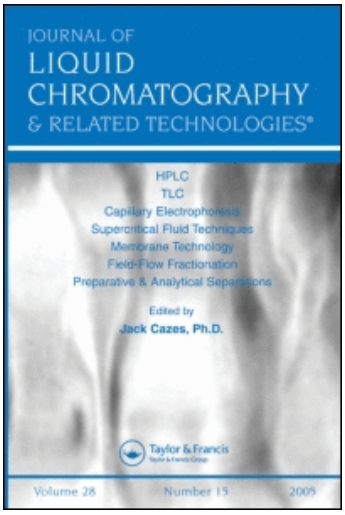

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Silver Ion as Modifier for High Performance Liquid Chromatography of Menaquinones

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SILVER ION AS MODIFIER FOR HIGH PERFORMANCE LIQUID CHROMATOGRAPHY OF MENAQUINONES

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ABSTRACT

The chromatography of the homologous series of menaquinones with 0 to 10 isoprenoid units on reverse phase high performance liquid chromatography is investigated with octyldecylsilica and C-8 silica supports. Addition of chloroform decreases retention (k') of all menaquinones and vitamin K_1 linearly with respect to log k'. The addition of silver ion decreases k' of menaquinones with 3 or more isoprenoid units with a non-linear relationship to log k'. There is a linear relationship at a given silver ion concentration between log k' and the number of isoprenoid units. There is no effect of silver ion on vitamin K_1, although there is one double bond in the side chain.

INTRODUCTION

Vitamin K is responsible for the carboxylation of specific glutamic acid residues on a precursor protein to yield a γ-carboxyglutamic acid (1). The vitamin K requirement can be met *in vivo* and *in vitro* by several 2-methyl-3-polyisoprenoid-1,4-naphthoquinones (2,3). The menaquinone series (Fig. 1) has, at the 3 position, a polyisoprenoid chain with all isoprenoid units unsaturated. The number of isoprenoid units can range from the synthetic menadione (n=o) to the naturally occurring, n=1 inclusively, up to n=12. Vitamin K_1, the vitamin K found in plants, has 4 isoprene units with the last 3 units saturated. The

similarity in the biological and chemical properties of K_1 and the MK-ns make separation, identification and quantitation difficult. The established biological and spectrophotometric procedures used to quantitate the vitamin K's cannot be used to identify a specific vitamin K or to quantitate a given vitamin K in a mixture of vitamin K's. Procedures to identify and quantitate the vitamin K's are necessary for studies on the metabolism of vitamin K. Reverse phase HPLC is a logical choice for the separation and quantitation of vitamin K's. Previous applications of HPLC to vitamin K quantitation have been restricted to a limited number of the naturally occurring vitamin K's (4,5). This paper studies the effectiveness of non-aqueous reverse phase HPLC on ODS and C-8 columns to separate the menaquinones (MK-n, n=0 to 10) and vitamin K_1. The hydrophobicity of MK-n, $n \geq 7$ is so large that elution from a Zorbax ODS column is not achieved with 100% methanol. A study was undertaken to determine the effectiveness of two different types of mobile phase modifiers to yield a mobile phase capable of eluting all vitamin K compounds. One modifier studied is chloroform, which generally decreases retention time (k') on an ODS column by decreasing the polarity of the mobile phase and thereby increasing the solubility of the non-polar solute into the mobile phase. The second modifier studied is silver ion which can reduce k' of non-conjugated unsaturated compounds by complexing with the double bond (6), thereby reducing the hydrophobic interaction with the ODS support. Argentation chromatography has been successfully used for the thin-layer chromatography of many alkenes, including

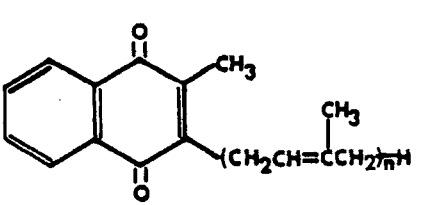

FIGURE 1. Structure of menaquinone (MK-n).

several of the menaquinone homologues (7). Application of argentation chromatography to HPLC has suffered from problems encountered in preparation of a stable support containing silver ion. Silver ion has been complexed to cation resins (8), impregnated into silicate (9-10), or bonded to aluminosilicate (12) in attempts to prepare a silver ion support suitable for HPLC. Such silver supports suffer from column deterioration, large particle size, or little control of the silver content. An alternate approach to argentation HPLC is the addition of silver ion to the mobile phase of reverse phase HPLC. Reverse phase argentation chromatography eliminates the problems associated with preparation of a silver support and permits the use of the materials and techniques used in reverse phase HPLC without modification. Reverse phase argentation HPLC has been applied to the separation of a few types of compounds containing double bonds (6,13-17). Silver ion is potentially well suited as a modifier to decrease the k' of the longer chain menaquinones, which contain one double bond in each isoprenoid unit of the side chain. The effects of chloroform and silver ion on the elution of the selected vitamin K compounds have been investigated first on an ODS column and then on a C-8 column.

MATERIALS AND METHODS

Apparatus

The chromatograph consisted of a DuPont Model 860 pump with an Isco model UA-5 absorbance monitor with 8 µl (2 mm path length) flow through cells and a Rheodyne model 7120 valve with 50 µl sample loop.

Reagents

Methanol (HPLC grade) and silver nitrate were obtained from J.T. Baker. Chloroform (HPLC grade) was obtained from Fisher Scientific. Vitamin K_1 was obtained from Sigma Chemical Co. and menadione was from ICN Biochemicals. The menaquinones, MK-1 through MK-10, were the generous gift of U. Gloor and F. Leuenberger, Hoffman-LaRoche & Co., Basle, Switzerland. 2,3-dimethyl-1,4-

naphthoquinone was obtained from Aldrich Chemical Co. All vitamin K compounds were purified by chromatography on the Zorbax ODS column prior to use.

Chromatography

A prepacked Zorbax ODS and a Zorbax C-8 column with a 7 micron particle size and dimensions of 4.6 mm ID x 25 cm were obtained from DuPont. The flow rate was 4 ml/min. Column temperature was ambient. Detection was at 254 nm.

RESULTS AND DISCUSSION

Initial attempts to obtain a good chromatogram of just menadione, MK-3 and K_1 on a Zorbax ODS column with methanol-water mixtures were not successful. The K_1 was either retained too long on the column (k' > 20) or not eluted at all. The totally porous nature and high carbon content of Zorbax ODS leads to a greater retention of the vitamin K compounds than observed for the pellicular support, Permaphase ODS (4). It was soon evident that non-aqueous mobile phases would have to be employed. Methanol, without modifiers, cannot elute MK$\geq$7. Methanol, without a modifier, is also not practical for MK-5 and MK-6 or K_1, as k' is greater than 12 (Table 1). The saturation at the 3 end isoprenoid units of K_1 is seen to dramatically increase the retention time of K_1 over that of MK-4, which also has 4 isoprenoid units. Changing the mobile phase from methanol to acetonitrile served only to increase k'. This is consistent with the solubility data of Rohrschneider (18) in that the non-polar solute, octane, is more soluble in methanol than in acetonitrile.

The desired separation of the homologous MK series requires the addition of a mobile phase modifier that will bring the k' of MK-10 close to the ideal upper limit of 10. This must be done without lowering the k' of the lower MK's very much below that of 1.0. The addition of chloroform to methanol was investigated as to its suitability to lower the k' of the longer chain MK's (Table 1). Increasing the non-polar characteristic of the mobile phase

by the addition of chloroform, as expected, decreased the k' of the MK's and K_1. The plot of log k' versus the concentration of chloroform (Fig. 2) gives a linear plot for all MK's and K_1. The relative position of K_1 between MK-5 and MK-6 is not affected by the chloroform concentration. There is a linear relationship between the number of isoprenoid units (n) and log k' at a given chloroform concentration (Fig. 3) for MK-1 through MK-10. Menadione does not have a 3 position side chain and thus, would be expected to be more polar than compounds with a 3 position side chain. The difference in k' of MK-0 through MK-10 is due to the interaction of the support with the 3 position side chain and thus the k' of MK-0 would not be related to the 3 position side chain. The difference between the observed k' of MK-0 and that predicted if the line in Fig. 3 were continued to zero (log k = 0.1) is an indication of the decrease in polarity by substituting R for the H in MK-0. This is substantiated by the k' of 2,3-dimethyl-1,4-naphthoquinone (k' = 0.7, log k' = 0.11). The usefulness of

TABLE 1

Effect of Chloroform on k' of Menaquinone on Zorbax ODS with Methanol Mobile Phase

	% Chloroform				
	0	15	20	25	30
K_1	14.50	4.61	3.45	2.65	1.97
MK-0	0.64	0.39	0.39	0.39	0.34
MK-1	1.39	0.81	0.71	0.65	0.59
MK-2	2.31	1.10	0.97	0.87	0.81
MK-3	3.92	1.65	1.42	1.16	1.00
MK-4	7.14	2.68	2.06	1.68	1.31
MK-5	12.89	4.06	3.03	2.32	1.72
MK-6	23.44	6.23	4.42	3.19	2.34
MK-7		8.81	6.39	4.45	3.09
MK-8		13.71	9.23	6.13	3.97
MK-9		21.13	13.47	8.48	5.28
MK-10		32.48	19.26	11.65	6.97

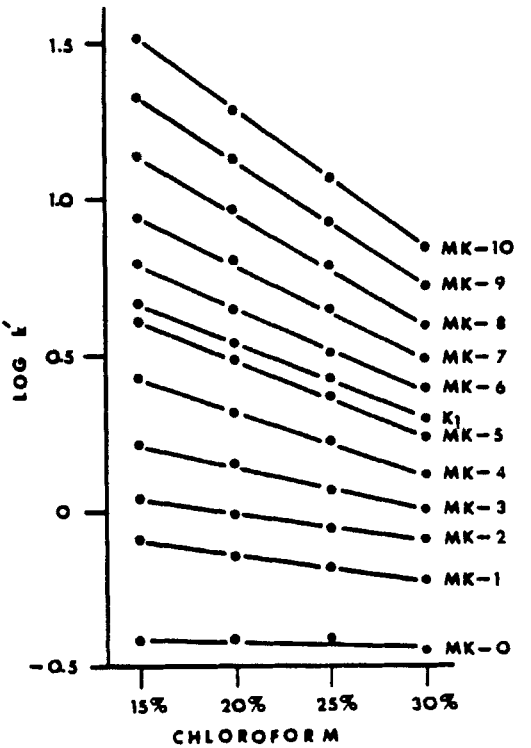

FIGURE 2. Log k' versus percent of chloroform in the methanol mobile phase for menaquinones on Zorbax ODS.

chloroform to obtain a good chromatogram of the MK-n, n=0 to 10, however, is questionable due to the jamming together of the lower MK-n ($n \leq 3$) as the amount of chloroform is increased. A modifier "better" than chloroform is clearly needed.

The "best" modifier to obtain separation of the homologous MK series would be one that decreases the k' of the higher members of the series proportionally more than the lower chain members, thereby decreasing the k' of MK-10 to around 10, while decreasing the k' of MK-0 to 3 only slightly. All non-polar modifiers would be expected to give results similar to those obtained with chloroform. A modifier that would be worthwhile to test is one that

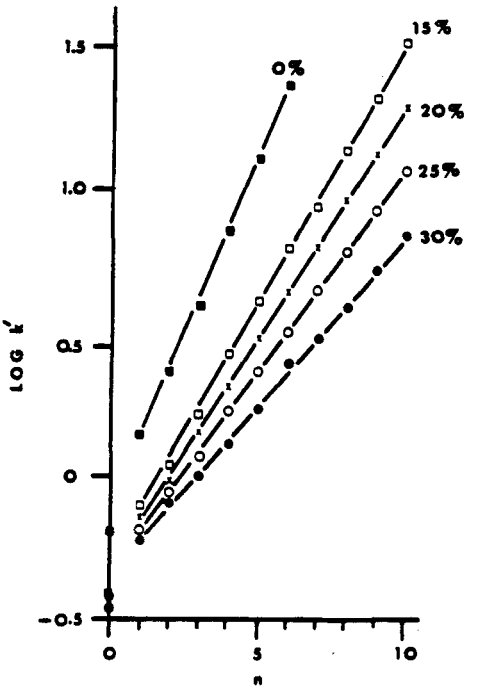

FIGURE 3. Effect of chloroform on log k' versus number of isoprenoic units (n) in the menaquinone side chain on Zorbax ODS.

would be expected to change k'of the menaquinones by means other than increasing the non-polar characteristic of the mobile phase. Silver ion is known to complex with double bonds that are not conjugated or sterically hindered (6). The use of silver ion as a mobile phase modifier has been previously reported (6,13-17) for compounds containing a few non-conjugated double bonds. The effect of silver ion on k' of the MK series would be expected to be greater on the higher members of the menaquinone series as they contain more double bonds than do the MK-n, n=0 to 3.

The effect of various concentrations of silver ion from 5 mM to 50 mM on k' of the MKs and K_1 were tested with methanol as the mobile phase (Table 2). The expected lowering of k' of MK=7 to

TABLE 2

Effect of Silver Ion on k' of Menaquinones on Zorbax ODS with Methanol Mobile Phase

	mM Silver Nitrate					
	5	10	20	30	40	50
K_1	14.06	13.13	13.05	13.87	14.00	14.32
MK-0	0.58	0.55	0.60	0.53	0.68	0.70
MK-1	1.33	1.16	1.15	1.08	1.30	1.36
MK-2	2.19	1.90	1.83	1.74	1.83	1.85
MK-3	3.64	3.16	2.65	2.28	2.23	2.08
MK-4	6.06	4.92	3.53	2.81	2.58	2.30
MK-5	10.00	7.63	4.90	3.63	3.18	2.75
MK-6	16.80	11.58	7.05	4.81	3.95	3.25
MK-7	27.39	17.37	9.35	6.29	4.75	4.05
MK-8				8.37	6.10	4.55
MK-9				11.10	7.68	5.48
MK-10				12.94	9.30	6.40

10 was observed as the concentration of Ag^+ reached 30 mM. There is no decrease in k' of MK-0 as $[Ag^+]$ is increased since there is no non-conjugated double bond with which the silver ion can complex. There is also little decrease in k' of MK-1 and K_1, possibly due to steric hinderance of the naphthoquinone ring. Thus, unlike with chloroform, the relative position of K_1 with respect to the other Mk's does change as the $[Ag^+]$ is changed. The slight effect of silver ion on k' of MK-0 to MK-3 results in a greater resolution of MK$\leq$3 at a $[Ag^+]$ which gives a k'=10 for MK-10 than obtained with the methanol-chloroform phases (Tables 1 & 2, Fig. 4, A & B).

The increase in k' of MK-0, 1,2 and K_1 at 40-50 mM $[Ag^+]$ was not expected and may be due to either a slight increase in the non-polarity of the mobile phase by the silver nitrate or by an interaction with the naphthoquinone ring making it less polar.

The relationship of log k' versus $[Ag^+]$ (Fig. 5) is linear for most of the MK-n for only a short concentration range. The non-linearity is probably due to approaching the maximum effect possible for a given MK-n. If a situation is produced at a given concentration of $[Ag^+]$ where all of the double bonds in a MK-n are

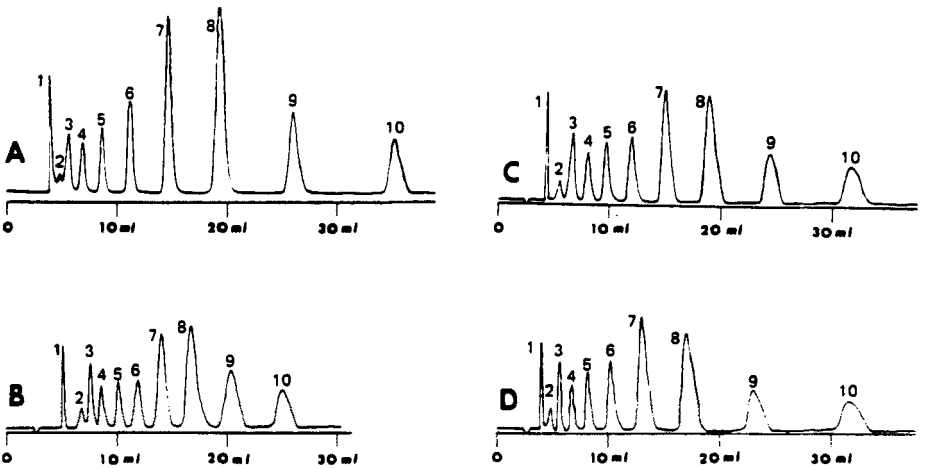

FIGURE 4. Selected chromatograms. A mixture of MK-1 to MK-10 was chromatographed under conditions to obtain a k' of approximately 10 for MK-10. The peak number is n, the number of isoprenoid units in the menaquinone. A, Zorbax ODS column with methanol-chloroform (75/25). B, Zorbax ODS column with 40 mM silver nitrate in methanol. C, Zorbax ODS column with 7.5 mM silver nitrate in methanol-chloroform (85/15). D, Zorbax C-8 column with 10 mM silver nitrate in methanol.

fully complexed with silver ion, then increasing [Ag^+] would not have any further effect on k' due to interaction of silver ion with a double bond. The secondary effect of silver ion to increase k' of the lower members of the MK-n series may have only a slight contribution. As for those MK-ns whose k' does increase at the higher [Ag^+], there is a leveling off of the k' before the increase is observed. The relationship for the homologous MK series at a given silver ion concentration is linear with respect to log k' (Fig. 6) for the number of isoprenoid units (n) in the side chain. The only deviation from linearity is seen with MK-1 where the decrease in k' is observed at low [Ag] concentration with essentially no effect on increasing [Ag^+] until 30 mM when k' starts to increase. The k' for MK-0 is not plotted as it cannot be considered like the other MK-ns in its reaction to silver ion, since there are no non-conjugated doubles with which the silver ion can complex.

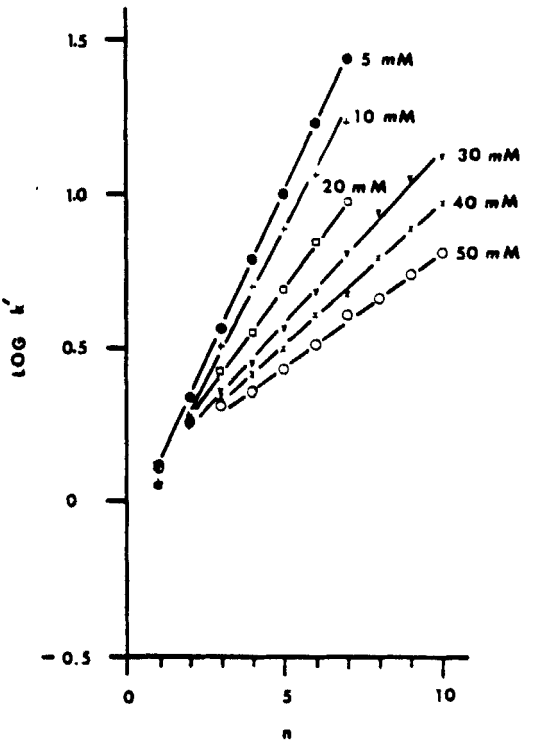

FIGURE 5. Log k' versus silver ion in the methanol mobile pahse for menaquinones on Zorbax ODS.

The possibility of utilizing both chloroform and silver ion as modifiers of the mobile phase was determined (Table 3). The effect of varying [Ag^+] even slightly had a dramatic effect on the k' of MK-6 through MK-10. The effect of both chloroform and silver ion was slightly synergistic. The silver ion double bond complex has a lessened affinity for the ODS support than does the non-complexed double bond material. A non-polar modifier such as chloroform, which decreases the polar interaction of solute with the ODS support, would be expected to decrease the k' of the more polar silver ion complex more than the non-complexed solute. The plot of log k' versus n (Fig. 7) once again gives a straight line.

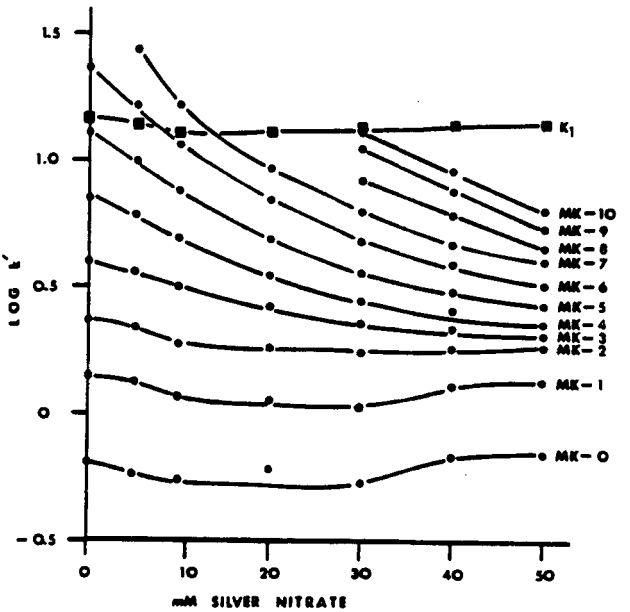

FIGURE 6. Effect of silver ion on log k' versus number of isoprenoic units (n) in the menaquinone side chain on Zorbax ODS.

TABLE 3

Effect of Silver Ion on k' of Menaquinones on Zorbax ODS with Chloroform/Methanol (15/85) Mobile Phase

	mM Silver Nitrate		
	5	7.5	10
K_1	4.77	4.65	4.84
MK-0	0.42	0.45	0.45
MK-1	0.87	0.87	0.87
MK-2	1.16	1.16	1.10
MK-3	1.68	1.61	1.52
MK-4	2.32	2.06	1.87
MK-5	3.19	2.71	2.39
MK-6	4.32	3.52	3.00
MK-7	5.94	4.61	3.81
MK-8	8.06	6.00	4.74
MK-9	11.13	7.94	6.06
MK-10	15.52	10.55	7.77

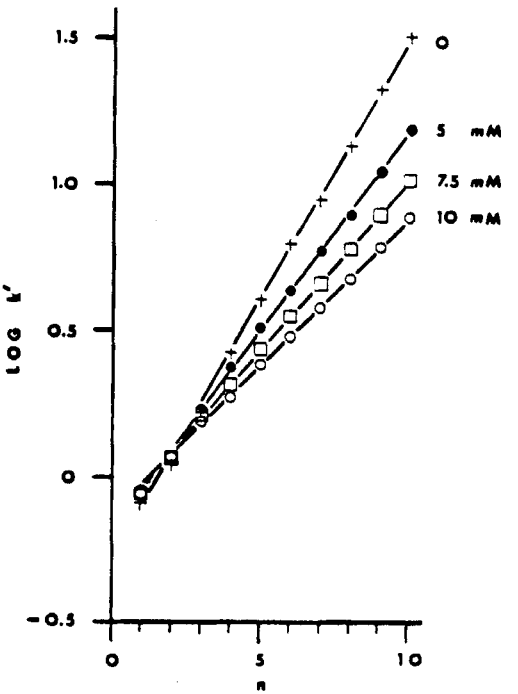

FIGURE 7. Effect of silver ion on log k' versus number of isoprenoid units (n) in the menaquinone side chain on Zorbax ODS with methanol-chloroform (85/15).

The combined use of a non-polar modifier with silver ion as a modifier is of value in identifying specific vitamin K's in a biological sample. The treatment of biological samples by chloroform or other non-polar solvents to extract vitamin K's will also extract numerous other lipid materials. The capability of changing the k' of the MK-n series by making the mobile phase more non-polar and/or by forming a silver ion complex with double bonds will allow the chromatographer to separate away the MK peaks from other lipids.

The possibility that a C-8 bonded silica gel would give better separation of the homologous series of menaquinones was investigated (Table 4). The retention of the menaquinone is less on the

TABLE 4

Effect of Silver Ion on k' of Menaquinones on Zorbax C-8 with Methanol Mobile Phase

	mM Silver Nitrate						
	0	5	10	15	20	25	30
K_1	3.15	3.18	3.18	3.18	3.18	3.24	3.27
MK-0	0.42	0.36	0.36	0.36	0.36	0.36	0.36
MK-1	0.48	0.49	0.52	0.52	0.54	0.52	0.52
MK-2	0.85	0.85	0.85	0.82	0.85	0.82	0.82
MK-3	1.27	1.21	1.12	1.00	0.97	0.97	0.97
MK-4	1.82	1.73	1.48	1.24	1.15	1.06	1.00
MK-5	2.78	2.48	2.00	1.61	1.42	1.24	1.06
MK-6	4.30	3.58	2.70	2.12	1.79	1.52	1.30
MK-7	6.70	5.21	3.70	2.79	2.24	1.88	1.58
MK-8	10.58	7.62	5.09	3.64	2.82	2.27	1.85
MK-9	16.79	11.36	7.12	4.82	3.58	2.79	2.22
MK-10	27.09	17.12	10.12	6.45	4.61	3.52	2.70

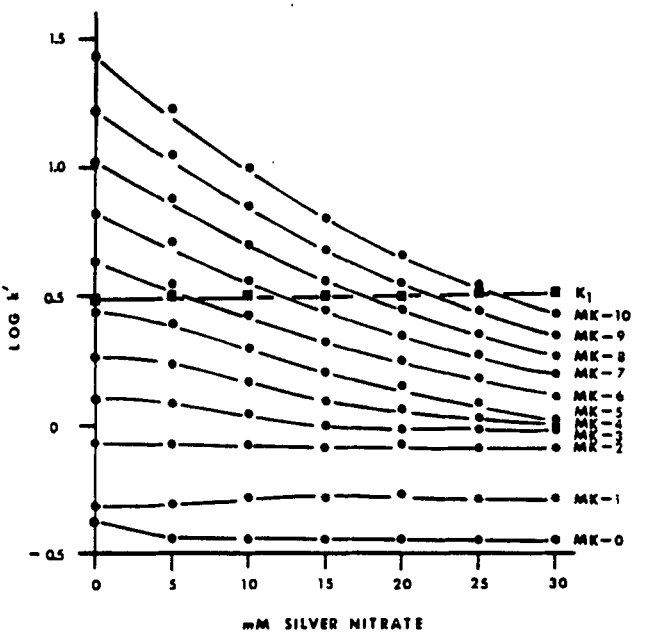

FIGURE 8. Log k' versus silver ion in the methanol mobile phase for menaquinones on Zorbax C-8.

C-8 support than on the ODS support. The MK-7 to MK-10 are eluted by methanol, but the MK-9 and MK-10 peaks are late and very broad. The effect of silver ion for the C-8 column is similar to that observed on the ODS column (Table 4). The plot of log k' versus concentration of silver nitrate is curved for all menaquinones (Fig. 8), except for MK-0, MK-1, MK-2 and K_1 which are minimally affected by silver ion. The plot of log k' versus the number of isoprenoid units (Fig. 9) is linear until the log k' for a given menaquinone approaches an apparent minimum.

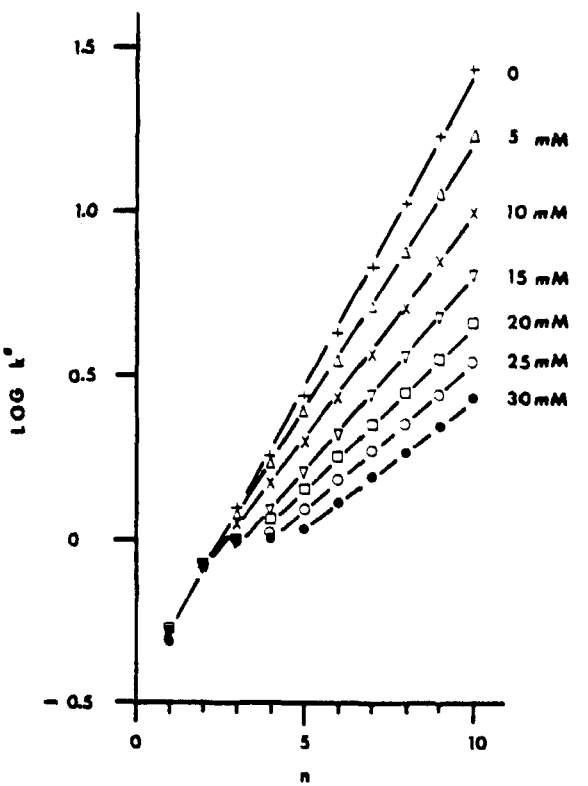

FIGURE 9. Effect of silver ion on log k' versus number of isoprenoid units (n) in the menaquinone side chain on Zorbax C-8.

The ODS and C-8 columns can both be used to separate the menaquinone series by adding silver ion to the mobile phase. There is no major difference between C-8 and ODS columns that would warrant recommending either column over the other. The C-8 column does require less silver nitrate to obtain a k' of $\leq$10 for MK-10 than does the ODS which may be of economic import. The addition of chloroform to the mobile phase, however, decreases the silver ion requirements for the ODS column making its behavior very similar to the C-8 column (Fig. 4, C & D). The technique of adding silver ion to the mobile phase is of definite practical importance to those interested in polyisoprenoids such as the menaquinone series. This technique is also potentially useful for studies on similar polyisoprenoids, such as the ubiquinones and the long chain polyisoprenoid alcohols.

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REFERENCES

1. Stenflo, J., Fernlund, P., Egan, W. and Roepstorff, P., Vitamin K Dependent Modifications of Glutamic Acid Residues in Prothrombin, Proc. Nat. Acad. Sci. USA, 71, 2730, 1974.

2. Matschiner, J.T. and Taggart, W.V., Bioassay of Vitamin K by Intracardial Injection in Deficient Adult Male Rats, J. Nutr., 94, 57, 1968.

3. Friedman, P.A. and Shia, M., Some Characteristics of a Vitamin K-Dependent Carboxylating System from Rat Liver Microsomes, Biochem. Biophys. Res. Commun., 70, 647, 1976.

4. Elliott, G.R., Odam, M. and Townsend, M.G., An Assay Procedure for the Vitamin K_1 2,3-Epoxide-Reducing System of Rat Liver Involving High-Performance Liquid Chromatography, Biochem. Soc. Transactions, 4, 615, 1976.

5. Donnahey, P.L., Burt, V.T., Rees, H.H., and Pennock, J.F., High-performance Liquid Chromatography of Menaquinone-4,2,3-epoxymenaquinone-4, Demethyl-menaquinone-4 and Related Compounds, J. Chromatogr., 170, 272, 1979.

6. Vonach, B. and Schomburg, G., High-performance Liquid Chromatography with Ag^+ Complexation in the Mobile Phase, J. Chromatogr., 149, 417, 1978.

7. Sommer, P. and Kofler, M., Physicochemical Properties and Methods of Analysis of Phylloquinones, Menaquinones, Ubiquinones, Plastoquinones, Menadione, and Related Compounds, Vitamins Hormones, 24, 349, 1966.

8. Merritt, M. and Bronson, G.E., Determination of 5-trans-prostaglandin E_2 in Prostaglandin E_2 via High Performance Liquid Chromatography of Their p-Nitrophenacyl Esters on a Silver Ion-loaded Cation Exchange Resin, Anal. Chem., 48, 1851, 1976.

9. Aigner, R., Spitzy, H. and Frei, R.W., Some Properties of Silver-Loaded Silica Gel Supports for Liquid Chromatography, Anal. Chem., 48, 2, 1976.

10. Aigner, R., Spitzy, H. and Frei, R.W., Separation of Drug Substances by Modern Liquid Chromatography on Silver Impregnated Silica Gels, J. Chromatogr. Sci., 14, 381, 1976.

11. Heath, R.R., Tumlinson, J.H. and Doolittle, R.E., Analytical and Preparative Separation of Geometrical Isomers by High Efficiency Silver Nitrate Liquid Chromatography, J. Chromatog. Sci., 15, 10, 1977.

12. Lam, S. and Grushka, E., Silver Loaded Aluminosilicate as a Stationary Phase for the Liquid Chromatographic Separation of Unsaturated Compounds, J. Chromatog. Sci 15, 234, 1977.

13. Janak, J., Jagaric, Z. and Dressler, J., Use of Olefin-Ag^+ Complexes for Chromatographic Separations of Higher Olefins in Liquid-Solid Systems, J. Chromatogr., 53, 525, 1970.

14. Schomburg, G. and Zegarski, H., Separation of Olefinic Compounds by Reversed-Phase Liquid Chromatography with a Mobile Phase Containing π-Complexing Metal Salts Like Silver Nitrate, J. Chromatogr., 114, 174, 1975.

15. Weber, D.J., High-Pressure Liquid Chromatographic Separation of 5,6-cis- and Trans-Prostaglandin A_2, J. Pharm. Sci., 66, 744, 1975.

16. Tscherne, R.J. and Capitano, G., High-Pressure Liquid Chromatographic Separation of Pharmaceutical Compounds Using a Mobile Phase Containing Silver Nitrate, J. Chromatogr., 136, 337, 1977.

17. de Ruyter, M.G.M. and de Leenheer, A.P., Effect of Silver Ions on the Reversed Phase High Performance Liquid Chromatographic Separation of Retinyl Esters, Anal. Chem., 51, 43, 1979.

18. Rohrschneider, L., Solvent Characterization by Gas-Liquid Partition Coefficients of Selected Solutes, Anal. Chem., 45, 1241, 1973.