Journal of Chromatography, 491 (1989) 27–36
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 4734

FORMATION OF CYCLIC ENOL ETHERS FROM A LABILE BIOLOGICAL PRECURSOR: AN EXAMPLE OF ANALYTICAL ARTIFACTS

SCOTT HARVEY, DONALD WIESLER and MILOS NOVOTNY*

Department of Chemistry, Indiana University, Bloomington, IN 47405 (U.S.A.)

(First received July 5th, 1988, revised manuscript received February 23rd, 1989)

SUMMARY

Three isomeric enol ethers are among those constituents apparently unique to mouse urine as identified by gas chromatographic analysis. These compounds appear to be artifacts arising from the cyclization and dehydration of 6-hydroxy-6-methyl-3-heptanone. Identification of the trimethylsilyl ether of 6-hydroxy-6-methyl-3-heptanone in the silylated ether extract of mouse urine indicates that the precursor keto alcohol is indeed present in the urine. Since similar heterocyclic compounds are often identified in urine samples analyzed by gas chromatography, formation of various analysis artifacts arising from analogous cyclization and dehydration reactions is likely.

INTRODUCTION

Numerous heterocyclic compounds have been reported as constituents of various physiological fluids which have been sampled by purge-and-trap techniques and analyzed by gas chromatography (GC) [1–9], including some (particularly cyclic enol ethers) which would not be expected to enjoy prolonged existence in an aqueous environment. Accordingly, we reason that many of these cyclic compounds may be analysis artifacts, being formed by dehydration of various hydroxy ketone, hydroxy aldehyde and hydroxy acid precursors initially present in such specimens. The high temperatures usually found in the injection port of a gas chromatograph can be expected to promote such dehydration reactions.

A series of three cyclic enol ethers of the nominal molecular weight 126 are among those constituents unique to mouse urine [1,2] and whole-body odors

0378-4347/89/$03.50

[10]. At this stage, it is not clear whether these compounds fluctuate with testosterone concentration in males. However, in female mice, the concentrations of the cyclic enol ethers oscillate in accordance with the estrous cycle, having a higher concentration during estrus than at other phases of the cycle [11,12]. Depressed urinary levels observed in pregnant and lactating mice further emphasize the endocrine dependency of the cyclic vinyl ethers in females [12,13]. There is a possibility that these compounds may be involved in chemical communication. The substances are invariably detected among the volatile constituents of mouse urine obtained by purge-and-trap sampling followed by GC analysis [1,2]; they have not been encountered in urine samples from other mammalian species analyzed in this laboratory. While their function is not presently clear, their ubiquitous presence in mouse urine may represent a fundamental biochemical process important in the physiology of the mouse.

The structures of these cyclic enol ethers [1,2], on the basis of their logical relationship via dehydration to the independently synthesized tautomeric mixture of 6-hydroxy-6-methyl-3-heptanone (open-chain hydroxy ketone) and 5,5-dimethyl-2-ethyltetrahydrofuran-2-ol (cyclic hemiacetal or lactol), are shown in Fig. 1. The lactol can undergo dehydration to form a double bond either inside or outside the ring. If dehydration occurs exterior to the ring, the resulting olefin can have a *cis* or *trans* configuration; hence, the appearance of three isomers with nearly identical mass spectra is explained.

A similar reaction has been reported in the literature and is known to occur under the dehydrating conditions found in the injection port of a gas chromatograph [14]. While studying the metabolism of 2-hexanone in the guinea pig, DiVincenzo et al. [14] identified 2,5-dimethyl-2,3-dihydrofuran in the sera of animals injected with this ketone, when the samples were analyzed by GC. It was further shown that the dihydrofuran derivative was a product of the de-

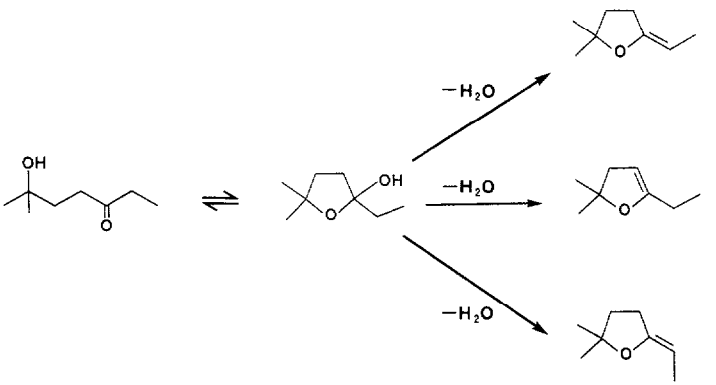

Fig. 1. Reaction scheme outlining the cyclization and dehydration of 6-hydroxy-6-methyl-3-heptanone to form the three cyclic enol ethers of molecular weight 126.

hydration of the cyclic tautomer of 5-hydroxy-2-hexanone that occurred upon injection of the sample into the GC inlet system.

Although Schwende et al. [2] showed that the three isomeric enol ethers originated from the synthetic lactol–hydroxy ketone mixture, the authors had not established the presence of the latter in urine. Our objective in the current study was to do so, as well as address the question of whether the mixture might be solely responsible for the abundance of the cyclic enol ethers seen in the purge-and-trap analysis of mouse urine. If the keto-alcohol were present at a concentration comparable to other urinary volatile constituents (roughly ppm levels [15]), it would be problematic to hypothesize that the keto-alcohol alone can account for the amounts of enol ethers observed. The keto-alcohol would not be expected to be sufficiently volatile, because of its molecular weight and rather polar nature, to be readily sparged into the Tenax pre-column; hence, contrary to observation, one would expect deceptively small peak areas of the enol ethers when analyzed by GC.

EXPERIMENTAL

The volatile constituents of mouse urine were analyzed by the previously described purge-and-trap method [16]. Urine samples of 1 ml, containing 0.300 g $(NH_4)_2SO_4$, were sparged with a 100 ml/min flow of purified helium. The volatile components were adsorbed on a precolumn packed with 3.0 mg Tenax-GC (60–80 mesh, Enka, Amsterdam, The Netherlands). After a 1-h sampling period, the precolumn was removed from the purge-and-trap apparatus and placed in the heated injection port (220–240°C) of a gas chromatograph. The volatile constituents were thermally desorbed from the Tenax precolumn for a 10-min period and condensed at the head of the analytical column. The column, 70 m × 250 μm I.D., coated with UCON 50-HB-2000, was temperature-programmed from 30 to 160°C at 2°C/min.

In order to detect the keto alcohol intermediate (Fig. 1) in mouse urine, we silylated the urinary extract with a mixture of hexamethyldisilazane (HMDS) and trimethylchlorosilane (TMCS) (commercially available under the name Tri-Sil from Pierce, Rockford, IL, U.S.A.). A 20-ml aliquot of a 24-h collection of male urine from the C57BL strain was used in this study. The urine was extracted with two equal volumes of diethyl ether. The combined ether extract was dried over anhydrous Na_2SO_4 for 12 h. After removal of Na_2SO_4 through filtration, the extract was concentrated, using a Kuderna-Danish apparatus, to a volume of about 1 ml. The concentrated urine extract was placed in a mixture of 1.0 ml dimethylsulfoxide (DMSO) and 300 μl of a 1:1 mixture of HMDS and TCMS. As the reaction proceeded, the diethyl ether was slowly removed in successive portions over a period of 4 h with a stream of dry nitrogen. At the end of the 12-h reaction period, the trimethylsilyl (TMS) derivatives were extracted with 4.0 ml of pentane. The solution was concentrated to

200 μl, as measured gravimetrically, with a stream of dry nitrogen and injected into a gas chromatograph provided with a 20 m$\times$0.25 mm I.D. glass capillary column, coated with an SE-30 elastomer.

A modified Varian 1400 gas chromatograph was used throughout the GC experiments, while the compound identification was carried out with a Model 5980A Hewlett Packard combined gas chromatograph–mass spectrometer using an Incos Nova 3 data acquisition system.

Supercritical fluid chromatography (SFC) of the synthetic mixture of cyclic enol ethers employed a Model 600 Series Lee Scientific (Salt Lake City, UT, U.S.A.) instrument. The separation was performed on a 10 m$\times$50 μm I.D. capillary column containing a 0.50-μm film of methylsilicone stationary phase (SB-Methyl-100, Lee Scientific). The mobile phase was supercritical nitrous oxide at 100°C, and detection was accomplished by the flame ionization method.

The donors of urine were normal male mice of the following strains: ICR albino (Ward's National Science Establishment, Rochester, NY, U.S.A.); BALB/cWt, C57BL, DBA/2, and AHE/J (Jackson Lab., Bar Harbor, ME, U.S.A.).

RESULTS AND DISCUSSION

A typical chromatogram of C57BL male mouse urine is shown in Fig. 2. The three cyclic enol ethers are indicated by arrows above chromatographic peaks (identifications of other components are described elsewhere [1,2]).

Some indirect evidence for the existence of the keto-alcohol can be inferred by examining the peak areas of the enol ethers found during the purge-and-

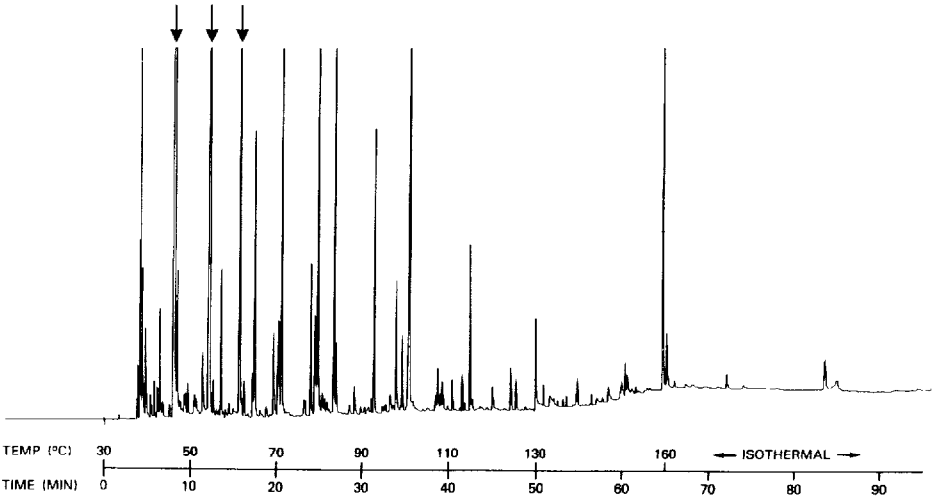

Fig. 2. Capillary gas chromatogram of the urinary volatile components of male mouse urine.

trap sampling and GC analysis of various mouse urine samples. If these three compounds arose from independent biochemical pathways, there would be no reason to expect the relative peak areas to remain constant among different samples that originate from the animals of different strains and sex. On the other hand, if the keto alcohol is present in the urine and gives rise to the enol ethers as an artifact of GC analysis, a constant ratio of the three peak areas would be expected. As seen in Table I, the ratios of the peak areas of the enol ethers remain fairly constant among different urine samples. Table I also provides the ratios of the enol ethers observed for the purge-and-trap sampling and GC analysis of three different aqueous samples spiked with 200 ppm of synthetic 6-hydroxy-6-methyl-3-heptanone [17]. It is evident that these samples decompose to give the three enol ethers with the same characteristic peak area ratios which are seen in the pooled urine samples. These data strongly argue against an independent biochemical origin of the enol ethers and toward the existence of a common intermediate.

Another goal of this study was to try to match unambiguously cyclic enol ether structure with each of the three GC peaks. The three compounds have been targets for ongoing synthetic efforts in this laboratory [17]. To date only one, the *endo* isomer, has been obtained under conditions excluding the other two. The tosylate derivative of 5,5-dimethyl-3-ethyl-tetrahydrofuran-3-ol was prepared and exposed to alumina at room temperature [17]. Barring double-

TABLE I

RATIOS OF PEAK AREAS OF THE CYCLIC ENOL ETHERS FROM DIFFERENT URINE SAMPLES AND DIFFERENTLY PREPARED AQUEOUS SOLUTIONS OF 6-HYDROXY-6-METHYL-3-HEPTANONE RELATIVE TO THE FIRST ELUTING COMPOUND

Sample type	Peak 1	Peak 2	Peak 3
ICR normal male[a]	1.0000	0.3157	0.1112
ICR normal male[a]	1.0000	0.3197	0.1115
ICR normal male[a]	1.0000	0.3130	0.1127
ICR normal male[a]	1.0000	0.3102	0.1058
ICR normal male[a]	1.0000	0.3173	0.1057
ICR normal male[a]	1.0000	0.3097	0.1057
BALB/cWT normal male[b]	1.0000	0.3106	0.1096
BALB/cWt castrate male[b]	1.0000	0.3035	0.1182
C57BL normal male[b]	1.0000	0.3074	0.1258
DBA/2 normal male[b]	1.0000	0.2846	0.0870
AHE/J normal male[b]	1.0000	0.3925	0.1208
200 ppm keto-alcohol	1.0000	0.3141	0.1327
200 ppm keto-alcohol	1.0000	0.3301	0.1492
200 ppm keto-alcohol	1.0000	0.3126	0.1127

[a]Urine samples from different individual ICR mice.
[b]Pooled 24-h urine samples collected from six to twelve mice.

bond isomerization (observed during our synthetic efforts to be frustratingly facile), one should, in agreement with our subsequent gas chromatographic–mass spectrometric (GC–MS) result, obtain only the *endo* isomer. The retention time and mass spectral characteristics of the synthetic compound matched the first-eluting and most abundant cyclic enol ether found in the purge-and-trap analysis of mouse urine. Unequivocal structure assignment has been possible only for this cyclic vinyl ether; assignments for the other two compounds are not yet possible.

Our strategy in attempting to detect the keto-alcohol intermediate in urine was to derivatize the molecule so as to preclude cyclization and, hence, the dehydration. Our choice of silylating agent for the tertiary hydroxyl group of the hydroxy ketone was influenced by several considerations: (1) too strong a silylating agent [*N*,O-bis(trimethylsilyl)trifluoroacetamide or *N*-trimethylsilylimidazole] would be expected to attack the carbonyl site of the molecule as well (through its enol form); (2) a common moderate agent, TMCS, even in the presence of HMDS, has been known to yield unwanted side-effects. Therefore, we settled on the method of Friedman and Kaufman [18] which employed DMSO as a solvent for silylation of tertiary alcohols. These investigators found that the reaction of 2-methyl-2-butanol with HMDS in DMSO proceeded rapidly and quantitatively to completion, with the TMS derivative forming a separate layer on top of the DMSO layer.

A chromatogram of the blank (Fig. 3, top) represents 40 ml of ether which was dried over Na_2SO_4, reduced in volume, and further treated in the same manner as the ether extract of mouse urine. The lower chromatogram in Fig. 3 shows the TMS derivatives of the diethyl ether extract of mouse urine. The labeled peak primarily attracted our attention; its mass spectrum is given in Fig. 4. The fragmentation pattern agrees well with that expected for the TMS ether of the keto-alcohol, as shown by the arrows. The *m/e* 73 and 75 fragments are characteristic of mass spectra of TMS derivatives of alcohols. Other peaks observed in Fig. 3 correspond to additional compounds from mouse urine which are carried through the extraction–derivatization scheme, by-products of the silylation reaction, and impurities which are concentrated from the organic solvents and reagents. Interestingly, we found no mass spectral evidence for the TMS derivative of the cyclic intermediate. Evidence for any di-TMS by-product derivative was likewise absent.

In the preparation for quantitation of the precursor in mouse urine, the purity of the synthetic keto-alcohol was first evaluated. Preliminary GC–MS studies of the synthetic mixture indicated the presence of 4-hydroxy-4-methylpentanoic acid lactone as a major impurity, an identification which was further verified by infrared spectrometry. Due to the demonstrated ability of SFC to analyze mixtures containing labile compounds [19, 20], this technique was utilized for determining the purity of the synthetic mixture. A separation of the crude synthetic mixture by SFC is shown in Fig. 5. In this chromatogram,

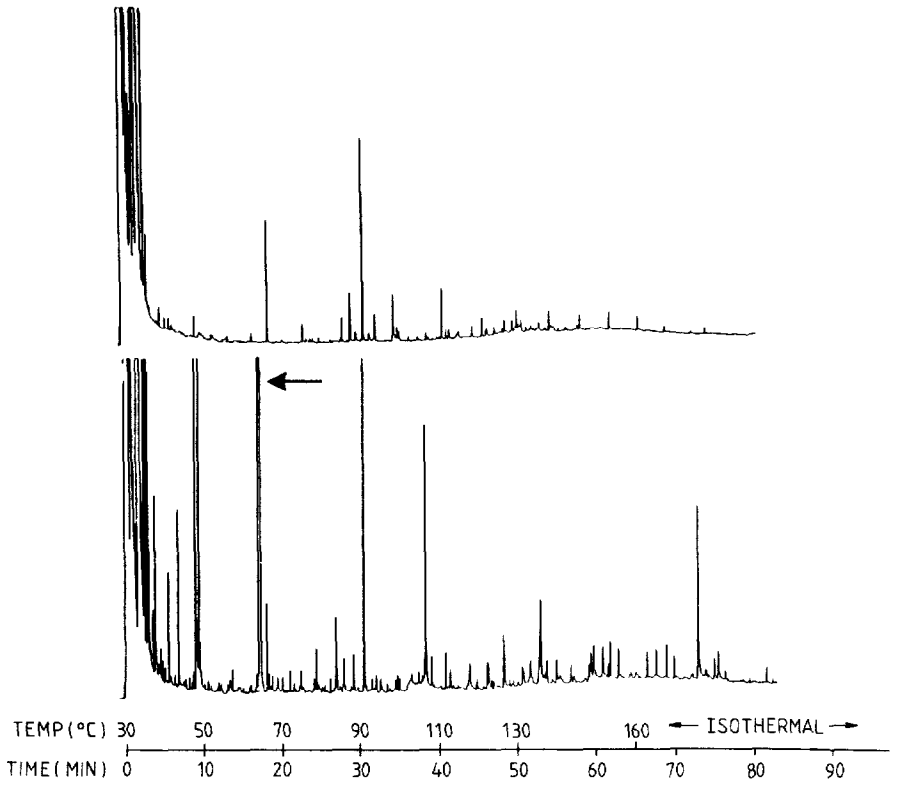

Fig. 3. Chromatogram of blank (top) and the trimethylsilyl ether derivatives (bottom) of a diethyl ether extract of mouse urine.

the lactone impurity elutes first, followed by what was initially believed to be the intact enol ether precursor. In a run at 75 °C (not shown here), the lactone impurity was followed by three peaks displaying the characteristic relative peak areas of the enol ethers, indicating that the keto-alcohol has undergone dehydration even under these milder chromatographic conditions. Likewise, the keto-alcohol was not eluted at 100 °C, but now a single peak believed to contain all three enol ethers was observed. By comparing the peak integration of combined dehydration products with that of lactone, an estimate of 80% keto-alcohol and 20% lactone impurity was obtained.

Aqueous solutions spiked with 6-hydroxy-6-methyl-3-heptanone and carried through the derivatization and analysis scheme allowed for an estimation of the concentration of about 160 ppm. This figure is well over 100 times those of the multifunctional mouse pheromones 3,4-dehydro-*exo*-brevicomin and 2-*sec*-butyl-4,5-dihydrothiazole [15,21,22]. The unusually large concentration of the keto-alcohol precursor thus accounts for the large quantities of enol ethers observed during the GC analysis of the mouse urine.

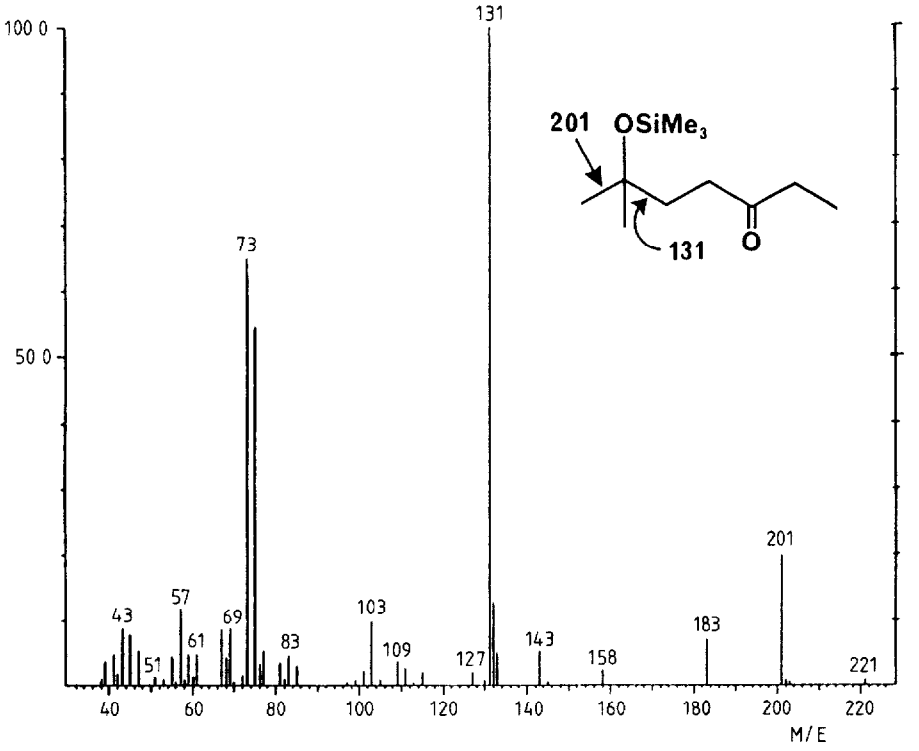

Fig. 4. Mass spectrum of the peak labeled in Fig. 3.

We realize the possibility exists that the derivatization reaction could have disturbed whatever equilibrium may exist in urine between the enol ethers and their precursor, resulting in ultimate conversion of enol ethers to silylated hydroxy ketone. Yet, literature data indicate that 2,5-dimethyl-2,3-dihydrofuran is readily hydrolyzed in aqueous solution [23], and it has been shown by NMR studies that 2,4-dimethyl-2,3-dihydrofuran dissolved in deuterated chloroform is gradually converted to 5-hydroxy-2-hexanone upon the addition of ^2H_2O [14]. We thus believe that the lactol–hydroxy ketone precursor, rather than the enol ethers themselves, predominates in the mouse urine.

Verification of the artifactitious nature of the enol ethers prompted us to review the many diverse urine samples we have analyzed in this laboratory for similar types of compounds. The identification of heterocycles which could arise from dehydration during sampling and analysis seems to be the rule rather than the exception among these samples. For example, Boyer et al. [5] found a series of lactones to be present in the urine of pine voles. The presence of γ-octanoic, γ-nonanoic, γ-decanoic, and δ-decanoic lactones may well be explained by the cyclization and dehydration of the corresponding hydroxy acids. Several furan derivatives tentatively identified from human and rat urine by

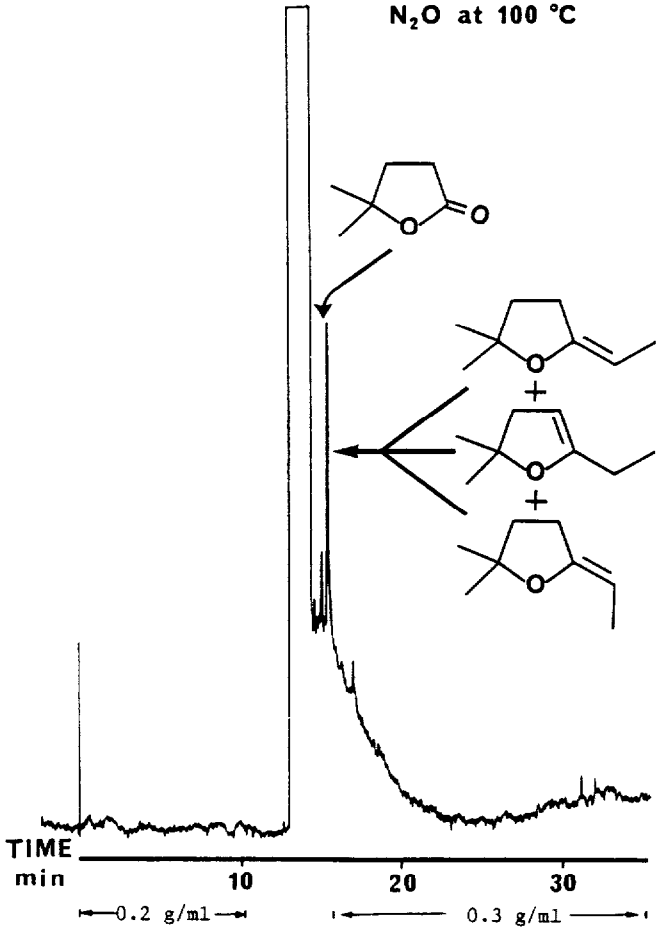

Fig. 5. Supercritical fluid chromatogram of crude synthetic 6-hydroxy-6-methyl-3-heptanone.

mass spectral data could likewise originate from the cyclization and dehydration of unsaturated hydroxy aldehydes and ketones [6–8]. Analysis of urines from deer, opossum, tomcat, and several special species of canids have also indicated the presence of various oxygen heterocycles [9]. It is not entirely clear whether these ethers and lactones are present in the urine, or if they are formed as artifacts. However, the present study emphasizes the fact that dehydration reactions of hydroxycarbonyl compounds may be of a more widespread occurrence than many investigators realize.

ACKNOWLEDGEMENT

This work was partially supported by Grant No. CHE 8605935 from the National Science Foundation.

REFERENCES

1 F.J. Schwende, Ph.D. Thesis, Indiana University, Bloomington, IN, 1983.
2 F.J. Schwende, D. Wiesler, J.W. Jorgenson, M. Carmack and M. Novotny, J. Chem. Ecol., 12 (1986) 277.
3 A. Zlatkis and H.M. Liebich, Clin. Chem., 17 (1971) 592.
4 A. Zlatkis, W. Bertsch, H.A. Lichtenstein, A. Tishbee, F. Shunbo, H.M. Liebich, A.M. Coscia and N. Fleischer, Anal. Chem., 45 (1973) 763.
5 M.L. Boyer, B. Jemiolo, F. Andreolini, D. Wiesler and M. Novotny, J. Chem. Ecol., 15 (1989) 649.
6 G.R. Rhodes, Ph.D. Thesis, Indiana University, Bloomington, IN, 1981.
7 M.L. Holland, Ph.D. Thesis, Indiana University, Bloomington, IN, 1982.
8 M.F. Yancey, Ph.D. Thesis, Indiana University, Bloomington, IN, 1987.
9 J.H. Raymer, Ph.D. Thesis, Indiana University, Bloomington, IN, 1984.
10 P.J. Apps, A. Rasa and H.W. Viljoen, paper presented at the 5th Conference "Chemical Signals in Vertebrates", Oxford, August 8–10, 1988.
11 F. Andreolini, B. Jemiolo and M. Novotny, Experientia, 43 (1987) 998.
12 B. Jemiolo, F. Andreolini and M. Novotny, in D. Duvall, D. Muller-Schwarze and R.M. Silverstein (Editors), Chemical and Biological Investigations of Female Mouse Pheromones, Chemical Signals in Vertebrates, Vol. 4. Plenum Press, New York, 1986, pp. 79–85.
13 B. Jemiolo, F. Andreolini, D. Wiesler and M. Novotny, J. Chem. Ecol., 13 (1987) 1941.
14 G.D. DiVincenzo, C.J. Kaplan and J. Dedinas, Tocixol. Appl. Pharmacol., 36 (1976) 511.
15 M. Novotny, S. Harvey, B. Jemiolo and J. Alberts, Proc. Natl. Acad. Sci. U.S.A., 82 (1985) 2059.
16 M. Novotny, M.L. Lee and K.D. Bartle, Chromatographia, 7 (1974) 333.
17 D. Wiesler, T.-M. Xie, S. Harvey and M. Novotny, submitted for publication.
18 S. Friedman and M.L. Kaufman, Anal. Chem., 38 (1966) 144.
19 J.C. Fjeldsted, R.C. Kong and M.L. Lee, J. Chromatogr., 279 (1983) 449.
20 K.E. Markides, S.M. Fields and M.L. Lee, J. Chromatogr. Sci., 24 (1986) 254.
21 B. Jemiolo, J. Alberts, S. Sochinski-Wiggins, S. Harvey and M. Novotny, Anim. Behav., 33 (1985) 1114.
22 B. Jemiolo, S. Harvey and M. Novotny, Proc. Natl. Acad. Sci. U.S.A., 83 (1986) 4576.
23 P. Dimroth and H. Pasedach, Angew. Chem., 72 (1960) 865.