Journal of Chromatography, 166 (1978) 189–199
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CHROM. 11,247

SELECTIVE FLUORESCENCE DETECTION OF CITRUS OIL COMPONENTS SEPARATED BY HIGH-PRESSURE LIQUID CHROMATOGRAPHY

HOWARD W. LATZ and DAVID A. ERNES
Department of Chemistry, Ohio University, Athens, Ohio 45701 (U.S.A.)
(Received May 10th, 1978)

SUMMARY

The analysis of citrus oils is aided by the use of selective fluorescence detection with high-pressure liquid chromatography. The differing fluorescence properties of the nonvolatile components present in the oil make detection at one excitation and emission wavelength unsuitable. Therefore, replicate chromatograms are obtained at particular wavelength combinations for the classification of those components based on peak intensity variations. From results with lime oil, this technique is successfully applied to lemon, grapefruit and bergamot oils.

INTRODUCTION

Historically, analysis of citrus oils was subdivided into the volatile components, such as the terpenes and alcohols, and the nonvolatile components including the coumarins, flavones and flavanones. The former group was investigated primarily with gas–liquid chromatography (GLC). Various methods were used to characterize the non-volatile components including gel permeation chromatography[1], thin-layer chromatography (TLC)[2,3], GLC combined with ultraviolet (UV) and nuclear magnetic resonance (NMR) spectroscopy[4], and high-pressure liquid chromatography (HPLC)[5–7]. Characterization of the individual components with the above methods was achieved by sample isolation and subsequent analysis. *In situ* fluorescence monitoring of TLC plates[8] facilitated the characterization of coumarins in citrus oils without sample isolation by observing peak intensity fluctuations resulting from wavelength changes. By judicious choice of these wavelengths, it was possible to selectively enhance the fluorescence of one group of coumarins while depressing the others. By employing selective fluorescence detection with HPLC, similar results are achieved.

EXPERIMENTAL

Apparatus and materials

A Model ALC 202 high-pressure liquid chromatograph with 6000 and 6000A pumps, U6K injector and 660 solvent programmer (Waters Assoc., Milford, Mass., U.S.A.) was used for all separations. Chromatograms were recorded on a Texas

Instruments Servo-Riter II recorder. A 0.110-ml minimum volume quartz flow cell (No. J4-7418) with flow cell adapter (No. J4-7392) was used with an Aminco-Bowman spectrophotofluorimeter (SPF) (American Instruments Co., Silver Springs, Md., U.S.A.) fitted with a xenon arc lamp and RCA 1P28 photomultiplier tube to obtain fluorescence data. Detection by UV absorption was achieved by employing the 254-nm attachment which was part of the ALC 202. The fluorescence flow cell was connected in series with the UV detector using PTFE tubing.

Since the output of the SPF's photomultiplier microphotometer (50 mV) exceeded the range of the recorder (10 mV), a 500-Ω variable potentiometer was connected in parallel and adjusted to synchronize the output of the microphotometer and the range of the recorder.

A Waters Assoc. 30 cm $\times$ 4 mm I.D. μPorasil column was used for all separations. The solvents employed, hexane and chloroform, were degassed. The lime, grapefruit, bergamot and orange oils were obtained from Fritzsche, Dodge and Olcott; the lemon oil from the Sunkist Growers Inc.

Procedure

Separation of the oils, except for the orange oil, was performed with diluted samples in chloroform (spectrophotometric grade) to obtain on-scale peaks. The chromatograms presented in this work are corrected for the void volume difference between the UV and fluorescence detectors to augment interpretation. A linearly programmed gradient initiated with 20% chloroform in hexane and ending with chloroform (with a flow-rate of 1.0 ml/min) was used to obtain all chromatograms. The program was run for 20 min starting upon injection with final conditions being retained to elute all components. Stop-flow results were obtained by periodically shutting down the pumps during a run and manually scanning the excitation and emission monochromators to optimize fluorescence.

RESULTS AND DISCUSSION

Analysis of lime oil — wavelength variation

Characterization of the coumarins in lime oil was achieved previously by column chromatography[9]. By employing HPLC, five of the major components have been identified on the chromatogram shown in Fig. 1. Identification was achieved by fraction collection followed by a comparison of spectral data with known values. Also, sample enrichment with authentic compounds verified the identifications.

Comparison of the chromatograms obtained using UV and fluorescence detection illustrates the selectivity and in some cases sensitivity obtained with fluorescence detection. It is notable that compounds eluting after 20 min can only be detected by fluorescence. Also, comparison of peak maxima aid in the identification of some peaks as in the case of 5,7-dimethoxycoumarin (peak D). Here the shoulder on the peak at 16 min in the UV chromatogram rather than the more prominent peak was identified as the coumarin by comparing the fluorescence peak maximum with the UV maximum.

Monitoring the fluorescence at one wavelength, however, is not sufficient to observe all of the coumarin derivatives. Specifically, only 5-geranoxypsoralen is observed along with the coumarins at the chosen wavelengths. Table I also shows the range of fluorescence maxima for these compounds, in the order of 100 nm for the

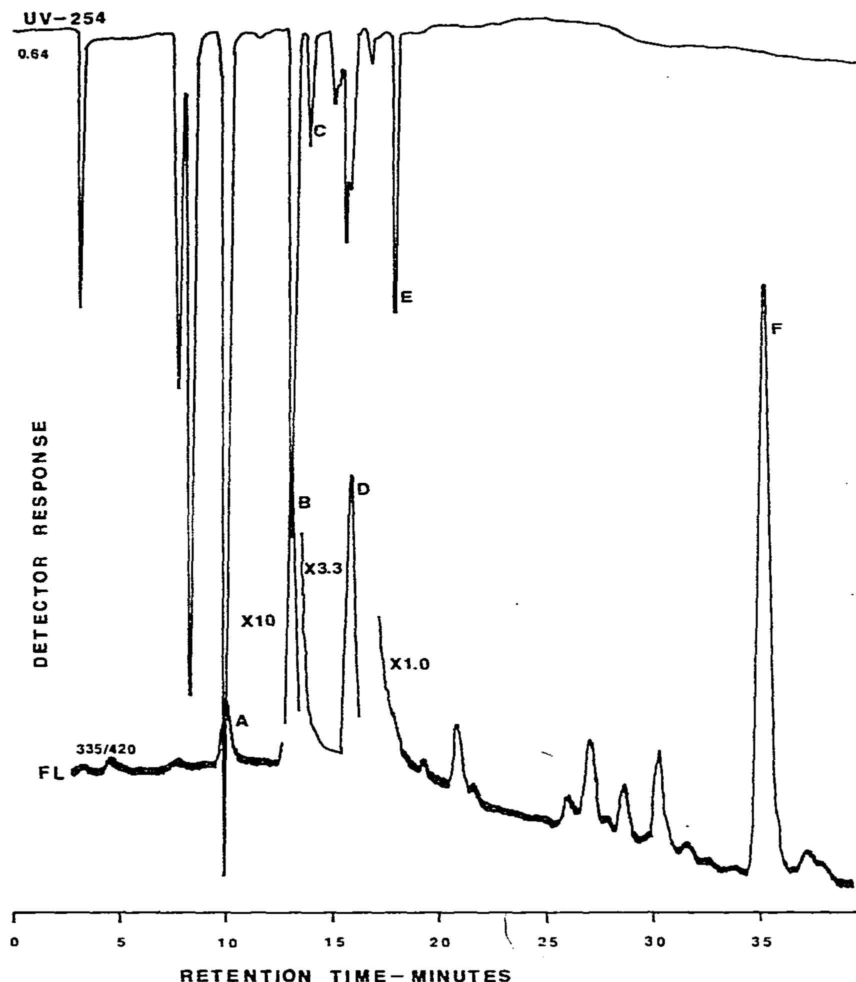

Fig. 1. UV and fluorescence (FL) chromatograms of expressed lime oil Extra (85 μg). Peaks identified are: A, 5-geranoxypsoralen; B, 5-geranoxy-7-methoxycoumarin; C, 5-geranoxy-8-methoxypsoralen D, 5,7-dimethoxycoumarin; E, 5,8-dimethoxypsoralen; F, flavanone (?).

emission maxima. Therefore, several chromatograms were obtained with the excitation and emission wavelengths varied individually, with results shown in Table II and an example in Fig. 2. This indicates that 5-geranoxypsoralen has an excitation maximum at *ca.* 305 nm and an emission maximum at *ca.* 440 nm. Both 5-geranoxy-7-methoxycoumarin and 5,7-dimethoxycoumarin show excitation and emission maxima at *ca.* 335 nm and 400 nm, respectively. The 5-geranoxy-8-methoxypsoralen is seen only as a shoulder on the 5-geranoxy-7-methoxycoumarin peak while 5,8-dimethoxypsoralen is resolved by the wavelength variation. With the emission wavelength at 335 nm, 5,8-dimethoxypsoralen progresses from an unresolved peak at 400 nm to a fully resolved peak at 480 nm. This is a consequence of the decreasing fluorescence of the coumarins along with the enhancement of the psoralen fluorescence at longer emission wavelengths.

TABLE I

FLUORESCENCE CHARACTERISTICS OF COUMARIN DERIVATIVES ISOLATED FROM LIME OIL*

Compound	*Excitation max. (nm)*	*Emission max. (nm)*	*Relative intensity*
5-Geranoxypsoralen	314	474	0.41
5-Geranoxy-7-methoxycoumarin	335	420	20.4
5-Geranoxy-8-methoxypsoralen	318	517	0.01
8-Geranoxypsoralen	312	460	0.01
5-Isopenteneoxy-8-methoxypsoralen	314	513	0.02
8-Isopenteneoxypsoralen	312	460	0.02
?	346	460	4.00
5-Methoxy-8-isopenteneoxypsoralen	317	506	0.02
5,7-Dimethoxycoumarin	335	420	28.8
5,8-Dimethoxypsoralen	318	514	0.02
5-(Dihydroxyisopentaneoxy)psoralen	314	474	0.68
5-Methoxypsoralen	320	462	0.7

* From ref. 9.

TABLE II

WAVELENGTH VARIATION OF LIME OIL COMPONENTS

Fluorescence wavelength (nm)		*Relative peak area* (cm² × 10²)*			
λ_{ex}	λ_{em}	*5-Geranoxy-psoralen*	*5-Geranoxy-7-methoxy-coumarin*	*5,7-Dimethoxy-coumarin*	*5,8-Dimethoxy-psoralen*
305	440	0.925	24.4	6.6	**
315	440	0.792	29.5	8.1	***
325	440	0.777	37.4	11.4	**
335	440	0.525	37.2	8.70	**
345	440	0.388	29.5	8.78	**
335	400	0.180	119	31.9	**
335	420	0.340	57.3	15.8	**
335	440	0.525	37.2	8.70	***
335	460	0.357	16.2	3.54	0.110
335	480	0.253	5.96	1.18	0.140

* Relative peak area = peak area (cm²) × meter multiplier setting.
** Peak unresolved.
*** Peak partially resolved but it was not possible to determine area.

The above study illustrates the trends shown by the coumarin derivatives found in lime oil. However, the maxima are estimates since the excitation and emission wavelengths were varied in steps of 5 and 20 nm, respectively. Therefore, stop-flow analysis was employed to obtain more accurate values for the maxima of the three groups of coumarin derivatives identified in lime oil: 5-alkoxypsoralens, 5,7-dialkoxycoumarins and 5,8-dialkoxypsoralens. The results of this investigation are shown in Table III. The differences between these values and those in Table I are a result of the solvent effects since ethanol was used for the results in Table I whereas this investigation employed a chloroform–hexane gradient. A separate experiment found that the

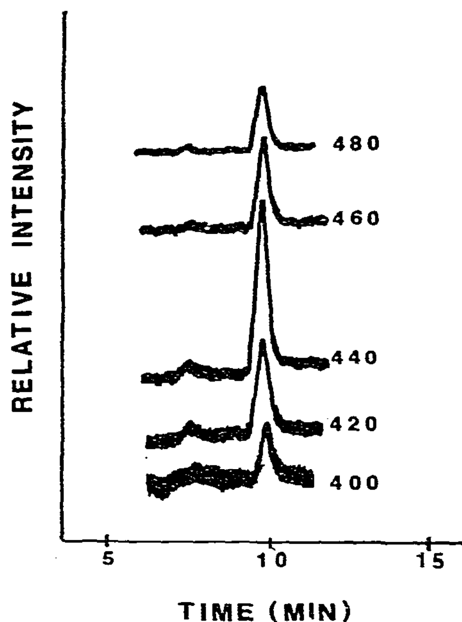

Fig. 2. Variation of the fluorescence of 5-geranoxypsoralen with changes in the emission wavelength. Excitation, 335 nm.

TABLE III

STOP-FLOW DETERMINATION OF THE FLUORESCENCE MAXIMA FOR REPRESENTATIVE COUMARINS IN LIME OIL

Compound	*Excitation max. (nm)*	*Emission max. (nm)*
5-Geranoxypsoralen	308	435
5-Geranoxy-7-methoxycoumarin	330	387
5,8-Dimethoxypsoralen	315	480

fluorescence maximum of a lime oil solution shifted to shorter wavelengths in hexane (389 nm) and chloroform (395 nm) compared to ethanol solutions (417 nm). The different shifts due to chloroform and hexane also show the necessity for stop-flow analysis for it would be difficult to determine a component's maxima in a solvent whose composition is constantly changing as is the case in this study.

The wavelength combination for the 5,7-dialkoxycoumarins was altered to 335 nm excitation and 400 nm emission due to the interference of scatter. The values obtained by stop-flow analysis produced a background that would prevent use of the 0.01 meter multiplier setting, thus reducing the sensitivity of the short-wavelength chromatogram.

Selective detection of lemon oil components

The overall composition of lemon oil is similar to that of lime oil as seen in Fig. 3, the lemon oil containing a lower percentage of total coumarins. Fluorescence detection at the 315/480 nm ($\lambda_{ex}/\lambda_{em}$) combination shows three major peaks designated 1-A, 1-B and 1-D, with shoulders 1-C and 1-E. A minor reproducible peak appears after

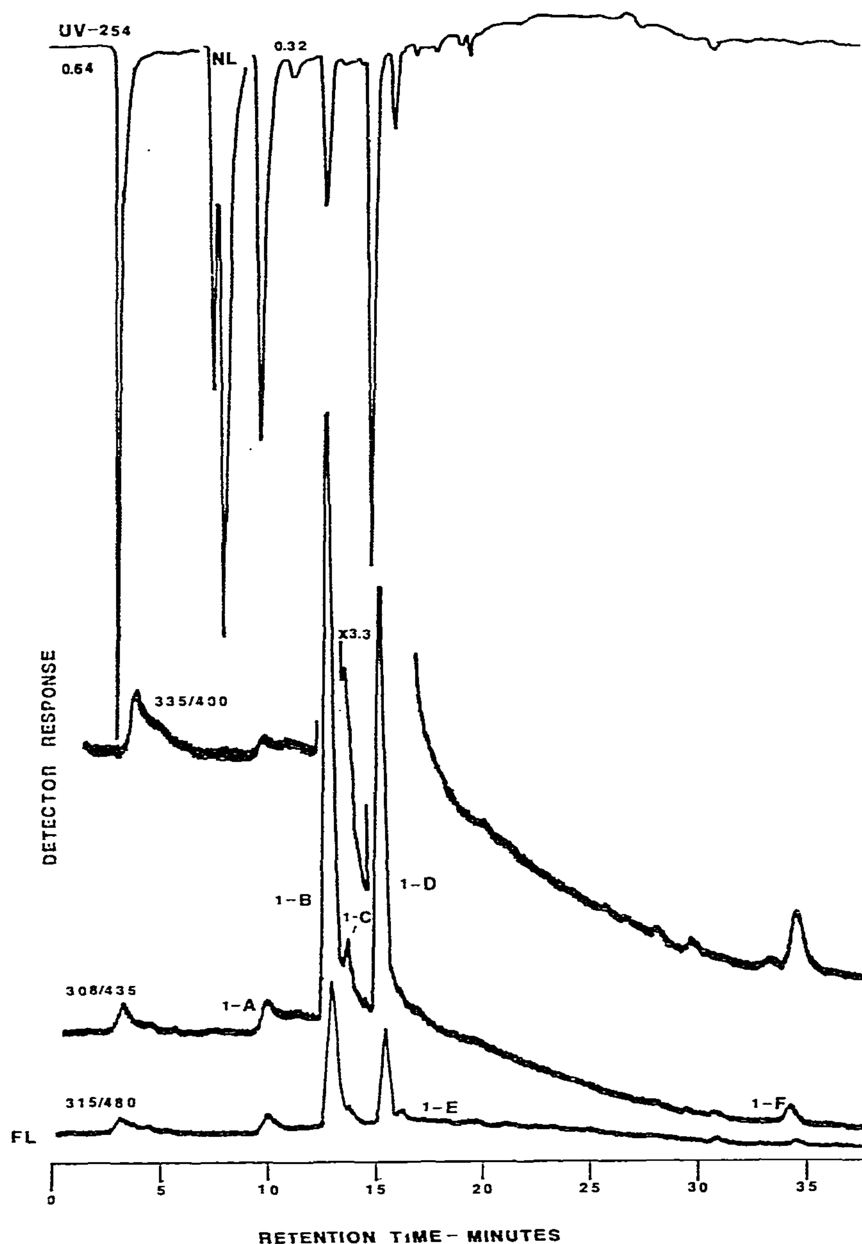

Fig. 3. Combined chromatograms of cold-pressed lemon oil California (340 μg) for selective detection. Peaks identified in text. NL, nonlinear scale.

4 min but is probably due to the fluorescence of one of the terpenes or sesquiterpenes also present in the oil. A dramatic increase is observed for peaks 1-B and 1-D at the 308/435 nm combination with the reduction of peak 1-E. Peaks 1-A and 1-C also increase at this setting with a new peak designated 1-F becoming evident at 34 min.

All of the above peaks except 1-A and 1-E increase at the final wavelength combination, 335/400 nm, peak 1-C being evident by a change in slope of 1-B. Assignment of peaks 1-B and 1-D as 5,7-dialkoxycoumarins is based on the increase seen by moving to shorter wavelengths. Peak 1-C behaves similarly indicating that it too is a dialkoxycoumarin. Peak 1-A has its maximum at 435 nm indicating a 5-alkoxypsoralen while peak 1-E may be a 5,8-dialkoxypsoralen based on its maximum at 480 nm. Peak 1-F behaves as a dialkoxycoumarin; however, assignment as such is questionable due to the difference in retention time compared with the other coumarins.

To verify the above assignments, a lemon–lime mixture was chromatographed and the results compared with the known composition of lemon oil[10]. Two of the coumarins in lime oil, 5-geranoxy-7-methoxy- and 5,7-dimethoxycoumarin, correspond to peaks 1-B and 1-D in the lemon oil chromatogram. Similarly, peak 1-A corresponds to 5-geranoxypsoralen. Previous work[11] postulated that a shoulder on the 5-geranoxy-7-methoxycoumarin peak was a third coumarin known to be present in lemon oil: 5-isopenteneoxy-7-methoxycoumarin. This is consistent with the results of this investigation for peak 1-C. Peak 1-E, present in both oils, was not identified in the lime oil investigation. However, it may be 5-methoxy-8-isopenteneoxypsoralen based on its emission maximum at 480 nm and its presence in both oils. It was noted from the lemon–lime comparison that the peak on the UV chromatogram that seems to correspond to peak 1-D consists of two unresolved peaks as was observed with lime oil. In this case, there was no partial resolution, probably a consequence of the lower concentration of 5,7-dimethoxycoumarin in the lemon oil.

A further portion of the nonvolatile fraction of lemon oil consists of the flavanones[11]. Possessing similar fluorescent properties as the coumarins, they may explain the appearance of peak 1-F. To check this assumption, bitter orange oil was chromatographed under identical conditions. The major fluorescent peaks appear after 20 min with several eluting close to 1-F. Being responsible for the fluorescence of orange oil, the flavanones probably account for the majority of the peaks in the fluorescence chromatogram and therefore may account for peak 1-F. From the lemon–lime comparison, it was noted that 1-F and F (from lime oil; *cf.* Fig. 1) eluted together which may explain the latter, as yet unidentified peak.

Selective detection of bergamot oil components

Analysis of bergamot oil yields a slightly more complex chromatogram, as seen in Fig. 4, especially with the fluorescence chromatogram. At 315/480 nm, two peaks predominate, designated 2-A and 2-D, along with several minor peaks, 2-B, 2-C, 2-E, and 2-F, the latter two appearing after 30 min. All of the peaks increase at the 308/435 nm combination, 2-B increasing more than 2-A but not more than 2-D. Little change is observed with 2-C at this setting. At the 335/400 nm combination, however, 2-C is obscured under the more intense 2-B. All the other peaks, except 2-A, increase at this setting.

These observations indicate that 2-B and 2-D are 5,7-dialkoxycoumarins while 2-A is a 5-alkoxypsoralen. Although 2-C does not vary much between the first two combinations, it is nonetheless a psoralen. Peaks 2-E and 2-F may be flavanones by the same argument presented with the lemon oil.

As with lemon oil, a bergamot–lime mixture was chromatographed as an aid in verifying the above assignments. The two coumarins present in both oils correspond to

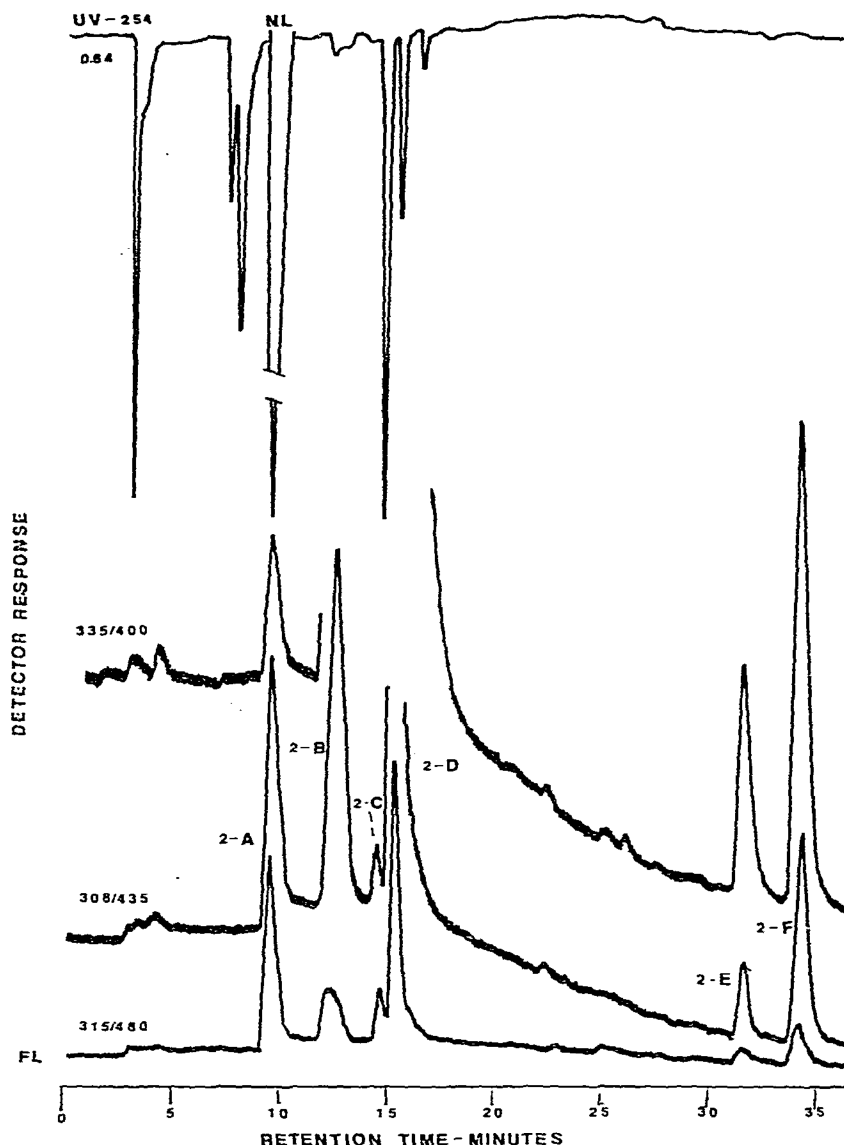

Fig. 4. Combined chromatograms of bergamot oil Extra (340 μg) for selective detection. Peaks identified in text. NL, nonlinear scale.

peaks 2-B (5-geranoxy-7-methoxycoumarin) and 2-D (5,7-dimethoxycoumarin). Peak 2-A corresponds to 5-geranoxypsoralen. The major constituent in bergamot oil, however, is 5-methoxypsoralen[4], present in trace amounts in lime oil[11]. From its behaviour, peak 2-C may be this psoralen since it appears as a minor constituent in lime oil.

Later work using HPLC-grade hexane (Fisher Scientific, Pittsburgh, Pa., U.S.A.) and a non-linear gradient (Profile 7) indicates that peak 2-B is an unresolved

doublet. Both peaks in the doublet behave as peak 2-B in Fig. 4 except that they are partially resolved at the 308/435 nm setting. A second sample of bergamot oil (Italian-Fritzsche Bro.) shows this same doublet at 308/435 nm except that the peaks are not of equal intensity. By comparison with a lime oil chromatogram run under the same conditions, the second peak was identified as 5-geranoxy-7-methoxycoumarin. The first peak therefore may be a coumarin not previously known to be present in bergamot oil. Results with lemon oil showed no deviations from the results presented here.

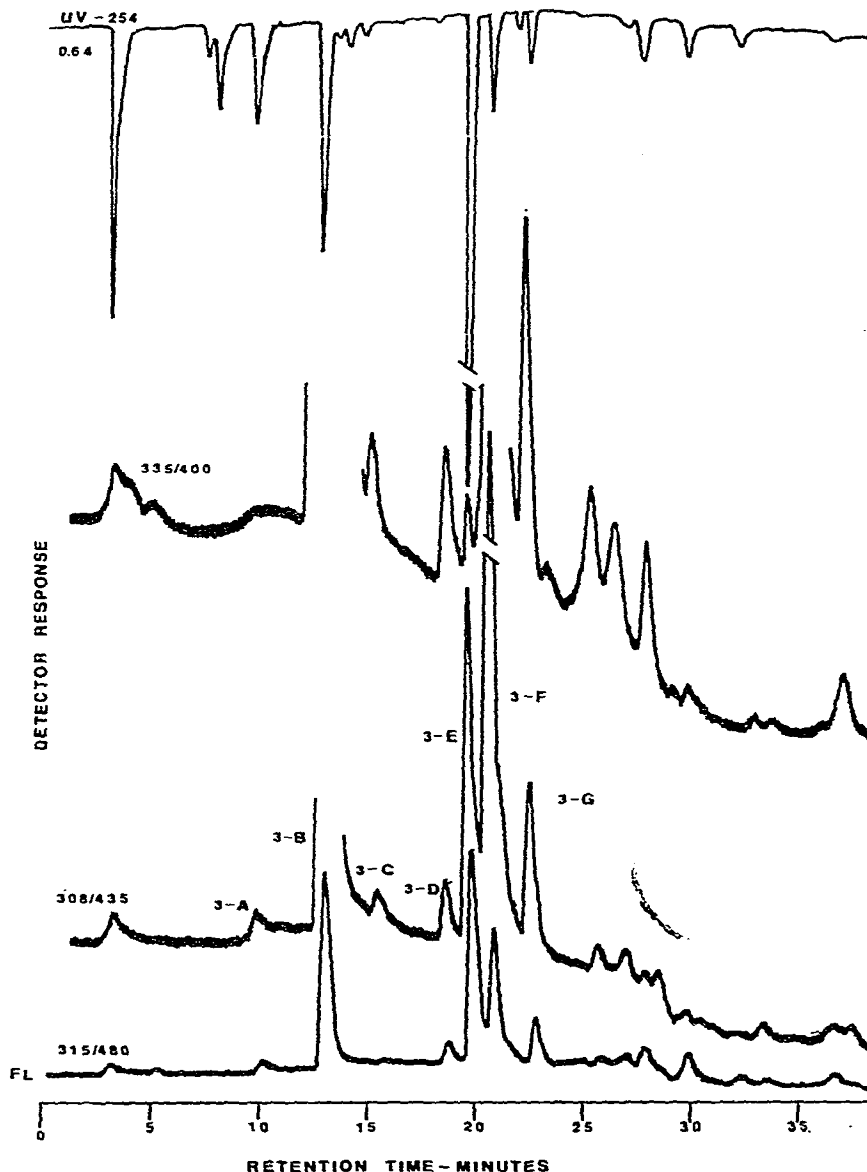

Fig. 5. Combined chromatograms of expressed grapefruit oil Florida (340 μg) for selective detection. Peaks identified in text.

Selective detection of grapefruit oil components

Grapefruit oil is the most complex oil studied thus far as can be seen from Fig. 5. Three major peaks are observed at the 315/480 nm setting, designated 3-B, 3-E and 3-F, with at least eleven apparent minor peaks distributed through the chromatogram. All peaks are enhanced to varying degrees at the 308/435 nm setting with the exception of some minor peaks around 30 min. Here peak 3-B becomes predominant while 3-F increases over 3-E. Also, a new peak, designated 3-C, is observed that was absent in the previous chromatogram. Further intensity changes occur at the 335/400 nm setting with all peaks increasing except 3-A and 3-E which are drastically reduced.

Peaks 3-A and 3-E have their emission maximum around 435 nm indicating 5-alkoxypsoralens. All of the other peaks have their maximum at 400 nm. Assignment as 5,7-dialkoxycoumarins, however, is questionable since several elute at the point postulated as flavanones in the orange oil chromatogram. Therefore, for a first approximation, peaks 3-B, 3-C and 3-E are coumarins and peaks beyond 25 min are flavanones based on retention data alone.

Verification of the above assignments can only be partially made since grapefruit oil contains many compounds not present in the other oils. Two compounds present in both, 5-geranoxypsoralen and 5,7-dimethoxycoumarin, can be identified as peaks 3-A and 3-C. From a grapefruit–lime mixture, peak 3-B corresponds to 5-geranoxy-7-methoxycoumarin. However, the UV chromatogram shows two peaks which are only partially resolved. Previous work[11] stated that 7-geranoxycoumarin present in grapefruit oil elutes at the same place on a thin-layer chromatogram. Although the fluorescence chromatogram suffers this same drawback, the UV chromatogram resolves the two components identifying peak 3-B as 7-geranoxycoumarin. This difference is most likely due to band broadening which may occur between the 10-μl UV cell and the 0.110-ml fluorescence flow cell. The remainder of the peaks are not present in the other oils so identification would be arbitrary.

CONCLUSION

This investigation has shown that by varying the wavelengths used to obtain fluorescence chromatograms with HPLC, an insight is gained as to the possible identity of the individual components. In each case presented, selective variation predicted the class of coumarin derivative responsible for each peak on the fluorescence chromatogram. The exception was with grapefruit oil where peak 3-B was identified as a 5,7-dialkoxycoumarin rather than a 7-alkoxycoumarin. This was a consequence of having to alter the wavelength combination because of scatter interferences. Since the 7-alkoxycoumarins fluoresce at an even shorter wavelength than the 5,7-dialkoxycoumarins, this study could not differentiate the two types. Also shown is the complementary nature of UV and fluorescence detection as illustrated by the identification of 5,7-dimethoxycoumarin on the UV chromatogram in lime oil and with the differentiation of 5-geranoxy-7-methoxy- and 7-geranoxycoumarin in grapefruit oil. In addition, the fluorescence chromatogram showed the postulated flavanones that were not visible in the UV chromatogram.

ACKNOWLEDGEMENT

The authors would like to thank Fritzsche, Dodge and Olcott, and the Sunkist Growers Inc. for supplying the oils used in this investigation.

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