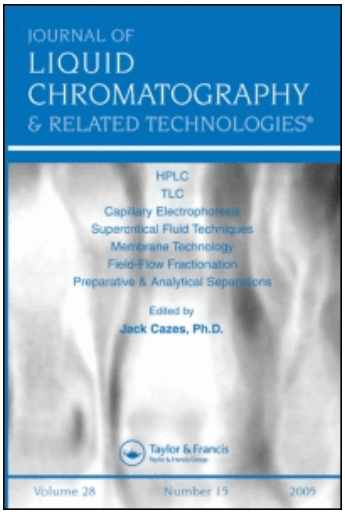

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Separation of Bacterial Menaquinones by HPLC Using Reverse Phase (RP18) and a Silver Loaded Ion Exchanger as Stationary Phases

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SEPARATION OF BACTERIAL MENAQUINONES BY HPLC USING REVERSE PHASE (RP18) AND A SILVER LOADED ION EXCHANGER AS STATIONARY PHASES

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ABSTRACT

Bacterial menaquinones were separated isocratically on a reverse phase Li Chrosorb RP18 5 µm and a silver loaded ion exchanger.
On octyldecylsilica support the separation of the menaquinones depends on their lipophilic character, on the silver column mainly on the number of double bonds in the isoprenyl chain. Comparing the runs of both columns the menaquinones were easily differentiated.

INTRODUCTION

Menaquinones (2 methyl 3 polyisoprenyl 1,4 naphthoquinones) play an important role in the bacterial electron transport, oxidative phosphorylation (10) and formation of endospores (5). The menaquinones are not evenly distributed among bacteria. They may vary in the length of their isoprenyl side chain and the degree of saturation. This variation is taxa specific. The value of menaquinones in bacterial taxonomy was clearly shown by Yamada (11), Minnikin (9), and Collins (3).

For the separation and identification of the different menaquinone species, various methods were employed. Thin layer chromatography on different layers (4), mass spectrometry (9), and gas chromatography after hydrogenation (2). Recent reports point up the value of high performance liquid chromatography for the separation of menaquinones. In this regard, reverse phase RP8 as stationary phase and methanol as mobile phase proved very effective (Collins, personal communication).
Also, RP18 and dichloromethane/acetonitrile (6) and RP18 and methanol/chloroform with silver ions as modifier (8) have been used sucessfully. Similar substances have been separated on silver loaded silicagel supports (1).

For routine analyses these methods have some disadvantages. The mobile phases containing silver nitrate are quite corrosive and have a high absorbance, and the silver of the silver impregnated silicagel bleeds from the support after some time, leading to a reduction of the retention time of the menaquinones. The separation of the ordinary menaquinones (menaquinones with isoprenyl side chains lacking saturated isoprenyl units) is sufficient with reverse phases (RP8 and RP18). However, these reverse phases cause problems in the separation of natural mixtures of menaquinones with isoprenyl side chains differing in degree of saturation and chain length. I now report the separation and identification of diverse menaquinone mixtures using stationary phases of different separation behaviours.

MATERIAL AND METHODS

The method of isolation of menaquinones has been described previously (7). Menaquinone mixtures of fully unsaturated menaquinones were obtained from Dr. D. Collins (Reading G.B.). Partly saturated menaquinones were iso-

lated from bacterial strains in which the type of menaquinone was known from the literature. Their identities were varified by mass spectrometry. Cis-menaquinones which arise by photoisomerisation were identified and isolated from the naturally occuring trans-menaquinones by silicagel thin layer chromatography using hexane/dibutylether 85:15 v/v as mobile phase (12). The chromatogramms shown in fig. 1 were obtained by injecting 1-5 µl of the menaquinone mixture in 2-chlorobutane. The following conditions were employed: Apparatus Desapro Milton Roy mininpump simplex with pulse dampening device and a Rheodyne 7125 injection valve. The stainless steel column 250x4 mm (Merck 50333) was filled with Li Chrosorb RP 18 5 µm. It was protected by a precolumn (Brownlee Labs. MPLC RP18) and kept at 30° C with a column oven (Jones Chromatography). The flow rate was 1 ml/min acetonitrile/tetrahydrofurane 70:30 v/v (Promochem Chrom AR), at a pressure of 80 atm. A Uvicord S detector (LKB) was used at 254 nm with a sensivity set at 0,005 abs. range. The chart speed was 1 cm/min using a Kontron W+W 600S recorder. The separations presented in fig. 2 were obtained by injecting 1 to 20 µl of the menaquinone mixture in methanol. The following conditions were employed: Apparatus Perkin Elmer LC-pump (series 1) with Rheodyne 7105 injection valve and stopflow fittings. The stainless steel silver column (250x4,6 mm i.d.) was custom-packed with silver loaded Nucleosil^{R} 10SA (Macherey, Nagel & Co, Düren F.R.G.) by Chrompack Nederland, Middleburg, The Netherlands. It was kept at 65° C by use of a precision thermostat (Lauda) and protected by a column inlet filter (Rheodyne). Methanol (Merck 6007) was used as eluat at a flow rate of 1,5 ml/min and 40 atm pressure. A Perkin Elmer LC-75 UV-detector with autocontrol was used at 269 nm, with sensivity set at 0,02 abs. range. The chart speed was 0,5 cm/min with a Varian A-25 recorder.

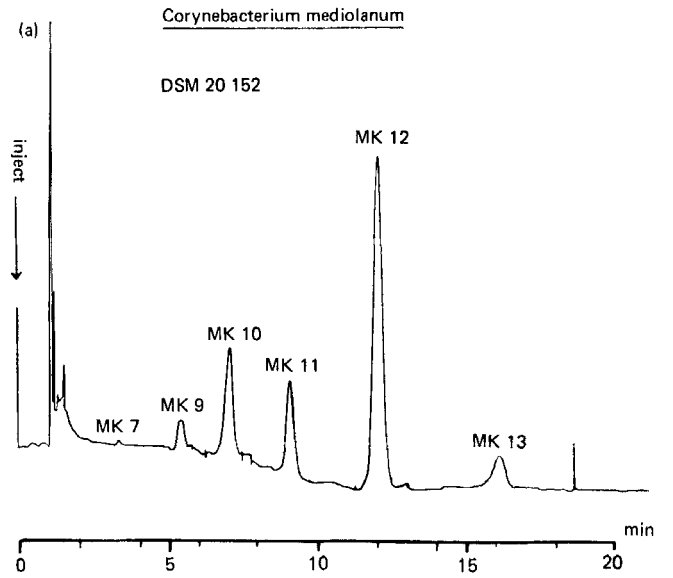


FIGURE 1A

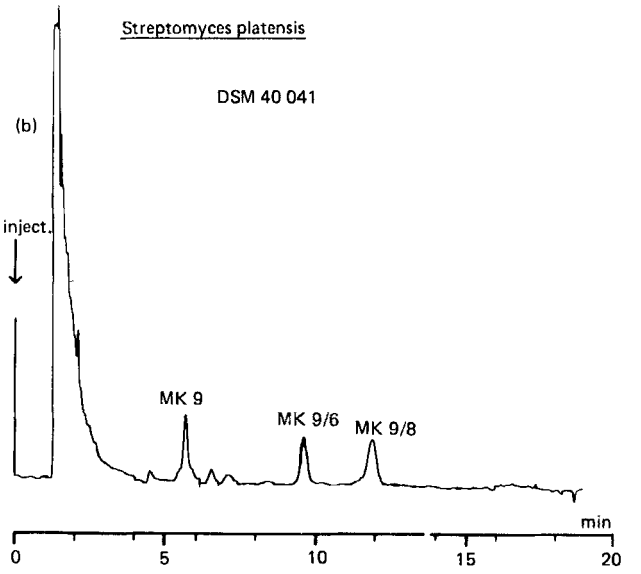


FIGURE 1B

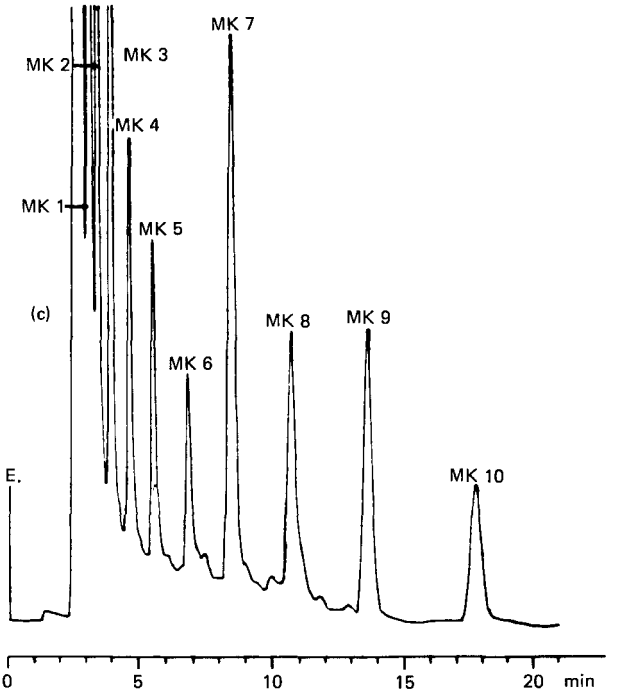


FIGURE 1

Separation of menaquinones (MK) on 5 µm Li Chrosorb^{R} RP18, column 250x4 mm, mobile phase ACN/THF, 70:30 v/v, flow rate 1 ml/min, temperature 35^{o} C, sample size 5 µl.

(a) menaquinones with an ordinary isoprenyl side chain (7-13 isoprenyl-units)

(b) menaquinones with nine isoprenyl units, three (MK9/6) or four (MK9/8) of them beeing saturated; MK9 internal standard

(c) menaquinone standard mixture MK1-MK10, conditions as before except the column, Beckman Ultrasphere ODS 250x4,6 mm

+DSM Deutsche Sammlung von Mikroorganismen (German Collection of Microorganisms)

RESULTS AND DISCUSSION

The best resolution of menaquinone homologues were obtained by separation on RP18 phase with acetonitrile/tetrahydrofurane 70:30 v/v at a flow rate of 1 ml/min. Homologues differing in number of isoprenyl units (MK1 to MK13, fig. 1a and 1c) as well as those with the same chain length but different degree of saturation (MK9(H6), MK9(H8), fig 1b) could be well separated. The theoretical plate heigth for MK9(H2) was 27 µm (9300 plated per 25 cm column). Under these conditions the loss of one double bond in the isoprenyl side chain resulted in an increase in retention time equivalent to 0,7 of an additional isoprenyl unit. This separation behaviour caused problems in differentiating menaquinones with a partly unsaturated isoprenyl side chain from those with a fully unsaturated one e.g. MK9(H6) and MK11 (3x0,7=2; 9+2=11, fig. 1a and 1b).

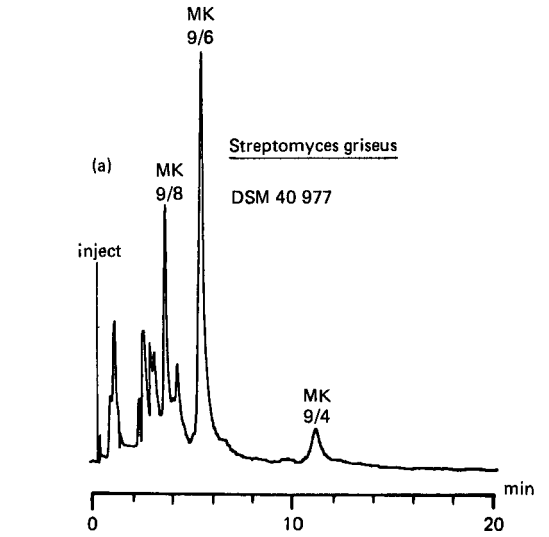
Therefore, it became necessary to separate the menaquinones on an additional stationary phase which had a different separation behaviour. The separation of menaquinones on octyldecylsilica (RP18) depends mainly on their lipophilic character. An increase in the retention time occurs by adding isoprenyl units or pairs of hydrogen atoms (eliminating double bonds).

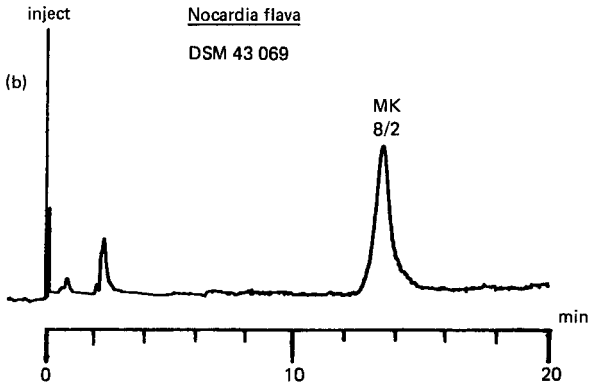
FIGURE 2

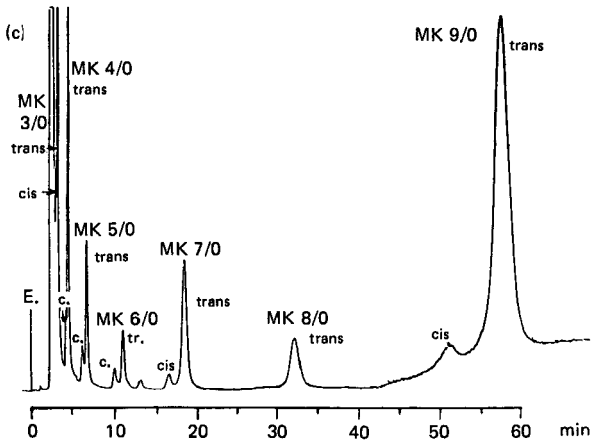
Separation of menaquinones on a silver loaded ion exchanger Nucleosil^{R} 10SA(Ag^{+}) column 250x4,6 mm, mobile phase methanol 100%, flow rate 1,5 ml/min, temperature 65^{o} C, sample size 10 µl.

(a) + (b) bacterial menaquinones with partly saturated isoprenyl side chains, MK9/4 (a) shows a shorter retention time than MK8/2 (b), both have the same number of double bonds

(c) menaquinone standard mixture MK1 - MK9







A totally different separation behaviour is obtained by using a silver loaded ion exchanger. In this case the bonds of double bond silver complexes compete with the solubility of the organic mobile phase. These bonds are so strong that only menquinones with 9 or less double bonds are eluted in reasonable retention times (MK9, 57 min at 65° C). Increasing the temperature resulted in boiling of the methanol use of a more lipophilic mobile phase had no effect on the retention time. The theoretical plate height for MK9(H2) was 60 µm (4100 plates per 25 cm column). As expected (Ag-TLC data), menaquinones with the same number of double bonds but longer isoprenoid chain length were eluted earlier thus, MK9(H4) eluted before MK8(H2) even though both contained seven double bonds (fig. 2a and 2b). Cis-menaquinones had shorter retention times than trans-menaquinones (fig. 2c). Menaquinones with the same number of isoprenyl units and double bonds but different points of saturation (isomers) were very well separated on the silver column; e.g. MK8(H4) of Micropolyspora brevicatena DSM 43024 and MK8(H4) of Nocardia autotrophica DSM 40011. The mass of both was varified by mass-spectrometry.

The results clearly show the value of HPLC for the separation and identification of menaquinones. By comparing the different elution profiles of the menaquinones isolated from bacteria and separated on both columns, the single peaks can easily be identified.

REFERENCES

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

2. Bratrakov, S.G., Panosyan, A.G., Rozynov, B.V., Konova, I.V. and Bergel`son, L.D., Menaquinones of Actinomyces olivaceus: The Structures of MK-9(H_6), MK-8(H_6), Bioorganicheskaya Khimiya, 2, 1538, 1976.

3. Collins, M.D. and Jones, D., Distribution of Isoprenoid Quinone Structural Types in Bacteria and Their Taxonomic Implications, Microbiological Reviews, 45, 316, 1981.

4. Dunphy, P.J., Phillips, P.G. and Brodie, A.F., Separation and Identification of Menaquinones from Microorganisms, J. Lipid Res., 12, 442, 1971.

5. Farrad, S.K. and Taber, H.W., Changes in Menaquinone Concentration During Growth and Early Sporulation in Bacillus subtilis, J. Bact., 117, 324, 1974.

6. Haroon, Y., Shearer, M.J. and Barkhan, P., Resolution of Menaquinones (Vitamin K_2) by High-Performance Liquid Chromatography, J. Chromatogr., 206, 333, 1981.

7. Kroppenstedt, R.M., Korn-Wendisch, F., Fowler, V.J. and Stackebrandt, E., Biochemical and Molecular Genetic Evidence for a Transfer of Actinoplanes armeniacus into the Family Streptomycetaceae, Zbl. Bakt. Hyg., I. Abt. Orig. C2 254, 1981.

8. Mack, D.O., Silver Ion as Modifier for High Performance Liquid Chromatography of Menaquinones, J. Liquid Chromatogr., 3, 1005, 1980.

9. Minnikin, D.E., Collins, M.D. and Goodfellow, M., Menaquinone Patterns in the Classification of Nocardioform and Related Bacteria, Zbl. Bakt. Hyg., I. Abt., Suppl. 6, 85, 1978.

10. Taber, H., Funktion of Vitamin K_2 in Microorganisms, 177, In Suttie J.W. (ed) Vitamin K Metabolisms and Vitamin K dependent Proteins, University Park Press Baltimore, 1980.

11. Yamada, Y., Inouye, G., Tahara, Y. and Kondo, K., The Menaquinone System in the Classification of Aerobic Gram-Positive Cocci in the Genera Micrococcus, Staphylococcus, Planococcus and Sporosarcina, J. Gen. Appl. Microbiol., 22, 227, 1976.

12. Yamano, Y., Ihenoya, S., Ohmae, M. and Kawabe, K., High-Performance Liquid Chromatography of Isoprenoid-quinones III: Photo-isomerization of Phylloquinone and Menaquinone-4, Yakugaku Zasshi, 99, 1102, 1979.