Rapid Analysis of Polymethoxylated Flavones from Citrus Oils by Supercritical Fluid Chromatography

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The analysis of citrus essential oils for their content of polymethoxylated flavones (PMFs) using packed column supercritical fluid chromatography (SFC) is described. Samples of sweet orange and mandarin oil were quantitatively analyzed and the resulting flavone levels compared to the values obtained using liquid chromatography. In addition, milligram quantities of all six individual flavone compounds were isolated from a sample of sweet orange oil. Flavone retention in SFC is explained on the basis of substituent dipolarity. Dipoles were calculated using molecular modeling techniques, and the effects of substituent positions or neighboring dipoles were investigated. It is postulated that steric hindrance plays a role in the retention mechanism.

Keywords: *Polymethoxylated flavones; sweet orange oil; mandarin oil; SFC*

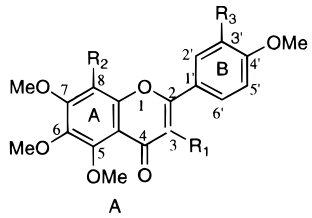
INTRODUCTION

Flavonoids are a group of naturally occurring compounds which are derivatives of the parent molecule, flavone. These compounds are present in many sources including citrus fruits (Sendra et al., 1978; Dugo et al., 1994; Mondello et al., 1993a,b; Castillo et al., 1994; Gaydou et al., 1994; Heimhuber et al., 1988; Bianchini and Gaydou, 1983; Bianchini and Gaydou, 1981; Rouseff and Ting, 1979; Dugo et al., 1995; Dugo et al., 1996), tobacco (Court, 1977), soya (West et al., 1978), and other plant species (Schaufelberger and Hostettmann, 1987; Strack et al., 1979; Galensa and Herrmann, 1980). The flavonoids present in citrus oils are mainly polymethoxylated flavones. The chemical structures of the polymethoxylated flavones present in sweet orange and mandarin peel oils are shown in Figure 1.

Determination of polymethoxylated flavones in citrus peel oils is usually performed using normal phase (Bianchini and Gaydou, 1980; Bianchini and Gaydou, 1981) and reversed phase liquid chromatography employing ultraviolet (Ting et al., 1979; Bianchini et al., 1987) and fluorescence detection (Rouseff and Ting, 1979). A mathematical optimization procedure for chromatographic separation of these compounds, which employs matrices to describe retention, has been proposed (Bianchini et al., 1987). More recently, analyses have been performed using SFC (Martinez-Verges, 1989) with both capillary (Hadj-Mahammed et al., 1993) and packed columns (Morin et al., 1991), which employed flame ionization, Fourier transform infrared (Williams and Fleming, 1989), and ultraviolet detection. In addition, supercritical fluids have been used in the separation of terpenes from citrus oils (Dugo et al., 1995) and the fractionation of a cold-pressed sample of lemon oil (Yamauchi and Saito, 1990).

In this paper we report the use of packed column SFC to analyze quantitatively sweet orange and mandarin essential oils, using an internal standard, and the resulting values were compared to those obtained using liquid chromatography. A sample of sweet orange essential oil was fractionated to isolate individual PMFs.

The retention order of the polymethoxylated flavones separated using an uncoated silica column can be explained in terms of methoxy and carbonyl substituent dipole moments calculated using molecular modeling techniques. The role of steric hindrance in the retention process has been investigated.



A

A) R_1 = H; R_2 = OMe; R_3 = H
B) R_1 = OMe; R_2 = OMe; R_3 = OMe
C) R_1 = H; R_2 = OMe; R_3 = OMe
D) R_1 = H; R_2 = H; R_3 = H
E) R_1 = OMe; R_2 = H; R_3 = OMe
F) R_1 = H; R_2 = H; R_3 = OMe

Figure 1. Chemical structures of polymethoxylated flavones (with substitution position labels): A, tangeretin; B, heptamethoxyflavone; C, nobiletin; D, tetra-*O*-methylscutellarein; E, hexamethoxyflavone; F, sinensetin.

MATERIALS AND METHODS

Materials. This investigation was carried out on Sicilian genuine cold-pressed sweet orange and mandarin essential oil samples. Spectrally pure polymethoxylated flavones, 5,6,7,8,4′-pentamethoxyflavone (tangeretin), 3,5,6,7,8,3′,4′-heptamethoxyflavone (heptamethoxyflavone), 5,6,7,8,3′,4′-hexamethoxyflavone (nobiletin), 5,6,7,4′-tetramethoxyflavone (tetra-*O*-methylscutellarein), 3,5,6,7,3′,4′-hexamethoxyflavone (hexamethoxyflavone), and 5,6,7,3′,4′-pentamethoxyflavone (sinensetin) were previously separated by semipreparative HPLC from sweet orange and mandarin oils (Mondello et al., 1993). The oils were analyzed by packed column SFC.

Approximately 2% w/w solutions of the essential citrus oils in HPLC grade ethyl acetate were prepared and used in the chromatographic optimization procedure. For component identification purposes, ethyl acetate solutions of the six pure

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PMFs were prepared (approximately 0.05% w/w in each PMF). To determine response factors relative to coumarin as an internal standard, six solutions containing coumarin (0.22 mg/mL) and a PMF were prepared in ethyl acetate by adding a quantity of PMF (1.1−2.3 mg) to 10 mL of a coumarin solution (0.22 mg/mL). The PMF content of the citrus oils was quantitatively determined by analyzing standard solutions prepared by adding 0.71 g of sweet orange oil or 1.72 g of mandarin oil to a solution of coumarin (5 mg) in ethyl acetate (20 mL). For the fractionation of sweet orange oil into its individual flavones, pure essential oil was chromatographed.

SFC. Packed column supercritical fluid chromatography was performed using a Hewlett-Packard G 1205A SFC system. CO_2 modified with small quantities of methanol at 50−60 °C was used as mobile phase, together with a Hewlett-Packard series 1050 multiple wavelength ultraviolet detector (MWD) fitted with a 13 μL high-pressure flow cell. Sample introduction was via a Rheodyne 7410-077 pneumatically actuated rotary injection valve fitted with a 5 μL internal sample loop. Air at 4 atm was employed as actuator gas. In the preparative work the injection valve was fitted with a 100 μL external sample loops, the entire contents of which were transferred on to the column by means of 4 and 15 s injection periods, respectively. The chromatograph was fitted with Phase Separations Ltd. 25 cm × 4.6 mm columns containing 5 μm particles of functionalized and unfunctionalized silica: S5W uncoated silica (P/N 830115); S5ODS2 octadecylsilyl (P/N 831915); S5CN nitrile (P/N 830915); S5NH_2 amino (P/N 831115). The ultraviolet detection wavelength was 315 nm.

Separation of Flavones. Conditions for the analysis of citrus oils were as follows: uncoated silica column, pressure 100 atm, temperature 40 °C, modifier 1.5%/min from 10% up to 30% thereafter held constant, flow rate of 2 mL/min for 4 min then gradient of 2 mL/min up to 5 mL/min thereafter held constant; octadecylsilyl column, pressure 100 atm, temperature 40 °C, modifier 10% throughout, flow 2 mL/min; nitrile column, as octadecylsilyl; amino column as octadecylsilyl.

Isolation of Flavones. For citrus oil fractionation, 100 μL of pure, undiluted sweet orange oil was injected into the chromatograph using the uncoated silica column under the following conditions: pressure 100 atm; temperature 40 °C; modifier 1.5%/min from 0% up to 30%, thereafter held constant; flow of 1 mL/min. The individual fractions were split using a six-way splitter valve (Upchurch, Oak Harbor, WA 98277) installed into the mobile phase stream immediately after the restrictor where fluid expansion occurs (Heaton et al., 1996) and collected in cooled vials containing approximately 5 mL of ethyl acetate. For the purity analysis of the isolated ethyl acetate solutions of the individual flavones, 20 μL aliquots of the flavone solutions contained in the collection vials were injected directly into the chromatograph under the same conditions as described for the analysis of citrus oils on the silica column.

Structure Optimization. The flavone structures were modeled and optimized using HyperChem (Hyperchem) molecular modeling software running on a Hewlett-Packard 486/25T personal computer.

Semiempirical geometry optimizations were performed using the complete neglect of differential overlap (CNDO) method for solving molecular wave functions (Hypercube, 1992). This allowed electron density contour maps indicating where electron density was most significant to be produced and the molecular parameters of bond lengths and charge separation for the most polar parts of the structures to be listed. This permitted the magnitude of intramolecular dipole moments to be computed and allowed the relationship between molecular structure, electron distribution, and retention to be investigated.

RESULTS AND DISCUSSION

Separation of Flavones in Sweet Orange and Mandarin Oils. Polymethoxylated flavones have been separated and measured in sweet orange and mandarin essential oils. Resolution and peak shape were better on the more polar uncoated silica and bonded phase amino columns and poorer for the less polar nitrile and octadecylsilyl phases, which gave rise to broad and overlapping peaks. This suggests that the polymethoxylated flavones are separated by a polar mechanism involving the stationary phase, i.e. the polar methoxy and carbonyl side groups are interacting strongly with polar silanol groups on the silica surface. The chromatogram obtained for sweet orange oil using the uncoated silica stationary phase under the optimized conditions of analysis is shown in Figure 2.

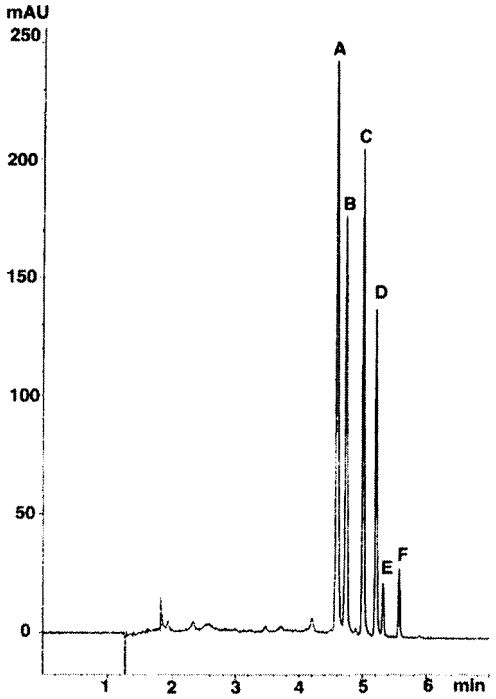

Figure 2. SFC separation of polymethoxylated flavones in sweet orange oil using a 5 μm uncoated silica stationary phase: A, tangeretin; B, heptamethoxyflavone; C, nobiletin; D, tetra-*O*-methylscutellarein; E, hexamethoxyflavone; F, sinensetin.

Table 1. Retention Data for Polymethoxylated Flavones in Sweet Orange Oil Analyzed Using SFC and HPLC (Dugo et al., 1994)

	flavone retention time (min)[a]	
flavone	SFC	HPLC
tangeretin	4.50	9.50
heptamethoxyflavone	4.67	11.30
nobiletin	4.91	13.70
tetra-*O*-methylscutellarein	5.11	15.90
hexamethoxyflavone	5.25	20.80
sinensetin	5.48	26.10

[a] Mean of three determinations.

This analysis allowed a complete separation of the six PMFs known to be present in sweet orange oil in less than 6 min. This represents a reduction in the analysis time by a factor of 4 compared to that obtained using HPLC with a silica stationary phase (Dugo et al., 1994). Principal retention times obtained using the silica column are compared to those obtained using HPLC (Dugo et al., 1994) in Table 1.

Table 2 reports the content of PMFs in both pure sweet orange and pure mandarin oils. The values obtained compared well with values obtained for the oils using HPLC (Dugo et al., 1994). The values fell between the upper and lower limits found in a number of sweet orange and mandarin oils. The differences in relative

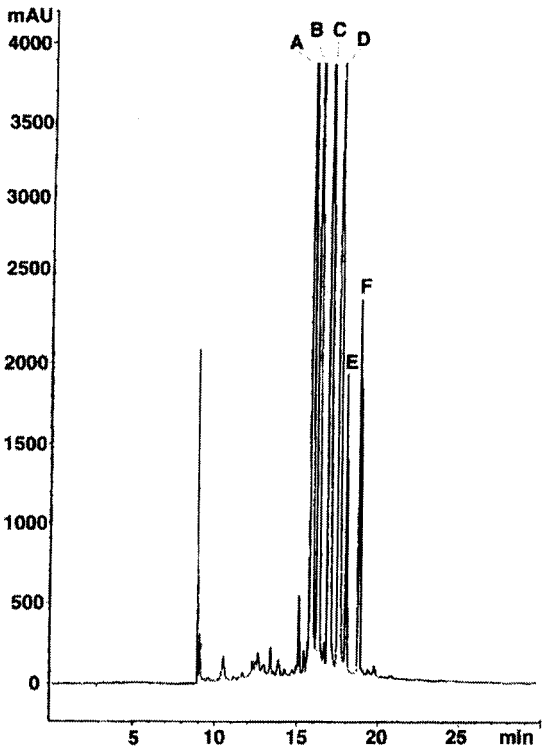

Figure 3. SFC chromatogram obtained during the fractionation of a sample of sweet orange oil. Peak identification: A, tangeretin; B, heptamethoxyflavone; C, nobiletin; D, tetra-*O*-methylscutellarein; E, hexamethoxyflavone; F, sinensetin.

Table 2. Content of Polymethoxylated Flavones in Sweet Orange and Mandarin Oils (g/100 g of Oil)

compound	sweet orange oil	mandarin oil
tangeretin	0.060	0.280
heptamethoxyflavone	0.071	0.050
nobiletin	0.039	0.066
tetra-*O*-mehylscutellarein	0.024	0.004
hexamethoxyflavone	0.007	0.000
sinensetin	0.006	0.001

abundance of the PMFs in citrus oils is characteristic of the citrus species, source, and production period, and this can form the basis for monitoring the authenticity of citrus oils.

Isolation of Polymethoxylated Flavones from Sweet Orange Oil. Milligram quantities of individual polymethoxylated flavones were isolated from a sample of pure sweet orange oil by successive preparative SFC runs. Due to the reduced mobile phase flow rate and modifier concentration, the analysis time was increased to 20 min. This allowed, however, the six polymethoxylated flavones to be separated with a comparable resolution to that obtained for the rapid, analytical scale separation. Figure 3 shows the chromatogram obtained during the fractionation of a sample of sweet orange oil. Injection of pure, undiluted oil into the chromatograph under the same conditions as for the analytical scale separation resulted in severe loss of resolution which would have made fractionation of the sample impossible.

The polymethoxylated flavone peaks are not quite resolved to baseline. This overlap meant that in order to isolate flavones of a high purity the peak fronts and tails needed to be "cut" from the eluant A stream and the overlapping parts of the solute band not collected in the collection vials. This enabled the purity of isolated flavones to be increased compared to that which would be obtained if the splitter valve was simply switched to divert the eluent to the next collection vial at the minimum point between the two peaks. This method suffers, however, from the disadvantage that fractional recovery values for individual flavones are reduced.

Ethyl acetate solutions of isolated flavones were analyzed to assess their purity and to determine the percentage recovery of the individual flavones. The fractional recoveries reflect the resolution of the flavones as the values are lower for those peaks which were less resolved. For example, the fractional recovery of tangeretin (0.64) is lower due to the poorer resolution from other components. Sinensetin, on the other hand, is recovered much more effectively (0.91) due to its baseline resolution from other components. The purities of the isolated fractions were estimated to be in excess of 95%. This was deduced by comparing the areas of the impurity peaks adjacent to the main peaks in the analytical chromatograms of these fractions. Although ultraviolet detection was employed, the peak areas give an indication of the relative amount of impurity present since the relative responses of the six flavones at 315 nm differ at most by only a factor of 2.

Retention Behavior of Polymethoxylated Flavones. The structures of the six polymethoxylated flavones present in sweet orange oil were optimized using the CNDO method. This permitted the production of electron density contour maps which indicated the electronic distribution within the molecules. Since the flavones modeled in this way were shown to be nearly planar, by producing a contour plot which represented the electronic density through the center of gravity and in the plane of the molecules, the part of the molecules possessing the highest electron density could be identified. Most of the electron density lies on the oxygen atoms of the methoxy groups and the carbonyl groups. This is to be expected in view of the high electronegativity of the oxygen atoms relative to the carbon atoms to which they are attached. Since this charge separation is present on the pendant side groups, the most efficient separation of these compounds is obtained using the more polar column, in particular when using uncoated silica as stationary phase. The polar nature of these side groups results in strong interactions with the silanol groups present on the silica surface. If an adsorption rather than a partition mechanism of flavone separation is assumed, the retention behavior and elution order of the PMFs in sweet orange can readily be understood. Although partitioning of the flavones between mobile and stationary phases will occur, the solubility of the flavones in the supercritical fluid is likely to be similar (Dugo et al., 1995). Since dipole−dipole effects are probably the predominant type of solute−stationary phase interaction, this assumption can be justified to a first approximation.

Carbon−oxygen charge separation and interatomic distances for each of the methoxy and carbonyl substituents were derived. The bond dipole moment was calculated by taking the product of these two quantities (Atkins, 1988; Barrow, 1979; Gilmore, 1975). The resulting values are listed in Table 3. The bond dipoles can be used to deduce the effect on neighboring dipoles of a methoxy substituent at various points of the flavone structure. By recognizing four trends inherent in this data, the elution order of the six polymethoxylated flavones in sweet orange can be explained and the role of steric factors in the retention process can be clarified.

Table 3. Bond Dipole Data for the Six Polymethoxylated Flavones Present in Sweet Orange Oil

substitution position	bond dipole, μ (10^{-30} cm)					
	tangeretin	heptamethoxyflavone	nobiletin	tetra-*O*-methylscutellarein	hexamethoxyflavone	sinensetin
8	8.0	8.0	10.1	0.0	0.0	0.0
7	9.6	9.4	9.6	8.0	9.6	9.7
6	10.4	10.3	10.2	10.0	10.5	10.7
5	9.3	9.2	9.4	9.2	9.1	9.1
4	13.3	12.0	13.3	13.2	12.1	13.7
3	0.0	9.8	0.0	0.0	9.9	0.0
3′	0.0	8.0	10.1	0.0	10.2	10.1
4′	8.0	6.5	10.0	8.0	10.2	10.0
total number of bond dipoles	6	8	7	5	7	6
sum of bond dipole, D (10^{-30} cm)	58.6	73.2	72.7	48.4	71.6	63.3

Trend 1. The presence of a methoxy substituent at position 3 causes: (a) a decrease of the polarity of the carbonyl bond dipole at position 4, as can be seen by comparing heptamethoxyflavone to tangeretin, heptamethoxyflavone to nobiletin, hexamethoxyflavone to tetra-*O*-methylscutellarein, and hexamethoxyflavone to sinensetin, and (b) a decrease of the polarity of the methoxy bond dipole at position 8, as can be seen by comparing heptamethoxyflavone to nobiletin; such an effect seems not to be observed when heptamethoxyflavone is compared to tangeretin. This is because the effect of the methoxy substituent at position 3 on the 8-position is made up for the opposite effect of the methoxy substituent at position 3′ on the same position; this last effect can be seen by comparing nobiletin to tangeretin.

Trend 2. The presence of a methoxy substituent at position 3′ causes: (a) An increase in the polarity of the methoxy bond dipole at the adjacent 4′-position, when at least one of the positions 3 or 8 is not substituted. The effect can be seen by comparing nobiletin to tangeretin, sinensetin to tetra-*O*-methylscutellarein, hexamethoxyflavone to tetra-*O*-methylscutellarein. (b) An increase in the polarity of the methoxy bond dipole at position 7, when position 8 is not substituted. This effect can be seen by comparing sinensetin to tetra-*O*-methylscutellarein, and hexamethoxyflavone to tetra-*O*-methylscutellarein. (c) An increase in the polarity of the methoxy bond dipole at position 8, when position 3 is not substituted, as can be deduced by comparing nobiletin to tangeretin, while this effect is not observed when a methoxy substituent is present at position 3 (compare heptamethoxyflavone to tangeretin). In fact, a methoxy substituent at position 3 causes a decrease in the polarity of the methoxy bond dipole at position 8, as seen in trend 1, and there is compensation of the two effects.

Trend 3. A methoxy substituent at position 8 generally has little effect on either the methoxy dipoles or the carbonyl dipole. This can be seen by comparing tetra-*O*-methylscutellarein to tangeretin, hexamethoxyflavone to heptamethoxyflavone, and sinensetin to nobiletin. However, an increase in the polarity of the methoxy dipole at the adjacent 7-position is observed, when tangeretin is compared to tetra-*O*-methylscutellarein. In this case, the methoxy substituent at position 8 is the fifth methoxy group of the molecule, while for nobiletin and heptamethoxyflavone, it represents the sixth and the seventh methoxy group, respectively; in these last two cases, it can be hypothesized that the introduction of the additional methoxy group virtually does not modify the electron distribution in the molecule.

Trend 4. The contemporary presence of methoxy groups at positions 3 and 8 causes a decrease in the polarity of the methoxy dipoles at positions 3′ and 4′, as can be deduced by comparing heptamethoxyflavone to all the other polymethoxylated flavones. The effect is due to the contemporary presence of both substituents, because it is not observed when only one of the two positions is substituted, as can be seen by comparing tetra-*O*-methylscutellarein to tangeretin, nobiletin to sinensetin, and sinensetin to hexamethoxyflavone.

The elution order of the six PMFs found in sweet orange oil can be explained using these four trends, together with additional information derived from the data contained in Table 3. Table 3 also lists the total number, n, of polar groups found in the six polymethoxylated flavones together with values of the sums, D, of these dipoles for each flavone.

Tangeretin contains several polar methoxy groups and a carbonyl group which can interact strongly with the polar silanol groups present on the surface of the silica stationary phase. The addition of a methoxy group to the 3′-position of tangeretin to form nobiletin causes an increase in polarity of the methoxy group at position 4′. This, together with the increase in polarity of the methoxy groups at position 8 offers potential for stronger dipole−dipole interactions with the stationary phase and therefore increased retention. The addition of this polar group is accompanied by an increase in the sum of bond dipoles, D, which is consistent with the increased retention observed.

The addition of a further methoxy group at position 3, producing heptamethoxyflavone, causes a general decrease in substituent polarity. This reduces the strength of the solute−stationary phase interactions, and therefore retention is decreased. The number of polar substituents is, however, increased by one, and the sum of the bond dipoles, D, is increased slightly, which suggests that an increase in retention might be expected. The decrease observed, therefore, is likely to be due to steric factors. The increased crowding of heptamethoxyflavone at positions 3 and 4 decreases the ability of the molecule to interact with stationary phase sites at these positions. The elution order of tangeretin, heptamethoxyflavone and nobiletin can, therefore, be explained on the basis of substituent dipolarity and steric hindrance.

The elution order of tetra-*O*-methylscutellarein, hexamethoxyflavone, and sinensetin can be explained similarly, since addition of a methoxy group to tetra-*O*-methylscutellarein at position 3′ (forming sinensetin) causes an increase in the polarity of the group at the 4′-position together with a slight increase at the 7-posi-

tion. The additional group together with the increased polarity of groups already present allow stronger solute−stationary phase interactions and thus increased retention. The sum of the bond dipoles, *D*, is also increased, which again is consistent with the increased retention observed.

Addition of a further methoxy group at position 3, forming hexamethoxyflavone, again causes a general decrease in substituent bond dipolarity. As was the case with heptamethoxyflavone, this reduces the interaction with the stationary phase. The additional methoxy group increases the sum of the bond dipoles, *D*, which, due to steric crowding about the 3- and 4-positions, does not cause an increase in retention.

The elution order of tangeretin, heptamethoxyflavone, and nobiletin together with that of tetra-*O*-methylscutellarein, hexamethoxyflavone, and sinensetin can thus be explained on the basis of substituent bond polarity. Although tangeretin, heptamethoxyflavone, and nobiletin possess an additional methoxy dipole at position 8, they are retained less than tetra-*O*-methylscutellarein, hexamethoxyflavone, and sinensetin. This can be attributed to steric hindrance when position 8 is occupied. In addition, Table 3 shows the sum of the bond dipoles, *D*, to be greater when a methoxy group is present at the 8-position. The decreased retention, therefore, is likely to be due to reduced accessibility of the methoxy dipoles at positions 7 and 8 when both positions are occupied. This will have the effect of reducing the ability of the solute to interact with the stationary phase at these positions. The magnitudes of the bond dipoles of the methoxy groups at positions 5, 6 and 7 are hardly affected by the substitution of a further methoxy at position 8. It is likely that the ability of the methoxy groups at positions 5 and 6, to interact with the stationary phase will be largely unaffected, and these positions are probably a sufficient distance from position 8 for steric effects to be small.

The above explanation of the elution order of the polymethoxylated flavones in sweet orange oil assumes that the solute−stationary phase interaction is the most important of all the factors that may influence retention. Solute solvation and volatility are not accounted for in this model. The optimized flavone structures and calculated dipoles effectively represent the molecules in the gas phase. The bond dipoles of the flavones, furthermore, are assumed to be independent of the proximity of the solutes to the stationary phase. Although the retention process is greatly simplified in the above explanation, the model does allow an insight into the factors important in the retention process to be gained.

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