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Comprehensive two-dimensional gas chromatography, retention indices and time-of-flight mass spectra of flavonoids and chalcones

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ABSTRACT

The applicability of comprehensive two-dimensional gas chromatography (GC × GC) for flavonoids analysis was investigated by separation and identification of flavonoids in standards, and a complex matrix natural sample. The modulation temperature was optimized to achieve the best separation and signal enhancement. The separation pattern of trimethylsilyl (TMS) derivatives of flavonoids was compared on two complementary column sets. Whilst the BPX5/BPX50 (NP/P) column set offers better overall separation, BPX50/BPX5 (P/NP) provides better peak shape and sensitivity. Comparison of the identification power of GC × GC-TOFMS against both the NIST05 MS library and a laboratory (created in-house) TOFMS library was carried out on a flavonoid mixture. The basic retention index information on high-performance capillary columns with a non-polar stationary phase was established and database of mass spectra of trimethylsilyl derivatives of flavonoids was compiled. TOFMS coupled to GC × GC enabled satisfactory identification of flavonoids in complex matrix samples at their LOD over a range of 0.5–10 μg/mL. Detection of all compounds was based on full-scan mass spectra and for each compound a characteristic ion was chosen for further quantification. This study shows that GC × GC-TOFMS yields high specificity for flavonoids derived from real natural samples, dark chocolate, propolis, and chrysanthemum.

1. Introduction

Phenolic compounds are one of the most important, numerous and ubiquitous groups of compounds in the plant kingdom. Although, there are more than 8000 different known structures [1], a basic aromatic ring skeleton with one or more hydroxyl groups is a common structural feature, including for instance, simple phenols, phenolic acids, coumarins, flavonoids, stibenes, and tannins [2]. Flavonoids, as the largest ubiquitous group of plant secondary metabolites, make up over half the total phenolics distributed in various foods and medicinal plants. Flavonoids are a group of structurally related compounds with a chromane-type skeleton, with a phenyl substituent in the C_2 or C_3 position. More than 4000 known flavonoids comprise 12 subclasses [3], mainly subdivided into flavone, flavonol, flavanone, flavanol, isoflavone, catechin, chalcone, anthocyanin and so on [4].

Methods for identification of flavonoids are of interest both because of the widespread occurrence of these compounds in different natural products, and their reported potential health benefits, such as antioxidant, anticarcinogenic, antiarteriosclerotic, anti-inflammatory, antimicrobial, antiallergic, and antiaging activities [5–11]. Their potential role in protection against cancer and coronary heart diseases [12] is reported.

In principle, a wide range of analytical methods can be used to determine flavonoids in natural compounds, but it is important to bear in mind that the complexity of the matrix, which generally contains these compounds, makes mandatory the use of separative techniques with high resolving power. The most used techniques have been chromatographic techniques (TLC, CE, HPLC, GC), combined with spectroscopic methods (UV, IR, MS, NMR, NIR) [2]. Currently, the first option for flavonoids analysis is typically HPLC using reversed-phase (RP) methods coupled to DAD or mass spectral detection [13–16]. HPLC-MS, combining efficient separation capacity and structural characterization of MS, is successfully used for natural samples [3,17].

Although HPLC remains the most dominant separation technique for flavonoids, and HPLC can detect flavonoids across one, or more subclass in one run, natural products and mixed diet may contain several or even all subclasses [18]. It is a challenge for single column HPLC, with limited peak capacity to achieve quantitative separation of all flavonoids from each other, and from co-extracted

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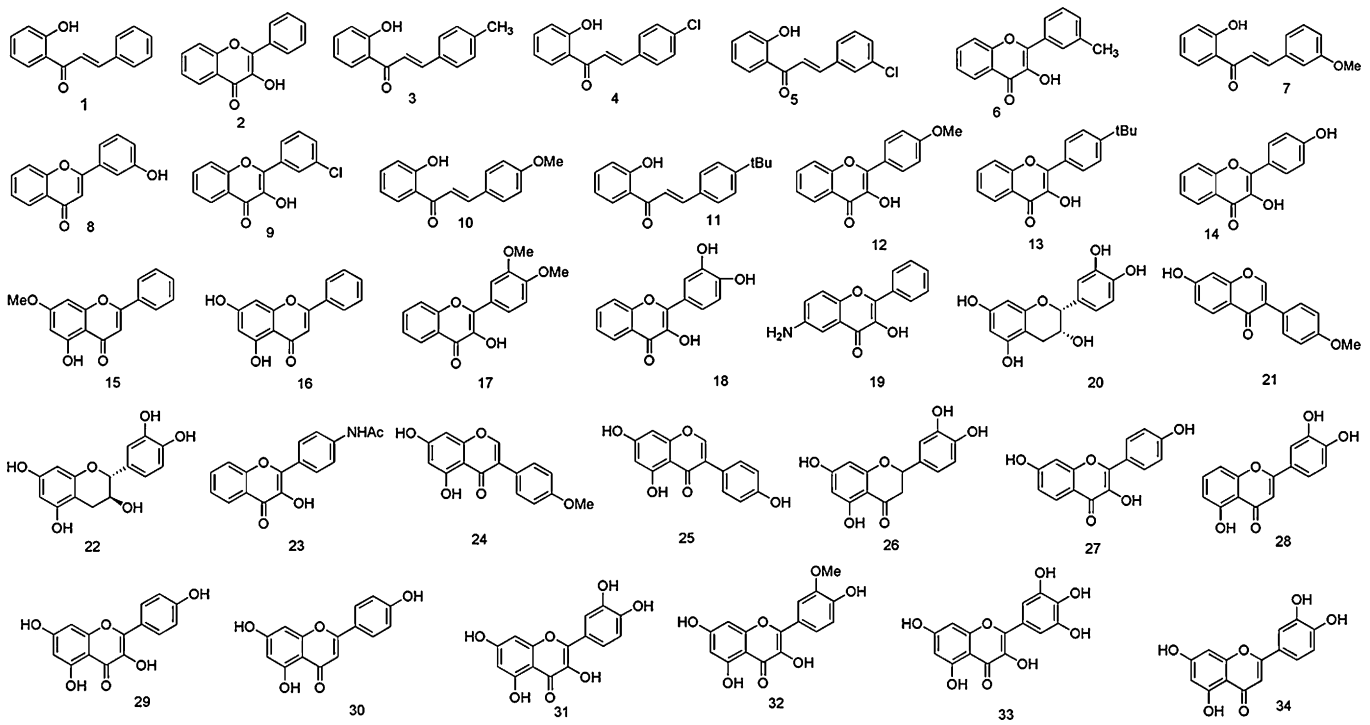

Fig. 1. Structures of flavonoids used in this study. See Table 1 for compound names.

chemical contaminants. This may compromise correct MS identification.

Gas chromatography (GC) with derivatization has been historically used for flavonoids analysis, especially before MS was available with HPLC. Most flavonoids and their metabolites are relatively polar, containing one or more functional groups (–OH, –COOH). Derivatization techniques provide enhanced gas phase volatility and stability for such polar and low volatility constituents. Normally, trimethylsilyl (TMS) derivatives of flavonoids are prepared to decrease polarity and to increase volatility and thermal stability of flavonoids, prior to GC analysis. TMS derivatives of flavonoids are stable, and give informative fragmentation ions under positive electron ionization (EI). The successful separation of TMS derivatives of flavonoids by GC followed by their mass spectrometric identification (GC–MS) has been reported by several researchers [19–21]. The ease of preparation and their excellent GC properties has led to an increased use of trimethylsilyl (TMS) derivatives for polar molecules for many compounds [22–26].

Comprehensive two-dimensional gas chromatography (GC × GC) has proved to offer significant advantages over conventional gas chromatography (1D GC) in terms of greatly increased peak capacity (separation power) and signal-to-noise ratio enhancement, and it also provides unique structured chromatograms when structurally related classes of substances (analogues, congeners, isomers) are analysed.

Briefly, GC × GC separation is based on two columns of distinctly different separation mechanisms, with a suitable interface (called a modulator) located near the coupling of the two columns. Further fractionation of the primary (first column, ^{1}D) separation, occurs on the short second (^{2}D) column, while preserving the previously achieved separation. Through use of a cryofocussing modulator, modulated peaks exhibit increased mass concentration due to the narrow focussed band of the chromatographic peak. Of signal importance to the analyst is the greater peak resolution achieved by GC × GC, effectively within the same time period as a conventional GC analysis, due to fast GC separation on the second column, i.e. within about 3–6 s. These very sharp peaks must be adequately detected, and identified, and for reliable area measurement about ten data points per peak are required [27]. This greater separation power means that many compounds are now completely separated, and this aids the correct MS identification of compounds separated in the GC × GC experiment.

Applications of GC × GC including petrochemicals [28], essential oils [29], amino acids [30], pesticides [31], food analysis [32], and forensic applications [33], amongst others.

The present study investigates the applicability of GC × GC coupled to both flame ionization (GC × GC–FID) and time-of-flight mass spectrometry detectors (GC × GC-TOFMS) for analysis of a wide range of flavonoids including flavones, flavonols, isoflavones, flavanonols, chalcones, and flavan-3-ols comprising a variety of substitution patterns and different numbers of derivatisable hydroxy groups. Selected flavonoid-rich complex natural materials are also analysed, with liquid/liquid extraction of the free flavonoids fraction, and derivatization with *N*,*O*-bis(trimethylsilyl) trifluoroacetamide (BSTFA) performed prior to analysis. Comparison between conventional GC and GC × GC, in terms of separation efficiency, sensitivity and identification power of TOFMS detection, is reported. A mass spectral compilation, a 10-peak MS ion abundance table, and retention indices are provided for all the compounds studied. This study illustrates the potential of GC × GC-TOFMS for flavonoids analysis, and detailed analysis of the complex profiles.

2. Experimental

2.1. Reagents

Analytical-reagent grade methanol and ethanol were purchased from Ajax Finechem Pty. Ltd. (Taren Point, Australia). HCl was from Merck (Darmstadt, Germany). Ultra pure water was supplied by a Milli-Q water purifier system from Millipore (Bedford, MA, USA). The derivatizing reagent was *N*,*O*-bis(trimethylsilyl) trifluoroacetamide (BSTFA) containing 1% trimethylchlorosilane (TMCS), and was obtained from Supelco (Bellefonte, PA, USA) and maintained

at 4 °C when not in use. HPLC grade methanol was from Merck (Darmstadt, Germany).

2.2. Flavonoid compounds

Most of the unnatural flavonoids and chalcones reported herein were prepared in the authors' laboratory and their syntheses have been reported elsewhere. (+)-Catechin (C), (−)-epicatechin (EC), kaempferol, isorhamnetin, myricetin, eriodictyol were from Sigma. The purity of these 34 compounds was higher than 98%, and they were stored at 4 °C until required.

The full list of available flavonoids and some of their precursor chalcones is given in Fig. 1.

Propolis capsules were from Nature's Care Pty. Ltd. and Australia Natural Pharmaceutical Pty. Ltd. Chrysanthemum was from Kambow Wholesaler (Australia), and Just Organic Dark Chocolate from Germany (purchased from a local store). All samples were stored at 4 °C in the dark prior to analysis.

2.3. Standard solutions preparation

Stock solutions (10–100 mg/L) were prepared by dissolving the flavonoid and chalcone reference compounds in methanol. They were kept at 4 °C in dark bottles sealed with PTFE/silicone caps.

2.4. Sample preparation and derivatization

Propolis, chrysanthemum and dark chocolate samples (approximately 1 g, weighed with analytical precision) were extracted with 45 mL of 70% aqueous ethanol (adjusted to pH 2 with concentrated HCl) using ultrasound assisted extraction for 60 min. The extraction mixtures were decanted into 50 mL plastic centrifuge tubes and centrifuged at 5000 rpm for 10 min. The upper layer was concentrated to 10 mL under reduced pressure in a rotary evaporator at 45 °C and then extracted with 30 mL (3 × 10 mL) ethyl acetate. The organic layer was collected and dried on a vacuum evaporator at 45 °C. The residue was dissolved with HPLC grade methanol to 10 mL as stock solutions. 100 μL aliquots of the methanol stock solution were dried by evaporation of the solvent under a nitrogen stream at 45 °C and then derivatized. For the silylation procedure, 50 μL pyridine and 50 μL BSTFA (containing 1% TMCS) were added to the dried extracts and the mixture heated at 100 °C for 30 min. This solution was directly used for GC analysis.

2.5. Instrumental analysis

2.5.1. GC-quadrupole MS analysis on a NP column

Single column GC–qMS analysis was first conducted on a 6890 GC and a 5973 MS system from Agilent Technologies (Nunawading, Australia). A BPX5 column (5% phenyl methylpolysilphenylene-siloxane; SGE Scientific, Ringwood, Australia) of dimensions 32 m × 0.25 mm ID × 0.25 μm film thickness (d_f) was used. The temperature program was 100 °C hold 1 min, heated at 30 °C/min to 210 °C hold 1 min, then 2 °C/min to 240 °C, 4 °C/min to 270 °C, then 5 °C/min to 310 °C, hold 5 min. Temperature of injector was set at 310 °C and 2 μL of the sample was injected in splitless mode with carrier gas (He) flow rate of 1.5 mL/min. The transfer line was at 280 °C and ion source at 230 °C, with electron ionisation (EI) mode set at 70 eV, and mass range 45–760 Da, and data collection rate of 20 Hz. Data acquisition and processing were performed by using Agilent MSD ChemStation software.

2.5.2. GC × GC-FID

The GC × GC experiment was conducted on an Agilent 6890 system (Palo Alto, CA, USA) equipped with a flame ionization detector (GC × GC-FID) retrofitted with a longitudinally-modulated cryogenic system (LMCS; Chromatography Concepts Pty. Ltd., Doncaster, Australia). Two complementary column configurations were used for flavonoid separation and identification in this experiment: a non-polar/polar phase set (NP/P), and a P/NP phase set for first (^{1}D) and second (^{2}D) dimensions, respectively. For the former, the ^{1}D NP column phase was a BPX5 phase of dimensions 30 m × 0.25 mm ID × 0.25 μm d_f, and the ^{2}D P column was a BPX50 phase (50% phenyl) of dimensions 1.0 m × 0.1 mm ID × 0.1 μm d_f, For the P/NP set, the ^{1}D and ^{2}D columns had the same dimensions as above, with P and NP phases of BPX50 and BPX5, respectively. The columns were from SGE Scientific (Ringwood, Australia). The injector and detector temperatures were 310 °C, and the sampling frequency was 100 Hz. 1 μL of the sample was injected in splitless mode. The modulation period was set to 4 s, 5 s or 6 s, and temperature of the modulator system (T_M) was varied stepwise from 0 to 150 °C during the optimization study. CO_2 was used as a coolant in the LMCS and nitrogen as a flush gas at a pressure of 15 psi. Agilent ChemStation software was used for data acquisition and processing.

2.5.2.1. Non-polar/polar column configuration (NP/P), BPX5/BPX50. The oven program for the NP/P column set was from 100 °C (hold for 1 min) to 180 °C at 20 °C/min, then to 326 °C at 3 °C/min. Hydrogen was used as a carrier gas at a flow rate of 1.2 mL/min.

2.5.2.2. Polar/non-polar column configuration (P/NP), BPX50/BPX5. The oven program for the P/NP column set was from 100 °C (hold for 1 min) to 210 °C at 30 °C/min, then to 234 °C at 3 °C/min, to 266 °C at 2 °C/min, and finally to 310 °C at 3 °C/min. Hydrogen was used as a carrier gas at a flow rate of 0.8 mL/min.

2.6. GC × GC-TOFMS

A LECO time-of-flight (TOF) mass spectrometer model Pegasus III (LECO Corp., St. Joseph, MI, USA) connected to an Agilent 6890 GC was used in GC × GC-TOFMS experiments. The modulator was a LMCS system, and subsequent to optimization, the cryotrap was operated isothermally at 120 °C with a modulation period of 4 s.

The column configuration used in this experiment was the 30 m BPX50 (0.25 mm ID; 0.25 μm d_f) as ^{1}D and 1 m BPX5 (0.1 mm ID; 0.1 μm d_f) as ^{2}D column set. Oven program for this column configuration was from 100 °C for 1 min, increased to 210 °C at 20 °C/min then to 320 °C at a rate of 2 °C/min. Helium was used as a carrier gas at a flow rate of 1.5 mL/min. Injector temperature was set at 310 °C, 1 μL sample volumes were injected in splitless mode. The transfer line T was 280 °C, the ion source set point T was 230 °C, the detector voltage was 1600 V, the filament bias applied EI voltage was 70 eV, and the data presentation rate was 100 Hz (spectra/s) over the mass range 45–760 Da. A separate GC × GC-TOFMS based library was generated for identification purposes. Data acquisition and processing were performed by ChromaTOF software (LECO).

3. Results and discussion

3.1. Derivatization of flavonoids

Trimethylsilyl derivatives are routinely employed in GC to increase the volatility and thermal stability of organic compounds containing active hydrogen. The combination of BSTFA and 1% TMCS (trimethylchlorosilane) is the preferred reagent for trimethylsilylation of organic compounds with OH groups. The efficiency of the derivatization procedure was investigated by analysing the individual flavonoids on the GC–qMS system. All of the flavones and isoflavones, chalcones, flavan-3-ols, flavonols (except 4′-acetamido-flavonol) and except the flavanonol eriodictyol presented single products with no measurable amount of

partially derivatised or underivatized products under the procedure employed.

The presence of two peaks for TMS derivatives of eriodictyol has been reported previously and was discussed in terms of a interconversion between flavanone and the corresponding chalcone. Two products of flavanonol **26** (tetra-TMS, characteristic ion, *m*/*z* 561, and penta-TMS, characteristic ion *m*/*z* 633), were observed in the presence of excess of silylating reagents at 100 °C for 30 min. The peak for the chalcone derivatives became more abundant upon application of higher temperature or with longer reaction time.

4′-Acetamido-flavonol **23** gave two products (bis-TMS, characteristic ion, *m*/*z* 424; and bis-TMS, then possibly loss of NAc, to give a characteristic ion *m*/*z* 382), with the peak (characteristic ion *m*/*z* 382) favoured in an approximate 3:1 ratio for derivatisation at 80 °C for 30 min. When the temperature and/or time of derivatization are increased the ratio is increased at 120 °C for 30 min, only the peak with characteristic ion *m*/*z* 382 was observed. But the bis-TMS, and the proposed NAc loss (*m*/*z* 382) was detected in measurable amounts under all the experimental conditions applied.

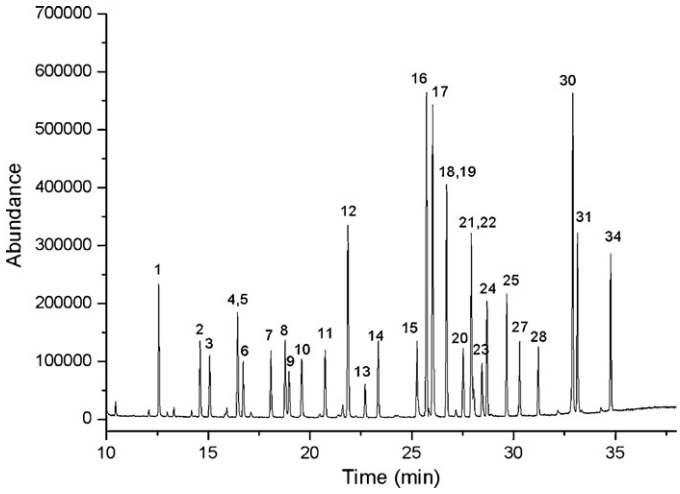

Fig. 2. Chromatogram of 30 TMS derivatized flavonoids on the 32 m BPX5 column using GC–qMS.

3.2. GC–qMS separation and identification

The standard mixture of flavonoid trimethylsilyl ethers at a concentration of 4–30 μg/mL were analyzed on the 32 m long BPX5 column. The GC analysis achieved for the 30 flavonoid aglycones in a relatively short run time is shown in Fig. 2.

Three co-elutions were observed; **4**/**5** (4-chloro-2′-hydroxychalcone/5-chloro-2′-hydroxychalcone, which are chalcone positional isomers), **18**/**19** (3′, 4′-dihydroxyflavonol/6-aminoflavonol), and **21**/**22** (7-hydroxy-4′methoxy isoflavone/catechin). Especially, **18** and **19** are totally overlapped, and under conditions of varying temperature program, they still showed one peak. However, **18** and **19**, and **21** and **22** could be separated by GC × GC (see later).

Table 1
Retention indices and mass spectra of derivatized flavonoids on BPX5 column.

Comp. no.	Compound name	No. of TMS groups[a]	Base ion[b]	RMM of TMS derivatives[c]	Ret Index[d]
1	2′-Hydroxychalcone	1	281	296	2216 ± 1
2	favonol	1	295	310	2311 ± 1
3	2′-Hydroxy-4-methylchalcone	1	295	310	2330 ± 3
4	4-Chloro-2′-hydroxychalcone	1	315	330	2387 ± 2
5	5-Chloro-2′-hydroxychalcone	1	315	330	2387 ± 2
6	5′-Methylflavonol	1	309	324	2401 ± 1
7	2′-Hydroxy-5-methoxychalcone	1	311	326	2454 ± 3
8	5-Hydroxyflavone	1	295	310	2480 ± 1
9	5′-Chloroflavonol	1	329	344	2487 ± 2
10	2′-hydroxy-4-Methoxychalcone	1	311	326	2508 ± 2
11	2′-Hydroxy-4-tert-butylchalcone	1	337	352	2539 ± 1
12	4′-Methoxylflavonol	1	325	340	2591 ± 3
13	4′-Tert-butylflavonol	1	351	366	2627 ± 3
14	4′-Hydroxyflavonol	2	383	398	2654 ± 2
15	Tectochrysin	1	325	340	2735 ± 1
16	chrysin	2	383	398	2755 ± 2
17	3′,4′-Dimethoxyflavonol	1	355	370	2769 ± 1
18	3′,4′-Dihydroxyflavonol	2	471	486	2800 ± 2
19	6-Aminoflavonol	2	382	397	2801 ± 3
20	epicatechin	5	368	650	2839 ± 1
21	7-Hydroxy-4′methoxy isoflavone	1	340	340	2855 ± 2
22	Catechin	5	368	650	2861 ± 1
23	4′-Acetamido-flavonol	2	424	439	2882 ± 1
24	4′-Methylgenistein	2	413	428	2896 ± 1
25	genistein	3	471	486	2951 ± 4
26-1	2′,4′,6′,3,4-Pentahydroxychalcone	5	633	648	2957 ± 4
26	Eriodictyol	4	561	576	2968 ± 5
27	Resokaempferol	3	471	486	2984 ± 2
28	5,3′,4′-Trihydroxyflavone	3	471	486	3040 ± 4
29	Kaempferol	4	559	574	3051 ± 4
30	Apigenin	3	471	486	3146 ± 2
31	Quercetin	5	647	662	3163 ± 1
32	Isorhamnetin	4	589	604	3177 ± 1
33	Myricetin	6	735	750	3213 ± 3
34	Luteolin	4	559	574	3278 ± 2

[a] Number of TMS groups of derivatives.
[b] Characteristic *m*/*z* ion.
[c] Relative molar mass of derivatives.
[d] Retention index.

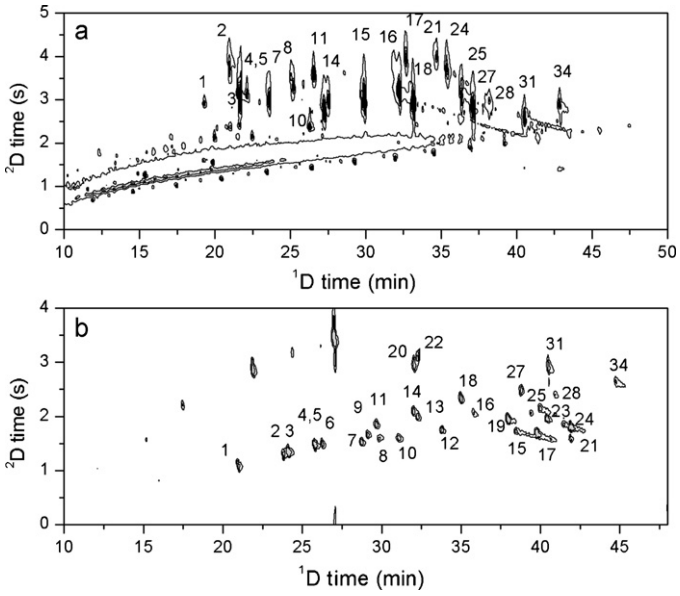

Fig. 3. GC × GC-FID contour plot of the TMS derivatized flavonoids on the NP/P and P/NP column sets. (a) 23 BSTFA derivatised flavonoids on a 30 m BPX5/1 m BPX50 (NP/P) column set. (b) 30 BSTFA derivatised flavonoids on a 30 m BPX50/1 m BPX5 (P/NP) column set.

Hexane solutions of *n*-alkanes C21–C40 were analysed under the above conditions. Linear temperature programmed retention indices (LTPRI) were calculated from the GC results of the separation of these mixtures and/or reaction mixtures of individual flavonoids as shown in Table 1. Linear interpolation within the alkane standard mixture according to the method of van den Dool and Katz was used, as reported in Girard's review [34]. Table 1 also provides the mass spectral base peak ion, molar mass and number of derivatised hydroxyl groups for each compound.

Identification of the components was initially attempted with the aid of the automatic system of processing GC–MS data supplied by NIST (US National Institute of Standards and Technology) and Wiley mass spectra libraries. However it was found in the process of GC–MS identification of the products of TMS derivatives that mass spectra are not available for all compounds in these libraries. Only three flavonoids (**1**, **20**, and **22**) in the GC–qMS chromatogram were identified against the NIST library at a match threshold of approximately 700. An in-house library was set up for all the compounds by injection of individual TMS flavonoids at a concentration of 4–30 μg/mL. In order to partially fill this significant information gap, mass spectra of all compounds are reported and presented in Supporting Information using TOFMS analysis.

3.3. GC × GC-FID separation and identification

The GC × GC separation of the standard mixture of flavonoids was initially accomplished by GC × GC-FID, investigating the two column configurations NP/P and P/NP, employing 5% phenyl- and 50% phenyl-methylpolysilphenylene-siloxane as the respective non-polar and polar phases, which are of adequate thermal stability and are suitable for use with derivatized samples. Whilst the BPX5/BPX50 column set offers reasonable overall separation but with rather broad peaks, the BPX50/BPX5 column set showed very narrow peaks with good peak shape and an acceptable spread over the two-dimensional separation space for the flavonoids samples (shown in Fig. 3). (Note that in Fig. 3a, contour plot shows a band eluting along the first dimension (^2t_R = 1–2 s), which also was observed in the derivatized reagent blank. Phase bleed (siloxanes) maybe be a possible cause of this.) This latter P/NP column

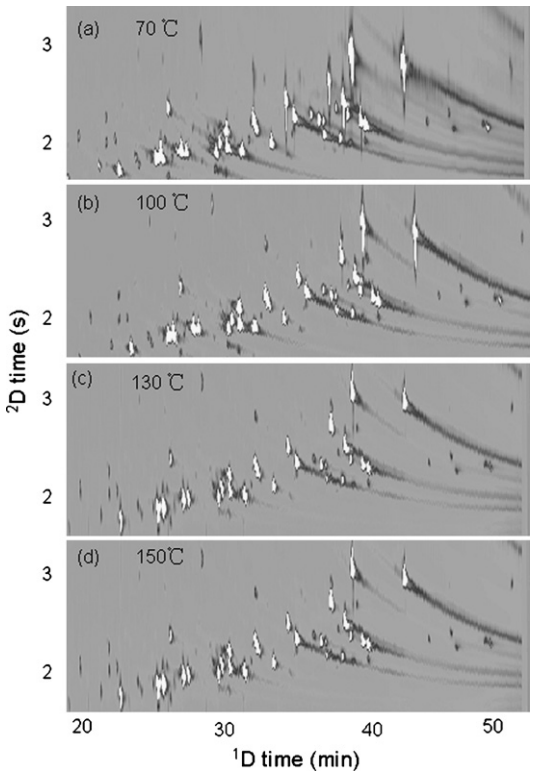

Fig. 4. GC × GC-FID contour plots showing the separation of 28 flavonoids using different isothermal modulation temperatures, T_M. (a) 70 °C; (b) 100 °C; (c) 130 °C; (d) 150 °C (30 m BPX50/1 m BPX5 (P/NP) column set).

set was applied for subsequent studies, and the modulation period and modulator temperature (T_M) were initially optimized. Comparison of modulation periods of 4 s, 5 s and 6 s revealed that peaks all eluted at ^2t_R < 4 s, and so a P_M value of 4 s is adequate.

Mitrevski et al. [33] reported that the peak shape of sterol BSTFA derivatives on the ^{2}D column depends on the T_M. Using the P/NP column set, the T_M study involved analyzing a standard mixture of flavonoids at isothermal modulator temperatures of 70, 100, 130, 150 °C presented in Fig. 4. The narrowest peak width at half height ($w_{1/2}$), for the first eluted compound **1**, with retention time of 21.5 min is obtained at 70 °C, but for the last eluted compound luteolin **34** with retention time of 45 min, the best T_M is found to be 150 °C and results in the narrowest peak. For the rest of the flavonoids the T_M producing narrowest peak widths tends to increment proportionally with increased retention times of the flavonoids. By applying a T_M program from 70 to 150 °C incremented in four steps by 20 °C, good separation for all the flavonoids was achieved as shown previously in Fig. 3b.

From the above results, a compromise isothermal T_M, was set at 120 °C, in order to avoid the necessity of manual adjustment of T_M while running batch samples.

3.4. GC × GC-TOFMS separation and identification of derivatised flavonoids on BPX50/BPX5 column set

Although GC × GC-FID is an ideal analysis method for good separation in complex matrices, it does not provide structural information for identification/confirmation of the analytes. Comprehensive 2D GC combined with TOFMS detection (GC × GC-TOFMS) demonstrates simultaneous chromatographic separation as well as providing mass spectrometric data to increase the reliability of identification of the compounds against either commercial or in-house libraries. Major ions for each compound are presented

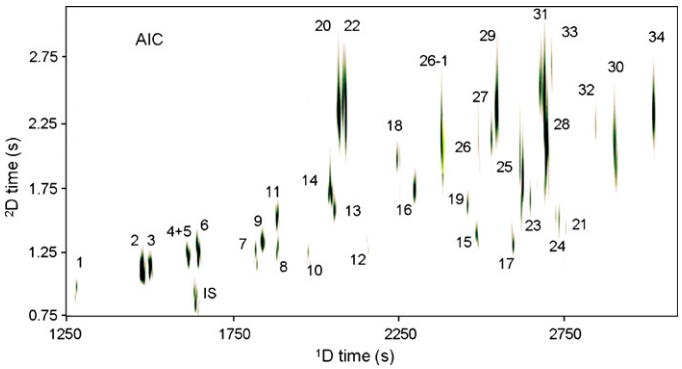

Fig. 5. GC × GC-TOFMS separation of 34 derivatized flavonoid mixture on BPX50-BPX5 column set (2D analytical ion chromatogram apex ion current or analytical ion current (AIC) plot.

in Supporting Information (Table S1) along with their corresponding mass spectra (Supporting Information Fig. S2).

Since mass spectral selectivity is determined by choice of the proper extracted ion(s) from the full mass spectra offered by TOFMS detection as established by spectral information, this parameter is equally relevant to both 1D and 2D operation.

According to prior GC × GC-FID knowledge, the BPX50-BPX5 P/NP column set was applied in subsequent GC × GC-TOFMS experiments. A 2D plot of the mixture of silylated flavonoids is displayed in Fig. 5. Table 2 reports the ^{1}D and ^{2}D retention times, and a GC × GC-TOFMS in-house mass spectral library match of all BSTFA-derivatized flavonoids is presented for the TOFMS spectra. This in-house library of authentic standards was tested for searching and identification purposes in subsequent experiments. Library spectra were recorded using a mixture of standard flavonoids at a concentration of 2–15 μg/mL, except for the internal standard (I.S), flavone (50 μg/mL).

The LOD quoted in Table 2 is for a minimum library match of 700, and often the library match value is significantly greater than this. The S/N ratio is uniformly much greater than a value of 3 which is a commonly accepted definition of LOD. However, if the library match is less than 700, even if the S/N > 3, the higher S/N value is taken as LOD. Use of a high mass quantification ion can lead to reduced sensitivity (see later, Section 3.4.2) due to reduced abundance for high mass ions. These effects lead to an apparently larger LOD than might be otherwise accepted.

The separation of the standard mixture of BSTFA derivatized flavonoids on a BPX50-BPX5 column configuration with no co-elution except **4** and **5** is presented in Fig. 5.

Three co-elutions were observed; **4**/**5**, **18**/**19**, and **21**/**22** using GC–qMS on a BPX5 column as shown in Fig. 2. However, **18** and **19**, and **21** and **22** could be separated by GC × GC-TOFMS, presumably due to the use of the polar BPX50 first dimension column here. However, chalcone positional isomers, **4** and **5** still co-eluted on either phase column. Alternative column sets will be required if separation of **4** and **5** is sought, but this will require a shape selective column such as a chiral or liquid crystal phase. Note that compounds **21** and **24** which have similar structures elute relatively near on the BPX5 column (Fig. 2) but are apparently strongly retained on the BPX50 phase (Fig. 5), and much later than component **22**. This is consistent for these two compounds.

3.4.1. The retention order of TMS flavonoids on BPX50/BPX5 column set

The 2D separation space is a chemical map of compound properties, which derive from the ability of the two columns to impose their individual separation mechanisms on the sample components. For the ^{2}D BPX5 phase column, a conventional non-polar phase, separation mechanism is largely based on the volatility (i.e. boiling-point (BP)). However, for the ^{1}D BPX50 (medium)-polarity phase column, separation will be primarily governed by the specific interaction(s) of the selected 'polar' column, but to some degree also by volatility. Hence, the location of peaks in 2D space provides some measure of the orthogonality of their separation by having two dissimilar columns possessing different separation mechanisms.

The number and position of hydroxyl groups, the number and position of other substituents, and the overall mass of flavonoids all have an effect on retention. Koupai-Abyazani and coworkers [35] summarized some rules regarding the influence of parameters on the retention order of TMS derivatised flavonoids on a non-polar RSL 200 BP column using GC-FID. In the present study, a large number of flavonoids were investigated on BPX50/BPX5 using GC × GC-TOFMS, and many compounds present were not included in Koupai-Abyazani's paper. Here, an attempt is made to analyze and discuss the results to supplement the general conclusions of Koupai-Abyazani on the influence that structure has on retention.

3.4.1.1. The influence of number of derivatised hydroxy groups on ^{1}D and ^{2}D separation. Koupai-Abyazani et al. reported the analysis of TMS derivatised flavonoids on a non-polar RSL 200 BP column using GC-FID, demonstrating that the retention times of the compounds in a particular group of flavonoids increase with the number of TMS ether groups [36]. In our case, the number of TMS groups revealed similar affects both on ^{1}D and ^{2}D relative position.

For instance, flavonols which all have aromatic rings, when separated on a ^{1}D BPX50 column, the retention order arises mostly according to the boiling points of the compounds, regardless of the aromatic (π–π) interactions, and this should correlate with number of derivatised groups. Hence, the elution order for TMS derivatives of flavonols are as follows, flavonol < 4′-hydroxyflavonol < 4′,7-dihydroxyflavonol < 4′,5,7-trihydroxyflavonol < 3′,4′,5,7-tetrahydroxyflavonol < 3′,4′,5,5′,7-pentahydroxyflavonol (i.e. 2 < 14 < 27 < 29 < 31 < 33) as shown in Table 2. The average ^{2}D retention for derivatized flavonoids with one through six hydroxy groups (and no other substituents) is 1.09 s, 1.53 s, 1.99 s, 2.12 s, 2.45 s, and 2.61 s, respectively.

3.4.1.2. The effect of chemical structure of flavonoids with various substituents on the A or B ring besides the hydroxyl group. Besides the hydroxyl group, methyl, methoxyl, *tert*-Bu (*t*-Bu), halogen, and acetamido were also investigated as substituents, and they also affect the retention order. By comparing the ^1t_R and ^2t_R of TMS derivatives as the flavonoids skeleton remains constant, it was found that flavonols with methoxyl and *t*-Bu substituent (compounds **12** and **13**, respectively) showed a reversal of elution order of their ^{1}D and ^{2}D separation, i.e. ^1t_R methoxyl > ^1t_R *t*-Bu, however ^2t_R methoxyl < ^2t_R *t*-Bu. The chalcones with methoxyl and *t*-Bu substituent **10** and **11** showed the same reversed ^{2}D separation (Table 2). In principle, compared with methoxyl (–OCH_3, MW 31), *t*-Bu (–$C(CH_3)_3$, MW 57) is more organic and less polar. Since the BPX50–BPX5 column set comprises a higher polarity first column and low polarity second column, the less polar flavonoid of this group should elute earlier in ^{1}D and later in ^{2}D.

Flavonols with hydroxyl, methoxyl and *t*-Bu were further compared. Taking the example of **14** (4′-hydroxyflavonol: ^1t_R = 1988s; ^2t_R = 1.700 s), **13** (4′-*tert*-butylflavonol: 2000s; 1.630 s) and **12** (4′-methoxylflavonol: 2092 s; 1.320 s), **12** has the greater ^{1}D retention time but shorter ^{2}D retention time. The TMS derivative of **12** is assumed to be more polar and more volatile since it elutes later on the polar BPX50 ^{1}D phase but earlier on the non-polar BPX5 ^{2}D phase. Note however that the interplay of polarity and volatility is a complex combination of compound and stationary

Table 2
Peak table and LOD of flavonoids using GC × GC-TOFMS on BPX50-BPX5 column set, with S/N for selected *m*/*z* ion value, match quality. For component names, refer to Table 1.

Comp. no.	R.T. (s)[a]	LOD (μg/mL)[b]	Quant. ion *m*/*z*	Quant. ion S/N ratio	Match[c]
1	1280, 0.970	0.51	281	50	856
2	1460, 1.090	0.27	295	64	800
3	1484, 1.110	0.51	295	30	750
4	1592, 1.170	0.96	315	110	856
5	1592, 1.170	1.37	315	110	856
6	1620, 1.190	1.16	309	205	807
7	1780, 1.240	1.61	311	78	767
8	1840, 1.270	0.96	295	30	745
9	1800, 1.300	1.16	329	124	833
10	1928, 1.270	1.51	311	44	790
11	1840, 1.530	1.61	337	117	773
12	2092, 1.320	0.55	325	30	734
13	2000, 1.630	0.96	351	97	736
14	1988, 1.700	1.6	383	216	855
15	2400, 1.370	6.02	325	12	720
16	2228, 1.710	2.43	383	19	760
17	2500, 1.300	1.1	355	47	730
18	2180, 1.970	1.64	471	153	787
19	2372, 1.640	2.47	382	67	834
20	2016, 2.290	1.72	368	24	805
21	2648, 1.400	1.1	340	45	865
22	2032, 2.240	2.44	368	21	935
23	2544, 1.650	7.4	382	60	800
24	2632, 1.460	1.14	413	48	750
25	2528, 1.740	1.37	471	223	873
26-1	2308, 2.030	6.46	633	12	879
26	2412, 1.940		561	50	826
27	2444, 2.210	1.1	471	80	744
28	2596, 2.040	4.11	471	145	786
29	2452, 2.220	3.81	559	22	890
30	2788, 2.000	7.96	471	60	849
31	2584, 2.450	4.23	647	15	855
32	2736, 2.180	6.2	589	42	732
33	2612, 2.610	8.08	735	20	785
34	2900, 2.200	5.1	559	78	980

[a] Retention times given in order of first then second dimension times.
[b] LOD concentration reported is the lowest concentration of mixture standards for which match statistic exceeds 700, and is reported for the quantification ion mass given in the table.
[c] Match (similarity) statistic reported is at their LOD.

phase properties, and this interpretation is not expected to be definitive.

3.4.1.3. The effect of flavonoid skeleton. Koupai-Abyazani et al. [37] reported that when the substitution pattern on the flavonoid skeleton remains constant, the observed elution order is: flavanone < isoflavone, flavonol < flavone on a non-polar column. In the present case, chalcones and flavan-3-ols were also examined. For instance, a comparison of hydroxy chalcone (**1**: 1280:s; 0.970 s), flavonol (**2**: 1460:s; 1.090 s) and flavone (**8**: 1840:s; 1.270 s), suggested that the elution order on both BPX50 and BPX5 are chalcone < flavonol < flavone. The retention time of **10**, **12** and **15** further support this conclusion. A comparison of catechin **22** (2032 s; 2.240 s), the corresponding chalcone of eriodictyol **26-1** (*m*/*z* 633: 2308 s; 2.030 s), and quercetin **31** (2584 s; 2.450 s), each of which possess five hydroxyl groups, suggests that the elution order on BPX50 and BPX5 both are flavan-3-ol < chalcone < flavonol. By further comparing the corresponding chalcone of eriodictyol **26-1** (*m*/*z* 633: 2308 s; 2.030 s, 5 OH groups), eriodictyol **26** (*m*/*z* 561: 2412 s; 1.940 s, 4 OH groups), and according to the literature [35], *m*/*z* 561 presented at a slightly greater retention time. This result was in good agreement with the result on the BPX5 column using GC–qMS. Isidorov and Szczepaniak [19] presented retention indices on a HP-5MS phase column for pinostrobin chalcone (2′,6′-dihydroxy-4′-methoxychalcone), pinocembrin (5,7-dihydroxyflavanone), 2′,4′,6-trihydroxy-4′-methoxychalcone, and naringenin (5,7,4′-trihydroxyflavanone), giving 2457, 2550, 2870, and 2897, respectively. This shows that chalcones should elute earlier than flavanones bearing the same number of OH groups on both mid-polarity and non-polar columns. Through the above comprehensive analysis, a primary conclusion is obtained that retention order of TMS silylated flavonoids with the same substituents on both BPX50 and BPX5 columns is flavan-3-ol < chalcone < flavonone < isoflavone < flavonol < flavone.

3.4.2. Mass spectral fragmentation of qMS vs.TOFMS

Time-of-flight and quadrupole mass analyzers are both important detectors for analysis and identification, and both may be applied in the GC field.

In both mass spectral systems, the molecular ions are weak or absent with the exception of 7-hydroxy-4′-methoxyisoflavone. In the qMS spectra of TMS derivatives, the $[M-CH_3]^+$ ion peak, commonly encountered for TMS derivatives as base or characteristic ion were obtained. Ferrer et al. [38] suggested an explanation for flavonoids to exhibit the M−15 ion as the base ion in the mass spectrum; it most likely arises from the close proximity of the trimethylsilyl group and the carbonyl group, promoting the loss of one methyl group in the EI source. These results may provide useful information for pre-directing the base peak of flavonoid TMS esters according to the OH group position, and its proximity to the carbonyl group.

In the TOFMS spectra, the $[M-15]^+$ ion may be formed as the base peak for TMS derivatives, with lower abundance of the molecular mass ion. However, a reduced intensity with increasing molecular mass and with a correspondingly more abundant trimethylsilyl ion at *m*/*z* 73 is seen in Fig. 6 for a range of compounds.

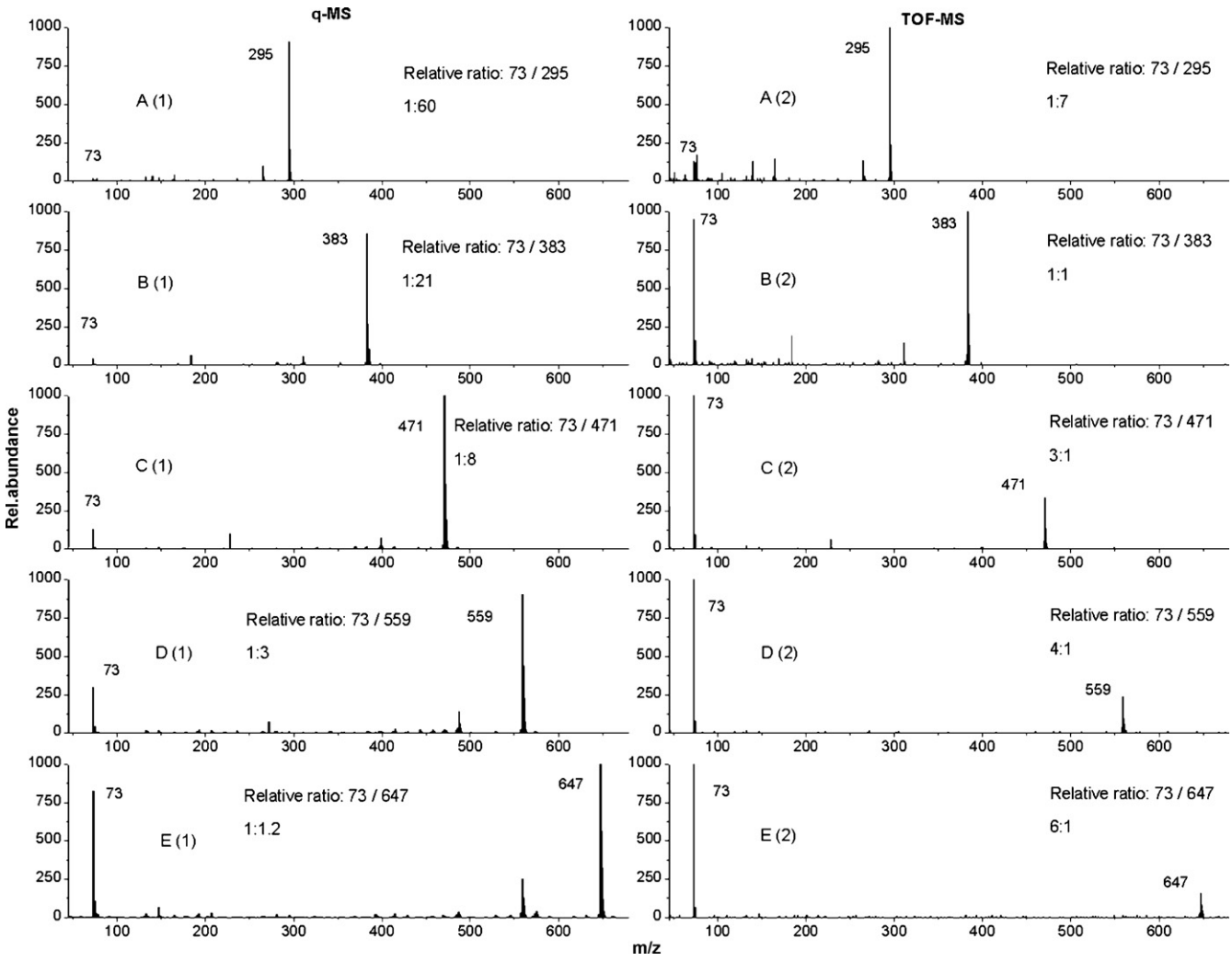

Fig. 6. Comparisons of TOFMS spectra and qMS spectra of typical respective compounds recorded at concentrations between 2-15 μg/mL. (1) q-MS, (2) TOFMS. (A) **2**, flavonol; (B) **14**, 4′-hydroxyflavonol; (C) **25**, genistein; (D) **29**, kaempferol; (E) **31**, quercetin.

The relative ratio of *m*/*z* 73 to the characteristic ion for compounds **2**, **14**, **25**, **29**, and **31** are 1:7, 1:1, 3:1, 4:1, and 6:1, respectively, as shown in Fig. 6A (2) to E (2).

In the qMS, although the characteristic peak is present most often as the base peak, the relative ratio of *m*/*z* 73 to the characteristic ion shows the same increasing trends, such that for compounds **2**, **14**, **25**, **29**, and **31** the ratio is 1:60, 1:21, 1:8, 1:3, and 1:1.2, respectively, as shown in Fig. 6A (1) to E (1).

Prior work on sterols analysis reported by Mitrevski et al. [39] also confirmed this difference in qMS and TOFMS data at higher mass; TOFMS ions in the system used here suffer a reduced ion abundance compared to lower mass ions in the spectrum.

3.5. Separation, 2D location and identification of flavonoids in natural samples

After identification of all the derivatised flavonoids on the 2D plot, creating a peak table and recording the TOFMS based library, and analyzing the spectral characteristics of derivatized flavonoids, the next experiments involved the separation and identification of flavonoids in selected samples – chocolate, propolis, and chrysanthemum. The complexity of the 2D apex ion current (AIC) plots are presented in Fig. 7. In particular, the propolis sample inset displays many small peaks, with some of approximately the same intensity as the flavonoids. Much more specific information can be obtained from extracted ion queries of the full scan TOFMS data, facilitated by the increased signal response obtained with prior separation by GC × GC. The full mass spectral capability provided by TOFMS allows choice of any ion in post-analysis data processing. As the samples were first studied in the full-scan mode, once the flavonoid of interest is confirmed, and then a suitable *m*/*z* ion, locked by predefined ^{1}D and ^{2}D retention times, can be used for quantification. In this case, it is relatively easy to confirm that the ion chosen for quantification does not suffer interference. Table 3 shows the calibration curve data and composition of target flavonoids present in tested samples corresponding to Fig. 7. Note that this table only reports the flavonoids for which standards were available, and therefore for which ^{1}D and ^{2}D retention, MS, and calibration data, were available. Good linear behaviour over the investigated concentration range (from 2 to 30 μg/mL) was observed, with values of r^2 greater than 0.9905 for all the target analytes. Mass spectra in conjunction with GC × GC positions confirmed the presence of 7 flavonoids in the propolis samples. Among them, the content of chrysin is comparatively large (4.02 mg/g), which is in agreement with the chemical composition reported from previous literature [40]. Of the available standards, the TMS esters of epicatechin, catechin, and the TMS esters of apigenin and luteolin were found in chocolate and chrysanthemum, respectively. Whilst expected flavonoids were found, and duplicate analyses were conducted, a more thorough analytical study with recovery and spiking data was

Table 3
Peak data table of the target flavonoids including retention times on each dimension, linearity data are for a concentration range of 2–30 μg/mL.

	R.T. (s)	Quant. ion[a]	Quant. ion S/N	R^2	Equation	Contents in test samples (mg/g)		
						Propolis	Chrysanthemum	Chocolate
Flavone	1608, 0.930	222	529					
EpiCatechin, penta-TMS	2020, 2.180	368	225	0.9990	$y = 24.9x - 1.0$	0.04		1.22
Catechin, penta-TMS	2036, 2.350	368	225	0.9944	$y = 22.5x - 0.2$	0.05		0.23
Chrysin, di-TMS	2232, 1.680	383	1054	0.9999	$y = 17.3x + 0.3$	4.02		
Tectochrysin,mon-TMS	2404, 1.370	325	765	0.9978	$y = 15.3x + 3.6$	1.04		
Kaempferol, tetra-TMS	2456, 2.390	559	498	0.9937	$y = 36.3x - 9.4$	0.04		
Quercetin, penta-TMS	2588, 2.440	647	224	0.9964	$y = 51.9x - 3.8$	0.01		
Isorhamnetin, tetra-TMS	2736, 2.240	589	277	0.9943	$y = 30.4x - 0.7$	0.02		
Apigenin,tris-TMS	2792, 1.930	471	284	0.9905	$y = 31.0x + 5.2$	0.16	0.06	
Luteolin, tetra-TMS	2896, 2.310	559	183	0.9979	$y = 47.4x + 5.8$		0.09	

[a] Quant. ion, i.e. characteristic ion for quantification.

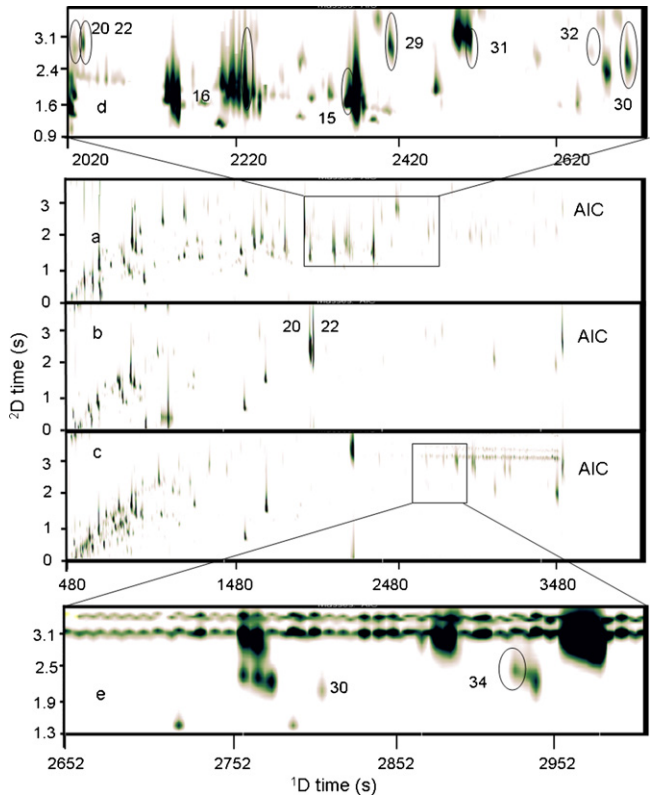

Fig. 7. 2D apex ion current (AIC) plots of (a) propolis, (b) dark chocolate, and (c) chrysanthemum extracts using GC × GC-TOFMS on the BPX50-BPX5 column set. Inset (d) shows the location of 8 identified flavonoids in the propolis sample, whilst inset (e) locates flavonoids **30** and **34** in the chrysanthemum extract.

not undertaken. Data should therefore only be considered indicative.

4. Conclusions

For the first time GC × GC hyphenated with FID and TOFMS has been reported for a broad suite of flavonoids and chaclones, for both standards and in complex natural products. This study shows that GC × GC can be successfully applied in flavonoids analysis, an application which is of a relatively high boiling point class of compounds, of a polar nature, and involves derivatization of between one a six hydroxyl functional groups. Although GC × GC coupled to FID lacks suitable identification capability, GC × GC with TOFMS is promising for screening, quantification and confirmation of flavonoids in complex matrices such as chocolate, propolis, and chrysanthemum. GC × GC provides greater separation power (peak positions in two dimensions), and TOFMS offers greater identification information content (full mass spectral library matches), as compared to conventional GC–MS.

In order to fill a significant gap in mass spectral information, both qMS and TOFMS in-house-created libraries were acquired for all the investigated TMS flavonoids compounds. Difference in qMS and TOFMS data at higher mass were observed, where lower relative abundances of ions were noted for TOFMS.

By analysis of an extensive suite of compounds, some rules regarding the influence of various parameters on the retention order of identically substituted TMS derivatized flavonoids on NP and P columns were deduced.

The results of this study demonstrated the potential of GC × GC-TOFMS with full spectral information for rapid and sensitive structural elucidation and identification of unknown compounds in a sample. The 2D position of flavonoids is specific to the individual compound, and the availability of full TOFMS spectra should allow new compounds (e.g. a previously unidentified flavonoid) to be located in a sample dataset once its retention and mass spectrum are established. The acquired GC × GC data can simply be reinterrogated to determine the presence of the compound [33].

This approach may be useful as an alternative tool for other polar and low volatility chemical studies in the pharmaceutical and natural product fields due to strong capabilities for separation and characterization of complex samples, for both qualitative and quantitative analysis.

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Supporting information is available from Journal of Chromatography A, **S1** = Table of data for mass spectral ion relative intensities for flavonoids and chalcones; **S2** = Mass spectra for flavonoids and chalcones.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.chroma.2010.10.093.

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