Supplements

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

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2.2. Isolation of thermophilic bacteria from hot springs

Bacterial strains were isolated from three thermal springs in Carlsbad (Czech Republic), namely Štěpánka, Mlýnský, and Vřídlo. Samples were collected into sterile 50 mL Falcon tubes and transferred to the laboratory under isotermic conditions, temperature during transportation was kept at least 50 °C. Enrichment cultivation was carried out as follows: 13.5 mL of water sample was mixed with 1.5 mL of YPD medium (dextrose 20 g/L, peptone 20 g/L, and yeast extract 10 g/L, pH 7.0) to achieve ten-fold dilution. Cultivation took place at a rotary shaker (150 rpm) at the temperature corresponding to the sample original spring source (58 °C or 42 °C) for 30 days. After the enrichment cultivation, samples were diluted to achieve 10^3 cells/mL cell count. Agar plates were inoculated with the diluted culture to obtain separate colonies.

Agars used in this study were: Reasoner's 2A agar (R2A, HiMedia, India) and Thermus 162 agar (HiMedia, India). Incubation of plates was at the temperatures of the springs (58 °C or 42 °C) for 1–3 weeks. Colonies with distinct different morphologies were transferred to plate count agar (PCA agar, HiMedia, India) and cultivated at 58 °C or 42 °C for 48 h for further identification and analysis. Molecular identification was based on 16S rRNA gene sequencing as described in our previous studies (Gharwalova et al., 2021; Kolouchova et al., 2021). 16S rRNA gene was amplified by PCR reaction using gene primers Fwd27 and

Rev1492. PCR products were purified using a High Pure PCR Product Purification Kit (Roche, Basel, Switzerland). Sequencing was carried out on an ABI PRISM 3130xl Genetic Analyzer (Applied Biosystems, Waltham, MA, USA). Editing and assembly of obtained sequences was done by Chromas Lite software (Technelysium Pty Ltd., Brisbane, Australia) and SeqMan (DNASTAR, Inc., Madison, WI, USA) respectively. The BLASTN program (NCBI, Bethesda, MD, USA) was used to search for 16S rRNA gene similarities in GenBank data library. Three bacterial isolates, designated *Geobacillus stearothermophilus* ST-YPD, *Geobacillus kaustophilus* ML-1, and *Geobacillus stearothermophilus* VR-1, were obtained from the springs (Table S1). In the cultivation experiments, the optimal growth conditions (temperature, media components) depending on their origin were used. The individual selected collection strains were not exposed to stressful conditions and at the same time, the menaquinone profile was not affected.

References

Gharwalova, L., Palyzova, A., Maresova, H., Kolouchova, I., Kyselova, L., Rezanka, T., Identification of homologous polyprenols from thermophilic bacteria. *Microorganisms, 9*(6) (2021). doi: 10.3390/microorganisms9061168

Kolouchova, I., Timkina, E., Matatkova, O., Kyselova L., Rezanka, T., Analysis of bacteriohopanoids from thermophilic bacteria by liquid chromatography–mass spectrometry. Microorganisms 9, 2062 (2021). doi: 10.3390/microorganisms9102062

Table S1. List of the cultivated bacterial isolates from three springs of Carlsbad (Karlovy Vary), Czech Republic and ten strains obtained from the Czech Collection of Microorganisms (CCM), Brno, Czech Republic.

Abbreviation	Cultiv. Temp.	Bacterium
CCM 4660/70	70	*Alicyclobacillus acidoterrestris* CCM 4660 (Apple-grape-raspberry juice)
CCM 4660/45	45	*Alicyclobacillus acidoterrestris* CCM 4660
CCM 2062/70	70	*Geobacillus stearothermophilus* CCM 2062 (no information about source)
CCM 5965/70	70	*Geobacillus stearothermophilus* CCM 5965 (Evaporated milk)
VR-1/58	**58**	***Geobacillus stearothermophilus* VR-1 (spring Vřídlo)** [a]
CCM 2062/55	55	*Geobacillus stearothermophilus* CCM 2062
ST-YPD/42	**42**	***Geobacillus stearothermophilus* ST-YPD (spring Štěpánka)** [a]
ML-1/58	**58**	***Geobacillus kaustophilus* ML-1 (spring Mlýnský)** [a]
CCM 4212/70	70	*Meiothermus ruber* CCM 4212 (Thermal pools, Hveragherti, Iceland)
CCM 4212/65	65	*Meiothermus ruber* CCM 4212
CCM 4212/55	55	*Meiothermus ruber* CCM 4212
CCM 3488/70	70	*Thermus aquaticus* CCM 3488 (Thermally polluted river near Brussel)
CCM 3488/65	65	*Thermus aquaticus* CCM 3488,

[a] These strains were obtained from the thermal springs in Carlsbad, Czech Republic, see GenBank: MT251887.1 (**ST-YPD**), GenBank: MT251886.1 (**VR-1**), and GenBank: MT251494.1 (**ML-1**).

Table S2. Describing data of technical replicates (three chromatographic analyses).

Peak No.	CCM 4660/70
1	0 ± 0
2	1.1 ± 0.07
3	52.4 ± 0.08
4	9.8 ± 0.05
5	7.4 ± 0.06
6	4.1 ± 0.04
7	4.7 ± 0.05
8	6.8 ± 0.04
9	3.1 ± 0.03
10	5.2 ± 0.08
11	2.8 ± 0.06
12	1.9 ± 0.04
13	0.7 ± 0.03
14	0 ± 0
15	0 ± 0
16	0 ± 0

As can be seen from this table, the chromatographic analysis shows at least one order of magnitude lower ±S.D., compared to the bacterial cultures, so it is not essential for the interpretation of the results.

Table S3. Comparison of the elution time by changing the composition of the mobile phase, i.e. the gradient, see original and optimized tR.

Menaquinone	tR (min)	Menaquinone	tR (min)
MK5	1.750	MK5	3.573
MK6	2.360	MK6	4.808
MK7	2.865	MK7	5.827
MK7(II. H2)	3.025	MK7(II. H2)	6.138
MK7(II. IV. H4)	3.225	MK7(II. IV. H4)	6.533
MK8	3.765	MK8	7.618
MK8(II. H2)	3.975	MK8(II. H2)	8.041
MK8(II. IV. H4)	4.225	MK8(II. IV. H4)	8.520
MK9	4.690	MK9	9.441
MK9(II. H2)	4.975	MK9(II. H2)	10.002
MK10	5.900	MK10	11.857
MK11	7.390	MK11	14.811
MK12	9.215	MK12	18.448
MK13	11.690	MK13	23.334
MK14	14.535	MK14	29.014
MK15	18.230	MK15	36.291

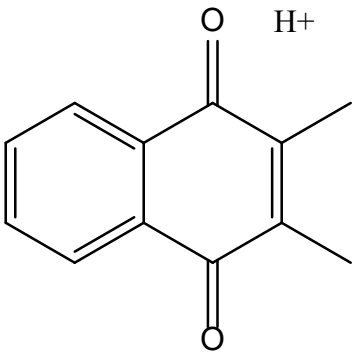

Fig. S1. Probable structure of the ion at 187.0755 Da (chemical formula $[C_{12}H_{11}O_2]^+$).

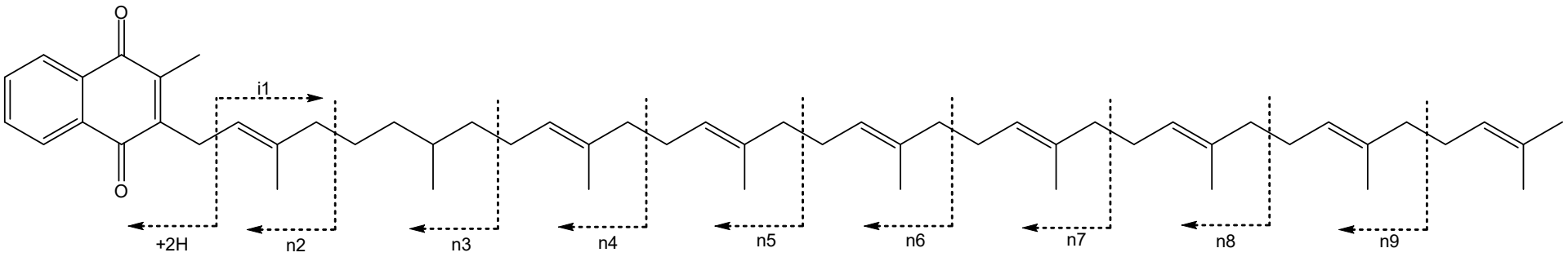

Fig. S2. Probable fragmentation of menaquinones by tandem mass spectrometry.

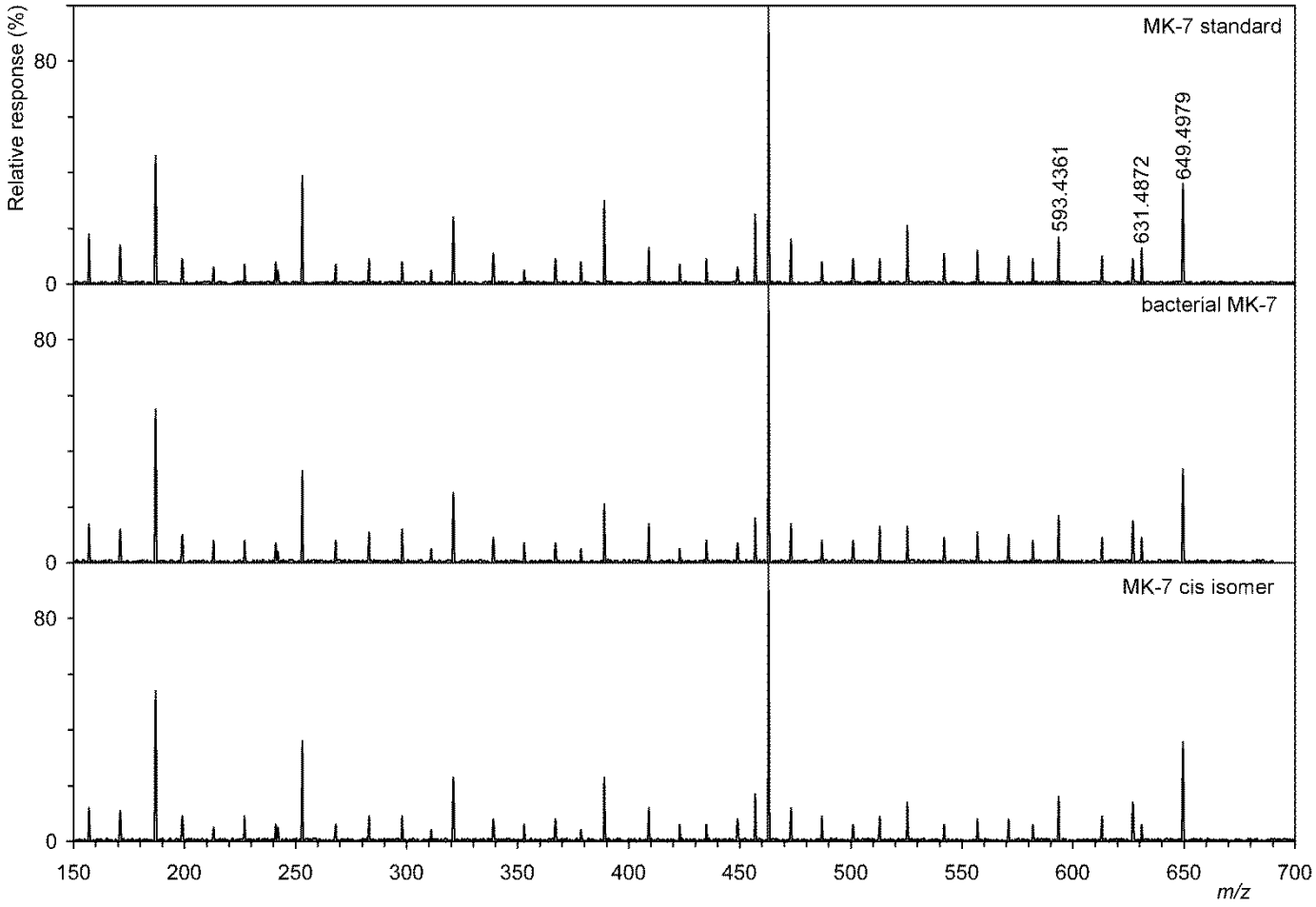

Fig. S3. Tandem MS of three MK-7, i.e. MK-7 standard, bacterial MK-7 and MK-7 cis isomer (peak at tR = 65.5 min from Fig. 5).

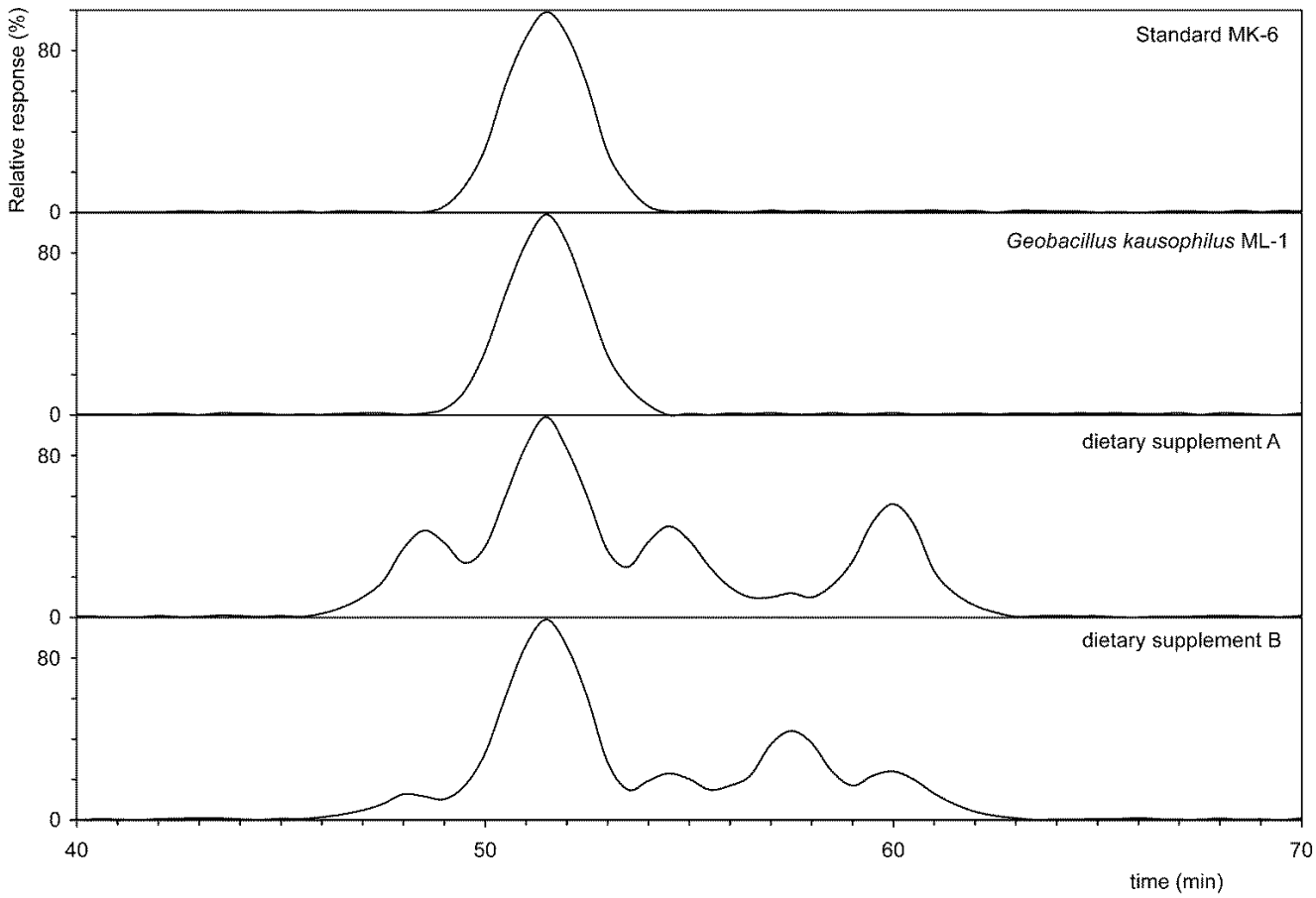

Fig. S4. Separation of menaquinones by COSMOSIL 2.5 Cholester column (cis/trans) of standard MK-6, *Geobacillus kaustophilus* ML-1, and two commercially obtained dietary supplements.

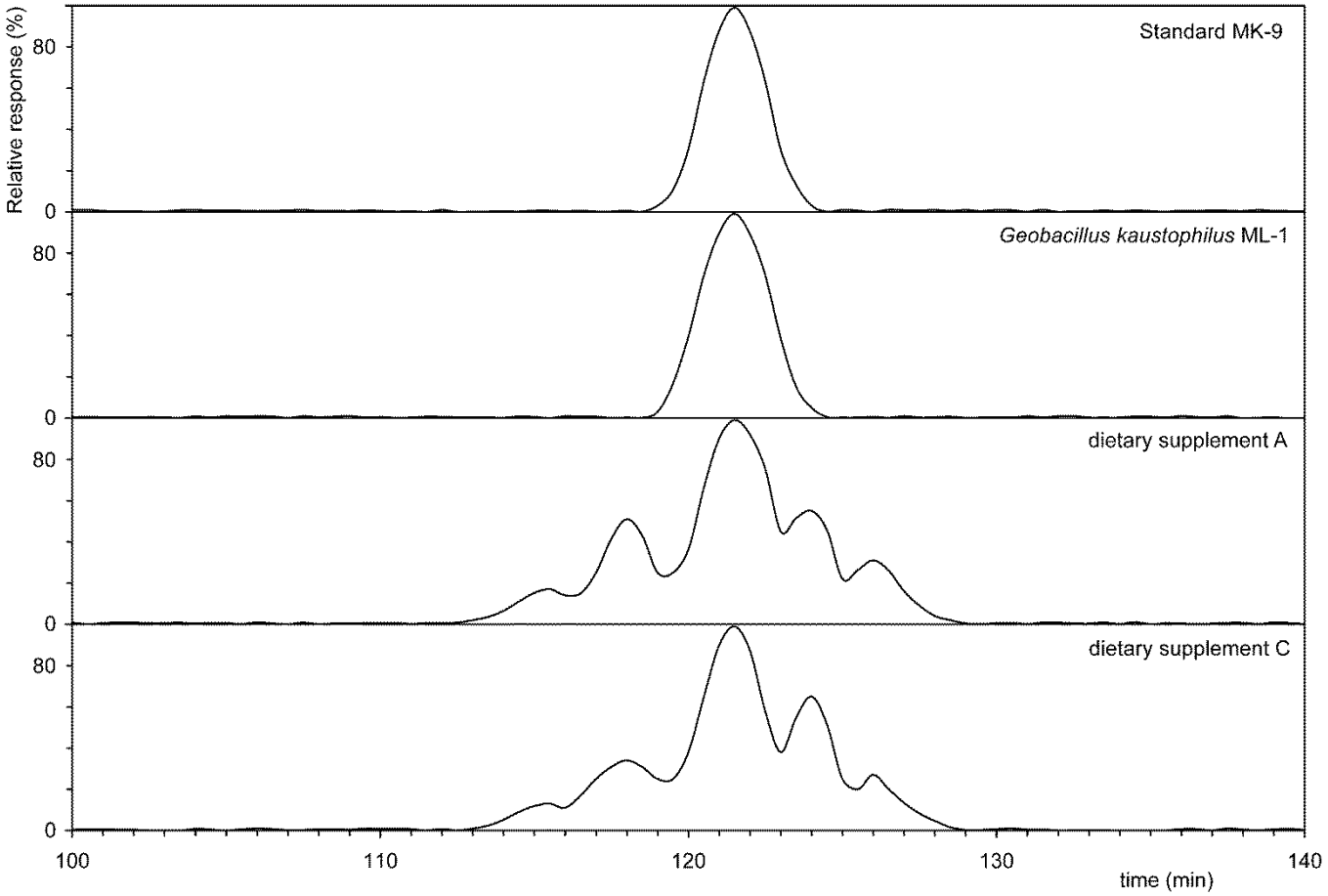

Fig. S5. Separation of menaquinones by COSMOSIL 2.5 Cholester column (cis/trans) of standard MK-9, *Geobacillus kaustophilus* ML-1, and two commercially obtained dietary supplements.