Proline nitroxide had an absorption band in the ultraviolet at 235 nm with extinction coefficient $\epsilon = 1363$ characteristic of nitroxides (Forrester et al., 1968). N-Hydroxyproline has no band in this region. The infrared spectrum, in accord with the known structure, had a triplet at 1350 to 1370 cm^{-1}, characteristic of a nitroxide NO vibration (Forrester et al., 1968). An nmr spectrum (Tom, 1973) also showed no unexpected bands.

STABILITY

Proline nitroxide was unexpectedly stable. It appeared to keep indefinitely in the solid state; in solution the stability depended on temperature and pH. In phosphate buffer (0.1 M) at pH 7, the signal of a 6.9×10^{-3} M solution of proline nitroxide disappeared after 80, 40, and 20 hr at 24°, 37°, and 50°, yielding first-order rate constants of 7.0×10^{-4}, 1.5×10^{-3}, and 4.3×10^{-3} min^{-1}, respectively (Tom, 1973).

The strength of the epr signal decreased upon acidification but thereafter disappeared more slowly than from alkaline media (Figure 2). The 50% loss of signal strength seen when the pH was adjusted to pH 5.2 from pH 9.7 was regained if the solution was immediately adjusted back to the original pH.

Proline nitroxide is an effective lipid antioxidant despite its lack of ready oil solubility. By a weight gain method (Olcott and Einset, 1958), samples of purified squalene and menhaden oil containing 0.065% (1 μmol/200 mg) proline nitroxide had induction periods of 2 months and 1 month, respectively, at 24° compared to controls which were rancid in 1 day.

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A Microcolumn Apparatus for Rapid Cleanup of 2,4-Dinitrophenylmethylamine Extracts for Carbamate Pesticide Analysis

A microcolumn apparatus has been devised and tested for cleanup of the reaction product of methomyl (an N-methyl carbamate insecticide) and 1-fluoro-2,4-dinitrobenzene before gas chromatography. Microcolumns packed with silica gel G-HR gave consistently good cleanup and quantitative recovery of 2,4-dinitrophenylmethylamine. Details of the apparatus and the cleanup procedure are described.

Microcolumn chromatography has been studied for cleanup and separation of some organophosphorus and organochlorine pesticide residues before gas–liquid chromatography (glc) (Kadoum, 1967, 1968a,b; Law and Goerlitz, 1970; Leoni, 1971). The types of columns used were disposable pasteur pipets (Kadoum, 1967); chromaflex columns of 6, 7, and 9 mm i.d. and a macrochromatographic column of 350 mm × 22 mm i.d. (Kadoum, 1968b); "disPo" disposable transfer pipets (Law and Goerlitz, 1970); and 30 cm × 4.2 mm i.d. columns (Leoni, 1971). The columns were packed with silica gel 950 (Kadoum, 1967, 1968a; Leoni, 1971), silica gel 923 (Kadoum, 1968b), and silica gel or deactivated alumina (Holden and Marsden, 1969; Law and Goerlitz, 1970). Recoveries reported ranged from above 90 to 100%.

Microcolumn cleanup was also used in a method developed for methomyl determination (Mendoza and Shields, 1974). The method consisted of alkaline hydrolysis of methomyl to produce methylamine and reaction of this product with 1-fluoro-2,4-dinitrobenzene (DNFB). The reaction extracts gave a large glc peak which interfered with that of 2,4-dinitrophenylmethylamine (DNPMA), a reaction product of DNFB and methylamine. We found the silica gel G-HR microcolumn satisfactory for cleaning up the DNPMA extracts before the glc analysis. However, elution was slow and time consuming.

We therefore devised a microcolumn apparatus attached to a vacuum source and using disposable pasteur pipets. The microcolumn apparatus was found to be a time-saving and efficient device. The design and use of this apparatus are described in this report.

MATERIALS AND METHODS

Preparation of Microcolumns. A 9-in. pasteur pipet was plugged tightly with a wad of glass wool. It was then filled with MN silica gel G-HR powder (Macherey Nagel and Co., Düren, West Germany) up to a height of 5 cm. (This gel is normally used in thin-layer chromatography.)
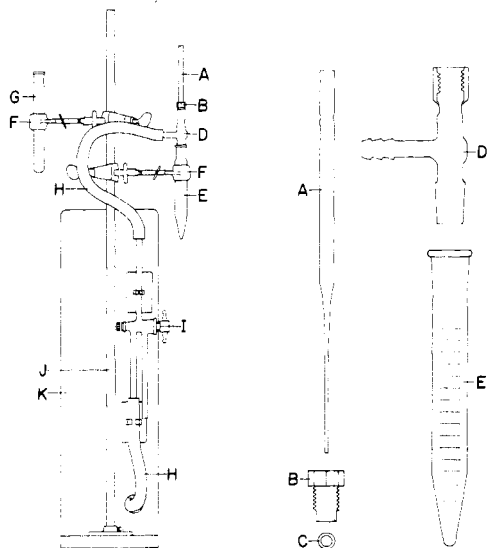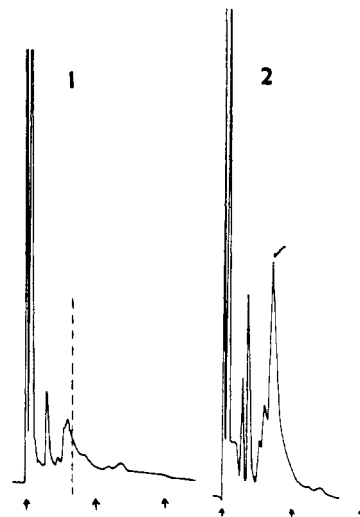
Figure 1. Microcolumn apparatus: (left) assembled; (right) a detailed view of the microcolumn and collecting tube; (A) 9-in. pasteur pipet; (B) nylon bushing; (C) Teflon back ferrule (0.25-in. i.d.); (D) modified feed tube adapter; (E) centrifuge tube; (F) clamp; (G) waste solvent or holding tube; (H) vacuum rubber hose; (I) three-way stopcock; (J) metal rod; (K) wooden back panel of the stand.
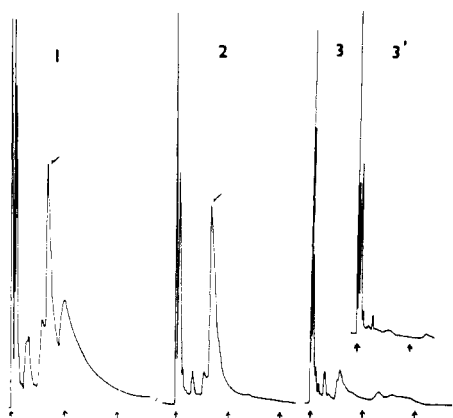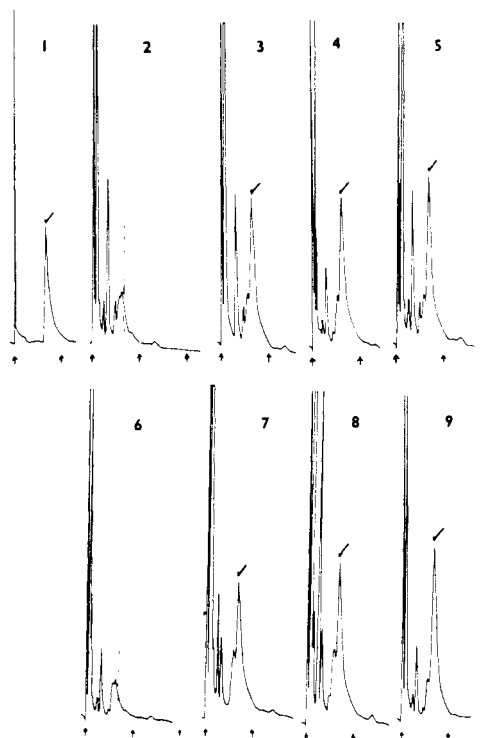

Figure 2. Typical chromatograms of extracts of DNPMA derived from methomyl and DNFB reaction: (1) without microcolumn cleanup; (2) with cleanup; (3) and (3') both blank reaction extracts with cleanup. Glc conditions are described in the text. Arrows at the bottom of each chromatogram indicate the time interval: 9 min between arrows.

Figure 3. Typical chromatograms of silica gel G-HR extracts (1) without methomyl and (2) with methomyl. Both extracts were cleaned up the microcolumn chromatography. The vertical broken line indicates where the DNPMA peak should appear. The time between arrows equals 9 min.

Figure 4. Typical chromatograms of methomyl that were detected by a tlc–ei technique, scraped off the plate, allowed to react with DNFB, and cleaned up by microcolumn chromatography: (1) standard DNPMA; (2) blank extracts obtained from a plate sprayed with indophenyl acetate (IPA); (3) IPA plus 5 μg of methomyl on a plate; (4) IPA on a plate, extracted and spiked with 5 μg of methomyl; (5) the same as 4 but spiked with 6.1 μg of DNPMA; (6) blank extracts from a plate sprayed with 5-bromoindoxyl acetate (5-BIA); (7) 5-BIA plus 5 μg of methomyl on a plate; (8) 5-BIA on a plate, extracted and spiked with 5 μg of methomyl; (9) the same as 8 but spiked with 6.1 μg of DNPMA.

The column was tapped to pack the dry powder and was assembled in the vacuum apparatus. It was rinsed with three 1.5-ml portions of benzene, or until the column gave a clean glc background, at a rate of 60–70 drops/min. The pipet was tapped gently to dislodge the gel adhering on the column and to further pack the gel to about 4.5 cm in height.

Microcolumn Cleanup of DNPMA Extracts. The DNPMA extract in benzene was concentrated in a centrifuge tube to about 0.5 ml under a gentle flow of nitrogen at 32°. The concentrate was transferred to the silica gel G-HR column. The centrifuge tube was rinsed with 0.5 ml of benzene; the rinse was combined with the extract already in the column. The extract was eluted under vacuum at a rate of 60–70 drops/min. The tube was rinsed again with three 1.5-ml portions of benzene. Each benzene fraction was transferred to the column when the meniscus reached the top of the gel. Elution was terminated after 11–15 min, when the meniscus of the last solvent fraction reached the top of the gel. Six samples could be processed at a time.

Microcolumn Chromatographic Apparatus. Figure 1 shows a diagrammatic illustration of a microcolumn apparatus used for cleanup. The vacuum source, which is not shown, may be a water pipe line or from a pump. The vacuum pressure was regulated by a three-way stopcock. A 0.25-in. Teflon back ferrule was used to seal and hold the pasteur pipet on the assembly.

Glc Analysis. A glc apparatus (Aerograph Hy-Fi Model

550) equipped with an electron capture detector was used. A glass column, 0.25 in. × 4 ft, was packed with Chromosorb W-HP (80–100 mesh) coated with 4% SE-30 and 6% QF-1. The column was used at 210–215°. The gas flow was adjusted to give DNPMA a retention time of about 9 min. Five-microliter samples of appropriate DNPMA or extract solutions were analyzed by glc. At attenuation 4, 1.5 ng of DNPMA gave about 0.5 fullscale deflection of the recorder pen. This amount was well within the linear detection range.

RESULTS AND DISCUSSION

The microcolumn apparatus was found invaluable in the glc method developed for methomyl residues in rapeseed oils (Mendoza and Shields, 1974). Consistently good cleanup and recovery of DNPMA were obtained from the microcolumn packed with silica gel G-HR. The apparatus is rapid and efficient in cleaning up the DNPMA extracts before glc analysis. It is routinely used in evaluating methods using DNFB and methylamine moieties of carbamate pesticides.

The following are illustrations of the application and performance of the microcolumn apparatus.

Figure 2 shows typical chromatograms of DNPMA extracts without (no. 1) and with microcolumn cleanup (no. 2) and reaction blank extracts (no. 3 and 3′) with cleanup. Accurate quantitation was not achieved when the peak which appeared after the DNPMA peak (marked with a check) was present. The peak on the front shoulder of the DNPMA peak did not interfere with quantitation unless its height was equal or higher than that of DNPMA. Chromatograms 2, 3, and 3′ show that a column cleanup procedure completely removed the broad interfering peak.

Figure 3 shows typical chromatograms of silica gel G-HR extracts without methomyl (no. 1) and with methomyl added (no. 2). The extracts were allowed to react with DNFB and cleaned up by microcolumn chromatography. Note that the gel did not give any interference.

In another study, methomyl was detected on tlc plates by a tlc-enzyme inhibition technique using indophenyl acetate on 5-bromoxindoxyl acetate and pig liver extracts. The site of inhibition was scraped off the plate, hydrolyzed, treated with DNFB, and cleaned up by microcolumn chromatography. Figure 4 illustrates that good cleanup and complete recovery of DNPMA were obtained after column chromatography. Good recovery of methomyl was achieved even after reaction with enzyme. Neither the enzyme, substrate, nor gel gave interfering glc peaks.

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Nitrite-Induced Inhibition of Purified Fractions of Chicken Muscle Cathepsin D

Isolation and purification of cathepsin D from chicken leg muscle indicated the presence of multiple forms of enzyme in the tissue. Two different fractions (A and B) of cathepsin D obtained during Sephadex G-100 gel filtration showed marked differences in their electrophoretic mobilities and response to treatment with sodium nitrite. At nitrite concentrations simulating those used in meat curing, only fraction A was inhibited, whereas fraction B was unaffected even at a higher concentration (1 mmol/0.8 mg of protein).

The potential risk of using sodium nitrite as a curing agent in meat processing is receiving considerable attention since, under favorable conditions, nitrite reacts with amines to form the nitrosamine group of carcinogenic and hepatotoxic compounds (Ender and Ceh, 1971; Wolff and Wasserman, 1972). Our current knowledge regarding the function of nitrite in meat curing is limited to its role in the enhancement of color (Giddey, 1966) and the inhibition of *Clostridium botulinum* (Emodi and Lechowich, 1969). However, besides bacterial spoilage, deterioration in quality during storage of meat products occurs by the action of hydrolytic enzymes in the tissue, thereby necessitating inactivation of the hydrolases during meat curing processes (Bandack-Yuri and Rose, 1961; Martins and Whitaker, 1968). The mode of action of nitrite on skeletal muscle lysosomal enzymes is, therefore, being carefully scrutinized in our laboratory.

Our results (Warrier et al., 1973; Harikumar et al., 1974) have demonstrated that enzymes associated with tissue autolysis, viz., cathepsin (EC 3.4.4.23), arylsulfatase (EC 3.1.6.1), and β-glucuronidase (EC 3.2.1.31), are readily inhibited by nitrite treatment at concentrations varying from 5 to 50 μmol. A combination of nitrite and mild heat treatment was found to be more effective than either of the single treatments in suppressing the activities of these enzymes. Among these the inhibition of cathepsin D merits special attention since it is known to be the major proteinase in muscle tissues (Weinstock and Iodice, 1969; Caldwell and Grosjean, 1971; Harikumar et al., 1974). The compounds which are known to inhibit cathepsin D, viz., tetranitromethane (Keilova, 1971), methyl esters of diazoacetylphenylalanine (Barrett, 1971), diazoacetylnorleucine (Smith et al., 1969), and heavy metal ions (Misaka and Tappel, 1971), have limited scope as food