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Analysis of Biologically Active Substances Using Modern Analytical Methods

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References

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Abstract

This work deals with selective isolation of hydrophobin SC3 using polytetrafluoroethylene microparticles and its subsequent identification. In the next part the whole process of characterization of azaphilone pigments of *Talaromyces albobiverticillius* was described. Last but not least attention was paid to the complex study of mead with a focus on finding suitable indicators for mead quality control.

Abstrakt

Tato práce se zabývá selektivní izolací hydrofobinu SC3 s využitím polytetrafluorethylenových mikročástic a jeho následnou identifikací. V další části byl popsán proces charakterizace azaphilovových pigmentů mořské houby *Talaromyces albobiverticillius*. V neposlední řadě byla věnována pozornost komplexnímu studiu medovin se zaměřením na nalezení vhodných ukazatelů pro kontrolu kvality medovin.

Keywords

Hydrophobin, azaphilones, phenolic compounds, mead, liquid chromatography, mass spectrometry

Klíčová slova

Hydrofobiny, azaphilony, fenolické látky, medovina, kapalinová chromatografie, hmotnostní spektrometrie

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1 Selective isolation of hydrophobin SC3 by solid-phase extraction and its subsequent identification using modern instrumental techniques

1.1 Introduction

Hydrophobins are small surface-active proteins with molar mass mostly between 7 and 15 kDa produced by filamentous fungi [1]. Their amino acid sequence usually contains eight cysteine residues ordered in a characteristic pattern which results in a compact molecular structure strongly stabilized by four disulfide bonds [2,3]. These proteins are characterized by the ability to assemble spontaneously into amphipathic monolayers at hydrophobic–hydrophilic interfaces. Based on their hydropathy plots, solubility and the type of layer they form, hydrophobins are divided into two classes [4]. Class I hydrophobins (e.g., hydrophobin SC3 from *Schizophyllum commune*) generate very insoluble assemblies, which can be solubilized only with harsh acid treatment utilizing e.g. trifluoroacetic [5] or formic acid [6,7]. Hydrophobin SC3 consists of 112 amino acids and contains 16–22 mannose residues that are probably linked to some of the threonine and serine residues within the amino-terminal part of the molecule [8]. Assemblies of class II hydrophobins (e.g. HFBI and HFBII from *Trichoderma reesei*) are also amphipathic but they can generally be solubilized with organic solvents or using detergents [9].

Hydrophobins are neither toxic nor immunogenic for humans [10], but they can, together with other amphipathic molecules, cause primary beer gushing (a spontaneous expulsion of beer or other carbonated beverages due to contamination with filamentous fungi) [11,12]. However, their properties make them interesting for use as stabilizers of emulsions and foaming agents [10]. Further, they are a matter of interest in other areas [2,13] such as surface modifications in material science and medicine, coating of fabrics or applications in food technology. They show a potential in the development of hydrophobic nanomaterials and in the solubilizing of lipophilic drugs [2,13].

Several isolation techniques for both classes of hydrophobins were previously reported [7,14–16]. Although, a more selective and efficient technique for specific isolation of class I hydrophobin has not been described yet.

1.2 Results and discussion

1.2.1 Isolation on PTFE microparticles

The selective isolation of hydrophobin SC3 from the model mixture of ten standard proteins was carried out using a microcolumn packed with PTFE microparticles and a subsequent elution using different solvents. Fig. 1 shows a Tris-tricine-SDS-PAGE analysis of fractions obtained from individual steps of hydrophobin SC3 isolation process. It demonstrates the excellent ability of the PTFE carrier to specifically isolate the hydrophobin SC3. Lane 1 represents the initial sample consisting of hydrophobin SC3 and nine model proteins. Although this protein mixture is relatively simple, the electropherogram contains many bands corresponding to various forms of the separated proteins e.g. dimers, degradation products etc. Lane 2 corresponds to the

flow-through fraction, where hydrophobin SC3 should not be present and is expected to be bound on the PTFE carrier. Band corresponds to hydrophobin SC3 is not visible on this lane which indicates good isolation efficiency. The next step in the isolation protocol was washing of the PTFE particles. The first washing step was performed with 80% ACN/0.1% TFA (Lane 3) that was very effective in removing many weakly bound proteins from the PTFE carrier. The second washing step with 1% SDS in water removed some additional, more strongly bound proteins and contaminants from PTFE particles with the exception of hydrophobin SC3 (Lane 4). This is in agreement with previous reports, where class I hydrophobins remained attached on PTFE surface in the presence of solutions containing SDS [7,17,18]. Finally, the hydrophobin SC3 was eluted with concentrated formic acid. Hydrophobin SC3 is almost completely isolated from other proteins in a very high purity (Lane 5). The difference between the expected molar mass of hydrophobin SC3 without signal sequence (~11 kDa) and the position determined on the gel (~19 kDa) is presumably caused by its higher level of glycosylation (Fig. 2) and high hydrophobicity. Both hydrophobicity and glycosylation of proteins have a significant influence on their electrophoretic mobility in SDS-PAGE [19] and the observed bands in the gel are usually shifted to higher molar masses.
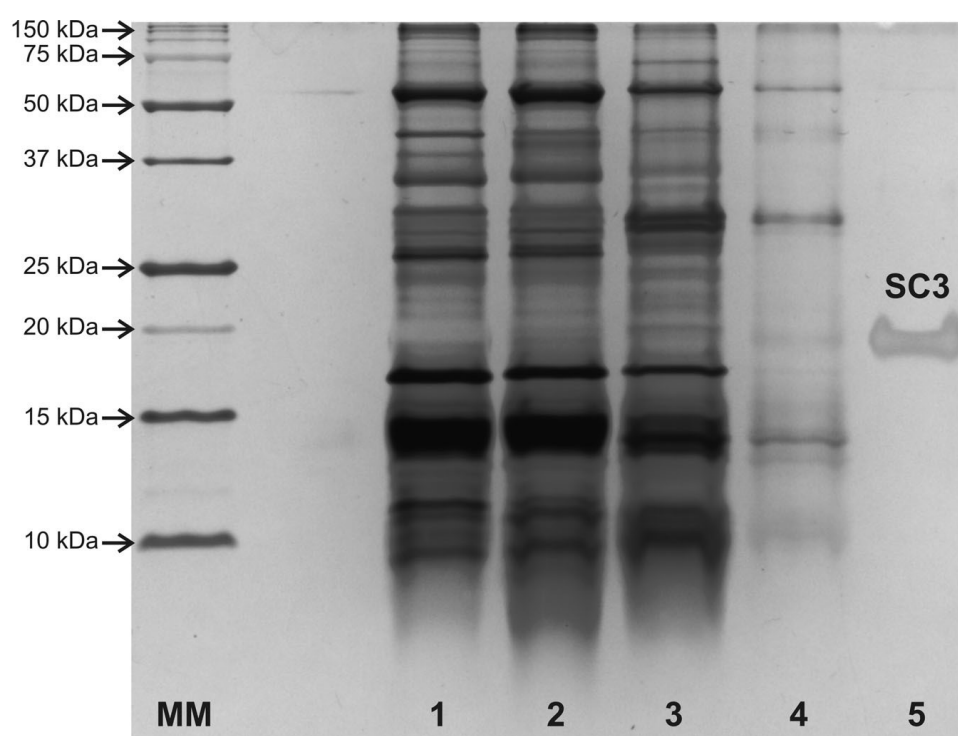
Fig. 1 Tris-tricine-SDS-PAGE analysis of fractions collected during the hydrophobin SC3 protein purification experiment from the model mixture of 10 proteins with PTFE microparticles loaded in microcolumn. Visualization: silver staining. Lanes: MM – marker of molar masses (10–250 kDa), 1 – model protein mixture, 2 – flow-through, 3 – first washing with 80% ACN/0.1% TFA, 4 – second washing with 1% SDS in water, 5 – elution with formic acid. The protein band in the lane 5 corresponds to the isolated hydrophobin SC

Results from Tris-tricine-SDS-PAGE confirmed a very strong affinity and isolation specificity of hydrophobin SC3 to PTFE microparticles, which is crucial for the protein's on-column isolation approach. In comparison to LLE, SPE provides several advantages, such as higher and more reproducible recoveries, decrease in organic solvent usage, cleaner extracts or tunable selectivity by the choice of SPE phase and solvent mixtures. SPE techniques have not previously been used for hydrophobin isolation and purification. Furthermore, high purity positively affects further analysis of these proteins in real samples (e.g. beer or malting barley). High purity is also very important for their direct utilization in other application areas (e.g. surface modification, nanomaterials or medicine).

1.2.2 MALDI-TOF-MS analysis of intact hydrophobin SC3

Linear MALDI-TOF/MS was used for analysis of hydrophobin SC3 on intact protein level. First, the effectiveness of dissolution of hydrophobin SC3 with TFA was verified and molar mass was determined (Fig. 2A). Monomeric hydrophobin SC3 was successfully detected in the MS spectrum in which the measured molar mass of various isoforms corresponds to intact glycosylated hydrophobin SC3. This is shown in detail in Fig. 2B, where the dominant peak distributions contains eight glycoforms of hydrophobin SC3 differing in the number of mannose residues. The highest detected isoform at *m/z* 14587 corresponds to hydrophobin SC3 containing 23 mannose residues and the lowest detected isoform contains 16 mannose residues (m/z 13452). This confirms the high level of glycosylation of hydrophobin SC3 reported previously [8]. Further, a minor distribution of isoforms with the loss of about 57 Da was observed in the Fig. 2B, which corresponds to the loss of a glycine residue at the N-terminus of the processed native form of hydrophobin SC3 protein sequence [20]. Figure 2C shows the mass spectrum of hydrophobin SC3 after the purification process on PTFE microparticles. Both spectra, before and after purification process (Fig. 2A and C), have a similar profile.
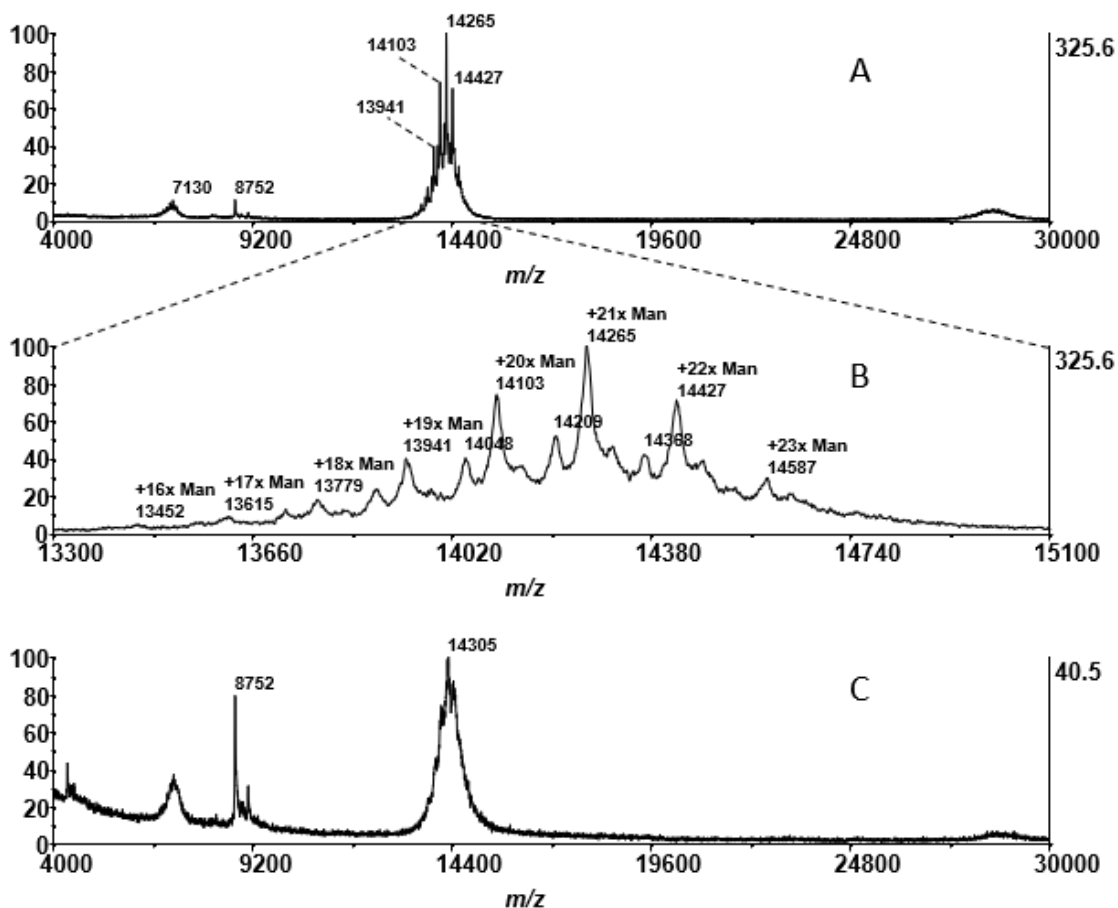
Fig. 2 Linear MALDI-TOF mass spectrum of the hydrophobin SC3. (A) Native hydrophobin SC3 dissolved in TFA; (B) detailed view of a indicating the presence of several glycosylated isoforms of hydrophobin SC3; C) hydrophobin SC3 after isolation procedure with PTFE microparticles.

1.2.3 Digestion of hydrophobin SC3 and its mass spectrometric analysis

The protein analysis of hydrophobin SC3 is extremely difficult due to its unfavorable properties such as low solubility, high hydrophobicity, very stable disulfide bonds and absence of basic residues (lysine, arginine). Protocol steps leading to the digestion of hydrophobin SC3 were performed under denaturing conditions that presumably minimize the steric/structural/hydrophobic factors hindering the completion of the particular reaction. Two detergents were used for denaturing of hydrophobin SC3: sodium deoxycholate and guanidine hydrochloride. Sodium deoxycholate seems to be more suitable denaturant thanks to its better compatibility with subsequent analytical steps. Guanidine hydrochloride was also used in a previous study [21] but from our observations the high amount of this detergent is not directly compatible with SDS-PAGE analysis used for monitoring of the digestion process. An important aspect in the protein analysis of hydrophobin SC3 is the effective disruption of disulfide bridges which keep the molecule of hydrophobins compact and highly resistant to digestion process. In our case, both reducing agents DTT and TCEP (always in the presence of

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a denaturing agent) at higher reaction temperature (80 °C) were used together to increase reduction efficiency. The reduced cysteines were alkylated using iodoacetamide. Another option for disruption of disulfide bridges is the oxidation of cysteines using performic acid [22], but MS spectra contained only a few peaks of peptides with a very low intensity after chymotryptic digestion. It was probably caused by the formation of negatively charged peptides, which decreased the ionization efficiency in the positive-ion mode.

The selection of a suitable enzyme or chemical reagent for protein cleavage is another important step in the digestion process. Trypsin and, to a lesser extent, Lys-C and Lys-N are among the most commonly used enzymes in proteomics [23]. Unfortunately, these enzymes are not suitable for the digestion of hydrophobin SC3 due to its complete absence of lysine and arginine residues as potential cleavage sites. Among others, chymotrypsin, pepsin and thermolysin were chosen for their general availability and low price. The first experiments with pepsin were not successful, because no specific peptides of reasonable size were obtained. That is why further experiments were focused on the application of chymotrypsin and thermolysin. Digestion in the presence of buffer B was first monitored with Tris-tricine-SDS-PAGE (Fig. 3). The region under 10 kDa in the electropherogram contains stained peptides, whereas the region of hydrophobin SC3 remained clear, which shows that both enzymes are able to digest hydrophobin SC3.
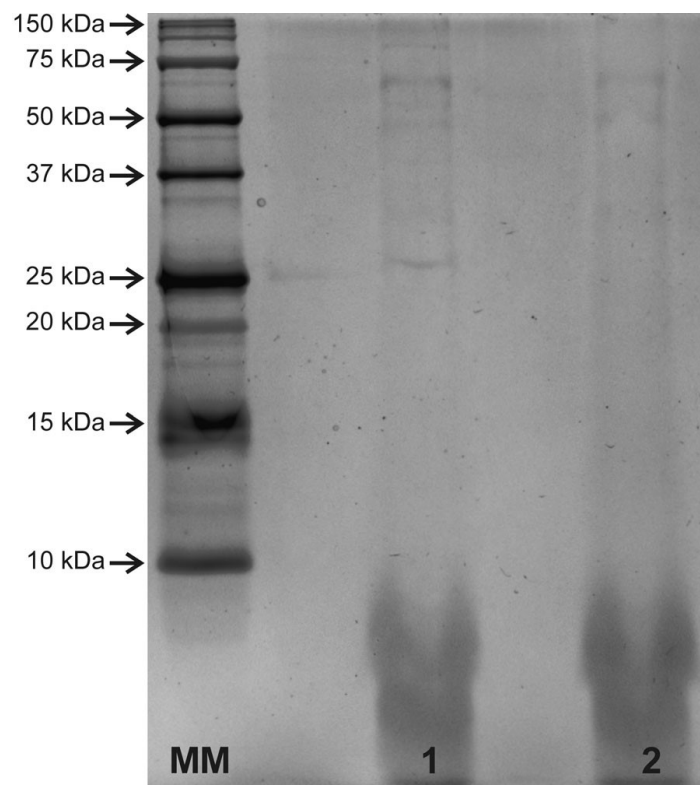

Fig. 3 Tris-tricine-SDS-PAGE analysis of the digestion mixtures of hydrophobin SC3 with chymotrypsin (1) and thermolysin (2). Visualization: silver staining. Molar mass marker (10–250 kDa) is in the left lane (MM).

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To obtain further information about the derived peptide mixtures, the purified digests were analysed using MALDI-Orbitrap mass spectrometry. Identification of the peptides from hydrophobin SC3 on MS level was done with MS-Fit database searching program with mass tolerance set to 5 ppm against hydrophobin SC3 protein sequence. Thermolysin digestion of hydrophobin SC3 produced mostly short non-specific peptide fragments (data not shown). In contrast to thermolysin, chymotrypsin produced more specific peptides. To obtain more comprehensive information about the obtained peptides, the peptide mixture after chymotryptic digestion was separated by C8 RP chromatography with a simple microgradient device [24,25]. The mass spectrum of the selected fraction (Fig. 4) contains not only signal of the native semispecific peptide (T)PPSTTTIAAGGTCTTGSL(S), but also the signal of its glycosylated forms with up to four mannose residues attached to it. An interesting observation during MALDI-Orbitrap mass spectrometric analysis were unusually abundant sodium and potassium adducts, which may relate to the specific amino acid sequence of hydrophobin SC3. Seven specific peptides originating from hydrophobin SC3 were identified with a sequence coverage of 67 % (based on the obtained MS data). MS/MS analysis obtained by MALDI-Orbitrap MS provided additional data that were processed using Proteome Discoverer program. Three peptides from hydrophobin SC3 were successfully identified during the database searching against SwissProt protein database using semispecific chymotryptic setting and SEQUEST HT as a searching engine. A detailed search against small contaminant database containing hydrophobin SC3 sequence yielded one more identified peptide (Table 1). Two chymotryptic peptides –(L)LGLLGIVL(S) and (F)NGLINIGCTPINIL(–) were common for both identification approaches. Analysis of MS/MS data thus extended the protein sequence coverage to 83 % and analysed peptides cover 93 amino acids out of 112 amino acids present in the mature form of hydrophobin SC3. This approach allows an increase in the protein sequence coverage, a confirmation of identity of the detected peptides and an improved analysis of post-translational modifications of hydrophobin SC3.

Table 1 Identified peptides of hydrophobin SC3 after enzymatic digestion with chymotrypsin. The identification was based on high mass accuracy MS and MS/MS data measured using MALDI-Orbitrap MS after reversed-phase separation of peptide mixture on C8 stationary phase. MS data were processed with MS-Fit v 5.10.19 database search program and MS/MS data were analysed using Proteome Discoverer 1.4 program. SwissProt was used as a searching database. Peptides with present XCorr value were successfully identified in MS/MS database searching. The asterisk denotes the peptide identified after more detailed analysis of measured data against SC3 sequence

m/z exp.	m/z theor. ([M+H]⁺)	ΔM [ppm]	Modifications	Start-end*	Missed cleavages	Sequence	XCorr
797,549	797,550	-0,82		79-86	3	(L)LGLLGIVL(S)	1,664
999,608	999,609	-0,82		80-89	3	(L)GLLGIVLSDL(N)	-
1227,679	1227,677	2,16	C5(Carbamidomethyl)	126-136	0	(L)INIGCTPINIL(-)	2,166
1511,823	1511,825	-1,05	C8(Carbamidomethyl)	123-136	1	(F)NGLINIGCTPINIL(-)	-
1692,810	1692,811	-0,46	C13(Carbamidomethyl)	44-61	0	(T)PPSTTTIAAGGTCTTGSL(S)	3,348
1856,015	1856,020	-2,53	C15(Carbamidomethyl)	83-100	3	(L)GIVLSDLNVLVGISCSPL(T)	3,595
1908,881	1908,879	1,27	C2,C3(Carbamidomethyl)	62-79	1	(L)SCCNQVQSASSSPVTALL(G)	-
2332,000	2332,000	-0,32	C10, C16, C17 (Carbamidomethyl)	101-122	0	(L)TVIGVGGSGCSAQTVCCENTQF(N)	-

*position refers to the sequence of the fruiting body protein SC3 no. P16933 in UniProtKB.
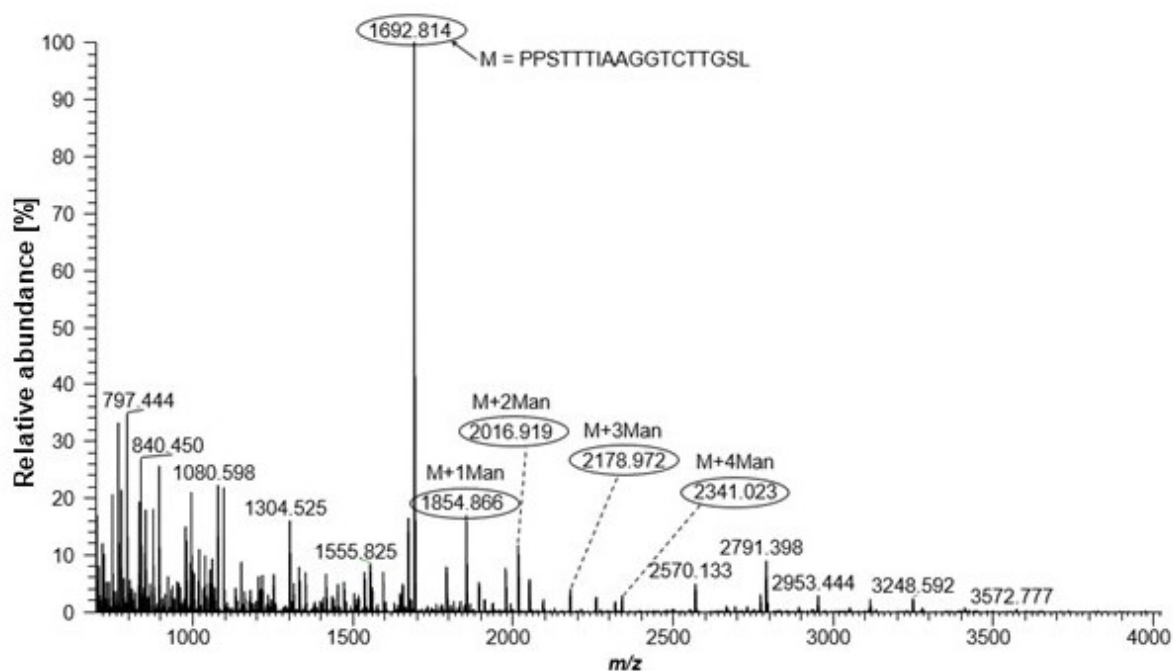
Fig. 4 MALDI Orbitrap mass spectrum of fraction no. 8 from the separation of chymotryptic peptide mixture of hydrophobin SC3 using C8 microcolumn RP LC. The denoted peak at *m/z* 1692.814 corresponds to peptide PPSTTTIAAGGTCTTGSL. Also the glycosylated forms of the peptide containing up to four mannose residues were observed.

1.3 Conclusion

SPE using hydrophobic PTFE resin in a microcolumn with acidic elution proved to be a simple, specific and effective method for the isolation of hydrophobin SC3 as the most common representative of class I hydrophobins. The enrichment demonstrated on hydrophobin SC3 from a protein mixture of ten proteins was shown to be a highly appropriate purification procedure of hydrophobins from protein mixtures. The analytical methods proposed herein can serve as a tool to obtain detailed information on the occurrence, behavior and properties of hydrophobins extracted from fungi and other samples, such as beer or malting barley. Improvements in the protocol for disulfide bridges reduction and enzymatic digestion led to the identification of about 83 % of the primary structure of the matured chain of hydrophobin SC3. MS and MS/MS analysis at the peptide level together with MALDI-TOF mass spectrometric analysis of intact hydrophobin SC3 provide further complementary information about its primary structure and post-translational modifications. An important advantage is represented by selectivity of the isolation procedure, thanks to which an application of additional separation steps is not required. In conclusion, the presented isolation method is easily applicable, reliable, cost-effective and complementary to other analytical techniques e.g. gel electrophoresis, LC, or MS.

2 Characterization of the azaphilone pigments produced by the marine-devided fungus *Talaromyces albobiverticillius* as potential colorant for the food industry

2.1 Introduction

There is a growing interest for the use of natural colours mainly from the consumers due to the harmful concerns associated with synthetic dyes and pigments. Natural pigments are derived from various sources, mainly from plants and microalgae and have applications in many foods and beverages. Special attention has been focused on filamentous fungi which are the potential producers of numerous shades of pigments ranging from yellow, red, reddish brown, bronze and maroon [26]. Fungal colorants can be chemically classified as carotenoids, melanins, polyketides, etc. in which the polyketides constitute the most representative class of pigments. Current industrial fungal productions are running at multi metric tons level with yellow-orange-red food colorants β-carotene and lycopene, biosynthesized by *Blakeslea trispora* [26-29]. Polyketide based pigments are structurally complex and involve pathways catalyzed by the enzymes polyketide synthases. The main classes of polyketide pigments include anthraquinones, hydroxyanthraquinones, naphthoquinones, and azaphilone structures, each of which exhibits an array of colour hues [30]. Since ancient times, azaphilone pigments produced by *Monascus sp.* have widely been used in the oriental countries (particularly Japan and China) to colour rice wine, koji, soyabean, cheese and meat. However, the use of Monascus pigments as food colorants is still forbidden in European countries owing to the time-to-time production of the mycotoxin citrinin [31] and also the production of the unwanted cholesterol-lowering drug mevinolin when added to foods [32].

On the other side, several nonpathogenic to humans *Talaromyces sp.* producing azaphilone series of yellow and red pigments without the production of mycotoxin seem to be an alternative to *Monascus* red pigments [33]. Azaphilones are interesting set of fungal secondary metabolites namely pigments with pyrone – quinone structures containing a highly oxygenated bicyclic core and a chiral quaternary center [34]. Studies have shown that some *Talaromyces sp.* such as *Talaromyces aculeatus, T. pinophilus, T. purpurogenus, T. funiculosus, T. amestolkiae, T. ruber* and *T. stolii* naturally produce polyketide azaphilone *Monascus* red pigments and their amino acid derivatives [35,36]. But, the later three species do not diffuse pigments into the culture medium and also *T. purpurogenus* produces mycotoxins such as rubratoxins A and B, rugulovasins and luteoskyrin which limits the biotechnological production of pigments by using this species [37]. Such compounds, for example, rubratoxin was produced in a high concentration in a rhubarb-wine contaminated with T. purpurogenus and induced an immediate liver transplant when consumed by a teenager [38,39]. Some other species, specifically *T. atroroseus, T. albobiverticillius, T. minioluteus*, and *T. marneffei* produce diffusing strong red pigments and some yellow pigments. One potential pigment producer among them, namely *T. albobiverticillius* collected from different sources produces several purple-red-orange azaphilone pigments such as monascorubramine (red), monascorubrin (orange), rubropunctatin (orange), PP-R (purple-red) [40-42] and

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a series of yellow-orange pigments such as monascin (yellow), mitorubrin (orange-yellow), mitorubrinic acid (yellow) or mitorubrinol (yellow) [33].

This study describes the pigment production from the marine derived fungus *Talaromyces albobiverticillius* strain 30548 isolated from the outer slope of the Réunion island coral reef (Indian Ocean) and the characterization of those pigments by high-performance liquid chromatography-diode array detection-electrospray ionization mass spectrometry (HPLC-PDA-ESI/MS), followed by the isolation of major compound(s) and the structure elucidation of a novel red azaphilone using NMR analysis.

2.2 Results and discussion

2.2.1 Extraction

An ideal solvent for fungal pigment extraction must have low toxicity and must be able to solubilize a range of target pigment molecules [43]. Initial extraction trials were conducted with the commonly used solvents from low to high polarity such as n-hexane, chloroform, ethyl acetate and ethanol successively which yielded differences in amounts of extracted pigments for both biomass and culture filtrate. Simultaneously, extraction was carried out using ethyl acetate and ethanol as single solvent extraction. On the basis of liquid chromatography-diode array detector (LC-DAD) chromatogram, among the used solvents, ethyl acetate as single solvent was found to be the best solvent for extraction of major pigmented compounds followed by ethanol. Indeed, *Monascus*-like polyketide pigments are hydrophilic in nature, slightly polar and so they are easily handled with polar solvents [44]. In non-polar solvents like n-hexane and chloroform, the extraction and recovery of pigments was very low and chloroform yielded two compounds (peaks 1 and 2) which were unpigmented. The yield from these solvents was very poor compared to ethyl acetate which yielded 12 different compounds and among them 10 compounds were pigmented (Fig. 5).

2.2.2 Optimisation HPLC-DAD-ESI/MS analysis

HPLC separations were performed on a C18 Kinetex (Phenomenex) column (100×2.1 mm-1.7 μm particle size); the mobile phases consisted of water (0.1% formic acid; mobile phase A) and acetonitrile, (0.1% formic acid; mobile phase B), using a gradient program as follows: 0 min, 5% B; 15 min, 95% B; 17 min, 95% B; 18 min, 5% B. The flow rate was 0.2 mL/min and the injection volume was 1 μL. The column oven temperature was 30 °C. The UV–vis spectra were acquired in the range of 200–600 nm, while the chromatograms were extracted at 470 nm and 360 nm (sampling frequency: 1,5625 Hz; time constant: 0.64 s). The MS was set as follows: Scan, both ESI positive (+) and negative (−); nebulizing gas flow (N$_2$): 1.5 L/min; Event Time: 0.3 s; Detector Voltage: 4.5 kV; m/z range: 60–600; Interface Voltage: ± 3.5 kV; Interface Temperature: 350 °C; DL Temperature: 250 °C; Heat Block: 400 °C.

Fig. 5 shows a typical representative chromatogram of the detected pigments (compounds No. 3–12) in *Talaromyces albobiverticillius* 30548 obtained from the EtOAc pigment extract and detected at the wavelength of 470 nm; in the same Fig. 5, it is also shown an insert representing the better detection for compound No.1 and No.2 obtained from the CHCl3 extract and recorded at the wavelength of 360 nm.
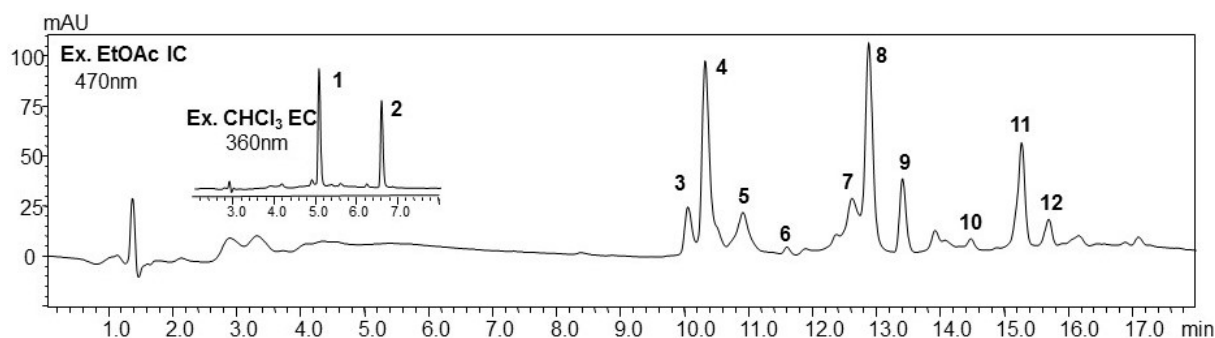
Fig. 5 Chromatogram showing the overall compounds detected by HPLC-PDA- ESI/MS in intracellular (IC) and extracellular (EC) extracts of *Talaromyces albobiverticillius.*

Table 2 presents all of the detected compounds, their corresponding retention times, PDA and MS data, with a relative tentative identification based on the obtained spectroscopic data and the comparison with literature data. Together with PDA, an on line MS detector operating in both ESI positive and ESI negative ionization mode was used in order to have a double confirmation of the mass values. Twelve different compounds were detected and four were tentatively identified as *Monascus*-type pigments (Table 2). The identified compounds 3, 5 and 8 are similar to the already known N-threoninemonascorubramine, N-glutaryl-rubropunctamine and PP-O respectively. Further, one compound was also characterized by NMR analysis and a new structure for this molecule, here named as 6-[(Z)-2-Carboxyvinyl]- N-GABA-PP-V, (or as N-GABA-PP-V), is provided for the first time based on PDA, MS and NMR data. .

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Table 2 Overall compounds detected by HPLC-PDA-ESI/MS in IC and EC extracts of *Talaromyces albobiverticillius* 30548, with reference to the chromatogram shown in Fig. 5.

| Compound number	Retention time	λ_{MAX} [nm]	ESI (+)	ESI (-)	Tentative identification
1	4.89	207, 362	255 $[M + H]^+$		n.i.
2	7.35	223, 390	269 $[M + H]^+$	267 $[M - H]^-$	n.i.
3	10.04	222,273,422, 511	484 $[M + H]^+$ 547 $[M + H + Na + CH_3CN]^+$	482 $[M - H]^-$	N-threonine-monascorubramine
4	10.32	221,273,425, 522	456 $[M + H]^+$ 498 $[M + H + CH_3CN]^+$	454 $[M - H]^-$ 496 $[M - H + CH_3CN]^-$	N-threonine-rubropunctamine
5	10.92	223, 430, 499	484 $[M + H]^+$ 440 $[M + H - CO_2]^+$	482 $[M - H]^-$ 438 $[M - H - CO_2]^-$	N-glutaryl-rubropunctamine
6	11.57	225, 409	375 $[M + H]^+$		n.i.
7	12.6	222, 280, 461	413 $[M + H]^+$ 475 $[M + H + Na + CH_3CN]^+$	411 $[M - H]^-$ 473 $[M - H + Na + CH_3CN]^-$	n.i.
8	12.8	223,286,458,470	413 $[M + H]^+$ 475 $[M + H + Na + CH_3CN]^+$	411 $[M - H]^-$ 473 $[M - H + Na + CH_3CN]^-$	PP-O
9	13.4	224, 287, 458	459 $[M + H]^+$	457 $[M - H]^-$	n.i.
10	14.4	225, 421	503 $[M + H]^+$	501 $[M - H]^-$	n.i.
11	15.2	224, 446	445 $[M + H]^+$	443 $[M - H]^-$	n.i.
12	15.6	225, 458	459 $[M + H]^+$		n.i.

Compound 3: Under the assumption that the α-amino acid threonine was incorporated into pigment, compound No. 3 was tentatively identified as N-threonine-monascorubramine; the corresponding [M+H]$^+$ *m/z* 484, and [M−H]$^-$ *m/z* 482, pseudomolecular ions mass values were consistent with the values reported [45] and the corresponding PDA data were also consistent with the reported values for *Monascus* type pigments [35].

Compound 4: Interestingly compound No. 4 was identified as a never previously reported compound. Under the assumption that the γ-amino acid, γ-aminobutyric acid was incorporated into pigment, the name of 6-[(Z)-2-Carboxyvinyl]-N-GABA-PP-V or simply as N−GABAPP-V was proposed for compound No. 4; the structure was determined on the basis of the obtained PDA and MS data and of a detailed NMR investigation. The PDA data are in agreement with the reported values for *Monascus* type pigments [35] and the compound showed corresponding [M+H]$^+$ *m/z* 498, and [M−H]$^-$ *m/z* 496, pseudomolecular ions (Fig. 6) which are consistent with the proposed structure (Fig. 7) for a compound having a molecular formula of $C_{27}H_{31}NO_8$, with a mass value of 497 amu. The PubChem database (PubChem CID: 44715338) reports the existence of a compound named as 4-{6-[(E)-2-Carboxyvinyl]-9α-methyl-3-octanoyl-2,9-dioxo-9,9α-dihydrofuro [3,2-g] isoquinolin-7(2H)-yl}butanoic acid with a *trans* configuration at the C10-C11 double bond, but no information was available/reported on the source or in any literatures for this compound. Therefore, this work reports for the first time on the characterization of the pigment N-GABA-PP-V derivative bearing a *cis* configuration at the C10-C11 double bond, in the investigated *Talaromyces albobiverticillius* species.
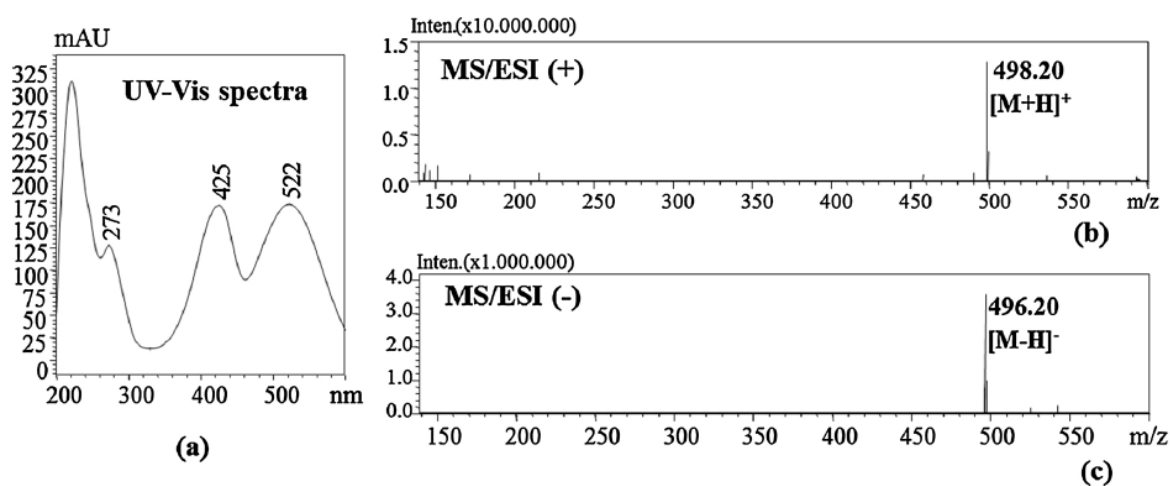
Fig. 6 Structural analysis of red pigments: (a) UV-Vis absorption spectrum of ethyl acetate extract, (b) positive ESI–MS *m/z* spectrum of compound No. 4, (C) negative ESI–MS spectrum.
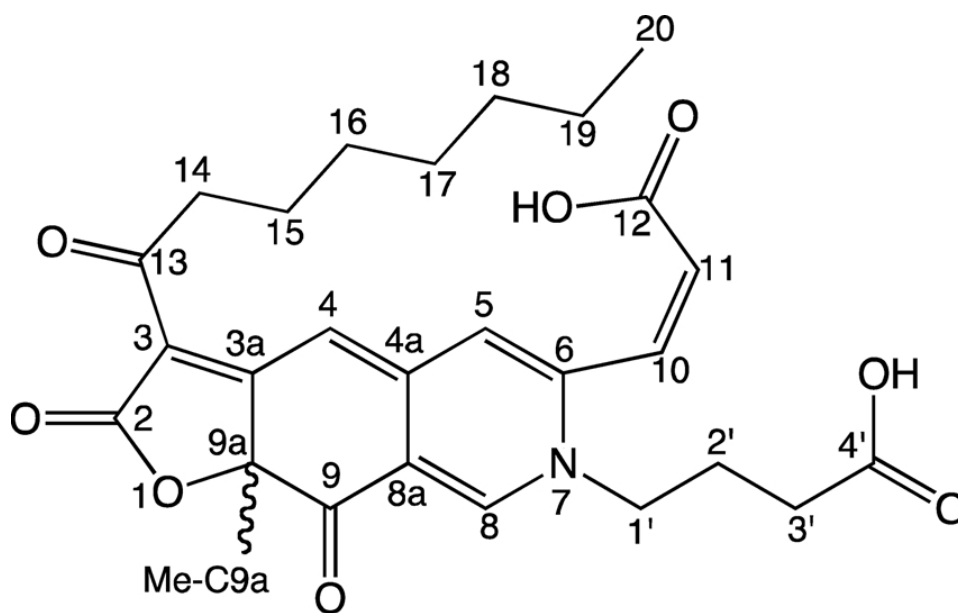
Fig. 7 Molecular structure and carbon atom numbering of compound No. 4. Proposed Name: N-GABA-PP-V, 4-{6-[(E)-2-Carboxyvinyl]-9a-methyl-3-octanoyl-2,9-dioxo-9,9adihydrofuro [3,2-g]isoquinolin-7(2H)-yl}butanoic acid.

Compound 5: Compound No. 5 was tentatively identified as N-glutaryl-rubropunctamine; it showed the $[M+H]^+$ *m/z* 484 and $[M-H]^-$ *m/z* 482 pseudomolecular ions, and UV–vis absorbance values in agreement with the literature reported values [36].

Compound 8: Compound No. 8 was tentatively identified as PP-O; it showed the corresponding $[M+H]^+$ *m/z* 413 and $[M-H]^-$ *m/z* 411 pseudomolecular ions, and UV–vis absorbance values in agreement with the literature reported values [35,46].

2.3 Conclusion

In this work, we isolated in the tropical marine environment of Réunion island, Indian Ocean, a different red pigment producing strain belonging to *Talaromyces albobiverticillius* to bring new information about the pigments produced. This research presents four out of twelve compounds were identified in the investigated pigmented extract from *Talaromyces albobiverticillius* 30548 using HPLC-PDA-ESI/MS and NMR: N-threonine monascorubramine ($C_{27}H_{33}NO_7$ formula weight 483,55), N-glutarylrubropunctamine ($C_{26}H_{29}NO_8$ formula weight 483,51), PP-O=((10Z)-12-carboxylmonascorubrin) ($C_{23}H_{24}O_7$ formula weight 412,43) and a new compound, a N-GABA-PP-V(6-[(Z)-2-Carboxyvinyl]-N-GABA-PPV) ($C_{27}H_{31}NO_8$ formula weight 497,53), pigment bearing a *cis* configuration at the C10-C11 double bond. This new compound will enlarge the list of 63 *Monascus* and *Monascus*-like pigments (24 O-containing compounds and 39 N-containing compounds) recently summarized [47]. The work will continue with large scale cultivation of *Talaromyces albobiverticillius* 30548 in fermenter, analysis of pigmented extracts HPLC/MS and finally isolation of still unknown compounds for additional NMR.

3   Analysis of phenolic compounds in mead

3.1   Introduction

The production of mead (honey wine) has been known since ancient times [48]. Mead is an alcoholic beverage, which contains between 9 to 18 % of ethanol by volume. It is obtained by alcoholic fermentation of mead wort with the possible addition of herbs, root spices, or fruit juices [49]. Process of mead wort fermentation, as well as its maturation, is time-consuming due to high sugar content, low pH, and low mineral content of honey, and varies from a few month to several years, depending on the method of mead wort dilution with water, as well as honey variety, yeast strain, yeast nutrition and control of pH [50,51]. Different types of mead are obtained depending on the proportion to which honey is diluted 1:0.5, 1:1, 1:2. and 1:3 (honey:water, v/v) [50]. Mead contains ethanol and many other compounds such as sugars, acids, vitamins, phenolic compounds, mineral, etc. [52].

Phenolic compound content in meads depends on honey and the ingredients used. Phenolic compounds are defined as the secondary metabolism products of the plants and they are the most common compounds. Some of these compounds are taken from plants to honey by bees (*Apis mellifera*) [53]. The chemical composition of honey, added ingredients to mead wort and technological applications influence the composition of meads [52]. The dispersion of phenolic groups (cinnamic acids, benzoic acids, flavonoids) shows varied profiles in honey from different floral sources [54,55]. Therefore, a characteristic distribution pattern of phenolic compounds should be found in unifloral honeys sourced from the corresponding plant sources. Honey flavonoids can originate from nectar, pollen or propolis [56].

In the Czech Republic is possible to buy mead of various qualities of Czech and foreign brands. Due to the insufficient legislative of quality of mead, unfortunately, there are also products with poor quality. Basic consumer is not able to recognise mead with good quality for the first view. For this reason, the aim of this work was to find indicators of the quality of mead, which could be used in the future for control of mead quality.

HPLC methods are the most suitable and common method for analyses of individual phenolic of honey and mead samples [57-60]. In this study we focused to find quick simple HPLC method to analyse the most common phenolic compounds in meads.

3.2   Results and discussion

3.2.1   Optimisation of RP-HPLC analysis

The analysis of phenolic compounds in biological matrices is typically performed using liquid chromatography coupled to a spectrophotometric detector or to a mass spectrometer. The major disadvantage of published HPLC methods is a long chromatographic run of about 40 – 100 min per analysis [52,61,62].

Optimisation was focused to find quick separation of 23 phenolic compounds (most common in honey/mead), which could be used for rutine quality control of meads. Parameters such as the concentration of formic acid in the mobile phase, the initial concentration of acetonitrile and the slope of the gradient were optimized. An Ascentis

Express C18 column was chosen for the chromatographic separation (150 mm × 3.0 mm × 2.7 μm), heated to 30° C. The mobile phase consists of 0.3% HCOOH (MP A) and acetonitrile (MP B). The flow rate was optimized to 0.6 ml/min, with a sample volume of 2 μl. The optimal gradient for the separation of individual components was as follows: 0 min - 10% B, 0.1 min - 23% B, 3 min - 24% B, 4 min - 50% B, 5 min - 60% B, 6 min – 10 % B. Quantitative analysis was performed by the method of multiple reaction monitoring of selected ions. MRM transition optimization was performed for individual standards by direct infusion in negative ion mode. Final optimized chromatographic separation is shown in Fig. 8.
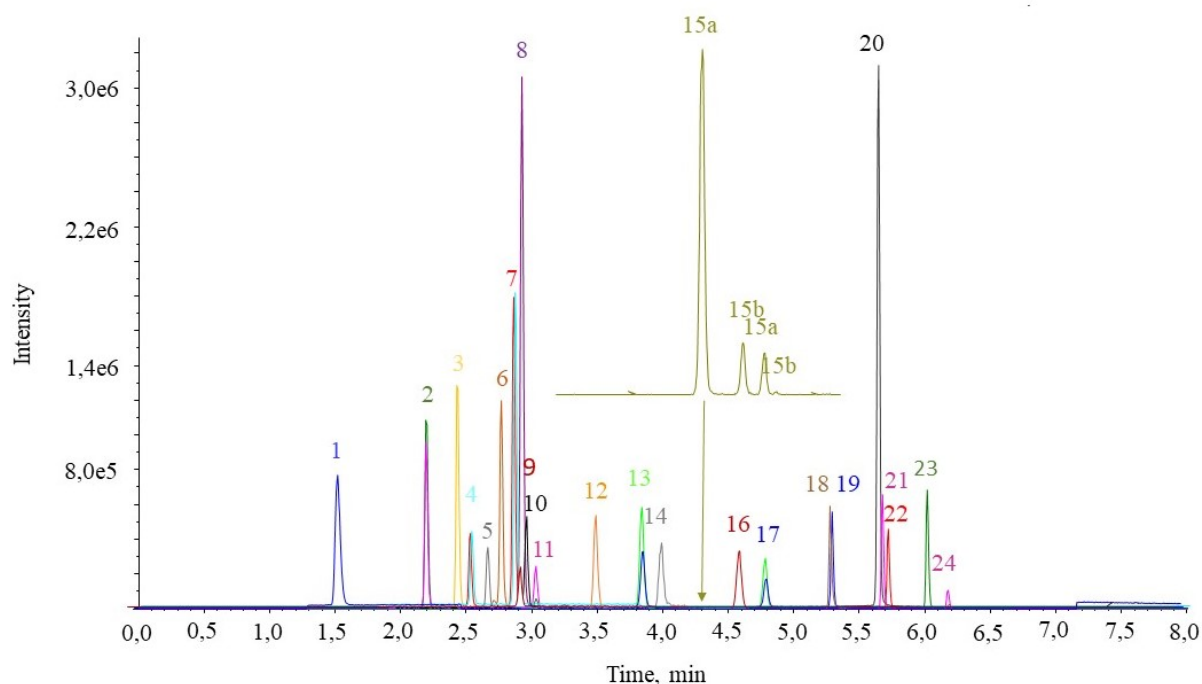
**Figure 8** Optimised chromatographic separation of 24 standards of phenolic compounds.

1 – gallic acid, 2 –protocatechuic acid, 3 – chlorogenic acid, 4– catechin, 5 – 3,4-dihydroxybenzaldehyde, 6 – 4-hydroxybenzoic acid, 7 – epicatechin, 8 –caffeic acid, , 9 –vanillic acid, 10 –syringic acid, 11 – gentistic acid, 12 – rutin, 13 –*p*-coumaric acid, 14 – vanillin, 15 – ferulic acid isomers (15a-*trans*, 15-*cis*), 16 – taxifolin, 17 –*m*-coumaric acid, 18 –*o*-coumaric acid 19 – myricetin, 20 –salicylic acid, 21 – luteolin, 22 – quercetin, 23–apigenin, 24 – hesperetin

Ascentis C18 (150 mm × 3,0 mm × 2,7 μm), MP A: 0,3 % HCOOH, B: Acetonitrile, 0,6 ml/min, gradient: 0 min, 10 % B; 0,1 min, 23 % B; 3 min, 25 % B; 4 min, 50 % B, 5 min, 60 % B, 6 min, 10 % B, 30 °C, 2 μl, MS/MS-MRM.

3.2.2 Optimisation of extraction

Different approaches to selected samples were tested to optimize the extraction of phenolic substances. First, the method described in the literature [63] was used using hand-made SPE columns with Amberlite XAD-2 sorbent. In addition, commercial SPE columns with octadecyl silica gel stationary phase were used. As a third variant, liquid-to-liquid extraction using ethyl acetate as the extraction system was chosen. For comparison, samples were also analysed without extraction. Fig. 9 is a graph describing the amount of phenolic compounds monitored by extraction methods in selected samples.

Both SPE variants provide approximately similar amounts of phenolic compounds, with Amberlite XAD-2 sorbent providing slightly better results. Non-extracted samples provided an even higher content compared to the SPE. The exception is sample 3, where the SPE-A method was slightly higher than without extraction. Liquid-to-liquid extraction has clearly been the most effective extraction method. For this reason, it was later used for all mead samples.
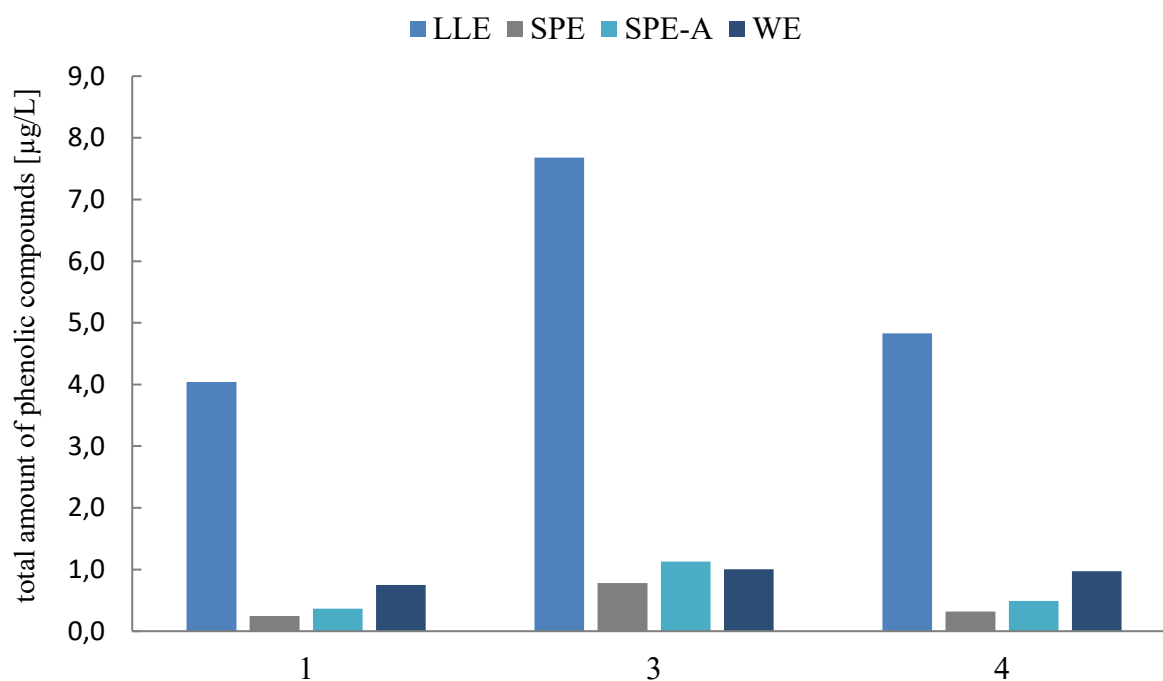
Figure 9 Comparison of the influence of different extraction methods on the total amount of extracted phenolic compounds in selected samples of mead.

LLE – liquid-liquid extraction, SPE – solid-phase extraction, SPE-A – solid-phase extraction using Amberlite sorbent, WE. – analysis without extraction

3.2.3 Analysis of real samples – statistical analysis

Principal Component Analysis, or PCA, is a dimensionality-reduction method that is often used to reduce the dimensionality of large data sets, by transforming a large set of variables into a smaller one that still contains most of information in the large set. The idea of PCA is to reduce the number of variables of a data set, while preserving as much information as possible.

In general, the scatterplot shows the objects perfectly scattered in 2D, where clusters of similar objects and outsiders can be found. The scatterplots in Fig. 10 and Fig. 11 show two clusters and several outsiders (samples significantly different from the others).

The first outsider (marked by green colour in Fig. 10 and 11) is sample No. 37. This sample contains an extremely high content of gentisic acid, *p*-coumaric acid and taxifoline, which according to the Fig. 10 have a markedly positive correlation with a factor1 and gentisic acid with a factor 2. This mead was fermented with addition of blackcurrant juice, which could have caused such high contents of mentioned phenolic compounds.

Another outsider - sample No. 14 (marked by orange colour in Fig 10 and 11) is shown in the same direction as the sample No. 37. The sample No.14 provided a very high content of gentisic acid and taxifoline, as well as a higher content of *p*-coumaric acid. These phenolic substances are also naturally found in honey. As it is a mead that has been fermented with the addition of grape juice, the higher content of phenolic compounds is due to the enrichment with fruit juice, which contains large quantities of these compounds.

The last outsider is sample No. 26 (marked by purple colour in Fig. 10 and 11). The scatterplot shows that this sample correlates positively with factor 1 due to the higher content of taxifoline and gentisic acid and negatively with factor 2 due to the very high content of 3,4-hydroxybenzaldehyde. This mead was prepared with addition of herbs, which caused high contents of almost all monitored phenolic substances, that were measured in this sample.

The pair of samples No. 19 and No. 25 (marked by red colour in Fig. 10 and 11) describes samples where an unusually high vanillin content was found. Due to Fig 11, vanillin strongly correlate to a factor 3. Such a high vanillin content in mead is naturally not possible. Synthetic vanillin was probably added to these two meads. Significantly lower content of other phenolic substances was also found in these two samples, therefore these meads were probable not produced from a sufficient amount of honey.

The blue cluster (Fig. 10 and 11) describes meads, which were enriched with herbs or fruit juices during preparation. Probably by a lesser amount than in the samples described above. Therefore, these meads also have a higher content of phenolic substances and were statistically evaluated as similar.

The black cluster (Fig. 10 and 11) consists of other meads, which were presumably fermented only from honey without other ingredients or with juices, with a lower content of phenolic compounds.
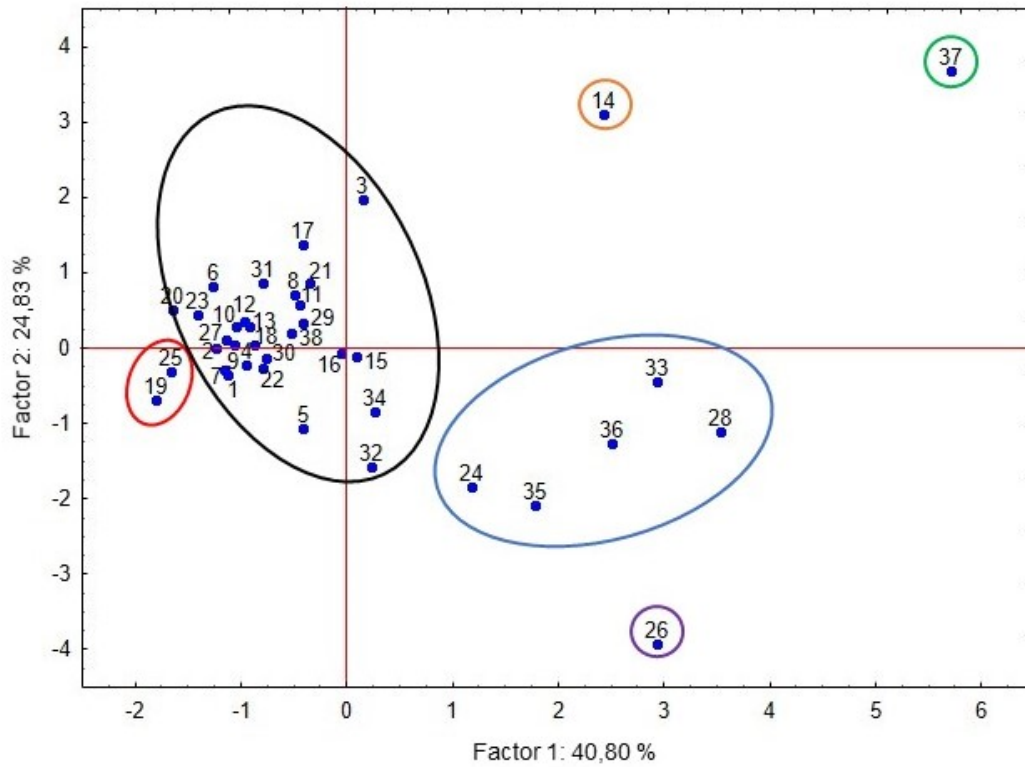
Figure 10 2D PCA-plot of the first and the second factor (STATISTICA, StatSoft).
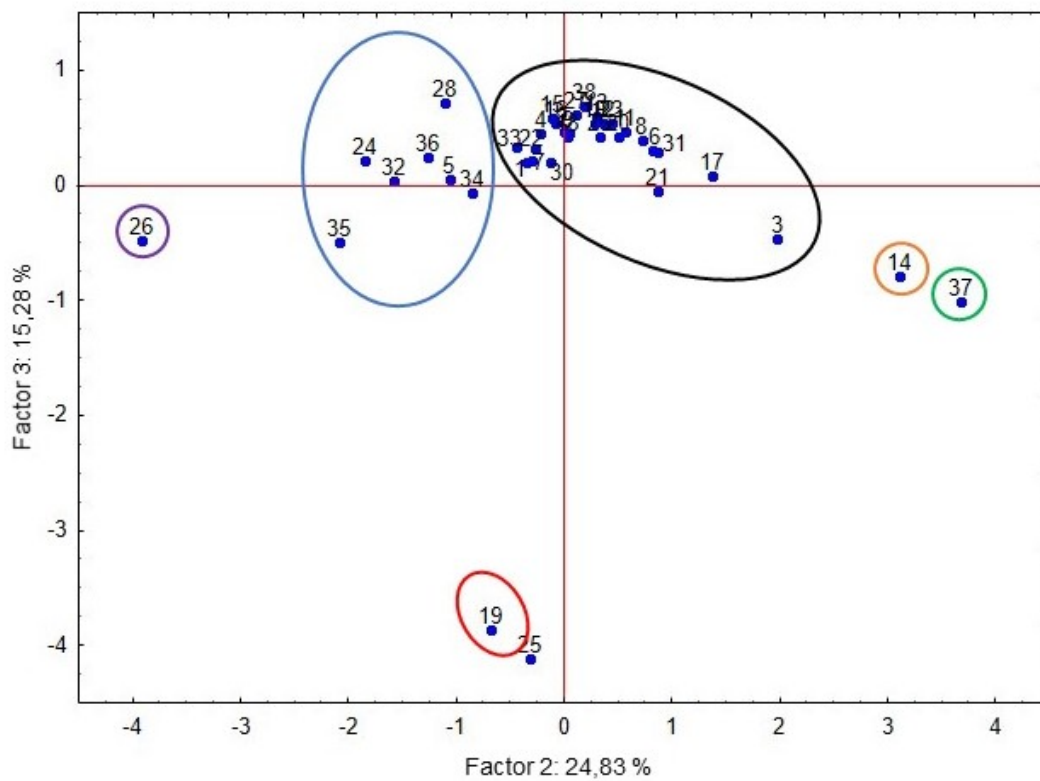

Figure 11 2D PCA-plot of the second and the third factor (STATISTICA, StatSoft).

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3.2.4 Antioxidant activity

Phenolic compounds are antioxidants, therefore contribute significantly to antioxidant activity. In order to have a more comprehensive view of individual mead, the antioxidant activity was also measured in all 38 monitored samples. Three spectrophotometric methods were chosen - determination of antioxidant activity using ABTS and DPPH radicals (Fig. 12) and using Folin-Ciocalteou reagent (Fig. 13).

Fig. 12 shows a graphical representation of antioxidant activity as an equivalent amount of Trolox/ml of mead. In most samples, a more pronounced antioxidant activity was measured using the ABTS radical. The opposite trend can be observed for the samples No. 19, 20 and 25, where higher antioxidant activity was measured using the DPPH radical. Overall, it can be stated that meads flavoured with fruit juices (No. 14, 15, 16, 18, 35, 37 and 38) or herbs (No. 26, 28, 36) have incomparably higher antioxidant activity compared to traditional mead. This increase is due to the very high amount of other substances showing antioxidant activity (vitamins, etc.) contained in fruits and herbs.

A similar trend can be observed in the intensity of antioxidant activity measured by Folin-Ciocalteou reagent in Fig. 13. The highest values were reached by mead containing fruit juices: No. 15 (lemon, orange), No. 16 (lemon, orange, cherry), No. 35 (sour cherry) and no 37 (blackcurrant).
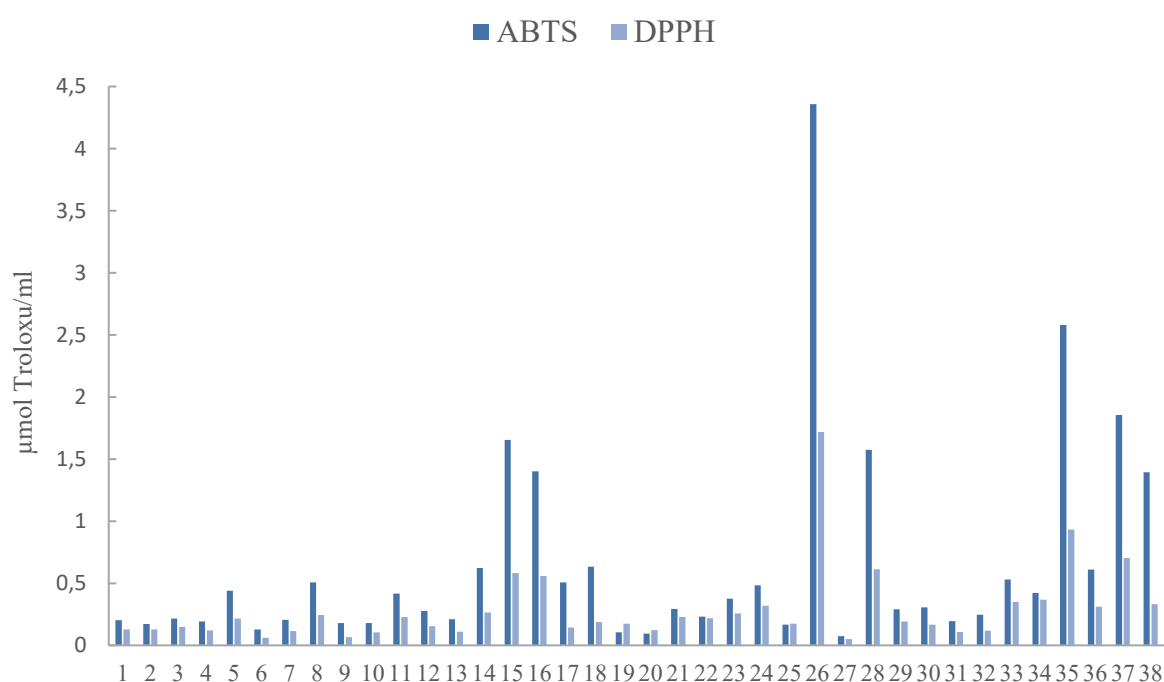
Figure 12 Comparison of antioxidant activity of mead by ABTS and DPPH radicals, expressed as equivalent amount of Trolox standard.
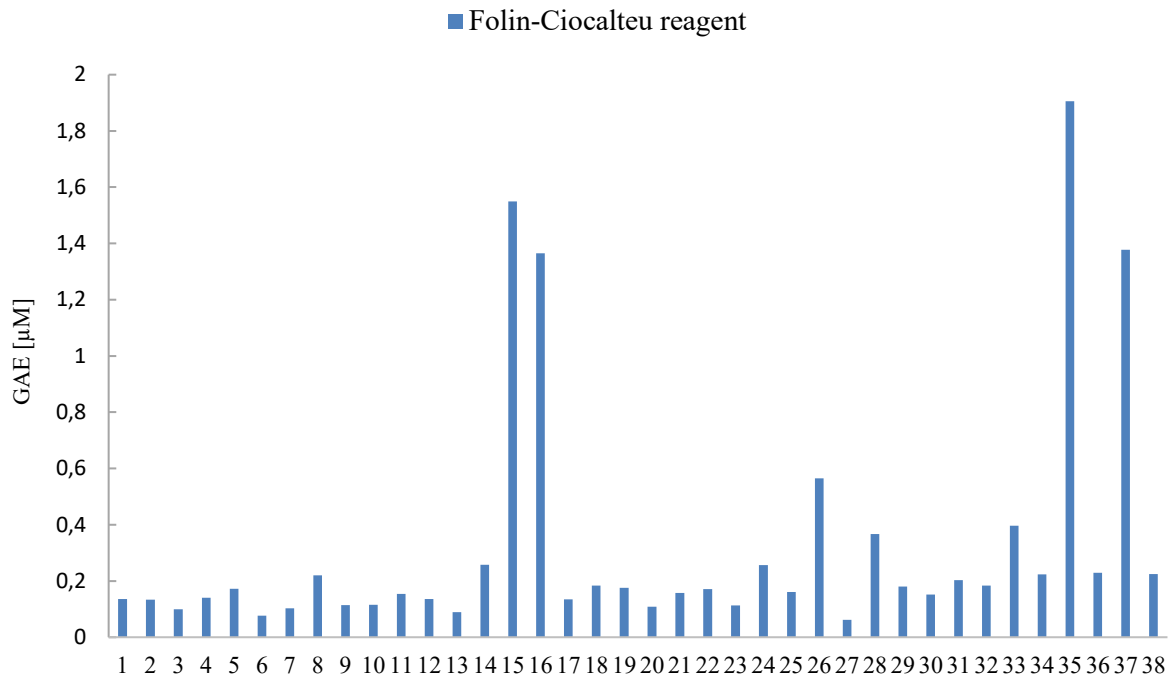
Figure 13 Total phenolic content determined using the Folin-Ciocalteou reagent, based on the equivalent amount of gallic acid

3.3 Conclusion

Mead is a very popular alcoholic beverage, especially during winter. Unfortunately, there is insufficient legislative of quality of mead. It is not easy to recognize product with good quality for average consumer. For this reason, we tried to find quick and simple method for quality control of mead.

In this work, a total of 23 phenolic compounds were analysed, which are most often represented in honey or mead. Both the extraction method and the subsequent separation by HPLC/MS/MS were optimized. Three types of extraction and the method without extraction were compared on three samples of mead. Liquid-to-liquid extraction with ethyl acetate was chosen as the most efficient. HPLC/MS analysis was performed according to an optimized 6-min. gradient. A column Ascentis Express C18 was used for separation. 0.3% HCOOH was chosen as mobile phase A, mobile phase B was acetonitrile with an initial concentration of 10 %.

All optimized parameters were used for the analysis of a total of 38 samples of mead, obtained both directly from beekeepers and purchased in stores. All samples were extracted and subsequently analysed by HPLC/MS/MS. The measured results were evaluated using a calibration curve and recalculated to the content of monitored phenolic substances in individual samples of mead. The obtained data were analysed using the multidimensional statistical method PCA. By this method, several clusters of mead samples with common features were obtained, as well as some remote points, for samples that differed significantly from the others. Two samples of mead were also found, which contained added synthetic vanillin.

Furthermore, the determination of antioxidant activity by ABTS and DPPH radicals and the determination of the total content of phenolic substances by Folin-Ciocalteu reagent were performed in all samples of mead. Although these methods do not provide information on individual phenolic substances, but they provide interesting information on the total content of phenolic substances and other antioxidants.

4 References

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5 List of Student´s Published Works related to presented dissertation

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