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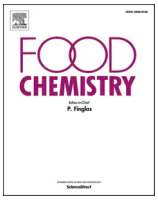

Off-line two-dimensional LC–tandem MS of menaquinones from thermophilic bacteria

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ABSTRACT

Thermophilic bacteria of four genera in contrast to the commonly used production strains such as *Bacillus subtilis*, produce homologs other than menaquinone (MK) with seven isoprene units. The number of isoprene units and the configuration of double bonds are essential factors for their biological activity. The goal was to obtain a strain of bacteria that produces a wide range of MK homologs and only all-*trans* geometrical isomers, which was the strain *G. kaustophilus*. Using off-line two-dimensional LC–tandem MS in columns with the RP18 phase and the COSMOSIL cholester phase (separation according to the geometric configuration of double bonds) it was shown that thermophilic bacteria grown at different temperatures produce only all-*trans* isomers of menaquinones from MK-5 (menaquinone with five isoprenyl units) to MK-15 (fifteen isoprenyl units). Therefore, *G. kaustophilus* appears to be a biotechnologically important strain produces only *trans* isomers and additionally homologs from 5 to 15 isoprene units.

1. Introduction

Menaquinones (MKs) (Fig. 1) are bacterial-derived sources of vitamin K that have a 2-methyl-1,4-naphthoquinone nucleus, and variable alkyl chains from 4 to 15 isoprenoid units. The side chains can exist as *cis* and *trans* isomers and can be partially saturated. The *trans* double bonds of the molecule form a linear structure, making the vitamin highly bioactive (Gnosis by Lesaffre USA, 2018; Sitkowski et al., 2018; Szterk, Bus, et al., 2018; Szterk, Zmyslowski, et al., 2018). Vitamin K with one or more *cis* double bonds exhibits only 1% of the biological activity of the *trans* isomer (Knauer et al., 1975; Lowenthal & Vergelrivera, 1979). The number of isoprene units of MK is an essential factor in its absorption and incorporation into the liver and that the improving effect of MKs on hypoprothrombinemia (Akiyama et al., 1995). MK-7 is used in respiratory chain transmission, coagulation function, and calcium homeostasis (Wilkens et al., 2021), bone healing, in preventing cerebrovascular and cardiovascular diseases, and in the fight against cancer cells, Alzheimer's, and Parkinson's disease (Mahdinia et al., 2019; Tarkesh et al., 2020). Humans cannot synthesize MK-7; its only synthesis sources are food or dietary supplements (Bergeland et al., 2019) and bacteria in the gut microbiome. The content of MK-7 from natural food sources, e.g., cheese, is too low to obtain preventive and therapeutic doses. To obtain the required concentrations, therefore, nutritional supplements are required.

Long-chain menaquinones are synthesized by organic synthesis but chemical reactions produce a mixture of *cis/trans* isomers (Baj et al., 2016). MKs are produced by bacteria (Yan et al., 2022) and are used in food production (Vermeer et al., 2018; Walther et al., 2021). The richest known source of natural vitamin K2 is *Bacillus subtilis* var. natto (Mahdinia et al., 2018). Recently, there has been significant interest in the overproduction of MKs via bacterial fermentation (Lal et al., 2022a; Zhang et al., 2021). For example, the MK-7 yield in *Bacillus subtilis* was significantly increased by surfactant Brij-58 supplementation (Yi et al., 2023).

Many articles have been published describing effective screening of MK-producing strains and optimization of fermentation conditions. For example, culture conditions can be optimized (Lal et al., 2022a, 2022b) or either engineered bacteria, or bacteria other than *Bacillus subtilis* var.

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natto can be used as production platforms (Kang et al., 2022; Ren et al., 2020; Yuan et al., 2020). The production of MKs in wild-type bacteria production achieves tens of mg of MK per 1 L (Kang et al., 2022). Engineered bacteria can produce MK-7 up to 1350 mg/L, but as a mixture of *cis/trans* isomers (Gao et al., 2021). Lal et al. (2022a) state that when cultivation conditions were optimized, the increase in all-*trans* MK-7 production was accompanied by an increase in the concentration of unwanted *cis* isomer. Unfortunately, commercial preparations are usually not analyzed for *cis* versus *trans* isomer composition, e.g., by HPLC, and thus for the actual biological activity. The information in the literature describes that MK-7 obtained by fermentation methods leads to variant isomer ratios due to different cultivation conditions (Sitkowski et al., 2018; Szterk, Bus, et al., 2018; Szterk, Zmyslowski, et al., 2018). A recent study focused on the impact of environmental and storage condition on the isomer profile of fermented MK-7 in order to ensure the preservation of the bioactive isomer (Lal et al., 2023).

Two options are possible for the analysis of molecular species of MKs. The first includes a more frequently used analysis of MKs using RP–HPLC (Xie et al., 2021). Identification is frequently performed by either the LC–UV method (HPLC with UV detector) or the LC–MS method. Soft ionization mass spectrometry is usually used, for example, atmospheric pressure chemical ionization (APCI), atmospheric pressure photoionization (APPI) (Geyer et al., 2004), or electrospray ionization (ESI) (Cao et al., 2020). Geyer et al. (2004) used APCI and APPI to analyze MK-4 and other quinones in about 2×10^7 cells, by means of the selected reaction monitoring mode (SRM). The most abundant ion in the MK-7 spectrum was detected at *m/z* 187.0759 (Bergeland et al., 2019), which represents the head of the 2-methylnaphthoquinone (Fig. S1). Separation of *cis/trans*-MKs has also been described and bespoke columns of type COSMOSIL Cholester (Szterk, Bus, et al., 2018; Szterk, Zmyslowski, et al., 2018) or HPLC with silver complexation (Bus et al., 2022) were used. The second method, lipidomic analysis using the shotgun method, is very often used for lipid analysis (Hsu, 2018; Law & Zhang, 2019; Rezanka et al., 2016; Rezanka et al., 2018). Unfortunately, to our knowledge, we have not found any reports describing the shotgun analysis of menaquinones.

This article presents the analysis of menaquinones from thermophilic bacteria obtained from a Czech Collection of Microorganisms (CCM, Brno, Czech Republic) or directly isolated from hot springs for which shotgun lipidomic analysis was first used. This is a particularly rapid method that allows a sample to be analyzed in a few minutes. Another advantage of shotgun analysis compared to LC–MS is that it displays the mass spectrum of the protonated molecule at a constant concentration of sample during direct infusion, allowing for scanning of precursor ion (PIS) and/or neutral loss (NLS). Furthermore, the two-dimensional off-line LC–tandem MS of menaquinones enables the separation of individual homologs with a wide range of side chain lengths, including partially saturated MKs. Using a COSMOSIL cholesterol phase column, it was possible to separate *cis/trans* isomers. The thermophilic bacteria analyzed in the study exclusively produced an all-*trans* mixture and wide range of MK homologs of MKs, regardless of the culture temperature.

2. Experimental methods

2.1. Chemicals and standards

Menaquinone 7 (vitamin K2 (MK-7), i.e. (all-E)-2-(3,7,11,15,19,23,27-heptamethyl-2,6,10,14,18,22,26-octacosaheptaenyl)-3-methyl-1,4-naphthalenedione)) was purchased from Sigma-Aldrich (now Merck, Prague, Czech Republic). Menaquinone 6 (MK-6, i.e. (all-E)-2-(3,7,11,15,19,23-hexamethyl-2,6,10,14,18,22-tetracosahexaenyl)-3-methyl-1,4-naphthalenedione) and menaquinone 9 (MK-9, i.e. (all-E)-2-(3,7,11,15,19,23,27,31,35-nonamethyl-2,6,10,14,18,22,26,30,34-hexatriacontanonaen-1-yl)-1,4-naphthalenedione) were purchased from Biosynth s.r.o., Bratislava, Slovakia. All other chemicals were purchased from Merck (Prague, Czech Republic). Four different dietary supplements of vitamin K2 (MK-7) in the form of hard pills, capsules, or vegetable oil solution were purchased in Czech pharmacies and were labeled **A**–**D**.

Fig. 1. Chemical structure of some menaquinones.

2.2. Isolation of thermophilic bacteria from hot springs

Three strains of thermophilic bacteria were isolated from three thermal springs: Mlýnský, Štěpánka, and Vřídlo in Carlsbad, Czech Republic. Other strains (Table S1) were obtained from the Czech Collection of Microorganisms (Brno, Czech Republic). Their cultivation, see Supplements, was previously described (Table S1) (Gharwalova et al., 2021). Strains isolated from thermal springs were identified using 16S rRNA gene sequencing; see Supplements.

2.3. Extraction of menaquinones

The lipid extraction procedure was performed according to the Bligh and Dyer method (Bligh & Dyer, 1959). The resuspended lyophilized cells (~10 mg) were extracted using a dichloromethane-methanol mixture (2:1, v/v) for 30 min with stirring. The dichloromethane phase was evaporated to dryness under reduced pressure. The menaquinone extract was purified using a Sep-Pak Vac Silica cartridge 35 cc (Waters, Milford, MA, USA; with 10 g of normal-phase silica), and nonpolar lipids were subsequently eluted with 5 mL hexane. The menaquinones were eluted from the cartridge with 20 mL of 2% diethyl ether in hexane. The total menaquinone eluate was evaporated, and the oil sample was dissolved in a propane-2-ol-hexane mixture (1:1, v/v), which was used for further analysis.

2.4. Analysis of menaquinones by shotgun mass spectrometry

An LTQ-Orbitrap Velos mass spectrometer (Thermo Fisher Scientific, San Jose, CA, USA) was operated in positive ionization mode with a heated electrospray interface (HESI). The MS scan range was performed in the FT cell and recorded within a window between 150 and 1500 Da. The mass resolution was 110,000, and the ion spray voltage was set at 3.0 kV in the positive ionization mode. The following parameters for the positive ionization mode were used: sheath gas flow, 17 arbitrary units (AU); auxiliary gas flow, 9 AU; ion source temperature, 290 °C; capillary temperature, 250 °C; capillary voltage, 45 V; and tube lens voltage, 155 V. Helium was used as a collision-gas collision-induced dissociation (CID) experiment. The CID normalization energy of 33% was used to fragment the parent ions. Flow injection analysis was used for sample introduction into the heated ESI–MS (H-ESI–MS) ion source. Hexane: isopropanol (50/50 v/v) was used at 100 μL/min flow rate.

2.5. Analysis of menaquinones by off-line two-dimensional LC–tandem MS

The MK analysis was performed in two modes. In the first mode, the retention times of the respective MKs and their structure by MS were determined. In the second mode, the relevant fractions were transferred to the COSMOSIL 2.5 Cholester column using a Rheodyne valve and the individual peaks were again identified by MS.

The HPLC equipment consisted of a 1090 Win system, a PV5 ternary pump and automatic injector (HP 1090 series, Hewlett Packard, USA), and a Luna Omega (Phenomenex, Aschaffenburg, Germany) high-performance liquid chromatography (HPLC) column (C18, 100 Å, 150 mm × 2.1 mm × 1.6 μm) with an injection volume of 10 μL. HPLC was performed at a flow rate of 0.33 mL/min for the menaquinones of all cultures. The mobile phase consisted of methanol (solvent A) and hexane:isopropanol (50:50, v/v, solvent B). The gradient profile was as follows: isocratic at 100% solvent A for 1.0 min, linear gradient to A:B (65:35 v/v) at 15 min, isocratic to 20.0 min, and hold for 6 min. The mobile phase was returned to solvent A for 5.0 min. The column temperature was 42 °C. The 10% HPLC flow was introduced into LTQ Orbitrap Velos; see above. Ninety percent of the flow containing fractions of individual menaquinones (full unsaturated MKs, approximately 50 μL for MK-5 to MK-12 and 66 μL for MK-13 to MK-15, i.e. intervals approximately 0.15 to 0.20 min) were transferred using a Rheodyne valve (Rheodyne, Rohnert Park, CA, USA, MXT715-000, 2-position 6-port switching valve with 100 μL stainless steel sample loop) to the second column.

The second column was COSMOSIL 2.5 Cholester (2.0 mm × 150 mm × 2.5 μm, Nacalai Tesque, Inc., Kyoto, Japan). The mobile phase consisted of 0.1% trifluoroacetic acid (TFA)-methanol:H_2O (90:10 v/v, solvent A) and 0.1% TFA-methanol (solvent B). The gradient profile was as follows: isocratic at 100% solvent A for 1.0 min, linear gradient to A:B (0:100%) at 99.0 min, isocratic to 120.0 min, and the mobile phase was returned to solvent A for 10.0 min. The flow of the mobile phase was 350 μL/min at a temperature of 42 °C.

LTQ Orbitrap Velos (Thermo Fisher Scientific, Bremen, Germany), a high-resolution hybrid mass spectrometer equipped with a heated electrospray interface (HESI) was used; see above.

2.6. Calibration

The commercially obtained MK-7 menaquinone standard was analyzed separately. The menaquinone stock standard was stored at −20 °C. The ion at *m/z* 187.0759 (from PIS) was used to generate a calibration curve using the peak area of the tandem MS corresponding to the different concentrations of the standard (1.0, 2.0, 5.0, 10.0, 20.0, 50.0, 100, 200, 500, 1000, 2000, 5000, 10,000, 20,000, and 50,000 fmol/μL). The linear regression of the calibration curve was calculated, as well as the signal/noise (S/N = 3) ratio. The assays were linear throughout the concentration range, ranging from 5 to 20,000 fmol/μL, with a mean coefficient of determination R^2 of 0.9981. The third point of the calibration curve (5.0 fmol/μL) corresponds to the lower limit of quantification (LLOQ). The detection limit (LOD) for MK-7 was determined and the concentration was 2.0 fmol/μL.

3. Results and discussion

3.1. Extraction and preliminary identification of MKs by precursor ion scan tandem MS

A thermophilic bacterial culture was prepared; the list of strains, including references, is given in Table S1. Lyophilized bacterial cells were extracted and total MKs were obtained. A large quantity of lipids was removed using Sep-Pak Vac Silica cartridges; see Experimental Methods.

Menaquinones were analyzed by ESI precursor ion scan tandem mass spectrometry (PIS MS/MS) at *m/z* 187.0759 (Szterk, Bus, et al., 2018), structure, see Fig. S1. Fig. 2 shows a shotgun analysis of the mixture of MKs of thirteen thermophilic bacteria cultivated at different temperatures; see also Table 1. Bacteria isolated from hot springs are marked in bold. Analysis of all thirteen strains cultured at different temperatures shows that most MKs were MK-7. Based on the calibration curve, it can be determined that MK-7 was contained in a relatively narrow concentration interval, from 0.20 to 2.52 mg/L. Furthermore, higher and lower homologs were identified in all strains, including MK-6 and MK-9. Also, partially saturated molecular species were detected. Tandem MS confirmed their structure; see below. Analysis of menaquinones by reverse phase HPLC with identification by ESI-MS, see Fig. 3 (*Geobacillus kaustophilus*), proved the presence of homologues up to MK-15.

3.2. Tandem MS of three MKs homologs

The proof of the structure of the MKs was performed using tandem MS. Fig. 4 shows that the menaquinone with the highest number of isoprene units could be MK-15. The MK-15 spectrum is presented, which, to the best of our knowledge, has not yet been published. In MS/MS, one of the dominant ions with an abundance of 45% of the base peak, see Fig. 4, is the ion $[M + H]^+$ at *m/z* 1193.9987. The calculated value using ChemDraw Ultra 22.2.0.3300 (PerkinElmer) for this monoisotopic ion $[C_{86}H_{128}O_2 + H]^+$ is 1193.9990. Tandem MS is shown in

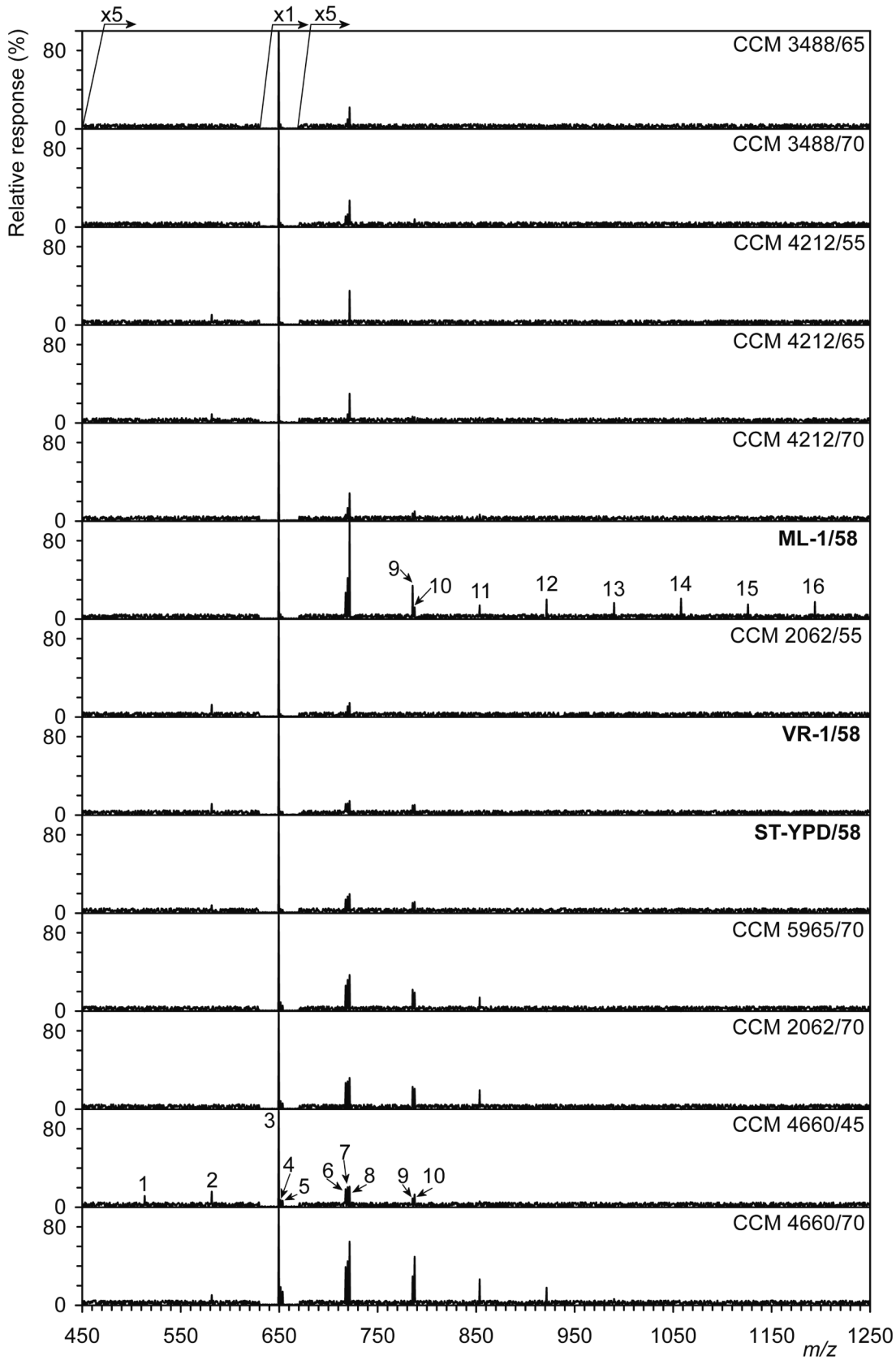

Fig. 2. Precursor ion scan (ion $[C_{12}H_{11}O_2]^+$) of menaquinones from thirteen strains of thermophilic bacteria. For full names of bacteria, see Table S1. Numbers 1, 2, 3, etc.; see Table 1.

Table 1
Content of MKs in the different bacteria (percent of total MKs).

Peak No.	Menaquinone	Chemical Formula	Exact Mass	CCM 4660/70	CCM 4660/45	CCM 2062/70	CCM 5965/70	**ST-YPD/58**[a]	**VR-1/58**[a]	CCM 2062/55
1	MK-5	$[C_{36}H_{49}O_2]^+$	513.3729	0 ± 0[b]	1.7 ± 0.4	0 ± 0	0 ± 0	**0 ± 0**	**0 ± 0**	0 ± 0
2	MK-6	$[C_{41}H_{57}O_2]^+$	581.4355	1.1 ± 0.2	2.3 ± 0.2	0.4 ± 0.1	0.2 ± 0.1	**1.3 ± 0.4**	**1.9 ± 0.7**	2.2 ± 0.6
3	MK-7	$[C_{46}H_{65}O_2]^+$	649.4981	52.4 ± 1.8	72.9 ± 2.7	68.8 ± 2.5	69 ± 2.6	**80.1 ± 3.1**	**81.7 ± 2.7**	87.2 ± 2.4
4	MK-7(II, H2)	$[C_{46}H_{67}O_2]^+$	651.5138	9.8 ± 0.6	5.3 ± 0.9	5.8 ± 1.1	6.3 ± 1.5	**3.5 ± 1.2**	**3.2 ± 0.5**	2.7 ± 0.4
5	MK-7(II, IV, H4)	$[C_{46}H_{69}O_2]^+$	653.5294	7.4 ± 0.9	4.9 ± 1	4.2 ± 0.4	3.9 ± 6	**2.8 ± 0.5**	**2.9 ± 0.3**	1.3 ± 0.2
6	MK-8	$[C_{51}H_{73}O_2]^+$	717.5607	4.1 ± 0.5	2.7 ± 0.6	3.7 ± 0.6	3.6 ± 0.4	**2.2 ± 0.6**	**1.9 ± 0.4**	0.9 ± 0.3
7	MK-8(II, H2)	$[C_{51}H_{75}O_2]^+$	719.5764	4.7 ± 0.7	3 ± 0.2	3.9 ± 0.7	4.4 ± 0.8	**2.7 ± 0.2**	**2 ± 0.2**	1.9 ± 0.5
8	MK-8(II, IV, H4)	$[C_{51}H_{77}O_2]^+$	721.5921	6.8 ± 0.6	3.1 ± 0.7	4.4 ± 0.3	5.1 ± 0.6	**3.1 ± 0.4**	**2.4 ± 0.3**	2.5 ± 0.6
9	MK-9	$[C_{56}H_{81}O_2]^+$	785.6233	3.1 ± 0.4	1.3 ± 0.2	3.2 ± 0.5	3 ± 0.7	**1.7 ± 0.3**	**1.6 ± 0.4**	0.5 ± 0.2
10	MK-9(II, H2)	$[C_{56}H_{83}O_2]^+$	787.6391	5.2 ± 0.9	1.9 ± 0.4	2.9 ± 0.4	2.6 ± 0.3	**1.8 ± 0.3**	**1.7 ± 0.3**	0.8 ± 0.1
11	MK-10	$[C_{61}H_{89}O_2]^+$	853.6860	2.8 ± 0.8	0.9 ± 0.5	2.7 ± 0.3	1.9 ± 0.2	**0.8 ± 0.2**	**0.7 ± 0.2**	0 ± 0
12	MK-11	$[C_{66}H_{97}O_2]^+$	921.7486	1.9 ± 0.3	0 ± 0	0 ± 0	0 ± 0	**0** ± 0	**0** ± 0	0 ± 0
13	MK-12	$[C_{71}H_{105}O_2]^+$	989.8112	0.7 ± 0.2	0 ± 0	0 ± 0	0 ± 0	**0** ± 0	**0** ± 0	0 ± 0
14	MK-13	$[C_{76}H1_{13}O_2]^+$	1057.8737	0 ± 0	0 ± 0	0 ± 0	0 ± 0	**0** ± 0	**0** ± 0	0 ± 0
15	MK-14	$[C_{81}H_{121}O_2]^+$	1125.9364	0 ± 0	0 ± 0	0 ± 0	0 ± 0	**0** ± 0	**0** ± 0	0 ± 0
16	MK-15	$[C_{86}H_{129}O_2]^+$	1193.9990	0 ± 0	0 ± 0	0 ± 0	0 ± 0	**0** ± 0	**0** ± 0	0 ± 0
Content of MK-7 (mg/L)[c]				0.63	0.35	1.49	1.34	**1.26**	**1.51**	1.33

Peak No.	**ML-1/58**[a]	CCM 4212/70	CCM 4212/65	CCM 4212/55	CCM 3488/70	CCM 3488/65
1	**0 ± 0**	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
2	**0 ± 0**	0.7 ± 0.2	1.5 ± 0.6	1.8 ± 0.5	0.7 ± 0.2	0.9 ± 0.3
3	**57.4 ± 1.7**	86.1 ± 2.5	85.6 ± 2.2	85.9 ± 3.1	83.4 ± 2.6	86.9 ± 2.3
4	**2.9 ± 0.5**	0.5 ± 0.2	1.7 ± 0.4	2.4 ± 0.2	3.7 ± 0.4	4.1 ± 0.4
5	**1.4 ± 0.3**	0.2 ± 0.1	1 ± 0.2	2.1 ± 0.6	1.9 ± 0.3	1.2 ± 0.2
6	**3.1 ± 0.4**	1.1 ± 0.6	0.6 ± 0.3	0 ± 0	1.8 ± 0.4	0.9 ± 0.2
7	**4.8 ± 0.8**	2.3 ± 0.3	1.5 ± 0.6	0.7 ± 0.2	2.2 ± 0.5	1.7 ± 0.3
8	**13.2 ± 1.4**	4.9 ± 0.4	5.1 ± 0.3	6 ± 0.8	4.5 ± 0.7	3.8 ± 0.5
9	**3.9 ± 0.3**	1.4 ± 0.5	1.1 ± 0.2	0.7 ± 0.3	0.5 ± 0.2	0 ± 0
10	**1.4 ± 0.2**	1.7 ± 0.3	1 ± 0.3	0.4 ± 0.2	1.3 ± 0.3	0.5 ± 0.2
11	**1.6 ± 0.4**	1.1 ± 0.2	0.9 ± 0.4	0 ± 0	0 ± 0	0 ± 0
12	**2.3 ± 0.2**	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
13	**1.9 ± 0.3**	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
14	**2.4 ± 0.5**	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
15	**1.7 ± 0.2**	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
16	**2 ± 0.3**	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
	2.52	0.78	0.45	0.20	1.26	0.83

[a]The strains obtained from the thermal springs in Carlsbad, Czech Republic. The full names can be found in the Supplements.
[b]Data are presented as arithmetic means from 3 cultivations (biological replicates) ± standard deviations (S.D.). Data of technical replicates (three chromatographic analyses), see Table S2.
[c]Values are based on 1 L of fermentation medium.

Fig. 4, and it can be seen that there is a gradual loss of ~ 68 Da, see also Fig. S2 (MKs cleavage scheme). Another key ion $[M + H\text{-}H_2O]^+$ at *m/z* 1175.9881 is a base peak marked as **i1** ion. This ion at *m/z* 1007.9312 confirms that the cleaved isoprenoid side chain is fully unsaturated. As follows from the text mentioned above, the ion at *m/z* 187.0759 (see Fig. S1), which was used for PIS, fully confirms the structure of the head group. Lower homologs of MKs were similarly identified, e.g., MK-9; see Fig. 4. In the tandem MS, the dominant ion is again **i1** at *m/z* 599.5556, then the ions $[M + H]^+$ and $[M + H\text{-}H_2O]^+$ at *m/z* 785.6233, respectively, at *m/z* 767.6125 and of course the ion at *m/z* 187.0759. An example of tandem MS of partially saturated MK, i.e. MK-9 (II,H2), is also shown in Fig. 4. Values of all key ions, i.e. $[M + H]^+$, $[M + H\text{-}H_2O]^+$, i1, nx, where n is the number of isoprene units, see Fig. S2 and × ranges from two to nine, are shifted by 2.0158 Da, which means that one of the double bonds is saturated. Based on the value of ions **i1** and **n2**, we assume the isoprene unit in the β position is saturated. However, this hypothesis could only be fully confirmed after further analyses, i.e. those already published (Azerad et al., 1967). In this case, obtaining a large amount of pure MK is necessary.

3.3. LC–MS of geometric isomers

Individual fractions obtained by column elution with the RP18 phase were collected using a Rheodyne valve and analyzed by two-dimensional off-line chromatography. The advantage of this arrangement is that it is unnecessary to collect fractions manually or by using a fraction collector. However, the disadvantage of the offline connection is the need to repeat the separation on the RP18 column as many times as the fractions were analyzed on the second column (COSMOSIL Cholester).

To separate geometric isomers, a column with a stationary phase of COSMOSIL Cholester (Nannapaneni et al., 2017; Sitkowski et al., 2018; Szterk, Bus, et al., 2018; Szterk, Zmyslowski, et al., 2018) or HPLC with silver complexation (Bus et al., 2022) was used. Identification of individual geometric isomers is not possible using MS, as noted previously by Szterk, Zmyslowski, et al. (2018) who stated: "*in the case of the use of HRMS, it is not possible to determine where in the isoprene chain of menaquinone-7 the cis configuration exists*". In contrast, the position of the *cis/trans* configuration of the double bond(s) can be determined by NMR (Sitkowski et al., 2018; Szterk, Bus, et al., 2018; Szterk, Zmyslowski, et al., 2018).

Sixty-six cultures of MKs-producing bacterial strains were analyzed for the presence of geometric isomers after elution from the COSMOSIL 2.5 Cholester column. Based on the similarity of the tR of a commercially obtained standard (Sigma-Aldrich and Biosynth see 2.1. Chemicals and Standards), it was found that MK-6, MK-7, and MK-9 from all bacteria, regardless of the cultivation temperature, contained only the geometric all-*trans* isomer. In the case of lower homolog (MK-5) and higher homologs (MK-8, MK-10–MK-15), only one peak in the chromatogram was obtained from the COSMOSIL Cholester column. These peaks were

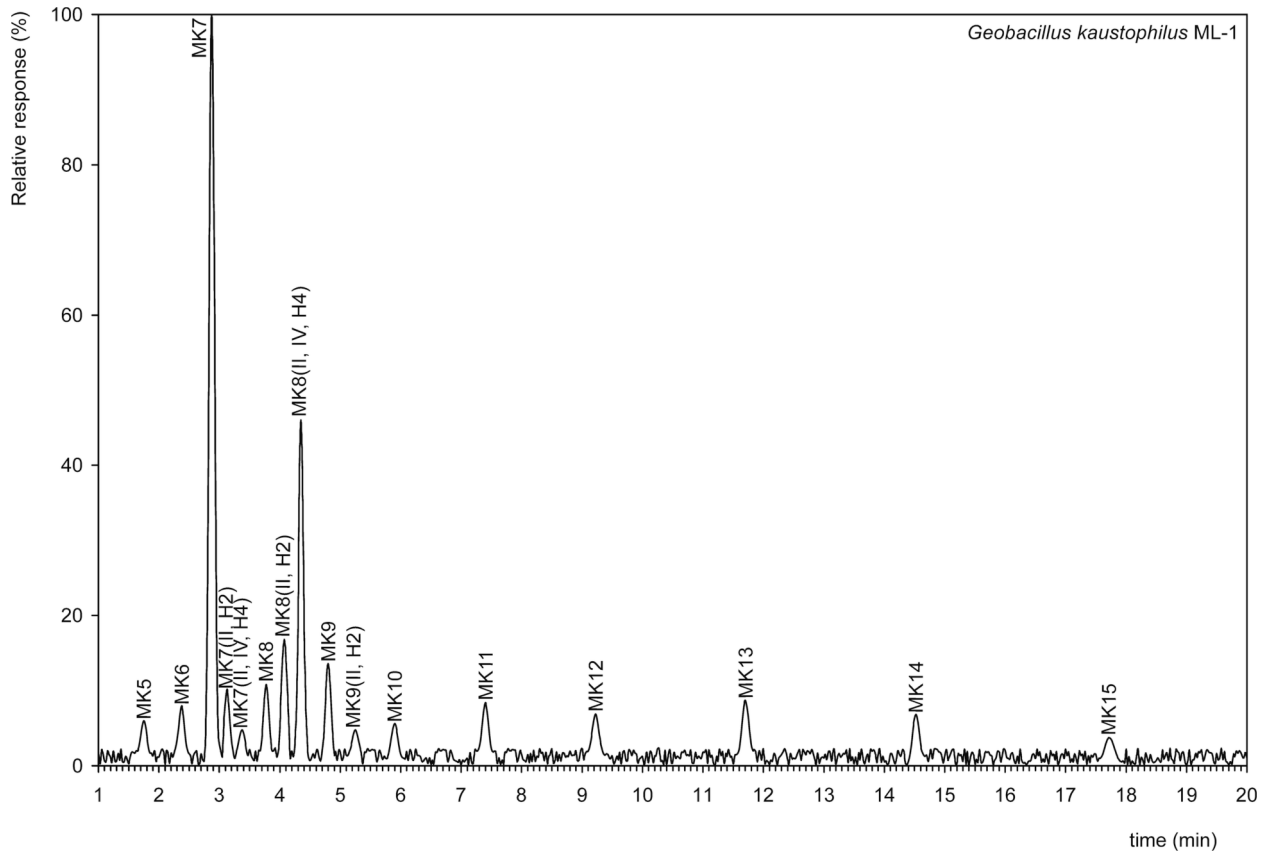

Fig. 3. Separation of menaquinones by RP–HPLC–MS of *Geobacillus kaustophilus* ML-1 detected by precursor ion at *m/z* 187.0755 (ion $[C_{12}H_{11}O_2]^+$).

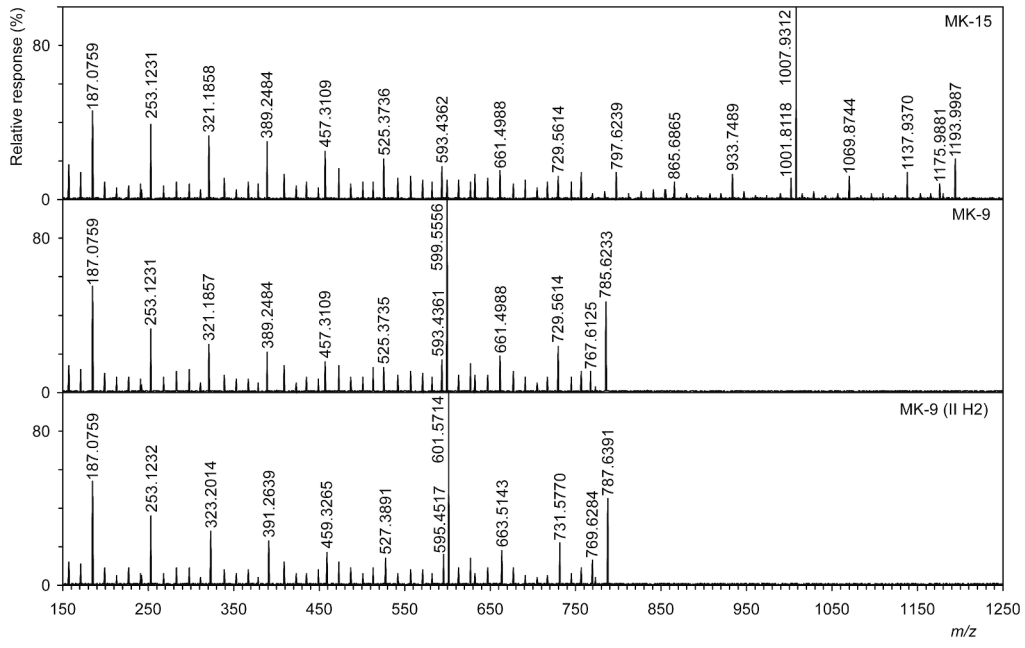

Fig. 4. Tandem mass spectra of three selected menaquinones, i.e. MK-15, MK-9 and MK-9 (II H2).

tentatively assumed also to be all-*trans* isomers. Determination of the positions and number of *cis* double bond(s) of other homologs of MKs is possible by NMR (Szterk, Zmyslowski, et al., 2018) or based on the agreement of tR with standards after analysis on a column with COSMOSIL Cholester stationary phase.

In the case of dietary supplements, the situation is entirely different for all four supplements in the form of hard pills, capsules, or vegetable oil solutions. Fig. 5 shows the chromatograms of two dietary supplements, i.e. hard pills and MK in vegetable oil. In these supplements, in addition to all-*trans* MK-7, several peaks were identified that had at least one *cis* double bond. However, their mass spectra (Fig. S3) are completely identical; see also the published data in the supplements of

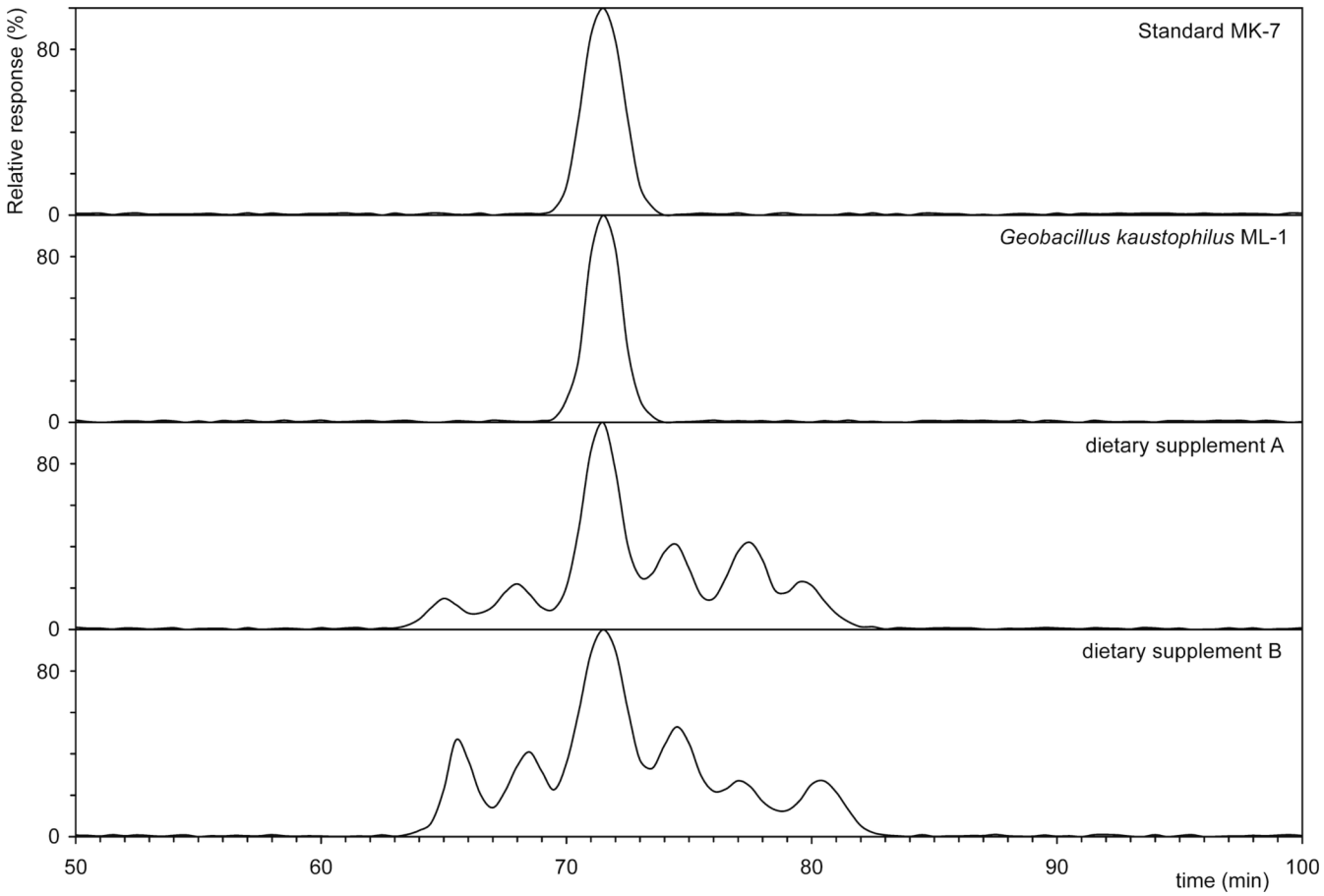

Fig. 5. Separation of menaquinones using the COSMOSIL 2.5 Cholester column (*cis/trans*) of standard MK-7, *Geobacillus kaustophilus* ML-1 and two commercially obtained dietary supplements.

Szterk et al. (2018). However, based on a comparison of the tR standards (MK-6, MK-7, and MK-9), bacterial MKs, and MKs isolated from nutritional supplements, it is possible to determine that only nutritional supplements contain a mixture of geometric isomers. Regardless of the text provided by the manufacturer, all four pharmaceutical products contained a mixture of geometric *cis/trans* isomers of MK-7, see Fig. S3, Fig. S4, Fig. S5 and Fig. 5. The results of these analyzes are completely consistent with results published previously (Bus et al., 2022; Szterk, Bus, et al., 2018; Szterk, Zmyslowski, et al., 2018).

Lal et al. (2022a) reported that geometric *cis* isomers were present in *B. subtilis* var. natto regardless of the composition of the fermentation medium. The *cis/trans* ratio was approximately 1:10 (see Table 3 of the paper Lal et al.). The absence of *cis* isomers in thirteen thermophilic bacteria strains can be explained in several ways. First, thermophilic bacteria cultivation was carried out at a higher temperature. At least to the best of our knowledge, no paper has described the effect of temperature on the production of *cis/trans* isomers of MKs. Furthermore, it is possible that even though *Geobacillus* belongs to the same family as *B. subtilis* (Bacillaceae), its metabolism is different. This is even more evident for *Meiothermus* and *Thermus* strains that belong to different bacterial classes (class *Bacilli* vs. class *Deinococci*).

4. Conclusion

Using shotgun lipidomics several dozen bacterial samples cultured at different temperatures could be accurately analyzed within a few hours. Two-dimensional offline LC–tandem MS also showed that thermophilic bacteria from four genera (*Alicyclobacillus*, *Geobacillus*, *Meiothermus*, and *Thermus*) cultivated at different temperatures (favorable for their growth) produce only all-*trans* geometric isomers of menaquinones. A newly developed method was used to analyze several tens of samples, consisting of the offline connection of two columns. On the column with the RP18 phase, MKs were separated according to the length of the side chain and the number of double bond(s). In the second column with COSMOSIL Cholester phase, *cis/trans* geometric isomers were separated.

In summary, this article describes the analysis not only of the presence of many homologs (MK-5, MK-6, MK-8–MK-15) but also of the presence of *cis/trans* isomers of MKs. Thermophilic bacteria have been shown to produce only all-*trans* MKs under optimal conditions for their growth. So far, the presence of *cis/trans* isomers has been demonstrated in only one bacterium, *B. subtilis* natto. The MKs of other bacteria have not yet been analyzed for the presence of *cis* isomers. This paper analyzed four additional strains of bacteria (*Alicyclobacillus, Geobacillus, Meiothermus* and *Thermus*). These results show that *G. kaustophilus* ML-1 is a potentially suitable strain of bacteria for the biotechnological production of MKs with a chain length other than seven prenyl units. These results can be further applied in the search for new production strains biosynthesizing MKs. In this regard, the strain *G. kaustophilus* ML-1, which produces a wide interval of homologs of MKs, appears promising.

CRediT authorship contribution statement

Elizaveta Timkina: Data curation, Methodology. **Irena Jarošová Kolouchová:** Data curation, Methodology. **Lucie Kyselová:** Data curation, Methodology. **Andrea Palyzová:** Data curation, Methodology, Writing – original draft. **Denis J. Murphy:** Software, Writing – review & editing, Supervision. **Tomáš Řezanka:** Conceptualization, Software, Writing – review & editing, Supervision.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2023.137112.

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