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CHEMICAL LABILITY OF THE TERTIARY AMINOPROPIOPHENONES OF EUGENOL AS CHARACTERIZED BY COMBINED GAS–LIQUID CHROMATOGRAPHY AND CHEMICAL IONIZATION MASS SPECTROMETRY

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SUMMARY

The lability of various silylated and acetylated derivatives of the tertiary aminopropiophenones of eugenol was characterized by combined gas–liquid chromatography and chemical ionization mass spectrometry. Because of decomposition during derivatization and/or during analyses, the acetylated and unreduced silylated derivatives of these metabolites are very undesirable for gas chromatography. Reduction of the tertiary aminopropiophenones of eugenol followed by silylation at room temperature yields "di"-silyl ethers which can be satisfactorily characterized by gas chromatography and combined gas–liquid chromatography and chemical ionization mass spectrometry on microgram quantities. The "di"-silyl ethers of these metabolites are easily decomposed by moisture, air and heat to yield the respective monosilylated nitrogen metabolites, which in turn decompose readily to yield the silylated allylic ketone and the secondary amines.

INTRODUCTION

Earlier reports[1] from this laboratory have described the production of nitrogen-containing metabolites from the allyl- and propenylbenzenes in foods, food additives and essential oils. Complete structural identification of these basic ninhydrin-positive metabolites[1] of the allylbenzene derivatives safrole[2], myristicin[3], eugenol[3], and elemicin[4] indicate that these nitrogen-containing metabolites are tertiary amino-propiophenones.

The postulated mechanism[3] for the biosynthesis of these tertiary amino-propiophenones suggests that the allylbenzene is first converted by an allylic oxidation to the allylic ketone. The allylic ketone could then condense with the secondary amines piperidine, pyrrolidine or dimethylamine in the presence of the appropriate enzyme system to yield the excreted tertiary aminopropiophenones[1-4]. The chemical reactivity of these various allylic ketones is very great[5]. These allylic ketones react very vigorously with water[5], secondary amines[5], sulfhydryl compounds and many other compounds of biological importance.

Production of transplantable tumors, fibrosarcomas and sarcomas in rats after administration of phenyl vinyl ketone[6], production of hepatic tumors[7-9], general fibrosis[3,4], mass adhesions, liver degeneration, and very abrupt pathological changes in experimental animals after administration of the allylbenzenes suggest that these environmental agents may be detrimental to individuals upon environmental exposure.

Earlier reports[2-4] described the general decomposition of the tertiary amino-propiophenones of safrole, myristicin and elemicin. These metabolites of elemicin[4] and eugenol decompose much more rapidly than those of safrole[2] or myristicin[3]. Recently combined gas–liquid chromatography and chemical ionization mass spectrometry (GLC–CIMS) was used to more easily study the production of the tertiary aminopropiophenones from safrole, myristicin, elemicin[4] and eugenol.

The present report describes the great degree of instability of the eugenol metabolites as characterized by GLC–CIMS. The presence of the easily oxidizable phenol group of eugenol coupled with the heat instability properties of the tertiary aminopropiophenones makes the eugenol metabolites very difficult and sometimes very uncontrollable metabolites.

EXPERIMENTAL

Materials

The synthetic tertiary aminopropiophenones of eugenol were prepared from 4-hydroxy-3-methoxyacetophenone (Aldrich Chemical Co.), paraformaldehyde, and the secondary amine hydrochloride using the Mannich reaction[10]. 3-N,N-Dimethyl-amino-1-(3'-methoxy-4'-hydroxyphenyl)-propan-1-one·HCl, m.p. 186–188°; 3-piper-idyl-1-(3'-methoxy-4'-hydroxyphenyl)-propan-1-one·HCl, m.p. 188–190°; and 3-pyrrolidinyl-1-(3'-methoxy-4'-hydroxyphenyl)-propan-1-one·HCl, m.p. 189–190°.

Methods

For the synthetic eugenol metabolites, the extraction procedure[3] was modified so that the basic fraction was obtained at pH 8.5 instead of the usual[3] pH 13. Using a chloroform–methanol (2:1) extraction at pH 8.5, about 75 % of the eugenol metabolite was recovered with one extraction. Two or more extractions using this solvent combination at pH 8.5 yielded most (> 90 %) of the synthetic eugenol tertiary aminopropiophenones.

Gas–liquid chromatography

A Tracor MT-220 gas chromatograph was used to characterize all eugenol metabolites prior to combined GLC-CIMS analyses. With this unit, using an 0.6 × 184 cm glass column packed with 3 % OV-1 on Gas-Chrom Q (80–100 mesh) with a helium flow of 50 ml/min, injector temperature of 250°, and flame ionization detector temperature of 250°, the silylated derivatives of the eugenol tertiary aminopropiophenones were chromatographed at 150° for 1 min, then with temperature program 150–180° at 5°/min. For the acetylated derivatives of the eugenol metabolites, the same conditions were used except that the material was injected on the column at 180°. After 1 min, the temperature is programmed from 180–240° at 5° or 10°/min as specified.

For GLC–CIMS a Varian Aerograph Model 1400 gas chromatograph with a 5 ft × 1/8 in. I.D. glass column packed with 3 % OV-1 on Gas-Chrom Q (80–100 mesh) and interfaced to the Finnigan 1015C Quadrupole chemical ionization mass spectrometer with a measured methane flow of 15 ml/min through the GC column was employed.

Using the combined GLC–CIMS system[4], the tertiary aminopropiophenones of eugenol after reduction[3] and/or silylation were injected in an appropriate volume of solvent (1–3 μl) on the OV-1 column at 150° with the ionizer off. After 1 min the column temperature was programmed from 150–200° at 10°/min. Finally, 2 min after injection, the ionizer was turned on and the computer control was initiated for collection of all data. For the acetylated derivatives, the sample was injected at zero time on the OV-1 column at 150° with the ionizer off. After 1 min, the column temperature was programmed from 150–250° at 10°/min. The ionizer was initiated at the specified time after injection (1, 2 or 4 min). All retention times are expressed as the time at which a particular peak reaches its maximum after zero time of injection.

The conditions for operation of the chemical ionization mass spectrometer were as described earlier for elemicin[4] and other allylbenzene derivatives.

Silylation

Careful silylation of the reduced eugenol metabolites with a 1:1 mixture of N,O-bis(trimethylsilyl)-trifluoroacetamide and trimethylsilylimidazole at room temperature overnight (16 h) yields the "di"-trimethylsilyl ether derivative of these tertiary aminopropiophenones. After reaction with the silylating reagent, it is desirable to remove the excess reagent with a stream of very dry nitrogen. The silylated derivative is then made up in spectrograde chloroform with silica gel preservative (Matheson, Coleman and Bell) (CX 1050 SG 5023) to a final concentration of about 5 μg/μl. Satisfactory silylation is also accomplished with the above conditions using Tri-Sil "Z" (trimethylsilylimidazole in dry pyridine, 1.5 mequiv./ml). Unless the sample is taken to dryness in order to remove the excess reagent, the reaction seems not to go to completion. Care must be taken to exclude moisture. As will be shown later, these silyl ethers decompose very easily to the monosilylated nitrogen metabolite and finally to the silylated allylic ketone.

Acetylation

The acetylated derivatives of the eugenol metabolites were prepared with an excess of acetic anhydride–pyridine (1:1) at 40° for 30 min. After reaction time, the excess reagent was removed with dry nitrogen and aliquots of spectrograde chloroform. The derivatives were then dissolved in spectrograde chloroform (5 μg/μl).

RESULTS AND DISCUSSION

CIMS is a very sensitive and fully descriptive method of identification and characterization of the tertiary aminopropiophenones formed from the various allylbenzenes[4]. As described for elemicin[4], the use of electron impact mass spectrometry for characterization of the silyl ether derivatives of the eugenol metabolites was very unrewarding. Even using 20 eV with the electron impact unit, one is unable to deter-

mine the number of silyl ether groups, the presence of the nitrogen moiety, or the presence of substitution on the aromatic ring.

The sample requirement for the silyl ether derivatives of eugenol is greater than that of the elemicin metabolite[4]. The large sample requirement seems to be caused by on-column continuous decomposition of these materials. At this time even with very careful handling, one must use 2–5 μg of a particular metabolite in order to have sufficient intact elution for CIMS.

As discussed earlier[2–4], all of the tertiary aminopropiophenones decompose by heat at varying degrees to yield the allylic ketone plus the secondary amine. In addition, the eugenol derivatives are very easily oxidized by air. Since traces of air oxidize the phenol, one must attempt to eliminate exposure to air as much as possible. If possible, the sample should be kept under nitrogen.

Increased stability to thermal decomposition has been accomplished by reduction of the carbonyl group to the secondary alcohol[2–4]. For all allylbenzenes except eugenol, it has been possible to analyze the reduced tertiary aminopropiophenones by GC with minimal decomposition without further derivatization. On the contrary, injection of the reduced eugenol metabolite yields no detectable materials that would elute from an OV-1 column even at 230°.

One must compromise on a satisfactory derivative with desirable GC properties and still maintain minimal thermal and hydrolytic decomposition of these derivatives. In all cases discussed, there is some thermal decomposition. The eugenol metabolites can be silylated without reduction. The piperidyl derivative of eugenol in the unreduced form (eugenol II) was silylated and then analyzed prior to GLC–CIMS
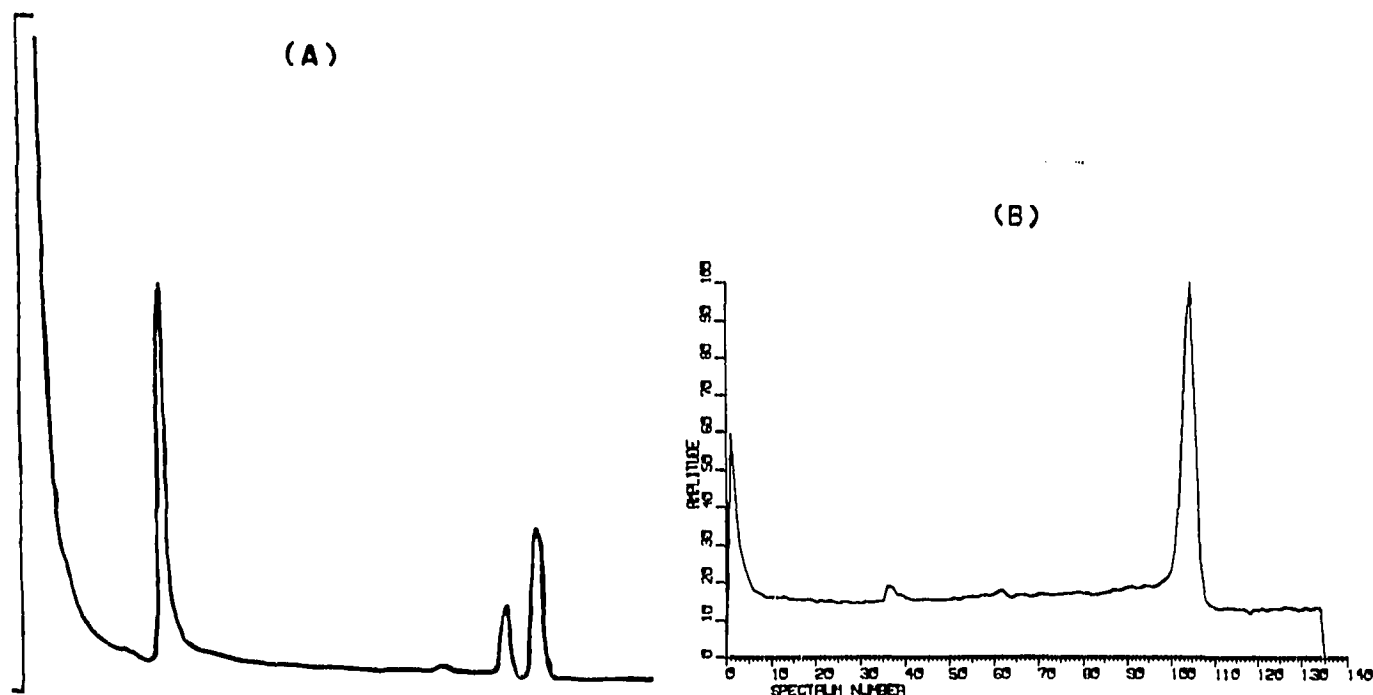

Fig. 1. (A) Gas chromatogram of the silylated derivative of unreduced 3-piperidyl-1-(3'-methoxy-4'-hydroxyphenyl)-propan-1-one, retention time: 12 min 15 sec. Major decomposition product: silylated 1-(3'-methoxy-4'-hydroxyphenyl)-3-propen-1-one, retention time: 3 min 45 sec. (B) Reconstructed chromatogram of combined GLC–CIMS analyses of silylated unreduced eugenol II.

analysis. Fig. 1A is the chromatogram of this analysis. This derivative also decomposes to yield the silylated allylic ketone and the secondary amine. The major component of this chromatogram is the silylated allylic ketone with a retention time of 3 min 45 sec. The intact unreduced silylated eugenol II has a retention time of 12 min 15 sec with a column temperature of 150° for 1 min and then programmed 150–200° at 5°/min. A precursor contaminant (retention time 11 min 30 sec) in a significant concentration makes the analysis of the silylated unreduced metabolite very undesirable by this means. In addition, without reduction of the carbonyl group, there is major decomposition of the desired metabolite as illustrated in Fig. 1B. By use of the computer limited mass search m/e 250–252, spectra 1–5 represent the silylated allylic ketone. Spectra 100–105 represent the elution profile of the intact unreduced eugenol II metabolite.

The representative chemical ionization mass spectra of the major decomposition product, silylated allylic ketone of eugenol, and the intact unreduced silylated eugenol II are illustrated in Fig. 2. The silylated allylic ketone (Fig. 2A) has a base peak and quasi-molecular ion $(M + 1)$ m/e of 251 with the recombination fragments m/e 265 $(M + CH_3^+)$, 279 $(M + C_2H_5^+)$, and 291 $(M + C_3H_5^+)$ with abundant 235, 223 fragments. The unreduced silylated piperidyl derivative (eugenol II) (Fig. 2B) has a base peak of m/e 98 with a quasi-molecular ion $(M + 1)$ of 336, recombination fragments 364 $(M + C_2H_5^+)$ and 376 $(M + C_3H_5^+)$, and common fragment m/e 279, 291, 265, 251, and 234 of the allylic ketone.

Because of increased thermal decomposition, precursor contamination and longer elution time, the silylated unreduced eugenol metabolites are much less desirable than the silylated reduced derivatives, as will be discussed later.

During the structural verification of the tertiary aminopropiophenones of safrole[2], the acetylated reduced metabolites were useful as additional means of identification; however, as will be seen, the acetates are not very satisfactory derivatives for GC. Attempts to acetylate the unreduced eugenol metabolites yielded upon GLC analyses only the acetylated allylic ketone. No detectable intact unreduced acetylated eugenol nitrogen-containing metabolite was detected. As is noted for all of the successfully acetylated derivatives of eugenol, the major product of acetylation upon GC analyses is the acetylated allylic ketone, the major decomposition product of the acetylated tertiary aminopropiophenones. Acetylation at room temperature for 24 h resulted in no detectable material that was eluted upon GC analysis except the above acetylated allylic ketone. Mild heating at 40° for 30 minutes yielded the diacetylated derivative of the reduced eugenol metabolite; however, during this mild heating and also probably during GC analysis, major decomposition takes place. In addition, a much higher temperature is required to elute the diacetate of the reduced eugenol metabolite.

As seen in Fig. 3, upon injection of 10 μg of the acetylated reduced eugenol metabolites, the major peak of this elution profile is the acetylated allylic ketone (retention time 2 min 15 sec). This sample contains only a very small quantity of the diacetate derivatives with retention times: I, 5 min 15 sec; III, 7 min 30 sec; and II, 8 min 15 sec. Fig. 3A represents the elution profile of the acetylated metabolite with the ionizer and computer initiated 1 min after injection. As described for the elemicin metabolites[4], greater intensification is accomplished by injection of the sample at zero time followed by initiation of the ionizer and computer 2 min 30 sec
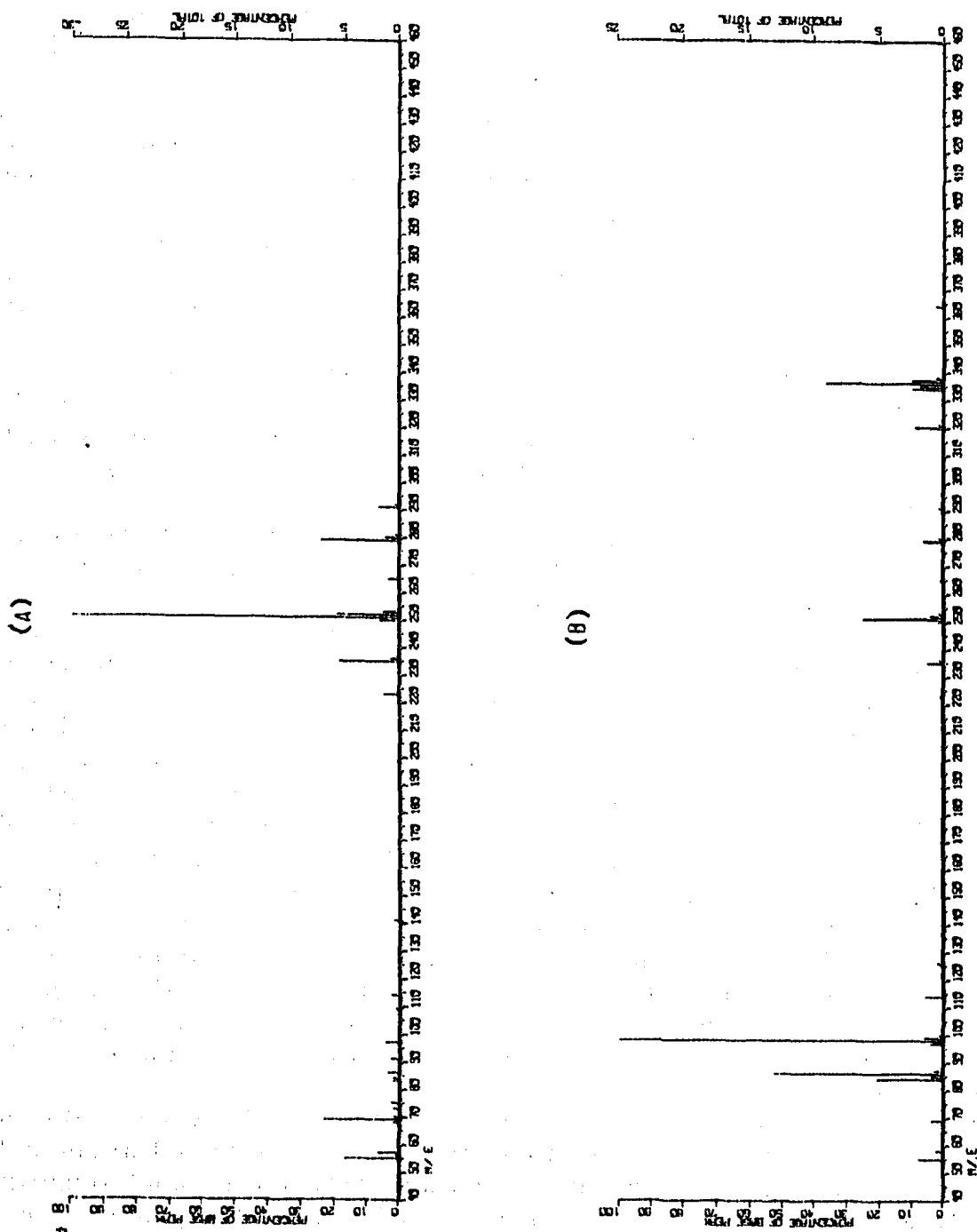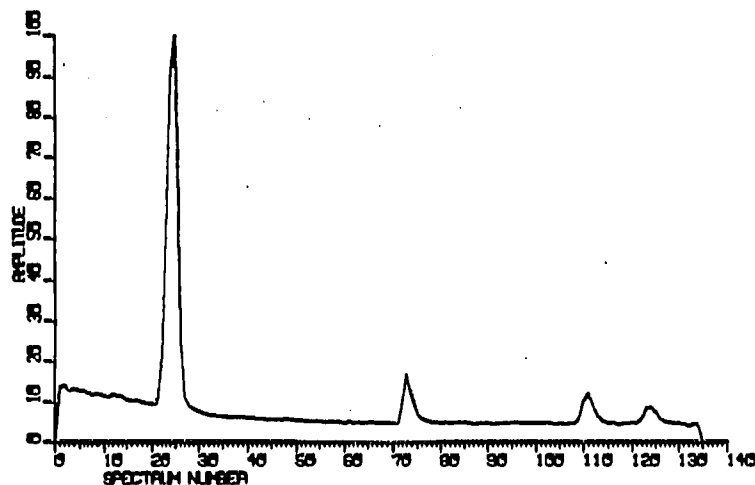
Fig. 2. (A) Chemical ionization mass spectrum of the silylated allylic ketone of eugenol. (B) Chemical ionization mass spectrum of the silylated unreduced eugenol II metabolite.
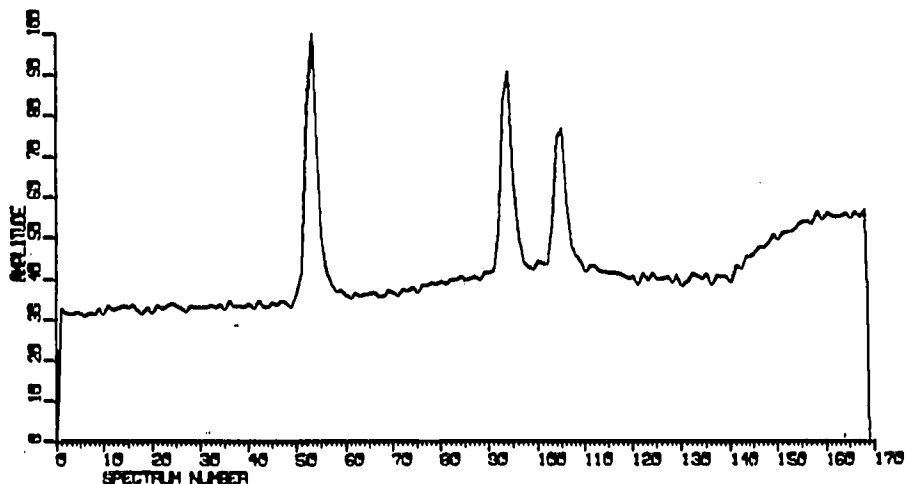
(A)

(B)

Fig. 3. (A) Reconstructed chromatogram of the reduced and acetylated eugenols I, II and III (10 μg total material); ionizer and computer initiated 1 min after injection. (B) Reconstructed ihromatogram of the reduced and acetylated eugenols I, II and III (10 μg total material); conizer and computer initiated 2 min 30 sec after injection.

after injection. Fig. 3B is the reconstructed chromatogram of the GLC–CIMS analyses 2 min 30 sec after injection. In this case (Fig. 3B) the major acetylated allylic ketone has eluted prior to initiation of the system.

The chemical ionization mass spectra of the acetylated allylic ketone and the diacetate of the reduced eugenol II are illustrated in Fig. 4. The acetylated allylic ketone (Fig. 4) has a base peak of m/e 179 with a quasi-molecular ion $(M+1)$ of m/e 221 and m/e 230 $(M+CH_3)$, m/e 249 $(M+C_2H_5{}^+)$, m/e 261 $(M+C_3H_5{}^+)$, and m/e 275 $(M+C_4H_7{}^+)$ recombination fragments.

Fig. 4. (A) Chemical ionization mass spectrum of the acetylated allylic ketone of eugenol. (B) Chemical ionization mass spectrum of the reduced and acetylated eugenol II metabolite.
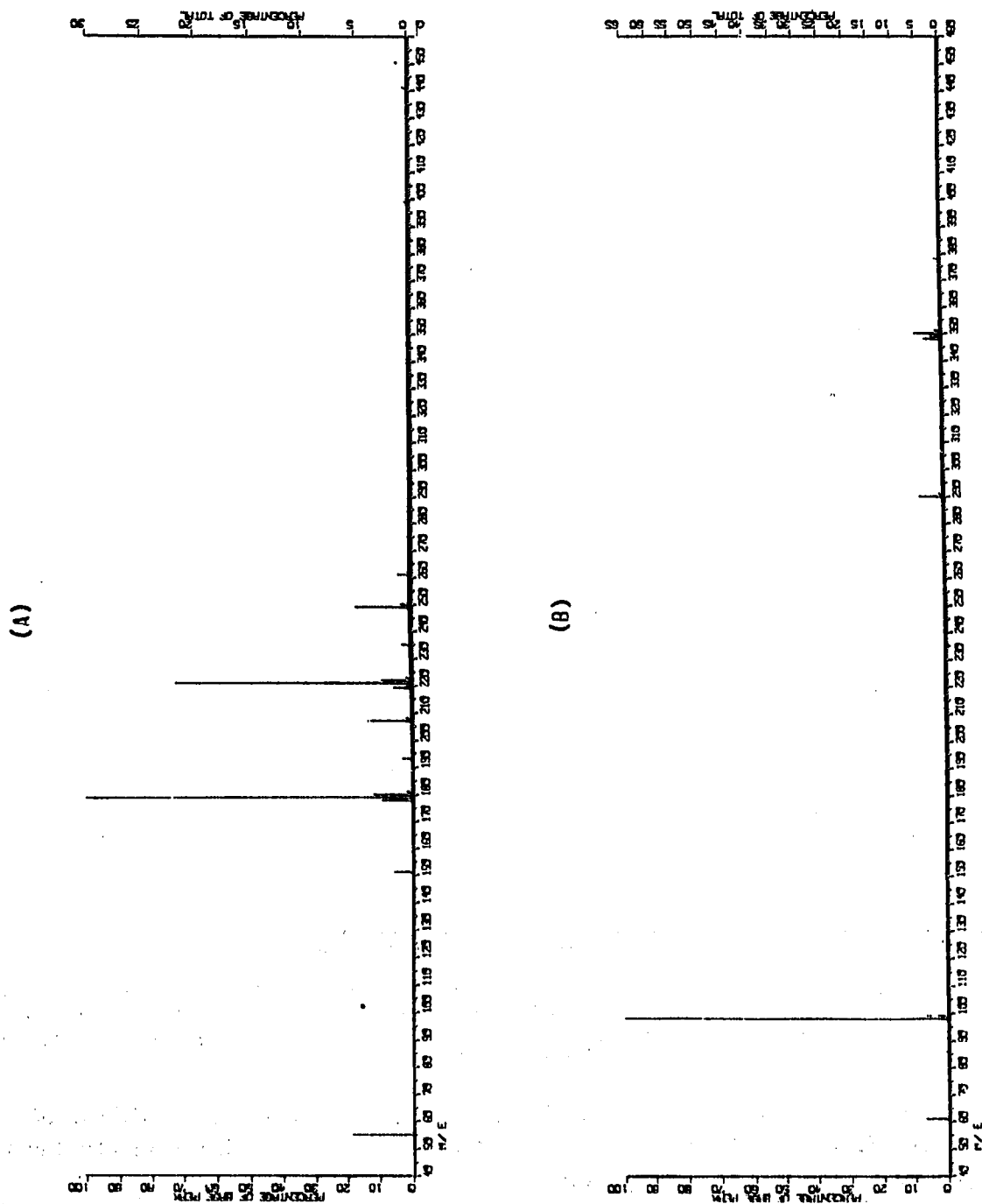
The reduced diacetyl derivative of the eugenol II metabolite (Fig. 4B) has, as expected, the base peak of m/e 98, a quasi-molecular ion $(M + 1)$ of 350, a recombination fragment m/e 378 $(M + C_2H_5^+)$, and a m/e 290 fragment.

Having similar lability properties to those of the silylated unreduced metabolite, the reduced and acetylated derivatives of the eugenol metabolite are very heat-labile during derivatization and during analyses. Because of this major decomposition to the appropriate allylic ketone even after reduction, the acetylated derivatives of the eugenol metabolite are not very desirable for GC analyses.

The most desirable derivatives for GC analyses of these eugenol metabolites are the "di"-silyl ethers of the reduced metabolites. On the contrary, care must be taken to minimize exposure to moisture and hydrolytic type solvents. Storage of these silylated derivatives is most satisfactory using spectrograde chloroform with silica gel preservative. Special care must be taken to remove moisture from the nitrogen gas used for evaporation of the excess reagent. Once the sample has been taken to dryness and dissolved in chloroform, minimal exposure to air and moisture is very desirable. For routine use, the desired sample is silylated in a small conical microflex vial (Kontes Glass Co.) without any heating. The excess silylating reagent is removed with a stream of very dry nitrogen. Immediately the vial is sealed using a microflex valve (Kontes Glass Co.) equipped with a syringe port and septum. Removal of the cap for access of sample is eliminated with this valve. Exposure of these silylated derivatives of the eugenol tertiary aminopropiophenones to air and moisture for periods of a few seconds ($>$ 10 sec) may result in complete hydrolysis of the "di"-silyl ethers. Use of acetone, methanol, chloroform with methanol preservatives, tetrahydrofuran and other solvents capable of carrying traces of moisture or capable of initiation of hydrolysis or exchange should be avoided. Pretreatment of the cured GC column with silylating reagent in order to minimize active sites is also very desirable for these polyfunctional metabolites. Excessive heating ($>$ 250°) of the injector port, column, and other components should be kept low in order to minimize thermal conversion of the silyl ether of the tertiary aminopropiophenones to the silylated allylic ketone and the secondary amine. GC and combined GLC–CIMS analyses should be completed as soon as possible after derivatization. With maximum care and storage, these silyl derivatives may be stored for only a few days without major decomposition and hydrolysis.

The sample requirements for the reduced and silylated derivatives of these eugenol metabolites are much greater than those reported for the elemicin metabolites[4]. At this time even with very careful handling, one must use 2–5 μg of a particular material in order to have sufficient intact elution of the desired materials for CIMS.

As indicated in Fig. 5A, the "di"-silyl ethers of the eugenol metabolites can be analyzed very satisfactorily by GC. A minimum amount of the silylated allylic ketone is produced prior and/or during the analyses. During exposure to moisture or prolonged storage, the "di"-silyl ethers hydrolyze to the "mono"-silyl ethers, which finally hydrolyze to yield non-elutive materials as monitored by GC except for the decomposition product, the silylated allylic ketone. As hydrolysis progresses, the thermal production of the silylated allylic ketone also increases (Fig. 5B). Considering the fact that the net end product of decomposition is the silylated allyl ketone, this suggests, as also seen for the unreduced silylated derivative, that the secondary
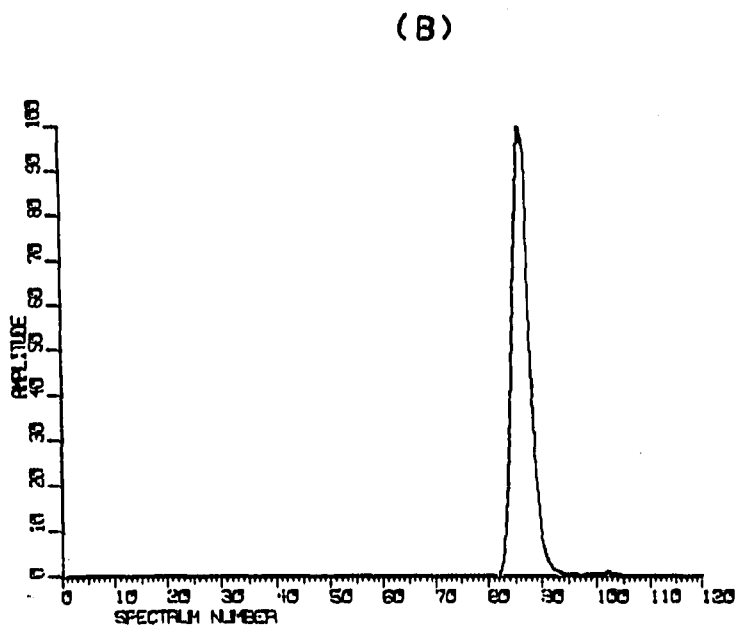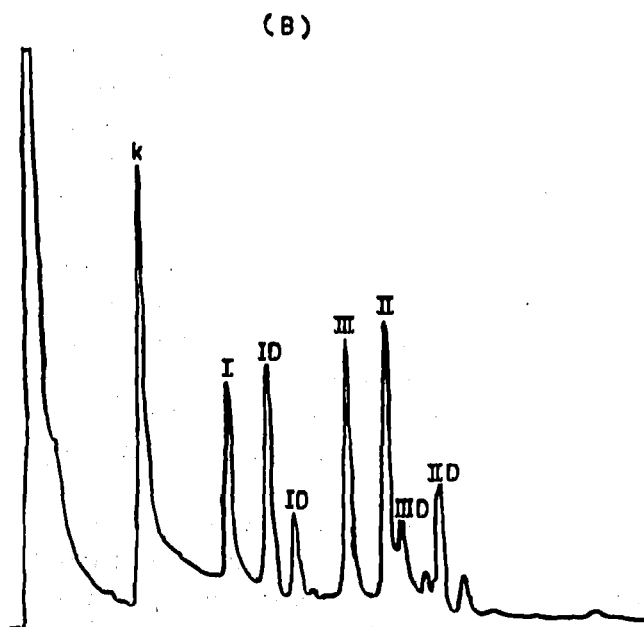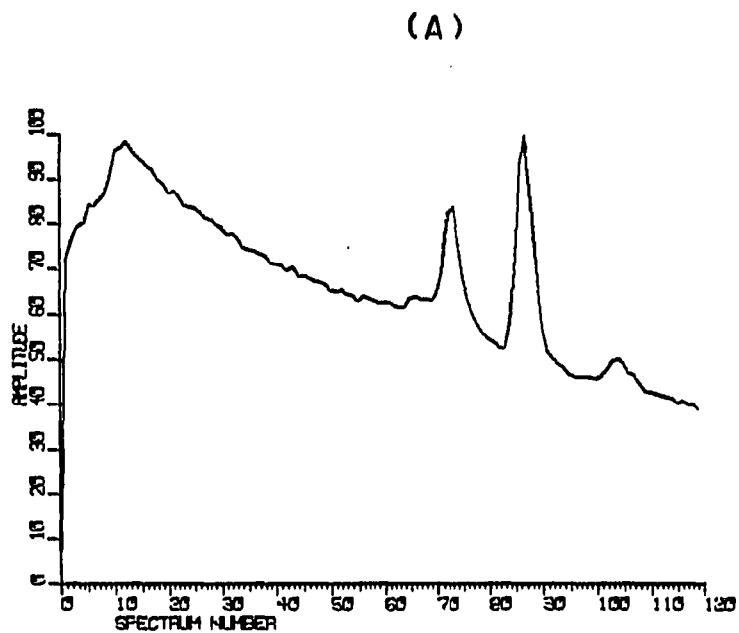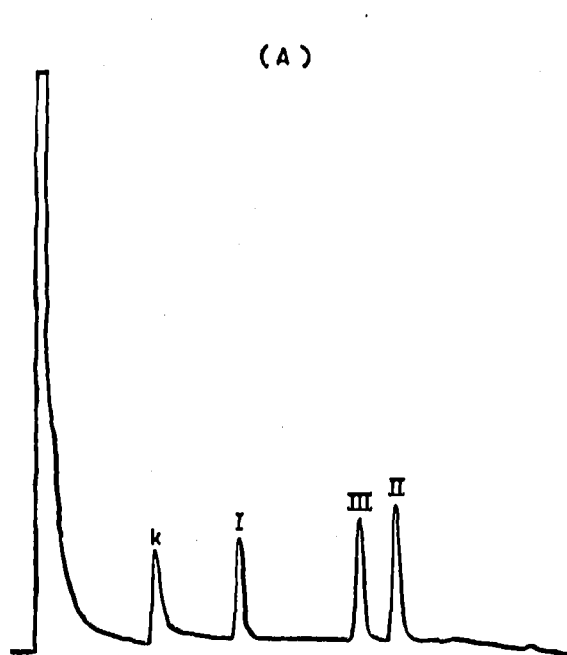
Fig. 5

Fig. 6

Fig. 5. (A) Gas chromatogram of the reduced and silylated metabolite of eugenols I, II and III prior to combined GLC–CIMS analyses using the MT-220 gas chromatograph. (B) Gas chromatogram of the reduced and silylated eugenols I, II and III after partial hydrolysis by moisture and storage using the MT-220 gas chromatograph. K = Allylic ketone; ID, IID and III D = decomposition products of I, II and III, respectively.

Fig. 6. (A) Reconstructed chromatogram with the combined GLC–CIMS system of the reduced and silylated eugenol II upon storage (5 μg total material). (B) Reconstructed chromatogram of 6A with a limited mass search m/e 409–411.
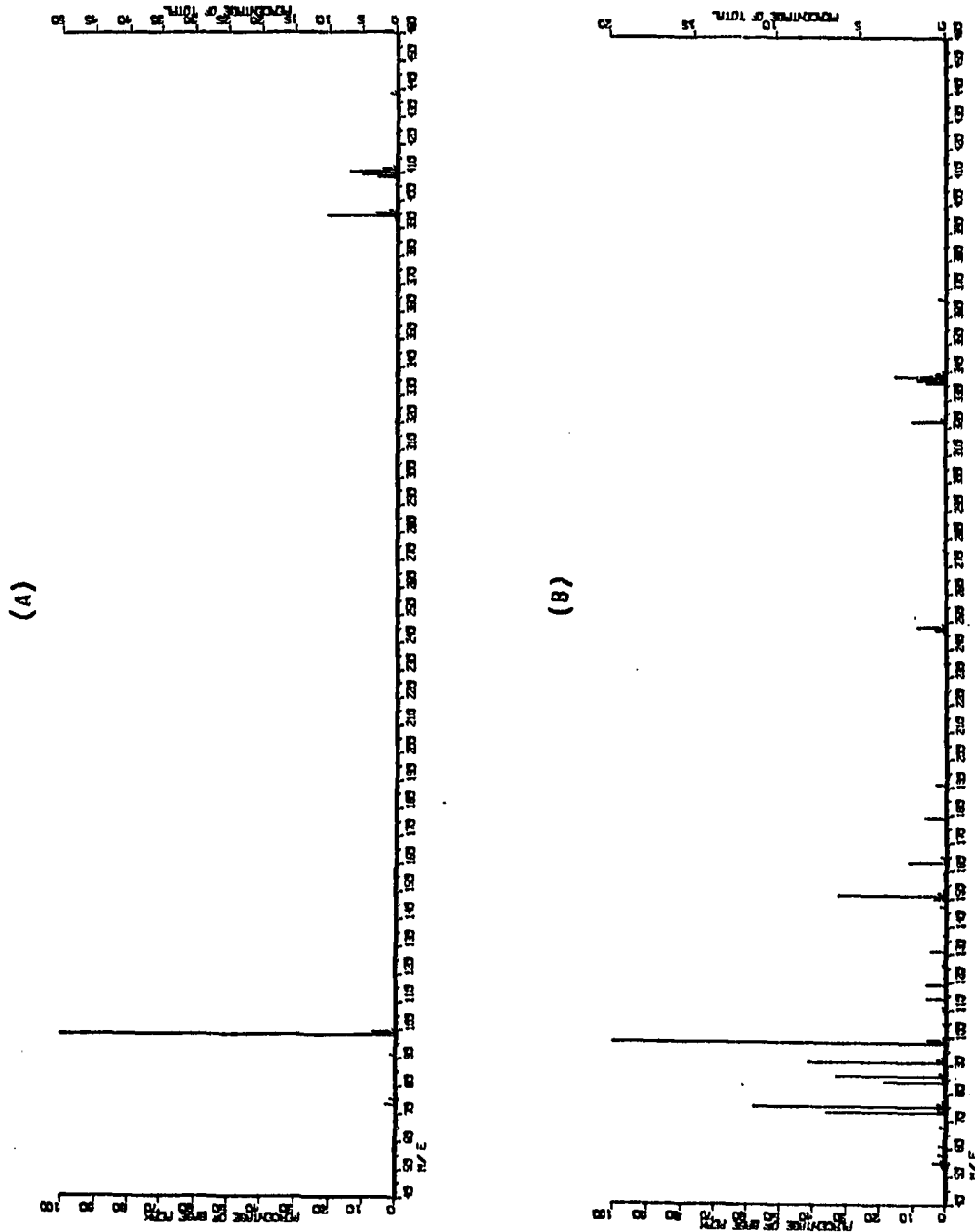
Fig. 7. (A) Chemical ionization mass spectrum of the reduced and silylated eugenol II (Fig. 6B): "di"-silyl derivative. (B) Chemical ionization mass spectrum of the reduced and silylated eugenol (Fig. 6A): "mono"-silyl derivative.

alcohol group reverts to the carbonyl group if not restricted by derivatization. Each of the "di"-silyl ether derivatives of the eugenol tertiary aminopropiophenones yield monosilylated species. As shown by the gas chromatogram prior to combined GLC–CIMS analysis, a relatively simple four-component mixture becomes very complex upon hydrolysis by atmospheric moisture. Each "di"-silyl derivative gives rise to at least two decomposition products prior to the formation of the silylated allylic ketone.

As illustrated with the reduced and silylated eugenol II, there are at least three components present (Fig. 6A) upon storage. The use of the limited mass search as
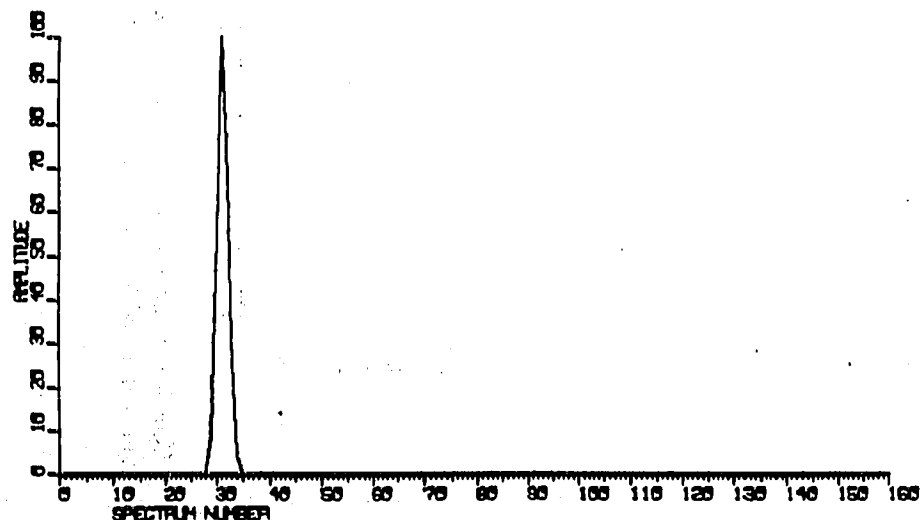
(A)
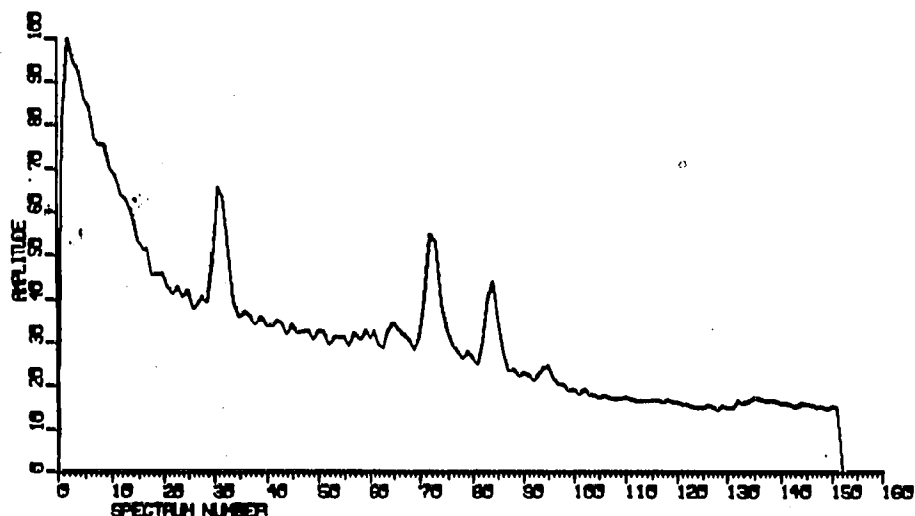
(B)

Fig. 8. (A) Reconstructed chromatogram of the reduced and Tri Sil 'Z' silylated eugenols I II and III. (B) Reconstructed chromatogram of 8A with limited mass search m/e 369–371.

reported for elemicin[4] is very helpful with mixtures of this nature. The silylated allylic ketone as located by limited mass search m/e 250–252 has a retention time of 3 min (spectra 8–25). The second decomposition or hydrolysis product with a retention time of 5 min 45 sec is the reduced tertiary aminopropiophenone with one silyl group (limited mass search, m/e 337–339). The initially desired "di"-silyl ether of eugenol II has a retention time of 7 min 15 sec as verified by the limited mass search m/e 409–411 (Fig. 6B). One other trace decomposition product of the "di"-silyl ether of eugenol II has a retention time of 8 min 15 sec (Fig. 6A, Spectra 101–109). Similar elution profiles were obtained for the reduced and silylated eugenol I and eugenol III upon storage.

Chemical ionization mass spectra of these decomposition products of eugenol II are given in Fig. 7. The spectrum of the silylated allylic ketone has already been described by Fig. 2A. The intact nitrogen-containing metabolites have a base peak m/e of 98. In addition (Fig. 7A), the "di"-silyl ether of the reduced eugenol II has a quasi-molecular ion $(M + 1)$ of m/e 410 with abundant m/e 424 $(M + CH_3)$, 438 $(M + C_2H_5^+)$, 394 $(M–CH_3)$, 73 and 91 and much less abundant 338, 320 and 251 fragments.

The monosilylation derivatives (Fig. 6A, spectra 70–79) have a quasimolecular ion $(M + 1)$ of m/e 338 with similar m/e 366 $(M + C_2H_5^+)$, 322 $(M – CH_3)$, 248, 151, 91 and 73 fragments (Fig. 7B).

The trace decomposition product (retention time 8 min 15 sec) has chemical ionization spectra as described for the monosilylated derivative (Fig. 7B).

As seen from the above chromatograms, the individual reduced "di"-silyl ether derivatives decomposed to the "mono"-silyl ethers and finally to the silylated allylic ketone.

Fig. 8 is the reconstructed chromatogram of the eugenol-reduced metabolite silylated with Tri-Sil 'Z'. The silylated allylic ketone is represented by spectra 1–17, the "di"-silyl ether of the N,N-dimethylamino derivative (I) by spectra 26–32, the "di"-silyl ether of the pyrrolidinyl derivative (III) by spectra 70–75, and the "di"-silyl ether of the piperidyl derivatives (II) by spectra 80–85. The chemical ionization mass spectra of silylation metabolites I and III are very similar to those reported for eugenol II (Fig. 7A) with the respective m/e 58 and 84 base peak and the appropriate quasi-molecular ion $(M + 1)$ and recombination fragments $(M + CH_3)$ and $(M + C_2H_5^+)$.

GLC–CIMS is a very useful method of structural verification and characterization of these polyfunction metabolites of eugenol, elemicin[4], and the earlier reported allylbenzene derivatives. Microgram quantities of the eugenol metabolite can be characterized satisfactorily after $NaBH_4$ reduction followed by silylation and GLC–CIMS.

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