Gas Chromatography of the Fat-Soluble Vitamins: A Review

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ABSTRACT

The application of gas liquid chromatography (GLC) as an analytical tool for the determination of the fat-soluble vitamins A, D, E and K has yet to be utilized to its full potential. A review of the published work of many researchers in this field is presented. GLC methods to measure the vitamin A isomers have not been developed to any appreciable practical extent. Liquid liquid chromatography might well be the technique of choice. In the field of vitamin D there are indications that a practical GLC analysis is feasible for pharmaceutical preparations. The GLC applications for vitamin E are diverse, well defined and generally widely accepted in research laboratory situations and for regulatory and quality control usage. Vitamins K_1 and K_2 have been measured with limited success in a few research laboratories, but the GLC methods have not developed on a practical basis. However GLC is used for measuring vitamin K_3 (menadione and menadione sodium bisulfite) on a fairly routine basis in quality control laboratories.

INTRODUCTION

Gas liquid chromatography (GLC) is a powerful analytical tool, but like all analytical instrumentation, it must be regarded as another means to be used in situations where it is superior to other instruments. In short, it must not be accepted *carte blanche* for every analytical problem.

Many research groups have attempted, with varying degrees of success, to use GLC as a means of identifying and quantitatively measuring the fat-soluble vitamins A, D, E and K. GLC has been most successfully applied on a practical basis to vitamin E. The overall success of GLC for analysis of vitamin A has been the least satisfactory of all the vitamins of the fat-soluble series.

Two papers appeared in December 1960 that signaled the advent of GLC as a possible means of determining the fat-soluble vitamins. Nicolaides (1) observed that α-tocopherol could be resolved partially from γ-tocopherol on a 5% silicone-gum rubber column packing during a study to determine the possible use of silicone-gum rubber as an immobile phase for GLC separation of lipids. Simultaneously Ziffer et al. (2) published their work aimed specifically at the GLC of vitamin D.

Ironically these early workers did not follow up their early advantage and leadership. It was left to others to develop GLC methods and applications for the fat-soluble vitamins. Eleven years after the initial reports, much remains to be done in completing the development of entirely suitable GLC applications to the fat-soluble vitamins. Despite this, however, there are areas of application where tremendous success has been achieved.

VITAMIN A

The term vitamin A, as used in this discussion, includes retinol, retinene, retinoic acid, retinyl acetate and retinyl palmitate. During 1963, Sheppard and Libby (unpublished results, 1963) attempted to develop a GLC method for the aforementioned vitamin A compounds. Stationary phases of 2.5% and 5% XE-60 and 1% and 5% SE-30 were tried together with column temperatures ranging from 170 C to 215 C. The palmitate derivative produced a single peak on the 5% SE-30 column at 215 C, but at ca. 200 C two peaks occurred with a shoulder appearing on the trailing edge of the second peak. Retinol produced multiple peaks on both SE-30 and XE-60 immobile phases, irrespective of per cent coating and temperature. The acetate derivative gave two well defined symmetrical peaks at 170 C on the 1% SE-30 column and produced a single peak exhibiting a trailing edge on the back side of the peak, typical of an absorption isotherm, on the 2.5% XE-60 column.

Japanese workers (3) reported in 1963 that retinol, retinyl acetate and retinyl palmitate undergo dehydration to form anhydro retinol during conventional GLC operating conditions. Their conclusions were supported adequately by IR, UV and thin layer analysis of collected fractions from GLC. Carroll and Herting (4) attempted to chromatograph all-*trans* retinol with little success. The all-*trans* retinol produced a large broad-based response, with several well defined apexes indicative of severe alteration of the parent compound, during the GLC analysis on a 3% SE-30 column maintained at 181 C.

Dunagin and Olson (5) confirmed the finding of Ninomiya et al. (3), who analyzed collected GLC fractions by UV analysis, that retinol and retinyl acetate were completely converted to anhydro retinol. This is no doubt the reason why previous workers had observed that the various analogs of vitamin A yielded multiple peaks during attempts to chromatograph them. However Dunagin and Olson developed the first technique to overcome the decomposition of vitamin A to anhydro retinol. To chromatograph retinol or retinyl acetate with a minimum of dehydration, solutions of β-carotene or hydroquinone were injected onto the column and the treated columns were conditioned at 250 C for several hours. Both reagents worked well as protective agents on glass bead columns, but only the β-carotene worked on the Gas-Chrom P columns. The β-carotene treatment was generally adequate for 3 days, whereas the hydroquinone lost its protective activity within 2-3 hr. Glass columns, 35 cm x 4 mm ID, packed with 0.1% or 1.0% SE-30 coated on 60-80 mesh Gas-Chrom P were used. Retinol and retinyl acetate were completely resolved (Fig. 1) with ca. 6% decomposition, which appeared as two minor peaks occurring within 2 min after sample injection with the following operating parameters: column, 150 C; detector, 165 C; injector, 230-250 C; flow rate, 880 ml/min. The method was usable down to <0.05-0.2 μg of retinol or its derivatives with a β-argon ionization detector, or both. Following the work of Dunagin and Olson, the efforts by American researchers to develop GLC methods for vitamin A have subsided.

Walle et al. (6) observed that if vitamin A was converted to trimethylsilyl ether, it would not interfere with GLC determination of calciferol in preparations containing vitamin A. Macek and Vanecek (7) developed and applied a GLC scheme to follow the production of the possible intermediates in the synthesis of pseudoionone, isophytol and vitamin A. A 20% SE-30 column was used isothermally at 170 C or temperature-programed from 160 to 190 C to determine all the intermediates except those formed during the last stages in the synthesis of vitamin A.

Vecchi and coworkers (8) made practical use of GLC in measuring vitamin A forms by using the trimethylsilyl ethers of vitamin A and its isomers as a means of monitoring a synthesis process on a manufacturing scale on 5% silicon QF-1 columns. Their operating parameters were as follows: column, glass, 2 m x 2.3 mm ID at 160 C; flash heater, 245-250 C; carrier gas, helium, 70 ml/min; column

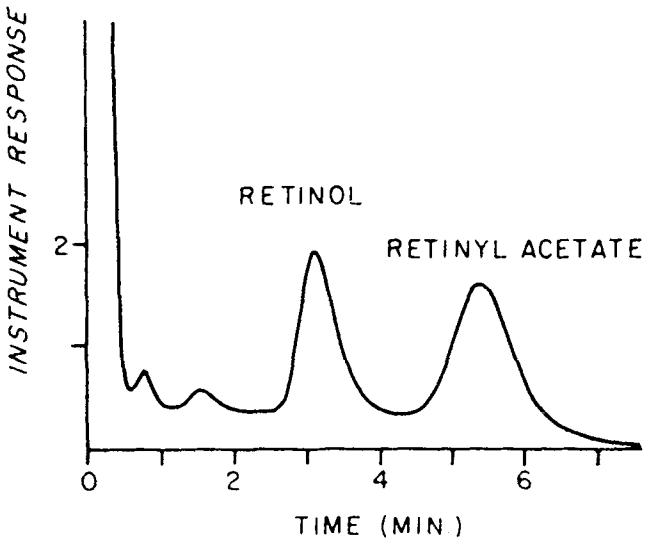


FIG. 1. Separation of retinol and retinyl acetate on β-carotene treated Gas-Chrom P (5).

packing, 5% silicon QF-1 coated on 80-100 mesh Gas-Chrom Q; detector, flame ionization presumably operated at approximately the same temperature as the flash heater. They were able to achieve complete resolution of 13-*cis*-vitamin A and all-*trans* vitamin A. The relative retention times based on all-*trans* vitamin A as 1.00 were as follows: 13-*cis*-vitamin A, 0.64; 11, 13-di-*cis*-vitamin A, 0.28; *retro*-vitamin A, 0.72 and 1.00. The analysis time was 25 min. The identification of the various GLC fractions was verified by mass spectrometry. The reproducibility of the method, based on 95% statistical certainty, was found to be 3.5% for all-*trans*-vitamin A and 13-*cis*-vitamin A and 3% for *retro*-vitamin A.

Hata and Hata (9) developed a GLC method to assay the vitamin A content of fish livers. The extracts were dissolved in benzene, and crystalline *p*-toluenesulfonic acid was added as a catalyst. The released fatty acids were esterified with diazomethane and determined by GLC. It was established that the catalyst did not alter the freed vitamin A fatty acids and that the wax esters and cholesterol esters did not release any fatty acids in the presence of the catalyst. Anhydrovitamin A was formed when the catalyst was used to release the fatty acids. The liver oil of bonito *(Katsuwonus pelamis)* was analyzed as an example. Based on the fatty acid analysis, the quantity of vitamin A present was calculated.

Shapira et al. (10) used GLC to study the intermediates in vitamin A production. At least one of the intermediates, *cis*-3-methyl-2-penten-4-yn-1-ol, underwent partial decomposition at temperatures exceeding 120 C. Their analyses for impurities in the intermediates were carried out on 12% polyethylene glycol 4000 or 12% polyethylene glycol adipate coated on 80-100 mesh Celite 545. These investigators did not devote any efforts toward the GLC analysis of vitamin A itself. A similar type of application previously was reported by Macek et al. (11).

Janecke and Voege (12) are often quoted in literature reviews regarding their studies of vitamin A. The cited report is a very short communication containing no research data per se, and indicates only that the fat-soluble vitamins can be determined on a 3% SE-30 column temperature programed from 100 to 270 C and on a 2.5% SE-30 column at 250 C.

VITAMIN D

The provitamins, the inactive precursors of the D vitamins, are cyclopentanophenanthrene derivatives. The main derivatives that occur in nature are ergosterol (provitamin D_2) and 7-dehydrocholesterol (provitamin D_3). Ergosterol is found in plants and 7-dehydrocholesterol in animals. This discussion is limited to the irradiation products of the provitamins, i.e., vitamins D_2 and D_3. Vitamin D_2 encompasses the terms calciferol, activated ergosterol, irradiated ergocalciferol and viosterol; vitamin D_3 is synonymous with cholecalciferol and activated 7-dehydrocholesterol. These terms are broadly used throughout the literature.

Interest in the development of GLC methods for the D vitamins has been caused by: (a) the inability of existing chemical methods to distinguish between vitamins D_2 and D_3; (b) the laborious clean-up procedures necessary to eliminate interferences from vitamin A and other substances during chemical analysis; (c) inability of the rat bioassay to distinguish between vitamins D_2 and D_3; and (d) the cost, labor and time required for bioassays.

Ziffer and coworkers (2) in 1960 were the first to report studies of the GLC behavior of the D vitamins. They used 0.75% SE-30 and 0.75% neopentyl glycol succinate (NPGS) immobile phases coated on 100-140 mesh Gas-Chrom P. The SE-30 columns (6 ft x 4 mm ID) were operated at 222 C and the NPGS columns (6 ft x 3 mm ID) at 210 C. Vitamins D_2 and D_3 yielded two GLC peaks each. UV and IR spectra of samples collected from the columns indicated that cyclization had occurred and that "pyro" and "isopyro" compounds were formed. They also observed that the "pyro" and "isopyro" compounds of the two vitamins do not occur naturally and that the relative retention times are quite distinct from those of the provitamins; thus GLC might be useful in studying irradiation mixtures. The relative retention time data reveals that, if both vitamins D_2 and D_3 were present in a sample, peaks would overlap and quantitation would be difficult under their GLC conditions. The influence of injector temperature on cyclization was investigated, and it was found that the ratio of products is only slightly dependent on injector temperature.

In 1963 Ninomiya et al. (3) reported their GLC experiences with vitamin D. The three columns evaluated were 1% Craig's polyester succinate on 80-100 mesh Anakrom A (silanized), 1% SE-30 on 80-100 mesh Anakrom A (silanized) and 1% (assumed) FS-1265 (QF-1) on 80-100 mesh Chromosorb W or Anakrom A. The glass columns were 200 cm x 0.6 cm ID. The formation of the "pyro" and "isopyro" cyclization compounds of vitamins D_2 and D_3 observed during GLC analysis by Ziffer et al. (2) was verified. It is evident from the retention time data with the three immobile phases that, at column temperatures sufficient to elute usable peaks (about 200 C or higher), vitamins D_2 and D_3 in a mixture would not be sufficiently resolved for quantitative analysis. On every column used α-tocopherol would interfere, and with the Craig's polyester succinate column α-tocopheryl acetate would also interfere with vitamin D analysis. It was evident that vitamin A eluted at temperatures significantly lower than vitamin D, especially on the Craig's polyester succinate and FS-1265 columns, and thus should not interfere with vitamin D analysis at 200 C or higher, even with the SE-30 column.

Concurrently in our laboratory (unpublished results, 1963-63), we were attempting to develop a GLC method for vitamins D_2 and D_3. Using 3 and 5% SE-30 on 100-120 mesh Gas-Chrom P (silanized) packed in glass columns (6 and 8 ft x 4 mm ID), we were never able to achieve a complete resolution of the two vitamin D forms. The isopyrovitamin D_3 and the pyrovitamin D_2 peaks overlapped sufficiently to circumvent proper quantitative analysis. We were able to duplicate the results of Ziffer et al. (2) using the 0.75% SE-30 packing, but we were never able to duplicate their efforts with 0.75% neopentyl glycol succinate columns.

Vessman and Ahlen (13) were first to report using a derivative (trimethylsilyl ether [TMS]) of vitamin D for

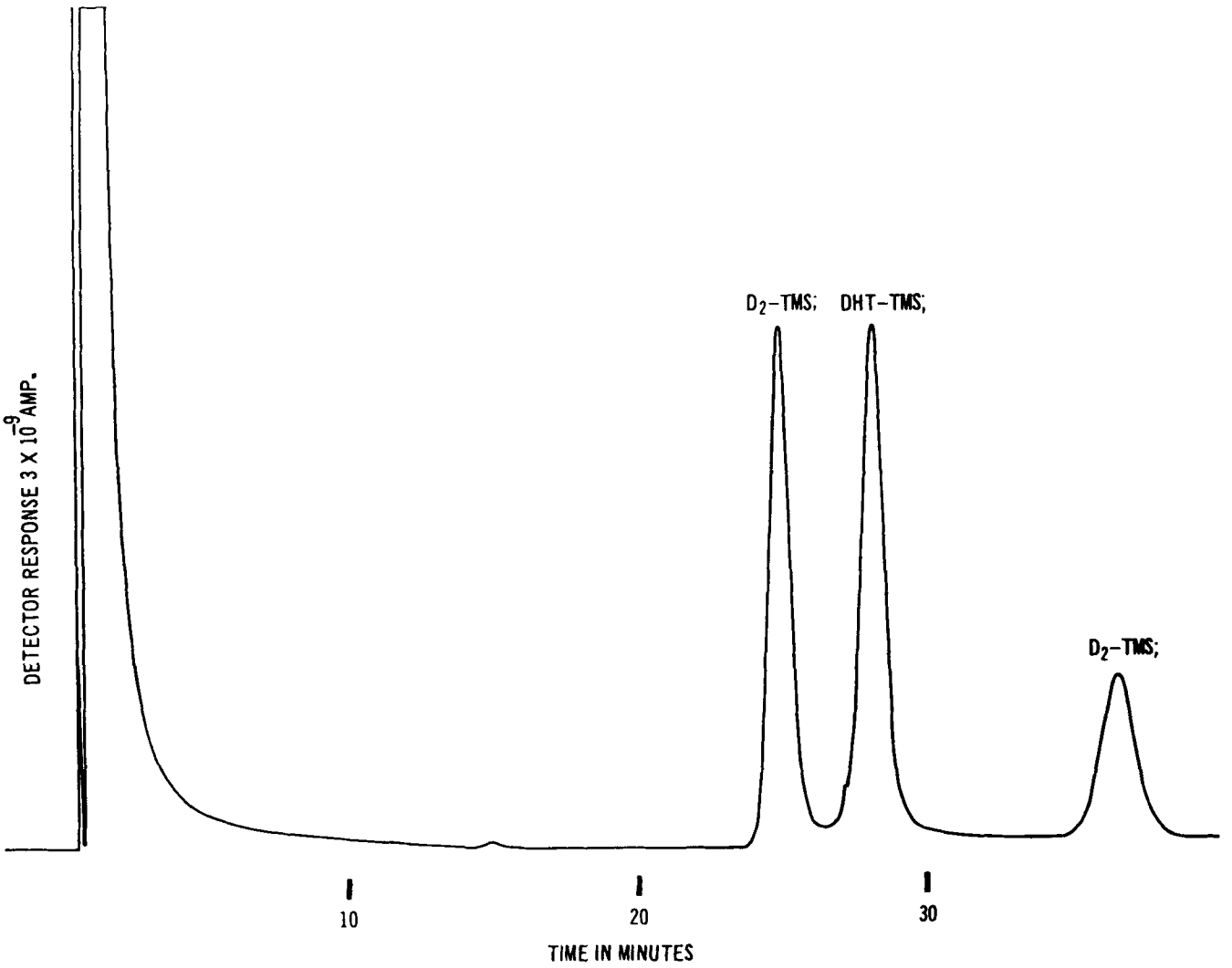


FIG. 2. Gas chromatographic separation of vitamin D_2 and dihydrotachysterol as trimethylsilyl ethers (14).

GLC analysis. They were also first to report efforts to exploit GLC specifically for quantitative analysis of vitamin D. They had concluded from the work of Ninomiya et al. (3) that on certain immobile phases vitamin A eluted at temperatures significantly lower than vitamin D, and they selected their immobile phases accordingly. They tried SE-30 and butanediol succinate (Craig's polyester succinate) stationary phases. The SE-30 immobile phase gave unsatisfactory resolution of pyro- and isopyrovitamin D_2 derivatives, but the polyester phase performed well, and they selected 3.4% butanediol succinate on Gas-Chrom P (glass column, 150 cm x 1/8 in. OD) operated at 210 C for the quantitative analysis of vitamin D_2. Cholesterol was used as an internal standard. The TMS derivative was formed, and the relationship between the area of the cholesteryl-TMS peak and that of the pyrovitamin D_2 derivative peak was used for quantitative calculations. Vitamin A palmitate eluted either with or in close proximity to the solvent peak. The TMS ether of cholesterol appeared intermediate between pyro- and isopyrovitamin D_2. However the resolution between the cyclization products and cholesteryl-TMS ether was sufficient for adequate quantitative analysis. From an inspection of their retention data, it is apparent that vitamin D_3 could have been measured effectively by the method, but the quantitative analysis would be difficult or impossible with mixtures of D_2 and D_3. If the ratio of vitamin A palmitate to vitamin D_2 was 30 or higher, the palmitate produced tailing that made measurements of the pyrovitamin D_2-TMS ether peak area more difficult. When 3 μg of vitamin D_2 per injection was used, a standard deviation of 1.5% was achieved and lower amounts gave diminished precision.

Nair et al. (14) reported the first successful attempt to measure the D vitamins in biological tissue by GLC. The provitamins and cholesterol were precipitated as the digitonides, and column chromatography was used for further purifying the extracts before GLC analysis. Studies were carried out in which the vitamin D was measured unreacted, as the trifluoroacetate (TFA), and as the trimethylsilyl ether (TMS). The GLC procedures were worked out for SE-52 and SE-52/β-cyanoethylmethylpolysiloxane (949) coated on the same inert support. Glass columns 6 ft x 5 mm ID were used routinely. The SE-52 columns were maintained at 213 C and the biphase columns at 225 C. It was reported that the structural alterations during GLC analysis of vitamins D_2 and D_3 are not affected by changes in temperature of the flash heater. GLC recoveries of 80-105% were obtained from blood and liver samples with the biphase column. Dihydrocholesterol and its TFA and TMS derivatives did not undergo cyclization upon GLC analysis. Typical chromatograms of TMS derivatives and the biphase column reproduced from their report are shown in Figures 2 and 3. The method was used routinely with 1-2 μg of vitamin injected onto the column. Much smaller samples could be analyzed at higher instrument sensitivity settings than those used routinely.

Murray et al. (15) recorded in a preliminary report the first attempt to avoid the formation of cyclization products of the D vitamins during GLC analysis by treating the

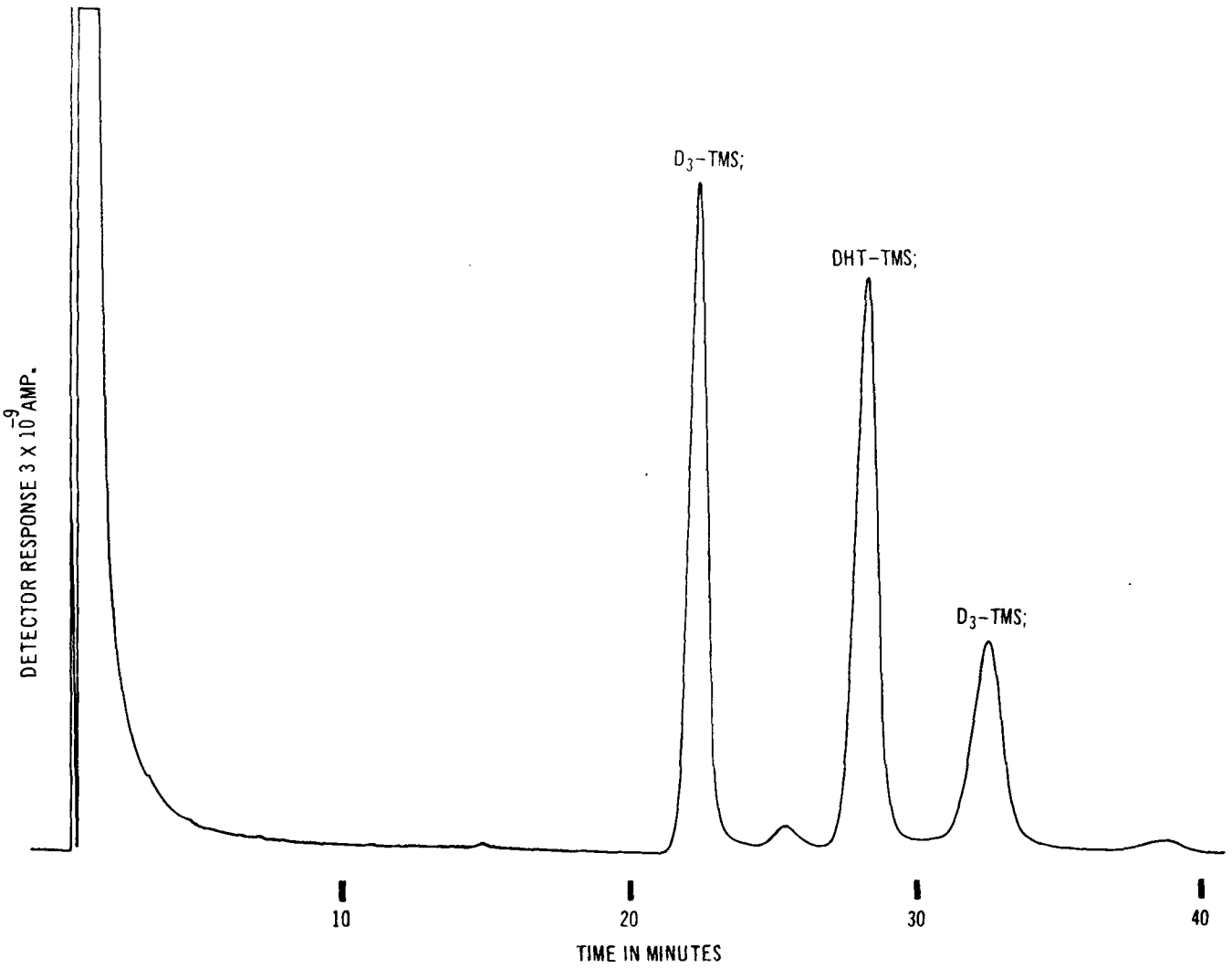


FIG. 3. Gas chromatographic separation of vitamin D_3 and dihydrotachysterol as trimethylsilyl ethers (14).

vitamin with antimony trichloride. The resulting compound was found to be stable during GLC, and it appeared to be isovitamin D based on UV spectra. Quantities down to 0.20 μg could be measured. GLC recoveries of 101% ± 2% were achieved for vitamin D_2 added to livers of deficient rats. A 4 ft glass column packed with 3% silicone oil on Celite maintained at 230 C and a flow rate of 60 ml/min was used. The sensitivity of this method is somewhat better than that reported by Nair et al. (14). Vitamin D_2 was used as the internal standard for vitamin D_3, and vice versa.

In 1966 Murray et al. (16) expanded their 1965 preliminary report (15) and included considerable detail about the sample extract clean-up procedures required. Two thin layer chromatographic steps—conversion of vitamin A to anhydrovitamin A and precipitation of sterols—were needed in addition to the key reaction of vitamin D with antimony trichloride. UV spectral data were presented in support of their contention that the provitamin was formed by the reaction of the D vitamins with antimony trichloride. The GLC resolution of the reacted vitamin D_2 and D_3 was not complete but was sufficient for analytical measurement.

Nair (17) reviewed the work of Ziffer et al. (2) in depth and showed the relationship among their studies, work in his own laboratory (14,18) and the chemistry of cyclization and derivative chemistry. This excellent review treats the specific aspects of the earlier research just cited in greater depth than does the present review. Nair and Bucana (19) successfully studied the intracellular distribution of vitamin D in rat liver, using the GLC method employing the trifluoroacetate and trimethylsilyl ether derivatives as discussed previously (14,17).

The study of Vessman and Ahlen (13) was expanded by Walle et al. (20) to include a closer study of vitamin D_2 rearrangement, which was found to be influenced by the presence of vitamin A among other factors. Stainless steel columns, 150 cm x 1/8 in. OD, packed with 1-3% butanediol succinate polyester on Gas-Chrom P (100-120 mesh) were operated isothermally at 200 C and temperature-programed at 2 C/min from 160 to 200 C, after which the temperature was maintained isothermally at 200 C. It was found that the separation of vitamin D derivatives was improved if vitamin A was present as the trimethylsilyl ether. The tailing of vitamin A palmitate was a problem during both isothermal and temperature programed analysis, whereas it was greatly reduced with the TMS derivative. The separation of the pyro- and isopyrovitamin D TMS ether derivatives from vitamin A TMS ether could be performed isothermally, but when the concentration ratio of vitamin A to vitamin D_2 exceeded 90, better precision was obtained by temperature programing. It was also found that measurements based on the sum of the pyro- and isopyrovitamin D_2 peaks gave somewhat lower values than measurements of the pyrovitamin D_2 peak alone. It was observed that, if large amounts of vitamin A were present in the test sample, rearrangement of vitamin D_2 was facilitated. Carrier gases, such as helium, which has a large thermal conductivity, gave more complete rearrangement than either argon or nitrogen, which have lower thermal conductivity. It was postulated that the differences ob-

tained with glass and metal GLC apparatus might be due partially to the different thermal conductivity of these materials. Only metal injectors gave a complete thermal rearrangement.

Avioli and Lee (21) described a GLC method capable of detecting 5 ng quantities from human serum. Sample extract clean-up procedures included precipitation of the sterols as digitonides and silicic acid column chromatography. No derivatives of vitamin D were used. The overall recovery of vitamin D added to serum by the clean-up procedures was 99.7% ± 2.4%. Sample size had little effect on the ratio of the artificial thermal products, thus confirming the observations of Nair et al. (14). Glass columns (4 ft x 3/16 in. OD) packed with 3.8% SE-30 on 80-100 mesh Diatoport S maintained at 215 C were used. The retention times relative to cholestane were: (a) pyrovitamin D_3, 1.68; (b) isopyrovitamin D_3 1.91; (c) pyrovitamin D_2, 1.87; and (d) isopyrovitamin D_2, 2.21. The sensitivity of this method was reported to be about a 40-fold increase over that reported by Murray et al. (16).

De Ritter (22) briefly discussed the work of Nair et al. (14) and the early work of Murray et al. (16) in his review of newer analytical techniques for vitamins during 1967.

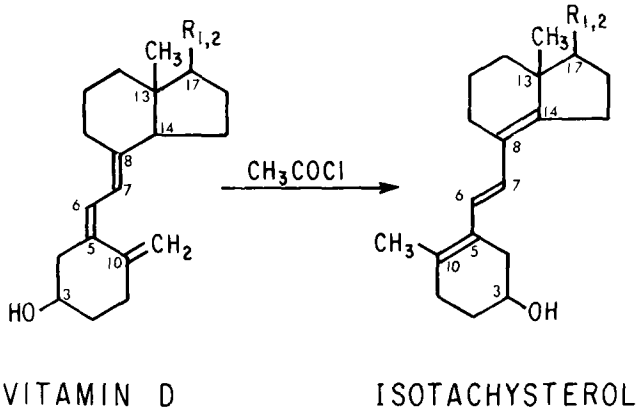
The mechanism of thermal cyclization was investigated by Nair and deLeon (23). It was found that the 5,6-*cis* configuration favors the formation of thermal isomers during GLC, whereas the 5,6-*trans* configuration does not. However, when the vitamin D-trifluoroacetate (TFA) derivative was dissolved in *n*-hexane, I_2 added, and the solution irradiated with UV light (black light), the 5,6-*trans*-vitamin D-TFA configuration was produced. Another interesting aspect of this study was the observation that inclusion of ubiquinone in the stationary phase produced a distinct improvement in the shape of the GLC peaks. It was concluded that column sites having a high affinity for double bonds are apparently saturated. The biphase column used in the previous studies of Nair et al. (14) was used in this study, except that 2.0 mg of ubiquinone$_{50}$ per 25 g of inert support was added.

Blondin et al. (24) observed that the 5,8-peroxides of ergosterol and 7-dehydrocholesterol gave a characteristic pattern of pyrolysis products in contrast to other sterols, which gave single peaks on 0.92% XE-60 columns. The 6 ft column was packed with 0.92% XE-60 on 90-100 mesh Anakrom AB maintained isothermally at ca. 230 C, when not temperature-programed.

Kobayashi (25-29) wrote a series of papers that have an important bearing on the theory and use of chemical isomerization of vitamin D_2 as a means of preventing thermal cyclization during GLC analysis. It was clearly established that vitamin D_2 in ethylene dichloride was readily isomerized to isotachysterol$_2$ by acetyl chloride (29). A GLC method was developed for the identification of vitamin D_2, 5,6-*trans*-vitamin D_2, isovitamin D_2, isotachysterol$_2$, tachysterol$_2$, lumisterol$_2$ and ergosterol (27,28). The column was stainless steel (75 cm x 0.4 cm OD) packed with 1.5% SE-30 coated on 60-80 mesh Gas-Chrom P (silanized) maintained at 220 C and a flow rate of 90-120 ml/min. The relative times for vitamin D_2, 5,6-*trans*-vitamin D_2, isocalciferol, isotachysterol$_2$, tachysterol, lumisterol$_2$, ergosterol and squalene were 1.59/1.86, 2.45, 2.45, 2.84, 2.77, 1.68, 2.05 and 1.0, respectively.

We were able to verify the work of Kobayashi and extended our studies to include vitamin D_3 in addition to vitamin D_2 (30). The isomerization reaction is shown in Figure 4. The column was glass, 8 ft x 4 mm ID, packed with 3% JXR on 100-120 mesh Gas-Chrom Q maintained at 265 C and a flow rate of 32 ml/min. Typical separations obtained are shown in Figure 5. With the flame ionization detector, we have been able to achieve peaks large enough for quantitative analysis with 0.15 μg quantities.

Nicolaides et al. (31) have found dihydrocholesterol



VITAMIN D ISOTACHYSTEROL

R_1 : $-CH(CH_3)-CH=CH-CH(CH_3)-CH(CH_3)_2$

R_2 : $-CH(CH_3)-(CH_2)_3-CH(CH_3)_2$

FIG. 4. Isomerization of vitamin D to isotachysterol; R_1: $CH(CH_3)$-CH=CH-$CH(CH)_3$-$CH(CH_3)_2$ and R_2: $CH(CH_3)$-$(CH_2)_3$-$CH(CH_3)_2$ (30).

present in the sterol ester fraction of human skin surface lipid. Its metabolic significance has not been elucidated. A 3% JXR column was used in the identification study.

Stereochemical studies on the microquantitative detection have been made by Nair and deLeon (32), extending their previous study (23). It was confirmed that the 9,10-secosterols having a 5,6-*cis* configuration contribute to the structural features favoring cyclization at elevated GLC temperatures. In the previous study it was established that the 5,6-*trans*-vitamin D was obtained by the iodine-catalyzed photoisomerization of vitamin D (5,6-*cis*) and did not exhibit any evidence of thermal cyclization under GLC conditions. However in the present study it was found that 5,6-*trans*-vitamin D undergoes double-bond rearrangement to the corresponding isotachysterol at high GLC temperatures. Thus the 5,6-*trans*-vitamin D analysis is in reality an isotachysterol analysis. The isotachysterols are stable under GLC temperatures, a fact established previously by Kobayashi (25,26) and in our own laboratory (30). However Nair and deLeon went a step further and formed isotachysteryl$_2$ and isotachysteryl$_3$ heptafluorobutyrates. A microquantitative method for the isotachysteryl esters was developed in which acyl or perfluoroacyl chlorides and vitamin D were used in the presence of ethylene dichloride as a solvent. Nanogram amounts of the vitamins D were detected as their isotachysteryl heptafluorobutyrates with a ^{63}Ni high temperature electron capture detector and a biphase column of 8% SE-52, 8% XE-60, and 4% coenzyme Q_{10} coated on 80-100 mesh Gas-Chrom P and maintained at 215 C was used. The glass columns were 6 ft x 5 mm ID. Coenzyme Q_{10} was included in the preparation of the column packing because highly symmetrical peaks could be obtained similar to those from older columns subjected to biological extracts. This effect was attributed to coenzyme Q_{10} which is retained on the column for an indefinite period, thus saturating sites having a high affinity for similar compounds possessing double bonds.

Blunt et al. (33) have used GLC analysis as one link in the identification of a biologically active metabolite of vitamin D_3 as 25-hydroxy-cholecalciferol. The column was glass, 4 ft x 0.25 in., packed with 3% W-98 on 80-100 mesh Diatoport S maintained at 240 C and a flow rate of 70 ml/min. The metabolite peak appeared in ca. 16 min. Vitamin D_3 occurred as two peaks at ca. 10 and 12 min.

Murray et al. (34) have adapted the method of Murray and coworkers (16) for the assay of vitamin D in multivitamin preparations. Column and thin layer chro-

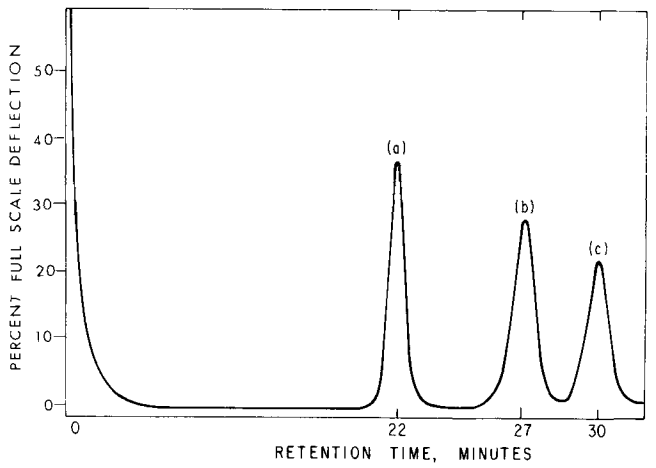


FIG. 5. Gas chromatogram of 2 μg each of (a) dihydrotachysterol D_2; (b) isotachysterol D_3; and (c) isotachysterol D_2 (30).

matographic procedures are used to remove vitamin A and other interfering compounds from the extracts. Recoveries of vitamin D_3 by the method were 100.5 ± 5.2% when vitamin A was not present in the vitamin preparation and 99.5 ± 5.2% in the presence of vitamin A. Vitamin A could be tolerated in the final test solutions in a ratio of 1:1 with vitamin D. When the method was used for the assay of multivitamin preparations, the coefficient of variation was 3.2%. Figure 6 is their published chromatogram representing 0.5 μg each of vitamins D_2 and D_3.

Wilson et al. (35) published GLC results utilizing the isotachysterol heptafluorobutyrate esters, as did Nair and deLeon (32), using a ^{63}Ni electron capture detector. The esters were prepared from heptafluorobutyric anhydride. The GLC column was not a biphase column as used by Nair and deLeon but rather a monophase column of 3.8% methyl silicone gum rubber W-98 on 80-100 mesh Diatoport S. The glass columns were 4 ft x 3 mm ID, maintained at 220 C and a flow rate of 60 ml/min. The increased sensitivity reported for the heptafluorobutyrate esters allowed for the estimation of at least 10 ng of vitamins D_2 and D_3. It appears that the usable sensitivity of the methods of Nair and deLeon (32) and Wilson and coworkers (35) using the isotachysteryl heptafluorobutyrate esters are similar: between 1 and 10 ng with a ^{63}Ni electron capture detector.

Petrova (36) reviewed the literature on vitamin D analysis in pharmaceutical preparations. The review includes chemical, biological and chromatographic methods. The chromatographic methods include paper, thin layer and GLC.

Edlund and Anfinsen (37) used GLC to determine vitamin D_2 in multiple-vitamin tablets and raw materials. Sample preparation was found to require saponification before extraction. Regisil (Regis Chemical Co.), Regisil-TMCS and trimethylsilylimidazole were about equally effective as silylating agents. The method utilizes an "internal-external" standard ratio procedure. The method is not effective for multiple vitamin preparations containing vitamin E or other materials that elute major peaks with the same retention time as silylated pyrovitamin D_2 and D_3 peaks. As the method is currently constituted, the multiple vitamin placebo must be available for preparation of the standard solution, unless the contribution of the placebo to the analysis has been previously established. The column used was glass, 6 ft x 0.08 in. packed with 3% OV-210 on 80-100 mesh Chromosorb W maintained at 250 C. A number of raw materials and tablets were analyzed by both the GLC and USP chemical methods. The GLC method had greater speed, precision and accuracy than the chemical method.

Canadian Food and Drug Directorate workers have extended the application of the GLC method (16,34) to water-dispersible preparations (38), to fortified nonfat dried milk (39) and to a more diverse range of pharmaceutical preparations (40). Panalaks (38) has reported that the previously required cleanup steps can be eliminated if the method is to be applied to water-dispersible preparations containing only vitamin D and a mixture of preservatives. This simplified version of the method has been used to study the stability of vitamin D in water dispersions. Total analysis time for vitamin D analysis of fortified nonfat dried milk products by GLC was ca. 7 hr. Average recovery of vitamin D added to the nonfat dried milk was 104%. The method was effective for both vitamins D_2 and D_3 added to the nonfat milk products. With the introduction of micro columns of silicic acid as a substitute for thin layer chromatography plates during sample extract clean-up, the GLC method was extended to a wider variety of pharmaceutical preparations (40).

The most recent GLC assay for vitamins D_2 and D_3 has been published by Feeter et al. (41). They have been able to accomplish satisfactory analysis in the presence of the tocopherols and vitamin A by treating the extracts with propionic anhydride to esterify all alcohols to their corresponding propionates. Trioctanoin was used as an internal standard. The solid support, 80-100 mesh Diatoport S, was propionated with propionic anhydride before the stationary phase was applied. Vitamin D_2 analysis was carried out on SE-52 columns, and vitamin D_3 was analyzed on SE-30 columns. The propionate derivatives underwent pyrolysis, forming the "pyro" and "isopyro" thermal rearrangement products. High sample levels of vitamin A were overcome by using higher levels of internal standard, which permitted use of higher attenuations in the vitamin D portion of the analysis with a consequent reduction of the magnitude of the solvent and vitamin A front. It was necessary to discard the chromatogram of the initial injection each day because its value was sometimes erroneous. Sample preparation procedures for this method are greatly simplified compared to previous methods. Glass columns 2.44 m x 0.63 cm were used for both vitamins D_2 and D_3. The SE-52 columns were maintained at 270 C, and the SE-30 columns were operated at 240-250 C. Carrier flow rate was 75 ml/min for both columns. The percentage of stationary phase was not determined by solvent stripping of the prepared packings but was based on the chemical ingredients used to prepare the columns initially; the immobile phase percentages would be 12% SE-52 and 4% SE-30. This is based on the assumption that all of the immobile phase in solution ended up on the inert support, a situation that normally does not occur. Nevertheless the method was quite successful and deserves consideration by investigators desiring to determine vitamin D_2 or D_3 in pharmaceutical products.

A recent article summarized the GLC methods and their application in our laboratory (42) for analysis of pharmaceuticals.

VITAMIN E

The discussion in this section is limited to the tocol series of the tocopherols, more specifically to those tocols commonly referred to as vitamin E. The various vitamin E analogs will be referred to by their Greek symbols in this review. More knowledge has probably been accumulated about the gas chromatographic characteristics of vitamin E and its subsequent analysis than for any other vitamin.

The indication that vitamin E might be amenable to GLC analysis was reported by Nicolaides (1) in 1960, when α-tocopherol was partially resolved from γ-tocopherol on a 5% silicone-gum rubber column in a study of low level silicone gum rubber columns for high temperature GLC of lipids.

The classic paper of Wilson et al. (43) was the first report solely devoted to GLC separation of the various vitamin E analogs. Their findings set the precedent for much of the subsequent work on the GLC analysis of vitamin E. Three immobile phases were tested: namely, 4% SE-30, 5% QF-1 and 4% polyethylene glycol adipate (PEGA), encompassing nonpolar to polar stationary phase characteristics. All columns were glass, 4 ft x 4 mm ID. Siliconized 100-120 mesh Celite was used as the inert support. The column temperatures were 250 C, 240 C and 250 C with flow rates of 40 ml/min, 50 ml/min and 50 ml/min, respectively, for SE-30, QF-1 and PEGA. The retention time for α-tocopherol, calculated from their data, was ca. 30.5, 13.1 and 16.0 min, respectively, for SE-30, QF-1 and PEGA columns. β- and γ-tocopherols were not resolved by any of the immobile phases tested. Tocopheryl acetates were prepared, and their gas chromatograms were characterized. Again the β and γ analogs could not be resolved from each other. The QF-1 packing gave a difference of 0.12 in relative retention volumes between the β- and γ-acetate derivatives, indicating that a highly polar column might possibly resolve these two analogs. No acetate derivative work using the PEGA column was reported.

Kofler et al. (44) evaluated 5% SE-30 and 5% Apiezon N stationary phases for the separation of the vitamin E analogs. The SE-30 column separated the groups of tri-, di- and monomethylated tocols and unsubstituted tocol. However, for the separation of the individual isomers, e.g., β-, γ- and δ_2-tocopherol, SE-30 as a stationary phase was not selective enough. Using SE-30, they were able to achieve almost a complete separation of α-tocopherol from α-tocopheryl acetate. The 5% Apiezon N stationary phase gave a separation of all tocols. The resolution of the two isomers β- and γ-tocopherol was partial. The retention times relative to the hydrocarbon n-$C_{28}H_{58}$ were 1.76 and 1.68 for γ- and β-tocopherol, respectively. Also of interest is that the α-tocopherol peak was the last peak to appear in the tocopherol series on the Apiezon N column. When a 5 m 5% Apiezon N column was used, α-tocopherol appeared in ca. 8 hr. However, on a 2 m column at 260 C, α-tocopherol appeared at slightly more than 2 hr. It was also found that the Apiezon N stationary phase would not separate α-tocopherol from α-tocopheryl acetate. The Apiezon N stationary phase used by these investigators appears to have excellent properties for the separation of the various natural vitamin E isomers but would not be so useful for pharmaceutical preparations. There was no indication of vitamin E alteration during GLC analysis.

Nair and Turner (45) reported their GLC experiences with vitamin E and vitamin K at the 36th AOCS Fall Meeting. The column was glass, 183 cm x 5 mm, packed with a stationary biphase of 2.5 mole % SE-52 and 2.5 mole % XE-60 coated on 60-80 mesh siliconized Gas-Chrom P. The operating parameters were: column, 228 C; detector, 250 C; flash heater, not specified but presumably approximately the same temperature as the detector; carrier gas flow rate, 75 ml/min; electrometer output, 3 x 10^{-8} amp; detector type, β-argon ionization with Sr_{90} foil. The biphase column did not separate β and γ-tocopherol isomers. The relative retention times with respect to α-tocopherol were 0.92, 0.92, 0.81, 0.49 and 0.28, respectively, for γ-tocopherol, β-tocopherol, Δ-tocopherol, Simon's metabolite and squalene. The α-tocopherol peak appeared at 32 min. The technique was applied successfully to the vitamin E isomers and closely related compounds in crude extracts from mammalian tissues. TMS ethers of the tocopherols were also used successfully. The elution times of the TMS derivatives of the various tocopherols were somewhat shorter than those for the unreacted tocopherols.

Schudel et al. (46) studied the synthesis of all-*trans*-δ_1-and- ε-tocopherol and the corresponding natural specimens in wheat bran. A GLC column of Apiezon N was used to help establish that the all-*trans* configuration of the side chain existed in nature.

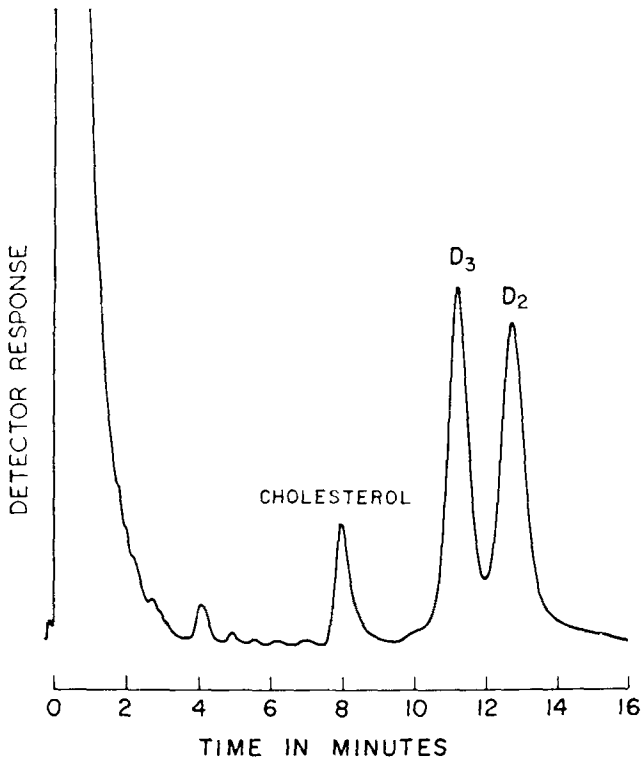


FIG. 6. Gas liquid chromatographic tracing of cholesterol, vitamin D_3 and vitamin D_2 (34).

An extensive study of the determination of α-tocopherol in animal tissues by GLC was carried out by Bieri and Andrews (47). The stationary phase SE-30 was evaluated by using concentrations of .75-7% coated on 80-100 mesh Chromosorb W in columns of 4 and 6 mm ID. Initial studies with *dl*-α-tocopherol indicated that the compound was not changed during GLC and that response deviated slightly from linearity in the 1-6 μg range with the β-argon ionization detector. Effective measurements were made in the range of 0.25-1.0 μg on a 1% SE-30 column. Detectable peaks were obtained at the 0.1 μg level, and 0.5 μg amounts gave a 67% full scale response. Calibration was achieved by comparing the response of an amount of standard α-tocopherol estimated to be close to the amount in the unknown. A DC cell voltage of 900 was found satisfactory for the tocopherols. The α-, γ- and δ-tocopherols were separated easily with relative retention times of 1.00, 0.83 and 0.68, respectively. It was found that α-tocopheryl quinone, which is of biological interest, came through the SE-30 column with a retention time only slightly different from that of α-tocopherol. On most columns tested, i.e., various percentages of SE-30, the separation of these latter two compounds generally resulted in a bicuspid peak. The 1% SE-30 packing proved to be the most satisfactory immobile phase. The retention times relative to cholestane on the 1% SE-30 column were 1.96, 2.11, 2.20, 2.27, 2.66 and 2.78, respectively, for cholesterol, *d*-α-tocopheryl quinone, *d*-α-tocopheryl acetate and cholesteryl acetate. The column was maintained at 230 C with an inlet pressure of 25 psi. This column was used to measure the tocopherol compounds in tissue extracts following extensive sample clean-up procedures involving saponification and an alumina and zinc carbonate column. Extensive data indicated the advantage of the GLC measurements over those obtained by the ferric chloride-bipyridyl chemical method (Emmerie-Engel).

Ninomiya et al. (3) included *dl*-α-tocopherol and *dl*-α-

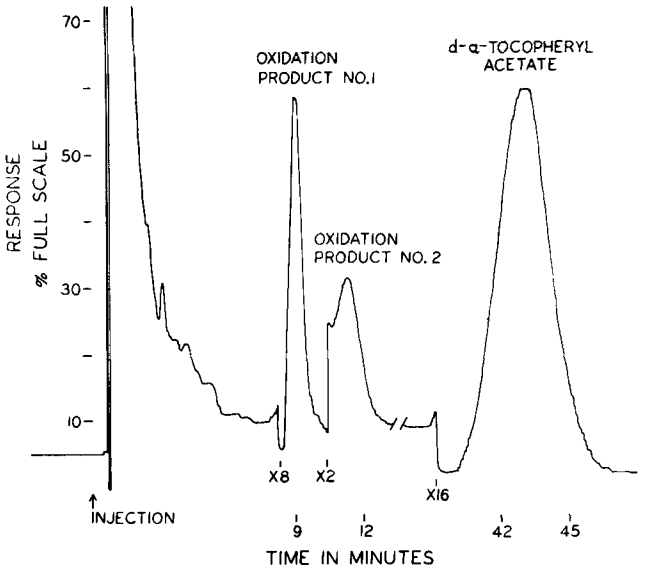


FIG. 7. Gas chromatogram on 5% SE-30 at 235 C of breakdown products from α-tocopheryl acetate when distilled above 160 C (Sheppard, Prosser and Strother, unpublished data).

tocopheryl acetate in their early studies on the GLC of the fat-soluble vitamins, previously discussed under vitamins A and D above. Excellent separation of the two α isomers was achieved by using SE-30 and FS-1265 (QF-1), but with Craig's polyester succinate the separation was nominal.

Conditions for the determination of the GLC relative retention volumes for the compounds occurring in a 14-stage vitamin E synthesis process have been reported by Yanotovski (48). The log relative retention volume vs. the number of carbon atoms in a homologous series was found to be linear. A column 120 cm long, packed with Apiezon L coated on Celite 545, was used. The column was maintained at 200 C with a carrier gas flow of 75 ml/min. Analysis time was 150 min.

Carroll and Herting (4) found that the *d*-isomers of monomethyl-, dimethyl- and trimethyltocols emerged in that order and were readily separated at 205 C on a 3% SE-30 immobile phase coated on silanized Gas Chrom P packed in a 6 ft x 1/8 in. glass column. The *d*-β-, *d*-γ and *dl*-δ_2-tocopherols were not effectively resolved from each other on the SE-30 column. The optical isomers of *dl*-δ_2-tocopherol and of *dl*-α-tocopherol appeared to have identical retention times. With a 3% QF-1-0065 column at 195 C (other column specifications same as the SE-30 column), δ-, β- and α-tocopherols were separated but not as well as on SE-30. The order of elution of γ- and β-tocopherol was reversed on QF-1-0065, but their retention times were almost identical. α-Tocopheryl acetate exhibited a greater relative retention time than the nonacetates on both immobile phases.

Libby and Sheppard (49) evaluated column loadings of 1-5% SE-30, 2.5-5% XE-60 and 5% SE-52. A 5% SE-30 on 100-140 mesh siliconized Gas Chrom P column was selected as most desirable, based on a number of factors such as resolution, sensitivity, reproducibility and analysis time. A glass column 8 ft x 3 mm ID was operated at 260 C. It was found that the column required saturation with 50 μg of each tocopherol on each assay day until a constant response was obtained. Heavy emphasis was placed on the necessity of calibration for each tocopherol analog in the sample size range as a part of GLC quantitative analysis. Linear response over a wide concentration range for each vitamin E analog was observed, but it was discovered that this resulted in specific response plots for each analog rather than a general response plot for the tocopherols.

Sheppard et al. (50) evaluated a vacuum distillation procedure for vitamin E isolation that had been recently designed for fatty acid work (51). α-Tocopheryl acetate, carbon-14 labeled in the methyl group at the 2 position, was used to determine the stability and recovery of the vitamin during vacuum distillation. Recovery of 20 mg of pure standards was 97%. Recovery of 0.5 mg or less of the labeled compound added to "stripped" lard was 98%, and recovery of the compound when 0.1 mg or less was added to butter was 82%. These recoveries were obtained at 140-160 C, 0.020 mm Hg and 60 min. Results of differential carbon-14 counting techniques indicated that the ester decreased in stability as temperature was increased much beyond 160 C. GLC analysis revealed two unidentified breakdown products. An unpublished GLC analysis showing the breakdown products is presented in Figure 7 (Sheppard, Prosser and Strother, unpublished data, 1965). This sublimation unit has the advantage over previous units, in that it can be completely disassembled for cleaning, has greaseless seals and can be stored in a laboratory desk drawer.

Ishikawa and Katsui (52) and Ishikawa et al. (53) developed a satisfactory GLC analysis for the tocopherols in soybean oil. The two reports refer to essentially the same procedure with the same general type of results. In the first study, mixtures extracted from soybean oil were found to contain no *d*-β-compound; thus the usual combined β-γ peak could be considered entirely due to *d*-γ-tocopherol. It was also noted that the use of trimethylsilyl esters brought the retention time close to that of squalene so that it could not be used as the internal standard. However they found that acetylating the tocopherols was superior to using the unreacted tocopherols themselves, and squalene could be used as an internal standard. The most important contribution of their second paper is the sample extract clean-up procedure using a Florisil column after saponification. In both reports a 1.5% SE-30 immobile phase coated on 60-80 mesh Chromosorb W packing was used. Stainless steel columns 225 x 0.4 cm and 450 x 0.4 cm were maintained at 250 C and a carrier gas flow rate of 80-100 ml/min. Excellent recoveries and analysis data were achieved. The practical application of the GLC method for tocopherol analysis of soybean oils is well defined and relatively simple to follow.

Bruschi (54) successfully applied simultaneous GLC analysis to vitamin E acetate and methyl testosterone content of pharmaceutical products containing both. Excellent results were achieved for both compounds. A silicone column, 2 m x 2.7 mm ID, was maintained at 250 C and a flow rate of 30 ml/min. Unfortunately the percentage of silicone immobile phase used was not established. Testosterone acetate was used as an internal standard. The sequence of elution following sample injection was solvent, methyltestosterone, testosterone acetate and vitamin E acetate. All compounds were separated completely.

Eisner et al. (55) studied the unsaponifiable matter in butter and 12 vegetable oils. The unsaponifiable matter was fractionated on a Florisil column. A third fraction contained the tocopherols in addition to high molecular weight alcohols and triterpenoid alcohols. A 6 ft x 1/4 in. ID glass column packed with 1.5% SE-52 on silanized 100-120 mesh Gas Chrom P was operated at 210 C with an inlet pressure of 12 psi. The order of tocopherol elution was δ,β or γ and α. They eluted in ca. 10-18 min. The presence of α- and δ-tocopherols was not definitely detected in the soybean oils examined. α-Tocopherol eluted with C_{28} normal alcohol under the GLC conditions imposed.

Nair et al. (56) achieved microquantitative separation of isomeric dimethyltocols with the SE-52/XE-60 biphase column used by Nair and Turner (45) as discussed above. γ-Tocopherol was separated from β-tocopherol as the paraquinones. The tocopherols react with ferric chloride, the reaction mixture is dissolved in α,α'-dipyridyl solution and the mixture is heated. This reaction appeared to be the

Emmerie-Engel reaction carried out on a microscale. The separation of the two isomers was complete under the GLC conditions used. The results reported for the separation of β- and γ-tocopherols are better than any other attempts reported in the literature. β-Tocopherol could not be separated from the synthetic isomer 5,7-dimethyltocol. When TMS derivatives were used, the 5,7-dimethyltocol could be separated distinctly from the γ- and β-tocopherol combined peak.

In a follow-up paper, Nair and Machiz (57) were able to obtain a complete separation of the isomeric monomethyltocols on the biphase column. Conversion of the monomethyltocols to the corresponding trimethylsilyl derivatives resolved all three positional isomers, including the 7- and 8-methyl isomers, which remained indistinguishable from each other both in the unaltered and *p*-quinone forms on the biphase column. Acetate, trifluoroacetate and pentafluoropropionate derivatives were also studied. Irrespective of the mode of preparation of the silyl ethers, using either bis(trimethylsilyl) acetamide or hexamethyldisilazane, the tocopherols were completely converted to the corresponding derivatives, as evidenced by the complete absence of the characteristic hydroxy band at 2.7 μ in the IR band.

GLC identification and estimation of tocopherols in wheat germ oil, soybean oil, whole wheat flour and corn meal has been studied extensively by Slover et al. (58). The tocopherols were converted to their TMS ethers. The separations were made at 235 C on 15 ft x 0.08 in. ID silanized columns packed with either 0.5% Apiezon L or 2% SE-30 on 110-120 mesh Anakrom (acid- and base-washed and silanized). Kovats Retention Indices were determined for tocol, tocoltrienol and all 14 possible methylated tocols and tocotrienols. Considerable effort was expended with various mixtures to ascertain that the method is capable of a high degree of accuracy and precision. A partial resolution of β- and γ-tocopherol TMS ethers was achieved on the 0.5% Apiezon N column with a total analysis time of ca. 80 min. When a 2% SE-30 column was used, the analysis time exceeded 60 min and the separation of β- and γ-tocopherol TMS ethers was exhibited as a doublet in which the apex of each compound was barely delineated. It was indicated that some tocopherol decomposition undoubtedly occurred during these analyses due to the long residual time on the column and the high column temperatures required. The conditions used were a compromise to give the best resolution with the decomposition held to a minimum. Attempts to separate the free tocopherols on Apiezon L were not completely successful because of variable sample loss on the column and tailing peaks.

Bunnel (59) published an excellent review of the early GLC applications for vitamin E analysis. A large table of retention times and column data is provided. This article should be reviewed by investigators entering the field of GLC analysis of vitamin E for the many valuable suggestions offered.

Pillsbury et al. (60) adapted the earlier work of Libby and Sheppard (49) to the analysis of the vitamin E content of pharmaceutical products. Recoveries of 100.0 ± 8.9% of known amounts of α-tocopheryl acetate and α-tocopheryl succinate added to vitamin E-free tablets were obtained. An extremely simple extraction system was perfected. Then 24 different samples of pharmaceutical products were analyzed. The samples included tablets, capsules, baby drops and injectables. The GLC results were compared with results obtained by the Emmerie-Engel chemical method. The overall mean percentage recoveries of label claim (± standard deviation) were 103.7 ± 11.7 and 95.0 ± 21.4, respectively, for the GLC method and the chemical method. Some of the products having label claims of α-tocopheryl acetate were shown not to be entirely α-tocopheryl acetate but a mixture of α-tocopheryl acetate and α-tocopherol. The strength of the method is in its overall speed of analysis and analog specificity.

A collaborative study was carried out by Sheppard et al. (61) in 1968 under the auspices of the Association of Official Analytical Chemists, in which three GLC methods and the Emmerie-Engel chemical method were compared for the determination of the vitamin E content of pharmaceutical products. One was the method of Pillsbury et al. (60), discussed above. The other two methods were basically variations of Pillsbury's method designed to increase the rapidity of analysis. Of the three GLC methods, that of Pillsbury et al. exhibited the highest precision and was the method of choice. The mean composite coefficient of variation obtained for all analogs and all laboratories was 10.2. In evaluating the GLC methods, all of which outperformed the chemical method, it was concluded that the precision achieved with the method of Pillsbury et al. outweighed the few minutes' reduction in total analysis time gained by the more rapid GLC methods. The mean composite coefficient of variation exhibited by the chemical method was 28.6%. The study included seven pharmaceutical formulations containing either α-tocopheryl acetate or α-tocopheryl succinate that were analyzed by nine collaborating laboratories. A means of identifying α-tocopheryl succinate was included in the study, since α-tocopherol and α-tocopheryl succinate had a relative retention time of 0.9 with respect to α-tocopheryl acetate. An acetylation reaction was attempted on the sample following an initial chromatographic analysis, if the peak observed exhibited a relative retention time of 0.9. The sample was then rechromatographed. If the peak shifted to the α-tocopheryl acetate, the sample contained the alcohol; if it did not shift, the sample contained α-tocopheryl succinate. All collaborators successfully used the test. The method was adopted by the Association of Official Analytical Chemists as an "official first action" method (62). The "official" method is included in the 11th Edition, *Official Methods of Analysis of the AOAC* (63).

Mahn and coworkers (64) determined the vitamin E in multivitamin products by GLC on a copper column 121.9 cm x 3 mm ID packed with 10% SE-30 on 100-120 mesh Aeropak 30 maintained at 245 C with a flow rate of 40 ml/min. α-Tocopheryl acetate appeared at 25.5 min. The method was successful for the analysis of tablets, capsules, injectables and creams. An extensive sample extract clean-up procedure was developed that is suitable for a wide variety of pharmaceutical products extending beyond the usual multivitamin products administered orally or by injection. The relative retention times were 0.85, 0.71, 0.75, 0.68 and 1.00, respectively, for α-tocopheryl acetate, α-tocopherol, α-tocopheryl succinate, vitamin D_2 and dotriacontane. The outstanding features of this method are the successful use of copper columns, the introduction of dotriacontane as an internal standard and a sample clean-up procedure suitable for extending the GLC method beyond the usual tablets and liquids to include specialized products such as multivitamin creams. A precision of ±2-3% was achieved.

Bowman and West (65) have developed a GLC assay for α-tocopheryl acetate in multivitamin products using a 3% SE-30 column. The column used was 2 m x 3 mm ID packed with 3% SE-30 on 100-120 mesh Gas Chrom Q maintained at 235 C with a flow rate of 50 ml/min. The internal standard technique was developed for the assay, using 3-(*p*-methoxyphenyl)-2,4,5-triphenylcyclopentadieneone. The sample preparation is more involved than that in the AOAC method (66) but less involved than that of Mahn et al. (64). It was estimated that one analyst with two gas chromatographs could handle 20 assays per day, whereas an experienced analyst was able to complete 3-5 chemical assays per day depending on the procedure followed. Assay precision was determined for each type of formulation by replicate assay of a single lot. In every case

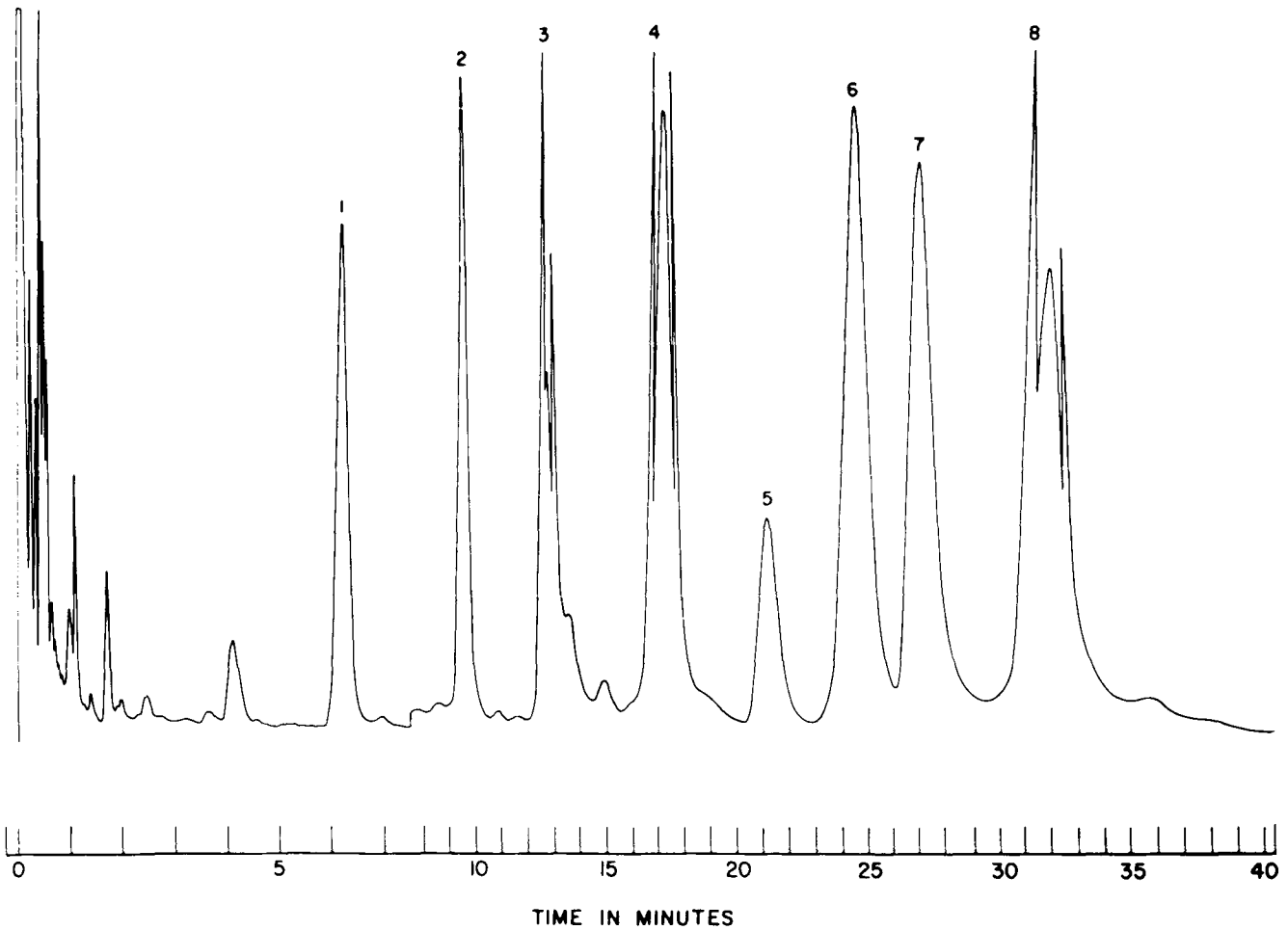


FIG. 8. Gas chromatogram of soya sludge unsaponifiables. Peak identification: (1) squalane; (2) squalene; (3) Δ-tocopherol; (4) β + γ-tocopherol; (5) α-tocopherol; (6) campesterol; (7) stigmasterol; and (8) sitosterol (70).

the coefficient of variation was less than 1.2%. When GLC assay results were compared with chemical assay results, the GLC values were consistently higher. A total of 20 samples encompassing a variety of pharmaceutical products were analyzed. The calculations and overall procedures used in this method and that of Mahn et al. (64) are similar. The outstanding feature of the method is the introduction of 3-(*p*-methoxyphenyl)-2,4,5-triphenylcyclopentadieneone as an internal standard in the GLC assay procedure.

Slover et al. (66) extended their previous study (60) and studied the validation of predicted retention data. Lipid from latex was extracted for its tocotrienol content. It was found that the predicted retention data and the peak identifications reported previously (58) were valid.

Hall and Laidman (67) have developed an improved analytical procedure to study the distribution of the tocopherols and of ubiquinone in ungerminated wheat grain. A comparison was made of several extraction procedures. Gradient elution column chromatography on acid-washed alumina columns efficiently separated the isoprenoid quinones and tocopherols into groups. The tocopherols were determined quantitatively by GLC analysis. Siliconized glass columns 5 ft x 4 mm packed with 4% SE-30 or 4% SE-301 on silicone-treated 100-120 mesh Gas-Chrom Z were used at 250 C with a carrier flow of 40 ml/min. The retention times of the tocopherols, tocotrienols and their acetates relative to that of α-tocopherol were similar to those reported by Wilson, Kodicek and Booth (43). Each tocopherol exhibited an individual nonlinear calibration curve, and the values were reproducible within ±4%. Quantities of tocopherol ranging from 0.2 to 1.0 μg could be determined conveniently by the hydrogen flame ionization detector.

Nair and Luna (68) have studied the identification of α-tocopherol from tissues by combined GLC, mass spectrometry and IR spectroscopy. The GLC vitamin E analysis was that previously reported from Nair's laboratory (45,56,57) in which the binary mixture of SE-52 and XE-60 was used. α-Tocopherol isolated from heart muscle was characterized by the combined system. The purpose of the study was to describe the identification procedures of α-tocopherol which form the basis of future biological studies.

Janecke and Voege (69) have developed a GLC quality control method for multivitamin preparations that purportedly assays both vitamin E and vitamin A in a single analysis. The vitamins react with N,O-bis(trimethylsilyl) acetamide. The column is programed from 100 to 270 C. It is indicated that the water-soluble vitamins can be determined by this method also.

GLC has been used to determine the tocopherol and sterol content in soya sludges and residues by Nelson and Milun (70). The method involves saponification of the sample followed by GLC analysis of the unsaponifiables. A glass column 6 ft x 3.5 mm ID packed with ca. 3% SE-30 coated on Gas Chrom Q was used. The column was maintained at 225 C with a carrier gas flow of 150 ml/min. Squalane was used as an internal standard. The relative retention times with respect to squalane were 3.40, 2.72, 2.05, 2.76, 3.90, 4.32 and 5.10, respectively, for α-tocopherol, β-tocopherol, Δ-tocopherol, campesterol, stigmasterol and sitosterol. A typical chromatographic separation of the unsaponifiables from sludge is shown in Figure 8. The β- and γ-tocopherols were not resolved. Compound identifications were confirmed by trapping GLC fractions and subsequent IR and mass spectral analysis. The non-

saponifiables were extracted by ether in a separatory funnel. Recovery data indicated that the method was very effective. The overall approach of the method was one of simplicity. The Nelson and Milun method is quite good and deserves consideration as a method of choice by investigators working in the area of legume oilseed products and byproducts who are seeking progress in their methodology.

A GLC method has been described by Siedenstrücker (71) for the identification and quantitative estimation of tertiary terpene alcohols occurring as intermediates in the syntheses of vitamins A and E. A 50 m x 0.25 mm steel capillary column coated with Apiezon L was used. Alcohols with molecular weights ≤224 were separated without preliminary reaction. The thermally unstable terpene alcohols with molecular weights >224 were silylated. It was observed that quantitative results may be lost when too much SiO_2, formed during combustion in the hydrogen flame ionization detector, deposits at the electrode.

Petrova (72) has recently reviewed the general area of vitamin E analysis. A section of the review is devoted to GLC analysis. Weglicki, Luna and Nair (73) used the GLC techniques previously developed in their laboratory (56,68) to study the sex and tissue differences in concentrations of α-tocopherol in mature and senescent rats. The biphase SE-30/XE-60 column was used to quantitate the α-tocopherol from the nonsaponifiable fraction. The α-tocopherol was measured as the trifluoroacetate derivative. In another study from the same laboratory that appeared in print at about the same time, Olweira et al. (74) reported data on the distribution of α-tocopherol in beef heart mitochondria. The α-tocopherol was identified by several independent procedures, one of which involved GLC. The GLC method was that of Nair et al. (56), previously discussed.

In the vitamin K work of Dialameh and Olson (75) α-tocopherol was used as an internal standard. On the 3.8% SE-30 column used, α-tocopherol appeared in 7 min. Prevot and Barbati (76) conducted a study of the analysis of hydroxyl compounds by GLC. They found the preparation of the TMS derivatives of the tocopherols to be very rapid and simple. The GLC method for the sterols was effective for measurement of the tocopherols. A 1.5 m column packed with 3% SE-30 coated on 60-80 mesh Chromosorb W HMDS and maintained at 255 C was used.

Slover et al. (77) have applied their previously discussed GLC techniques (58,66) to analyze the tocopherol content of foods. A wide variety of seeds, fats and oils were tested. Detailed procedures are described for extraction, saponification and partial purification by thin layer chromatography. The individual tocols and tocotrienols were identified and estimated as their trimethylsilyl ethers by GLC on SE-30 or Apiezon L columns maintained at 235 C. In a concurrent paper, Slover et al. extended their application of the GLC method (78) to determine the tocopherol content of 11 wheats, the flours from them and products made from the flours. In addition, 10 pooled consumer product samples from each of 10 U.S. cities were analyzed. These studies are of excellent analytical quality and represent the application of GLC to a very practical problem. Slover (79) presented a summary and discussion of the recent literature on the tocopherols in natural, processed and prepared foods at the 1970 AOCS-ISF World Congress. The discussion is centered around the literature appearing since 1964. A wide variety of foods and fat data has been compiled from a large number of sources. Although a considerable amount of the tabulated data was obtained by GLC analysis, it is not a methods paper.

Nelson et al. (80) have made some improvements in their previously published method for GLC analysis of tocopherol and sterol content of soya sludges and residues (70). The major modification was the conversion of the tocopherols and sterols to butyrate esters prior to GLC, whereas in their previous report no derivative was formed. The use

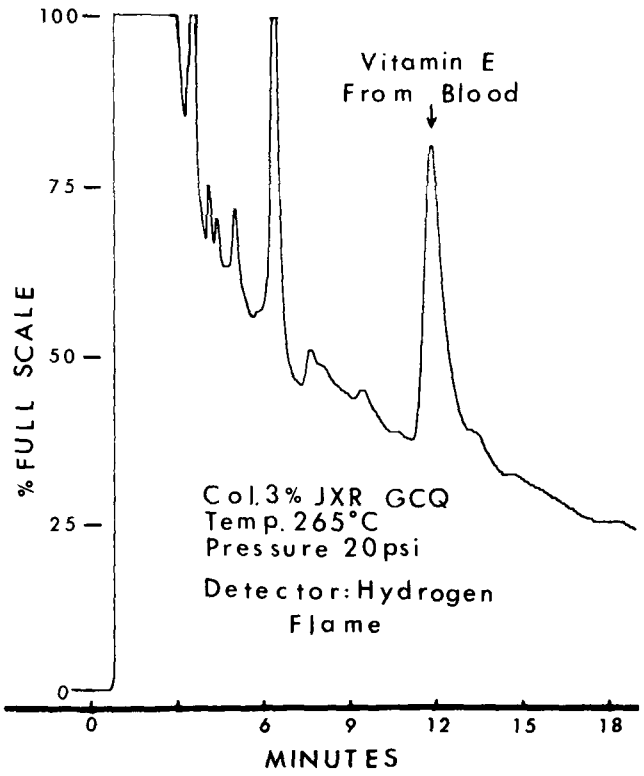


FIG. 9. Gas chromatogram of α-tocopherol in blood samples (Sheppard, Quaife and Friedman, unpublished results, 1969).

of the butyrate esters eliminated interference from materials encountered in the sludges and residues during GLC analysis. It was found that derivatization of the tocopherols to the acetate, propionate and the dimethyl or trimethylsilyl ethers did not succeed in eliminating these interferences. Also, the SE-30 immobile phase was reduced to 1%. The GLC conditions were revised slightly to column temperature of 235 C and a carrier gas flow of 170 ml/min. Cholesteryl isovalerate was used as an internal standard, whereas squalane has been used previously. The retention times relative to cholesteryl isovalerate were 0.46, 0.61, and 0.75, respectively, for δ-tocopheryl butyrate and β- and γ-tocopheryl butyrate. The new method exhibited improved precision over the previous method: coefficients of variation were 1.0% and 3.3%, respectively, for total tocopherol in soya sludge.

Bieri et al. (81) have used the gas chromatograph to determine the α-tocopherol in human erythrocytes. The α-tocopherol was oxidized to α-tocopherylquinone prior to GLC analysis. The columns used were glass, 180 cm x 3 mm ID, packed with 3% QF-1 on 80-100 mesh silanized Supelcoport maintained at 230 C and a carrier gas flow rate of 50-70 ml/min. The α-tocopherylquinone peak appeared in 7-8 min under these GLC conditions. It was necessary to prime the column at the start of the day with overloads of α-tocopherylquinone to saturate the active sites. Linearity of response was observed over the range of 0.025 to 0.25 μg of α-tocopherylquinone. Carbon disulfide was used as the carrier solvent. Sample preparation included thin layer chromatographic clean-up procedures. Two samples required a full day for total processing, but eight samples were easily completed in a 2 day period. No attempt was made to determine the presence of other tocopherols in erythrocytes. The retention times relative to α-tocopherylquinone for β-, γ- and δ-tocopherylquinones were 0.91, 1.09 and 1.00, respectively.

Kovensky and Day (82) have used GLC for individual determination of α-tocopherol, α-tocopheryl acetate or α-tocopheryl acid succinate in vitamin E capsule preparations. The column used was glass, 5 ft x 2 mm ID, packed

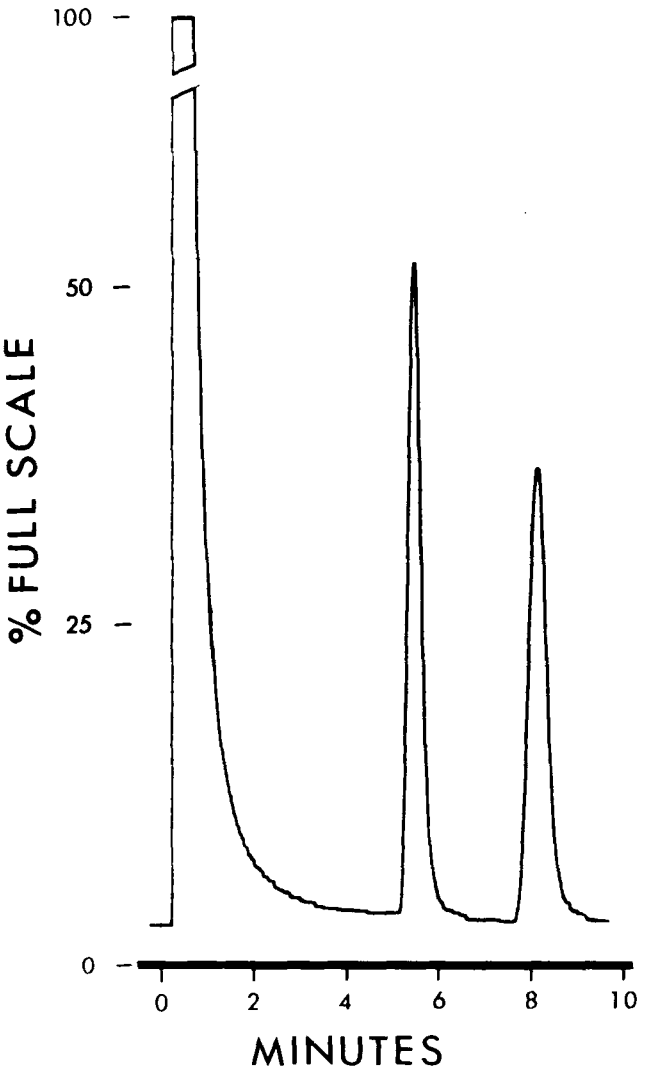


FIG. 10. Chromatogram of 0.5 μg/μl of menadione (ca. 6 min) and butylated hydroxy anisole (BHA, ca. 9 min) in *n*-hexane on a 1% neopentyl glycol succinate column at 145 C (98).

with 3% OV-1 on 60-80 mesh Chromosorb W acid-washed maintained at 260 C with a carrier gas flow of 54 ml/min. Hexadecyl palmitate was used as an internal standard. The average retention times for tocopherol, tocopheryl acetate, tocopheryl acid succinate and hexadecyl palmitate were 2.48, 2.85, 2.64 and 4.85 min, respectively. When the relative retention times are taken into consideration, this method is extremely limited in that only one analog of vitamin E can be measured in a given GLC analysis. Also, since the retention times are so closely grouped, analog identification by the method would be tenuous. Of interest in this method is the introduction of OV-1 immobile phase into the vitamin E field. For applications in pharmaceutical analysis, the time required for analysis is extremely rapid. Recovery rates of 101.5 ± 3.0% were reported. The analytical data presented indicated that the method has excellent precision ranging from ca. 1% for α-tocopherol to 2% for the α-tocopheryl acetate.

Lehmann and Slover (83) have recently described a GLC method for the determination of the tocopherols present in plasma. This method is a modification and adaptation of their previously discussed method for separation and identification of tocopherols in foods (77). The glass columns, 15 ft x 0.125 in. OD, were silanized and then packed with 0.5% Apiezon L on 100-125 mesh Gas Chrom Q and operated isothermally at 250 C with a carrier gas flow rate of ca. 25 ml/min. The method includes 5,7-dimethyltocol as an internal standard. Only α- and γ-tocopherols were detected in human plasma. A comparison of methods was carried out. The chemical method generally gave higher answers than the GLC, which were attributed to other reducing substances in plasma. The applicability of the GLC method was tested with blood from a series of human donors.

We have recently published an extensive review of our GLC methods for vitamin E measurements in a variety of items, i.e., pharmaceuticals and biologicals (84). This method was used to obtain the blood tocopherol measurements reported by Quaife and Friedman (85). Figure 9 is a typical gas chromatogram of α-tocopherol in those blood samples (Sheppard, Quaife and Friedman, unpublished results, 1969).

VITAMIN K

Vitamin K in this discussion includes 2-methyl-3-phytyl-1,4-naphthoquinone (K_1), 2-methyl-3-difarnesyl-1,4-naphthoquinone (K_2), 2-methyl-1,4-naphthoquinone (menadione or K_3), 2-methyl-1,4-naphthoquinone sodium bisulfite (menadione sodium bisulfite), 2-methyl-1,4-naphthoquinone diphosphoric acid ester tetrasodium salt (menadione diphosphate), 2-methyl-1,4-naphthohydroquinone diacetate (K_4) and 2-methyl-1,4-amino-1-naphthol hydrochloride (K_5). The common names in parentheses will be used throughout this discussion.

The initial report indicating the possibility of GLC analysis of vitamin K_1 was published by Nair and Turner (45). Their data showed very clearly that vitamin K_1 can be resolved from a mixture of tocopherol metabolites, tocopherols and vitamin K_1. The biphase column and operating parameters used were the same as for vitamin E. Under those conditions, vitamin K_1 appeared 53.2 min after sample injection. The relative retention time of vitamin K_1 was found to be 1.66 relative to α-tocopherol. It can be calculated from their data that the retention time is 6.05 relative to squalene, which is currently used by some workers as an internal standard in performing GLC analysis of fat-soluble materials, especially in the vitamin field. They also discovered that vitamin K_1 would not react with a mixture of hexamethyldisilazone and trimethylchlorosilane in chloroform to form the silyl ether. Carroll and Herting (4) and Libby et al. (86) subsequently verified that on the silicone polymer type of columns vitamin K_1 follows the vitamin E series as reported originally by Nair and Turner.

Ninomiya and coworkers (3) reported their evaluation of three column packings (1% SE-30 on 80-100 mesh silanized Anakrom A, 1% Craig's polyester succinate on 80-100 mesh silanized Anakrom A and 1% FS-1265 on 80-100 mesh Chromosorb W) for GLC separation of the fat-soluble vitamins including vitamins K_1-, K_3- and K_4-diacetate. On these columns vitamin K_1 required 200-220 C, vitamin K_4-diacetate required 150-180 C and vitamin K_3 required 90-130 C depending on the column packing and flow rates. Their chromatogram of vitamin K_1 on SE-30 was characterized by a broad peak preceding the main peak which had enough disturbance on its leading edge to suggest a possible breakdown product and distinct indications of two peaks intermeshed with the trailing edge. Unfortunately they did not provide chromatograms of vitamin K_1 on Craig's polyester succinate and FS-1265. One very surprising aspect of their experience with vitamin K_3 is that on all three stationary phases tested, two distinct peaks appeared that were not completely resolved. This is the only report found in the literature that indicates anything other than a single well defined peak for vitamin K_3. The vitamin K_4-diacetate peaks were excellent. This is the only known GLC work on this particular vitamin K analog.

Carroll and Herting (4) found that vitamin K_1 yielded two peaks, 8.6 min apart, having a 4:1 area ratio, on the 3% SE-30 column previously described under vitamin E. The

first peak occurred at 41.7 min. Vitamin K_1 gave a single peak on QF-1-0065 immobile phase at 195 C (previously described under vitamin E) in 27.8 min.

Dugan and Lundgren (87) developed a GLC vitamin K_3 method to detect the presence of menadione in extracts of *F. ferooxidans* obtained while studying coenzyme Q_6. One peak in the extract was tentatively identified as menadione based on retention time. They were unsuccessful in obtaining chromatograms for vitamin K_1. Their 5 ft x 1/8 in. OD glass columns were packed with 5% Dow silicone immobile phase coated on 60-80 mesh Chromosorb W and operated at 180 and 205 C. Vitamin K_3 appeared in 1 min at 205 C and in ca. 1.7 min at 180 C column temperatures. An electron capture detector was used since it "takes advantage of the electron capture system in that 'extensive' procedures are eliminated because of the inability of the detection system to sense most naturally occurring substances in low concentrations, i.e., alcohols, aldehydes, acids" (87). No preparation of halogenated derivatives was indicated.

Libby and Sheppard (88) carried out experiments to determine what column packing was most useful on a practical basis for menadione. Five stationary phases were evaluated: SE-30, XE-60, neopentyl glycol succinate (NPGS), neopentyl glycol adipate (NPGA) and neopentyl glycol sebacate (NPG Seb). The ideal column, in terms of peak confirmation and compound sensitivity, was a 6 ft x 4 mm ID glass column packed with 1% NPGS stationary phase coated on silanized 100-140 mesh Gas-Chrom P. As little as 4 ng of menadione could be detected with the hydrogen flame and 20-40 ng could be detected with the β-argon (^{226}Ra-foil) ionization detectors. Water-soluble derivatives could be analyzed by converting them to menadione. The GLC response to increasing quantities of menadione was found to be linear through 40 μg with the upper limit of linearity not established.

Cornelius and Yang (89) developed a GLC method to measure vitamin K_5 content of dried carrots. Vitamin K_5 was studied in foods because of its possible use as a food preservative and as a sensitizing agent for microorganisms subjected to ionizing radiation. Two approaches were tried, one to determine vitamin K_5 as its trimethylsilyl ether (TMS) derivative and the second to determine it as vitamin K_3. A 5 ft x 1/8 in. glass column packed with 3% OV-1 (STAP, Varian Aerograph) coated on 100-120 mesh AW-DMCX Chromsorb W was used with a 25 ml/min carrier gas flow rate at 175 C. A chart speed of 25 in./hr was used to obtain retention times of 16 mm for vitamin K_5 TMS, 132 mm for vitamin K_5 and 5.4 mm for vitamin K_3 which, when converted to a time scale, were 2.5, 20.8 and 8.5 min, respectively. Vitamin K_5 decomposed in the presence of water with time; thus it was necessary to determine both compounds on the food extracts. The vitamin K_3 peak occurred well before the solvent front was completely eluted and the instrument returned to the baseline.

Libby et al. (88) studied the GLC characteristics of vitamin K_1 and K_2. Four stationary phases were tested: SE-30, XE-60, QF-1 and a binary mixture of SE-30 and QF-1. All of these immobile phases produced satisfactory peaks for vitamin K_1 under similar operating conditions. However SE-30 was selected for the majority of the research primarily because vitamin K_1 could be chromatographed in combinations with the tocopherols without interference. It was found that the same extraction procedure could be used for both vitamins; thus being able to resolve vitamin K_1 from the tocopherols has practical merit. The columns used for the majority of the study were glass, 6 ft x 4 mm ID, packed with 1% SE-30 coated on silanized, 100-120 mesh Gas-Chrom P operated at 250-255 C. When small quantities of vitamin K_1 were measured, the peak had a small shoulder on the ascending slope. It was observed during this study that, when the columns were allowed to cool after use or were stored, they became unreliable for further vitamin K_1 analysis. Reproducibility was lost and multiple peaks were observed; thus the column had to be discarded and a new one prepared. The method has been applied successfully to a few regulatory samples of injectables to verify chemical results. GLC responses with 0.5 μg of vitamin K_1 were sufficiently large for quantitative analysis. When carrying out the original study, we were equipped with 0-5 mv strip chart recorders, but using newer instrumentation equipped with better electrometers and 0-1 mv recorders we have been able to achieve vitamin K_1 detection at 1-2 ng levels. Quantitative analysis requires at least 5 ng and is far superior at 15-20 ng, considering electrometer noise and chart measurements. Our results with vitamin $K_{2(35)}$ have been unsatisfactory to date. On the basis of molecular weights and differences in the side chain, it would normally be expected that vitamin K_1 and $K_{2(35)}$ could be resolved from each other under the same GLC conditions. Unfortunately vitamin $K_{2(35)}$ did not resolve as a single peak. There is always the possibility that the particular batch of vitamin $K_{2(35)}$ that was used was not pure, but in view of some of the problems noted with vitamin K_1 the GLC behavior of vitamin $K_{2(35)}$ is not too surprising.

Vetter and coworkers (90) obtained excellent vitamin K_1, vitamin $K_{2(35)}$ and vitamin $K_{2(45)}$ peaks by chromatographing the TMS derivatives of the dihydrovitamin. The structure and purity of the derivatives were confirmed by both GLC and mass spectrographic data. They were able to achieve a complete resolution of *cis*- and *trans*-TMS-dihydrovitamin $K_{1(20)}$. For vitamin $K_{1(20)}$ and TMS-dihydrovitamin $K_{1(20)}$, 5% Apiezon L or 10% UCW-98 immobile phase coated on 80-100 mesh Gas-Chrom Q was packed in a glass 2 m x 2.2 mm column operated at 250 C for UCW-98 and 275 C for Apiezon L. Vitamin $K_{1(20)}$ exhibited two peaks, whereas the TMS-dihydrovitamin $K_{1(20)}$ exhibited a single peak. A similar column packing of 3% silicon UCW 98 produced peaks of excellent chromatographic quality at 280 C for the TMS derivatives of both vitamins $K_{2(25)}$ and $K_{2(45)}$. It was further determined that the TMS ethers of the reduced vitamins K_1 and K_2 were well suited to GLC analysis. The problems of thermal conversion were attributed to the formation of chromenols and chromanols.

Russian workers have published their experiences (91,92) with GLC of a series of vitamins K and ubiquinones. Two stationary phases, SE-30 and Apiezon, were used as 10% coating on 100-120 mesh Celite 545 in 1.2 m x 6 mm ID columns at a variety of isothermal temperatures between 150 and 250 C. A dependency of the log of retention time on the number of isoprene units in a molecule was observed. It was postulated that a series of vitamin K and ubiquinones could be identified by means of plots of the retention time vs. the number of isoprene units. There were no indications that thermal conversion of the quinones to chromenols and chromanols occurred under their operating conditions.

Silvestri and Staibono (93) and Hubbard and Sheppard (94) published reports devoted to the analysis of vitamin K_3. Silvestri and Staibono were concerned with water-soluble vitamin K_3 in the form disodium disulfate. The GLC analysis was a determination of menadione following hydrolysis and oxidation of the water-soluble forms. Menadione appeared in 12 min on a glass column, 6 ft x 1/8 in., packed with 1.5% SE-30 on Chromosorb W-HMDS, maintained at 130 C and a carrier gas flow of 25 ml/min. A wide variety of dosage forms was tested with analytical errors of less than ±5%. The paper by Hubbard and Sheppard (94) reported the application of the Libby and Sheppard method (88) to the measurement of menadione and menadione sodium bisulfite content of pharmaceutical products. The GLC method was compared to the National

Formulary chemical method (95). Nine capsules, tablets and injectable types were analyzed. A comparison study of the data revealed no significant differences between the results; thus the GLC method was equivalent to the NF method. However the GLC had a distinct advantage over the NF methods, in that sample processing and analysis time was reduced by approximately half. Menadione sodium bisulfite was converted to menadione, which was determined by GLC. When NF menadione standard was added to samples containing a known amount of menadione, the mean recovery and standard deviation were 98 ± 3%. The samples ranged in potency from 0.1 mg per tablet to 7.2 mg per milliliter, indicating that the GLC method has a good practical range of application. The recovery of claim for all samples was 109 ± 2.2% for the NF methods and 113 ± 3.6% for the GLC method.

Dialameh and Olson (96) have described a GLC method for the identification and quantitation of 2-tocopherol, phytyl-ubiquinone, vitamin K_1 and homologs of the vitamin K_2 (menaquinone) family. Samples used for quantitative determinations were in the range of 0.02 to 1.0 μg. This is an excellent achievement considering that they not only worked with pure compounds but also obtained samples from liver that had to undergo a series of isolation steps. Glass columns, 122 cm x 3 mm ID, packed with 3.8% silicone gum rubber (SGR UC-W-982) coated on 80-100 mesh Diatoport S were used. The columns were used under a variety of temperatures, depending on the compound(s) to be analyzed, ranging from 235 to 295 C with a carrier gas flow rate of 25 ml/min. Relative tetention times of 1.00, 1.10, 1.62 and 1.95, respectively, were obtained for 2-tocopherol, phytyl ubiquinone, phylloquinone and menaquinone at 260 C on their 3.8% silicone gum rubber column. They did not encounter the problems of thermal conversion of the quinones to chromenols and chromanols as reported by Vetter and coworkers (90). The TMS-ether of phylloquinone was not found useful because of the susceptibility of the TMS-ether derivative to hydrolysis and of the resulting hydroquinone to oxidation. Furthermore TMS-ether derivatives did not appreciably improve the usefulness of the GLC analysis of lipophilic quinones. Their method is predicated on the direct GLC of the menaquinone series without resorting to formation of derivatives as the method of choice. A position that they justified scientifically by their data is that, unless there is a decided analytical advantage to using a derivative, the analysis should be done directly. Another important aspect of their work, especially for biological studies, is that they were able to use the system on a preparative scale to collect samples for subsequent chemical investigations.

The original vitamin K_3 study of Libby and Sheppard (88) has been developed into a routine GLC assay of menadione and menadione sodium bisulfite content of pharmaceutical products (97). Ten laboratories collaboratively studied the method and compared it with the NF methods (95). The data obtained indicated that overall the NF analysis is subject to a larger variation than the GLC analysis. There was no statistically demonstrable difference ($P>0.05$) between the average results by the two methods for the three pairs of samples used in the study. There was no statistically demonstrable difference ($P>0.05$) in the precision of the two methods for products containing 1,5 and 10 mg. The analytical time for the GLC method, including sample preparation, was considerably shorter than for the NF method. This basic method is currently used by some industrial quality control laboratories. Following the collaborative study report, attempts have been made to reduce the variation of the GLC method still further (98) by the evaluation of a number of compounds for possible use as an internal standard in the GLC method for menadione and menadione sodium bisulfite. Butylated hydroxy anisole (BHA) was found a most satisfactory compound for use as an internal standard. The retention time for BHA is ca. 9 min when the menadione peak appears at 6 min. The inclusion of BHA in future collaborative studies is expected to reduce the between laboratory variation. Figure 10 is a typical menadione-BHA GLC chromatogram.

Two articles have appeared recently that summarize the earlier attempts to analyze vitamin K and our efforts toward solution of the GLC analytical problems involved (99,100). However these articles are not full reviews of the subject in general and are methods for the application of GLC vitamin K analysis to practical problems as used in our laboratory.

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