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Application of comprehensive two-dimensional gas chromatography to the quantification of overlapping faecal sterols

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Abstract

Standard solutions containing a mixture of seven sterols and 5α-cholestane as internal standard, and sample mixtures that comprised varying ratios of sterol and stanols from green lip mussel tissue and dried cow faeces were analysed by using comprehensive two-dimensional gas chromatography (GC × GC). Quantitative results were compared with single-column GC analysis. The latter samples included sterols of interest, but which cannot be readily obtained elsewhere. It may also mimic potential environmental samples where dairy production and aquaculture (oyster, mussel cultivation) share the same catchment; environmental sterol signatures may exhibit characteristics of both sample types comprising this mixture. Whereas single-column GC–flame ionisation detection was unable to reliably quantitate target sterols, the GC × GC experiment permitted small amounts of sterols and stanols to be detected and separated. Likewise GC–MS analysis was unable to detect some of the minor sterols which coeluted on a single column. The GC × GC mode allows complete separation of several important sterols and stanols, such as 24-ethylcoprostanol, campesterol and 24-methylenecholesterol, demonstrating the enhanced resolving power of the GC × GC system. Separation of 24-ethyl-epi-coprostanol from several algal-derived interfering components was achieved, leading to higher degree of confidence in the quantitative analysis of faecal sterols. The effects of a number of operating variables—column length, carrier flow-rate and elution temperature—on component resolution and presentation of data in the two-column analysis are described.

Keyword: Sterols

1. Introduction

Nutrient enrichment of waters is partly due to animal wastes. For the past 100 years researchers have used microorganisms such as *Escherichia coli* and *Streptococci* to determine the faecal contamination in water systems. These methods have proven to be limited in their applications. In the last 25 years some analysts have adopted faecal sterols, as chemical biomarkers, to determine the sources of faecal contamination contributed either by humans or animals [1]. Much work has concentrated on coprostanol (5β-cholestan-3β-ol) because it is one of the major sterols present in human faeces [2]. It is formed by bacterial reduction of cholesterol in the intestines of higher animals such as humans. It is not

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naturally present in aerobic or unpolluted waters [3]. 5β-Stanols such as coprostanol do not occur naturally in fresh or marine water or in fully oxic sediments because only anaerobic bacteria appear capable of hydrogenating Δ^5-sterols to 5β-stanols. However, under conditions of anoxia, Nishimura [4] found relatively small amounts of 5β-stanols in sediments (and mobilised sediments) not contaminated by faecal pollution. In animals such as humans, pigs, cats, cows, sheep, rodents, etc., the presence of certain 5β-stanols and sterols can be used to determine the source of faecal pollution; the ratio of 5β-stanols relative to each other and their sterol precursors varies between species [1–7]. For example, coprostanol is not found in invertebrates and birds, other than chickens where the stanol/sterol ratio is diagnostic, while in herbivores such as cows, 24-ethylcoprostanol is most prevalent. Hence, faecal sterol profiles, if not the individual sterols, are source-specific [5–8]. In a recent study by Elhmmali et al., faecal sterols and stanols were employed, together with bile acids, as tracers to assess sewage discharges and other faecal sources along the Avon River in Bristol, UK [9].

Faecal sterols are normally analysed by either gas chromatography–electron-capture detection (GC–ECD), GC–flame ionisation detection (FID) or GC–mass spectrometry (MS), after they have been extracted and derivatised. Leeming et al. [10], using GC–MS, noted co-eluting sterols originating from a variety of plants and/or animal sources when trying to trace faecal contamination in receiving waters. However GC–MS, while being able to determine the presence of co-eluting contaminants, cannot always accurately quantify their individual amounts. GC–ECD is a sensitive detection method for the trace faecal sterols in water samples [11], but it suffers from being sensitive to interferences arising from the derivatising agent. GC–FID suffers from the presence of co-eluting (interfering) naturally occurring compounds and it may be insufficiently sensitive for trace level solutes. The presence of unresolved components compromises the accurate measurement of these components in single-column GC. Increased resolving power is desirable, and single capillary column analysis is unlikely to offer the required separation of closely eluting compounds.

Multidimensional gas chromatography (MDGC) techniques provide increased resolution for limited regions of a chromatographic analysis, using two separate conventional capillary columns. MDGC finds particular application in essential oil analysis and especially for chiral separation, due to the complex nature of the materials, and the need for highly efficient separation for specific analysis goals. Gordon et al. used a dual oven system for tobacco essential oils [12] and Mondello et al. [13] characterised enantiomeric monoterpene distributions in lemon oil using a chiral second column in MDGC. Krock and Wilkins [14] employed multiple parallel cryogenic traps to separate closely eluting heart-cuts of petroleum samples, and MDGC has been used for polychlorinated biphenyl (PCB) congener analysis [15–17]. For sterol analysis, there are few reports on dual-column GC. Gerhardt et al. [18] used two 4 m × 2 mm I.D. double-looped U-shaped glass columns packed with 1% SP-1000 (an acid modified Carbowax phase) to separate derivatised and underivatised faecal neutral steroids in a faecal slurry. It appears that this application has not been revisited with higher resolution capillary columns in MDGC.

Bertsch [19] recently reviewed MDGC technology and applications. Comprehensive two-dimensional gas chromatography (GC × GC) offers a significant extension to MDGC, using two directly connected columns arranged in series in the GC with a "modulator" located near the column junction. Fig. 1 shows this arrangement, employing the longitudinally modulated cryogenic system (LMCS; described elsewhere [20]) which was used in the present study. The second column provides "fast GC" analysis (short length, narrow I.D., and thin phase film so that peaks elute within a few s). A cryotrap acts as both a collection zone and fast reinjection device, resulting in a series of sharp pulses eluting from the second column for each peak entering the cryomodulator from the first column. Two columns of different phase provide multidimensional separation. Higher sensitivity and greater resolution are now well-recognised properties of the GC × GC method [21].

Beens et al. [22] and Frysinger et al. [23] have shown that GC × GC analysis gives improved quantitative results for selected compounds because better resolution leads to increased certainty of analysis, supported by studies on complex samples such as petroleum [24], pyrolysates [25] and essential oils [26,27]. Lewis et al. [28] reported that true detector

baseline in GC × GC provides improved quantitative analysis of atmospheric organic constituents. In our previous study [21] it was found that the "selective mode" of the LMCS [29] was applicable to sterol compounds in a standard mixture comprising resolved compounds. However, it was not useful when compounds with similar polarity overlapped, as separation could not be achieved on the second column. For complex samples, the comprehensive mode provides better analysis. The objective of this present study was to further develop the GC × GC method by applying it to samples of environmental interest. A mixed sterol sample derived from mussel and cow was chosen because it offers a complex sterol suite with the mussel sterol located within the cow-derived sterols.

2. Experimental

2.1. Reagents

Dichloromethane was AR reagent grade (Merck). Seven standards representative of faecal sterols were used. 5β-Cholestan-3β-ol (coprostanol, COP) was obtained from Matreya (Pleasant Gap, USA). 5β-Cholestan-3α-ol (epicoprostanol, EPI), 5-cholesten-3β-ol (cholesterol, CHL), 3β-hydroxy-5α-cholestane (dihydrocholesterol, DHL), 24β-ethylcholesterol (β-sitosterol, B-SIT), 3β-hydroxy-24-ethyl-5,22-cholestadiene (stigmasterol, SROL) and 24α-ethyl-5α-cholestan-3β-ol (stigmastanol, SNOL: the 5α-stanol of β-sitosterol) were from Sigma–Aldrich (Castle Hill, Australia). The internal standard (I.S.) was 5α-cholestane (Sigma–Aldrich). The derivatising agent used was bis(trimethylsilyl)trifluoroacetamide (BSTFA) with 1% trimethylchlorosilane (TMCS) (Alltech, Baulkham Hills, Australia).

2.2. Instrumental conditions

Fig. 1 illustrates a schematic diagram of the GC × GC setup incorporating the LMCS (Chromatography Concepts, Doncaster, Australia) with liquid CO_2 cryogen. The LMCS unit was retrofitted to an Agilent Technologies Model 6890 gas chromatograph (Little Falls, DE, USA) with an FID system operated at 5 Hz under normal mode and at 50 Hz when GC × GC is used. General details of operation are described elsewhere [20]. The split/splitless injector was operated in splitless mode. The cryogenic modulator operation is commenced by the 6890 GC events table, using a controller to select the modulation period. A 4-s modulation cycle time for GC × GC was used.

The column ensemble comprising a low polarity first column (column 1) and a medium polar second

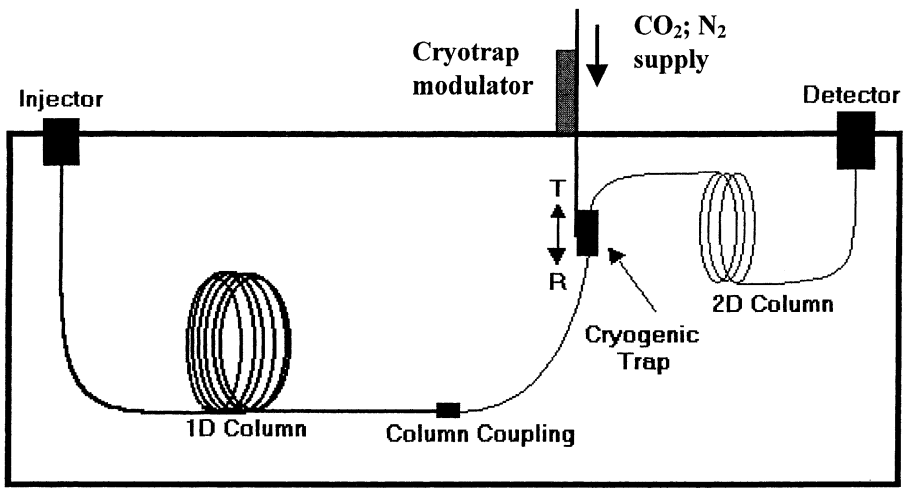

Fig. 1. Schematic diagram showing the GC × GC instrument design with a moving cryogenic trap located between the columns. For normal capillary GC operation, the modulator operation is not applied. Columns (dimensions) 1 and 2 constitute normal and fast elution columns, respectively.

column (column 2) coupled in tandem. Column 1 was a 30 m BPX5 (5% phenyl polysilphenylene) column, of 0.25 mm I.D. and 0.25 μm film thickness (d_f) and column 2 was a 2 m BPX50 (50% phenyl polysilphenylene) column, of 0.1 mm I.D. and 0.1 μm d_f (both columns from SGE International, Ringwood, Australia). GC operating conditions were, unless otherwise stated: oven temperature programmed from 50 °C (1 min hold) to 280 °C ramped at 30 °C/min, then 2 °C/min to 320 °C (40 min hold) giving 68.67 min total run time. Pulsed splitless (1 min) injections were made at a hydrogen carrier pressure of 4.0 p.s.i. and a pulse splitless pressure of 60 p.s.i. (1 p.s.i.=6894.76 Pa). The injector temperature was 320 °C and the flame ionisation detector temperature was 340 °C. Injections of 1 μl were made.

2.3. Standards and mixture preparation

The sterol standard solution concentration used in this study was 2.0 μg/ml of each of the seven sterols and 5.0 μg/ml of 5α-cholestane internal standard made up in dichloromethane. Sterol standard solutions and the internal standard were added into GC vials and evaporated to dryness with nitrogen. Derivatisation agent (BSTFA–1% TMCS, 200 μl) was quantitatively added to each vial and heated in a thermal block at 60 °C for 60 min. To each vial, 800 μl of dichloromethane was then quantitatively added, giving a final volume of 1000 μl.

2.3.1. Green lip mussel and cow sample mixtures

Three "test mixtures" comprising varying sterol ratios prepared from two different sterol sources were obtained from the CSIRO Division of Marine Research, Hobart, Australia. These samples were green lip mussel tissue and dried cow faeces, which were mixed together in ratios of 25:75, 50:50 and 75:25, respectively. The original cow faecal sample was made from 103 mg dry mass of cow faeces which had 22.0 μg of 5β(H)-cholan-24-ol internal standard added to it during solvent extraction; it was then combined with green lip mussel in the ratios listed above. Sample extracts were evaporated to dryness with nitrogen prior to the addition of 100 μl of BSTFA–1% TMCS, then heated for 60 min at 60 °C, then made up to 500 μl with dichloromethane. These two samples were of interest since they contain sterols, which co-elute in single column GC analysis. This would provide a test of the separation capabilities of the GC × GC system and should allow more accurate quantification.

2.4. GC–MS analysis

A Fisons MD800 benchtop quadrupole GC–MS system was used for identification of the sterols and stanols from the green lip mussel and cow samples. Scan acquisition from *m/z* 50–550 was applied. Agilent Technologies HP1 and HP5 non-polar capillary columns (50 m × 0.32 mm I.D., 0.17 μm d_f, cross-linked 100% methyl silicone or 5% phenyl–methyl silicone fused-silica columns) were interchanged depending on the constituents to be analysed. GC–MS analysis conditions were: He carrier gas, with head pressure of 70 kPa, splitless injection with injector temperature 290 °C. A temperature program of 50 °C for 1 min to 200 °C with a ramp rate of 25 °C/min, then 0.8 °C/min to 260 °C then to 310 °C at 30 °C/min hold for 10 min. Injections of 1 μl were used. Neither of the columns could separate all the components under the conditions listed; GC–MS results will not be further specifically discussed here.

2.5. Data processing

Quantitation was undertaken using Excel spreadsheet programming with macro instructions to collect together the individual peaks of a particular component, and report the total area, height and average retention of each compound. Operationally, GC × GC data were first integrated (note that selection of integration parameters may be non-trivial to ensure all peak pulses, especially small peaks, are properly recorded), then the file exported by Chemstation and read into the excel program. Peak pulses of a specific component are identified by defining a retention time difference threshold based on the modulation period, and for 4-s modulation this was set to between 0.065 and 0.068 min. This groups together those pulses that differ by this time difference, and so should be the same component. These can then be extracted from the total data set. Of course, this procedure will not be suitable where compounds are not resolved in the second dimension, although incorporation of consideration of the gaussian peak shape is possible to

ensure that the grouping of peak pulses is reasonable. Manual checking of data is needed to confirm that reliable area measurement is performed.

For single dimension and two-dimensional contour plot presentation, Chemstation (Agilent Technologies) acquired data were converted to ASCII format then imported into the Origin (Microcal, Northampton, USA) graphics program, or the Transform (Fortner Research, Boulder, CO, USA) program, respectively.

3. Results and discussion

3.1. Separation of four overlapping sterol compounds from green lip mussel and cow faecal mixtures

Fig. 2A–C show chromatograms of samples with the ratios of 25:75, 50:50 and 75:25 mussel:cow for the normal GC mode using the dual column set, but without the CO_2 provided to the trap. Results illustrate that for all three samples analysed, there was considerable overlap between the peaks for 24-ethylcoprostanol, 24-methylenecholesterol and 24-ethyl-epicopsrostanol. The presence of 24-methylenecholesterol (peak 2), a sterol predominant in green lip mussel, was not evident as a separately defined peak in the 25:75 sample, although it was known to be present. In the 50:50 and 75:25 samples it was clearly evident. In general, the normal GC mode gave no baseline resolved peaks, and barely five peak maxima could be recognised in the region of interest. The tallest peak for all three samples had a response between 20 and 30 pA.

Fig. 3A–C show partial chromatograms for 25:75, 50:50 and 75:25 mussel:cow samples using the cryogenic pulsing GC × GC mode which are approximately the same regions as shown on the expanded inset diagrams in Fig. 2A–C. Fig. 4 is an expanded presentation of Fig. 3B (50:50 sample) and the individual pulsed peak envelopes for each individual compound can be seen more clearly, shown approximately by the lines drawn on the trace. Peak envelopes may be deduced based on the relationship between successive pulses of a compound (they should differ by the modulation period), and the expected peak profile trend mapping out an approximate Gaussian distribution. This suggests that at least nine constituents can be successfully resolved and/or identified when the comprehensive mode was adopted. These nine compounds were found amongst the peaks numbered 1 to 5 on the poorly resolved normal chromatograms of Fig. 2. All except the 24-methylenecholesterol component are from the cow faecal sample. Also highlighted is campesterol which could not be seen in the normal mode, but was detected between 24-methylenecholesterol and 24-ethyl-epi-coprostanol when comprehensive GC was employed. The presence of 24-methylenecholesterol was always apparent in all three samples, and the tallest peak using the comprehensive mode gave a response between 140 and 220 pA. Note that a true detector baseline is obtained for all comprehensive chromatograms, since between resolved peak pulses the detector returns to its baseline response. Data for each component given by the data system for normal chromatograms shown in Fig. 2, in the expanded region of interest, are tabulated in Table 1. Note that none of these components are resolved, so “quantitative data” are derived from protocols inherent in the data system such as dropping vertical lines at peak valleys or skimming routines which allocate nominal areas to the overlapping peaks. Clearly such data should be considered semi-quantitative at best.

Unlike normal GC, where peak co-elution makes quantification difficult, and most likely inaccurate, this study demonstrated that GC × GC allowed quantitative determination of all nine compounds found in the region of interest present in the environmental samples. Table 2 presents the percentages of peak areas reported for each of the peaks, calculated by summing the total pulsed peak areas of each peak and compares them with the total peak area for the four peaks of interest in this region of the chromatogram (peaks identified as 1, 3, 4 and 5). The area percentages of 24-methylenecholesterol, 24-ethylcoprostanol, and 24-ethyl-epi-coprostanol were consistent with the sample ratios 25:75, 50:50 and 75:25, given that 24-methylenecholesterol was only present in the mussel sample. Comparison of Tables 1 and 2 illustrates the value of quantification by using GC × GC. With normal GC, 24-methylenecholesterol cannot be estimated in the 25:75 sample since no separate peak is seen; most of its area, along with that of the underlying campesterol component, is allocated to the peak for 24-ethyl-epi-coprostanol. Likewise, in the 50:50 sample the 18% area given to 24-methylenecholesterol underestimates its true area,

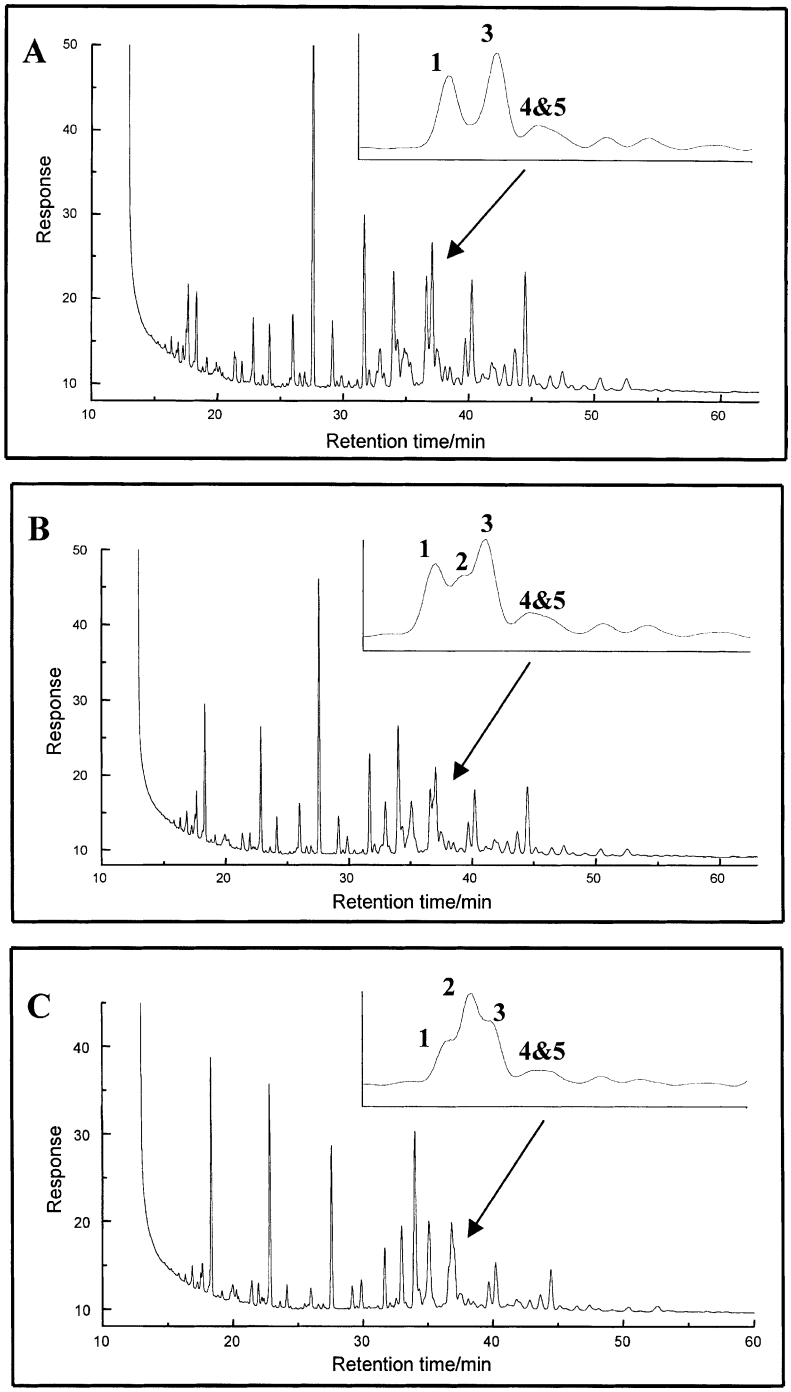

Fig. 2. Normal GC traces of sterol components in green lip mussel and cow samples. During analysis the cryofluid was not supplied to the cryotrap. (A) Green lip mussel/cow sample ratio of 25:75. (B) 50:50 sample. (C) 75:25 sample. Insets show expanded regions of interest: 1 = 24-ethylcoprostanol; 2 = 24-methylenecholesterol; 3 = 24-ethyl-epi-coprostanol; 4 and 5 = unknowns. For 25:75 sample, peak 2 (24-methylenecholesterol, a green lip mussel sterol) was not evident as a separate peak.

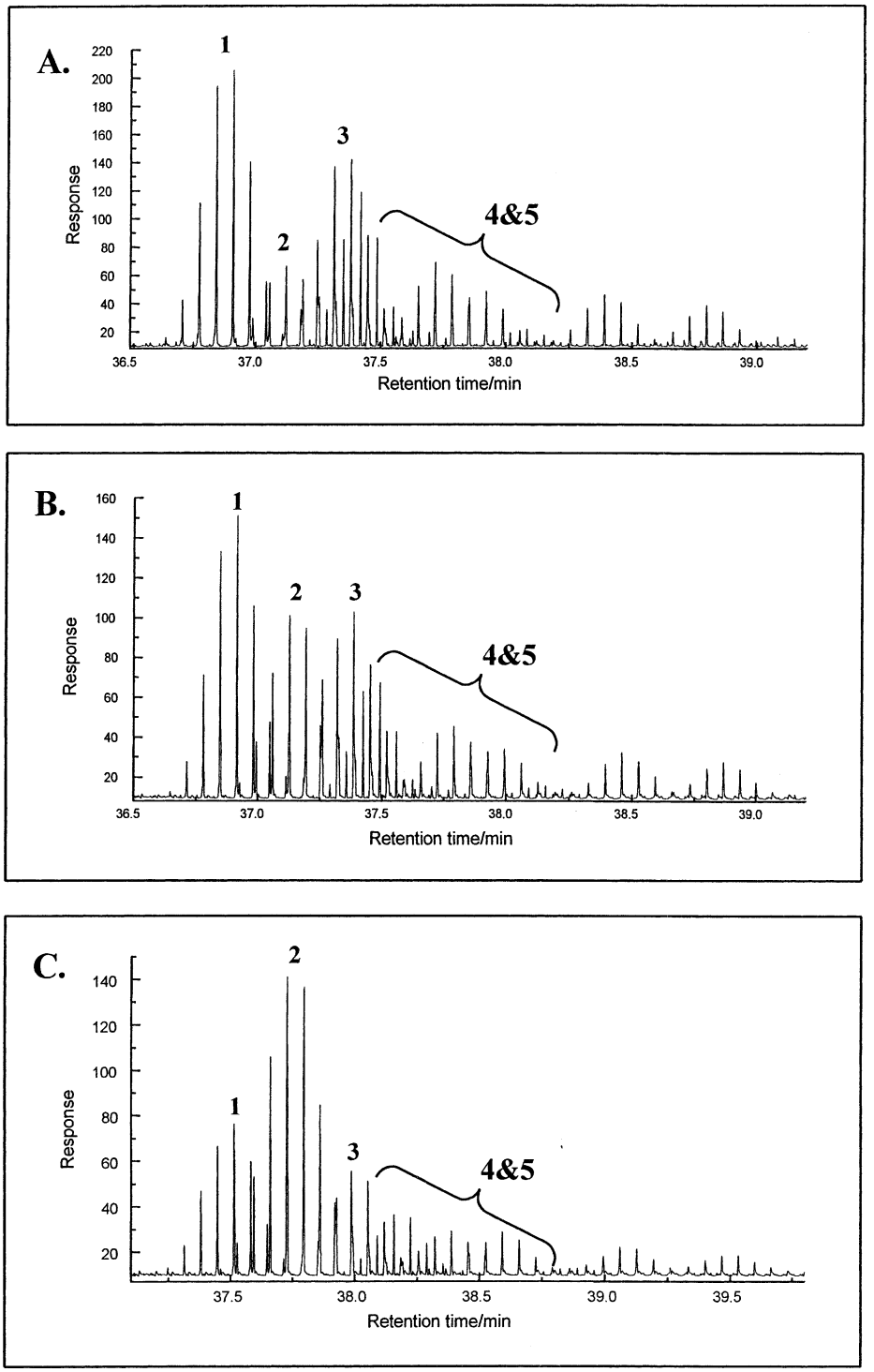

Fig. 3. Cryogenic pulsed GC traces of sterol components in green lip mussel and cow samples, of the same regions shown in insets in Fig. 2A–C. (A) 25:75 sample. (B) 50:50 sample. (C) 75:25 sample.

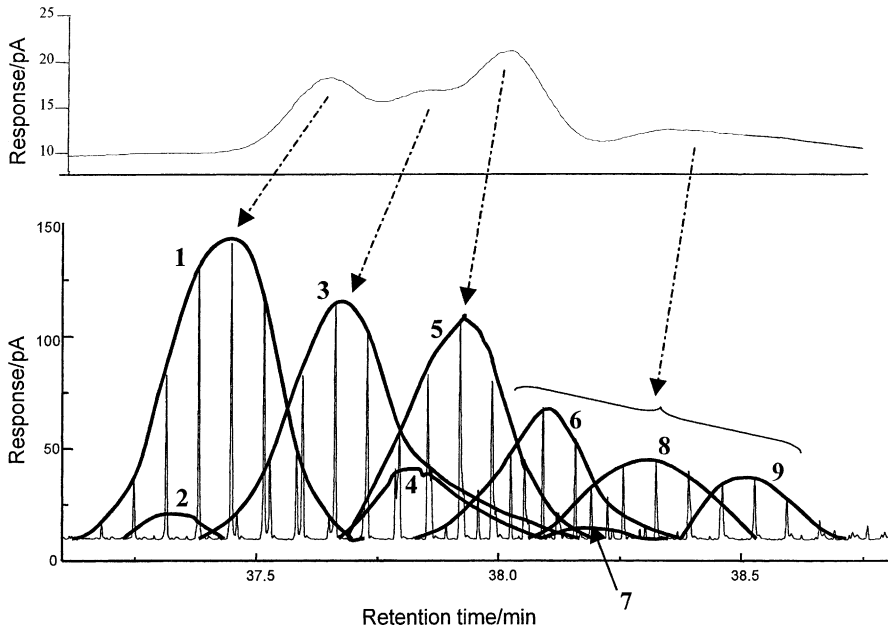

Fig. 4. Expanded diagram of Fig. 3B (50:50 sample) illustrating the pulsed peak distribution in the sterol sample mixture. There are nine recognisable compounds present in the pulsed cryogenic chromatogram of this sample shown approximately by outline envelopes. Relative peak areas were calculated to be: 1: 35.0%; 2: 1.1%; 3: 24.8%; 4: 24.1%; 5: 6.8%; 6: 12.1%; 7: 1.5%; 8: 11.3%; 9: 8.1%. Note that peak number assignment is not the same as used in Figs. 2 and 3. Here, peaks 1, 3 and 5 correspond to peaks 1, 2 and 3 in Figs. 2 and 3.

and 24-ethyl-epi-coprostanol is about 100% larger than GC × GC data suggests it should be. Only in the 75:25 sample can 24-methylenecholesterol be adequately measured when normal GC is used, however both 24-ethylcoprostanol and 24-ethyl-epi-coprostanol components are in error. Note that in all cases it is possible to observe and estimate the campesterol peak in GC × GC, but it is impossible to see the peak for this component in normal GC analysis.

Results obtained show the trends expected, that is, the proportions of the three compounds varied accordingly with the ratios of these constituents present in each sample. Fig. 5 presents the trend in variation in the three target compounds in the three samples. The ratio of 24-methylenecholesterol, which is only present in the green lip mussel, increases linearly from the 25:75 to 75:25 ratio samples, and is almost exactly three times larger in the 75:25 sample than in the

Table 1
Areas reported for sterols of interest by using normal GC

Peak	25:75			50:50			75:50		
	t_R (min)	Area	Area %	t_R (min)	Area	Area %	t_R (min)	Area	Area %
1	36.66	195 000	44	36.65	110 100	33	36.66	47 400	19
2	NA	NA	NA	36.89	59 300	18	36.86	137 800	55
3	37.11	250 300	56	37.09	159 800	49	37.00	66 900	27
4	37.48	98 500		37.47	60 300		37.73	27 500	
5	38.13	31 000		38.12	20 100		38.12	9900	
Σ1–3		445 300			329 100			252 100	

See Fig. 2. Peaks: 1=24-ethylcoprostanol, 2 = 24-methylenecholesterol, 3 = 24-ethyl-epi-coprostanol, 4, 5 = unknowns. NA—for 25:75 sample, 24-methylenecholesterol was not detected as an identifiable peak. Area % is given based on total of peaks 1, 2 and 3.

Table 2
Areas reported for sterols of interest by using cryogenic pulsed comprehensive GC

Peak	25:75		50:50		75:50	
	Average t_R (min)	Area %	Average t_R (min)	Area %	Average t_R (min)	Area %
1	36.85 (0.14)	44	37.48 (0.13)	35	37.49 (0.05)	23
2	36.77 (0.18)		37.53 (0.32)		37.51 (0.16)	
3	37.17 (0.05)	17	37.76 (0.004)	33	37.74 (0.27)	55
4	37.24 (0.27)	8	37.93 (0.004)	7	37.98 (0.14)	7
5	37.29 (0.14)	31	37.99 (0.004)	24	37.99 (0.001)	15
6	37.36 (0.14)		38.18 (0.19)		38.14 (0.10)	
7	37.61 (0.14)		38.35 (0.31)		38.31 (0.05)	
8	37.70 (0.14)		38.39 (0.25)		38.38 (0.05)	
9	37.84 (0.10)		38.58 (0.19)		38.56 (0.09)	

See Fig. 3. Peaks: 1 = 24-ethylcoprostanol; 3 = 24-methylenecholesterol; 4 = campesterol; 5 = 24-ethyl-epi-coprostanol; 2, 6–9 = unknowns. (Note: peaks 1 and 2 may exchange positions due to the method of retention assignment for their peak pulse profile). Area % values are calculated on the basis of the four identified components. Values in parentheses for t_R are RSD values based on three replicates.

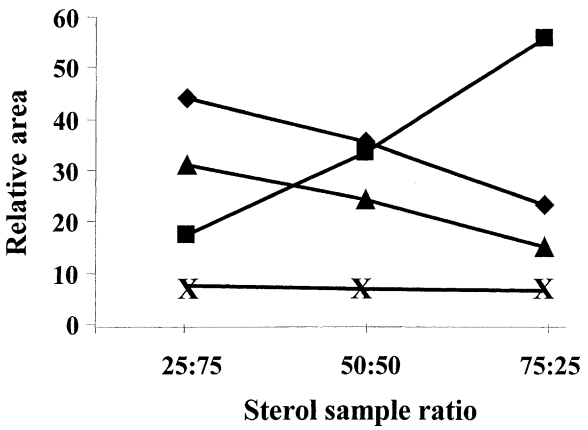

Fig. 5. Variation in relative response areas for the four major sterols in the three sample mixtures. 24-Ethylcoprostanol (◆), 24-methylenecholesterol (■), campesterol (×), and 24-ethyl-epi-coprostanol (▲).

25:75 sample as required (i.e., a ratio of 1:2:3 in the three mixed samples). The other target compounds decrease monotonically in the three samples from 25:75, to 50:50 to 75:25, since they occur predominantly in the cow samples.

GC × GC data are normally presented in two-dimensional figures, either as contour or surface plots. Two-dimensional plots, given for selected contour response heights, showed that six instead of nine peaks could be seen in the region of the three interested compounds. Since contour plots are based on peak heights, peaks with responses less than the lowest threshold chosen for the contour plot or co-eluting with other compounds will not be seen on these plots. However, of the six peaks observed in the two-dimensional plot in Fig. 6, the campesterol peak is clearly nestled between 24-methylenecholesterol and 24-ethyl-epi-coprostanol. It is not surprising therefore that normal GC will not report the presence of this component unless GC–MS is used, and only provided it allows a unique ion for campesterol to be used for analysis and reporting purposes.

GC × GC results demonstrate that the complexity of analysing faecal sterols in environmental samples is much greater than suspected from single-column GC analysis.

The high temperature application of sterol analysis generally means that the target solutes elute after the upper temperature of the programmed temperature analysis is reached. Thus the compounds elute into the modulation zone and then onto the second column under isothermal conditions. The later eluting compounds therefore have increasingly wider ^{1}D peaks, and comprise more ^{2}D pulsed peaks, than the earlier solutes. The later peaks also will be increasingly retained on the second column since their retentions are largely controlled by their reduced volatility. Thus in the two-dimensional plot of retention space (Fig. 6) the sterol elution trend defines a strongly increasing ^2t_R retention zone, rather than a zone expected to be

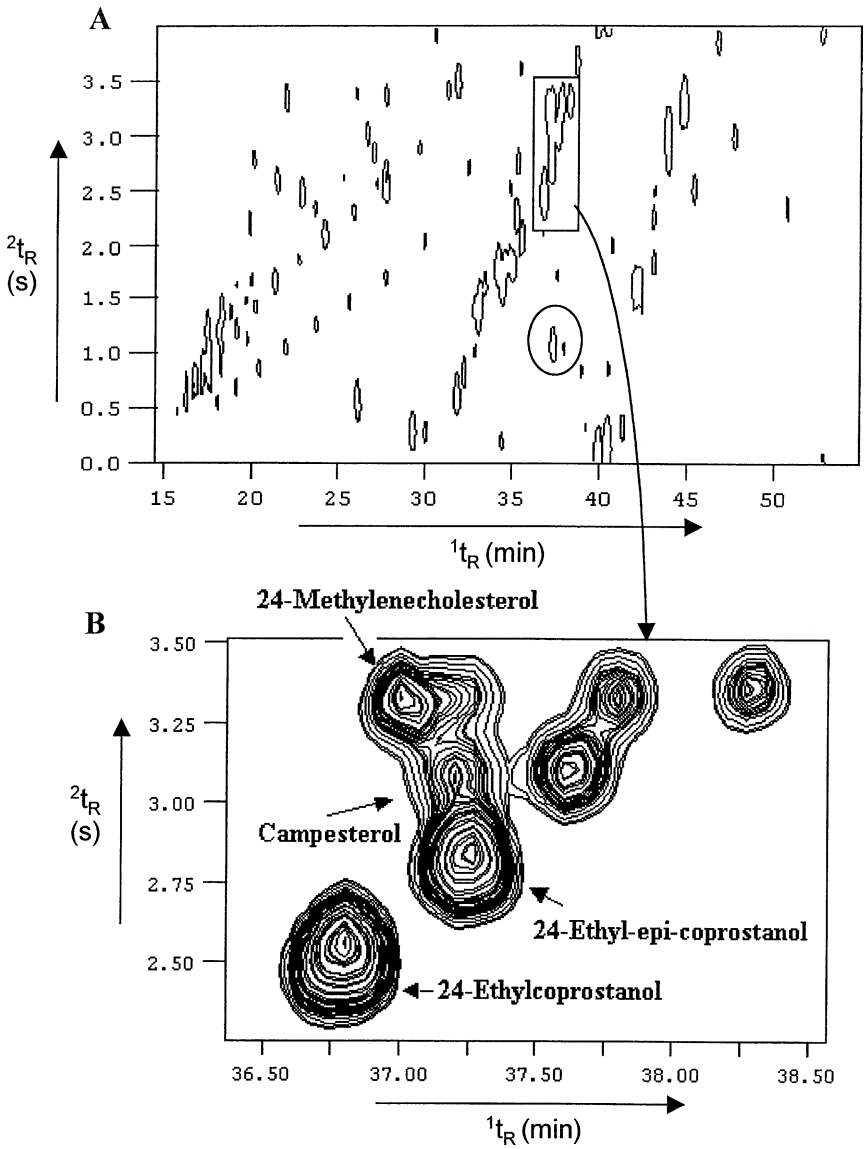

Fig. 6. (A) Full two-dimensional separation space presentation for the sterol sample using the comprehensive GC × GC data from Fig. 3A. (B) Expansion of the two-dimensional separation space region of interest from (A). In the region of 24-ethylcoprostanol, 24-methylenecholesterol and 24-ethyl-epi-coprostanol, seven instead of the anticipated nine compounds can be seen due to the contour levels used to generate the plot. Campesterol is located between 24-methylenecholesterol and 24-ethyl-epi-coprostanol; the other three peaks are unknowns. The larger peak circled in (A) is the peak that tends to "swap" positions with the target sterol, 24-ethylcoprostanol in the first dimension as these two compounds essentially have equivalent ^1t_R times. However, their ^2t_R times are significantly different.

located at relatively constant ^2t_R time if they were to elute under constant temperature programmed condition. Both ^1t_R and ^2t_R are defined as the retention time on the ^{1}D and ^{2}D columns, respectively. Additionally in the two-dimensional plots shown here, the later eluting compound has a much broader contour plot in both the first and second dimensions than is seen for the early compounds, again as a consequence of the

isothermal elution condition. For example, two broad peaks in ^{2}D are seen at ∼45 min in Fig. 6A.

3.1.1. Effect of experimental parameters

Ong et al. [30] and Dallüge et al. [31] reported correlation of GC × GC two-dimensional plots with various experimental conditions. The effects of some parameters were also studied here.

^{2}D columns of length 86 cm and 2 m were compared. There are three points to note. First, at a constant carrier pressure the longer second column results in a lower flow-rate and therefore a longer retention time, with t_R values increasing by about 10 min for the longer column. Second, pulsed peaks are narrower on the shorter ^{2}D column due to both greater carrier flow and shorter residence time on the column. Consequently they have greater heights. The peaks enter the cryogenic modulator with broader widths when the longer second column is used, and so they also give more pulsed peaks. Third, significantly better resolution is obtained on the longer ^{2}D column as evidenced for the resolution of 24-ethylcoprostanol and the unknown peak (not shown here).

Table 3 reports data on retention, area and heights of peaks when carrier pressure is varied. Peak areas generally were very comparable but with slight increases as pressure increased, presumably due to injection sampling variation, while summed peak heights dramatically increased. Solutes have shorter retention times at higher carrier pressures, and since they elute earlier, their peak widths will be narrower; increased mass flux results in an increase in the amount of zone-compressed solute pulsed to the second column. Shorter retention on the second column also reduces peak broadening. Both of these factors lead to the height increases recorded. Two-dimensional separation plots for the 2.0 μg/ml standard showed that "wrap-around" occurred (where solute retention on ^{2}D is longer than the pulse period used). With a lower the flow-rate more wrap-around occurs on ^{2}D. The chromatograms in Fig. 7 illustrate different separation performance of compounds for varied flow-rates. Later-eluting peaks give more pulsed peaks, and in the GC × GC plots (not shown here) give elongated and broader contours than the earlier-eluting peaks. These sterol compounds eluted after isothermal conditions were established, accounting for increasingly broad peaks in the first dimension, and increased retention on ^{2}D. Interestingly, the apparent resolution of the pairs of peaks shown in expanded insets on Fig. 7 appear to increase as the carrier flow is increased. This may seem to be a contradiction to normal expectations where efficiency and hence resolution will decrease as flow velocity increases. For the standards, a higher flow-rate leads to better resolution and better analytical sensitivity.

Table 3
Compilation of data for different carrier flow-rates, using a 2 m ^{2}D column and 2.0 μg/ml of sterol standard solution with 5.0 μg/ml of 5α-cholestane internal standard (refer to Fig. 7)

	Carrier pressure				
	3.0	3.5	4.0	5.0	7.3
t_R (min)					
I.S.	33.42	30.93	29.04	26.42	22.47
COP	37.03	34.30	32.15	29.09	25.10
SROL	44.63	41.09	38.53	34.53	29.65
Peak area (pA × s)					
I.S.	160	161	174	178	179
COP	100	99	116	120	120
SROL	46.9	46.8	56.9	57.6	61.2
Peak height (pA)					
I.S.	639	763	954	1130	1496
COP	425	462	661	805	1011
SROL	134	177	250	309	405

Note: peak height is total peak height of all pulses; peak area is the summed peak area of all peak pulses for the indicated compound; t_R is given as the time to the maximum peak pulse.

At higher isothermal temperature, retention times of eluting compounds will decrease and there are qualitative differences in the solute resolution. Peak heights increased while peak areas effectively remained unchanged as final temperature increased. For the sterol standard, GC × GC results correlated well with the normal GC results for peak areas, with peak heights in the comprehensive mode again showing much greater sensitivity over the normal GC mode. With increasing temperature, fewer pulsed peaks are observed. For example, for cholesterol, 300 °C gave 11 pulses per compound, 305 °C 10 pulses, and 320 °C six pulses. The later-eluting sterols gave more pulsed peaks—stigmastanol had 15 pulses whilst 5α-cholestane had 10 pulses at 300 °C. Analysis of the green lip mussel and cow sample mixtures gave good correlations with elution of the standards in the two-dimensional contour plots for the sterol standards

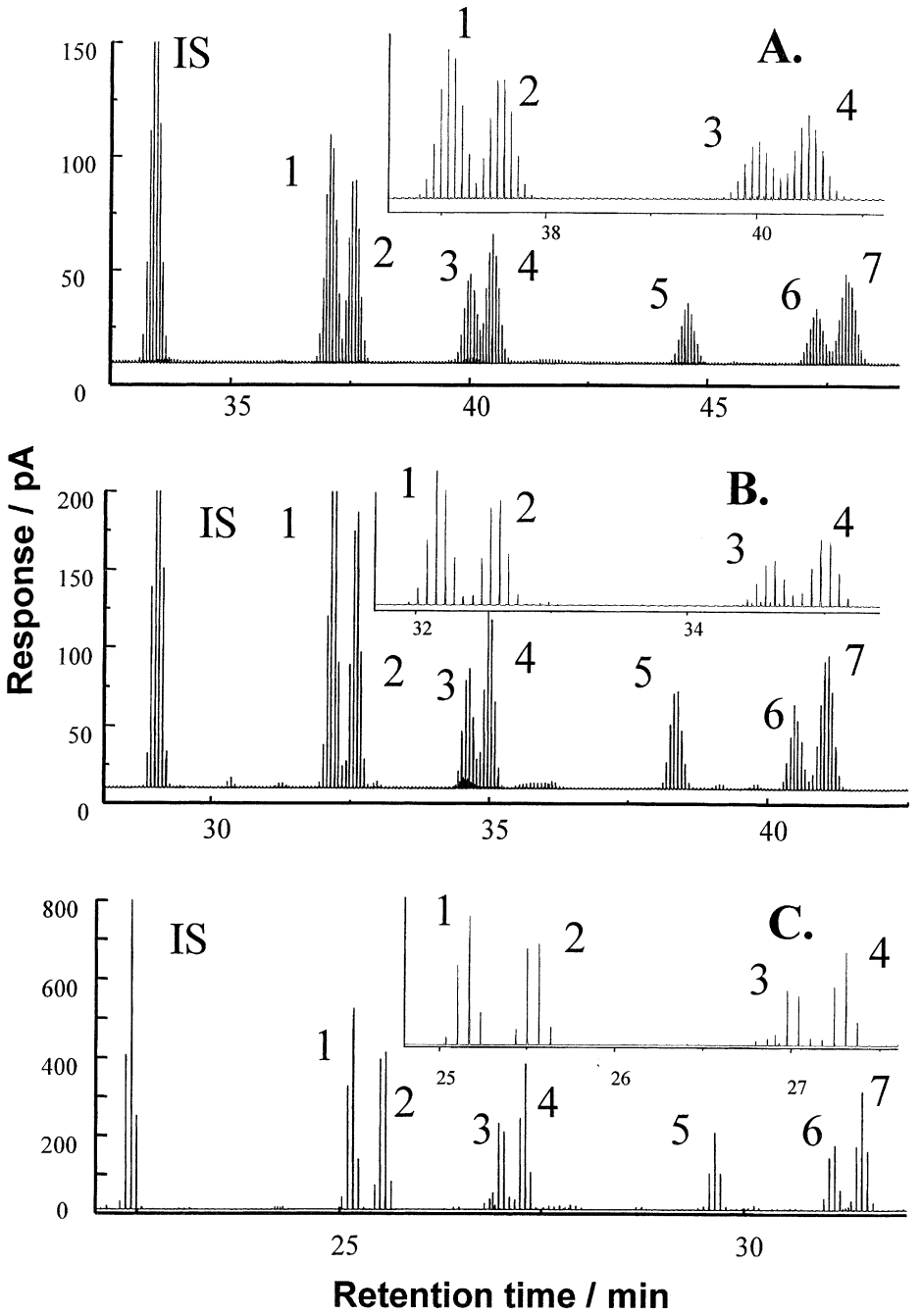

Fig. 7. Effect of carrier gas pressure on selected separations of components using pulsed cryogenic modulation for a sterol standard solution. I.S.: 5α-Cholestane; 1: COP; 2: EPI; 3: CHL; 4: DHL; 5: SROL, 6: B-SIT; 7: SNOL. The expanded inset separations shown are for the pairs COP and EPI, and CHL and DHL, and for selected carrier pressures: (A)–(C) = 3.0, 4.0, and 7.3 p.s.i., respectively. Modulation period was 4 s.

for all three temperatures. Qualitative comparison of the real samples showed that in spite of more pulses at 300 °C, and higher sensitivity at 320 °C, 305 °C gave the best result with respect to resolution than the other temperatures. Whilst results suggest 305 °C would be the optimum upper isothermal temperature to use, 320 °C was chosen for most analyses in this study since it gave reduced analysis time.

4. Conclusion

From this study it is demonstrated that GC × GC technology provides greater confidence in quantitative analysis for sterol analysis than conventional single-column GC. Samples of environmental relevance, comprising varying ratios of green lip mussel tissue extract and cow sterol and stanol concentrations showed that the comprehensive mode gave nine detectable compounds amongst the constituents of interest (24-ethylcoprostanol, 24-methylenecholesterol and 24-ethyl-epi-coprostanol), as opposed to five apparent but incompletely resolved peaks with the normal GC analysis. Studies of selected GC × GC parameters showed that column lengths, carrier flow-rates (pressure setting) as well as maximum oven temperature in temperature program analysis influenced component sensitivity and separation. Whilst a 2 m second dimension column did not give best sensitivity, it provided better resolution amongst the components of interest. A slower carrier flow-rate of 0.6 ml/min (pressure of 4.0 p.s.i.) also allowed for enhanced resolution. While the oven temperature program final temperature of 305 °C was found to be the optimum, 320 °C was used throughout this study to reduce analysis time. Overall, the GC × GC method permitted almost complete separation of peaks of interest which co-elute in normal capillary GC analysis, and revealed other peaks in this same region which were obscured in the lower resolution single column technique. This results in more reliable and accurate quantification of these components.

Although not directly reported here, in the complex region of the chromatogram of interest, GC–MS did not indicate the number of unresolved peaks in the single column GC trace. Hence there is no knowledge of how many components one may need to "deconvolute". GC × GC offers better separation, and a clear indication of the complexity of the region. GC–FID offers precise quantitation over a wide concentration range. Subject to considerations of non-linear effects [29], there is every likelihood that GC × GC–FID will therefore offer better quantification than GC–MS.

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