5 EXPERIMENTAL RESULTS AND DISCUSSIONS

5.1 Experimental Conditions used in the Analysis of the Essential Oils

In the optimised GC×GC system, 0.1 µl of essential oil sample was manually injected at a split ratio of 1:125. The essential oils were used as supplied without any dilution. The injector, operating in the split mode, was kept at 250°C. The first column (28.6 m × 250 µm I.D. × 0.250 µm d_f, HP-1, Hewlett Packard Corp., USA) was temperature programmed from 30°C (2 min. hold) – 160°C at 1°C/min, then ramped to 250°C at 5°C/min (5 min hold) for all the samples except *Tagetes minuta*. For this essential oil the temperature was programmed from 30°C (2 min hold) - 210°C at 1°C/min, then ramped to 250°C at 5°C/min (5 min hold). The second column (1 m x 100 µm I.D. x 0.1µm d_f, Rtx-1701 or Rtx-Wax, Restek International, USA) was temperature programmed from 60°C (2 min hold) – 190°C at 1°C/min, and ramped to 250 at 5°C/min. For *Tagetes minuta* it was raised up to 230°C at 1°C/min and ramped to 250 at 5°C/min. The FID detector was held at 300°C for all the runs. The carrier gas was H_2 and N_2 was used as a make-up gas. A constant inlet pressure of 110 kPa was used throughout.

Data acquisition was performed *via* a computer interfaced with the 6890A gas chromatograph (Agilent Technolgies, Wilmington, DE, USA) by SCB-68 interface board (National Instruments, USA). LabView Version 2.0x comprehensive two-dimensional gas chromatography operating program (National Instruments, USA) was used to acquire the GC×GC data. This software also translates the raw data after each analysis into text format for export to other software. Chromatograms were plotted by the use of transform (Research Systems, Noeys Version V2.0) and MATLAB Version 6.0.0.88 Release 12 (Mathworks Inc., USA) software packages.

5.2 The Power of Comprehensive Two-Dimensional Gas Chromatography

As already explained in the previous chapters, comprehensive two dimensional gas chromatography (GC×GC) has distinct advantages over linear gas chromatography, *viz.*, high peak capacity, increased sensitivity, higher resolution power, and the provision of two independent retention times for reliable component identification [1].

The high peak capacity in GC×GC is a result of the added separation dimension, which simplifies the analysis of complex samples. In GC×GC, members of the same homologous series are progressively separated due to their volatility differences in the first non-polar dimension and show similar second dimension retention times. The second dimension separation, independent of the first dimension separation, enables the compounds to form a class-type arrangement Accordingly, groups of peaks are formed occupying well-defined first and second dimension retention times, depending on the complexity and make-up of the sample analysed. Non-polar compounds, like hydrocarbons, form the first group occupying the left hand side of the two dimensional space. These are followed by medium polar components, such as monocyclic aromatic compounds, aldehydes and ketones, which exhibit higher second dimension retention times than hydrocarbons. More polar compounds including polycyclic aromatic compounds, alcohols and acids show the longest second dimension retention times and form their own distinct group in the right hand side of the GC×GC chromatogram [1, 2]. This is exemplified in figure (5.1), where components of a standard mixture were separated into their respective classes by GC×GC.

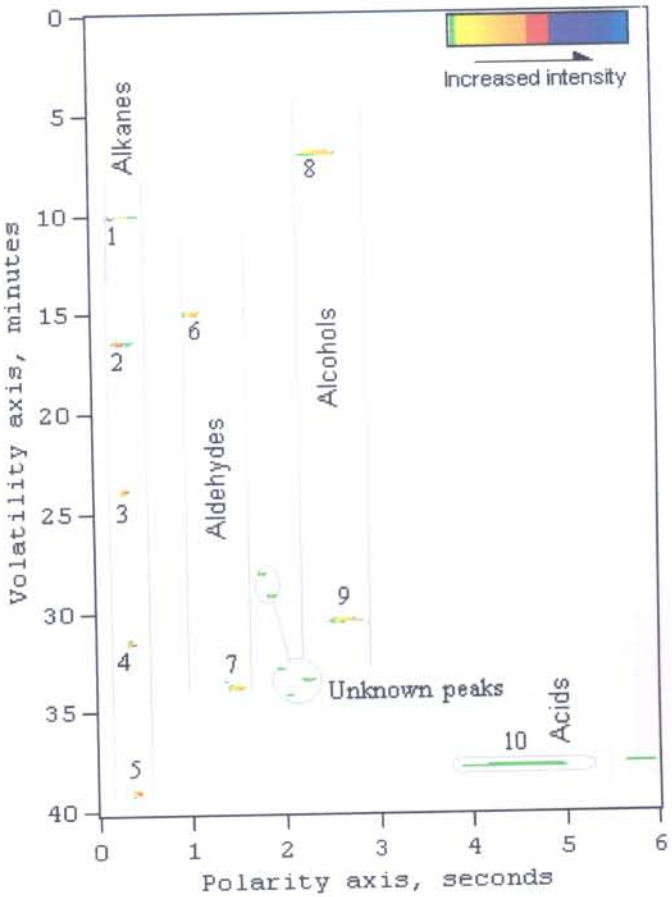

Figure 5.1 Component classes of a standard mixture separated by GC×GC[1], forming distinct groups: (**1 – 5**) are C-8 to C-12 n-alkanes, (**6**) Heptanal, (**7**) Citronellal, (**8**) 3-methyl-1-butanol, (9) Linalool, and (**10**) is Octanoic acid.

From the two alternatively used second dimension columns, the more polar Rtx-Wax column produces peaks that are well spread in the two-dimensional separation space. In the case of the medium polar Rtx-1701 column the peaks are more crowded in the second dimension, as can be clearly seen from figure (5.2). It is also evident in this figure that components with similar volatility are separated in the second dimension due to their polarity differences. The advantage of GC×GC in this case is self-evident. Had a linear system been used, for example, the circled peaks in the figure would have not been separated clearly. As each group has almost the same first dimension retention times, the compounds would have co-eluted as single peaks.

[1] GC×GC conditions: GC oven 30°C (2 min. hold) – 110°C at 2°C/min., auxiliary oven: 60°C (2 min. hold) – 140°C at 2°C/min. The second dimension column was Rtx-Wax.

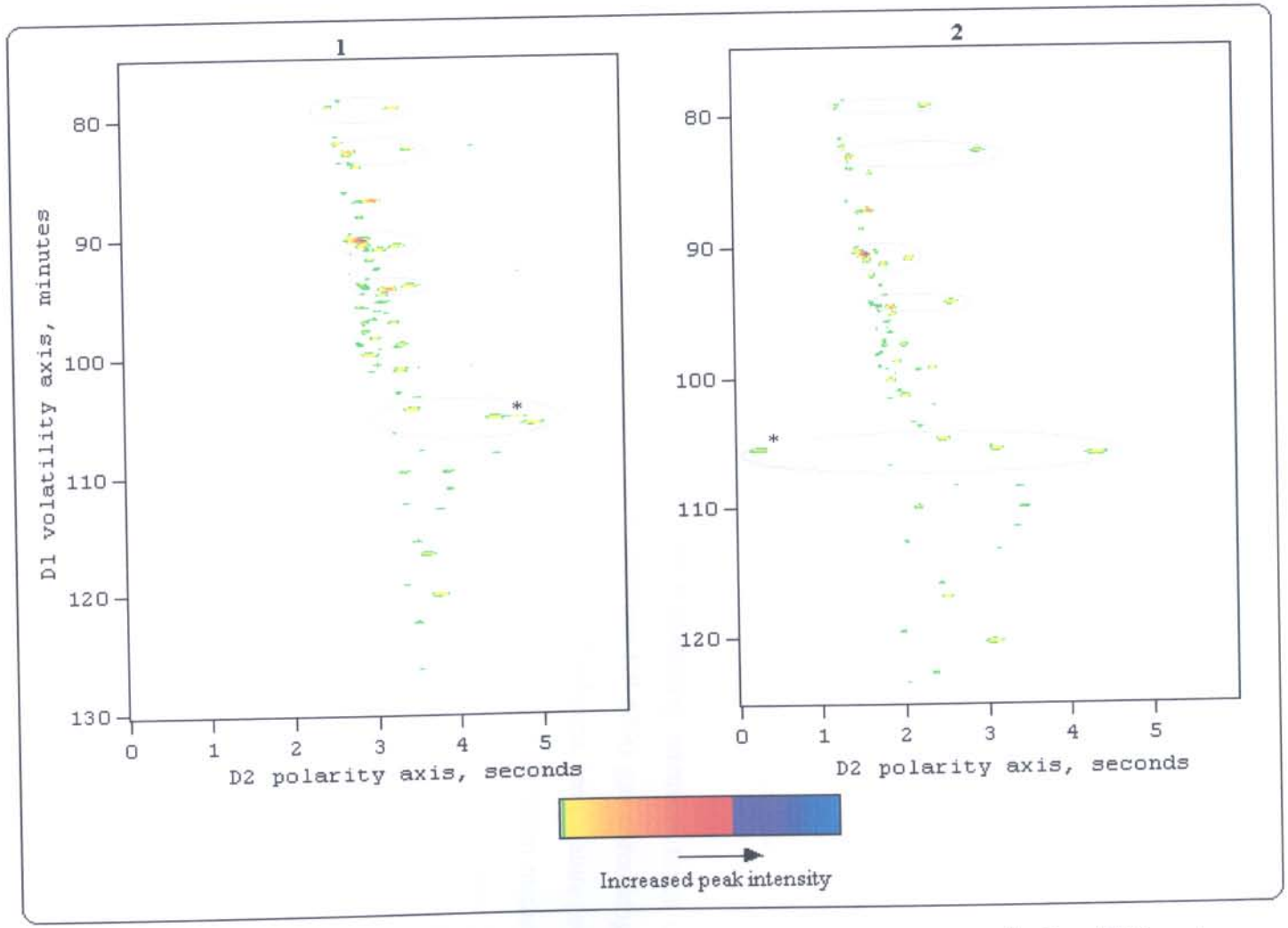

Figure 5.2 Extracted parts of GC×GC two-dimensional plots of Bourbon Geranium. (**1**) Rtx-1701 and (**2**) Rtx-Wax second dimension columns, and (*) Wrap-around has occurred for this compound in the case of Rtx-Wax second dimension column.

5.3 Qualitative Identification of Essential Oil Components by Gas Chromatography – Time-of-Flight Mass Spectrometry (GC-TOFMS)

Some of the most important parameters obtained from a chromatogram are the retention times, often used for the identification of components. Retention times are functions of many chromatographic parameters such as temperature programming rate, column dimension, stationary phase type, film thickness and linear gas velocity (inlet pressure). A change in any of these parameters brings about a change in retention times. The attempt and success of using retention times for the purpose of qualitative identification of compounds, therefore, depends on maintaining all these factors as constant as possible.

Retention time locking is a new method of reproducing retention times of identical components from instrument to instrument (between HP 6890 gas chromatographs) and from one laboratory to another using the same nominal columns, temperature programming rates and dead time, regardless of the column exit pressure and detection method used [3]. Reproducing retention times between GC-FID and GC-MS runs has been notoriously difficult because, in the latter case, the column exit pressure is normally at 0 kPa, giving rise to changes in absolute and relative retention times. Retention times are locked by adjusting the column head pressure using the RTL calibration procedure of the RTL HP ChemStation Software (ChemStation Rev.A.08.03 [847], Agilent Technologies). Adjusting the head pressure also adjusts the dead time, so that dead times also become the same when pressures are locked.

Instrument to instrument analysis methods are translated using the 'Mxlator' method translation software (GC Method Translation Software Version 2.0a, Hewlett Packard). Two methods are mutually translatable if the columns used have the same stationary phase and the same phase ratio. For exact reproduction of retention times, a constant speed gain, S, of one ($S = t_{R1}/t_{R2}$) is selected [3]. Other values of S are used to expand or compress the time axis of a chromatogram with full maintenance of peak positions.

The method translation software calculates the inlet pressure of the instrument being locked to a given chromatogram. The calculated pressure gives an approximate, but not an exact

match of the original retention time. Exact retention times are only obtained by 'retention time locking' the new method with the original method. In this procedure, a range of inlet pressures (around the calculated value) is plotted against experimentally observed retention times and the correct inlet pressure is found by interpolation to match the exact retention time of the original method [4].

The locking of the first dimension GC×GC retention times with those from a GC-MS has not been attempted before and deals with even larger difficulties in column exit pressure. In this case the exit pressure of the GC×GC first dimension is at around 100 kPa above atmospheric (required to drive the 100 μm I.D. second dimension column).

The potential rewards of successfully locking GC×GC and GC-TOFMS retention times are large, as it would effectively give both a polarity retention time as well as an accurate-mass mass spectrum of single components eluting from the (first dimension) gas chromatogram.

In this project, an attempt was made to use retention times of components analysed on a gas chromatography – Time-of-flight mass spectrometry (GC-TOFMS) to identify the same components analysed by GC×GC, using the retention time locking (RTL) and method translation procedures.

A hydrocarbon standard mixture was run using the optimised GC×GC system. Then, to get the initial GC-TOFMS target pressure the GC×GC parameters with the dead time and the GC-TOF parameters were put into the GC method translation tool. From the software the initial calculated pressure was about 16 kPa. Using this initial pressure a series of five runs were made at ±10% and ±20% of the target pressure (12, 14, 16, 18 and 20 kPa[2]) to help retention time lock the method. Then, all the retention times of the hydrocarbons with the associated pressures were entered into the RTL Software. This procedure determines, by interpolation, the value of the head pressure that has to be used in the new method, *i.e.* the GC-TOFMS for reproduction of the first dimension GC×GC retention times [4].

[2] The pressures used are the nearest whole number pressures to the calculated ±10% and ±20% of the target pressure. The GC-TOFMS accurate mass instrument used was not controlled by the ChemStation software. Under the software used only whole number pressure values are allowed.

Out of the five GC-TOFMS runs made the retention times of most of the hydrocarbons from the 14 kPa run were more close to the GC×GC retention times (run at 110 kPa), as can be seen from table (5.1). Moreover, at this pressure the retention time of nonane, C-9, from GC-TOFMS exactly locks with its GC×GC retention time. Based on this, a test run of the actual essential oils was done, but the retention times were a lot lower than the GC×GC retention times. Accordingly, more runs were made at higher GC-TOFMS inlet pressures. At 18 kPa the retention times of the essential oil components were close enough to enable the correct correlation of peaks between the two instruments. This pressure was used for all the essential oil runs and subsequent component identifications, although the retention time correlation was not entirely satisfactory.

Table 5.1 Retention times from the original GC×GC method and GC-TOFMS runs

Hydro-carbon	GC×GC t_R (min.), at 110 kPa	GC-TOFMS retention times (min.) at different pressures in kPa				
		12	14	16	18	20
C-8	11.1	11.09	10.96	10.83	10.45	10.17
C-9	**20.14**	20.40	**20.14**	20.04	19.35	18.75
C-10	32.54	32.94	32.60	32.46	31.61	30.85
C-11	46.60	47.19	46.77	46.73	45.65	44.70
C-12	60.70	60.66	60.26	59.99	59.38	58.84
C-13	74.64	75.29	74.78	74.75	73.64	72.64
C-14	87.78	88.51	87.95	87.91	86.77	85.79
C-15	112.10	112.74	112.18	112.08	111.04	110.14

Note the good retention time correlation between GC×GC retention times and those of GC-TOFMS at 14 kPa.

5.3.1 Identified Essential Oil Components

The essential oil components were identified using their GC×GC[3] and GC-TOFMS retention time correlations and accurate-mass mass spectra (designated as 'a' in the tables) and from relative retention of the compounds reported in the literature. In the case of lemongrass (*Cympobogon citratus) and Artimisia afra* additional identification of the components was obtained from GC×GC-TOFMS[4] runs of the two samples (designated as

[3] The GC×GC runs were made using the HP-1 – Rtx-1701 column set.

[4] The GC×GC-TOFMS runs were done by courtesy of Leco (USA) using Rtx-5 (30 m × 250 μm × 0.25 μm) first dimension column and DB-WAX (1 m × 100 μm × 0.1 μm) second dimension column. Oven temperature: 55°C (0.2 min. hold) – 155°C at 1°C/min. Second oven: 75°C (0.2 min. hold) – 170°C at 1°C/min.

'b' in the tables). Peaks observed only in the GC×GC chromatograms but not in the GC-TOFMS runs were left out (especially for *Tagetes minuta*, Bourbon Geranium, and *Cymbopogon flexuosus*). Some peaks identified from the GC×GC-TOFMS runs, that could not be correlated with any GC×GC counterparts were also left out from the list (tables (5.2) to (5.6)).

Table 5.2 Identified Essential Oil Components of Bourbon Geranium (*Pelargonium capitatum ×p. radens)*

Peak no.	GC×GC		GC-TOFMS	Difference		Identified Components	Method of identification
	t_{r2}, sec	t_{r1}, min	t_r, min	Δt_r, min	%		
1.	1.7	22.60	22.50	0.10	-0.44	α-pinene	a, [ref. 5, 6]
2.	2.0	30.00	30.00	0.00	0.00	β-myrcene	a, [ref. 6]
3.	2.1	30.90	30.89	0.01	-0.03	phellandrene	a
4.	2.5	33.00	33.08	-0.08	0.24	p-cymene	a
5.	2.3	33.80	33.80	0.00	0.00	β-ocimene	a, [ref. 6]
6.	2.2	34.20	34.25	-0.05	0.15	Limonene	a
7.	3.5	44.00	45.26	-1.26	2.86	Unknown	---
8.	2.9	45.50	45.77	-0.27	0.59	Unknown	---
9.	2.9	47.70	47.74	-0.04	0.08	Unknown	---
10.	4.1	50.70	51.60	-0.90	1.78	p-Menthan-3-one	a, [ref. 5, 6, 7]
11.	4.2	62.00	61.08	0.92	-1.48	Citronellol	a, [ref. 5, 6,7]
12.	4.1	62.40	62.48	-0.08	5.90	Citronellol acetate	a
13.	4.2	65.50	67.92	-2.42	5.42	Geraniol	a, [ref. 5, 6, 7]
14.	3.5	68.40	69.20	-0.80	3.20	Citronellyl formate	a, [ref. 5, 6, 7,]
15.	3.5	71.60	72.32	-0.72	2.23	Unknown	---
16.	3.3	78.90	79.68	-0.78	0.99	Citronellyl propionate	a, [ref. 5, 6]
17.	3.5	82.40	82.53	-0.13	0.16	α-copaene	a
18.	2.8	82.70	83.38	-0.68	0.82	β-bourbonene	a, [ref. 5, 6]
19.	3.0	86.80	87.55	-0.75	0.86	*Trans* caryophyllene	a, [ref. 5, 6, 7]
20.	2.9	90.10	91.14	-1.04	1.15	Guaiadene	a, [ref. 5, 6]
21.	3.2	94.20	95.16	-0.96	1.02	β-cadinene	a
22.	3.1	94.70	95.48	-0.78	0.82	Gurjunene	a
23.	3.2	97.10	97.62	-0.52	0.54	Unknown (similar to 91.14)	---
24.	3.0	98.30	98.86	-0.56	0.57	Germacrene D	a, [ref. 5, 6]
25.	3.3	98.90	99.42	-0.52	0.53	Calamenene	a
26.	3.0	99.70	100.37	-0.67	0.67	σ-cadinene	a, [ref. 7]
27.	3.4	101.00	101.51	-0.51	0.50	Citronellyl butyrate	a, [ref. 5, 6]
28.	4.5	105.00	105.04	-0.04	0.04	Unknown	---
29.	5.0	105.50	106.18	-0.68	0.64	Unknown	---
30.	3.7	116.50	116.89	-0.39	0.33	Geranyl butyrate	a, [ref. 5, 6, 7]
31.	3.9	119.90	120.45	-0.55	0.46	Unknown	---

(a) Peaks identified by their GC×GC and GC-TOFMS retention time correlations and mass spectra.
[ref.] References in square brackets indicate literature where these compounds have been reported.

Table 5.3 Identified Essential Oil Components of Kakiebos (*Tagets minuta*)

Peak no.	GC×GC retentions		GC-TOFMS	Difference		Identified Components	Method of identification
	t_{r2}, sec	tr_1, min	t_r, min	Δt_r, min	%		
1.	*	*	4.30	*	*	4-methyl-pentene	*
2.	*	*	4.80	*	*	Cyclohexane	*
3.	1.6	14.3	14.01	0.29	-2.03	Ethyl-2-methyl butyrate	a, [ref. 8, 9, 10]
4.	1.8	27.2	27.01	0.29	-1.06	Sabinene/alpha-pinene	a, [ref. 8, 9, 10]
5.	2.0	34.5	34.47	0.03	-0.09	Limonene	a, [ref. 9, 10]
6.	2.1	36.5	36.41	-0.11	0.30	*Cis*-ocimene	a, [ref. 8, 9, 10]
7.	3.1	37.5	37.52	-0.02	0.05	Dihydrotagetone	a, [ref. 8, 9, 10]
8.	2.6	41.4	41.09	0.11	-0.27	Geranial	a
9.	3.1	48.5	48.19	0.21	-0.43	Unknown	---
10.	3.3	49.9	49.63	0.07	-0.14	*Cis*-tagetone	a, [ref. 8, 9, 10]
11.	3.6	50.5	50.53	-0.03	0.06	*Trans*-tagetone	a, [ref. 8, 9, 10]
12.	3.6	51	50.75	-0.05	0.10	Unknown	---
13.	4.4	57.9	58.10	-0.50	0.87	(2-methylprop-1-enyl)-cyclohexa-1,5-diene	a
14.	4.4	58.8	59.03	-0.43	0.73	Unknown(isomer of 58.10	a
15.	3.9	61.3	61.35	-0.35	0.57	Ocimenone (isomer unknown)	a, [ref. 8]
16.	4.1	62.2	62.17	-0.27	0.44	Carvacrol	a
17.	5.2	81.8	81.73	-0.23	0.28	Unknown	---
18.	2.8	87.1	87.05	-0.25	0.29	*Trans* caryophylline	a, [ref. 8, 10]
19.	3.0	91.2	91.20	-0.40	0.44	Unknown	---
20.	4.3	105.2	105.11	-0.51	0.49	Unknown	---
21.	4.7	155.5	154.71	-0.21	0.14	Piperitone	a
22.	4.6	161.7	160.06	-0.16	0.10	Unknown	---
23.	5.1	161.7	160.52	-0.22	0.14	Unknown	---
24.	5.2	166.3	164.56	-0.06	0.04	Unknown (base peak 83)	---
25.	5.2	171.0	167.86	0.34	-0.20	Unknown (base peak 83)	---

* Peaks not observed in the GC×GC chromatogram.

(a) Peaks identified by their GC×GC and GC-TOFMS retention time correlations and mass spectra.

[ref.] References in square brackets indicate literature where these compounds have been reported.

Table 5.4 Identified Essential Oil Components of Lemongrass (*Cymbopogon flexuosus)*

Peak no.	GC×GC retention		GC-TOFMS	Difference		Identified Components	Method of identification
	t_{r2}, sec	t_{r1}, min	t_r, min	Δt_r, min	%		
1.	1.2	24.1	23.68	0.42	-1.74	Camphene	a, [ref. 11, 12]
2.	2.8	28.3	27.76	0.54	-1.91	6-methyl-5-heptene-2-one	a, [ref. 12, 13]
3.	2.7	30.2	29.96	0.24	-0.79	Myrcene	a, [ref. 6, 11,12, 13 ,]
4.	1.6	34.5	34.21	0.29	-0.84	Limonene	a, [ref. 6,11, 12, 13]
5.	2.8	40.1	39.63	0.47	-1.17	4-nonanone	a, [ref. 13]
6.	3.0	44.4	44.18	0.22	-0.50	Linalool	a, [ref. 6, 11, 12, 13]
7.	3.5	49.1	48.67	0.43	-0.88	Unknown	---
8.	4.4	55.0	54.60	0.40	-0.73	Unknown (similar to 48.67)	---
9.	4.5	62.5	63.10	-0.60	0.96	Neral	a, [ref. 6, 11, 12, 13]
10.	4.5	66.7	67.70	-1.00	1.50	Geranial	a, [ref. 6, 11, 12, 13]
11.	0.5	74.3	74.70	-0.40	0.54	Methyl geranate	a
12.	0.9	79.1	82.58	0.22	-0.27	Geranyl acetate	a, [ref. 6, 11, 12, 13]
13.	2.5	98.7	98.59	0.11	-0.11	Cadinene (unknown isomer)	a, [ref. 12, 13,]
14.	4.0	105.6	105.13	0.47	-0.45	Unknown	--

(a) Peaks identified by their GC×GC and GC-TOFMS retention time correlations and mass spectra.

[ref.] References in square brackets indicate literature where these compounds have been reported.

Table 5.5 Identified Essential Oil Components of Lemongrass (*Cymbopogon citratus*)

Peak no.	GC×GC		GC-TOFMS	Difference		Identified Components	Method of identification
	t_{r2}, sec	t_{r1}, min	t_r, min	Δt_r, sec	%		
1.	1.9	28.10	27.86	0.24	-0.85	6-Methyl-5-hepten-2-one	a, b, [ref. 6, 14]
2.	0.7	29.20	---	---	---	2,3-Dehydro-1,8-cineols	b
3.	0.5	30.10	30.47	-0.37	1.23	β-Myrcene	a, b, [ref. 6, 14, 15]
4.	0.9	34.60	---	---	---	Cymene	b, [ref. 15]
5.	1.3	35.50	---	--	---	Eucalyptol (1,8-cineole)	b, [ref. 6, 14]
6.	0.6	36.20	36.18	0.02	-0.06	Limonene	a, b, [ref. 6, 14]
7.	0.7	37.60	37.47	0.13	-0.35	(Z)-β-ocimene	a,b, [ref. 6, 14, 15]
8.	2.2	42.50	42.59	-0.09	0.21	α-Thujone	b, [ref. 6]
9.	2.1	43.10	---	---	---	Nonanal	b, [ref. 6]
10.	2.0	43.80	---	---	---	Citronellal	b, [ref. 6, 15]
11.	2.1	44.40	---	---	---	Verbenol	b, [ref. 14]
12.	1.9	44.40	44.17	0.03	-0.07	Linalool	a, b, [ref. 6, 14, 15]
13.	2.0	46.10	46.05	0.05	-0.11	Decanal	a, b
14.	2.4	48.90	48.76	0.14	-0.29	α-terpineol	a, b, [ref. 6, 14, 15]
15.	2.1	52.20	52.27	-0.07	0.13	Methylcyclohexyl-ethanal	a
16.	2.1	54.70	54.77	-0.07	0.13	Oxiranecarboxaldehyde, 3-methyl-3-(4-methyl-3-pentenyl)	b
17.	2.4	62.10	---	---	---	Nerol	b, [ref. 6, 14, 15]
18.	3.3	62.10	63.18	-1.08	1.74	Neral	a, b, [ref. 6, 14, 15]
19.	2.5	65.70	---	---	---	Geraniol	b, [ref. 6, 14, 15]
20.	3.4	66.30	67.66	-1.36	2.05	Geranial	a, b, [ref. 6, 14, 15]
21.	2.1	70.70	---	---	---	2-Undecanone	b, [ref. 6, 14]
22.	1.9	71.80	72.08	-0.28	0.39	Geranyl formate	a, b
23.	5.2	73.80	74.78	-0.98	1.33	2,7-Dimethyl-2,6-octanediol	a, b
24.	5.5	78.50	79.17	-0.67	0.85	Epoxy-linalooloxide	a, b
25.	1.5	81.20	---	---	---	Neryl acetate	b
26.	1.9	82.50	82.89	-0.39	0.47	Geranyl acetate	a, b, [ref. 6]
27.	1.2	89.90	---	---	---	Caryphyllene	b, [ref. 15]
28.	2.3	96.80	97.04	-0.24	0.25	2-Tridecanone	a, b, [ref. 14]
29.	3.0	105.20	---	---	---	Caryophyllene oxide	b
30.	2.9	109.40	---	---	---	Selina-6-en-ol	b

(a) Peaks identified by their GC×GC and GC-TOFMS retention time correlations and mass spectra
(b) Peaks identified by GC×GC-TOFMS.
[ref.] References in square brackets indicate literature where these compounds have been reported.

Table 5.6 Identified Essential Oil Components of African Wormwood (*Artemisia afra)*

Peak no.	GC×GC		GC-TOFMS	Difference		Identified Components	Method of identification
	t_{r2}, sec	t_{r1}, min	t_r, min	Δt_r, min	%		
1.	1.5	14.3	---	---	---	Santolina triene	b
2.	1.1	14.9	---	---	---	5-methyl-5-hexen-2-one	b
3.	1.1	15.6	---	---	---	Lilac alcohol B	b
4.	1.3	21.1	---	---	---	Tricyclene	b, [ref. 16]
5.	1.4	22.3	---	---	---	α-pinene	b, [ref. 16, 17]
6.	1.6	23.5	23.65	-0.15	0.64	Camphene	a, b, [ref. 16, 17]
7.	1.8	26.7	---	---	---	Sabinene, thujene	b, [ref. 16]
8.	3.0	27.9	---	---	---	1-Butanol, 3-methyl-, propanoate	b
9.	2.9	30.9	---	---	---	1-Octen-3-ol	b
10.	2.3	32.8	33.03	-0.23	0.70	p-cymene	a, [ref. 16, 17]
11.	2.3	33.8	33.96	-0.16	0.47	1,8-cineole	a, b, [ref. 16, 17]
12.	2.8	38.2	38.31	-0.11	0.29	Isopropyl triazole	a
13.	2.9	42.1	---	---	---	α-Terpinoline	b, [ref. 16]
14.	3.9	43.1	44.27	-1.17	2.71	α-thujone	a, b, [ref. 16, 17]
15.	3.8	44.5	45.38	-0.88	1.98	β-thujone	a, b, [ref. 16, 17]
16.	4.1	47.2	47.48	-0.28	0.59	Camphor	a, b, [ref. 16, 17]
17.	4.0	51.5	---	---	---	Borneol	b, [ref. 16]
18.	3.1	53.5	---	---	---	α-Thujenal	b
19.	2.7	54.8	55.04	-0.24	0.44	α-terpineol	b, [ref. 16]
20.	3.5	64.5	---	---	---	Myrthenal	b, [ref. 16]
21.	4.4	70.7	---	---	---	*Cis*-Caryophyllene	b

(a) Peaks identified by their GC×GC and GC-TOFMS retention time correlations and mass spectra.
(b) Peaks identified by GC×GC-TOFMS.
[ref.] References in square brackets indicate literature where these compounds have been reported.

Retention time locking makes peak identification more accurate and allows comparing of results from different instruments. It permits transferring of methods from one column to another. It makes communication of results a lot easier and it is a good way of checking that temperature, flow, and column are working properly [4]. The procedure of retention time locking and method translation is fairly user friendly once you have the appropriate instruments and the necessary software. The level of accuracy obtained for retention time locking of different one-dimensional GC systems, with the same or different detectors, is very high as reported in the literature [3, 18].

The difficulty of trying to lock GC×GC columns with the GC column in the GC-TOFMS system is evident as can be seen from the results in the tables above. One of the requirements in RTL is to use the same nominal columns, but GC×GC uses not one but two different polarity and dimension serially coupled columns. Separation is not only a function of volatility but also a function of polarity. Basically, it is only possible to match the first

column of the GC×GC with the GC-TOFMS column. Discrepancies in retention times can arise due to the short polar second dimension column. Another issue is the temperature of the second dimension column, which was maintained at higher temperatures than the first dimension column. Retention time as a function of temperature therefore might be slightly affected by this temperature difference.

The determination of accurate retention times of components is also affected by the peak shape. In determining the retention times of standards it is possible to regulate the peak shapes by injecting the right amount of sample to obtain only non-overloaded, Gaussian shaped peaks. In using actual samples difficulties arise due to variations in the quantity of the individual components in the sample. Some of the components produce fairly Gaussian shaped peaks, others produce totally overloaded peaks with typical 'fronting'. The shapes of such peaks could be improved by injecting dilute samples, but this is done at the cost of losing information on the minor peaks.

In practice in retention time locking of these two systems was not simple since quite a few factors affect the procedure. The search for better understanding of these factors to get better retention time correlation requires more work and it is too early to give any concluding remarks regarding the matter. More work is still underway to solve the problem.

5.4 GC×GC Run to Run Reproducibility and Overall Efficiency

The efficiency of a comprehensive two-dimensional gas chromatographic system can be measured by the complexity of samples that can still be effectively separated into the individual chemical components. Reliable and reproducible results can only be achieved if the proper functioning of all parts of the separation system is ensured. The modulator has to effectively trap, focus and re-inject sub-samples quantitatively into the second column. The detector has to have a broad dynamic range and has to be very sensitive so that it can effectively detect all solutes, which are present in the sample in a wide range of concentrations. The columns used must be stable over a wide temperature range, have a low bleed and be able to effect good separation of the analytes.

To check on the separation effectiveness of the GC×GC system, the precision and consistency of some chromatographic peak parameters can be monitored. These parameters include: reproducibility of retention times, peak widths and peak shapes.

5.4.1 Retention Times and Peak Width Reproducibility

Retention time is a function of column dimension, stationary phase chemistry, film thickness, temperature programming rate and carrier gas velocity. Under constant conditions of temperature programming rates and linear gas velocity, a certain column or a set of coupled columns must produce reproducible retention times. The consistency of the retention times is most important especially in instances where retention times are used for pattern recognition, or if reliable identification of individual components using standards is being performed [19].

To test the efficiency of the GC×GC system utilised in this project, a series of replicate runs were performed using the HP1-RtxWax column set under optimised conditions. Then, in order to compare the retention times in both dimensions and the second dimension peak widths, some peaks were randomly chosen. The selected peaks represent the different volatility and polarity ranges exhibited by the essential oil components.

Table 5.7 GC×GC run-to-run reproducibility* of selected peaks (*Cymbopogon flexuosus*)

Peak no.	Compound name	D1 retention times, minutes			D2 retention times, seconds			D2 peak widths (W_h), milliseconds		
		Mean	STD	%RSD	Mean	STD	%RSD	Mean	STD	%RSD
1.	Camphene	24.10	0.09	0.32	0.82	0.07	9.13	48.00	2.45	5.10
2.	6-methyl-5-heptene-2-one	28.20	0.14	0.41	2.76	0.08	2.90	120.00	5.48	4.56
3.	Limonene	34.60	0.09	0.22	1.12	0.07	6.68	52.00	4.00	7.69
4.	4-nonanone	40.06	0.10	0.23	1.72	0.07	4.35	72.00	4.00	5.56
5.	Linalool	44.44	0.16	0.31	4.72	0.12	2.47	205.00	10.00	4.88
6.	Unknown	52.44	0.16	0.30	3.02	0.32	10.76	144.00	4.90	3.40
7.	Unknown	54.98	0.12	0.14	3.38	0.12	3.45	142.00	7.48	5.27
8.	Geranyl acetate	82.66	0.39	0.40	3.04	0.30	9.89	120.00	6.12	5.10
9.	Cadinene	98.86	0.10	0.10	1.86	0.08	4.30	69.00	2.00	2.90
10.	Unknown	105.64	0.12	0.11	3.10	0.11	3.53	120.00	5.48	4.56
Mean				**0.25**			**5.75**			**4.90**

*The values in the table are the mean of five GC×GC runs. More results for all the essential oils are given in the Appendix.

As can be inferred from table (5.7), the first dimension retention times showed good reproducibility with a mean relative standard deviation of only 0.25%. The first dimension retention times stayed fairly constant and the actual deviation in the retention times was in the range of 6 – 12 seconds (0.1 – 0.2 minutes). For example, for the first peak the retention times for the five runs were: (24.1), (24.2), (24.2), (24.0) and (24.0) minutes[5].

The second dimension retention times and peak widths showed greater variation than that observed in the first dimension retention times. This is mainly because the second dimension retention times, peak shapes and widths were, to a certain degree, affected by the modulator performance. One of the problems of working with the dual stage jet modulator is the precise setting of the cooling rate, pulsing frequency and heating of the hot pulses. Shifting in retention times and irregular peak shapes occurred whenever there were slight differences in modulator performance due to inefficient trapping, too much cooling or inefficient heating of the trapped fractions. Occasional cold spots in the modulator bracket, before or after the two modulation stages or between the two stages, create irregularities in retention times (especially first dimension retention times) and peak shapes.

The precision of second dimension retention times is affected by the reproducibility of modulation start time between the different runs. As it was difficult to monitor the modulation start time with the software used in this project, the retention times had to be realigned for some of the runs after the completion of each run [19]. The peak widths at half height observed in all the runs were quite impressive, and range between 45 milliseconds for peak number one and 200 milliseconds for peak five (table (5.8)) which was the broadest of all the selected peaks.

Shellie *et al.* [19] reported that the first dimension retention times and peak widths at half height show excellent reproducibility for runs done in the same day. As is shown in table (5.8), the retention times and peak widths at half height reproduce almost precisely. Variations in retention times and peak widths are more visible when comparisons are made between runs done on different days, as is the case in table (5.7).

[5] The retention times and peak width at half height reproducibility study was done for all the oils, and more results are given in the Appendix.

Table 5.8 Comparison of peaks from runs done in the same day (***Cymbopogon flexuosus)***

Peak no.	Compound name	t_{r1} in minutes			D2 W_h in milliseconds		
		Run 1	Run 2	Δt_r	Run 1	Run 2	ΔW_h
1.	Camphene	24.0	24.0	0.0	45	45	0.0
2.	6-methyl-5-heptene-2-one	28.0	28.1	0.1	110	120	10.0
3.	Limonene	34.5	34.5	0.0	50	50	0.0
4.	4-nonanone	39.9	40.0	0.1	70	70	0.0
5.	Linalool	44.2	44.3	0.1	200	200	0.0
6.	Unknown	52.5	52.5	0.0	140	135	5.0
7.	Unknown	55.0	55.0	0.0	140	140	0.0
8.	Geranyl acetate	82.9	82.3	0.6	110	*	*
9.	Cadinene	98.9	98.8	0.1	65	65	0.0
10.	Unknown	105.7	105.7	0.0	110	110	0.0

*The geranyl acetate peak in the second run gave a very broad and flat peak due to sudden change in the performance of the modulator (improper trapping and re-injection).

5.4.2 Second Dimension Retention Times *vs.* the Polarity of Second Dimension Columns

The time sample components stay in the second dimension column of a GC×GC system is influenced by the second column dimensions (length, inner diameter and film thickness) the temperature and the linear flow rate of the carrier gas. Under fixed column dimensions, only the column temperature and linear flow rate can be manipulated. Running the second dimension column at higher temperatures than the first dimension column helps to reduce the retention time of the more polar compounds, but this reduces the separation efficiency of the less polar components. Reduced temperature differences between the two columns increases retention time in the second dimension column, but improves the second dimension separation between medium polar components [20, 21].

The second dimension retention times, as a function of component class, showed differences between the two second dimension columns used. Non-polar and medium-polar compounds like non-oxygenated terpenes showed minimum second dimension retention times on both columns. Compounds with high hydrogen bonding ability but with moderate dipole moments (*e.g.* alcohols) showed high retention times on the Rtx-Wax second dimension column and moderate retention on the Rtx-1701 second dimension column. Compounds with large dipole moments but with low hydrogen bond ability (ketones and aldehydes) displayed high retention times on the Rtx-1701 second dimension column and moderate retention times on the Rtx-Wax second dimension column [22].

Table 5.9 Comparison of second dimension retention times of some components of the essential oil of *Cymbopogon flexuosus*

Peak no.	Compound name	t_{r1}, minutes	t_{r2}, seconds	
			Rtx-1701	Rtx-Wax
1.	Camphene	24.1	1.2	0.7
2.	Limonene	34.4	1.6	1.0
3.	4-Nonanone	40.0	2.8	1.6
4.	Linalool	44.3	3.0	4.5

The variation in the second dimension retention times observed in table (5.9) is a function of the chemical class to which the components belong. Camphene and limonene, non-polar terpene hydrocarbons, showed minimum second dimension retention in both columns. Nonanone, a ketone, is retained more in Rtx-1701 while linalool (an alcohol) has higher second dimension retention in Rtx-Wax.

In some cases more polar compounds may show second dimension retention times greater than the modulation period (>6 seconds), complicating the determination of second dimension retention times. In such cases adding the modulation period to the second dimension retentions and subtracting the same amount of time from the first dimension retentions can adjust the retention times. In cases of wrap-around the modulation period can be increased to find the actual second dimension retention times. A bigger temperature difference between the two dimensions can also be used to elute all solutes in one modulation period (6 sec. in this case). Wrap-around, besides giving wrong retention times may affect the quantification and identification of components if the wrap-around is causing overlapping of peaks [19, 22, 23]. The essential oils analysed here did not show any extensive wrap-around, only occasional wrap-around was observed like the peak in figure (5.2), marked with an asterisk.

5.5 Qualitative Comparison of Lemongrass Samples

One of the uses of comprehensive two-dimensional gas chromatography is the pattern recognition of different samples for quality control and forensic investigations [24]. Qualitative comparison of the same products of different origin can be accomplished by comparing the detailed GC×GC chromatograms of the samples. In this way, differences can

be observed by overlaying the different chromatograms on top of each other or by specifically looking at the patterns of peaks obtained from the different samples. Differences in peak intensities may also indicate the quantitative differences between the same compounds from sample to sample.

The chemical composition of essential oils from the same type of plant may differ both qualitatively and quantitatively depending on the place of origin, extraction method used, harvest season and weather. This difference plays a role in the pricing of the oils [25, 26]. Knowing the qualitative and quantitative chemical composition of an essential oil helps to determine whether the oil is pure, or a blend, or has been adulterated with synthetic lower cost compounds. If the gas chromatographic analysis of a known oil gives extra peaks, which have not been reported before, it is possible that the oil might have been tampered with. Synthetic additions of compounds, which are part of the sample, can be detected by the trace amounts of synthesis impurities, especially when the impurities are not naturally found in the essential oil [26].

The citral rich (60 - 85%) essential oil of lemongrass is dominated by monoterpene hydrocarbons and oxygenated monoterpenes (alcohols, aldehydes, esters and ketones) in various proportions [6, 14, 15]. The quality of this oil depends on the amount of (*cis* and *trans*) citral it contains; the minimum market requirement is 75% [, 14, 15]. In this study, a total of seven different lemongrass essential oil samples, obtained from six farms, were analysed and their GC×GC chromatograms were compared. To make the comparison, seven peaks, that visibly showed noticeable variations in their peak intensities, were chosen and their relative peak intensities were compared.

Table 5.10 Normalised peak areas (FID responses, arbitrary units) of selected compounds from the essential oil of lemongrass

Compound name	Sample number						
	1	2	3	4	5	6	7
Myrcene	0.135	0.066	0.093	0.273	0.141	0.243	.049
Limonene	0.088	0.038	0.061	0.328	0.182	0.267	0.037
Ocimene	0.101	0.056	0.191	0.172	0.148	0.269	0.063
Neral (*cis*-citral)	0.084	0.113	0.120	0.136	0.189	0.167	0.191
Geranial (*trans*-citral)	0.077	0.082	0.115	0.131	0.164	0.165	0.267
Undecanone	0.079	0.019	0.121	0.227	0.289	0.216	0.050
Geranylacetate	0.165	0.284	0.332	0.033	0.014	0.043	0.130

The relative peak intensities of the selected compounds were found to vary amongst all seven samples. Although samples 1 and 2 were from the same farm, the components from the two samples showed significant differences in their relative peak intensities (table (5.10) and figure (5.3)). These two samples also showed the lowest relative (*cis/trans*) citral content of all of the seven samples compared (figure (5.4)). Samples 4 and 6 showed high relative contents of myrcene and limonene. Sample 7 showed the lowest relative amounts of myrcene and limonene of all the seven samples, but it had the highest relative (*cis/trans*) citral content followed by samples 5 and 6 (figure (5.4)). For all of the seven samples, the relative amount of undecanone and geranylacetate varied greatly. For example, the relative ratio of undecanone between sample 5, with highest relative undecanone content, and sample 2, with lowest relative undecanone content was 15 : 1 (table (5.10)).

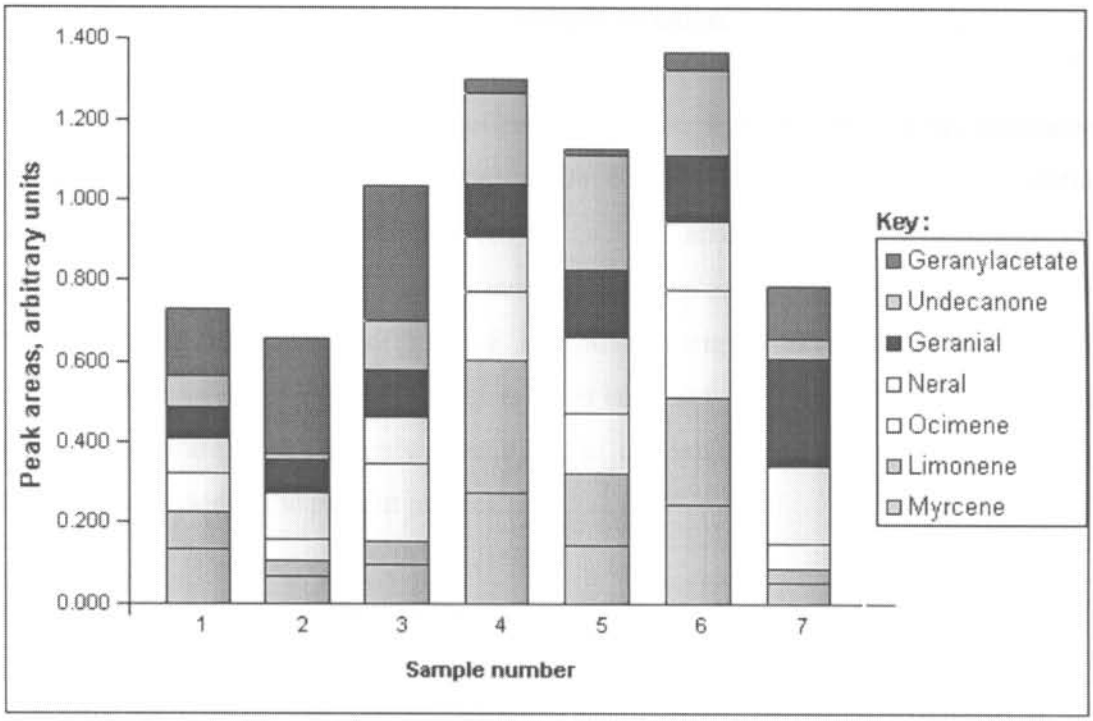

Figure 5.3 Normalised peak areas (FID responses, arbitrary units) of selected compounds of the essential oils of lemongrass. (Peak areas not corrected for run-to-run variation in 0.1 µl nominal injection volume).

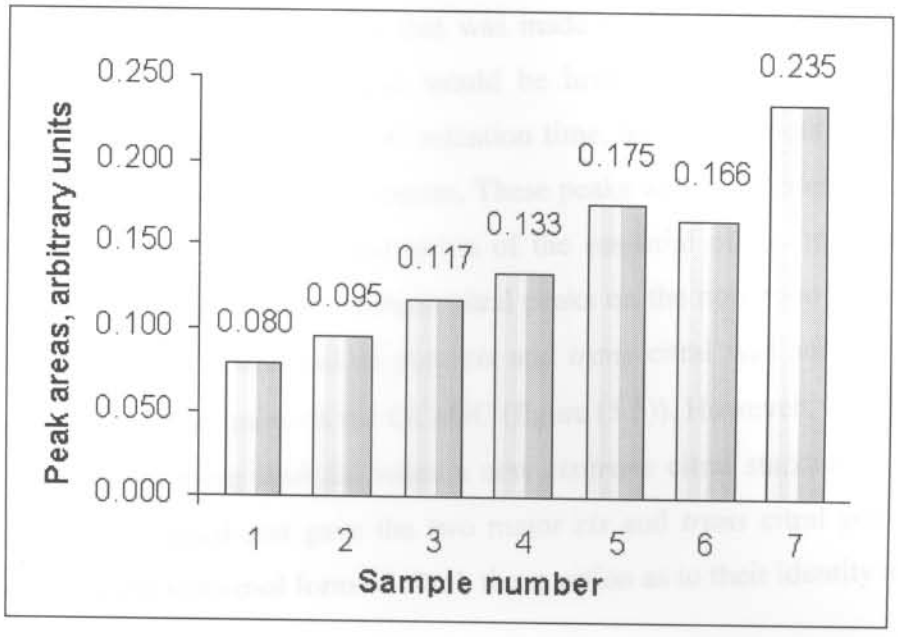

Figure 5.4 *cis* + *trans*-Citral sample-to-sample variation.

The sole purpose of the above comparisons was to determine if there are any qualitative[6] or relative quantitative differences between the components of the different essential oil samples. The information obtained from the GC×GC analyses was able to provide enough information to make the above comparisons. To make a complete assessment and determine the percentage abundance of all the individual compounds requires the quantitative determination of all the components present in each sample. In the absence of quantitative analysis software, that has only recently been available from the company ZOEX, this could not be done in the present project.

5.6 Observation on the *cis/trans* Citral Peak Pair

The high resolving power of comprehensive two-dimensional GC as a function of the two separation parameters, *viz*. volatility and polarity, is able to isolate compounds with very similar characteristics. Compounds of the same volatility but with slight polarity differences are easily resolved. The use of a chiral second dimension column will also help to separate enantiomers [27]. From the beginning of this project, we observed two peaks with first

[6] Taking sample 6 as a reference (it is an export quality oil), no new peaks were observed in any of the other lemongrass (*Cymbopogon citratus*) essential oils that have not been seen in sample 6.

dimension retention times similar to those of the *cis* and *trans* citral peak pair in lemongrass. We were keen to find out whether those peaks were possibly the result of a continuous keto-enol tautomerism process that was made visible by the very fast second dimension analysis. Such a transformation would be invisible in a slow GC run if it happened at a rate much faster than the GC retention time, as the GC peak would merely reflect the average movement of the two isomers. These peaks were not observed during the GC-TOFMS analyses done for the identification of the essential oil components, due to their overlap with the more abundant *cis/trans* citral peaks on the non-polar DB-1 column. To further investigate this, a standard of pure *cis* and *trans* citral was analysed, and the results showed the same peak pairs on the GC×GC (figure (5.5)). However, we still had our reservations and became more doubtful when a new *cis/trans* citral standard (95% citral, Sigma Aldrich) was analysed and gave the two major *cis* and *trans* citral peaks only. If those peaks were not the keto-enol form of citral, the question as to their identity arose.

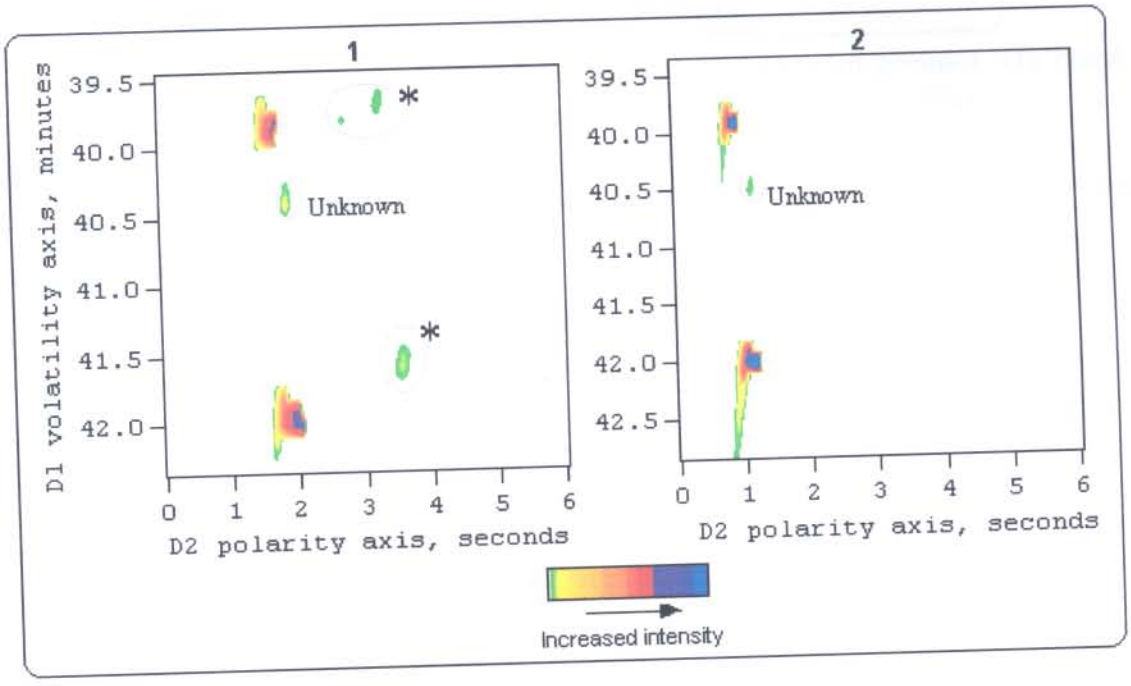

Figure 5.5 *cis/trans*-Citral standard: (**1**) Old standard, (*) Extra peaks in old *cis/trans* citral standard and (**2**) New standard without the extra peaks.

The volatility of the two alcohols, nerol and geraniol, is very close to that of the *cis*-citral (neral) and *trans*-citral (geranial), respectively. Therefore, a standard of these alcohols was

co-injected with the new *cis/trans* citral standard to determine if the alcohol peaks elute in the same position as the peaks obtained from the old *cis/trans* citral standard (figure (5.6)).

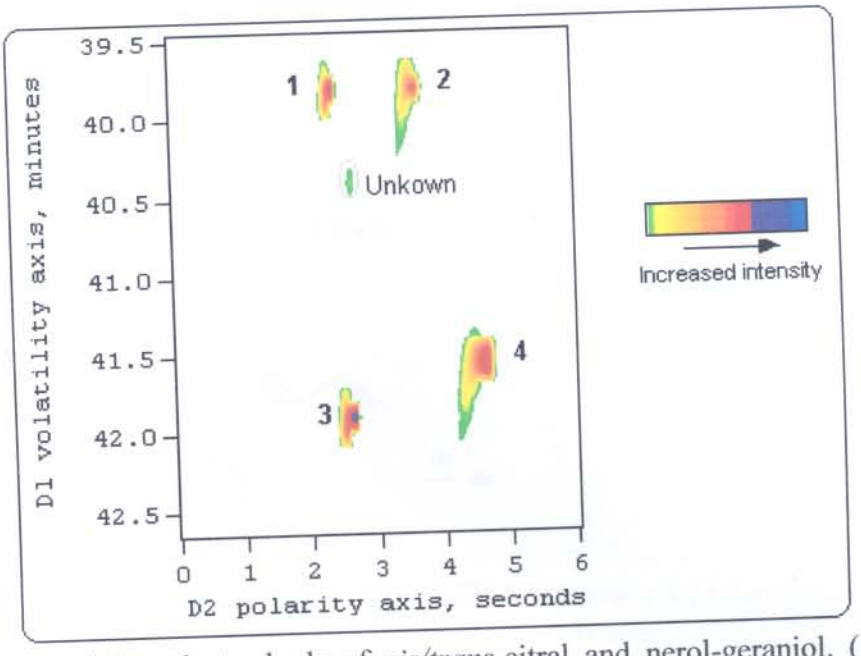

Figure 5.6 Co-injected standards of *cis/trans*-citral and nerol-geraniol. (1) Neral, (2) Nerol, (3) Geranial and (4) Geraniol. Second dimension column is Rtx-Wax.

As expected, the alcohol peaks eluted at the same retention times as the unknown peaks in the old *cis/trans* citral standard. These peaks in the lemongrass essential oil sample were identified as nerol and geraniol, respectively, by GC×GC-TOFMS. Nerol and geraniol have indeed been identified in the essential oil of lemongrass [6, 14, 15]. The fact that these peaks cannot be seen in a conventional GC on a DB-1 column, clearly demonstrates the power of GC×GC for essential oil analysis. Normally, *cis/trans* citral is catalytically hydrogenated to give these alcohols [28] as in figure (5.7).

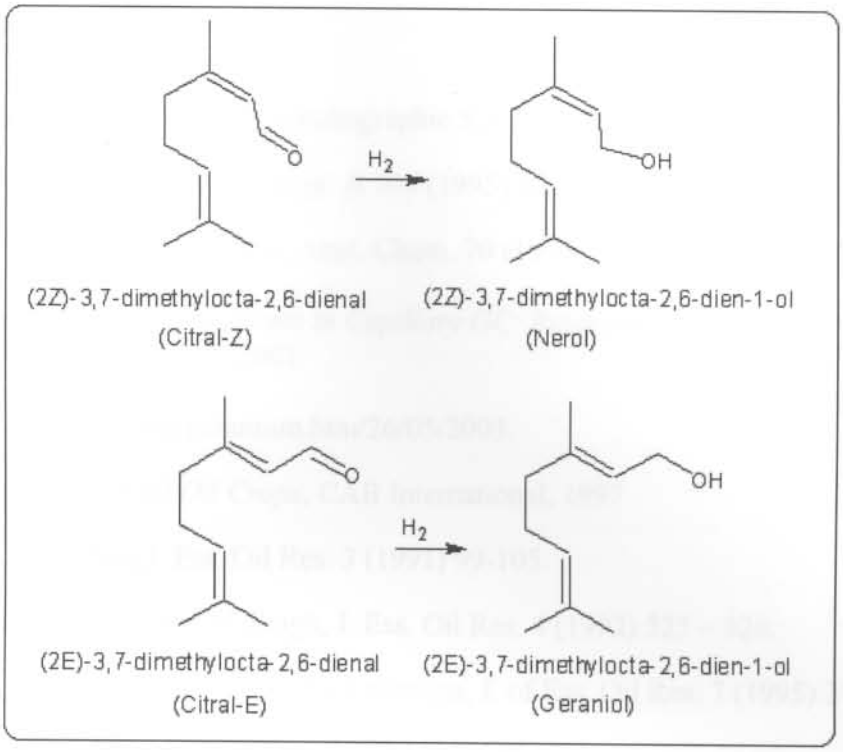

Figure 5.7 Catalytic hydrogenation of *cis* and *trans*-citral to nerol and geraniol, respectively [28].

Without running the pure standard of *cis/trans* citral, it could be considered that the partial reduction to nerol and geraniol was an artefact produced by the hydrogen carrier in the hot injector. Obtaining only the aldehyde peaks from the pure standard disproves this argument and proves that the small amounts of nerol and geraniol are indeed contained in the lemongrass essential oil (and in the impure old standard).

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