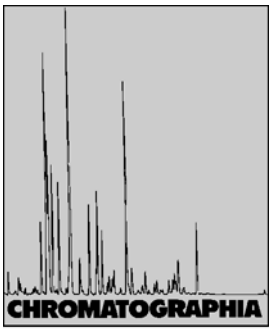
Application of Solid-Phase Micro-Extraction and Comprehensive Two-Dimensional Gas Chromatography (GC x GC) for Flavour Analysis



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M. Adahchour[1]* / J. Beens[1] / R. J. J. Vreuls[1] / A. M. Batenburg[2] / E. A. E. Rosing[2] / U. A. Th. Brinkman[1]

[1] Department of Analytical Chemistry and Applied Spectroscopy, Vrije Universiteit, De Boelelaan 1083, 1081 HV Amsterdam, The Netherlands; E-Mail: adahchou@chem.vu.nl
[2] Unilever Research Laboratory Vlaardingen, Oliver van Noortlaan 120, 3133 AT Vlaardingen, The Netherlands

Key Words

Comprehensive two-dimensional gas chromatography
Cryogenic modulation
Solid-phase micro-extraction
Flavours
Foodstuffs

Summary

Headspace solid-phase micro-extraction (HS SPME), comprehensive two-dimensional GC (GC × GC), and flame ionization detection (FID) have been examined for their suitability and compatibility for rapid sampling, separation, and detection of garlic flavour volatiles. This approach (HS-SPME-GC × GC-FID) is distinctly superior to use of one-dimensional GC, i. e., HS-SPME-GC-FID. Direct comparison of the experimental results showed that a 10 – 50-fold increase in sensitivity is obtained, separating power is substantially enhanced, and the peak capacity is up to ten times higher. As a consequence, much more detailed flavour analysis can be performed; this results in better information about the aroma-active compounds.

Introduction

Food analysis is important for evaluation of nutritional value, for quality control, for detection of adulteration of fresh and processed products, and for monitoring of food additives. It is also a tool for product improvement; for example, flavour, which is a combination of taste and olfactory sense, is a crucial factor in consumer acceptance of foods [1].

A technique suitable for the analysis of flavours in food must be able to isolate all relevant aroma compounds with good recoveries while the formation of artefacts should be avoided or, at least, limited as much as possible. Next to isolation, the most important aspect is the identification of the food constituents which contribute to aroma. These are usually present in trace to ultra-trace amounts and comprise a wide range of classes of chemical compounds [2]. In recent years solid-phase micro-extraction (SPME) has become an elegant option for sample preparation in aroma analysis, enabling rapid sampling at low cost with ease of operation and adequate sensitivity [3, 4].

The analysis of flavours is very demanding, because of the wide range of odour thresholds of the individual compounds – because man is much more sensitive to some than others [2]. One-dimensional capillary gas chromatography (1D GC) is routinely used to separate the volatile constituents of food samples. The complex nature of these samples requires long GC run times to obtain the maximum separating power, and even then coelution frequently occurs and is a major challenge for complete qualitative analysis, even with the use of definitive confirmation technologies such as mass spectrometry (MS). As mentioned above, the aroma-active compounds, or key flavours, are usually present in ultra-trace amounts and are not usually the major volatile constituents of the food. When using GC with olfactometric detection the human nose can often detect a distinctive smell where the chromatogram produces a flat baseline [2]. Even more frustrating, a substance that does elute at the proper retention time is not necessarily responsible for that smell – the aroma-active compound(s) might well be hidden by artefacts present at higher concentrations.

Obviously, there is a distinct need for a greater separating power; this can be achieved by using a multidimensional GC (MDGC) procedure [5, 6]. Unfortunately, because of the considerable increase in analysis time, conventional MDGC heart-cutting methods are limited to the analysis of only a few discrete target regions of a chromatogram. In addition, they require sophisticated instrumentation and experienced analysts.

Comprehensive two-dimensional gas chromatography (GC × GC) is a new and extremely useful alternative technique that uses a valve-less on-column interface

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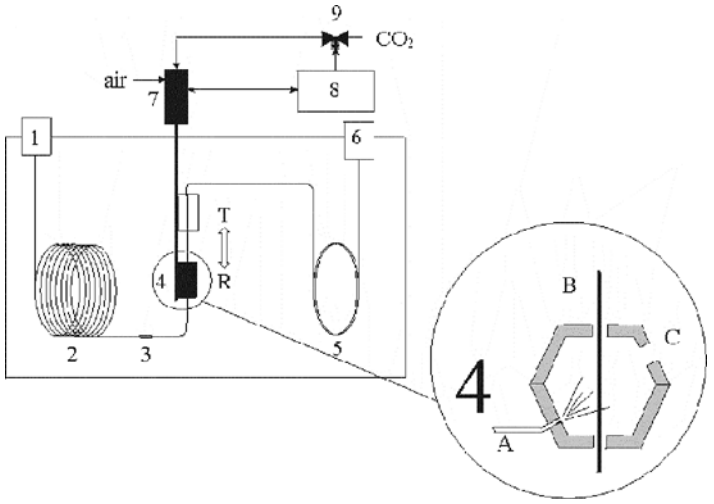

Figure 1. Schematic diagram of the GC × GC system and the cryogenic modulator. **1** = Injector; **2** = first-dimension column; **3** = press-fit connector; **4** = modulation chamber; **5** = second-dimension column; **6** = detector; **7** = pneumatic ram; **8** = modulation controller; **9** = two-way CO_2 valve; **T** = trapping position; **R** = release position. Insert shows (**A**) CO_2 inlet with orifice, (**B**) top of second-dimension GC column, and (**C**) CO_2 outlet.

between the two GC columns and a high-speed separation in the second dimension to combine the advantages of increased separating power and reduced analysis time [7–11]. The state-of-the-art of GC × GC has recently been reviewed [12, 13]; these papers reflect the early focus on petroleum and mineral oil products. Briefly, in GC × GC, two independent GC separations are applied to the entire sample. The sample is first separated on a normal-bore high-resolution capillary GC column in the programmed-temperature mode. A modulator at the junction between the two columns accumulates and focuses successive small portions of eluate from this column and, next, causes its transfer to the second-dimension column, which is short and narrow to enable rapid separation within the available time span of a few seconds before the next eluate fraction arrives. The second-dimension chromatograms are essentially a series of adjacent high-speed chromatograms. The reconstructed final chromatogram is usually presented as a two-dimensional contour plot, with the two time axes representing the retention on each of the two columns, and colour intensities indicating the peak heights. A major advantage is that the enhanced peak capacity – which is, ideally, equal to the product of the peak capacities of the two columns – of a properly designed GC × GC system is generated in the same time span as a conventional high-resolution 1D-GC separation. As well as the improved separating power, GC × GC also results in better analyte-identification capability than standard GC techniques.

In most GC × GC arrangements the columns are selected to provide a 'boiling-point' separation on the first, usually non-polar, column, and a more selective separation on the second, more polar, column – with the actual choice being determined to give maximum differentiation of chemical compounds of the sample being studied. As a consequence, the two retention times reflect both volatility and polarity.

In this paper, we discuss the use of GC × GC for the qualitative analysis of food samples such as garlic powder and briefly compare the results with those from 1D GC. As in most published GC × GC studies flame ionization detection (FID) was used to meet the stringent demands of rapid response to the second-dimension separation. Some early results from the use of time-of-flight MS are also presented.

Experimental

Headspace SPME Sampling

The SPME holder for manual operation, and the fibres used in this investigation were a gift from Supelco (Bellefonte, PA, USA). Two fibres were used – one coated with 100 μm polydimethylsiloxane (PDMS) and the other with 50/30 μm Stable Flex divinylbenzene-carboxen-polydimethylsiloxane (DVB-CAR-PDMS). Before first use they were conditioned by insertion into the GC injector, which was kept at a suitable conditioning temperature for each fibre – 250 °C for 1 h for PDMS and 270 °C for 4 h for DVB-CAR-PDMS.

For each headspace (HS) SPME analysis, samples were placed in 4-mL glass vials sealed with silicone rubber septa. The SPME needle pierced the septum and the fibre was extended through the needle to bring the stationary phase into contact with the headspace of the sample. The fibre was withdrawn into the needle after the sampling time, which was varied from 1 to 30 min. Finally, the SPME needle was removed from the vial and inserted for 1 min in the injection port of the gas chromatograph. The compounds extracted were thermally desorbed at 250 °C for 1 min and transferred directly to the (first-dimension) GC column.

Before each micro-extraction, the fibres were cleaned to prevent carryover of high-boiling material. To that end, the fibres were inserted in the injection port, which was kept at the conditioning temperature of each fibre, for at least 20–30 min.

GC x GC System with Cryogenic Modulator

GC Conditions

Gas chromatography was performed with a Hewlett-Packard (Agilent, Palo Alto, CA, USA) HP6890 instrument with split/splitless injector and an FID capable of producing a digital signal at a sampling rate of 200 Hz. The first-dimension 30 m × 0.32 mm i. d. column was coated with 0.25 μm HP1 (Varian-Chrompack, Middelburg, the Netherlands). It was coupled with a press-fit connector to the second-dimension 1.5 m × 0.10 mm i. d. column, which was coated with 0.1 μm DB5 or BPX50 (SGE Europe, Milton Keynes, UK). The flow was set to 1.0 mL min^{-1} by use of a column head-pressure of 180 kPa helium (99.999% purity; Hoekloos, Schiedam, The Netherlands). The columns were temperature programmed at 4° min^{-1} from 30 °C (2 min isothermal) to 220 °C (5 min isothermal). A schematic diagram of the GC × GC system is presented in Figure 1.

Modulator

The cryogenic modulator was home-made, and has been described in detail

elsewhere [14]. It was cooled by use of the Joule-Thompson effect with expanding liquid carbon dioxide (HoekLoos; technical grade). The temperature of the modulator was regulated at approximately 100° below the oven (and, thus, column) temperature. The modulator consisted of a cooling chamber 3-cm long. Modulation was achieved by moving the chamber at a constant speed up and down along the top 4 cm (i. e. the part marked by T↔R in Figure 1) of the second-dimension column, to effect trapping (T) and remobilization (R) of the small fractions eluting from the first-dimension column. It should be noted here that the cold gas is sprayed directly on to the capillary column. The escaping gas from the chamber prevents ambient air (and water!) from entering the chamber, so no freezing problems are encountered inside the chamber. A modulation period of 8 s was used.

For direct identification of the numerous compounds separated by GC × GC, it is desirable to couple this technique to a mass spectrometric detector. As mentioned above, for accurate detection of the very narrow second-dimension peaks, a fast mass spectrometer is required. At present, only TOFMS can scan at sufficiently high scan rates (up to 500 scans s^{-1}).

TOF MS Analysis

GC × GC-TOFMS was performed with an HP6890 gas chromatograph coupled to a Pegasus II time-of-flight mass spectrometer (LECO, St Joseph, MI, USA). The mass spectrometer was operated at a spectrum storage rate of 50 spectra s^{-1}, with an ion-source temperature of 180 °C and a transfer-line temperature of 280 °C. The multichannel plate voltage was set at 1800 V. A modulation period of 6 s was used, which means that peaks could be modulated 2 or 3 times. All other conditions were the same as for the GC × GC-FID experiments.

Data Handling

Data acquisition was performed by means of a Hewlett-Packard Chemstation. The raw data were exported in .csv format and then converted to ASCII matrices by use of a home-made conversion programme (Ph. Marriott, RMIT, Melbourne, Australia). Contour plots and second-dimension chromatograms of these files were generated by use of Neosys Transform (Creaso, Apeldoorn, the Netherlands).

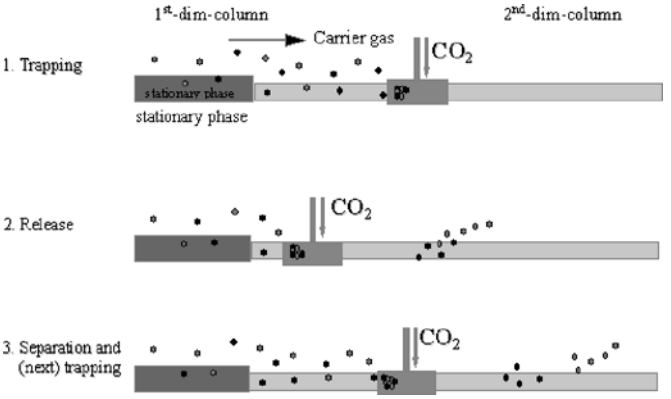

Figure 2. Schematic diagram of the cryogenic modulation process.

Results and Discussion

Modulation

Because comprehensive GC is not yet widely used, a rather detailed explanation of the technique is required including the various steps in the modulation process and important operating conditions.

In GC × GC the effluent from the first-dimension column is separated into a large number of small fractions, each of which is subsequently separated on the second-dimension column. The key component of the arrangement that enables the focusing and transfer of the small and narrow fractions from the first column onto the second is the modulator interface between the two columns (Figure 1). The cryogenic modulation process is represented schematically by Figure 2. This process is occurring continuously during analysis, and can be divided into three phases.

Trapping Phase: During analyte accumulation, solutes move towards a section cryogenically cooled by the modulator, which is 'held' at a position a few centimetres from the inlet of the second-dimension column.

Release Phase: After a pre-set time the modulator is rapidly moved to a new position slightly further upstream. This exposes the previously focused zone to the thermal environment of forced flow convection in the GC oven. The focused narrow band is now re-volatilized at a rate depending on the temperature of the oven and the thickness of the stationary phase (generally ~10 ms). Meanwhile, the next fraction from the first column is trapped in the cooled section upstream, so that it cannot interfere with the previous fractions.

Separation and Trapping Phase: While the fraction injected into the second column in the previous step is subjected to (rapid) chromatographic separation, the modulator returns to its initial position carrying the next refocused fraction. The time needed for the total process is called the modulation time. Modulation times are typically from 2 to 10 s, as will be discussed in some detail below.

Analytes co-eluting as single peaks from the first-dimension column are modulated into a series of separate second-dimension chromatograms which are reassembled to form the raw GC × GC chromatogram. The number of modulations across a first-dimension peak depends on its width and on the demands made by the second-dimension separation plus detection. In Figure 3, the arrows delimit the modulation periods. Ideally each peak is modulated 3–5 times to ensure that the first-dimension separation is maintained as completely as is possible. The time allowed for running of the second-dimension separation cannot, however, be too short. In actual practice, therefore, a compromise must be reached and, frequently, modulation across a peak is limited to two or three fractions. Because the raw GC × GC chromatogram so obtained is difficult to interpret, conversion of the data into a more user-friendly format is needed. From the HP Chemstation the file is exported and saved in an ASCII comma-delimited (*.csv) file format. By use of a home-made conversion programme, '2D-GC converter', this file is converted and transformed into a two-dimensional array according to the modula-

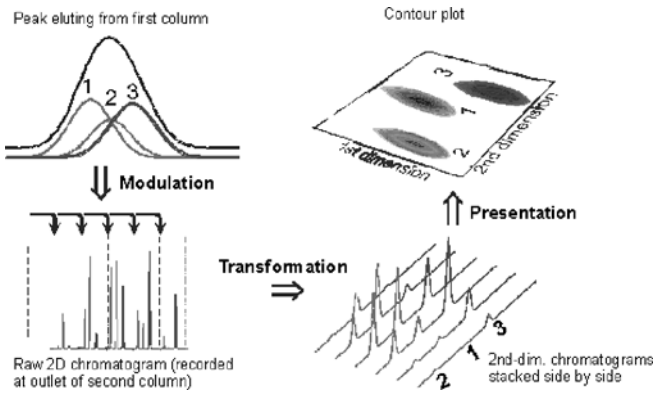

Figure 3. Construction of a GC × GC chromatogram.

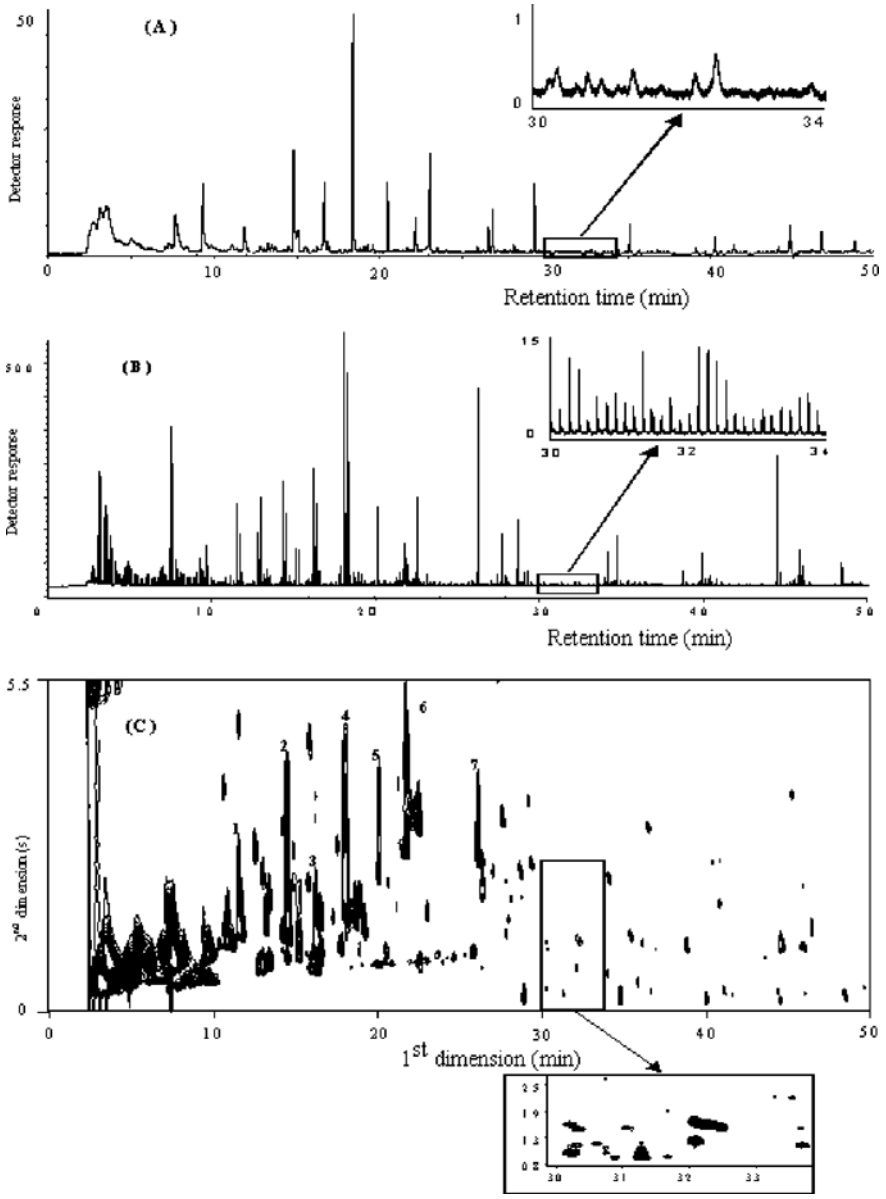

Figure 4. Comparison of (**A**) conventional 1D-GC and (**B** and **C**) GC × GC chromatograms, with FID detection, obtained from garlic powder. A raw GC × GC chromatogram is shown in **B**, a contour plot in **C**.

tion and detector frequencies. Finally, a contour plot of this file is generated by use of Neosys Transform. To emphasize once again that in GC × GC the separations in the two dimensions can, and should, be made independent of each other, the order of elution of the three peaks of Figure 3 has been made different in the two dimensions.

SPME

Because this study deals primarily with the general practicability of GC × GC combined with SPME for the trace-level analysis of flavours, the selection of suitable sample preparation conditions was studied only briefly. Two types of commercial SPME fibre were tested, 100 µm polydimethylsiloxane (PDMS) and 50/30 µm Stable Flex divinylbenzene-carboxen-polydimethylsiloxane (DVB-CAR-PDMS); the test sample was garlic powder. In the experiments the same sampling, desorption, and (one-dimensional) GC conditions were used for the two fibres. The temperature during sampling was kept constant at room temperature (25 °C) and the sampling time was varied between 1 and 30 min. Thermal desorption was performed by inserting the fibre for 1 min into the injection port of the gas chromatograph at 250 °C. The results showed that throughout the chromatograms, and irrespective of the experimental conditions used, peaks were higher for the DVB-CAR-PDMS fibre than for the PDMS, fibre. A considerable increase of extraction efficiency was observed – approximately 2- to 20-fold. One should note here that this increase becomes greater when analyte volatility increases. The better performance of the recently introduced carboxen-based fibre probably reflects the combination of an outer 50 µm layer of DVB blended with PDMS, which traps larger and somewhat less volatile analytes, while enabling the smaller, more polar analytes to pass through and be retained by the inner 30-µm layer of carboxen, also blended with PDMS [15, 16].

Comparison of Chromatographic Results

A typical HS SPME chromatogram obtained from garlic powder (0.5 g in a 4-mL SPME vial), separated by conventional 1D-GC, is shown in Figure 4A. For this analysis the comprehensive, i. e. GC ×

GC, equipment was used but with the modulation switched off, so all compounds passed through both columns. Because the separation on the second column is a mere 6–8 s compared with up to 50 min on the first column, the result is essentially the same as for a conventional 1D-GC analysis [17]. The peaks are approximately 10–15 s wide, and the responses are relatively low – from 0.5 to 50 pA. Close inspection reveals that under the conditions used co-elution occurs in many parts of the chromatogram.

In GC × GC, on the other hand, the cryo-modulation not only traps the eluate fractions but also effects re-focusing; with second-dimension peak widths of, typically, less than 200 ms. This zone compression results in considerably larger peaks. To demonstrate this, Figure 4B shows the result obtained when the same sample is subjected to GC × GC using an 8 s modulation period, viz. 4 s in the trapping (T) and 4 s in the release (R) positions (cf. Figure 1). This means that only two modulations will be taken for most first-dimension peaks. A rather long modulation time was preferred (i. e., some first-dimension separation was sacrificed) to achieve the maximum peak-height enhancement. As expected, the traces in Figures 4A and 4B closely resemble each other. In the latter, however, the peaks are at least 10–20 times higher (1–600 pA). The benefits of GC × GC are made especially clear by study of peaks which emerge just above the baseline in Figure 4A; a distinct response is obtained for these when they are modulated, i. e. in Figure 4B. The 30–34 min region of Figures 4A and 4B is a suitable example, especially if one notes the different *Y*-axis scales. In this instance, the signal-to-noise (*S*/*N*) ratios can be shown, by calculation, to be improved 25–30-fold on going from GC to GC × GC analysis. Examination of Figure 4B also clearly reveals that this type of presentation is not easily inspected and/or interpreted; this confirms our earlier observations made with regard to Figure 3, above. The almost invariably preferred contour plot of the same sample analysis (Fig. 4C) is a distinctly superior presentation. This is admirably illustrated by comparing the insert of Figure 4C with that of Figure 4B – whereas at best 5–7 peaks can be discerned in the latter insert, approximately 20 can easily be distinguished in the contour plot.

From the contour plot it is clear that, with the exception of a few compounds in the 3–5 min region, all peaks elute from the second column between 0 and 5.5 s. This implies that the 8-s modulation can be reduced to approximately 6 s in this analysis and that the number of modulations per first-dimension peak can be increased to enable better preservation of the integrity of the first-dimension separation. Further reduction of the modulation time cannot be achieved because peaks introduced during a specific modulation period, might then elute from the second-dimension column only during the next cycle and, consequently, adversely affect resolution at that stage. This partly, at least, explains the crowded character of the lower left-hand corner of the contour plot of Figure 4C (cf. below). Finally, it should be noted that identical GC conditions were used for 1D-GC and the first-dimension of the GC × GC separation; detection was by means of FID.

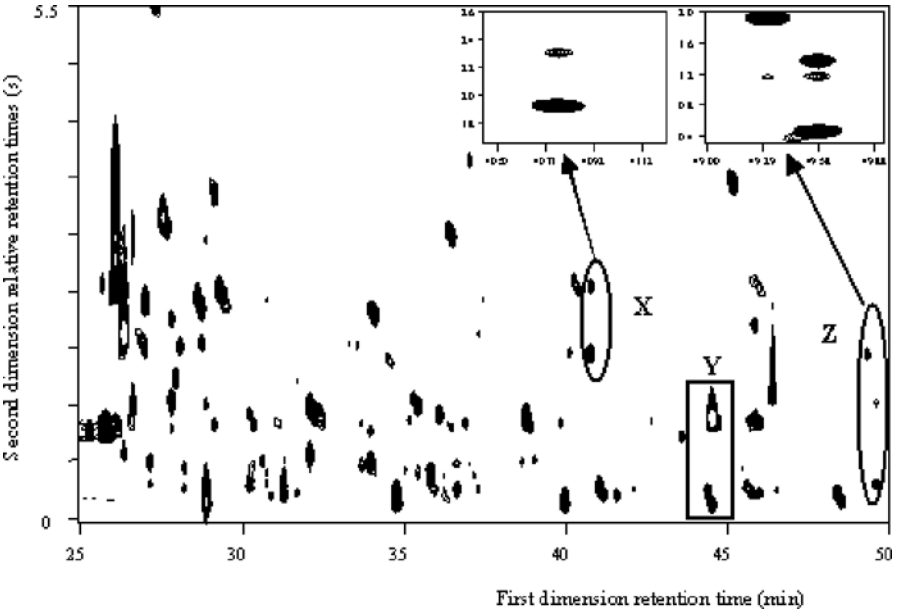

Figure 5. Part of the GC × GC data from Figure 4C presented as a contour plot. Regions marked **X** and **Z** are expanded in the inserts.

In Figure 4C, two distinct regions can be distinguished. On the left-hand side (< 25 min) many intense peaks are visible; the retention of these on the second column tends to increase when their retention on the first column increases. Many peaks in this region are rather broad in the second-dimension direction although some in-between peaks are well defined and substantially narrower. The second region (> 25 min) features fewer peaks with proper peak shapes and satisfactory distribution in the separation plane. This difference no doubt partly reflects the different nature of the compounds present in this complex sample – in terms of their volatility and polarity. Closer inspection of the unduly broad peaks revealed that the broadening is not caused by tailing or overloading of the system, because the peaks are symmetric in the *Y*-axis direction. Most probably, the broadening indicates that the pertinent compounds did not elute within the allotted modulation time but required one or more extra rounds – they are too strongly retained and, hence, elute during later modulation periods.

There are two ways of solving this problem. One is to control and properly adjust the temperature of the individual columns by using a dual-oven system. This will also enable further optimization of the second-dimension separation for particular first-dimension groups of peaks. The modulation time(s) should, of course, be adapted accordingly. The alternative is to select a second-dimension column which is less polar than BPX50 but has closely similar adsorption characteristics for the analytes of interest. The combined use of both options to achieve optimum analyte resolution will be the subject of forthcoming studies.

For further demonstration of the power of a GC × GC analysis under these experimental conditions it is best to turn our alteration to the right-hand side of the contour plot of Figure 4C. This is shown in more detail in Figure 5. Three different examples are indicated. The possibility of separating two co-eluting peaks is shown

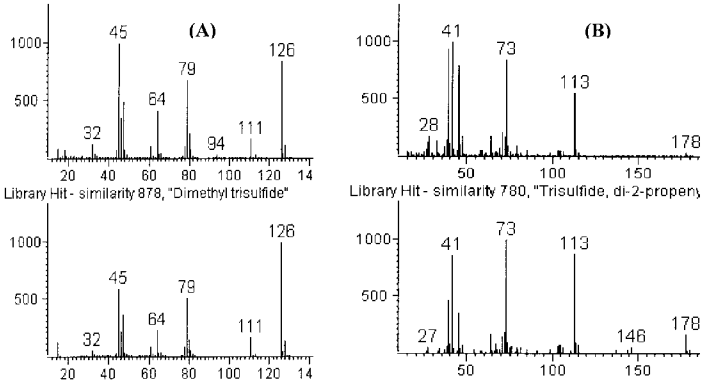

Figure 6. Mass spectra (**top**, experimental; **bottom**, library) of peaks 2 (**A**), and 7 (**B**). Peak assignment is given in Table I.

Table I. TOFMS-based identification of peaks 1–7 of Figure 4C.

Analyte	Peak no.	t_R (s)		Major ions*
		1st dimension	2nd dimension	
Disulphide, methyl 2-propenyl	1	325	0.8	**120**, 73, 41
Dimethyl trisulphide	2	397	1.2	**126**, 45, 79
Benzene, 1-methyl-3-(1-methylethyl)-	3	504	0.8	**119**, 91, 134
Diallyl disulphide	4	575	1.5	**41**, 146, 81
Trisulphide, methyl 2-propenyl	5	665	1.9	**41**, 73, 152
3-Vinyl-1,2-dithiocyclohex-4-ene	6	737	2.3	**111**, 144, 79
Trisulphide, di-2-propenyl	7	917	2.0	**73**, 41, 113

* The base peak is printed in bold.

for the region marked X – co-elution in the first-dimension is followed by baseline separation in the 5.5-s second-dimension separation, even through the retention time difference is a mere 0.35 s. Detecting the relatively weak second peak (the FID responses indicate a 100:15 mass ratio) no longer creates any problems. A seemingly similar example is presented in the region marked Y. Again, there was almost total co-elution on the first column, with R_S = 1.05, but almost excessive resolution R_S = 4.6 after the second separation. More importantly, however, three rather than two peaks are now seen to be present in the part of the chromatogram indicated. The extreme usefulness of such improved peak separation/recognition hardly needs emphasizing. The final example, the region marked Z, underscores this statement. What, initially, was a single peak, shows up as three separate peaks after the second-dimension run or even, upon careful focusing, as five easily distinguishable peaks with an additional sixth being partly resolved, Again, the clear recognition of very minor peaks is the most dramatic gain which can become a major tool in flavour analysis where, so far, odour perception and instrumental analysis frequently do not match (as discussed above). This is the more true because the combined examples demonstrate that good peak shapes are obtained, with peak widths of the order of 10–15 s in the first dimension and 100–150 ms in the second. Retention times are very repeatable, as other authors have reported – typical standard deviations of the second-dimension retention times are of the order of 0.1–0.2 s.

Analyte Identification

The detection of the minor sample constituents discussed in the previous section is a challenging task which can be solved at least partly by GC × GC with its inherently high sensitivity, because of peak refocusing during modulation, and its capability of resolving minor peaks during the second-dimension run. It should be noted that, for the examples presented in Figure 5, even mass spectrometric (MS) detection in 1D GC would not enable recognition of all peaks because:

(i) co-elution is too close; and
(ii) intensity differences between 'major' and 'minor' peaks are too large.

This also makes us aware that one pressing problem has not yet been solved – MS-based analyte identification compatible with the rapid second-dimension GC run. Although we shall not discus this topic in detail, it is appropriate to mention that the recent introduction of commercial time-of-flight (TOF) MS instruments is a major step forward – such instruments can acquire up to 500 mass spectra per second.

As an example data relating to seven major spots in Figure 4C are reported in Table I. With one exception (No. 3), the identified compounds were well-known sulphur-containing flavour components. As an illustration the experimental and library mass spectra of peaks 2 and 7 are shown in Figure 6. As stated above, the mass spectra of the two analytes were totally obscured by interferences in 1D-GC-MS. The high quality of the spectra obtained from GC × GC-TOFMS is clearly apparent. It should be noted that identification was achieved by treating the seven peaks of Figure 4C as unknowns. In such circumstances identification is performed by first calculating the total retention time, which is the sum of the retention times from the first- and second-dimension separations. Next, the peak in the raw chromatogram at this total retention time is identified on the basis of its mass spectrum. These results should suffice to demonstrate that, next to the separation and detectability problems referred to above, the analyte identification problem of the new technique can also be solved satisfactorily.

Conclusions

SPME in headspace mode combined with GC × GC is a convenient and straightforward means of significantly enhancing the separating power and enabling the analyte recognition urgently required in the flavour analysis of food, exemplified by garlic powder in this study. The main benefits are:

(i) improved resolution for the entire sample compared with both 1D-GC and heart-cut MDGC;
(ii) improved analyte detectability as a result of efficient refocusing during cryo-modulation;
(iii) improved peak capacity as a result of the baseline-resolution of many more peaks; and
(iv) the briefly explored combination with a TOFMS as a third dimension to this 2D separation system opens the way to the use of sophisticated identification procedures.

These advantages are, of course, essentially the same as claimed earlier for petrochemical analysis and the determination of chlorinated micro-contaminants. This strongly suggests that SPME-GC × GC-TOFMS might well become a powerful, and user-friendly, tool for further unravelling of the often incompletely known aroma-active compound profiles of a wide range of foods. This is the current research theme of our group.

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