussion on the informing power of hyphenated analytical separations with mass spectrometry (MS). A single GC column has an informing power of about 500-600 (i.e. can separate 500-600 peaks (compounds) in a single analysis). Complex sample have a high likelihood of multiple peak overlaps, and according to Davis and Giddings (6-8) may demand multidimensional separations. Electron induced ionisation GC/MS increases informing power to about 6.6x10⁶ due to the discrimination of the MS dimension; MS is able to provide considerable identification
power due to the use of library matching. GC/MS-MS further extends informing power to an amazing $6.6 \times 10^9$, due to the selectivity of the second MS dimension.

Similarly, GC can provide significantly more resolving power by selecting small zones of eluting peaks from a first column, then separate them on a second (orthogonal) column, by choice of different polarity phases. Consider selection of a complex group of peaks eluting within a zone equivalent to 10 peaks wide (about 1 min of effluent, possibly comprising 50 overlapping peaks). Applying this to a $^2$D column with a peak capacity of 500, should allow separation of each sampled peak. But can we develop a method that can do this with one injection, in real time? The $^2$D column cannot provide a capacity of 500, within a 1 min analysis time (in a manner that could permit the next fraction from the $^1$D column, and every subsequent fraction thereafter).

An elegant analysis that almost demands MDGC, is that of enantioselective analysis. 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, e-$^2$D column, for complete chiral separation. Ideally, the two fully resolved enantiomers will be completely resolved from any matrix compounds transferred with the enantiomeric compounds (9). Use of an e-$^1$D column is not appropriate; a wider transfer effluent zone that encompass the two resolved enantiomers will capture more matrix compounds which have to be resolved from the enantiomers.

Two methods most closely achieve the rapid sequential 2D analysis for all compounds in a sample. Our first approach is based on a method called rapid targeted MDGC, which selects contiguous zones of effluent from a first column (10,11). About 1 min of effluent from the $^1$D column is collected in a device we call a longitudinally modulated cryogenic system (LMCS). The cryotrap is moved away from the cooled zone to release components to the $^2$D column. A peak capacity of 40+ peaks in a 1 min sampled zone is realised (the re-injection band is negligibly narrow; a fast 0.1 mm ID $^2$D column of about 5+ m long generates very fast $^2$D analysis). The second is to use comprehensive 2D GC (GC×GC) (12,13). Here, a much shorter, narrow bore $^2$D column (ca 1 m long; 0.1 μm ID), and a modulator (we use the LMCS for this) performs a repetitive “collect – pulse” operation from the $^1$D to $^2$D column. A number of modulations arise for each $^1$D peak, according to the modulation ratio $M_R$ selected (14), and each modulation (2D separation) should ideally be completed within the same timeframe as the modulation period. A further method that involves multiple trapping elements (15) each separately eluted through a conventional column, or to different detectors, is not a rapid method.

Using advanced separations for essential oil samples

Targeted MDGC has been not used for many applications to date, but included alleged allergens in personal products (16), where the problem is 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. With better resolution we can achieve better analysis certainty.

GC×GC has been used for analysis of a broad range of essential oils. In a typical application, we used GC×GC/TOFMS to compare volatile headspace compounds in a variety of pepper and related aromas (17). Other applications have been reported in the review series by Adahchour et al. (18,19) such as ginger, hops, sandalwood, wine, and these works are an excellent overview of the technology of GC×GC.
Incorporation of olfactometry with GC×GC and MDGC

Olfactory analysis using GC (GC-O) is commonly used to determine odour activity of individual compounds in a mixture. Well-established procedures can be used for assessing odour quality and relative importance of odour-active compounds eluting from the GC (20). The overall aroma of a sample is a convoluted combination of many interacting odorants. The individual separated molecules in GC-O will present their own odour signatures. It is difficult to determine the recombined odour simply from individual separated compound(s), due to complex interactions and physiological effects (i.e. threshold, volatility, receptor biology). Odour assessment is more complex than might be acknowledged by the above comment. Combining I values, MS and odour profiling is a powerful multidimensional strategy. Where peak overlaps arise, precisely allocation of the olfactory result to analytical data (e.g. assignment of chemical structure to an aroma compound) is difficult. Inadequate resolution leads to uncertainty of compound identity, and is an opportunity for application of multidimensional separation technologies. Recent research serves to demonstrate the capabilities of some new techniques available to chromatographers.

Begnaud and Chaintreau (21) described the development of an arrangement that quantitatively trapped compounds within a capillary column loop, isolated them on-line from preceding and following peaks, and introduced the target region into the 2D column for effective resolution from interfering matrix compounds (Figure 1A). This allows both MS and/or olfactory analysis. A small split flow (~ 10%) to a monitor detector achieves correct 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 operates in two ways (Figure 1B). The first is a reverse direction loop through the cryotrap (Fig 1B(i)). The second (Fig 1B(ii)) as originally proposed, passes the loop through the same direction as the incoming strand. The former arrangement allows smaller loop size, with fewer movements of the cryotrap to permit the solute to enter, be trapped then remobilised into the second dimension.

**Figure 1.** (A) Schematic diagram of a ‘double cool-strand loop modulator’ incorporating a looped column (L) through a longitudinally modulated cryogenic system (LMCS). Target regions can be selected, sampled and passed to the second dimension (2D). The 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.
A common approach to implementing MDGC is via a micro-fluidic pressure-balanced Deans switch system, (Figure 2). 1D column eluate is directed to a monitor detector; when a target region elutes from the 1D column, flow is diverted to the start of the 2D column (see Marriott (22)). A cryotrapping device incorporated into the system, to cryo-focus the heartcut analyte band at the head of the second column, would be a preferred option, particularly for short, thin-film columns, to maximise performance.

![Figure 2](image-url)  
**Figure 2.** (A) Schematic diagram of the physical design of a Deans switch system. (B) is the representation of the Deans switch used in Figure 4.

A second case study incorporates GCxGC with GC-O for coriander leaf volatiles (23). Coriander character-impact odorants were located by CharmAnalysis™ aroma profiling in GC-O. The GC×GC distribution of components permitted identification of series of related compounds. Each series (E-2-alken-1-ols; alkanols; E-2-alkenals; Z-2-alkenals; Z-4-alkenals; alkanals; alkanes) forms an approximate linear relationship within the 2D space, confirmed by using GC×GC-time-of-flight mass spectrometry (TOFMS) (Figure 3). Such plots allow prediction of individual compound locations, and confirms of their presence. I values, MS and reference compound injection were variously used for identification.

This study did not employ GC×GC-O to evaluate odour activity, although this group has investigated this approach, finding that the ‘modulation’ process can enhance the perception of volatile aroma compounds concentrated by the cryotrapping step. But it is difficult for the assessor to assign specific peaks. Other researchers (24) claimed olfactory detection can reliably be used with GC×GC, demonstrating identification of many compounds; this is yet to be confirmed. Further study focused on essential oils from four hop varieties, and the presence of a potent ‘spicy, woody, cedarwood’ odour (25). The following summarises our hyphenated method progression:

(i) GC-O located the odour-active region. The GC had considerable complexity in this region (oxygenated sesquiterpenes). GC/MS analysis could not adequately identify a single component responsible for the odour perception. Peak overlap prevented olfactory (CharmAnalysis™) response correlation with particular peak abundances.

(ii) GC×GC analysis quantitatively separated the target region, revealing up to 13 resolved components. It was possible to compare relative peak abundances in GC×GC analysis of eight samples of hop oils, with the CharmAnalysis™ results; in this case, a greater %Charm value could be correlated with a specific peak increase...
in the oil extract ($R^2 = 0.95$). However this still is inadequate for providing confirmatory evidence that the suspected peak is correctly assigned to the odour.

(iii) MDGC and selecting the appropriate zone allowed the target region to be passed to the $^2$D column, with separation of all the peaks. Each peak was submitted to olfactory assessment. The woody odour was tentatively assigned to 14-hydroxy-\(\beta\)-caryophyllene by its mass spectrum and retention index acquired with GC×GC/TOFMS.

**Figure 3.** GC×GC analysis of Coriander oil. 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.

**Development of micro-prep capillary MDGC with MS and NMR spectroscopy**

MDGC is usually best accomplished with MS detection, which works best with well resolved peaks with a single peak entering the detector. Overlapping peaks can still be analysed (background subtraction, deconvolution, or quantified by selected ion monitoring using identified unique ions); none of these techniques are necessary with if pure GC peaks, achieved by using MDGC. With pure peaks there should be no limitation to the detection tools that can be on-line or off-line hyphenated with GC. We must consider: (i) can detection be directly hyphenated with MDGC? (ii) what procedure can deliver the separated solute to the spectroscopic system? (iii) what is the relative sensitivity needed to provide adequate detection of target molecules?

For (i), on-line hyphenation requires compatibility with the separation step, for real-time data acquisition. Some methods cannot accomplish this; others may have suitable sensitivity for GC hyphenation, but the interface may be unwieldy. Therefore, we resort to situation (ii). We pioneered a Deans switch device to direct flow and selected compound to a FID or a cryotrap at the end of the LMCS-MDGC system (26). Point (iii) is a critical consideration if there is a significant mismatch between the amount of solute that can be reliably injected into the MDGC system, and sensitivity of detection. The latter is normally the limiting step (of lower sensitivity) so multiple injections/trappings of solute is required, and/or overloading of the MDGC system. A pure isolated compound from the MDGC system then allows any spectroscopic method for its identification. The focus is on chemical identification, since once
identified and the pure spectra obtained, this can be added to a database (e.g. MS, or I values) for identification in other samples. Collected residue can be analysed by MS, GC/MS, GC×GC (to get retention position in 2D space), FTIR, high-resolution MS, or optical methods. Wilkins et al. were early adopters of MDGC with simultaneous detection, such as MS and FTIR (27).

Our recent work (26) demonstrated such an approach, integrating a novel approach for MDGC, with cryotrapping and off-line NMR spectroscopy, initially presented for qualitative separation of geraniol from a region where many compounds coeluted on the 1D column. Geraniol was collected by using a Deans switch at outlet of the MDGC, cutting the single component to an external cryotrap (Figure 4). The system operates automatically, apart from manual elution of the cryotrap tube into an NMR tube. Automatic injection and multiple sample repetitions (up to 100) can accumulate sufficient solute (~ 78 μg) to perform 1H NMR in both 1D and 2D modes. NMR identification, supported by MS information, makes absolute structural configuration possible. Geraniol was chosen to demonstrate our approach, and a 'blind' study of randomly selected compounds is now needed.

**Figure 4.** Procedure developed for MDGC with NMR. DET 1 provides a monitor signal for the total sample. The looped LMCS cryo-system selects specific target regions, to facilitate separation of the target zone on the 2D column. The Deans switch diverts the target (geraniol) to an external cryotrap, to collect the trapped geraniol for subsequent NMR analysis.

Figure 5 demonstrates the method for isolation of geraniol from a complex mixture of essential oils. Geraniol is completely resolved from the matrix components, allowing collection of pure component from a complex sample, and analysis by NMR – but any desired spectroscopic technique may be used.

**Conclusions**

Hyphenated analysis for flavour monitoring, detection and discovery is a very robust, continually advancing, topic. For volatile compounds, GC/MS is used reliably, often providing the answer demanded by the analyst. It is increasingly recognised that complex samples require more than a single column separation. The power of MS is often insufficient to provide unambiguous measurement for grossly overlapping
compounds. We must resort to higher peak capacity or MDGC methods. Various new approaches have been introduced in our laboratory to address the above limitations.

Figure 5. Data acquired for the system shown in (Figure 4) (A). FID 1 monitors the 1D result, to indicate the appropriate target sampling region. (B) Both preceding and following zones are resolved from the target region, shown in expanded format. FID 2 is the result for the target region. (C) Geraniol is diverted to the external cryotrap. Compounds are identified as follows: linalyl acetate (LA), pulegone (P), carvone (C).

We maximise resolution and improve capabilities for detection. Case studies include coriander leaf oil showing trends in chemical class retention in GC×GC, analysis of
hop oils with woody character by using GC-O correlated with GC×GC data, and finally MDGC-O. A new study demonstrated the power of a micro-scale MDGC separation method, with a loop modulator and a micro-fluidic Deans switch to isolate a pure geraniol component, followed by NMR analysis. Truly, the methods utilising multidimensional separations and hyphenation with advanced spectroscopic procedures will permit many new and exciting future studies for flavours.

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References