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QUALITATIVE AND QUANTITATIVE *IN SITU* FLUORIMETRY OF CITRUS OIL THIN-LAYER CHROMATOGRAMS

BROOKS C. MADSEN AND HOWARD W. LATZ*
Department of Chemistry, Ohio University, Athens, Ohio 45701 (U.S.A.)
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SUMMARY

The development of direct fluorimetric scanning for thin-layer chromatogram analysis has introduced a versatile tool which has diversified applications in the analysis of citrus oils and potential for analysis of a wide variety of other natural products. The characterization of citrus oils from fluorescence and fluorescence quenching profiles which result from the presence of crystalline components in the oils can be accomplished. Quantitative determination of two coumarin derivatives, 5-geranoxy-7-methoxycoumarin and 5,7-dimethoxycoumarin, by *in situ* fluorimetry of lemon, lime, and bergamot oil thin-layer chromatograms provides an accurate and rapid procedure for quality control based on coumarin derivative content.

INTRODUCTION

Recently the application of direct fluorimetric scanning of thin-layer chromatograms with commercially available instruments has received considerable attention. In an early stage of development, *in situ* fluorimetry has been utilized primarily in bio-medical analysis. This development has paralleled that of fluorimetry which also is used extensively in bio-medical analysis and research[1]. Application of *in situ* fluorimetry to complex natural product mixtures such as citrus oils has not been explored. The application of fluorimetry in combination with thin-layer chromatography indicates that many widely diversified and thus far unexplored applications of fluorimetric scanning of thin-layer chromatograms are possible. An early application of *in situ* fluorimetry was made to demonstrate the usefulness of the technique in air polution studies[2]. KLAUS[3] has investigated the effect of spot shape in direct chromatogram analysis. JÄNCHEN AND PATAKI[4] summarized the various procedural features available for fluorimetric scanning of thin-layer chromatograms and demonstrated the methods with applications to amino acid derivative mixtures and nucleotide mixtures. ZÜRCHER *et al.*[5] have investigated reproducibility in quantitative thin-layer chromatography when direct spectrophotometry and fluorimetry are employed. This versatile technique has a great potential for both qualitative and quantitative analysis.

* Address correspondence to this author.

Expressed citrus oils contain considerable amounts of crystalline components which can be separated by thin-layer chromatography and which exhibit fluorescence upon excitation by UV radiation[6–9]. An extensive examination of the luminescence properties of expressed lime oil and individual coumarin derivatives isolated from expressed lime oil[10] has shown that the presence of 5-geranoxy-7-methoxycoumarin (I) and 5,7-dimethoxycoumarin (II) are responsible for the fluorescence characteristics of lime oil. The amounts of I and II present in expressed lime oil are responsible for the variations in fluorescence emission intensity observed from ethanol solutions of lime oil samples from different geographical regions. Quantitative fluorimetric scanning of thin-layer chromatograms results in a rapid and accurate method for the determination of the amounts of I and II present in lime oil and other citrus oils.

The characterization of citrus oils from fluorescence and fluorescence quenching profiles obtained from thin-layer chromatograms resulting primarily from the presence of crystalline components in the oils can be accomplished. *In situ* fluorimetry combines the advantages of thin-layer chromatography with those of fluorimetry which include greater selectivity and sensitivity when compared to absorption spectrophotometry and densitometry.

EXPERIMENTAL

Apparatus and materials

An Aminco Bowman Spectrophotofluorometer (SPF) with a high-pressure mercury–xenon arc source lamp, RCA 1P28 multiplier phototube, motorized thin-film scanner (American Instrument Co., Silver Springs, Md.) and a Speedomax G Model S recorder, equipped with a Disc Chart Integrator Model 203 (Leeds and Northrup Co., Philadelphia, Pa.) was used to obtain all emission and quenching profiles and quantitative data. The photomultiplier microphotometer (PMP) supplied with the SPF is designed to be connected with a 50-mV, 1-sec response recorder. Therefore, to operate the recorder with a 0-mV to 5-mV span, it is necessary to apply one-tenth of the PMP output voltage to the recorder. This was accomplished by connecting a 500 Ω variable potentiometer in parallel with the output resistance of the PMP which allows the desired signal range to be applied to the recorder.

Precoated analytical layer chromatoplates, Silica Gel F-254 (Merck) (supplied by Brinkmann Instruments Inc., Westbury, N.Y.), were used to obtain thin-layer chromatographic separations in a Brinkmann sandwich developing chamber. Singly redistilled solvents were used to develop all chromatograms.

Thin-layer chromatography and direct chromatogram analysis

The following procedure was used to obtain thin-layer chromatograms and profiles from the chromatograms in the subsequent studies. Samples were applied 2.0 cm from the bottom of a thin-layer plate. Eight or nine samples were applied to each plate. The plate was then developed to 15 cm above the origin spot line with ethyl acetate–*n*-hexane–acetic acid (50:150:1). The developed chromatogram was then dried in the dark for at least 2 h.

Direct chromatogram analysis is based on the measurement of emission from components which exhibit fluorescence on the thin-layer plate. When the chromatoplate is placed on the thin-film scanner with the adsorbent layer face down, radiation

from the source lamp is carried through the excitation monochromator of the SPF and appropriate optics and is directed onto the adsorbent layer. This radiation can cause excitation of components chromatographed on the thin-layer plate or can cause excitation of a fluorescent indicator added to the adsorbent layer prior to preparation of the chromatoplate. In either case a portion of the visible fluorescence emission, which is transmitted through the glass plate backing of the adsorbent layer, is carried by a fiber optic to the emission monochromator of the SPF then to the multiplier phototube. If the components under investigation exhibit native fluorescence then an emission profile is obtained by scanning the thin-layer chromatogram in the direction of plate development using the excitation wavelength which will excite the greatest number or most prominent components and an emission wavelength which will monitor fluorescence from the greatest number or most prominent components. If the components under investigation do not exhibit native fluorescence but do absorb UV radiation, their presence can be detected in an adsorbent layer which contains a fluorescent indicator. The components which absorb UV radiation will quench the emission of the fluorescent indicator and a quencing profile is obtained when the chromatogram is scanned utilizing excitation and emission wavelengths which correspond to maximum excitation and emission respectively for the fluorescent material previously added to the adsorbent layer.

The plates were scanned in the same direction as development to obtain qualitative profiles. Quantitative data were obtained by scanning individual chromatogram spots in directions parallel to and perpendicular to the direction of plate development. Scanning was accomplished by orientating the plate on the scanner with the adsorbent layer face down so the scan passed through the center of the chromatogram spot(s) of interest.

Direct qualitative thin-layer chromatogram analysis

Emission monochromator wavelength settings of 403 nm, 440 nm, and 490 nm were used to obtain the various fluorescence emission profiles. Excitation at 313 nm or 365 nm produces the most intense emission when a mercury–xenon lamp is employed. An excitation wavelength of 272 nm and an emission wavelength of 520 nm were used to obtain the various fluorescence quenching profiles. This wavelength combination makes it possible to measure the maximum emission from the fluorescent indicator in the adsorbent layer with the described instrumental set-up. The slit program employed when quenching profiles were recorded was 5–4–0.5–5–5 where the slit widths expressed in millimeters are for the excitation monochromator slit, excitation side of the lens and mirror assembly slit, exit aperture of the lens and mirror system slit, emission monochromator slit, and multiplier phototube shutter slit, respectively. Emission profiles were obtained with slit program 5–4–0.1–5–5. This slit arrangement gives a small improvement in resolution for emission profiles, however, equivalent quenching profiles are obtained with either slit program. The wider exit aperture slit is favored in quenching studies because it allows measurements to be made at a higher PMP meter multiplier setting which reduces instrumental noise and also is not as sensitive in recording minor variations due to "grain" in the adsorbent layer.

Thin-layer chromatograms for the expressed citrus oil samples were obtained from 0.2 to 0.5 μl of lime oils, 1.0 μl of lemon, bergamot, and grapefruit oils, and

2.0 μl of sweet and bitter orange oils. Profiles for mixtures of citrus oils were obtained from chromatograms of 1.0 μl of each oil mixture. Lemon oil–grapefruit oil mixtures, 1:1, 2:1, and 4:1; lemon oil–lime oil mixtures, 1:1 and 4:1; and grapefruit oil–lime oil, 4:1 were investigated.

Direct quantitative thin-layer chromatogram analysis

The influence of time on fluorescence intensity for I and II was investigated under conditions to be used for quantitative determinations. The thin-layer chromatograms were obtained from appropriate amounts of I and II. The first scans of the chromatogram spots were started immediately after completion of plate development. Chromatogram spots were then scanned at approximately 15-min intervals for 5 h. The plate was aligned so that each scan would pass through the center of the individual spots. The center of the spot was located by manual adjustment of the plate position until a maximum signal was registered by the PMP. Quantitative *in situ* fluorescence emission and quenching were measured with slit program 5–4–0.5–5–5.

Known amounts of I and II were subjected to thin-layer chromatography. *In situ* fluorescence analytical curves were obtained from the resulting chromatograms. Fluorescence quenching analytical curves were obtained for 5-geranoxypsoralen (III) and 5,8-dimethoxypsoralen (IV) using a similar procedure. The quenching analytical curves were obtained using 272-nm excitation and 520-nm emission monochromator wavelength settings. The emission analytical curves were obtained with 335-nm excitation and 440-nm emission monochromator settings. While maximum excitation is not obtained from the mercury–xenon lamp at 335 nm this is a more suitable wavelength than 313 nm or 365 nm for quantitative analysis because photodecomposition due to the intense radiation does not become significant.

Determination of the amounts of I and II present in citrus oils was accomplished by analyzing up to five samples on the same thin-layer plate with four or five standards. Standard solutions which contain both I and II were prepared. The concentration of each component was such that an analytical curve for each could be obtained which is applicable for analysis of I and II in the particular citrus oil being analyzed. Standard solutions with the following concentrations of I and II, respectively, in ethanol were prepared: (A) 25.0 μg/ml, 10.9 μg/ml; (B) 49.9 μg/ml, 10.9 μg/ml; and (C) 8.32 μg/ml, 21.8 μg/ml. Ethanol solutions of the citrus oils which contained 2.0 mg/ml, 10 mg/ml, 20 mg/ml and 500 mg/ml of expressed lime, bergamot, lemon, and distilled lime oils, respectively, were prepared. Four aliquots ranging from 1.0 to 4.0 μl of solution A or B were spotted to obtain an analytical curve for the analysis of the amounts of I and II present in lemon or expressed lime oils. The amounts of each lemon or lime oil solution to be spotted were determined from a preliminary analysis on each sample. Depending on the approximate amounts of I and II present in each sample, 2.0, 2.5, or 3.0 μl of each lemon oil solution and 1.0, 1.5, 2.0, or 4.0 μl of each expressed lime oil solution were spotted. Five aliquots ranging from 1.0 to 5.0 μl of solution C were spotted along with 3.0 μl of each distilled lime oil solution and 1.5 μl of each bergamot oil solution for the analysis of I and II in these oils.

Relative fluorescence emission intensity (*RE*) determined from chromatogram profile peak areas was obtained as follows. A baseline was drawn tangent to the minima which occur between successive peaks in each profile. The geometric area (*GA*) expressed in square chart units, under the baseline was converted to integrator counts.

The conversion factor is 3.02 integrator counts per square unit of chart paper. The total integrator count (*IC*) for each peak includes this background or blank area. The relative emission intensity was determined using the following expression

$$RE = [IC - GA(3.02)]MM \quad (1)$$

where *MM* is the PMP meter multiplier setting. Recorder chart speed was two inches per minute, which represents approximately one inch of chart paper per inch of thin-layer chromatogram scanned. Peak areas as small as one square centimeter and as large as twenty square centimeters were obtained under the described conditions.

Relative fluorescence quenching intensity (*RQ*) from chromatogram profile peak areas was determined by triangulation. A baseline was drawn tangent to the maxima which exist when no absorbing component is present on the adsorbent layer. Peak height (*PH*) to the baseline was determined. The peak width at one-half peak height (*WHP*) and peak height were used to determine the peak area. The relative quenching was determined using the following expression

$$RQ = (PH)\ (WHP)\ (3.02)MM \quad (2)$$

where all terms have been previously defined. The 3.02 factor is introduced to convert peak area into integrator counts which allows *RQ* to be compared to *RE*.

Relative fluorescence emission and fluorescence quenching intensity from chromatogram profile peak heights (*RH*) were determined as follows

$$RH = (PH)MM \quad (3)$$

where the peak height, expressed in chart units, is measured from the baseline drawn as previously described to the peak maximum for emission or peak minimum for quenching.

RESULTS AND DISCUSSION

Direct qualitative fluorimetry of citrus oil thin-layer chromatograms

Fluorescence emission and fluorescence quenching profiles for various citrus oils are presented in Figs. 1 to 4. The fluorescence emission profiles in Figs. 1 and 2 represent typical but not necessarily the most characteristic profiles for each oil. Comparison of two emission profiles of grapefruit oil, Fig. 1A, and two emission profiles of bitter orange oil, Fig. 2A, reveals that the presence of the greatest number of components is detected when two or more profiles are obtained for each oil using different characteristic emission monochromator settings. The variations obtained are generally limited to increases or reductions in the relative emission intensity emanating from each component spot. It is not intended that these profiles reflect any correlation with respect to intensity and therefore to the amounts of individual components present. In extreme cases it may be possible to completely resolve one component from another in the emission profile through the judicious choice of both excitation and emission wavelengths. This technique has been investigated in studies related to air pollution[2]. An emission maximum of 440 nm for two spots predominates in lemon, lime, and bergamot oil thin-layer chromatograms. This emission is due to the presence

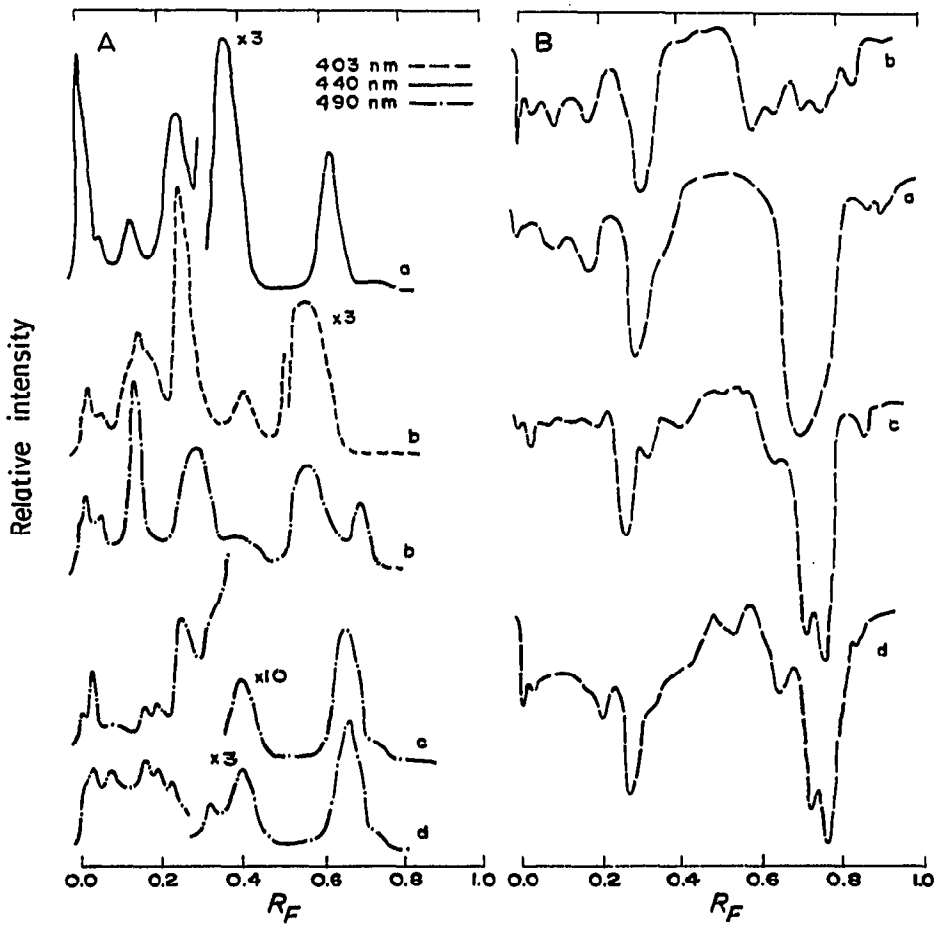

Fig. 1. Fluorescence (A) and fluorescence quenching (B) profiles of expressed citrus oils. (a) Bergamot; (b) California grapefruit; (c) Persian lime; (d) West Indian lime.

of I and II. The much lower relative fluorescence intensities of other types of coumarin derivatives result in less prominent emission from these components. Expressed citrus oils can be characterized to some extent by measuring emission profiles which result from the use of various emission wavelengths because of the presence of a number of 5-alkoxypsoralens, 5,7-dialkoxycoumarins, and 7-alkoxycoumarins. Each shows a characteristic emission spectrum with maxima at 490 nm, 440 nm, and 403 nm, respectively.

The citrus oils in turn exhibit profiles which are generally characteristic of each particular kind of oil. In addition, minor variations are observed in profiles obtained from different samples of each kind of citrus oil. Expressed lime oil samples show the greatest variations. Three distinctly different profiles, Figs. 1A, c and d and 4A, were obtained from samples of lime oil. However, classification into subgroups based on individual profiles after examination of only one sample from various geographical regions cannot be justified. Even classification of each lime oil sample into the Mexican or Tahitian group[11] cannot be made without reservation. Only two samples of expressed grapefruit oil and of expressed bergamot oil were examined. However, characteristic profiles are obtained for each oil. Only minor variations are observed in the profiles obtained for samples of lemon oil. The only sample of bitter

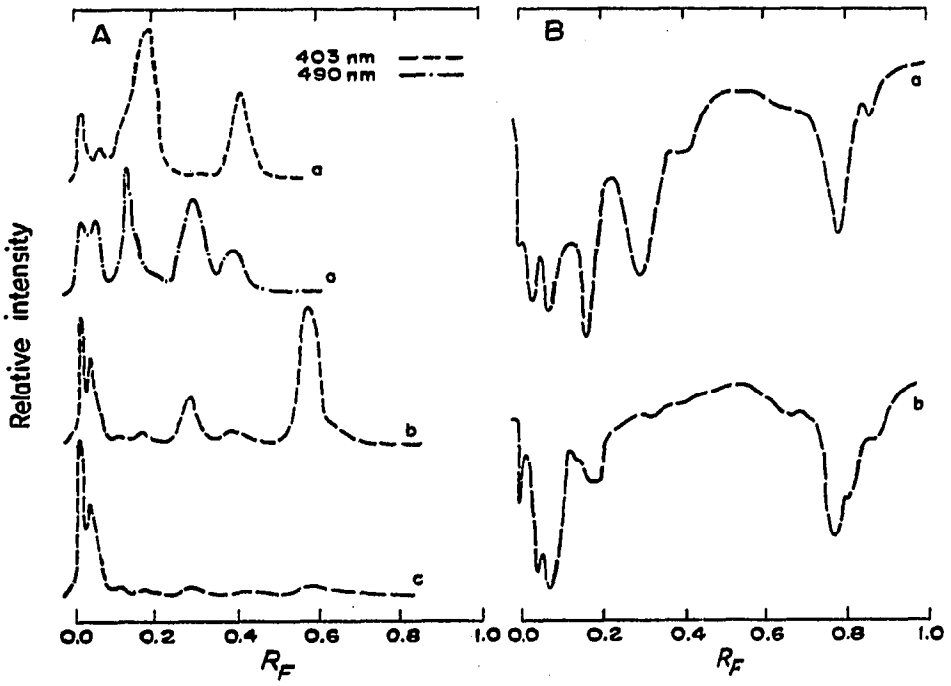

Fig. 2. Fluorescence (A) and fluorescence quenching (B) profiles of expressed orange oils. (a) West Indian bitter orange; (b) orange, North American standard; (c) Florida orange.

orange oil investigated has emission and quenching profiles which are greatly different from those obtained from various samples of sweet orange oil.

Comparison of the various emission and quenching profiles of the citrus oils reveals an increased complexity of expressed lemon and lime oils compared to the other oils. Lemon and lime oils while exhibiting similar profiles do show variations which allow for differentiation between the two. The emission and quenching profiles of expressed lemon oil samples are characterized by the presence of two well-resolved peaks at R_F 0.00 and 0.03. In the same region of expressed lime oil thin-layer chromatograms only two of five samples investigated yielded a profile similar to the lemon oil profile. Lime oil at R_F 0.70 and 0.75 exhibits two partially resolved peaks in the quenching profile. These can be attributed to the presence of III and citral and/or other oxygenated terpenes which are capable of absorbing short-wavelength UV radiation. In the corresponding region of the lemon oil quenching profile only a shoulder due to the presence of III is observed on the prominent peak which again results from the presence of citral and/or other oxygenated terpenes. This difference may be due to the larger amount of III present in lime oil compared to lemon oil. Prominent peaks which correspond to the presence of I and II do not occur in the quenching profiles. The fluorescence of these two components upon excitation with short-wavelength UV light apparently is great enough that emission from these components will compensate for loss of emission from the fluorescent indicator in the adsorbent layer. In the emission profiles of both lemon oil and lime oil the presence of III is indicated by a shoulder at R_F 0.7 on the very intense peak due to the presence of I. The peak for III is most apparent when measurement of the emission profile at 490 nm instead of 440 nm is employed. These are prominent differentiating features observed in the emission and quenching profiles for expressed lemon and lime oils.

A general feature which distinguishes expressed lime oil emission profiles from other citrus oils is present in the first quarter of the chromatogram scan. A relatively high background emission is observed. This background emission is not as pronounced in the emission profiles of other citrus oils. The thin-layer chromatograms of expressed lime oils, when examined under 365 nm UV light, show considerable streaking in this region while the other citrus oil thin-layer chromatograms are relatively free from streaking when the same plate development procedure is used.

Expressed bergamot and orange oils exhibit profiles of less complexity presumably because of the smaller number of coumarin derivatives present[6]. The presence of at least seven well-defined peaks or shoulders on peaks in the emission profiles for the samples of orange oil and bergamot oil indicates that the coumarin composition of these oils may be much more complex than has been reported.

The emission and quenching profiles obtained can be of considerable value in detecting mixtures of two oils. Lemon oil–grapefruit oil mixtures can be detected from the quenching profile, Fig. 3, where at R_F 0.4 a strong absorption appears at a 1:1 mixture of the two oils and becomes progressively stronger as the relative amount of lemon oil is increased. The value of this observation is limited to situations where grapefruit oil is adulterated with a large amount of lemon oil. A more meaningful relationship exists when comparisons are made of the peaks which appear at R_F 0.3 and 0.4. Both peaks are present in lemon oil profiles where the latter is generally more intense. However, only the peak at R_F 0.3 is present in grapefruit oil profiles. Therefore, the presence of even small amounts of grapefruit oil in lemon oil can be detected by observing an increase in intensity of the peak at R_F 0.3 with respect to the peak at 0.4.

The emission profiles of lemon oil–grapefruit oil mixtures are characterized by variations which occur over the 0.5 to 0.7 R_F region. These profiles, obtained with emission monochromator settings of 490 nm, 440 nm, and 403 nm, reflect the presence

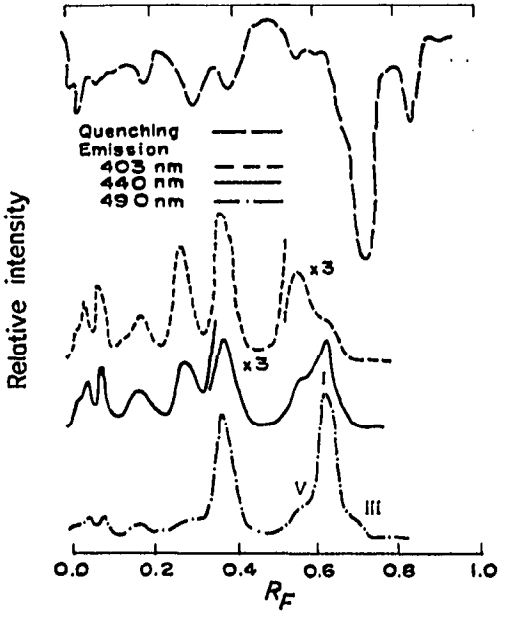

Fig. 3. Fluorescence and fluorescence quenching profiles of a California lemon oil and California grapefruit oil mixture, 4:1.

of III, I, and 7-geranoxycoumarin (V) in the mixture. As shown in Fig. 3 the presence of V is readily detected in the presence of I when a comparison is made of the profiles obtained at 403 nm and 440 nm.

Quenching profiles of lemon oil–lime oil mixtures cannot be used readily to detect the presence of a mixture. This is because of the similarity in profiles obtained for the individual oils and because samples of lime oil have been shown to exhibit variations in individual profiles. Some resemble lemon oil more closely than others. However, a mixture of lemon oil and lime oil can be suspected if the characteristic quenching profiles from R_F 0.6 to 0.8 are not observed. The emission profiles of lemon oil and lime oil are also very similar and no truly distinct characteristics are observed. The detection of lemon oil–lime oil mixtures is best evaluated from quantitative data obtained for I and II, which are present in both oils. These data are presented and evaluated in the section which considers quantitative applications of the technique.

The lime oil–grapefruit oil mixture was not extensively investigated. However this mixture can be detected from observations similar to those already discussed for lemon oil–grapefruit oil mixtures.

Thin-layer chromatography of the coumarin derivatives isolated from expressed lime oil[10] and run simultaneously with samples of expressed lemon and lime oils makes possible the tentative identification of the crystalline components responsible for many of the peaks in the emission and quenching profiles of the two oils. Table I identifies some of the coumarin derivatives which are responsible for peaks in expressed lemon and lime oil profiles and designates peaks in Fig. 4 which have been attributed to the presence of these components. Identifications were based on results obtained from *in situ* fluorimetry and by visualization of the citrus oil and crystalline component thin-layer chromatograms under UV light. A number of less prominent peaks appear which are possibly due to the presence of other not yet identified coumarin derivatives. Some components may not yield characteristic peaks in the emission and/or quenching profiles because of the small amounts present or weak native fluorescence.

An improvement in resolution of the individual components on the citrus oil

TABLE I

IDENTITY OF COUMARIN DERIVATIVES RESPONSIBLE FOR THE EMISSION AND QUENCHING PROFILES FOR EXPRESSED WEST INDIAN LIME OIL AND EXPRESSED CALIFORNIA LEMON OIL

Coumarin derivative	*Peak number*	
	Lime oil (Fig. 4A)	*Lemon oil (Fig. 4B)*
5-Geranoxypsoralen (III)	1	1
5-Geranoxy-7-methoxycoumarin (I)	2	2
5-Geranoxy-8-methoxypsoralen	3	3
5-Isopentenoxy-8-methoxypsoralen	4	
8-Geranoxypsoralen		5
5,7-Dimethoxycoumarin (II)	6	6
5-Methoxy-8-isopentenoxypsoralen / 8-Isopentenoxypsoralen	7	
5-Methoxypsoralen	8	
5,8-Dimethoxypsoralen (IV)	9	
5-Dihydroxyisopentanoxypsoralen	10	10
5-Methoxy-8-dihydroxyisopentanoxypsoralen	11	11

thin-layer chromatograms can be accomplished using a developing solvent such as ethyl acetate–*n*-hexane–acetic acid 30:270:1 and developing the plate three times instead of only the single development with 50:150:1 solvent. The improvement is most apparent in the quenching profile. However, a single development is adequate to characterize the different citrus oils.

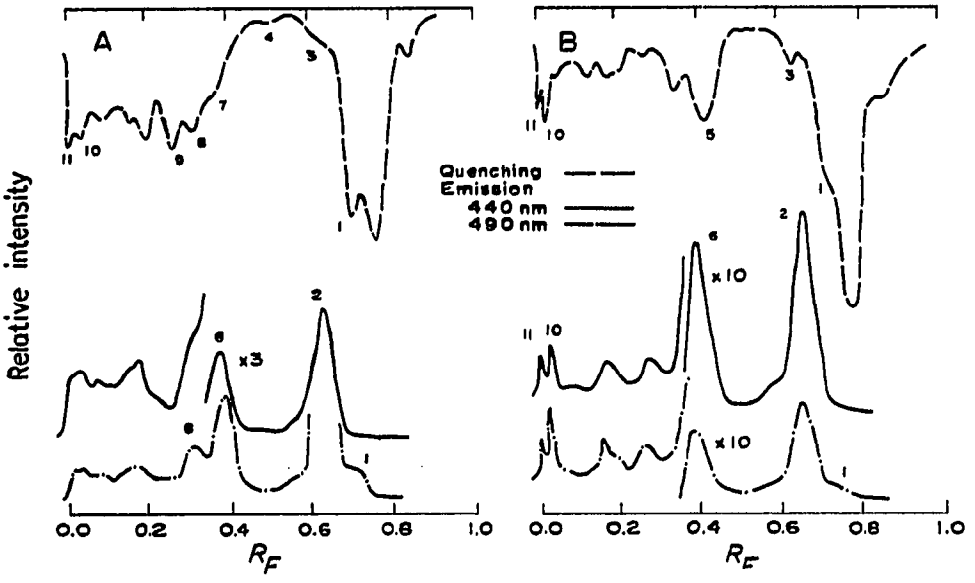

Fig. 4. Fluorescence and fluorescence quenching profiles of Florida lime oil (A) and California lemon oil (B). The numbered peaks are identified in Table I.

The same peaks appear in emission profiles obtained with excitation from a xenon lamp at 330 to 350 nm and from the mercury–xenon lamp at 313 nm or 365 nm. However, the intensity of various peaks is changed when different lamps and different excitation wavelengths are used. The mercury–xenon lamp is of greatest value for characterization purposes because the high intensity at 313 nm and 365 nm makes it possible to detect trace components. This advantage is limited if measurements are made on thin-layer chromatograms with high fluorescent backgrounds. Both lamps give identical quenching profiles using 272-nm excitation. However, the mercury–xenon lamp makes the use of a higher meter multiplier setting on the PMP possible.

Under the conditions employed to develop the chromatograms, the emission profiles can be used only for qualitative characterization of each individual oil. No extensive significance should be applied to the relative intensity of the various peaks in the profile because the emission from many of the components is highly quenched due to the relatively high concentration of the various components present. In addition, the fluorescence efficiency for individual coumarin derivatives has been shown to vary over a wide range. Variations in intensity observed when emission profiles are recorded with different emission monochromator settings can be significant for determination of the type of coumarin derivative present. For example, in the lemon oil–grapefruit oil mixture profiles, Fig. 3, recorded at 403 nm and 440 nm, the relative intensity for the peaks of R_F 0.5 and 0.6 is reversed. This indicates the presence of two components which are characterized by different emission wavelength maxima.

Evaluation of experimental parameters for direct quantitative fluorimetry of citrus oil thin-layer chromatograms

An initial investigation of various parameters which can affect quantitative *in situ* fluorimetry of thin-layer chromatograms was conducted. These parameters include the influence of time on fluorescence intensity, component spot alignment with respect to the exit aperture slit, the relationship of profile peak area and peak height to concentration for purposes of obtaining linear analytical curves, and methods of area determination for the profile peaks obtained from both emission and quenching measurements.

An investigation of the influence of time on fluorescence intensity for I and II revealed an initial decrease in intensity followed by an essentially constant intensity up to approximately 5 h when measurements were discontinued. This indicates that quantitative measurements should be made only after allowing the developed thin-layer chromatogram to dry in the dark for a minimum of 1 h. Apparently the "wetness" of the adsorbent layer exerts an influence on fluorescence emission intensity. The precaution of drying the plate in the dark is to prevent any possible photodecomposition of the components.

The length of the exit aperture slit of the lens and mirror assembly, which is approximately 5 mm, and the dimensions of the source lamp radiation covering this slit, which are determined primarily by the excitation side of the lens and mirror assembly slit, limits the area of the chromatogram spot that is subjected to the excitation radiation. This plus the small area monitored by the fiber optic does not allow measurement of the entire emission from one chromatogram spot during a single scan. Therefore individual spots should be aligned manually with respect to the exit aperture slit before an actual scan is made. This alignment should be such that maximum emission intensity can be measured. While some variation is observed when the same spot is consecutively rescanned, this does not appear to be a serious disadvantage. Best results are obtained from an average of duplicate or triplicate scans of each chromatogram spot.

A restricted but usable linear range for the relationship of peak area to concentration has been reported using the Aminco thin-film scanner[12]. These authors were concerned with lateral diffusion of the spots during plate development. They used scored thin-layer plates which limited the lateral diffusion to the length of the exit aperture slit. In the present investigation it was found that it is not necessary to use scored thin-layer plates. Fig. 5 shows typical analytical curves for I and II. Both peak

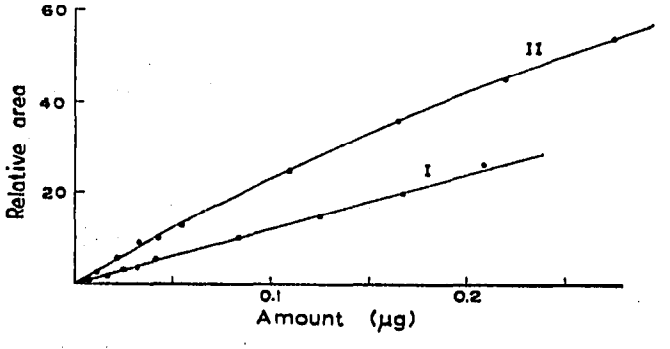

Fig. 5. Fluorescence analytical curves for 5-geranoxy-7-methoxycoumarin (I) and 5,7-dimethoxycoumarin (II).

area and peak height can be used to monitor emission intensity for analysis. These curves represent relationships of emission intensity to amount of component present where only part of the total chromatogram spot emission is measured. If care is taken to scan the chromatogram spot so the maximum emission intensity is measured then analytical curves are obtained which are capable of yielding accurate results in the analysis of unknowns.

The utility of fluorescence quenching in analysis of III and IV was investigated. A usable linear analytical curve can be obtained using either peak area or peak height to monitor the amount of quenching. Fig. 6 shows the typical behavior which is observed. The sensitivity of fluorescence quenching is not as great as for fluorescence emission measurements in many cases. However, in this case the emission intensity of III is quite low and that of IV undetectable at the concentrations where quenching is applicable.

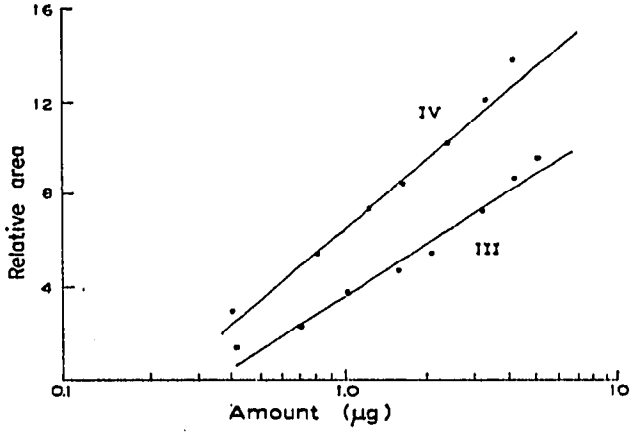

Fig. 6. Fluorescence quenching analytical curves for 5-geranoxypsoralen (III) and 5,8-dimethoxypsoralen (IV).

Comparison of profile peak areas determined by triangulation with values obtained from the integrator indicate that for profile peaks which result from measurement of emission the areas obtained from the integrator are more consistent. However, because a small decrease in a large integrator response is usually observed when quenching of plate fluorescence is measured, the triangulation procedure is favored.

Determination of 5-geranoxy-7-methoxycoumarin and 5,7-dimethoxycoumarin in citrus oils

The fluorescence emission profiles of expressed lemon, lime, and bergamot oils show two prominent peaks which are due to the presence of I and II. In addition, distilled lime oil thin-layer chromatograms exhibit two blue weakly fluorescent spots which correspond to those in the previously mentioned oils. An adjustment in the amount of citrus oil spotted on the thin-layer plate is necessary before quantitative determination of I and II can be accomplished because quenching of the measured emission is observed at the concentrations of I and II present when the whole oils are chromatographed. Application of a more dilute sample to the thin-layer plate,

while not significantly decreasing the intensity of the emission due to I and II, results in a decrease in the emission from other component spots. Therefore, the emission due to the presence of I and II is essentially free from interference of other components even in situations where complete separations from other components in the thin-layer chromatogram have not been accomplished. Only in the analysis of bergamot oil does appreciable interference arise due to the relatively large amount of III present.

Attempts to duplicate fluorescence emission analytical curves for I and II were of only limited success. Apparently minor variations in adsorbent layer thickness and conditions of plate development cause variations in chromatogram spot shape which make it difficult to obtain reproducible results from day to day and from plate to plate. This difficulty may be due to the fact that only a portion of the total potential emission can be measured with the instrument used. However, relationships similar to those shown in Fig. 5 are easily obtained. The slope of the analytical curves vary by as much as 25 % from plate to plate. For this reason quantitative determination of I and II was performed by spotting up to five citrus oil samples and four or five standards on each plate. This procedure makes it possible to obtain an analytical curve directly from the thin-layer chromatogram used in the analysis.

Chromatogram spots were scanned parallel to and perpendicular to the direction of plate development. Peak areas obtained by scanning in the direction of plate development are somewhat greater than those obtained by scanning perpendicular to the direction of plate development. This behavior is observed because the chromatogram spots of both I and II are elliptical in shape with the major axis in the direction of plate development. Peak heights obtained by parallel and perpendicular scanning of the same spot exhibit insignificant differences.

The amounts of I and II present in the citrus oil samples can be determined from peak area or peak height obtained either by scanning the individual chromatogram spots in a direction parallel to or perpendicular to plate development. Results obtained by these four procedures are presented for two selected citrus oils in Table II. The results obtained by scanning the chromatograms perpendicular to the direction of plate development are generally somewhat lower than those obtained from parallel scanning. A slight improvement is observed in the comparison of experimental and

TABLE II

A COMPARISON OF THE AMOUNT OF 5-GERANOXY-7-METHOXYCOUMARIN AND 5,7-DIMETHOXYCOUMARIN DETERMINED BY *in situ* FLUORIMETRY FROM CHROMATOGRAM PEAK AREA AND HEIGHT AND FROM DIFFERENT DIRECTIONS OF SCANNING

	5-Geranoxy-7-methoxycoumarin (%)[a]		*5,7-Dimethoxycoumarin* (%)[a]	
	California lemon	*Florida lime*	*California lemon*	*Florida lime*
Parallel scan				
Peak area	0.152 ± 0.002	0.690 ± 0.014	0.0606 ± 0.0020	0.203 ± 0.006
Peak height	0.152 ± 0.002	0.709 ± 0.012	0.0610 ± 0.0015	0.179 ± 0.002
Perpendicular scan				
Peak area	0.130 ± 0.003	0.667 ± 0.027	0.0568 ± 0.0005	0.185 ± 0.002
Peak height	0.131 ± 0.004	0.662 ± 0.012	0.0561 ± 0.0006	0.178 ± 0.003

[a] Average of triplicate analysis.

TABLE III

EVALUATION OF *in situ* FLUORIMETRY FOR THE DETERMINATION OF 5-GERANOXY-7-METHOXYCOUMARIN AND 5,7-DIMETHOXYCOUMARIN IN CITRUS OILS

	5-Geranoxy-7-methoxycoumarin		*5,7-Dimethoxycoumarin*		*Solution fluorescence relative intensity*	
	Av. (%)[a]	*RD*[b] (%)	*Av.* (%)[a]	*RD*[b] (%)	*Experimental*	*Calculated*
Expressed lime oil						
Oil lime	1.58	0.7	0.455	0.7	5.50	4.53
Persian	2.95	2.4	0.465	2.6	7.97	7.36
Mexican	3.54	2.5	0.656	1.5	10.7	9.12
West Indian	4.66	7.3	0.842	12.7	13.2	11.92
Florida	0.690	2.0	0.203	3.0	2.14	1.99
Expressed lemon oil						
Italian	0.134	2.2	0.0585	1.2	0.486	0.442
California	0.152	1.3	0.0606	3.3	0.501	0.485
California (dewaxed)	0.129	0.8	0.0578	1.4	0.455	0.429
North American standard (sample 1)	0.152	1.3	0.0297	3.7	0.400	0.396
North American standard (sample 2)	0.158	1.9	0.0471	1.5	0.506	0.458
Expressed bergamot oil						
Italian	0.100	4.0	0.258	1.2	1.10	0.948
Extra	0.0754	5.8	0.155	3.9	0.761	0.601
Distilled lime oil						
West Indian natural	0.00040	7.5	0.00319	6.5	0.0138	0.0100
Extra	0.00087	27.6	0.00365	1.7	0.0124	0.0123

[a] Average of triplicate analysis.
[b] Relative deviation.

calculated fluorescence intensities of the citrus oils discussed below when the amounts of I and II are determined from chromatogram scans parallel to the direction of plate development. Results obtained for other citrus oils from peak areas and chromatogram scanning in the direction of plate development are presented in Table III. The amounts of coumarin derivatives I and II present in the citrus oil samples were calculated as follows:

$$\%\mathrm{I} = \frac{\mu\mathrm{g\ I}}{\mu\mathrm{g\ oil}}(100\,\%) \tag{4}$$

$$\%\mathrm{II} = \frac{\mu\mathrm{g\ II}}{\mu\mathrm{g\ oil}}(100\,\%) \tag{5}$$

where μg I and μg II are determined directly from the appropriate analytical curve obtained from the same thin-layer plate.

The quantitative analysis of individual coumarin derivatives has not been the subject of extensive investigations[13,14]. The amounts of the individual components present in citrus oils are not accurately known and can vary from sample to sample.

A comparison can be made between the relative fluorescence intensity from ethanol solutions of citrus oils, determined as previously described[10], and ethanol solutions of components primarily responsible for the citrus oil emission. The fluorescence from ethanol solutions of lemon, lime, and bergamot oils can be attributed to the presence of I and II in each oil. The contribution of fluorescence intensity due to I and II can be determined if the relative fluorescence intensity of the two components in ethanol is known and if the concentrations of the two components in the oil sample under investigation are known. Calculation of a relative fluorescence intensity (*RI*) for citrus oils is based on the following equations:

$$RI_{\mathrm{I}} = \frac{20.4\ (\%\,\mathrm{I})}{10} \tag{6}$$

$$RI_{\mathrm{II}} = \frac{28.8\ (\%\,\mathrm{II})}{10} \tag{7}$$

$$RI_{\mathrm{I}} + RI_{\mathrm{II}} = RI_{\mathrm{citrus\ oil}} \tag{8}$$

where the experimental numerical factors 20.4 and 28.8 are the relative fluorescence intensities from ethanol solutions of I and II (ref. 10). The factor of 1/10 is introduced in eqns. 6 and 7 to relate the relative fluorescence intensities of I and II measured for 0.1 μg/ml solutions and the percent of I and II to the relative intensity of 1.0 μg/ml citrus oil solutions. Agreement between the calculated relative fluorescence intensity for each citrus oil calculated from eqns. 6, 7, and 8 and an experimentally measured relative intensity is used to determine if the method for determination of the concentrations of I and II in each citrus oil is indeed reasonable and accurate. An evaluation of the method using the above comparison is presented in Table III, where agreement between calculated and experimental relative intensities is observed to be quite good. The assumption made prior to making this comparison is that the measured solution fluorescence characterized by excitation at 335 nm and emission at 420 nm of the four citrus oils is due primarily to the fluorescence of I and II. The results presented in Table III in addition to verifying this assumption have also established the reliability and accuracy of the method.

Determination of the concentrations of I and II in this limited number of samples has revealed that these components are present in widely varying amounts in expressed lime oil. This variation is not observed for expressed lemon oil. In no sample of lime oil did the amount of I or II represent less than three times the corresponding component in lemon oil. This observation indicates that *in situ* fluorimetry can be valuable for detecting mixtures of lemon oil with expressed lime oil simply by determination of the concentrations of I and II. It will be necessary to investigate a much larger number of samples before definite concentration limits can be formulated.

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