Journal of Chromatography, 563 (1991) 271–282
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam

CHROMBIO. 5652

Design of a gas chromatograph with parallel radioactivity and mass spectrometric detection

Application to the identification of the major metabolite of *d*-limonene associated with α2u-globulin

P. A. RODRIGUEZ*, R. TAKIGIKU, L. D. LEHMAN-McKEEMAN, M. L. FEY, C. L. EDDY and D. CAUDILL

The Procter and Gamble Co., Miami Valley Labs., P.O. Box 398707, Cincinnati, OH 45239-8707 (U.S.A.)

(Received August 15th, 1990)

ABSTRACT

A Perkin Elmer 3920 gas chromatograph, equipped with a versatile inlet system (*i.e.* an injector/trap), was interfaced to a radioactivity detector and a mass-selective detector (H/P 5970B) to identify ^{14}C-labeled compounds. The use of a pre-trap as a demountable, programmable-temperature injector, in conjunction with the injector/trap, allowed the introduction of 0.5-ml samples of rat kidney cytosol extracts to 0.32 mm I.D. capillary columns. The instrumentation greatly facilitated the identification of the major radiolabeled metabolite of *d*-limonene associated with the male rat-specific protein α2u-globulin as 1,2-*cis*-*d*-limonene oxide.

INTRODUCTION

Limonene (*p*-mentha-1,8-diene) is a monoterpene naturally found in a wide variety of foods [1]. The most prevalent dietary isomer is *d*-limonene [(+)-R-limonene], although both the 1-[(−)-*S*] and racemic (dipentene) forms have been observed in many natural products [2]. For example, *d*-limonene comprises from 50–97% (w/v) of citrus oils [3]. Because *d*-limonene is perceived as having a distinct "orange" character, orange oil-derived *d*-limonene is widely used as a perfuming and flavoring material in products designed to deliver orange-like aromas [4].

Although humans routinely consume limonene without adverse effects, when dosed orally to male rats, *d*-limonene causes the male rat-specific protein α2u-globulin to accumulate in phagolysosomes of renal proximal tubule cells [5]. This toxicity, seen exclusively in adult male rats, is described as hyaline droplet nephropathy.

This male rat-specfic nephropathy is apparently induced by a wide variety of hydrocarbons, *e.g.* decalin, unleaded gasoline, isophorone, *p*-dichlorobenzene

0378-4347/91/$03.50

and 2,4,4-trimethylpentane. Numerous investigations have shown that this nephrotoxicity is dependent on the production and accumulation of α2u-globulin [6,7]. Furthermore, it has been shown that *d*-limonene or its metabolite(s) bind reversibly to α2u-globulin, and this binding appears to be a prerequisite to the accumulation of the protein in the kidney [8]. Therefore, to understand the importance of the binding, it is essential to identify the limonene-related species interacting with the protein.

Metabolite identification efforts often begin with a search for differences in the chromatographic profiles of fluids obtained from control animals and animals fed a test substance. Once the profile differences point to regions of interest, the identification effort may proceed either by interfacing the analytical column to a mass spectrometer or by pursuing any number of off-line alternatives. Ultimately, the success of the identification effort depends, to a large extent, on the complexity of the chromatographic profile.

The chromatographic profile of a complex mixture can be simplified by the use of selective or specific detectors. Because radioactivity detectors (RADs) are highly sensitive detectors that can be made to be specific for a given radiolabel, they can be very useful, in conjunction with radiolabeled test substances, in facilitating the identification effort. Radiolabeled chemicals have been used previously in work aimed at identifying the metabolites of *p*-dichlorobenzene [9] and 2,2,4-trimethylpentane [10] associated with α2u-globulin. Like the metabolites of those compounds, the metabolite(s) of *d*-limonene were found to be volatile. Therefore, the analytical method of choice was gas chromatography (GC). To simplify the identification effort, we used ^{14}C-labeled *d*-limonene and instrumentation designed to obtain mass spectra of radioactive compounds detected on-line by a flow-through RAD. In this communication we report the instrumentation and the approach used to identify the major metabolite of *d*-limonene associated with α2u-globulin.

EXPERIMENTAL

Instrumentation

The instrumentation, shown schematically in Fig. 1, consists of a gas chromatograph (Model 3920, Perkin-Elmer, Norwalk, CT, U.S.A.), equipped with an injector/trap [11], a 50 m × 0.32 mm I.D. OV-101 fused-silica capillary column (Hewlett-Packard, Avondale, PA, U.S.A.), a flow-through RAD [12] and a mass-selective detector (MSD, H/P 5970B, Hewlett-Packard, Palo Alto, CA, U.S.A.).

Interface of GC, MSD and RAD

Details of the interface between RAD and MSD are shown in Fig. 2. A column effluent splitter (A), made from an SGE glass-lined union and two two-hole graphite-vespel ferrules, was assembled and housed in the heated detector manifold module of the PE 3920. Both ends of the union were drilled to allow place-

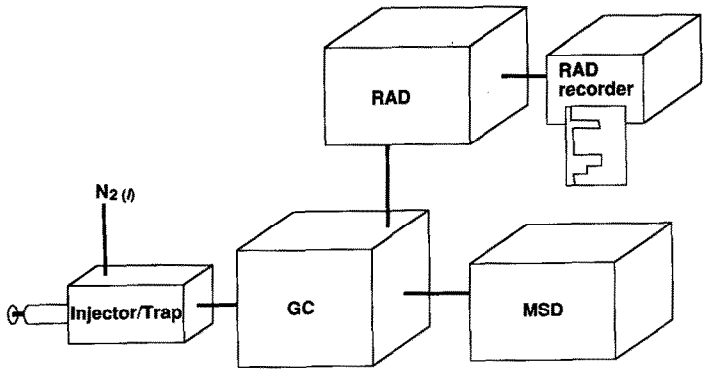

Fig. 1. Block diagram of injector/trap–GC–RAD–MSD instrumentation.

ment and sealing of two pieces of fused-silica tubing. The column and a piece of 0.32 mm I.D. fused-silica tubing, carrying helium make-up gas, were connected to the inlet side of the union. Two pieces of fused-silica tubing, 55 cm × 75 μm I.D. and 15 cm × 0.32 mm I.D., were connected to the outlet side of the union. The 55 cm-long piece (B) served as restrictor for the direct inlet of the MSD.

The restrictor (B) is protected by a heated casing (C), constructed by wrapping

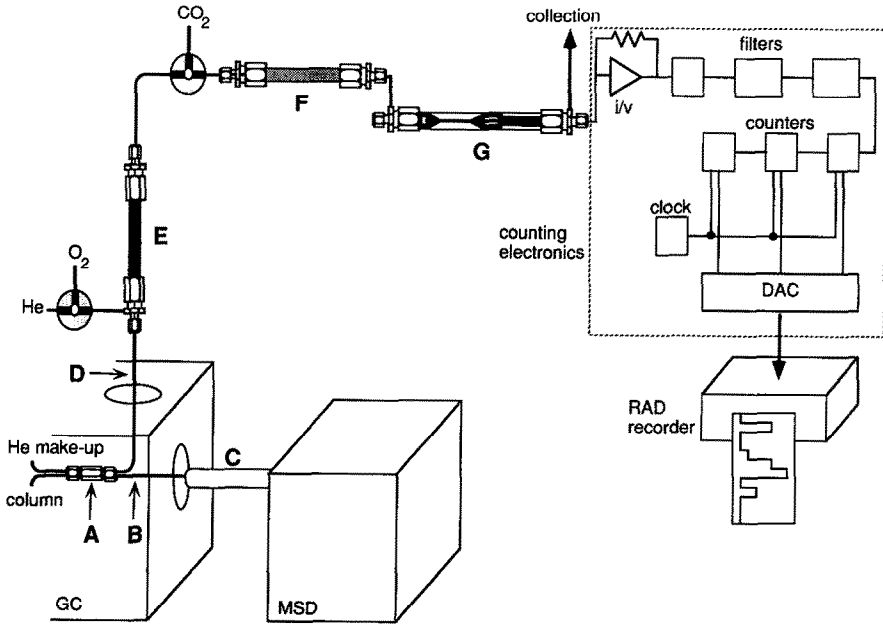

Fig. 2. Schematic diagram of GC–RAD–MSD interface. A, SGE union; B, fused-silica restrictor for MSD; C, heated casing for restrictor; D, transfer line to FID base; E, combustion furnace; F, drying tube; G, proportional counter tube.

glass tape on a 0.76 mm I.D. × 1.6 mm O.D. piece of ss-tubing followed by glass-coated nichrome wire. A thermocouple was potted to the tubing mid-section with ceramic cement, and the assembly insulated by a ceramic sleeve (Omega Engineering, Stamford, CT, U.S.A.). The restrictor assembly temperature was kept at 175°C by means of a Variac. This assembly exits the manifold through a hole drilled on the side of the GC facing the MSD. The restrictor is sealed to the MSD by means of a specially machined nut. This nut has an extended back and was drilled to accept the 1.6 mm restrictor casing.

The 15-cm-long piece (D) was connected to the RAD via the flame ionization detector (FID) base of the GC. The FID tip was removed and replaced with a 6-cm-long section of glass-lined tubing potted to the base of an FID tip with ceramic cement.

Radioactivity detector

The RAD consists of a combustion furnace with provisions for adding helium make-up gas and a trickle of oxygen, and a counter tube with associated quench-gas and electronics. The glass-lined tubing replacing the FID jet was used as the interface to the furnace: it was inserted into a modified 1.6- to 6.4-mm ss-reducing union used as the furnace inlet. The union was modified to accept a tube carrying make-up gas.

The furnace (E) was made by packing a 14 cm × 6 mm O.D. × 4 mm I.D. quartz tube with 2.5 g of a 5:1 mixture (w/w) of CuO and ground quartz ((1–3 mm CuO wire, 0.4-mm particles).

Glass-coated Nichrome wire (3.9 m of 4.4 ohm/30 cm) was wrapped around the quartz tube and potted with high-temperature ceramic cement. A thermocouple, placed at the midpoint of the tube, provided a temperature readout. The furnace was insulated with a ceramic sleeve (Omega Engineering), and kept at *ca.* 650°C by means of a Variac.

Helium, at *ca.* 60 ml/min, and oxygen, at *ca.* 1 ml/min, were introduced at the base of the furnace (E), where they mixed with the column effluent. After combustion, the gases containing radioactive CO_2 from the sample were mixed with quench gas (CO_2, 6 ml/min) and dried in a tube packed with $MgClO_4$ (F). Radioactivity in the dry gas stream was counted with a low-volume proportional counter (G). The output of the counter was converted to a histogram by circuitry described previously [12].

Isolation of d-limonene-related species from the renal protein fraction

[^{14}C]*d*-Limonene (17.5 mCi/mmol, Wizard Labs, Davis, CA, U.S.A.) was diluted with *d*-limonene (Sigma, St. Louis, MO, U.S.A.) and administered orally to rats at a dosage of 3 mmol/kg (2.5 mCi/kg). The kidneys were removed 24 h after dosing, homogenized in two volumes of Tris–HCl buffer (10 m*M*, pH 7.4, Sigma) and centrifuged at 10 000 *g* for 15 min. The supernatant was then centrifuged at 100 000 *g* for an additional hour. Renal proteins were isolated from the resulting

cytosolic fraction by Sephadex G-25 gel-permeation chromatography (PD-10, Pharmacia, Piscataway, NJ, U.S.A.). The α2u-globulin was purified from this protein fraction by reversed-phase high-performance liquid chromatography (HPLC) as described previously [8].

The compound(s) associated with the purified protein were extracted quantitatively with two volumes of methylene chloride. To obtain sufficient radioactivity for counting, we injected 0.5 ml of the extract by means of a pre-trap and injector/trap.

Procedures

To perform mass balance studies, a pre-trap was used as a demountable, programmable vaporizing injector. The pre-trap is shown in Fig. 3. It was made from a 1.0-ml Glenco syringe, and was packed with deactivated glass wool (inlet section, D, *ca.* 50% of volume) and Tenax TA (E). To load the sample, portions (50–75 μl) of the extract were carefully aspirated into the pre-trap to avoid wetting the Tenax portion of the trap. This operation was followed by flushing most of the methylene chloride from the trap with helium. These operations (load/flush) were repeated until all of the sample was loaded onto the pre-trap. To establish the volatile fraction of the radioactivity in the extract, a helium stream (*ca.* 15 ml/min) was used to backflush the trap contents into a scintillation cock-

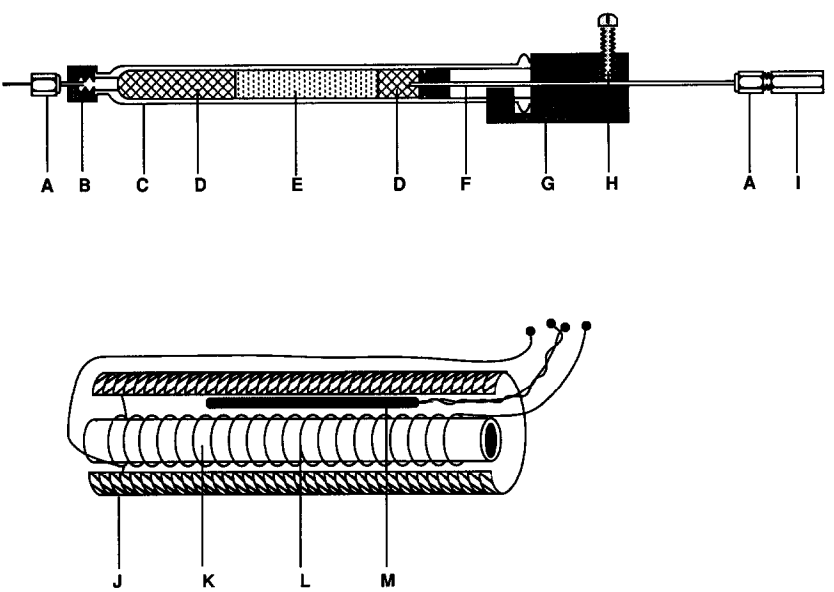

Fig. 3. Pre-trap and heating assemblies. A, Swagelok nut, 1.6 mm; B, vespel nut, 6.25 mm-28 threads/25.4 mm; C, 1-ml Glenco syringe barrel; D, glass wool; E, Tenax TA; F, SS tubing, 1.6 mm; G, machined Nylon® syringe holder; H, Nylon set screw; I, female to female adaptor, 6.25 mm-28 threads/25.4 mm to 1.6 mm nut; J, ceramic insulating sleeve; K, aluminum tube; L, glass-coated Nichrome heating wire; M, Thermistor sensor.

tail. The pre-trap was flushed for 30 s prior to ramping the temperature (at *ca.* 100°C/min) to 170°C to desorb volatile analytes. Heating was done by inserting the pre-trap into a low-mass heating sleeve. When the desorption was complete (*ca.* 10 min), the pre-trap was cooled and disassembled. All pre-trap components were loaded into scintillation vials and counted using liquid scintillation techniques.

Analyses were performed by attaching the pre-trap to the injector/trap, followed by connecting a helium source (adjusted to deliver 15 ml/min) to the back of the pre-trap. After flushing for 30 s, the temperature of the pre-trap was increased to 170°C by means of the heating sleeve. Volatile compounds desorbed from the pre-trap were transferred, via the injector/trap, to the capillary column. The initial column temperature, 50°C, was held constant for 4 min, followed by programming the temperature, at 8°C/min, to a final temperature of 250°C. The final temperature was kept constant for 4 min. The output of the column was split so that *ca.* 85% reached the RAD while the remaining fraction reached the MSD.

To obtain samples of authentic *cis*-(+)-limonene oxide, we purified a mixture of commercially available *cis–trans* isomers (Aldrich, Milwaukee, WI, U.S.A.) by means of a spinning-band distillation column [13]. Pressure within the column was maintained at 500 mmHg and the distillation was performed at 113°C. Percent enrichment for *cis* or *trans* isomers was monitored by GC–FID. Early fractions were redistilled to optimize the enrichment for the lower-boiling isomer. Enrichment for the higher-boiling isomer was accomplished by successive removal of the lower-boiling isomer.

RESULTS AND DISCUSSION

Design criteria for GC–RAD–MSD instrumentation

The instrumentation was designed to obtain mass spectra while simultaneously detecting ^{14}C-labeled compounds eluting from a capillary column. The design minimizes ambiguities in the assignment of peak identities by optimizing peak band-widths and by minimizing time delays between detectors. In addition, by diverting most of the mass to the RAD, we were able to perform mass balance studies while minimizing the effect of column temperature on the split of material between detectors.

RAD band-width. The output of the RAD is a histogram, the resulting peak-width is determined by the internal volume of the counter tube (*ca.* 6 ml) and the counting time selected in the digital counter (6 s). Because the peaks undergo exponential dilution in the counter tube, after one counting period, and with a flow-through rate of *ca.* 70 ml/min, about 30% of a narrow peak would remain in the tube. Consequently, most radioactive peaks will be 12 s wide, with most of the radioactivity contained within one 6-s counting period.

Time delay between detectors. When a detector used in parallel with the MSD has negligible or no dead volume, *e.g.* the FID, the time difference between peaks

detected by that detector and the MSD depends only on the make-up gas flow-rate and diameter of connecting lines. When using the FID, and when the flow-rate out of the FID branch of the splitter is greater than 2.0 ml/min, the difference between a peak detected by the FID and the MSD is less than 0.01 min.

However, when the RAD replaces the FID, the minimum delay between the two detectors is determined by the volumes of the furnace, drying tube and connecting lines. To minimize time delays, a make-up gas (*i.e.* helium flowing at 60 ml/min) was introduced at the base of the furnace. Thus, the minimum delay was *ca.* 2 s. The maximum delay is given by the sum of the delays contributed by the system volume and the counting period chosen (6 s). Because it is possible for a peak to enter the counter tube immediately after a counting period, the maximum delay between the two detectors is ca. 8 s.

Effect of splitter on mass balance. When the flow-rate out of the splitter was $\geq$ 3.0 ml/min, $\geq$85% of the sample was sent to the RAD. We chose to send most of the sample to the RAD because the samples were expected to have about 1 μg of mass associated with the radioactivity found in the typical extract (*e.g.* 15 000 dpm, 7 nCi or 250Bq in 0.5 ml of methylene chloride). Therefore, while we would have sufficient mass for identification, we would require virtually all of the radioactivity to obtain a detailed profile while counting on-the-fly.

In addition, the high RAD/MSD split ratio (*ca.* 85:15) minimizes the effect of temperature on column flow. As the temperature increases during the run, the column flow-rate would decrease, thereby affecting the fraction of sample reach-

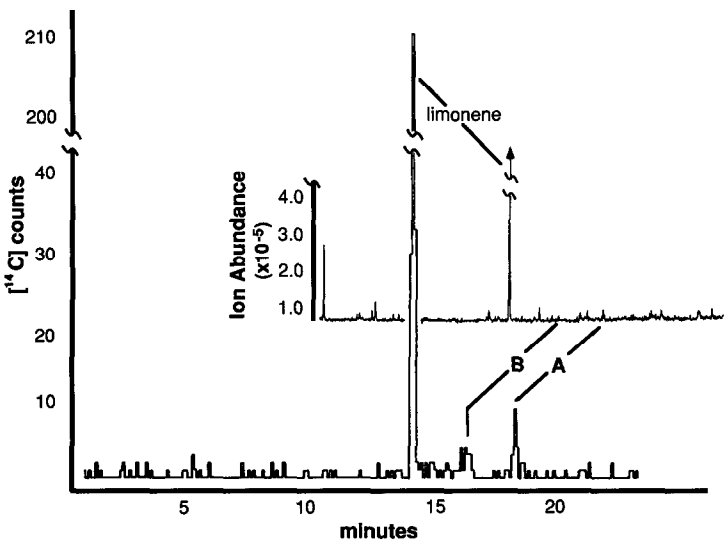

Fig. 4. Radiochromatogram and total-ion chromatogram of ^{14}C-labeled *d*-limonene. A and B are radiolabeled impurities, corresponding to 6 and 3%, respectively, of the radioactivity detected.

ing each detector. To minimize the effect those changes would have on mass balance studies, the MSD restrictor was selected to draw *ca.* 0.5 ml/min.

Because the make-up gas ensured a minimum flow-rate of 2.7 ml/min to the RAD at the highest column temperature, 84% of the mass eluting from the column would reach the RAD. At the lowest column temperature the flow-rate out of the splitter was *ca.* 4 ml/min, thus, *ca.* 89% of the sample would reach the RAD. Therefore, variations in column flow-rate due to changing temperature during programming did not affect, to a significant extent, the fraction going to the RAD.

Mass balance and analysis of d-limonene standard

A radiochromatogram and a total-ion chromatogram (TIC) of the ^{14}C-labeled limonene used to dose animals are shown in Fig. 4. By collecting radioactive CO_2 out of the RAD we accounted for 80% of the radioactivity injected. After correction for the split (11–15% of the injection to the MSD), the 80% recovered out of the RAD respresents $\geq$ 90% of the expected radioactivity. We did not find any residual radioactivity in the pre-trap, indicating that there were no non-volatile components in this sample.

While limonene accounts for *ca.* 90% of the radioactivity in the sample, there are at least two other radioactivity-containing peaks in the chromatogram (A and B). While there was insufficient mass for identification, the peak at 18.4 min (A, Fig. 4) had prominent ions at *m/z* (%) 82(100), 54(60), 91,93(30), 94,95(20), 107,108(30). The retention time and prominent ions suggest a mono-substituted alcohol derivative of *d*-limonene.

The chromatograms shown in Fig. 4 serve to illustrate the high sensitivity attainable with the instrumentation. The mass of impurity A reaching the MSD is slightly less than 0.2 ng. Also, because the split ratio is 11–15% to the MSD, the amount reaching the RAD is *ca.* 2 ng. Therefore, at this specific activity (17.5 mCi/mmol), the signal-to-noise ratio of the RAD suggests a detection limit comparable to that shown by the MSD.

Identification and mass balance of the major metabolite

A radiochromatogram and TIC of compounds associated with the α2u-globulin are shown in Fig. 5. Although the TIC shows four distinct regions with many partially resolved peaks, the radiochromatogram clearly shows the regions of interest. The compound corresponding to the major radioactive peak was identified as the 1,2-*cis*-*d*-limonene oxide by comparison of its mass spectrum [*m/z*(%) 43(100), 67(56), 81(20), 82(17), 84(10), 91(10), 93(30), 95(14), 108(16), 109(27), 137(21), 152(2)] and retention time with the authentic compound. The mass spectrum and structure are also shown in Fig. 5. The identity of the authentic compound was verified by high-resolution ^1H NMR (2D-^1H NOE NMR), IR [14] and optical rotation [14,15]. The authentic compound was obtained by spinning band distillation of a mixture of *cis* and *trans* isomers. The *cis*-epoxide, reported

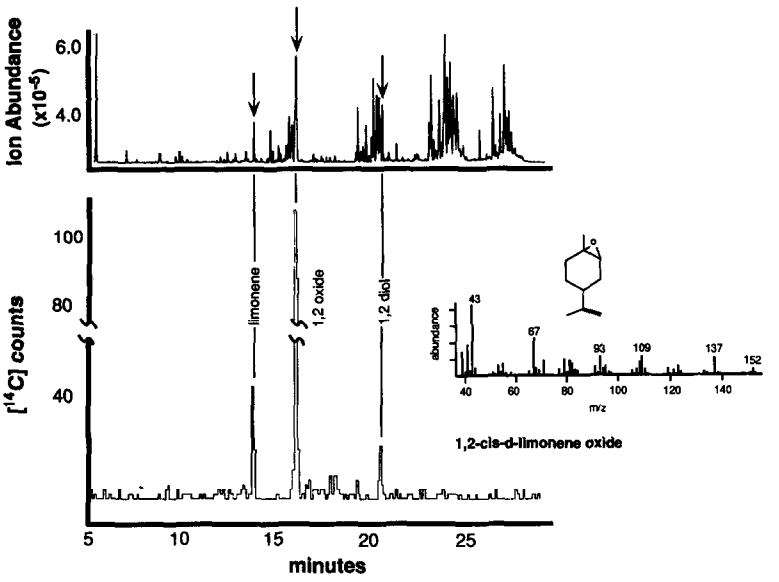

Fig. 5. Radiochromatogram and total-ion chromatograms of a 0.5-ml sample of kidney cytosol extract obtained at pH 7.4. The mass spectrum of the major radiolabeled species, 1,2 *cis*-*d*-limonene oxide, is also shown.

to be the lower-boiling isomer [13], distilled first in the spinning band column and required multiple passes to achieve $\geq 75\%$ isomeric purity.

To obtain the radiochromatogram shown in Fig. 5, a total of 10 770 dpm were injected. We recovered 7300 dpm from the RAD or 68% of the total. This recovery is near-quantitative after correction for split (11–15% to the MSD) and residual in the pre-trap (*ca.* 10%). The oxide (retention time, t_R = 16.2 min, Kovats index = 1126) accounts for 79% of the radioactivity, *d*-limonene (t_R = 14 min, Kovats index = 1031) accounts for 13% and a 1,2-diol (t_R = 20.8 min, Kovats index = 1352) accounts for 7%. While the diol probably originates from hydrolysis of the oxide during sample work up, the presence of *d*-limonene suggests that it too is involved in the renal protein overload produced in the male rat as a result of *d*-limonene exposure. In addition, two other minor radiolabeled species are found in the region between 16.5 and 18.4 min. Because of the high specific activity of these two radiolabeled species, we conclude they must come from the radiolabeled limonene standard shown in Fig. 4. However, we did not have sufficient mass in this run for positive identification.

The hydrolysis of the 1,2-*cis*-*d*-limonene oxide to a 1,2-diol provides additional evidence of identity. When a mixture of the two isomeric 1,2-oxides (*cis* and *trans*) is subjected to mildly acidic conditions, only the *cis* isomer reacts to form a 1,2-diol, while the *trans* isomer remains unchanged. This behavior is shown in Fig. 6. The chromatogram of an enriched sample of the oxides, obtained after

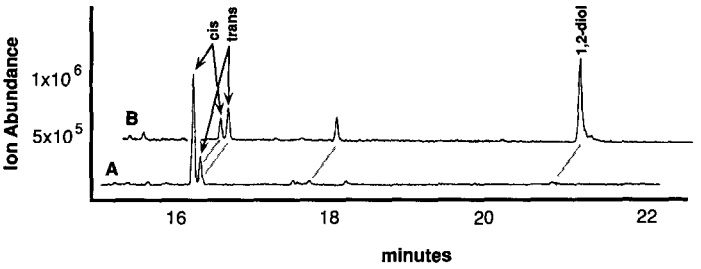

Fig. 6. Total-ion chromatogram of a mixture of 1,2-limonene oxides subjected to neutral (A) and acidic (B) conditions.

extracting a neutral solution (pH 6.8) with methylene chloride, is shown in the trace-labeled A. The ratio of *cis* to *trans* isomers, 4:1, is nearly the same as that measured from a standard solution of the oxides in methylene chloride, indicating that virtually no hydrolysis has taken place since only small peaks appear in the 17.4–18.1 min region and in the region *ca.* 20.6 min.

After extracting a mildly acidic solution (pH 3.2) with methylene chloride we obtained the chromatogram shown in B. The *cis/trans* ratio changed from 4:1 to less than 1:1 in the acidic sample. Furthermore, while the abundance of the *cis* isomer decreases, a correspondingly large peak appears (*ca.* 20.6 min, Kovats index = 1340). The mass spectrum and molecular weight (170) are congruent

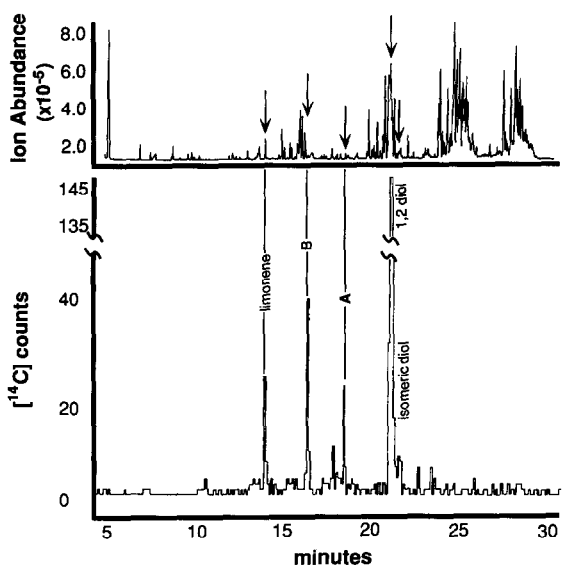

Fig. 7. Radiochromatogram and total-ion chromatogram of a 0.5-ml sample of kidney cytosol extract obtained at *ca.* pH 1.

with that of a 1,2-limonene diol [*m/z* (%) 152(23), 137(15), 109(24), 108(31), 71(100) and 43(92)]. While the experiments were not done to generate exact kinetic data, this change took place in less than 1 h at room temperature.

A chromatogram of a metabolite extract, obtained under acidic conditions, is shown in Fig. 7. The major radioactive peak is the 1,2-diol identified earlier. The RAD points to the center peak of a partially resolved portion of a group of peaks in the chromatogram, greatly simplifying the assignment of identity. Several minor radioactive peaks are also observed in the chromatogram. The peak eluting at 14 min is limonene (7% of radioactivity shown), while the peaks eluting at 16.2 min (B) and 18.4 min (A) are the unknowns previously noted in Fig. 5. Again, these unknowns do not have large masses associated with them since there are no significant peaks present in the TIC. However, because of the higher mass injected in this analysis, we could get a reasonable mass spectrum of compound A. This compound has ions at *m/z*(%) 82(100), 54(50), 91(14), 107(20), 108(30) and 93(36), identifying it as the impurity present in the radiolabeled standard.

There is another interesting radioactive peak appearing in Fig. 7 after the 1,2-diol. It has a similar mass spectrum [*m/z*(%) 170(18), 152((34), 137(68), 109 (41), 108(10) and 43(100)] and forms with time at the expense of the 1,2-diol. The mass spectrum suggests an isomeric diol.

CONCLUSIONS

The monitoring of capillary GC effluents by an MSD and RAD greatly facilitated the attainment of structural and mass balance information on radiolabeled, volatile metabolites. Minimizing the time lag and band-width differences between detectors resulted in a fuller realization of the resolving power of the technique.

We also demonstrated the use of pre-traps as demountable, programmable-temperature injectors to facilitate mass balance and prevent contamination of the chromatographic system. The pre-traps coupled to the injector/trap made possible the injection of 0.5-ml samples of an extract into a capillary column while maintaining analytical accuracy on materials as volatile as limonene.

The major metabolite associated with the formation of hyaline droplets in the kidney of male rats, after oral administration of *d*-limonene, was identified as the 1,2-*cis*-*d*-limonene oxide. Parent *d*-limonene was also found as a minor component associated with α2u-globulin.

ACKNOWLEDGEMENTS

We like to thank W. F. Erman for many valuable discussions, K. Dyehouse-Noahr and S. C. Lee for 2D-^{1}H NOE NMR, S. Buring-Stuard for NMR and R. A. Schneider for organic synthesis assistance.

REFERENCES

1 S. van Straten and H. Maarse, *Volatile Compounds in Food Qualitative Data*, Institute CIVO Analysis TNO, Zeist, 5th ed., 1983, p. 45.
2 T. E. Furia and N. Bellanca, *Fenaroli's Handbook of Flavor Ingredients*, Vol. 2, CRC Press, Cleveland, OH, 2nd ed., 1975, p. 319.
3 S. Rangana, V. S. Govindarajan and K. V. R. Ramana, *CRC Crit. Rev. Food Sci. Nutr.*, 18 (1983) 313.
4 O. Sticher, in H. Wagner and P. Wolff (Editors), *New Natural Products and Plant Drugs with Pharmacological, Biological or Therapeutical Activity*, Springer-Verlag, Berlin, 1977, p. 137.
5 R. L. Kanerva, G. M. Ridder, F. R. Lefever and C. L. Alden, *Food Chem. Toxicol.*, 25 (1987) 345.
6 C. L. Alden, R. L. Kanerva, G. M. Ridder and L. C. Stone, in M. A. Mehlman, C. P. Hemstreet, J. J. Thorpe and N. K. Weaver (Editors), *Renal Effects of Petroleum Hydrocarbons,* Princeton Scientific Publishers, Princeton, 1984, p. 107.
7 J. A. Swenberg, B. Short, S. Borghoff, J. Strasser and M. Charbonneau, *Toxicol. Appl. Pharmacol.*, 97 (1989) 35.
8 L. D. Lehman-McKeeman, P. A. Rodriguez, R. Takigiku, D. Caudill and M. L. Fey, *Toxicol. Appl. Pharmacol.*, 99 (1989) 250.
9 M. Charbonneau, J. Strasser, E. A. Lock, M. J. Turner, Jr. and J. A. Swenberg, *Toxicol. Appl. Pharmacol.*, 99 (1989) 122.
10 E. A. Lock, M. Charbonneau, J. Strasser, J. A. Swenberg and J. S. Bus, *Toxicol. Appl. Pharmacol.*, 91 (1987) 182.
11 P. A. Rodriguez, C. L. Eddy, G. M. Ridder and C. R. Culbertson, *J. Chromatogr.*, 236 (1982) 39.
12 P. A. Rodriguez, C. R. Culbertson and C. L. Eddy, *J. Chromatogr.*, 264 (1983) 393.
13 A. Kergomard and H. Veschambre, *C. R. Acad. Sci.*, 279 (1974) 155.
14 W. F. Newhall, *J. Org. Chem.*, 29 (1964) 185.
15 S. G. Traynor, B. J. Kane, M. F. Betkouski and L. M. Hirshy, *J. Org. Chem.*, 44 (1979) 1557.