Journal of Chromatography, 202 (1980) 127–130
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 13,239

Note

Semipreparative high-performance liquid chromatographic separation of singlet oxygen derived limonene hydroperoxides

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(Received August 12th, 1980)

The reaction of singlet oxygen ($^1O_2$) with (+)-limonene (1) is well known[1,2], but the hydroperoxides (3a–8a) that are formed, as evidenced by iodometric titration and infrared, have never been isolated and characterized. Instead, their structures have been inferred indirectly by reduction to the corresponding alcohols (3b–8b). A previous report on the TLC separation of limonene hydroperoxides[3] was of limited value for our purposes since their mixture was poorly resolved and little characterization work was done. High-performance liquid chromatographic (HPLC) techniques have recently been used with success in the separation of some alkyl hydroperoxides[4] by reversed phase with acetonitrile–water, and in the separation of lipid[5,6] and cholesterol[7] hydroperoxides employing both normal- and reversed-phase columns.

We wish to report the complete HPLC resolution of the limonene hydroperoxides (3a–8a) employing columns with Whatman Partisil 10 and Partisil 5, and also with these two columns in tandem. Characterization of the hydroperoxides by direct spectroscopic techniques, reduction to known alcohols, and gas–liquid chromatographic (GLC) thermograms will be published elsewhere.

EXPERIMENTAL

The hydroperoxides were prepared from (+)-limonene by the rose bengal sensitized singlet oxygen reaction of Schenck *et al.*[1]. The solvents were from Burdick & Jackson Labs. (Muskegon, MI, U.S.A.), "distilled in glass," and were all degassed under aspirator vacuum for 1 min prior to use.

The HPLC system was a Waters Model ALC/GPC 201 which included a M-6000 pumping system, a M-U6K universal injector and a M-R 401 differential refractometer. The columns were Whatman Partisil-PXS consisting of 25 cm × 4.6 mm I.D. stainless steel tubing packed with 10- or 5-μm microparticulate silica. The Partisil 10 column was placed before the Partisil 5 column when they were used in tandem. Two Partisil 5 columns were less useful because of the resulting operative higher pressure. A guard column, consisting of 7 cm × 2.1 mm I.D. stainless-steel tubing and packed with Whatman HC-Pellosil, was used in all cases. A flow-rate of 2 ml/min was employed for all separations.

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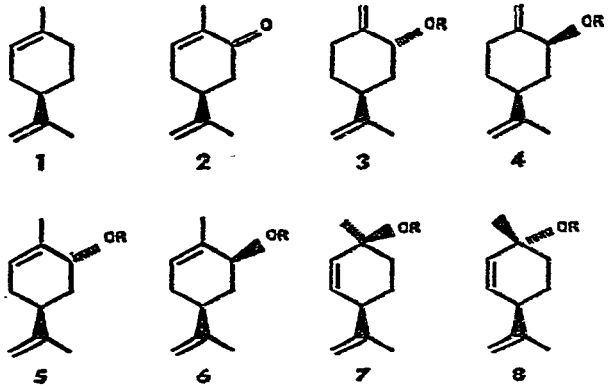

a: R=OH
b: R=H

RESULTS AND DISCUSSION

The best solvent system previously found[8] for the separation of a complex mixture of monoterpene alcohols, ethyl acetate–methylene chloride (2.5:97.5), was utilized initially for the elution of the hydroperoxides (3a–8a). The results showed that they eluted much faster than their respective alcohols (3b–8b), with a retention time similar to that of carvone (2). In order to increase the capacity factor (k')* of the hydroperoxides, the polarity of the solvent was reduced to ethyl acetate–methylene chloride (0.25:99.75), and a considerably better separation was obtained (Fig. 1A). Reducing the polarity further to 100% methylene chloride was slightly better overall

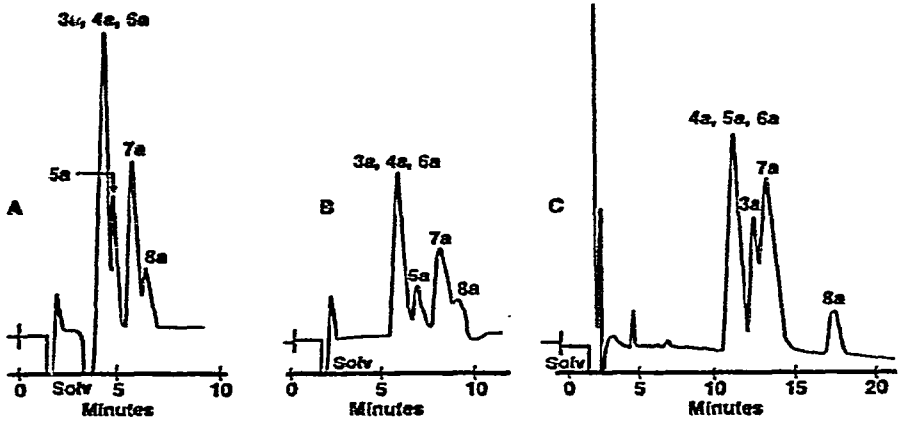

Fig. 1. HPLC separation of limonene hydroperoxides, 3–4 μl. A, ethyl acetate–methylene chloride (0.25:99.75); B, 100% methylene chloride; C, ethyl acetate–hexane (4:96).

* The capacity factor is defined here as $(V_x - V_0)/V_0$ where V_0 is the void volume and V_x is the elution volume of the peak of interest.

(Fig. 1B); in this case, 5a separated from 3a, 4a and 6a. Next, various levels of ethyl acetate–hexane were tried and the best separation in the series was obtained with 4% ethyl acetate. As shown in Fig. 1C, a much better separation of 7a and 8a was achieved and also, 3a was separated from 4a, 5a and 6a. Levels of chloroform in toluene were also employed, and the system chloroform–toluene (10:90) allowed the separation of 4a from 3a, 5a, and 6a. The best overall separation was obtained with ethyl acetate–toluene (0.5:99.5) (Fig. 2). In this case, 4a and 6a were separated from each other and from 3a and 5a. Conditions previously employed by Chan and Levett[5] for lipid hydroperoxide separation, ethanol–hexane (0.75:99.25), proved useless for the limonene hydroperoxides.

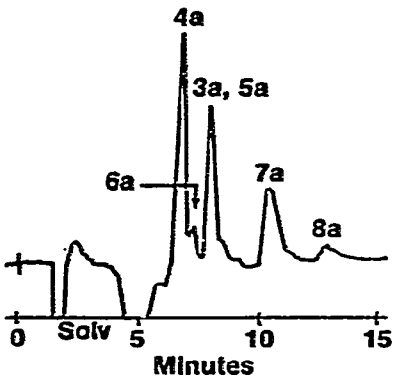

Fig. 2. HPLC separation of 4 μl of limonene hydroperoxides with ethyl acetate–toluene (0.5:99.5).

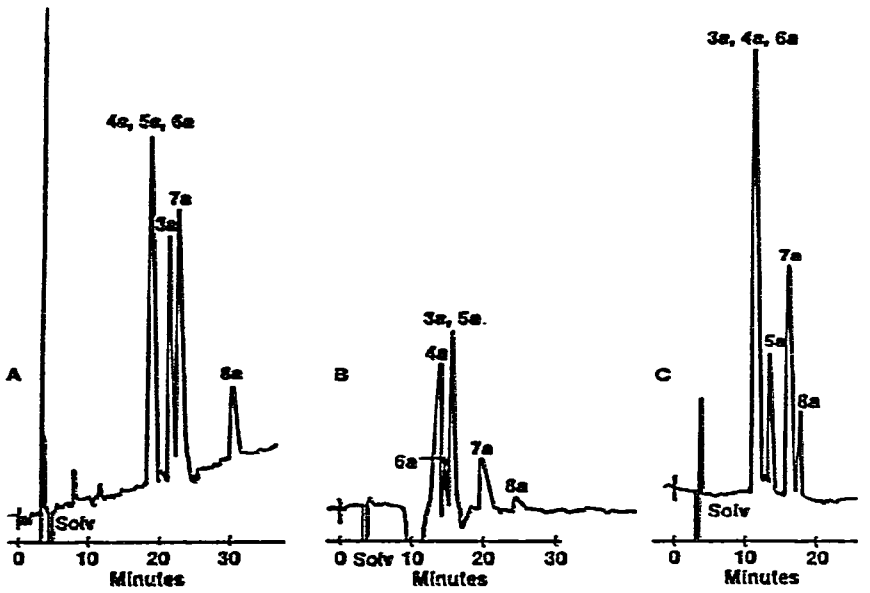

Fig. 3. Hydroperoxide separations on two columns of Partisil 10 and 5 in tandem. A, ethyl acetate–hexane (4:96), 2.5 μl; B, ethyl acetate–toluene (0.5:99.5), 10 μl; C, 100% methylene chloride, 5 μl.

Column packing size and length were also investigated. It was found that Partisil 5 gave better resolutions than Partisil 10, and that the use of Partisil 10 and 5 columns in tandem improved the separations over those achievable on them individually. Chromatograms for separations using ethyl acetate–hexane (4:96), ethyl acetate–toluene (0.5:99.5) and 100% methylene chloride with the tandem columns are shown in Fig. 3A, B and C.

For preparative purposes, all the solvents except toluene could readily be removed by rotary evaporation at 28°C, full aspirator vacuum, without affecting the hydroperoxides. Removal of toluene, however, required a temperature of 33°C which resulted in a rearrangement of the hydroperoxides if volumes were reduced below 0.5 ml. Consequently, when toluene was used, isolation of the materials first required concentration of each fraction to 0.5 ml followed by rechromatography twice with a different solvent, resulting in removal of most of the toluene. All the compounds were separated with the mixture of ethyl acetate–toluene (0.5:99.5) except hydroperoxides 3a and 5a (Fig. 3B). However, these two were separated upon rechromatography of this fraction using 100% methylene chloride (Fig. 3C) or ethyl acetate–hexane (4:96) (Fig. 3A).

The order of elution for each *trans/cis* pair of the alcohols (3b–8b) is *trans* before *cis* both by HPLC and GLC[8]. However, for the hydroperoxides, the HPLC order of elution for the first two pairs, 3a:4a and 5a:6a, is *cis* before *trans* and for the last pair, 7a:8a, it is still *trans* before *cis*. This order is found for all the hydroperoxide runs in which resolution is achieved between any of the isomeric pairs.

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