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Origin and control of multi-peak formation in the analysis of trimethylsilyl derivatives of flavanone aglycones by capillary column gas chromatography

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ABSTRACT

Trimethylsilyl derivatives of flavanone aglycones give double peaks in their gas chromatograms. The origin of these peaks, from an isomerization between the flavanones and their corresponding chalcones, has been examined using gas chromatography–mass spectrometry and UV spectrophotometry. The effects of derivatization temperature, time and capillary column injection technique on the rate of the interconversion have been defined.

INTRODUCTION

Flavanones (**I**) are one of the most common groups of flavonoid aglycones, and have been extensively studied by natural product chemists [1–6]. Unlike other flavonoids, *e.g.*, flavones, isoflavones and flavonols, which are fully unsaturated, flavanones contain a partially reduced heterocyclic C-ring. For this reason, the ether linkage of the C-ring of flavanones is more labile than the corresponding bond in fully unsaturated flavonoids, an effect that gives rise to the different chemical properties commonly observed in the flavanone group of natural products. In this paper we examine consequences of these reactivity differences on the behaviour of the flavanones during derivatization and analysis by gas chromatography (GC).

GC is a successful technique for the analysis of most flavonoid compounds, but complications have been encountered with flavanone substrates. Packed-column GC has been used for the separation of a variety of flavonoid derivatives [7–14] and for their identification in several plants [15–17] and fruits [18,19]. Trimethylsilyl (TMS) derivatives of the flavonoid aglycones (flavones, isoflavones and flavonols) generally show a single peak in GC analysis, whereas flavanones exhibit multiple peaks [8,10]. Furuya [9] suggested that the flavanones may undergo dehydration after B-ring cleavage or other chemical changes on the column leading to multiple peak formation. Narasimhachari and Von Rudloff [7] proposed that these peaks arise from the isomerization between the flavanones (**I**) and the corresponding chalcones (**II**) during GC analysis as a result of the high temperature necessary for chromatography. Their assignment was based on the isolation of the eluted compounds and a comparison of their UV spectra with those of standard materials.

We have recently reported a successful capillary column GC separation of methyl and TMS derivatives of flavonoid aglycones [20]. In this study, it was observed that the conversion of the TMS derivatives of the flavanone aglycones into the corresponding chalcones occurs both in the derivatization mixture (prior to injection) and in the injection port during GC analysis. Hence the ratio of peaks due to the TMS derivatives of the flavanone and chalcone varied with both derivatization and GC conditions. We report here a systematic investigation of the effect of the derivatization time,

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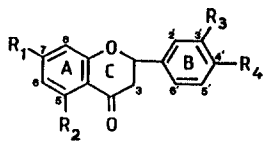
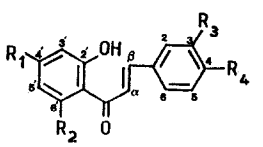


(I)



(II)

Ia: R_1, R_2, R_3, R_4 = H
Ib: R_1, R_2, R_4 = OH; R_3 = H
Ic: R_1, R_2, R_3 = OH; R_4 = OCH_3
Id: R_1, R_2, R_3, R_4 = OH

IIa: R_1, R_2, R_3, R_4 = H
IIb: R_1, R_2, R_4 = OH; R_3 = H
IIc: R_1, R_2, R_3 = OH; R_4 = OCH_3
IId: R_1, R_2, R_3, R_4 = OH

temperature and capillary column injection technique on the rate of the flavanone–chalcone interconversion, using GC, combined GC–mass spectrometry (GC–MS) and UV spectrophotometry.

EXPERIMENTAL

All the flavanones and 2′-hydroxychalcone were gifts from the AFRC Institute of Plant Science Research and John Innes Institute (Norwich, UK). Naringenin, hesperetin and eriodictyol chalcones were purchased from A-Apin Chemicals. These compounds were used without further purification. Pyridine (silylation grade) was obtained from Pierce. 1,1,1,3,3,3-Hexamethyldisilazane, 98% (HMDS), trimethylchlorosilane (TMCS) and methanol, 99.9% (spectrophotometric grade), were purchased from Aldrich.

Derivatization

GC and GC–MS. A 1–2-mg amount of each flavanone or chalcone was dissolved in 0.1 ml of anhydrous pyridine in a screw-capped vial and 0.1 ml of HMDS and 0.05 ml of TMCS were added. The mixture was shaken vigorously for 1 min and allowed to stand at room temperature for 30 min (mild conditions) or heated at 60°C overnight (vigorous conditions). After centrifugation, 0.5–2 μl of the solution was used for injection into the gas chromatograph.

UV spectrophotometry. A 1-mg amount of each flavanone or chalcone was dissolved in 0.4 ml of pyridine in a screw-capped vial and 0.4 ml of HMDS and 0.2 ml of TMCS were added. The mixture was shaken vigorously for 1 min and allowed to stand at room temperature for 30 min. The supernatant solution was separated by centrifugation. An aliquot of 0.05 ml was removed and the remaining solution was heated at 60°C overnight. Aliquots (0.05 ml) were removed from the sample after 2, 4, 6, 8, 10 and 24 h at 60°C. The solvent and excess of reagents in each aliquot were evaporated in a stream of dry nitrogen. The residue was dried *in vacuo* and the dry residue was dissolved in 5 ml methanol for UV spectrophotometry.

Gas chromatographic analysis

GC analysis was carried out on a Phillips PU 4400 series gas chromatograph equipped with a flame ionization detector. The output from the detector was recorded using a Phillips Analytical Chromate PC data system or a chart recorder. A 50 m × 0.25 mm I.D. RSL 200 BP capillary column (0.2 μm film thickness) (Alltech) was maintained isothermally at 280 or 250°C (for flavanone and 2′-hydroxychalcone, respectively) for GC separation. The linear velocity of the oxygen-free nitrogen carrier gas was 17.5 cm s^{-1} (97 kPa) and the split flow-rate was set to 30 ml min^{-1}. GC–MS was carried out on a Varian 3400 gas chromatograph directly interfaced to a Finnigan MAT ion trap mass spectrometer (ITMS) operated via a Walters International Baby AT/PC. A 30 m × 0.24 mm I.D. DB-5 capillary column (0.25 μm film thickness) (J & W Scientific) was used for analysis at an isothermal temperature of 280 or 250°C (for flavanone and 2′-hydroxychalcone, respectively). The injector and transfer line temperatures were maintained at 300 and 275°C, respectively. The carrier gas was helium (83 kPa) and the split flow-rate was set at 30 ml min^{-1}. The conditions for MS were electron impact ionization under automatic gain control at a trap temperature of 150°C. UV spectra were recorded on a Pye Unicam SP8-500 UV–VIS spectrophotometer or a Hitachi 557 double-wavelength, double-beam spectrophotometer. The following conditions were applied: cell path length, 1 cm; scan range, 500–240 nm; scan speed, 120 nm min^{-1} (20 nm cm^{-1}); and slit width, 2 nm.

RESULTS AND DISCUSSION

In our initial investigation of the derivatization and capillary GC analysis of flavonoid aglycones [20], the gas chromatograms obtained for TMS

derivatives of flavones, isoflavones and flavonols generally showed a single peak under mild derivatization conditions. However, TMS derivatives of flavanones (naringenin, **Ib**, hesperetin, **Ic**, and eriodictyol, **Id**) produced double peaks, a small peak followed by a major one, under these conditions. Growth of the minor peak at the expense of the major peak occurred when the derivatization mixture was stored at room temperature for several days. This observation differs from earlier reports [8,10] which suggested that the high temperature necessary for GC analysis alone accounted for the formation of multiple peaks in the chromatogram. Heating the derivatization mixture at 60°C showed that the rate of the interconversion of the two peaks increases significantly at this temperature, such that the reaction was complete within 24 h compared with several days at room temperature. The rate of the conversion is faster for the TMS derivative of hesperetin (**Ic**) than for the TMS derivatives of eriodictyol (**Id**) and naringenin (**Ib**). Hence, after heating overnight at 60°C, chromatograms obtained for the TMS derivatives of hesperetin (**Ic**) and eriodictyol (**Id**) contained a single peak at the retention time of the original minor peak. In contrast, for the derivatization of naringenin (**Ib**) two peaks, a major peak followed by a small one, were still present at this stage, and further prolonged heating was needed in order to simplify the chromatogram to a single peak.

In GC–MS experiments on the TMS derivatives of the flavanones, the expected double peaks were present in the total ion current traces. The mass spectra associated with the two peaks showed that for all the flavanones, the second peak corresponded to the derivatized flavanone, whereas the first peak gave a simple spectrum containing a strong ion (base peak) 72 dalton above the main peak in the mass spectrum of the silylated flavanone. This second component arises from ring opening and further TMS derivatization of the C-ring, *i.e.*, the formation of the corresponding derivatized chalcone in the presence of excess of silylating reagents. For example, in the mass spectrum of the TMS derivative of eriodictyol, **Id**, the derivatized chalcone [eriodictyol chalcone (**IId**), first peak] is identified by a strong ion at m/z 633 (100%) and the TMS derivative of eriodictyol, **Id**, (second peak) by an ion at m/z 561 (100%). The base peak in each spectrum arises from loss of a methyl group [21]. Fig. 1 shows the GC–MS total ion current traces for the TMS derivative of eriodictyol under the mild derivatization conditions (i) and after almost complete conversion to the chalcone (ii), and the corresponding mass spectra of the derivatized chalcone (Fig. 1a) and flavanone (Fig. 1b).

In order to confirm that the flavanones are converted into the corresponding chalcones, derivatization experiments (under both mild and vigorous

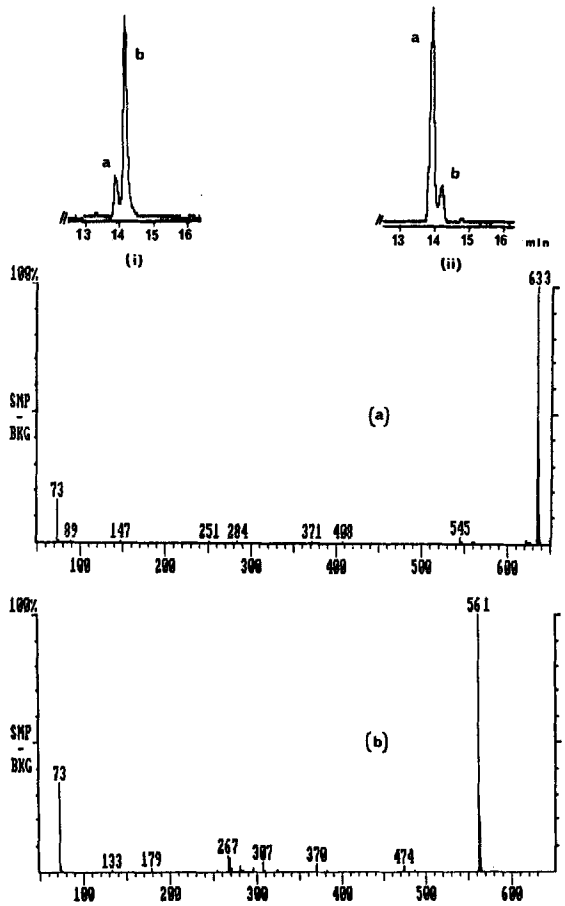


Fig. 1. GC–MS total ion current traces after the TMS derivatization of eriodictyol under (i) mild derivatization conditions and (ii) after almost complete conversion to the chalcone, and the corresponding mass spectra of the derivatized (b) flavanone and (a) chalcone. GC–MS conditions: column, bonded-phase DB-5 fused-silica capillary (30 m × 0.24 mm I.D.; 0.25 μm film thickness); column temperature, 280°C isothermal; linear velocity of helium carrier gas, 25 cm s^{-1}; transfer line temperature, 275°C; electron impact ionization under automatic gain control at a trap temperature of 150°C.

conditions) were carried out using the naringenin, hesperetin and eriodictyol chalcones, (**IIb**, **IIc** and **IId**) as starting materials. The gas chromatograms of the TMS derivatives of the chalcones obtained under the mild derivatizing condition also showed double peaks where the first peak was considerably stronger than the corresponding peak observed when the starting material was a flavanone. In this instance, heating the sample also caused the growth of the first peak at the expense of the second, as observed for flavanones. Injection of a mixture of each TMS derivative of flavanone and its corresponding chalcone in HMDS–TMCS silylating reagent showed two peaks in the gas chromatogram, confirming that the double peaks in the TMS derivatization of the flavanones have the same retention times as the corresponding peaks for the TMS derivatization of the chalcones.

Confirmation of the interconversion of flavanones and their corresponding chalcones under the derivatization conditions was sought by UV spectrophotometry. The absorbance maximum for flavanones occurs in the range 270–295 nm whereas the absorbance maximum for the chalcones is usually in the range 340–390 nm [4]. Changes in the UV spectra of TMS derivatives of naringenin (**Ib**), hesperetin (**Ic**) and eriodictyol (**Id**) as a function of time at 60°C revealed a decrease in absorbance for the flavanone and an increase in absorbance for the corresponding chalcone. Fig. 2 shows the UV spectra for the conversion of TMS derivative of hesperetin into the corresponding chalcone with time in the derivatization mixture at 60°C. These results are consistent with the data obtained from the GC analysis, demonstrating that the conversion of flavanones into the chalcones does not arise solely because of high temperature used in GC. The calculated pseudo-first-order rate constants for the TMS derivatization of naringenin, hesperetin and eriodictyol are $2.8 \cdot 10^5$; $3.1 \cdot 10^5$ and $3.7 \cdot 10^5$ s^{-1}, respectively.

The effect of different capillary column injection techniques (split, splitless and cold on-column injection) was studied using the trimethylsilylation reaction of eriodictyol (**Id**) as a model system to determine the behaviour of the flavanone aglycones. Because of differences in the injector residence times in the split and splitless techniques, kinetic experiments were carried out under the following conditions: split mode, splitless mode, splitless mode (with glass-wool in the injection port) and cold on-column injection.

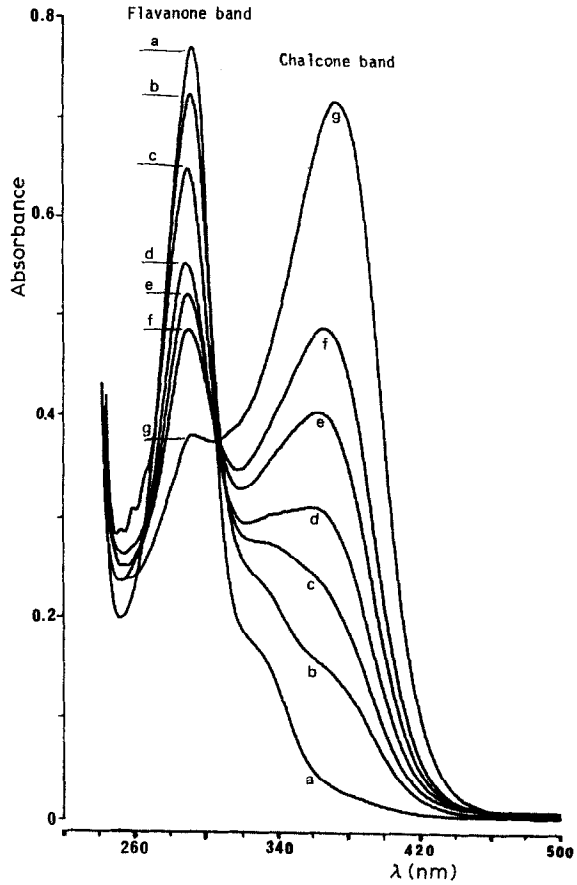


Fig. 2. UV spectra for the conversion of the TMS derivative of hesperetin into the corresponding chalcone in the derivatization mixture at 60°C with time. Spectra (a)–(g) show the increase in absorbance for chalcone and the decrease in absorbance for flavanone after (a) 0, (b) 2, (c) 4, (d) 6, (e) 8, (f) 10 and (g) 24 h.

The results for a series of injections during the TMS derivatization of eriodictyol (**Id**), at various times after addition of the derivatization reagents at 60°C, showed that the rate of the conversion of the TMS derivatives of the flavanones into the corresponding chalcones follows the order splitless mode (with glass-wool) > splitless mode > split mode > cold on-column injection. Injections under splitless (with glass-wool) conditions could cause the reaction to proceed rapidly because of two factors: the longer residence time (*ca.* 30 s) in the hot injection port and the presence of the large surface area of the

glass-wool. Even without the use of glass-wool packing, under splitless conditions the conversion rate is faster than in the split mode because of the longer injector residence time. The rate of the conversion is slowest in cold on-column injection, as expected from the behaviour of the flavanones in HMDS–TCMS solution.

In contrast to the behaviour of naringenin, hesperetin and eriodictyol, the parent compound, flavanone (**Ia**), and its corresponding chalcone (2′-hydroxychalcone, **IIa**), exhibited different gas chromatograms. An injection of the flavanone (**Ia**) in silylating reagents (mild conditions) produced a single peak in the gas chromatogram. Heating the sample overnight at 60°C did not cause any of the rapid changes in the height of the peak observed for other flavanones, producing only a small additional peak at higher retention time. When the sample was stored at room temperature for 3 weeks, the growth of the small peak and the appearance of a new peak at a longer retention time was observed. In the same way, the gas chromatogram of the TMS derivative of the corresponding chalcone (2′-hydroxychalcone, **IIa**) also showed a single peak under mild derivatization conditions. A small secondary peak appeared at lower retention time following vigorous conditions for derivatization. This peak became larger as the sample was left at room temperature for 3 weeks. The second and third eluting peak obtained from flavanone, with silylating reagents, have retention times identical with those of the two peaks observed for the TMS derivative of 2′-hydroxychalcone in their gas chromatograms. GC–MS studies of these compounds showed that the mass spectra of these two peaks in the gas chromatogram of the TMS derivative of 2′-hydroxychalcone are similar, both showing ions at m/z 296 ($M^{+\cdot}$), and 281 (base peaks, $[M - 15]^+$). The presence of two GC peaks with similar mass spectra for the TMS derivative of 2′-hydroxychalcone may be attributed to *cis*- and *trans*-isomerization of the open C-ring in the derivatized chalcone. This isomerization was not observed for naringenin, hesperetin and eriodictyol chalcones because of steric hindrance arising from the presence of bulky TMS groups on the B-ring, which disfavour the formation of the *cis*-isomer. The mass spectrum of the first-eluting peak in the gas chromatograms of flavanone (silylating conditions) showed an ion at m/z 224 corresponding to flava-

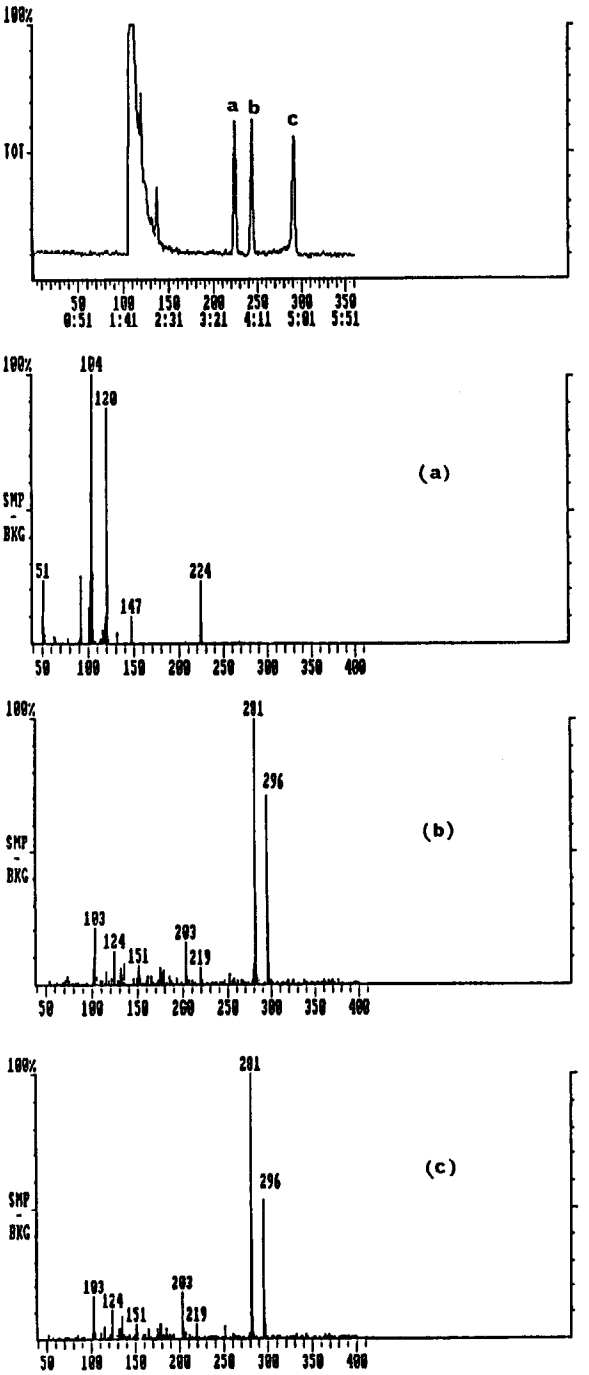


Fig 3. GC–MS total ion current trace of the TMS derivatization products of flavanone and the corresponding mass spectra of (a) the flavanone and (b and c) the derivatized *cis*- and *trans*-chalcone isomers. GC–MS conditions: column temperature, 250°C isothermal; other conditions as in Fig. 1. Time in min:s (top chromatogram); mass units (spectra a, b and c).

none, but the second- and third-eluting peaks exhibited mass spectra that were comparable to that for the TMS derivative of 2′-hydroxychalcone. The identical retention time in both instances and the similarity of the mass spectra of the last two peaks in the gas chromatogram obtained from flavanone and those of the TMS derivative of 2′-hydroxychalcone suggest that the flavanone is slowly converted into the TMS derivative of corresponding chalcone (2′-hydroxychalcone) under the silylating conditions used. Fig. 3 shows the GC–MS total ion current trace of the parent compound, flavanone (after vigorous derivatization conditions and 3 weeks at room temperature), and the corresponding mass spectra of the flavanone (Fig. 3a) and derivatized *cis*- and *trans*-chalcone isomers (Fig. 3b and c).

Interconversion of TMS derivatives of flavanones and the corresponding chalcones is observed in both the derivatization mixture (prior to injection) and during GC because of the high temperature necessary for analysis. The derivatization temperature, time and capillary column injection technique all have an influence on the rate of the interconversion. This phenomenon is very slow for the parent compound, flavanone, and gives rise to two characteristic peaks for chalcone under the conditions reported here, whereas for the TMS derivatives of other flavanones investigated, the conversion rate is faster and only one peak is observed for the corresponding chalcone. The observations in this study provide a basis for the optimization of the derivatization procedure for the identification of these compounds by capillary column GC, especially in complex biological systems.

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