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GC-MS ANALYSIS OF *RAMALINA CAPITATA* (ACH.) NYL. EXTRACT[†]

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Abstract. *This is the first report on the GC-MS profile of the ether-soluble fraction (ESF) of the methanol extract of the lichen Ramalina capitata (Ach.) Nyl.* (*Ramalinaceae*). *The profile was dominated by orcinol (22.9 %) and its monomethyl ether (30.9 %), which accounted for more than a half of the GC-MS analyzable fraction of ESF. Significantly lower amounts of structurally related sparassol (5.8%) and atraric acid (0.9 %) were also detected. Additionally, ESF contained methyl linoleate, methyl linolenate and methyl palmitate (17.3 %, 7.3 % and 5.0 %, respectively).*

Key words: *Ramalina capitata (Ach.) Nyl., volatiles, GC-MS, orcinol, orcinol monomethyl ether*

1. INTRODUCTION

Lichens are a symbiotic association between fungi and photosynthetic algae (and/or cyanobacteria). They produce a lot of (more than 1,500) unique chemical compounds – "lichen substances" – as a result of adaptation for life in marginal habitats (Temina *et al*., 2010). Some of these metabolic products appear only in lichens and play a dominant role in their system (Huneck, 1999). These secondary metabolites fall into various chemical classes (Dayan and Romagni, 2001). Lichen substances have many ecological and biological activities on viruses, microorganisms, algae, bryophytes, higher plants and human race (Huneck, 1999).

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Dedicated to Professor Radosav Palić on the happy occasion of his 70th birthday.
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Lichens have been used for commercial purposes in perfume, dye, alcohol, food, and drug industries (Kirmizigul *et al.*, 2007). Also, lichen communities can be used as biomonitors of change in ecosystems (Garty, 2001).

The genus *Ramalina* Ach. (family *Ramalinaceae*) has a widespread distribution and contains over 240 species. Many papers have previously been published on the investigation of chemical composition of secondary metabolites of *Ramalina* species. Some of those studies were mainly focused on the identification and isolation of usnic acid that has a variety of biological activities (Cansaran *et al.*, 2007). The secondary metabolites of lichens are located in the cortex and medulla. Among the compounds which are located in the cortex, usnic acid, which belongs to the type of benzofuran compounds, is present in almost all examined *Ramalina* species, while majority of pale gray species contain depside atranorin (Aptroot and Bungartz, 2007). The most common major medullary compounds are salazinic acid, sekikaic acid, homosekikaic acid, divaricatic acid, or rarely norstictic acid, lecanoric acid, protocetraric acid and orsellinic acid.

Some *Ramalina* species are used as food in some Central and South Eastern Asian countries and also for medical purposes, in perfumery and cosmetics (Richardson, 1974).

According to the SciFinder search of the CAS databases, as well as Google Scholar (up until January 2015), there is only a limited data (content of usnic acid (Cansaran *et al.*, 2007)) regarding chemical composition of *Ramalina capitata* (Ach.) Nyl. (syn: *Ramalina capitata* var. *strepsilis* (Ach.) Motyka, *Ramalina polymorpha* var. *capitata* Ach., *Ramalina strepsilis* (Ach.) Zahlbr.). As a part of our ongoing interest in the chemistry of genus *Ramalina*, the aim of this study was to determine the GC-MS profile of the diethyl ether soluble fraction (ESF) of the methanol extract of *R. capitata*. Since it is very difficult to collect a sufficient amount of *R. capitata*, methanol was chosen as a primary solvent that gives the highest yield (Stojanovic *et al.*, 2013) and is sufficiently polar to not extract waxes. To the best of our knowledge, there are no data regarding volatile profile of this species.

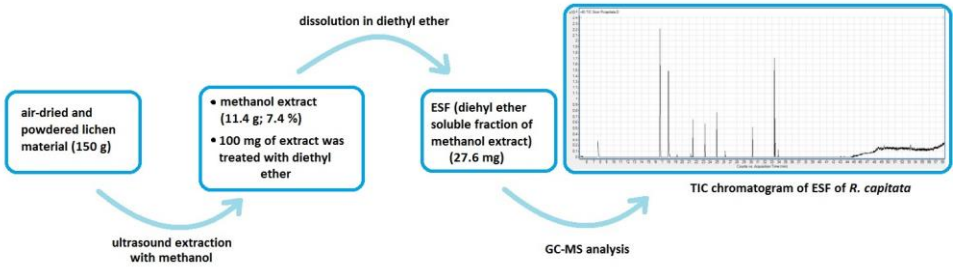
2. Materials and Methods

2.1. Lichen material

Ramalina capitata was collected in May 2013, at mountain peak Babin zub (1,650 m above sea level, coordinates 43° 23' N, 22° 40' E; Stara planina mountain, Serbia), from the population growing on rock habitat (red sandstone, silicates). The voucher specimen has been deposited in the Herbarium collection at the Department of Biology and Ecology, Faculty of Science and Mathematics, University of Niš under the acquisition number 9374.

2.2. Extraction

The lichen material was air-dried without exposure to direct sunlight for 10 days and stored at ambient temperature (25° ± 2°C) prior to further treatment. Powdered lichen material of *R. capitata* (150 g) was first subjected to ultrasound-assisted extraction with methanol (950 mL; 30 min in ultrasound bath – UZK 8; Maget, Bela Palanka, Serbia); after that, the extract was left in the dark (room temperature) for additional five days.



Scheme 1 The procedure of sample preparation for GC-MS analysis

The crude extract was filtered and the methanol was evaporated under reduced pressure in a rotary evaporator. The extract yield was 7.4 %. The dry residue (100 mg) was dissolved in diethyl ether and filtered through 0.45 µm filter. In order to measure the yield of the ether-extract, the ether was evaporated and gave 27.6 mg (2.0 % of lichen material) of the residue (diethyl ether soluble fraction of methanol extract, ESF). ESF was re-dissolved in diethyl to give solution with concentration of 10 mg/ml, which was further analyzed by GC-MS analyses. The entire procedure is shown in **Scheme 1**.

2.3. GC–MS analyses

Diethyl ether soluble fraction of methanol extract of *R. capitata* (ESF) was analyzed in triplicate using a 7890/7000B GC-MS/MS triple quadrupole system (Agilent Technologies, USA, equipped with a Combi PAL auto sampler). The fused silica capillary column HP-5MS (5% phenylmethylsiloxane, 30 m x 0.25 mm, film thickness 0.25 µm) was used. The injector and interface operated at 250 and 300 °C respectively. The temperature program: 70 °C for 2.25 min, 5 °C min^{-1} to 300 °C, then isothermally held for 10 min. The carrier gas was helium with a flow of 1.0 mL min-1. The 5 µL of samples were injected split ratio 2 : 1. Post run: back flash for 1.89 min, at 280 °C, with helium at 50 psi. MS conditions were as follows: ionization voltage of 70 eV, acquisition mass range 50-650, scan time 0.32 s. The percentage composition was computed from the TIC peak areas. Constituents were identified by comparison of their linear retention indices (relative to C_8 – C_{40} alkanes on the HP-5MS column) with literature values (Dool and Kratz, 1963; Adams, 2007; Stein, 1990) and their MS with those from Wiley 6, NIST02 and Mass Finder 2.3, by the application of the AMDIS software (the Automated Mass Spectral Deconvolution and Identification System, Ver. 2.1, DTRA/NIST, 2011).

3. Results and Discussion

Identity and relative abundance of the volatile compounds of the ether soluble fraction of the methanol extract of *R. capitata* were determined by GC-MS analysis. The results are given in **Table 1**.

Table 1 Composition (%) of the GC-MS analyzable fraction of ESF of *R. capitata*

Compound	*RI*	*RL*	*Retention time (min)*	*Relative abundance (%)*
Orcinol monomethyl ether (*syn.* 3-methoxy-5-methylphenol)	1319	1317*	16.676	30.9
Orcinol (*syn.* 5-methylbenzene-1,3-diol)	1369	1369*	17.959	22.9
Sparassol (*syn.* methyl 2-hydroxy-4-methoxy-6-methylbenzoate)	1589	1580*	23.216	5.8
Unknown	1668	-	24.96	7.6
Atraric acid (*syn.* methyl *β*-orcinolcarboxylate or methyl 2,4-dihydroxy-3,6-dimethylbenzoate)	1727	1706*	26.24	0.9
Methyl palmitate (*syn*. methyl ester hexadecanoic acid)	1922	1921**	30.174	5.0
Methyl linoleate (*syn.* methyl (*Z*,*Z*)-9,12-octadecadienoate)	2091	2095**	33.34	17.3
Methyl linolenate (*syn*. methyl (Z,Z,Z)-9,12,15-octadecatrienoate)	2097	2100***	33.44	7.3

RI: Experimental retention indices relative to C_8-C_{40} *n*-alkanes
RL: Literature values of the retention indices
* Stojanović *et al.*, 2011
** Adams, 2007
*** Zhao *et al.*, 2009
syn.-synonym
Unknown MS, 70 eV, *m/z* (rel. int.): 55 (100 %), 67 (93 %), 81 (85 %), 82 (73 %), 54 (65 %), 96 (60 %), 68 (57 %), 69 (57 %), 95 (52 %), 83 (38 %)

Eight identified compounds represented 97.7 % of GC-MS analyzable fraction of ESF of *R. capitata*. Their chemical structures are given in Fig. 1.

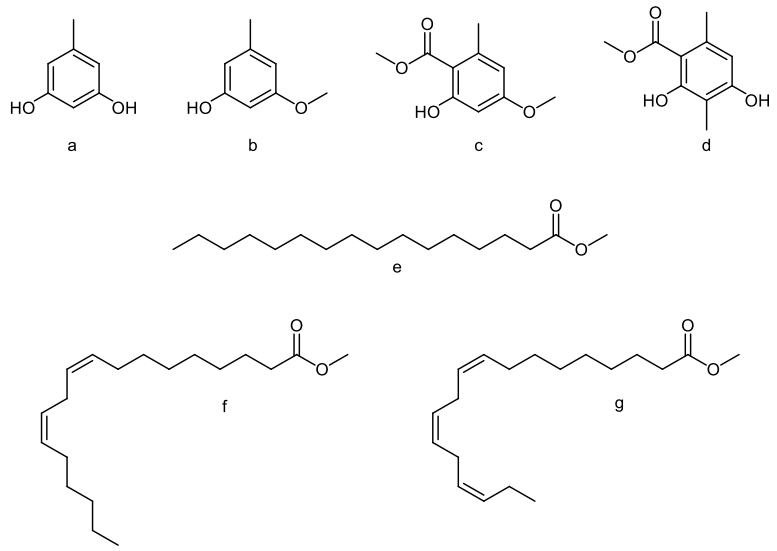
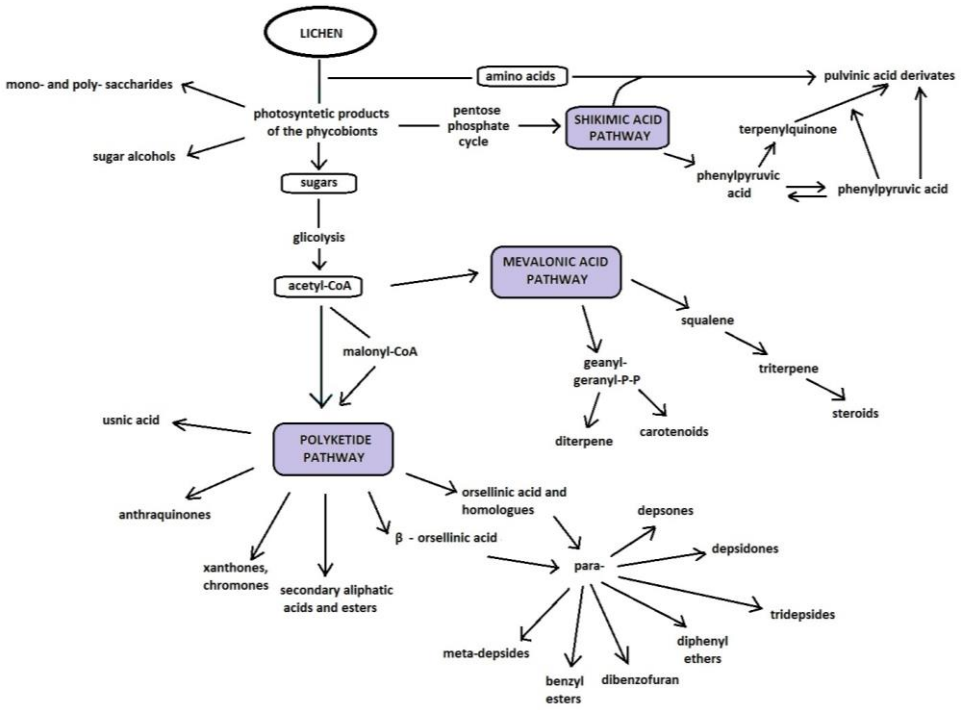


Fig. 1 The chemical structures of *R. capitata* constituents: a) orcinol, b) orcinol monomethyl ether, c) sparassol, d) atraric acid, e) methyl palmitate, f) methyl linoleate, g) methyl linolenate

The major components detected in ESF of *R. capitata* are monocyclic aromatic compounds: orcinol (22.9 %) and its derivate orcinol monomethyl ether (30.9 %), comprising together 53.8 %. Sparassol was present in much lower relative amount (5.8 %). Large relative amounts of atraric acid and orcinol were previously detected in *Evernia prunastri* (L.) Ach. and/or *Parmelia sulcata* Taylor (up to 30% of the GC-MS analyzable fraction of the studied samples) (Stojanović, 2011). Atraric acid is also represented in the high shares (12.1 – 47.7 %) in the extracts of *Platismatia glauca* and *Pseudevernia furfuracea* (Mitrović *et al.*, 2014). Unlike these lichen species (both belonged to Parmeliaceae family), *R. capitata* contained much lower relative amount of atraric acid (0.9 %). This quantitative variation of the produced secondary metabolites present within different species is the result of biosynthetic pathway diversity. The secondary metabolites are generally produced by one of three major pathways – polyketide, shikimic acid and mevalonic acid pathways (Scheme 2) (Chooi, 2008). The produced compounds are modified by various enzymes during the biosynthesis resulting in a highly diverse collection of molecules in both structure and function (Deduke *et al*., 2012). Therefore, dominance of secondary metabolites is various among species due to their different metabolism. *R. capitata* predominantly biosynthesizes derivates of orcinol and, to a lesser extent, derivates of *β*-orcinol (atraric acid).



Scheme 2 Adapted scheme of probable pathways leading to the major groups of lichen metabolites (Chooi, 2008)

The GC-MS analyses of ESF of *R. capitata* also showed the presence of methyl esters of common fatty acids. Methyl linoleate was found as a major ester (17.3 %;) followed by methyl linolenate (7.3 %) and methyl palmitate (5.0 %). Previous study has shown relatively small amounts of methyl palmitate and methyl linolenate (0.5 – 5.7 %) in several other lichen species (*Pseudevernia furfuracea* (L.) Zopf, *Evernia prunastri* (L.) Ach. and *Letharia vulpina* (L.) Hue; populations from Turkey). The mentioned lichen samples were characterized by high relative amounts of linoleic acid (34.4 – 47.9 %). Similarly, a considerable relative amount of atraric acid (8.6 – 14.9 %) was detected in tested lichen species (Kirmizigul *et al.*, 2007). Methyl palmitate was detected in small amounts in *Platismatia glauca* and *Pseudevernia furfuracea* while methyl linoleate was detected only in *P. furfuracea* (Mitrović *et al.*, 2014).

In addition to fatty acid esters, the unknown compound is also present (7.6 %). Since the identification was conducted based on mass spectra from a database, this unidentified compound might be diene or unsaturated alcohol. Considering both, the mass spectra (Fig. 2) and the retention indices, it is assumed that it is unsaturated alcohol. However, geometric isomer of unsaturated alcohol is not determined due to the fact that spectra are almost identical and literature values of retention indices for isomers differ for a unit.

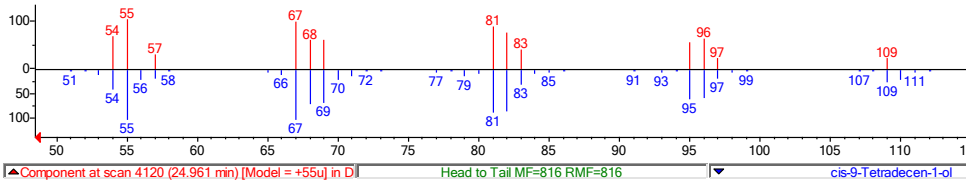


Fig. 2 Head to Tail Mass spectra of unknown compound and (*Z*)-9-Tetradecen-1-ol (NIST MS Search)

In this study, we did not reveal the presence of usnic acid, which was detected in *R. capitata* in previous work (Cansaran *et al.*, 2007). A possible cause could be that the concentration of usnic acid in the examined fraction was below the detection limit.

4. CONCLUSION

This report presents the GC-MS profile of the ether soluble fraction of methanol extract of *R. capitata*. Orcinol (22.9 %) and orcinol monomethyl ether (30.9 %) were dominant components. Methyl esters of fatty acids (linoleic, linolenic and palmitic acids) constituted around one third of the GC-MS analyzable fraction of the examined lichen extract (17.3 %, 7.3 % and 5.0 %, respectively). These results contribute to the knowledge of the chemical composition of the ether fractions of the methanol extract of *R. capitata* which could be analyzed by GC-MS.

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GC-MS ANALIZA EKSTRAKTA LIŠAJA *RAMALINA CAPITATA* (ACH.) NYL.

GC-MS profil etarske frakcije (EF) metanolnog ekstrakta lišaja Ramalina capitata *(Ach.) Nyl.* (*Ramalinaceae*), *predstavljen je po prvi put. GC-MS profilom su dominirali orcinol (22,9%) i njegov derivat – orcinol monometil etar (30,9%), koji su zajedno sačinjavali polovinu EF isparljive/stabilne pod uslovima GC-MS analize. U značajno manjoj količini su detektovani sparasol (5,8%) i atrarna kiselina (0,9%). Takođe, EF je sadržavao metil-linoleat, metil-linolenat i metil-palmitat (17,3%, 7,3% i 5,0%, redom).*

Ključne reči: *Ramalina capitata (Ach.) Nyl., isparljiva jedinjenja, GC-MS, orcinol, orcinol monometil etar*