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Trehalose, mannitol and arabitol as indicators of fungal metabolism in late cretaceous and Miocene deposits

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ABSTRACT

Trehalose, mannitol and arabitol are the main saccharides of extant fungal metabolism, but their occurrence and distribution in geological materials have rarely been considered. Here, we identify these sugars in Miocene lignites and for the first time in Late Cretaceous mudstones and coals. The co-occurrence of trehalose, mannitol and arabitol in the sedimentary rocks investigated suggests their fungal origin, because these three saccharides are major compounds present in most modern fungi, including the very common mycorrhizal and wood-rotting groups. Therefore, we conclude that these sugars should be treated as new fungal biomarkers (biomolecules) present in geological rocks. Trehalose and mannitol are major compounds in total extracts of the samples and a sum of their concentration reaches 4.6 μg/g of sample. The arabitol concentrations do not exceed 0.5 μg/g, but in contrast to trehalose, the concentration correlates well with mannitol ($R^2 = 0.94$), suggesting that they have the same, translocatory role in fungi. Based on the trehalose vs. mannitol and arabitol distributions in Cretaceous samples and their comparison with data for modern fungi, we preliminarily conclude that the coal seams from the Rakowice Małe section were formed during warmer climatic periods than the overlying sediments. Furthermore, no DNA could be isolated from the samples of lignites and overlying sediments, whereas it was abundant in the control samples of maple, birch and oak wood degraded by fungi. This indicates an absence of recent fungi responsible for decay in lignites and implies that the saccharide origin is connected with ancient fungi.

Other sugar alcohols and acids like D-pinitol, quinic acid and shikimic acid, were found for the first time in sedimentary rocks, and their source is inferred to be from higher plants, most likely conifers. The preservation of mono- and disaccharides of fungal origins in pre-Palaeogene strata implies that compounds previously thought as unstable can survive for tens to hundreds of millions of years without structural changes in immature rocks unaffected by secondary processes.

1. Introduction

Biomolecules, natural products of living organisms are relatively rare in sedimentary organic matter (OM). This is mainly connected with the fact that their low stability leads to early metabolic and diagenetic destruction or conversion. Nevertheless, in favorable conditions (low maturation, reducing redox potential, no secondary processes) biomolecules can be preserved hundreds of millions of years, and were occasionally reported from sediments across the Mesozoic and Cenozoic (e.g. Otto and Simoneit, 2001; Otto et al., 2002; Marynowski et al., 2007a, 2007b; Talbot et al., 2016; Rybicki et al., 2017).

Saccharides, ubiquitous biomolecules in both plant and animal kingdoms, are common in marine and terrestrial environments (Klok et al., 1984; Moers et al., 1989; Hernes et al., 1996; Wakeham et al., 1997; Amon and Benner, 2003; Comont et al., 2006; Jia et al., 2008), but were only incidentally described from geological materials older than Holocene (e.g. Moers et al., 1994; Fabbri et al., 2009). However, in euxinic conditions polysaccharides can be preserved through sulfur-

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Table 1
Concentration of saccharides identified in the Rakowice Małe section (μg/g of sample, and μg/g TOC in parentheses).

Sample	TOC [%]	Arabitol	α-Glucose	Mannitol	β-Glucose	Sucrose	Trehalose	Shikimic acid	Pinitol
Rc3	3.7	0.003 (0.10)	0.03 (0.81)	0.01 (0.28)	0.04 (1.08)	0.03 (0.81)	0.20 (5.41)	–	0.03 (0.81)
Rc4A	1.7	–	0.03 (1.76)	–	0.03 (1.76)	0.03 (1.76)	0.03 (1.76)	–	–
Rc5A	10.6	0.05 (0.47)	0.01 (0.09)	0.03 (0.28)	0.03 (0.28)	0.03 (0.28)	0.14 (1.32)	–	–
Rc6A	5.2	0.08 (1.54)	0.03 (0.58)	0.02 (0.38)	0.04 (0.77)	0.06 (1.15)	0.15 (2.88)	–	–
Rc7	3.6	0.01 (0.28)	0.04 (1.11)	0.03 (0.83)	0.06 (1.67)	0.04 (1.11)	0.78 (21.7)	–	–
Rc8B	11.0	0.06 (0.55)	0.03 (0.27)	0.10 (0.91)	0.16 (1.45)	0.04 (0.36)	0.29 (2.64)	–	–
Rc9B	49.0	0.31 (0.63)	0.05 (0.10)	2.09 (4.27)	0.07 (0.14)	–	0.06 (0.12)	–	–
Rc10B	8.0	–	0.06 (0.74)	0.14 (1.75)	0.09 (1.13)	0.03 (0.38)	1.09 (13.6)	–	–
Rc10BW	38.5	0.45 (1.18)	0.14 (0.36)	4.28 (11.1)	0.16 (0.42)	0.09 (0.23)	1.95 (5.06)	–	–
Rc11	2.3	–	0.05 (2.17)	0.02 (0.87)	0.08 (3.48)	0.06 (2.61)	1.49 (64.8)	–	–
Rc12	0.3	–	0.04 (13.3)	0.01 (3.33)	0.05 (16.7)	0.27 (90)	1.32 (440)	0.20 (66.7)	–
Rc13	2.5	–	0.01 (0.40)	–	0.01 (0.40)	–	0.29 (11.6)	–	–
Rc14C	1.9	–	0.02 (1.05)	0.01 (0.53)	0.03 (1.58)	0.02 (1.05)	0.03 (1.58)	–	–
Rc14CK	2.5	–	0.06 (2.40)	0.01 (0.40)	0.01 (0.40)	0.06 (2.40)	0.41 (16.4)	–	–
Rc15C	24.5	–	0.04 (0.16)	–	0.08 (0.33)	–	–	–	–
Rc16C	0.8	–	0.03 (3.75)	–	0.03 (3.75)	0.05 (6.25)	0.02 (2.50)	–	–

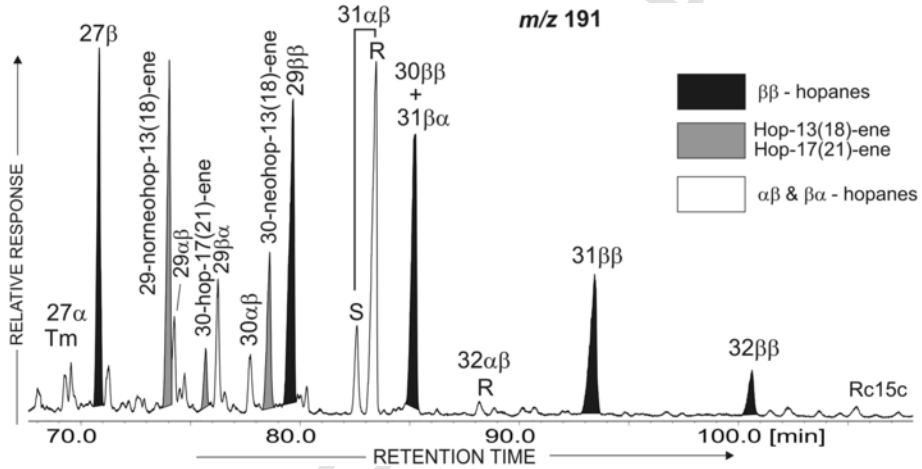

Fig. 1. Mass fragmentogram (*m/z* 191) showing an example of the hopane distribution (sample Rc15C). Hopanes are marked by a shortened notation indicating their stereochemistry at the C-17 and C-21 positions and total number of carbon atoms.

ization processes. Experimental study has shown that the reaction of glucose and cellulose with H_2S leads to formation of organic sulfur compounds (Moers et al., 1988). Later, van Kaam-Peters et al. (1998) and Sinninghe Damsté et al. (1998) demonstrated using isotope and molecular studies that sulfurized carbohydrates can constitute an important part of kerogen.

Nonetheless, not all data can be considered as credible. Reports from the sixties about occurrence of sugars in Precambrian and Cambrian rocks should be treated as historical studies showing the detection of contamination by saccharides dissolved and migrated in water (e.g. Palacas et al., 1960; Swain et al., 1970). Only recently Marynowski et al. (2018) identified primary α- and β-glucose in a middle Jurassic fossil wood and over a dozen mono- and disaccharides in Miocene detritic lignites and xylites. The disaccharides occurrence (sucrose and trehalose) is especially of interest, because this shows that the glycosidic bond between saccharide monomers can survive for millions of years.

The source of saccharides in the geological record is not clear. It is likely, that glucose found in Mesozoic wood and sedimentary rocks could be a remnant of cellulose degradation (Rybicki et al., 2017; Marynowski et al., 2018). Other mono- and disaccharides from Miocene lignites can be degradation products of biopolymers such as hemicellulose and cellulose, but would potentially also occur as free sugars preserved in xylites and detritic coals (Marynowski et al., 2018).

Here we demonstrate the co-occurrence of mannitol, arabitol, trehalose, and other sugars in the Late Cretaceous sections from the Rakowice Małe area (SW Poland), as well as their presence in some Miocene detritic lignites and xylites. We propose that these compounds, when present as dominant saccharides, can be used as indicators of fungal metabolism, as is typical for many modern fungi (e.g. Koide et al., 2000; Simoneit et al., 2004; Hybelbauerová et al., 2008).

2. Geological settings

The Late Cretaceous sedimentary rocks, exposed in the Rakowice Małe area (sandstone quarry "Rakowiczki") contain the youngest (Santonian) part of the structural depression of the North-Sudetic Basin. This sedimentary basin comprises rocks of ages from Carboniferous to Cretaceous, and is strictly connected with a suite of structural depressions, which accompanied the European Variscide orogen (Chrząstek and Wojewoda, 2011). The Santonian sedimentary rocks belong to the Czerna Formation (Milewicz, 1997), where the oldest part is the Nowogrodziec Member. The Nowogrodziec Member is represented by clays, mudstones and sandstones with coal intercalations. These sedimentary rocks are described as swamp and lagoon deposits formed in a subtropical climate (Milewicz, 1997; Leszczyński, 2010). Generally, the Czerna Formation is represented mainly by sandstones with coal and clay intercalations. This formation has an origin in a river delta located near the front of the Variscian orogen (Milewicz, 1997). The section in

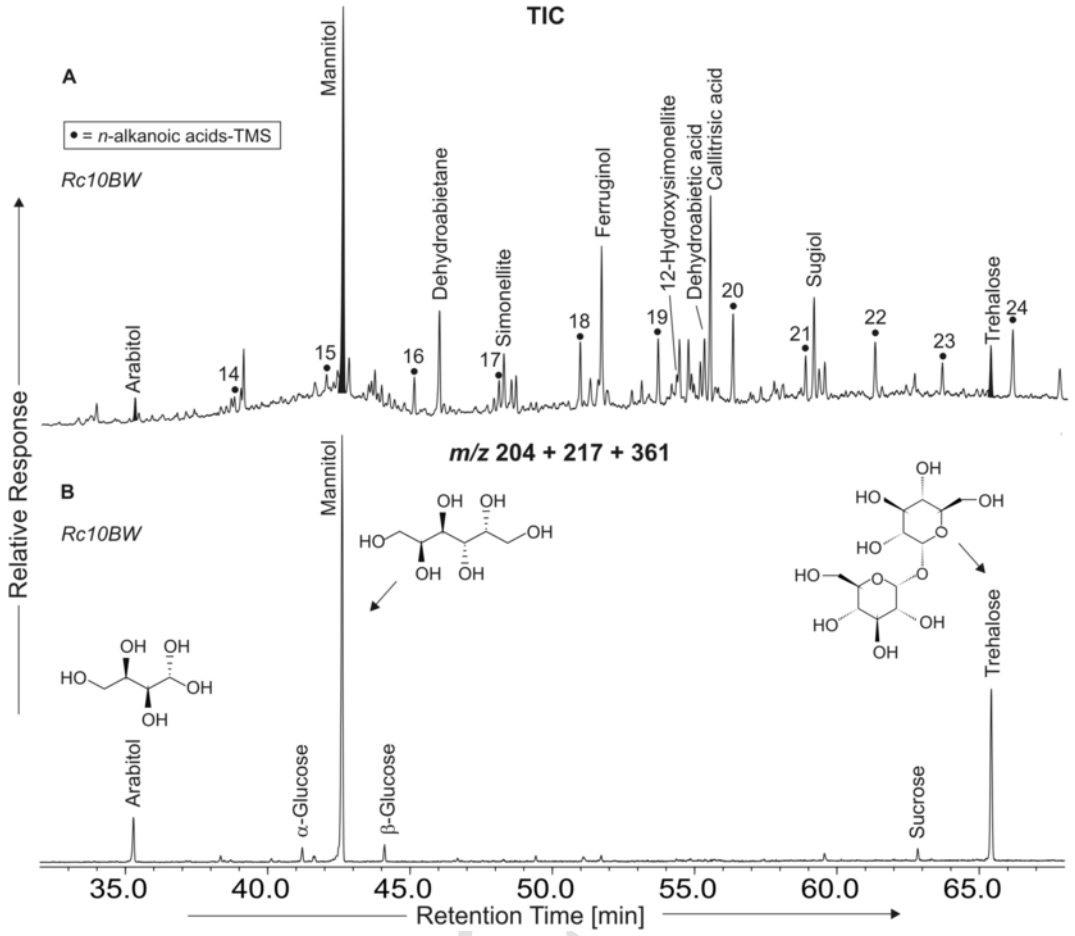

Fig. 2. GC–MS data for a total extract of a detritic coal sample: (A) Total ion current (TIC) trace with mannitol and trehalose as important compounds, and (B) summed mass chromatogram *m/z* 204 + 217 + 361 of the same (Rc10BW) sample.

the Rakowiczki quarry exposes the Coniacian sandstone, of the Nowogrodziec Member sedimentary rock series, as well its contact with the main sedimentary series of the Czerna Formation (Leszczyński, 2010).

Additionally, other sampling sites are the Konin area (outcrops Jóźwin IIB and Drzewce) and the Jaroszów Mine. Jóźwin IIB and Drzewce are outcrops of exploited Miocene lignites located in the Konin area. In the Jaroszów Mine the exploited rocks are clays with two thin lignite beds. Currently, only the younger lignite bed is exposed on the surface. Its age is correlated the same as the lignites from Konin. For details regarding the Konin area see Widera (2016) and regarding the Jaroszów Mine see Urbański et al. (2011).

3. Samples and methods

3.1. Samples

Two groups of samples were investigated using geochemical methods. The first group was Late Cretaceous (Coniacian/Santonian) samples from the inoperative "Rakowiczki" sandstone quarry (51°9′56.57"N; 15°32′33.85″E). Series of Santonian sedimentary rocks are exposed above the exploited deposit of Coniacian sandstone, and between them is an erosion hiatus. The thickness of the Santonian sedimentary rocks is 13 m at the sampling location, and they consist mostly of sandstones, dark grey mudstones and shales with coal (lignite) intercalations and some fossil plant debris. In summary, 23 samples were collected from the section and 16 were selected for geochemical analysis. The detailed description of the section and location map was given by Leszczyński (2010).

The second group was Miocene lignites from the Konin area, central Poland (Jóźwin IIB - 52°25′41.10"N; 18°10′10.20″E and Drzewce mines) and the Jaroszów mine (50°59′39.00"N; 16°27′31.50″E), SW Poland. These coals have a low maturity (huminite reflectance for the Konin coals is between 0.16 and 0.22% R_r; Fabiańska and Kurkiewicz, 2013), and the xylites have an abundant (holo)cellulose content (Bechtel et al., 2007; Fabbri et al., 2009; Marynowski et al., 2018). Two xylite samples and two detritic coals were selected.

Three control samples of wood (maple, birch and oak trunks) degraded by fungi were sampled for DNA tests. Moreover, five extant wood-degrading fungi were sampled and analyzed.

3.2. Total organic carbon content

The total carbon (TC) and total inorganic carbon (TIC) contents were determined using an Eltra CS-500 IR-analyzer with a TIC module at the Faculty of Earth Sciences, Sosnowiec. The TC was determined by using an infrared cell detector for CO_2, which evolved from the combustion of organic matter under an oxygen atmosphere with the simultaneous thermal decomposition of carbonates. The TIC content was determined by an infrared detector for CO_2 that was derived from the carbonates by reaction with 15% warm hydrochloric acid. The total organic carbon (TOC) was calculated as the difference between the TC and TIC. The instrument was calibrated utilizing the Eltra standards.

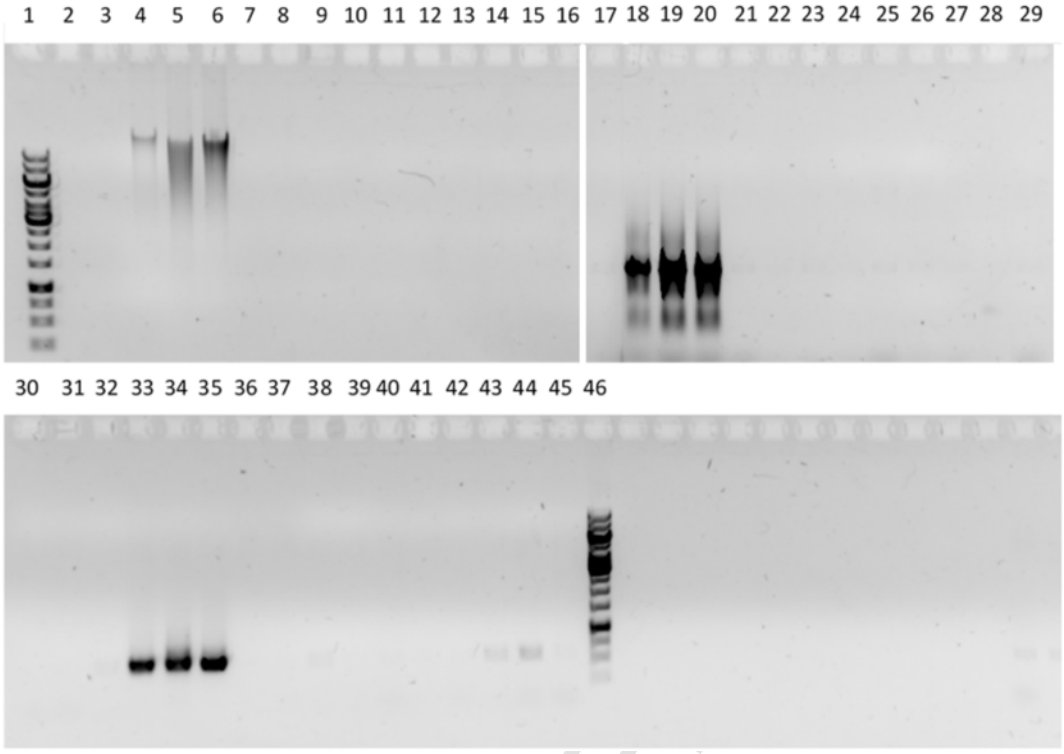

Fig. 3. Agarose gel electrophoresis of probes obtained after attempts of total DNA isolation from all the samples examined (2–16), bacterial 16SrRNA PCR products (17–30) and fungal 28SrRNA PCR products (31–45). 1. DNA 1 kb marker; 2. blank sample 1 (1.7 ng/μl); 3. blank sample 2 (3.6 ng/μl); 4. rotten birch wood (39.9 ng/μl); 5. rotten maple wood (87.6 ng/μl); 6. rotten oak wood (63.6 ng/μl); 7. RC $6A_1$ (2.6 ng/μl); 8. RC $6A_2$ (4.6 ng/μl); 9. RC 7_1 (2.6 ng/μl); 10. RC 7_2 (1.9 ng/μl); 11. RC $9B_1$ (1.6 ng/μl); 12. RC $9B_2$ (1.5 ng/μl); 13. RC $10B_1$ (7.7 ng/μl); 14. RC $10B_2$ (4.9 ng/μl); 15. RC $14CK_1$ (1.6 ng/μl); 16. RC $14CK_2$ (1.8 ng/μl); 17. blank sample 1; 18. rotten birch wood; 19. rotten maple wood; 20. rotten oak wood; 21. RC $6A_1$; 22. RC $6A_2$; 23. RC 7_1; 24. RC 7_2; 25. RC $9B_1$; 26. RC $9B_2$; 27. RC $10B_1$; 28. RC $10B_2$; 29. RC $14CK_1$; 30. RC $14CK_2$; 31. blank sample 1; 32. blank sample 2; 33. rotten birch wood; 34. rotten maple wood; 35. rotten oak wood; 36. RC $6A_1$; 37. RC $6A_2$; 38. RC 7_1; 39. RC 7_2; 40. RC $9B_1$; 41. RC $9B_2$; 42. RC $10B_1$; 43. RC $10B_2$; 44. RC $14CK_1$; 45. RC $14CK_2$; 46. DNA 1 kb marker.

3.3. DNA tests

A PowerSoil DNA isolation kit (MoBio Laboratories, Carlsbad, CA) was used to isolate total DNA from selected samples of the Rakowice Małe section (RC6A, RC7, RC9B, RC10B, RC14CK). Modern rotten trunks of birch, maple and oak were used as a control material (a positive control). DNA was extracted and purified according to the manufacturer's protocol with some modifications. The 350–400 mg samples of the rock or the rotten wood were placed into bead tubes for extraction in duplicate. Two bead tubes did not contain any sample (blanks). All tubes were incubated at 65 °C for 10 min and then shaken horizontally in a MoBio vortex adapter for 20 min at maximum speed. The remaining steps were performed as directed by the manufacturer. The final samples of extracted DNA were stored at −20 °C. The presence of DNA was checked by agarose gel electrophoresis and its concentration was measured with a Thermo Scientific™ NanoDrop 2000C. Using the total DNA isolated from the microbial community as template, (i) the full-length bacterial 16S rRNA gene fragments were amplified by polymerase chain reaction (PCR) using PCR Mix Plus (A&A Biotechnology) with the universal primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5'-GGTTACCTTGTTACGACTT-3′); and (ii) a fragment of the 28S rRNA gene was amplified using the primer pair NL1 (5' GCA TAT CAA TAA GCG GAG GAA AAG 3′)/NL4 (5′ GGT CCG TGT TTC AAG ACG G 3′) for detection of fungi (Cui et al., 2013). For the primer pair NL1/NL4 the following amplification conditions were used: 94 °C for 2 min, followed by 30 cycles of 94 °C for 30 s, 52 °C for 30 s and 72 °C for 1 min, with a final extension at 72 °C for 7 min. For the primers 27F and 1492R the following amplification conditions were used: 95 °C for 5 min, followed by 20 cycles of 95 °C for 30 s, 53 °C for 30 s, 72 °C for 90 s; and then 15 cycles of 95 °C for 30 s, 46 °C for 30 s, 72 °C for 1.5 min; with a final extension at 72 °C for 10 min. The PCR products were submitted to agarose gel electrophoresis. Tests were provided at the Institute of Biochemistry and Biophysics, PAN, Warszawa.

3.4. Organic petrology

Five samples rich in TOC (Rc7, Rc8B, Rc9B, Rc10BW, Rc15C) were selected as a representative group for organic petrological studies. Random reflectance measurements were carried out on 100 points according to the procedures described in ISO 7404-5 (2009) on polished chips. All the petrographic observations and analyses were carried using an optical microscope Axio Imager.A2m at the Faculty of Earth Sciences, Sosnowiec.

3.5. Extraction, separation and derivatization

Powdered samples (ca. 15 g) were extracted using a dichloromethane (DCM)/methanol mixture (1:1 v:v) with an accelerated Dionex ASE 350 solvent extractor. Column chromatography was performed using activated silica gel (110 °C for 24 h) and extract aliquots were separated into aliphatic, aromatic and polar fractions by modified column chromatography (Bastow et al., 2007). The eluents used for collection of the three fractions were: *n*-pentane (aliphatic), *n*-pentane and DCM (7:3, aromatic), and DCM and methanol (1:1, polar). All spectroscopically pure solvents were of super-dehydrated grade. Aliquots of the polar fractions and total extracts were converted to trimethylsilyl (TMS) derivatives by reaction with *N,O*-bis-(trimethylsilyl)trifluoroacetamide (BSTFA), 1% trimethylchlorosilane, and pyridine for 3 h at 70 °C. An Internal standard (ethyl vanillin)

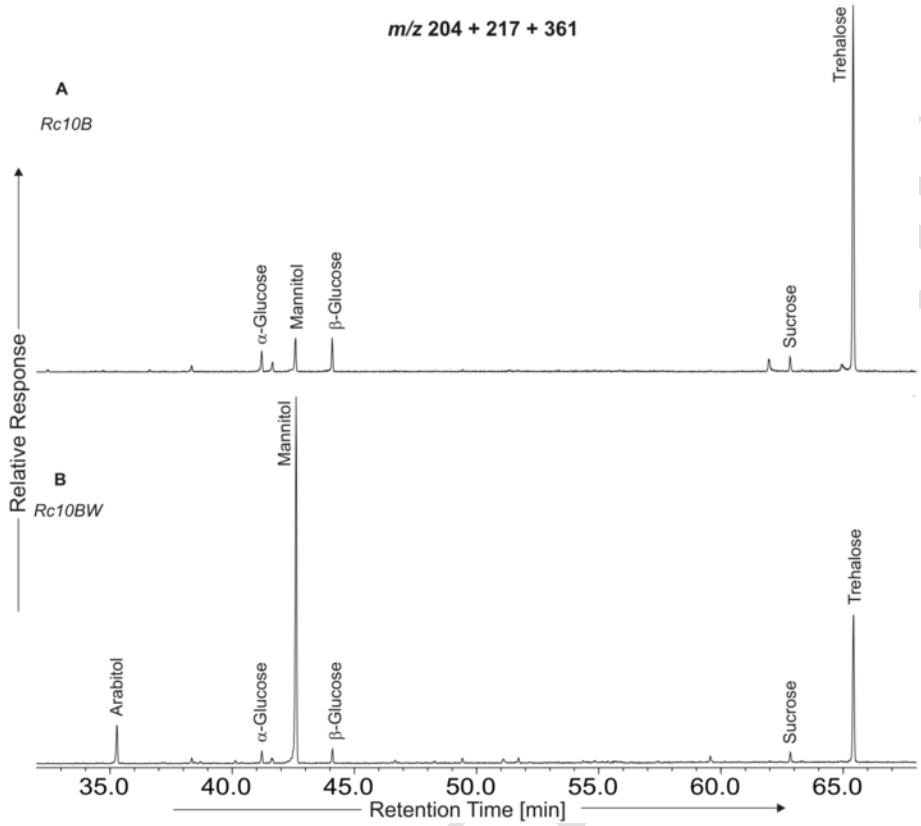

Fig. 4. Summed mass chromatograms *m/z* 204 + 217 + 361 of: (A) the mudstone (Rc10B) where trehalose is the dominant sugar, and (B) coal seam (Rc10BW) within the Rc10B mudstone where mannitol dominates.

was added to the total extracts before derivatization. The excess reagent was evaporated off under a stream of dry nitrogen gas and the sample mixture dissolved in an equivalent volume of *n*-hexane. A blank sample (silica gel) was analyzed using the same procedure (including extraction and separation on columns). Saccharides were not detected in the blank sample. In case of wood-degrading fungi, total extracts were analyzed using the same method as rock extracts.

3.6. Gas chromatography - mass spectrometry

Gas chromatography - mass spectrometry (GC–MS) analyses were carried out with an Agilent Technologies 7890A gas chromatograph and Agilent 5975C Network mass spectrometer with Triple-Axis Detector (MSD) at the Faculty of Earth Sciences, Sosnowiec. Helium (6.0 Grade) was used as a carrier gas at a constant flow of 2.6 ml/min. Separation was obtained on a fused silica capillary column (J&W HP5-MS, 60 m × 0.25 mm i.d., 0.25 μm film thickness) coated with a chemically bonded phase (5% phenyl, 95% methylsiloxane), for which the GC oven temperature was programmed from 45 °C (1 min) to 100 °C at 20 °C/min, then to 300 °C at 3 °C/min (hold 60 min), with a solvent delay of 10 min.

The GC column outlet was connected directly to the ion source of the MSD. The GC–MS interface was set at 280 °C, while the ion source and the quadrupole analyzer were set at 230 and 150 °C, respectively. Mass spectra were recorded from 45 to 550 da (0–40 min) and 50–700 da (> 40 min). The MS was operated in the electron impact mode, with an ionization energy of 70 eV. All GC–MS analysis were performed at the Faculty of Earth Sciences, Sosnowiec.

An Agilent Technologies MSD ChemStation E.02.01.1177 and the Wiley Registry of Mass Spectral Data (9th edition) software were used for data collection and mass spectra processing. Mono- and disaccharides were identified based on comparison of mass spectra and retention times with those of standards and data published by Medeiros and Simoneit (2007). For identification of saccharides, the following standards were used: (from Sigma-Aldrich) D-glucose, L-(−)-arabitol, D-(+)-arabitol, mannitol, erythritol, threitol, sucrose, shikimic acid, pinitol, quinic acid, tricarballylic acid and (from Fluka) trehalose. Ethyl vanillin (Sigma-Aldrich) was used as internal standard.

4. Results

4.1. Vitrinite reflectance

The dominant maceral in all samples is vitrinite, preserved usually as large but highly porous fragments. Inertinite was identified in one sample only as compacted / shattered cells. Mean vitrinite reflectance values measured for 5 samples (100 counts each) ranged from 0.39% to 0.45% Rr (Table S1 in Supplementary Material section). Such values are characteristic for lignites to sub-bituminous coals, where the maximum temperature influence on the OM never exceeded 50 °C (Hunt, 1995).

4.2. General geochemical data

Total organic carbon values for the Cretaceous samples are diverse and range from 0.3 to 49% wt. (Table 1). In the Rakowice Małe section one thin coal seam can be distinguished (Rc9B; 49% wt. TOC), and one mudstone horizon (Rc10B) with coal intercalations (Rc10BW; 38.5% wt. TOC). The organic matter (OM) in the Rakowice Małe section is immature (see section 4.1). The Carbon Preference Index (CPI) values are >3 for all samples indicating not only terrestrial higher plant input, but also supporting the immaturity of the OM (Table S2). Hopanes

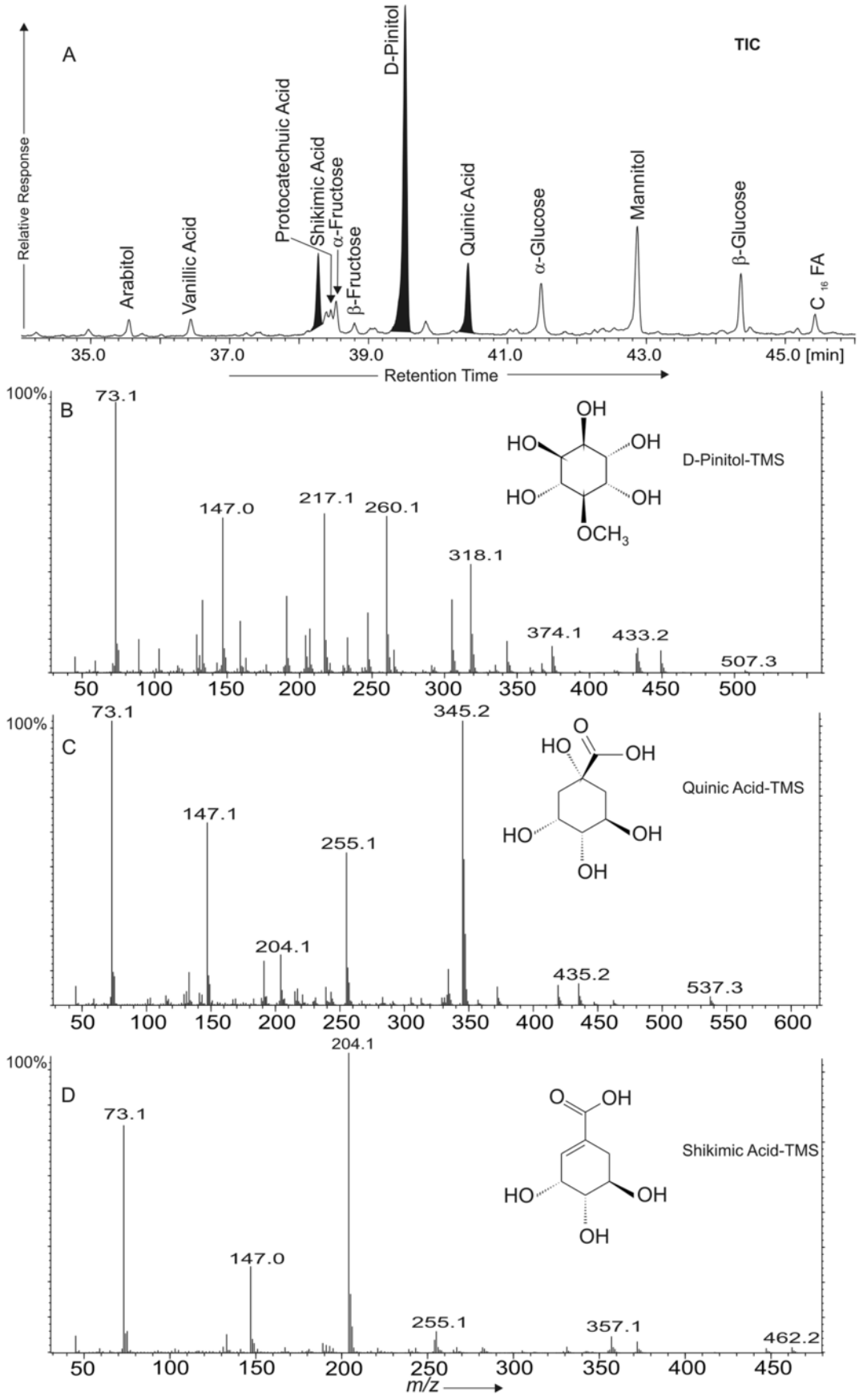
A
TIC
Relative Response
Arabitol
Vanillic Acid
Protocatechuic Acid
Shikimic Acid
α-Fructose
β-Fructose
D-Pinitol
Quinic Acid
α-Glucose
Mannitol
β-Glucose
C 16 FA
35.0
37.0
39.0
41.0
43.0
45.0 [min]
Retention Time
B
100%
73.1
147.0
217.1
260.1
318.1
374.1
433.2
507.3
D-Pinitol-TMS
C
73.1
345.2
147.1
255.1
204.1
435.2
537.3
Quinic Acid-TMS
D
204.1
73.1
147.0
255.1
357.1
462.2
Shikimic Acid-TMS
m/z

Fig. 5. TIC chromatogram of a Miocene detritic coal sample showing the distribution of saccharides (A), and the mass spectra of D-pinitol (B), quinic acid (C) and shikimic acid (D), as the trimethylsilyl derivatives.

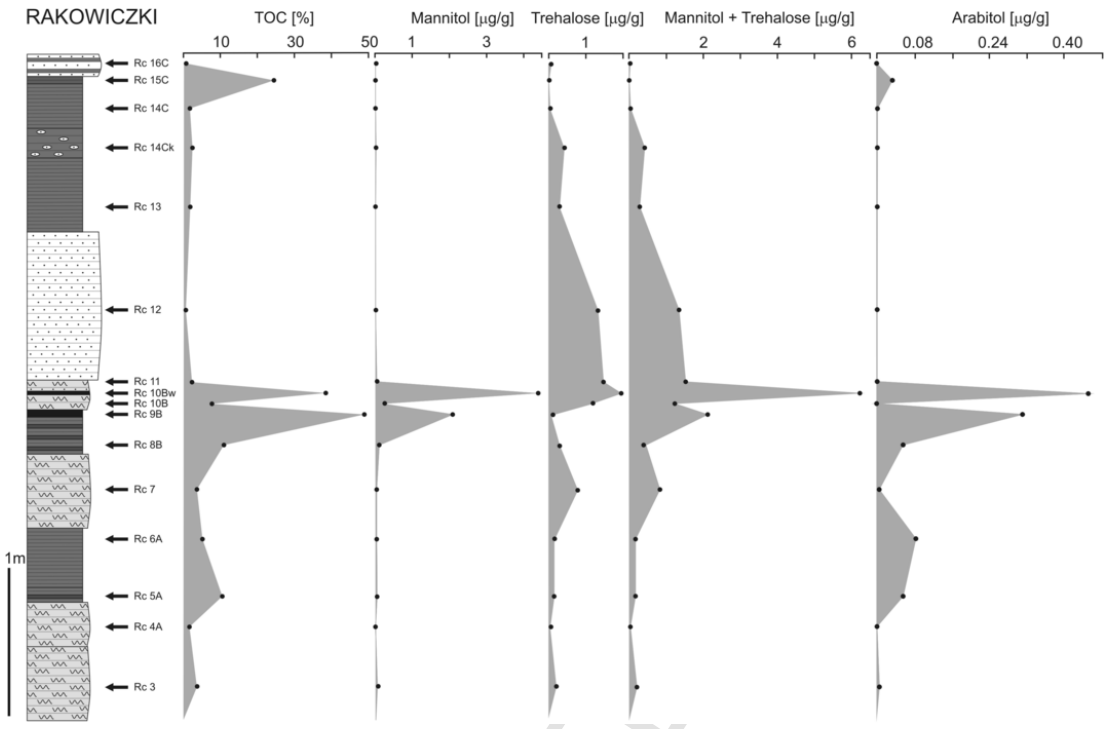

Fig. 6. Composite plot of the simplified Rakowice Małe section showing depth trends of the bulk TOC and the saccharide data. For the detailed section see Leszczyński (2010).

with the biological precursor 17β(H),21β(H) configuration and hopenes predominate in all samples (Fig. 1), which is, together with the 22R-homohopane dominance [22S/(22S + 22R) ~ 0.2; Table S2], characteristic for immature OM (Peters et al., 2005). The limited range of the hopanes (C_{27}, C_{30} and C_{31} high) with hopenes is typical of an origin from degraded terrestrial vegetation as in peats and soils (Quirk et al., 1984; Ries-Kautt and Albrecht, 1989). Moreover, the samples contain conifer biomolecules like ferruginol, dehydroabietic acid, and callitrisic acid (Fig. 2A) which typically occur only in immature OM (Marynowski et al., 2007a, 2007b). Other compounds characteristic for coniferous higher plants identified in these samples are dehydroabietane, simonellite and retene, while tetracyclic diterpenoids were scarce or not present. Tricarballylic (1,2,3-propanetricarboxylic) acid was identified in two TOC-rich samples (Rc9B and Rc10BW).

The Miocene lignites have very low maturities and the details were described by Marynowski et al. (2018).

4.3. DNA tests

The results of the DNA manipulation performed in this study are shown in Fig. 3. Lanes 2–16 present the electrophoretic separation of probes obtained after attempts of total DNA isolation from all the Cretaceous and blank samples analyzed. No detectable DNA bands were obtained from the rock samples (Fig. 3, lanes 7–16). The DNA concentration measured by the NanoDrop spectrophotometer in the Cretaceous samples (lanes 7–16) were comparable to those of the blanks (Fig. 3, lanes 2, 3), and resulted from contamination and the high sensitivity of the analytical method (see the legend of Fig. 3). The samples of rotten wood of birch, maple and oak constituted a positive control for the experiment. In this case 39.9 ng/μl, 87.6 ng/μl and 63.6 ng/μl of DNA were obtained, respectively, and DNA bands were observed after electrophoretic separation (Fig. 3, lanes 4–6). Next, all the samples after DNA isolation were used as templates to the PCR reaction to detect and distinguish bacterial and fungal DNA. The results clearly show that the samples of the rotten wood contain both fungal and bacterial DNA, indicating the presence of bacteria and fungi in the examined material. Lanes 18–20 and 33–35 in Fig. 3 present, respectively, the PCR products specific for bacteria and fungi. On the contrary, no DNA bands and consequently no PCR product bands were obtained from the rock samples after electrophoretic separation (Fig. 3, lanes 21–30 and 36–45).

4.4. Saccharide distribution and concentration

The saccharides and saccharols detected in the samples of Miocene coals and Cretaceous sedimentary rocks are: glycerol, α- and β-glucose, sucrose, trehalose, erythritol, arabitol and mannitol (Figs. 2 and 4). In addition, D-pinitol, quinic acid, and shikimic acid were identified in two Cretaceous samples and one Miocene detritic coal from Jaroszów (JR7z), based on co-elution with authentic standards and mass spectral fragmentation patterns (Fig. 5). Their mass spectra are presented in Fig. 5B, C and D. The distribution of saccharides in the Cretaceous samples is diverse. Trehalose is the major sugar (maximum concentration 1.95 μg/g of sample) in most of the samples, dominating over mannitol, sucrose and glucose (Table 1). However, in the case of two organic-rich coal samples (Rc9B and Rc10BW) mannitol is the main sugar at 2.09 and 4.28 μg/g of sample (Figs. 2, 4 and 6 and Table 1). Glucose is present in all samples at moderate to low concentrations, while sucrose was identified in some samples at usually low concentrations (Table 1). Arabitol and erythritol are present only in some samples (not exceeding 0.5 μg/g of sample), but there is a good correlation ($R^2 = 0.95$) between the arabitol and mannitol concentrations in the Cretaceous rocks (Fig. S1). No such correlation is observed between trehalose and mannitol ($R^2 = 0.2$; Fig. S1).

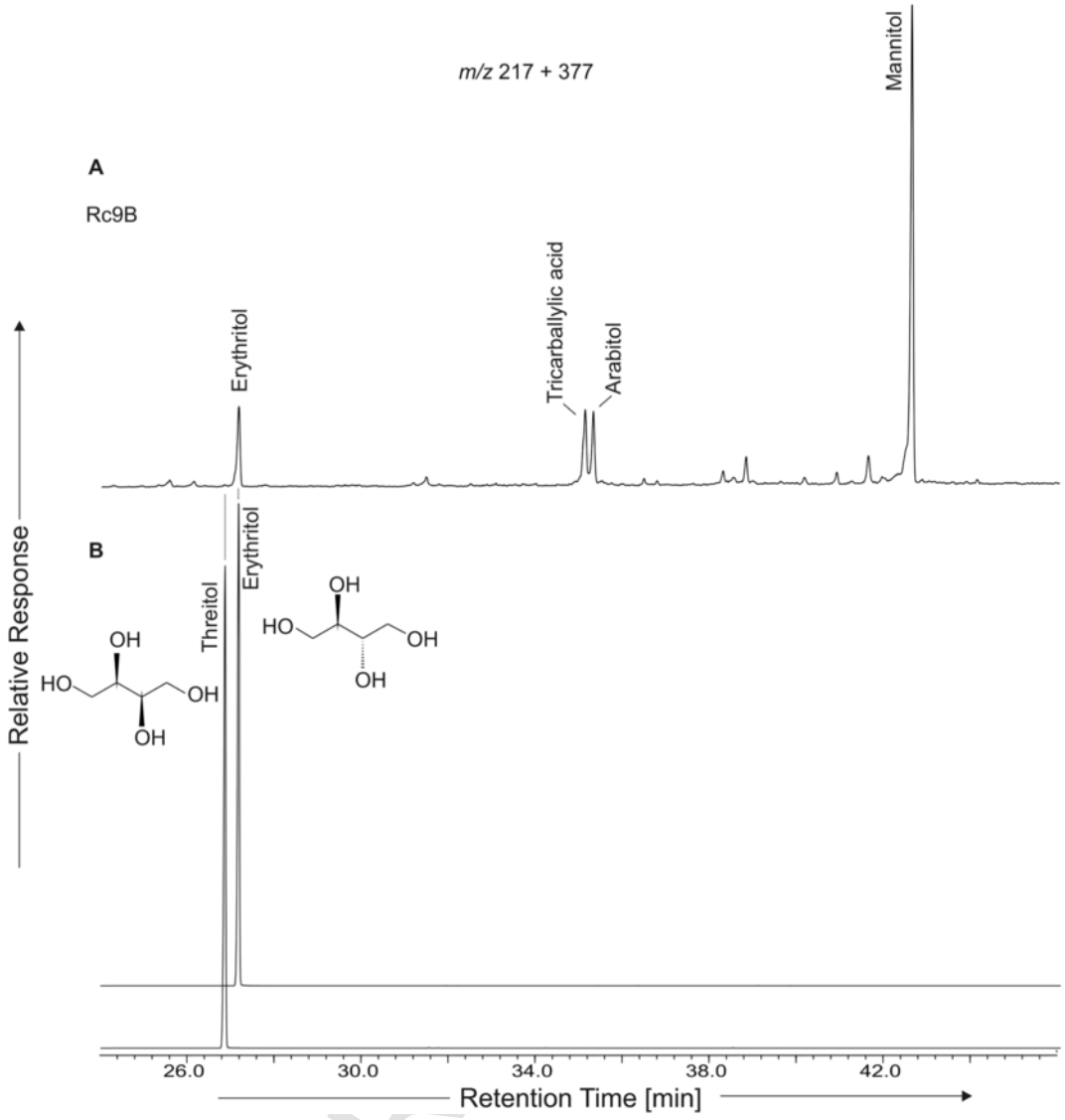

Fig. 7. Summed mass chromatograms *m/z* 217 + 377 of: (A) sample Rc9B with saccharols and tricarballylic acid as major compounds, and (B) erythritol and threitol standards.

5. Discussion

5.1. Primary character of fungi in late cretaceous and Neogene samples

The confirmation of the primary origin of fungal saccharides in an investigated geologic section is an important issue, because modern fungi can potentially contaminate sedimentary rocks or even occur in low rank coals (Haider et al., 2015).

That is why we attempted to isolate total DNA from the Cretaceous rock samples and samples of rotten wood of birch, maple and oak as a positive control. The results of the DNA tests clearly show the presence of bacteria and fungi in the samples of rotten wood, whereas no detectable DNA was obtained from the rocks. These results could indicate that low numbers of microorganisms are present in the examined material. Many efforts have been undertaken to examine autochthonous microflora of lignite (e.g. Strąpoć et al., 2008, 2011; Haider et al., 2015). Culture-independent techniques were employed for molecular analyses of fresh raw coal samples (Strąpoć et al., 2008, 2011). For example, enrichment techniques were required in many approaches to detect and find methanogenic *Archaea* (Green et al., 2008; Strąpoć et al., 2008, 2011; Opara et al., 2012; Barnhart et al., 2013). However, the identified sugars are major compounds in extracts of the TOC-rich Cretaceous samples, and the lack of isolatable DNA suggests their primary origin, connected with fungal 'blooms' during coal formation.

5.2. Origin of mannitol, trehalose and other saccharides in sedimentary rocks

Perylene, detected in sedimentary rocks, is the compound commonly regarded as a biomarker for wood degrading fungi (e.g. Grice et al., 2009; Marynowski et al., 2013). Its biological precursor according to Itoh et al. (2012) is 4,9-dihydroxyperylene-3,10-quinone (DHPQ), biosynthesized by *Cenococcum geophilum* Fr., an ectomycorrhizal fungus.

In contrast, saccharides of a fungal origin have never been identified in rock samples. It is widely known that trehalose, mannitol and arabitol when present together, are characteristic for different fungi, including mycorrhizas and mycorrhizal (Martin et al., 1988; Schubert et al., 1992; Koide et al., 2000; Nehls et al., 2010), entomopathogenic (Bidochka et al., 1990), wood-rotting (Croan, 2000; Hybelbauerová et al., 2008), as well as root-rot (Asiegbu, 2000) species (see also Solomon et al., 2007). Their role in fungi is still not fully recognized,

but includes carbohydrate storage, reserve carbon source, as well as membrane and macromolecule protectants toward different types of stress (Thevelein, 1984; Asiegbu, 2000; Ruijter et al., 2003; Elbein et al., 2003; Solomon et al., 2007; Ferreira et al., 2007). It is also noteworthy that some types of fungi, i.e. white rot fungi, will degrade lignin relative to carbohydrates (Robertson et al., 2008). Most recently Marynowski et al. (2018) identified elevated levels of trehalose and mannitol in some Miocene detritic coals and xylites, and preliminarily interpreted them as fungal biomarkers. The presence of these compounds in the Late Cretaceous samples as major saccharides (Figs. 2 and 4) undoubtedly suggests a fungal origin. Taking into account the ubiquitous occurrence of these sugars in extant fungi (Table S3) and fungal spores, and their preservation potential in rocks as old as Cretaceous, we conclude that trehalose, mannitol and arabitol should be treated as new fungal biomarkers (biomolecules). In addition, in samples enriched with mannitol and arabitol (Rc9B and Rc10BW), tricarballylic acid was identified (Fig. 7). It is a mycotoxin compound common in modern fungi (Freire and Sant'Ana, 2018).

5.3. Implications for differences in saccharide distribution and organic matter preservation

Differences in the distributions of trehalose, mannitol and arabitol were observed in the Rakowice Małe section. Mannitol and arabitol are present at higher concentrations in the TOC-rich samples, while trehalose dominates in all other rock horizons (Table 1; Fig. 6). Differences in saccharide distribution were also noted in extant wood-degrading fungi (Table S3).

Koide et al. (2000) reported that the role of trehalose and mannitol is diverse in fungi. Trehalose is a storage carbohydrate and its accumulation rises in winter, while mannitol plays a translocatory role, increasing in concentration during warmer seasons. The good correlation between mannitol and arabitol for the Rakowice Małe samples, also observed for airborne fungal spores (Bauer et al., 2008), suggests that their role in fungi would be similar. If the trehalose, mannitol and arabitol concentrations reflect colder vs. warmer periods, then the coal seams of the Rakowice Małe section formed during warmer climatic stages, which seems reasonable. Generally, the correlation of the saccharide distribution with temperature may be useful in paleoclimate reconstruction, but that requires additional study, especially since diverse sugar distributions are also dependent on fungi types (e.g. P-type vs. S-type; see Asiegbu, 2000). Moreover, Bondarenko et al. (2017) found that some alkaliphilic fungi change their carbohydrate composition at a very high pH (glucose is substituted by mannitol and arabitol). The high concentration of fungal biomarkers in this Late Cretaceous section suggests their important role in this period of time. The Cretaceous is generally considered a time of warm climate (e.g. Steuber et al., 2005), which together with increased humidity would have enhanced fungal 'blooms'. The swamp to lagoonal sedimentary environment of this rock section (Leszczyński, 2010) would also have provided excellent conditions for intensified growth of both wood-rot and mycorrhizal fungi. It is worth mentioning that arbuscular mycorrhizal colonies are typical representatives of modern peat swamps (e.g. Tawaraya et al., 2003), and thus potentially responsible for the occurrence of fungal sugars in coal samples.

The occurrence of trehalose as a major saccharide and the presence of sucrose in some samples imply that disaccharides can survive in sedimentary rocks as old as the Cretaceous. Other compounds like: isoprenoidal GDGTs (Schouten et al., 2003; Littler et al., 2011; Jenkyns et al., 2012), sterols (Melendez et al., 2013), hypericinoid pigments (Wolkenstein et al., 2008; Wolkenstein, 2015), polyketide-derived spiroborate pigments (Wolkenstein et al., 2015), or polar diterpenoids (Marynowski et al., 2007b) have been reported in Palaeozoic and Mesozoic sedimentary deposits. These are other groups of compounds previously thought as unstable which were preserved in pre-Palaeogene strata. This indicates that under favorable conditions (immature OM, lack of secondary processes like oxidation, biodegradation etc.) not only simple biological compounds like fatty acids, but also labile, polar natural products, can survive tens or even hundreds of millions of years without structural alteration.

5.4. Other sugars and their origin

In addition to fungal saccharides some of the Cretaceous and Miocene samples contain sugar alcohols and acids (i.e., D-pinitol, quinic acid and shikimic acid) (Fig. 5; Table 1), not previously described in sedimentary rocks. These compounds are natural products often present in modern plants that form peat bogs. For instance, D-pinitol is a well-known metabolite common in conifers (e.g. Savidge and Förster, 2001). Also, quinic and shikimic acids were identified in *Pinus radiata* (Cranswick and Zabkiewicz, 1979) and *Ginkgo biloba* (Singh et al., 2008) extracts, reflecting their common occurrence in the higher plant kingdom. These compounds supplement the list of saccharides identified in lignites and other sedimentary rocks (Rybicki et al., 2017; Marynowski et al., 2018; see Medeiros and Simoneit, 2007).

Erythritol and threitol are two natural tetrols (saccharols) with a similar structure and mass spectra (see Medeiros and Simoneit, 2007). However, they can be distinguished by the slightly different retention time on the DB-5 capillary column. Here we compared authentic standards of both compounds with the Miocene and Cretaceous samples. In both cases erythritol is present in the samples (Fig. 7). Emygdio et al. (2018) reported the identification of threitol besides arabitol and mannitol in São Paulo, Brazil ambient air particles and connected that with a fungal origin. It is highly probable, that the identified compound by Emygdio et al. (2018) is in fact erythritol, also described from some modern fungi (e.g. Gunde-Cimerman et al., 2009; Rakicka et al., 2016).

6. Conclusions

Trehalose, mannitol and arabitol were identified in Late Cretaceous and Miocene sedimentary rocks using GC–MS analysis. Their origin was interpreted as fungal, based on correlation with diverse types of modern fungi. We believe that the co-occurrence of these three saccharides in sediments indicates fungal metabolism and they can be regarded as fungal biomarkers (biomolecules). Other saccharols and sugar acids including: D-pinitol, quinic acid and shikimic acid were found in geological materials for the first time. The origin of these compounds is interpreted to be from conifers and other higher plants. Based on the trehalose, mannitol and arabitol distributions we preliminarily conclude that the coal seams of the Rakowice Małe section (Cretaceous) were deposited during warmer climatic periods. Preservation of mono- and disaccharides in pre-Palaeogene strata suggests that under favorable sedimentary conditions, compounds previously thought as unstable can survive unchanged for tens to hundreds of millions of years.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.coal.2018.11.003.

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