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Analysis of Vitamin K_1 in Some Green Leafy Vegetables by Gas Chromatography

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A gas chromatographic method has been developed for the assay of naturally ocurring vitamin K_1. The vitamin was extracted with hexane from the freeze-dried plant, purified on an alumina column, and assayed directly by gas chromatography. Lettuce, cabbage, and spinach were found to contain 0.51, 0.76, and 3.30 mg/100 g (dry weight basis), respectfully. Convenient levels of sensitivity for the assay were 0.05–1.0 μg although lower levels are detected.

The K vitamins have an essential but not completely understood role in the hepatic synthesis of prothrombin and proconvertin which are required for the blood clotting process. There is also evidence to indicate that K vitamins are involved in electron transfer in the oxidative chain and associated phosphorylation (Cantarow and Schepartz, 1962). Vitamin K_2 (2-methyl-3-difarnesyl-1,4-naphthoquinone), from intestinal microflora, appears to provide adequate amounts for human needs. Nutritional requirements for vitamin K_1 (2-methyl-3-phytyl-1,4-naphthoquinone) have not been established, probably because all its functions are not completely understood. An assay method for vitamin K_1 would be an important tool to use in exploring the functional and related nutritive requirements of vitamin K_1 in man. Little attention has been given analytical assays for K_1 in biological systems. Schilling and Dam's (1958) colorimetric method was applied only to alfalfa, and interfering substances could affect the accuracy of the method. Aaron and Winefordner (1972), in their publication of a phosphorimetry method for pure K_1, reviewed the literature and found few quantitative determinations of naturally occurring K_1. The comprehensive review of gas chromatography (GLC) of the fat-soluble vitamins by Sheppard et al. (1972) discussed the results of various GLC assays for K_1 and analogues. Initially, Nair and Turner (1963), working with lipid extracts and synthetic mixtures, and later Carroll and Herting (1964) and Libby et al. (1967), using synthetic mixtures, showed the feasibility of separating K_1 by GLC. A number of other workers, reviewed by Sheppard et al. (1972) and cited by Aaron and Winefordner (1972), explored the possibilities of GLC assay. Sheppard and Hubbard (1971) described a GLC method for K_1 in liver tissue but did not report any results. Problems of thermal breakdown of pure K_1 were reported by Vetter et al (1967), although they obtained excellent resolutions of their Me_4Si derivatives of the reduced K_1. The elegant work of Dialameh and Olson (1969) in separating and applying direct GLC analysis of K_1 from liver tissue showed the feasibility of using GLC for assaying naturally occurring K_1. However, their method required a long separation procedure. A simple quantitative method with reported assay results for K_1 in human foods has not been demonstrated.

The purpose of the present work was to develop a simple, sensitive assay of naturally occurring vitamin K_1. An extraction and one-step separation procedure proceeded the direct GLC assay of extracts from three commonly eaten vegetables–spinach, lettuce, and cabbage.

EXPERIMENTAL SECTION

Materials. (1) Fresh spinach, iceberg lettuce, and head cabbage were obtained from local produce markets and immediately separated into individual leaves, frozen, and freeze-dried. (2) Vitamin K_1 (2-methyl-3-phytyl-1,4-naphthoquinone) used as a standard without further purification was obtained from Sigma Chemical Co, St. Louis, MO. Its purity was checked by GLC. (3) Dotriacontane from Eastman Kodak C., Rochester, NY, was purified by recrystallization from ether and its purity checked by GLC. (4) Ether was freshly distilled before use. (5) Hexane 95% from Matheson, Coleman & Bell Division of the Matheson Co., Norwood, OH, was distilled through a 20 plate still. (6) Heptane from Eastman Kodak (EK 2215) was used

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without further purification. (7) Alumina from M. Woelm Eschwege, Germany, neutral activity Grade 1, was made Grade 3 by the addition of 8% by weight water prior to use. (8) Silica gel (Silicar cc-7) 100–200 mesh was obtained from Mallinckrodt Chemical Works, St. Louis, MO. (9) Dexsil 300GC (a 16000 to 20000 molecular weight polymer composed of units with a carborane isosahedral cage structure connected by siloxane units) is available from Analabs, Inc., North Haven, CT.

Gas Chromatography (GLC). A glass column, 2 mm i.d. × 2.1 m long, was packed with 80/100 mesh Chromosorb G (DMCS treated and acid washed) coated with 2.5% by weight of Dexsil 300GC. The column extended into the injector and detector ports forming an all-glass system. The column was operated isothermally at 290 °C. The injector was at 270 °C. The hydrogen flame detector was at 265 °C. The carrier gas nitrogen had a flow of 40 mL/min through the column at 25 °C.

Hydrogen and air flows were adjusted for maximum sensitivity. The unit, a Varian Model 1440 flame ionization gas chromatograph, was operated at 10^{-11} amp/mV with attenuation between 1 and 16 X.

Sample, Preparation, and Determination. Spinach, lettuce, or cabbage (1–2 kg) from local markets was separated into individual leaves, frozen, and then freeze-dried 30–40 h at plate temperatures of 27–32 °C at an ultimate vacuum of 100 μm in a laboratory freeze-drier (Repp Industries, Inc., Gardiner, NY). The dried leaves were crushed to form a homogeneous mixture of fine leaf particles and were protected from light and stored at −20 °C. The material obtained from subsequent extractions and separations was protected from light and kept at room temperature.

Individual samples (30 g dry weight) of spinach, lettuce, or cabbage were extracted with 750 mL of hexane in a Soxhlet extractor for 3 h and concentrated in portions in vacuo on a rotary evaporator using a 40 °C water bath. A 10-mL aliquot equal to 12 g of dry material was placed on the 2.5 cm i.d. × 25 cm long glass column containing 50 g of Woelm alumina (neutral activity, grade 1) with 8% by weight water as described by Dam and Sondergaard (1967). This aliquot yielded a good working range (50–300 μg) of K_1 for GLC analysis. The column was packed by pouring a slurry of hexane and alumina onto the column washing the walls with hexane and letting the alumina settle with a minimum covering of hexane. Material was eluted using 175 mL of the following: (1) hexane, (2) hexane containing 1.5% ether (v/v), (3) hexane containing 4% ether (v/v), and (4) hexane containing 6% ether (v/v). Different flow rates did not seem to influence the separations obtained. Each eluant was concentrated separately on a rotoevaporator using a 40 °C water bath. The material was quantitatively transferred from the rotovac flask with portions of heptane to a foil covered vial and the volume was adjusted to 500 μL. Samples were first analyzed by GLC to check for interfering peaks at the retention time of the internal standard. Then 100 μg of dotriacontane in 500 μL of heptane was added to the samples as an internal standard.

A standard curve was prepared by injecting 2 μL of standard solutions containing 0.04–2.0 μg of vitamin K_1, and plotting concentrations of vitamin K_1 against the respective peak areas (peak height times peak width at half-height). The response was linear over this range. A standard curve for dotriacontane over the range 0.05 to 0.4 μg was similarly obtained and was linear over that range. Vitamin K_1 in unknown samples was determined from the peak area of a 2-μL injection compared to the standard K_1 curve. The amount of K_1 per eluant (each of the four eluants was assayed separately) was calculated from the equation $K = K_f D V_t / D_f V_i$ where K = μg of K_1 in each eluant, K_f = μg of K_1 found in the injected sample read from its peak area on the standard K_1 curve, D = μg of dotriacontane expected in the injected sample, D_f = μg of dotriacontane found in the injected sample read from its peak area on the standard dotriacontane curve, V_t = total volume of eluant (μL), and V_i = injected volume of sample (2 μL). The total amount of K_1 found per dry weight of vegetable sample taken for assay is the sum of K_1 found in each eluant and is expressed as mg of K_1 per 100-g sample.

The effects of light exposure on vitamin K_1 were determined by exposing a solution of K_1 (1 mg/mL) in heptane in a glass vial at room temperature to the fluorescent and indirect sunlight of the laboratory. Samples were taken after 2–9 days of exposure and assayed.

Columns were eluted with hexane alone, followed by successive and increasing amounts of a polar solvent in hexane with both alumina and silica gel. The alumina column was prepared as previously described. The silica gel (Mallinkrodt Silicar cc-7) was similarly prepared. Different flow rates did not apparently influence separations of K_1. Spinach extracts and standard solutions of K_1 were used to compare benzene and ether as polar constituents of the hexane solution used for elution and purification of K_1 prior to assay by GLC. Benzene in 1.5, 4, 6, and 8% by volume in hexane was compared to ether in 1.5, 4, and 6% by volume in hexane as eluting solutions. Each column was assayed by the proposed GLC method, and total quantities of K_1 were calculated.

Alumina and Silica Gel Chromatography. Samples of K_1 from standard solutions and from spinach extracts were chromatographed on alumina and on silica gel columns then assayed to determine which would be the best column to use for quantitative work. Samples from spinach were prepared as follows. A sample (8.8 g) of dried spinach leaves was extracted with hexane (350 mL) and concentrated to dryness. A heptane solution containing 400 μg of standard K_1 was added and the volume adjusted to 25 mL with heptane. This solution was used for the following experiments. An aliquot (10 mL) was placed on an alumina column (25 g) and eluted as previously described under sample preparation with hexane followed by hexane containing 1.5, 4, and 6% ether (v/v). These eluants (A_1, A_2, A_3, and A_4) were concentrated individually and adjusted to 1-mL volumes. Samples (500 μL) taken from each of these four eluants were combined and placed on a silica gel column (20 g) and eluted as described for the alumina column giving four fractions (AS_1, AS_2, AS_3, and AS_4). The procedure was repeated with a second 10-mL aliquot from the 25-mL flask of spinach extract except that it was first chromatographed on silica gel obtaining four fractions (S_1, S_2, S_3, and S_4). Samples (500 μL) from S_1 through S_4 were combined and chromatographed on alumina obtaining four fractions (SA_1, SA_2, SA_3, and SA_4). There were thus four sets of samples A, AS, S, and SA each with four subsamples. The 16 samples were each adjusted to 1 mL volumes containing 100 μg of the internal standard dotricontane and were then assayed by the proposed GLC method. The purpose of these separations was to find the best column or column system without losing or altering K_1.

Mass Spectral Analysis. A sample of vitamin K_1 in 30 μL of heptane prepared from lettuce and previously analyzed to contain 2.4 μg of K_1 was concentrated in an argon stream to 4 μL and injected into the gas chromato-

Table I. Vitamin K_1 in Some Leafy Vegetables[a]

sample	no. of detms.	mean, mg/100 g[b]	range, mg/100 g
spinach	4	3.30 ± 0.03	3.26-3.33
lettuce	3	0.51 ± 0.05	0.47-0.56
cabbage[c]	5	0.76 ± 0.08	0.75-0.79

[a] Values reported are based on dry weight of sample. [b] Mean is followed by standard deviation. [c] Values not corrected by the internal standard.

graph under the same conditions of its previous assay except the hydrogen flame was off. At the previously determined retention time for K_1, the sample was collected in a 2 mm i.d. tapered glass tube 10 cm long protected from light and connected with Teflon tubing to the flame tip. The tube was then sealed at one end, washed with a few microliters of pentane, centrifuged, and sealed. A standard sample of K_1 (4 μg/μL of heptane) was collected in the same way.

The K_1 samples collected from the gas chromatograph were introduced via probe into a VG Micromass Model 7070F double-beam focusing mass spectrometer. Multiple spectra were obtained by electron ionization (EI) for each sample. The spectra of K_1 from lettuce was compared to both the spectra of standard K_1 and to the spectra of K_1 published by Elliot and Waller (1972).

RESULTS AND DISCUSSION

The three fresh vegetables assayed—spinach, iceberg lettuce, and head cabbage—were chosen because they are common to most American diets and were thus likely to have nutritional importance. A single homogeneous freeze-dried sample of each vegetable was prepared and portions taken for assay. Standard K_1 was not added to these samples. The internal standard dotriacontane was added after final sample preparation just before the GLC assay. The results of these assays for vitamin K_1 are given in Table I. The tracings from the chromatograph of the 1.5% ether–hexane eluant (containing most of the vitamin K_1) of these vegetables are shown in Figure 1. K_1 was also found in lesser amounts of the other eluants. Total K_1 is the sum of K_1 found in each eluant. A peak in the cabbage sample interfered with the dotriacontane internal standard so that K_1 could not be accurately corrected in that sample. The chromatograms were usually run for 45 min in which time other peaks were eluted from the column; these are not shown. No suitable standard was found for correcting for losses in extraction, purification, and concentration of the samples.

Recoveries of standard K_1 added to spinach extracts averages 92% for 80 μg added to spinach samples containing ca. 56 μg of K_1. Standard solutions of K_1 in 100- and 1000-μg amounts were recovered in yields of 90–97%. Individual assays tended to show more variation than the averaged data indicates.

A Soxhlet extraction of the freeze-dried plant leaves with hexane removed K_1 along with other materials which interfered with the GLC assay. Column chromatography using alumina or silica gel was explored as a method for purification of the extract. Dam and Lewis (1937) reported no recovery of K_1 from their alumina chromatography and concluded it was destroyed by such a strong absorbent. Good recovery of K_1 was obtained with the spinach extracts using a lower activity neutral grade III Woelm alumina with hexane–benzene solutions as elution solvents as described by Dam and Sondergaard (1967). Hexane, used alone, eluted the interfering carotenoids, leaving chlorophyll on the column while successive elutions of hexane with increasing amounts of benzene (2–8%) removed K_1 along with other components separable by GLC. Freshly distilled ether, substituted for benzene as a polar solvent, worked as well as benzene and was chosen for use in all the assays.

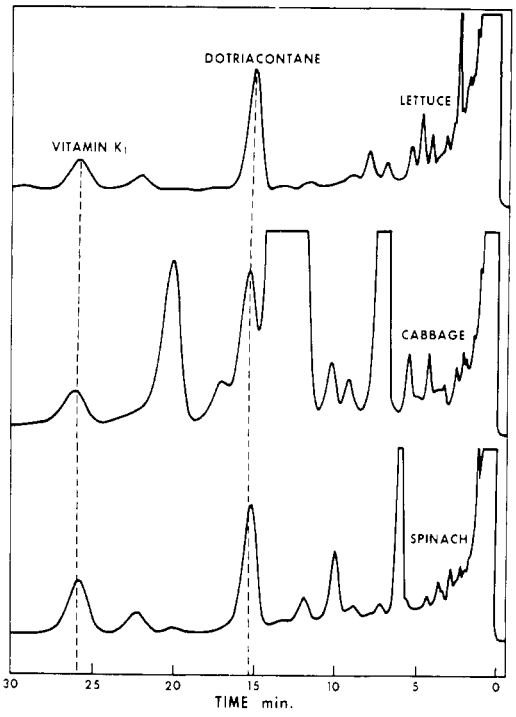

Figure 1. Gas chromatograms of extracts from lettuce, cabbage, and spinach.

Silica gel was used by Dialameh and Olson (1969) as a TLC absorbent as part of their long separation scheme for purifying an extract containing K_1 from liver tissue. A comparison of K_1 recovery in hexane extracts of spinach applied to silica gel vs. alumina showed a 29% higher recovery from alumina although standard solutions of K_1 (0.1 and 1.0 mg of K_1) averaged 92% recovery from both alumina and silica gel. An assay of 1 mg standard K_1 solution passed through alumina and then through silica gel assayed at 97% of that obtained from the original assay. Another experiment was made to determine the effects on and recoveries of K_1 from the two columns. Four sets of samples were obtained: K_1 through (1) alumina, (2) silica gel, (3) alumina, then silica gel, and (4) silica gel, then alumina. The GLC assays of these fractions showed that 90% of the K_1 was recovered from alumina chromatography and 92% from silica gel. Rechromatographed material from alumina through silica gel gave 87% recovery while 83% recovery was found for material chromatographed first through silica gel, then alumina. The major portion was found in the 1.5% ether–hexane fraction. Recovery through either column was about the same. Multiple chromatography seemed to offer no advantages in purification. Alumina chromatography tended to give a cleaner mixture for GLC separation of the spinach extract, so alumina was chosen for purifying all the leafy vegetables assayed.

Although samples were protected from light and stored in the freezer, there was some light exposure at room temperature during sample preparation and concentration. A 2-day exposure to laboratory light conditions at room temperature resulted in neglibible loss of K_1, while exposure for 9 days resulted in a 50% loss. Therefore, the conditions of preparation and assay should not result in losses due to light or heat exposure. Some samples protected from light and stored in a freezer were determined 4 months

later after their initial assay and had not deteriorated.

Proof of identity of the K_1 peak from GLC was shown by collection of the peak from a lettuce sample for mass spectral determination. The spectra from a lettuce sample agreed with the spectra obtained from a standard sample of K_1 similarly collected and the spectra published by Elliot and Waller (1972). Vitamin K_1 (MW 450.68) is converted to the hydroquinone form during its introduction via probe into the mass spectrometer. The molecular ion 452 and the M − 1 ion 451 were found for the standard K_1 and the lettuce sample. Some spectral scans showed an impurity with the parent at 425 although the major portion of the sample was K_1. The impurity was not identified but the method of sample collection, i.e., the lower temperature of the flame tip compared to the column temperature could well allow peak spreading and thus contamination of the K_1. Temperature programming and different column temperatures for the GLC assay showed only one peak for the vitamin.

Two GLC peaks for K_1 were observed when samples of standard K_1 passed through alumina or silica gel were combined with standard K_1 which had not been through either of these absorbents. The retention times were extremely close and separation was minimal. Apparently the K_1 changes on column chromatography. The change does not affect the results of the assay. The author has no explanation for the difference in retention time for the column chromatographed K_1 vs. the nonchromatographed compound.

The direct GLC method eliminated problems of hydrolysis and multiple peak formation often encountered in using Me_4Si–ether derivatives. There was no thermal breakdown of chromatographed K_1 as experienced by Vetter et al. (1967) or Libby et al. (1967). No extraneous peaks were observed. The quantitative sensitivity for the method as used was about 0.05 μg for K_1, although detection levels were lower. The Dexsil 300GC used to coat the Chromosorb G proved to be very stable; no breakdown was seen over 6 months use.

Spinach, lettuce, and cabbage—common to the American diet—were assayed for K_1 to demonstrate this method of general feasibility and relative simplicity. The assay of other material may require modifications from the procedure used here. The method eliminates a series of elaborate isolation steps and was quantitative for the samples assayed. The identity of the peak measured for K_1 was confirmed by mass spectral analysis.

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