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IMPROVED METHOD FOR BERGAPTEN DETERMINATION BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

Different high-performance liquid chromatography methods were examined for determinations of bergapten in natural or treated citrus oils including bergamot, lime, lemon, orange, tangerine, petitgrain and grapefruit. Comparisons among normal bonded-phase partition chromatography (Carbowax 400/Corasil), porous-layer particle adsorption chromatography (Corasil II) and totally porous microparticle adsorption chromatography (μ-Porasil and Zorbax-Sil) are discussed. In the most effective method for quantitative assay of bergapten in all the above citrus oils a Zorbax-Sil column equipped with specially constructed low dead-volume fittings was employed. A sensitivity better than 0.5 ppm for bergapten can be obtained using 254-nm ultraviolet absorbance detection.

INTRODUCTION

Many citrus oils are commonly used in flavor and fragrance manufacture. However, phototoxic furocoumarins, of which bergapten is the most important, are present naturally in some oils at levels usually below 1% (refs. 1–5). The industries concerned have made great efforts to remove or to minimize bergapten content but have been hampered by lack of a sensitive and reliable method for its determination.

In the past, Cieri[1] and Evers and Sieczkowski[6] used thin-layer chromatography (TLC) and ultraviolet (UV) detection to estimate bergapten in several oils, but quantitative data obtained from the TLC method are of questionable reliability. Gas chromatographic (GC) methods have been utilized[7,8]. However, Everett[8] found that reproducibility was poor when analyses were attempted at lower levels of bergapten.

Recently, Stermitz and Thomas[9] published a high-performance liquid chromatographic (HPLC) method for separation of furocoumarins extracted from spring parsley. Based on this technique, Porcaro and Shubiak[10] developed a quantitative procedure for determination of bergapten content in bergamot oils. Both methods employed adsorption chromatography on columns of Corasil I (ref. 9) or Corasil II (ref. 10) and the combination of chloroform and *n*-hexane as the mobile phase without an added modifier. Initially, we attempted to duplicate the work of Porcaro and

Shubiak[10], but difficulties were encountered until a suitable amount of mobile phase modifier was used. Because high-performance adsorption chromatography requires considerable time and effort to prepare solvents and adsorbent and to equilibrate the column in order to maintain column reproducibility[11–13], an attempt was made to use partition chromatography with permanently bonded phases. This approach is more straightforward if the necessary separations can be achieved.

A reversed-phase partition chromatographic column, Bondapak C18/Corasil (Waters Ass., Framingham, Mass., U.S.A.) was first tried, but bergapten co-eluted with other constituents in a bergamot oil. A normal partition chromatographic packing Carbowax 400/Corasil was tested and found to perform the required separation for most of the samples. However, the separation obtained was not reproducible with packings from different production batches, and further, the resolution obtained was inadequate for the analysis of some oils. Consequently, the micro-particle adsorbent columns, μ-Porasil (Waters Ass.) and Zorbax-Sil (DuPont, Wilmington, Del., U.S.A.) were evaluated with the goal to determine bergapten content in all citrus oils using a single method. After careful manipulation of the chromatographic conditions, a much improved chromatographic method employing a Zorbax-Sil column was developed.

In this paper the different chromatographic methods are covered and the results of analyses from each method are compared in dealing with seven types of citrus oils. The advantages of each method are also discussed.

EXPERIMENTAL

Apparatus

Pumping systems, detectors and injectors used in this study are tabulated in Table I. A 5-μl high-pressure syringe, Hamilton Model HP305 from Hamilton, was used.

TABLE I

APPARATUS USED IN THIS STUDY

Apparatus	*Type*			
Pumping system	Waters Ass. Model C903	Waters Ass. Model 6000	Varian-Aerograph Model 4100	DuPont Model 841
Injector	Varian septum	Varian septum	Varian high pressure	DuPont septum
Detector (UV, 254 nm)	Varian	Varian	Varian	DuPont

Samples

The recrystallized bergapten reference standard was prepared by Mr. Robert Trenkle of International Flavors and Fragrances (IFF), and its purity was shown to be greater than 99% by TLC, GC and LC. Carefully weighed samples were dissolved in a minimum amount of chloroform before appropriate dilutions were made with *n*-heptane. Different citrus oil samples, either natural or treated, also supplied by IFF,

were diluted with chloroform, if necessary, to obtain adequate resolution of the bergapten peak.

Solvents

All solvents used were spectro quality. Water in solvents was removed either by distillation for lower-boiling solvents (tetrahydrofuran (THF), chloroform, *n*-hexane and ethylacetate) or by passage through a large silica gel column (*n*-heptane and isooctane). Isopropanol (IPA) and methanol were used without treatment. In subsequent work we have purified the lower-boiling solvents by both distillation and passage through silica gel to insure more consistent results.

Columns

Columns employed in this study with column specifications are shown in Table II. For columns of Carbowax 400/Corasil and Corasil II, the stainless steel tubing was washed with solvents and dry-packed using the tap-fill procedure[14]. Prepacked columns of μ-Porasil and Zorbax-Sil were purchased from Waters Ass. and DuPont, respectively. The adsorption columns were conditioned by passing dry chloroform through the column at high flow-rate for several hours. Standard, commercially available stainless-steel reducing Swagelok fittings (Crawford Fitting Co.) were used for all columns except the Zorbax-Sil column on which the standard DuPont Model 841 fittings were utilized when used in that instrument. Virtually zero dead-volume fittings were made for the Zorbax-Sil column in our laboratory by boring the shoulder on the 1/4 in. O.D. end down to the bottom. The fitting on the injector end of the column was further modified by filling the injector connection with silver solder and drilling a small hole (approx. 0.03 in. I.D.) through to clear the syringe needle, so that the dead volume was much reduced. This fitting modification allowed the 1/4 in. O.D. column end to extend into the fitting completely to the point of reduction to the small diameter opening.

Data calculations

For quantitative measurements, an external standard computer method[15] was used for the Carbowax 400/Corasil short column. Manual peak area triangulation methods were used for the other columns.

Procedure

Partition chromatography. Using a bergamot oil, the bergapten reference standard and a pumping system (Nodel 6000, waters Ass.), separation of bergapten from the other constituents present in the sample was tried on the 2 ft. × 1/8 in. O.D. Carbowax 400/Corasil column under various chromatographic conditions. IPA–*n*-heptane (3:97) was chosen as the optimum mobile phase. The peak from the bergamot oil sample corresponding to bergapten in retention time was collected and analyzed by mass spectrometry to insure that it was the single desired component. Under the conditions established, different amounts of bergapten reference solutions were successively injected in order to determine the useful linear range of the detector. After samples had been analyzed, the mobile phase was changed to THF–*n*-heptane (5:95) or THF–isooctane (5:95) and a longer column (5 ft. × 1/8 in. O.D.) was used to provide increased resolution.

TABLE II

CHROMATOGRAPHIC CONDITIONS CHOSEN IN THIS STUDY

Column	*Dimensions*	*Particle character*	*Particle size (μm)*	*Chromatographic type*	*Mobile phase used*	*Flow-rate (ml/min)*	*Number of theoretical plates*
Carbowax 400/Corasil	2 ft. × 1/8 in. O.D. 5 ft. × 1/8 in. O.D.	Bonded in ester form on porous layer support	35	Bonded-phase partition	IPA–*n*-heptane (3:97) or THF–*n*-heptane (5:95)	0.5	1300
μ-Porasil	30 cm × 4.0 mm I.D.	Totally porous	10	Adsorption	Isooctane-ethyl acetate-isopropanol (80:1:1)	1	2700
Zorbax-Sil	25 cm × 2.1 mm I.D.	Totally porous	5	Adsorption	Isooctane-ethyl acetate-isopropanol (80:1:1)	0.4	2500
Corasil II	4 ft. × 1/8 in. O.D.	Porous layer	35	Adsorption	Chloroform–*n*-hexane (1:3) + 0.125% methanol	1	900

Micro-particle adsorption chromatography

Based on the results obtained from the partition columns, a μ-Porasil column and a Zorbax-Sil column were individually examined under several different chromatographic conditions using the DuPont 841 and the Varian-Aerograph (Walnut Creek, Calif., U.S.A.) 4100 pumping systems. The most suitable conditions found in each case are shown in Table II. The low dead-volume fittings were installed on the Zorbax-Sil column in the Varian instrument.

Porous-layer particle adsorption chromatography

In an effort to confirm the literature method[10], Corasil II was tried using the C903 pumping system (Waters Ass.) and the chloroform–*n*-hexane mobile phase as reported[10]. Different amounts of methanol were added to the mobile phase as a modifier, and the separations were again attempted. Samples were analyzed under the conditions indicated in Table II for the purpose of comparison with other columns.

RESULTS AND DISCUSSION

A typical chromatogram obtained using the Carbowax 400/Corasil short column for bergapten determination is shown in Fig. 1. Each analysis was completed in about eight min. The mass spectrum of the peak collected from the column corresponding to the bergapten retention time was identical to the spectrum obtained from the bergapten reference standard. The reproducibility and the detectability achieved using this chromatographic system are ±1.5% and 3 ppm, respectively. No evidence of stationary phase bleeding or change in retention time was observed during the three months of study on this column using IPA–*n*-heptane (3:97). The manufacturer originally recommended to use not more than 10% alcohol, because the stationary phase, Carbowax 400, is chemically bonded to the Corasil support in the silicate ester form and can be hydrolyzed by water or alcohols in excessive concentration. More recently, the manufacturer has recommended that alcohol be avoided altogether. Based on this recommendation 5% THF was found to be an adequate replacement for IPA in the mobile phase. Though this is a simple and rapid analytical method, it was found not applicable to the more complex samples, especially the lemon oils, which always contain components eluting very near bergapten. Although it requires more time, the longer Carbowax 400/Corasil column (5 ft. × 1/8 in. O.D.) yielded better resolution as shown in Fig. 2. The bergapten peak in lemon oil, however, was still only partially resolved. Furthermore, difficulty was encountered with variations in column efficiency among packings from the manufacturer's three different lots which were evaluated. The Carbowax 400/Corasil column showing the best separation was one in which IPA–*n*-heptane (3:97) was used for a period of three months after which time THF–*n*-heptane (5:95) or THF–isooctane (5:95) was used.

Although the μ-Porasil column is very efficient, the sample resolution around bergapten was not adequate in case of the lemon oil sample (Fig. 3). However, for most other oils this column produces acceptable results. Using the μ-Porasil column, the major peak eluting just before bergapten from the lemon oil sample was isolated and analyzed by mass spectrometry. The mass spectrum shows a fragmentation pattern characteristic of the psoralen nucleus, similar to that of 5-geranoxy psoralen

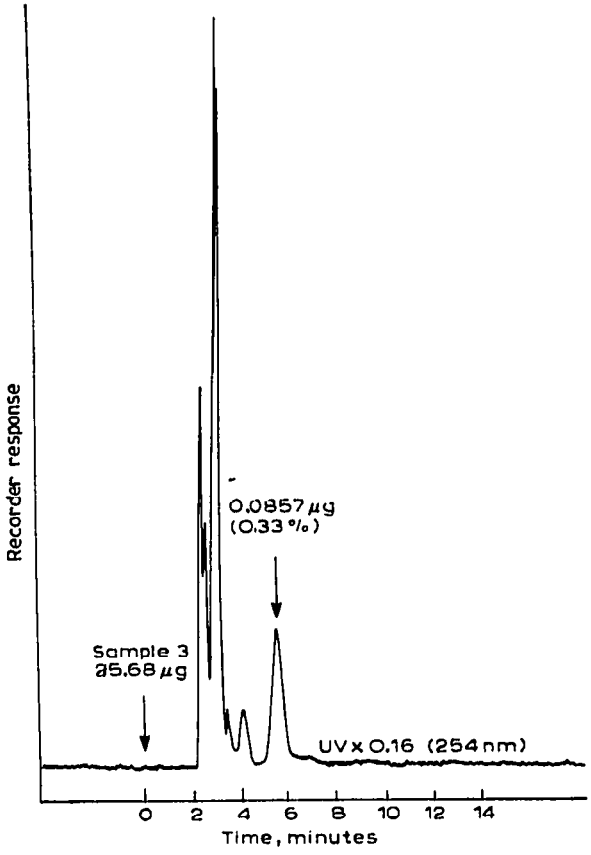

Fig. 1. Analysis of a natural bergamot oil. Column, Carbowax 400/Corasil partition column (2 ft. × 1/8 in. O.D.). Solvent, IPA–*n*-heptane (3:97). Flow-rate, 0.5 ml/min. Pumping system, Waters Ass. Model 6000.

(bergamottin). It is not bergamottin, however, based on the elution volume compared to an authentic standard. The back-pressure with the µ-Porasil column was very low, 400 p.s.i., which allowed the use of a septum injector.

Using the Zorbax-Sil column in the DuPont 841 instrument, the bergapten determination could be successfully done in all the samples except the lemon oil in which the bergapten peak was again not completely resolved. When the same column was mounted with standard Swagelok reducing fittings in the Varian 4100 pumping system, considerably more peak tailing was observed. It was at this point that the low-dead-volume fittings were made and installed on this column resulting in much improved peak shape. In fact, resolution of the bergapten peak in the lemon oil sample (Fig. 4) was superior to that achieved with the same column operated in the DuPont 841 instrument. Under these optimized conditions, a number of citrus oil samples were analyzed. It is obvious that the dead volume in the column system is an extremely important contributor to the performance of the small-particle high-performance columns, and much reduced dead volume fittings are urgently required.

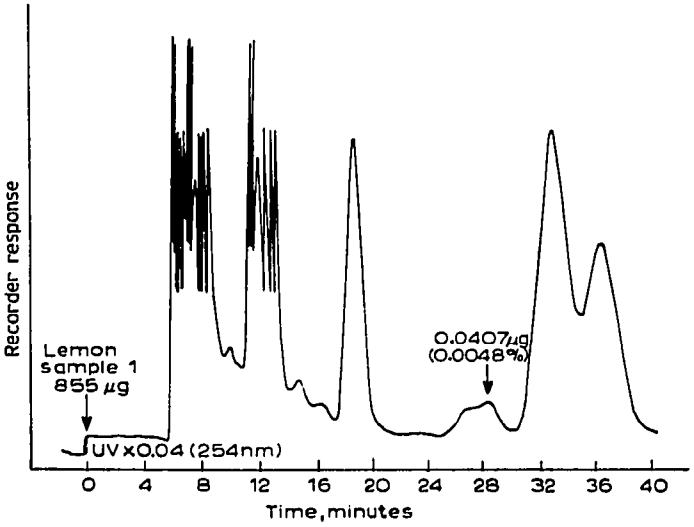

Fig. 2. Analysis of California lemon oil. Column, Carbowax 400/Corasil partition column (5 ft. × 1/8 in. O.D.). Solvent, THF–isooctane (5:95). Flow-rate, 0.5 ml/min. Pumping system, Waters Ass. Model C903.

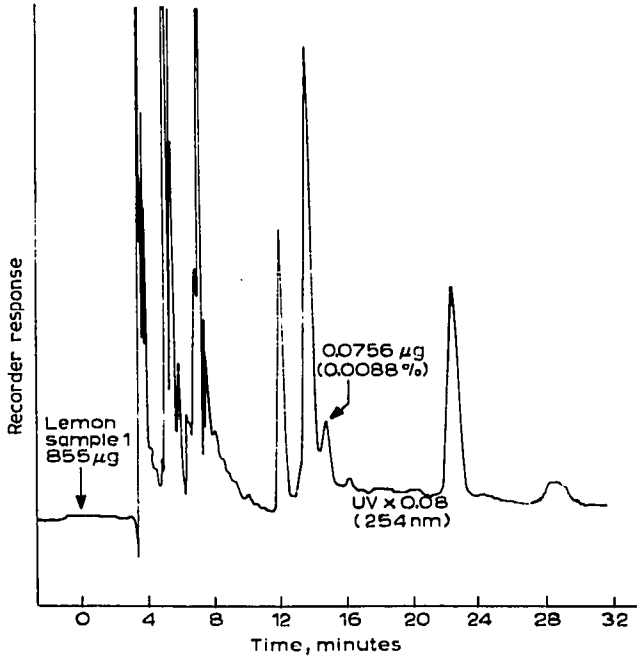

Fig. 3. Analysis of California lemon oil. Column, µ-Porasil (30 cm × 4 mm I.D.). Solvent, isooctane–ethyl acetate–IPA (80:1:1). Flow-rate, 1.0 ml/min. Pumping system, Varian Model 4100.

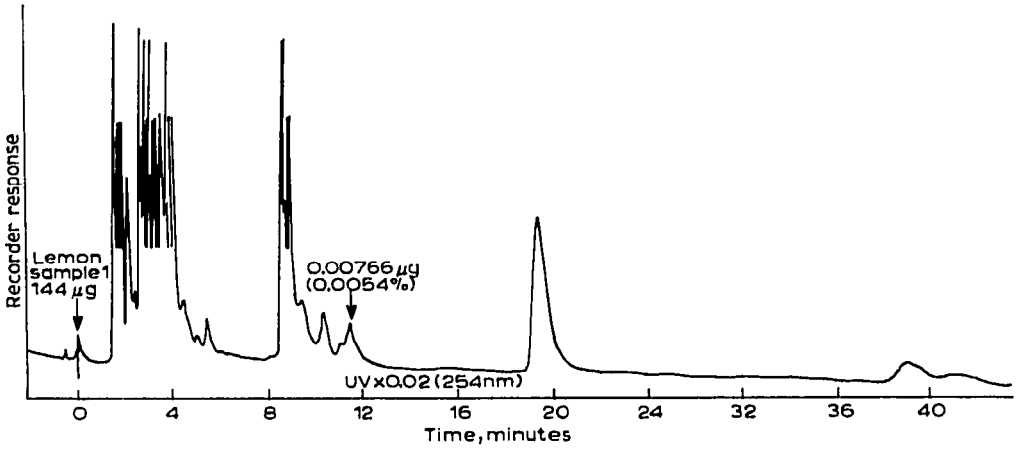

Fig. 4. Analysis of California lemon oil. Column, Zorbax-Sil (25 cm × 2.1 mm I.D.) with low dead-volume fittings in the Varian Model 4100 pumping system. Solvent, isooctane–ethyl acetate–IPA (80:1:1). Flow-rate, 0.4 ml/min. Pumping system, Varian Model 4100.

It has been found that the syringe-type pump of the Varian 4100 provides an excellent baseline without pumping noise or pulses which enables us to monitor the eluent using a sensitivity of 0.02 absorbance units full scale. The largest injected sample, 4 mg, along with the minimum detectable quantity (from the linear portion of the standard curve), $2 \cdot 10^{-3}$ μg, leads to the calculation of the detection limit as $2 \cdot 10^{-3}$ μg/4 mg = $5 \cdot 10^{-5}$% or 0.5 ppm in a citrus oil. Of course, larger sample sizes may be injected, though at some risk of losses in column efficiency, to obtain a detectability lower than 0.5 ppm. Under the conditions employed, the column back-pressure was about 1800 p.s.i. for the 5-μ Zorbax-Sil column. As a result, a high-pressure injector or a sampling valve is needed.

It should be pointed out that the fine particle adsorbent columns, characterized by high efficiency and large column capacity because of the total porosity, may show increased back-pressure, peak tailing or a decreased plate number after a period of use or if the column is severely overloaded. In such a case, the column should be washed with chloroform and/or tetrahydrofuran at high flow-rate for several hours. If this treatment fails to restore the column efficiency, the column may have to be discarded. This limited column life coupled with some variability even among the new prepacked commercially available columns means that the separation method will probably have to be "fine-tuned" and checked frequently with a reference sample to insure reliable results over the long term.

Investigation of the Corasil II column with the reported mobile phase consisting of chloroform–*n*-hexane (1:3) revealed that the analytical results could not be reproduced until 0.125% methanol was used as a modifier in the mobile phase in order to control adsorbent activity. Porcaro and Shubiak[10] were probably able to obtain their results due to the fact that the solvent used happened to contain a constant amount of water and/or ethanol functioning as the modifier. We found that the resolution was very poor when 0.25% methanol was used, and peaks were eluted too slowly if 0.05% methanol was used, illustrating the sensitivity of the separation to this parameter.

Analysis of several samples on the Corasil II column indicated that it would only be useful for the oils giving very simple chromatograms because of the limited separating power. The plate number for the bergapten peak was calculated to be approximately 900.

Table III shows the bergapten content in the samples obtained using the above-described methods. Results from each method are in reasonable agreement except for the lemon oil samples 1 and 2. Results of these samples obtained using method D (Zorbax-Sil mounted with the low dead-volume fittings designed in this laboratory) and A (Carbowax 400/Corasil, 5 ft. × 1/8 in. O.D.) are lower than the other methods, due to increased separation of interfering materials from bergapten. This co-eluted material was not encountered in other oil samples.

Comparison of results between method D and method A (Carbowax 400/Corasil, 2 ft. × 1/8 in. O.D.) indicates that some data are in good agreement (samples 3 and 4) and some not (sample 5). Results obtained from method D are more reliable than those obtained from method A, because the former method shows far greater resolution.

Taking into account some natural variability, the bergapten levels determined in this study for bergamot oil (samples 3 and 4) are in good agreement with the estimations of Cieri[1] and Porcaro and Shubiak[10]. The results of lime oil (sample 8) also remain in the range of the values Cieri[1] obtained. This is the first quantitative report of the bergapten content in bitter orange oil, grapefruit oil and lemon oil. Bergapten was not detectable in samples of petitgrain absolute, tangerine oil, Florida orange oil and the treated lemon and treated bergamot oil samples. Figs. 5, 6, and 7 show chromatograms of grapefruit oil, bergamot oil, and petitgrain absolute, respectively. TLC methods do not approach the HPLC method in resolution, speed, accuracy and ease of quantification.

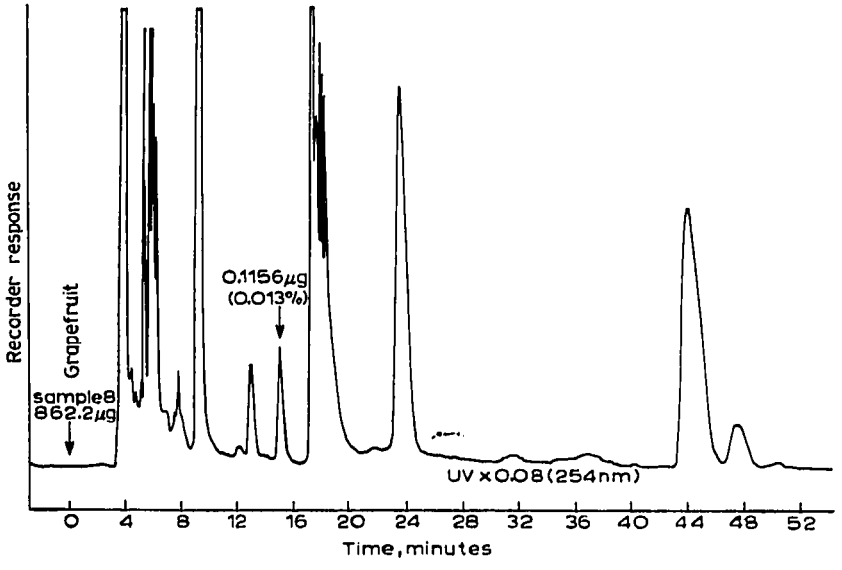

Fig. 5. Analysis of a grapefruit oil on the μ-Porasil column. Other conditions as in Fig. 3.

TABLE III

BERGAPTEN CONTENT (%) OF SAMPLES ANALYZED UNDER DIFFERENT CHROMATOGRAPHIC CONDITIONS*

Method	*Sample*								
	Cal. lemon oil (1)	*Cal. lemon oil (2)*	*Bergamot oil (3)*	*Bergamot oil (4)*	*Treated bergamot oil (5)*	*Mexican lime oil (6)*	*Persian lime oil (7)*	*Grapefruit oil (8)*	*Bitter orange oil (9)*
Carbowax 400/Corasil, Waters Ass. 6000 (A)	0.0048		0.33	0.33	0.025**				
μ-Porasil, Varian 4100 (B)	0.0088**		0.28		0.010	0.21		0.013	
Zorbax-Sil, DuPont 841 (C)	0.0064**	0.0029			0.014		0.39	0.013	0.073
Zorbax-Sil, Varian 4100 using low dead-volume fittings (D)	0.0054	0.0011	0.31	0.28	0.013	0.27	0.33	0.012	0.069

* Bergapten was not detected in the following oils (detection limit, 0.00005% or 0.5 ppm): tangerine, Florida orange oil, petitgrain, treated bergamot and treated lemon.

** These values are somewhat high due to unknown components eluting with bergapten.

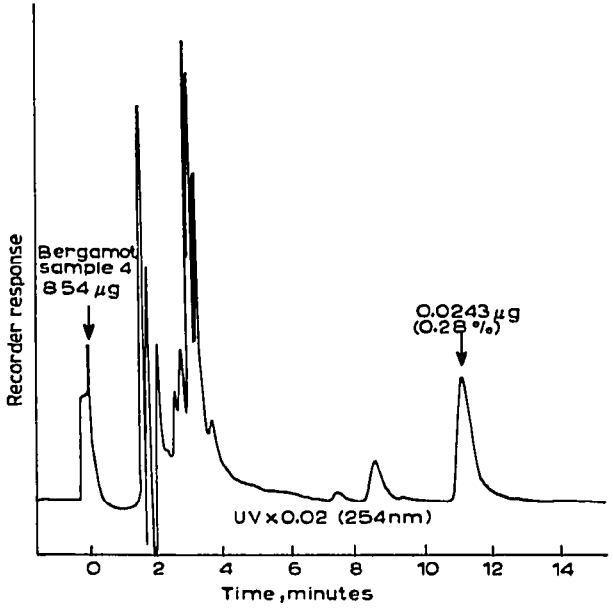

Fig. 6. Analysis of a bergamot oil on the Zorbax-Sil column with low dead-volume fittings. Other conditions as in Fig. 4.

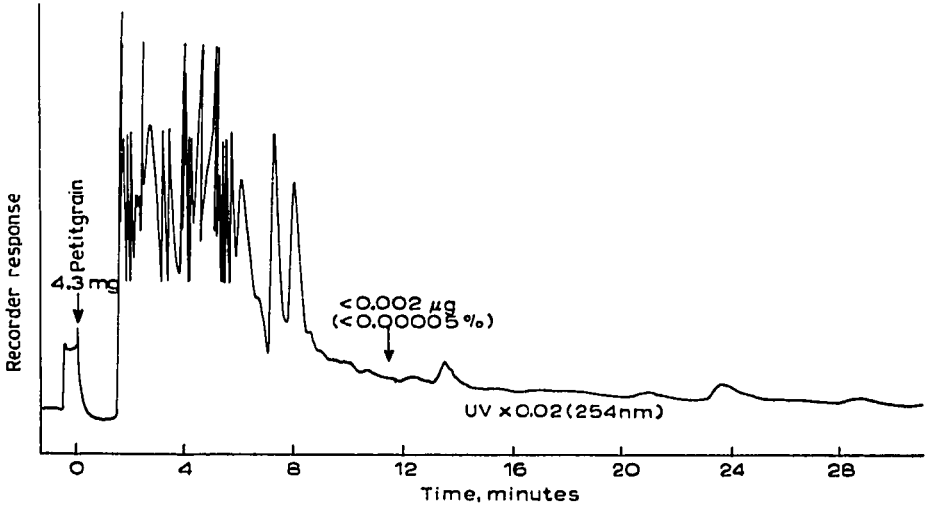

Fig. 7. Analysis of petitgrain absolute on the Zorbax-Sil column with low dead-volume fittings. Other conditions as in Fig. 4. No bergapten was detected.

CONCLUSIONS

Our experience indicates that the 5- or 10-μm totally porous adsorbent columns offer the greatest resolving power for the determination of bergapten in several citrus oils. A detectability of at least 0.5 ppm was attained. The column and condi-

tions which we feel are best suited for this determination are a Zorbax-Sil column with low dead-volume fittings; the solvent system isooctane–ethyl acetate–IPA (80:1:1) at a flow-rate of 0.4 ml/min; a pulseless positive displacement pump and UV detection. The μ-Porasil column used under the same conditions yielded acceptable results for all samples except the lemon oil and has the advantage that it can be operated successfully at pressures well below 1000 p.s.i. The short bonded-phase partition Carbowax 400/Corasil column will give bergamot oil analyses in about 8 min, but erroneously high values are occasionally obtained due to incomplete resolution of bergapten. The increased sample capacity of the totally porous adsorbents further makes possible about a fourfold decrease in the lower limit of detection as compared to the partition column and the Corasil II column. The porous-layer particle adsorbent Corasil II does not provide sufficient resolution to compete with the two previously mentioned methods.

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