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Ultra-high-performance liquid chromatography profiling method for chemical screening of proanthocyanidins in Czech hops

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ABSTRACT

Hops represent an important natural source of bioactive polyphenols, particularly proanthocyanidins, which can contribute to prevention of several civilization diseases, owing to their antioxidant and radical scavenging activity. We have developed a high-throughput ultra-high-performance liquid chromatography time-of-flight mass spectrometry profiling method, which can be used for monitoring of bioactive proanthocyanidins in hops. The method was applied for analysis of hops of four Czech varieties (Saaz, Sladek, Preminat and Agnus) from the 2011 crop (9 localities, 11 samples) and the 2012 crop (24 localities, 40 samples). Hop samples were extracted by acetone and the analytes were separated on the Acquity UPLC BEH Shield RP18 column. Partial validation of the method revealed a satisfactory intra-day repeatability of the method for retention times (relative standard deviation within 1.39%) as well as areas under the peaks (within 9.89%). Experimental data were evaluated using principal component analysis and cluster analysis. Significant amounts of di-, tri- and tetramer proanthocyanidins consisting of (epi)catechin and (epi)gallocatechin were found in the hop samples. The dependence of the proantocyanidin composition on both the variety and the growing locality was observed. Specifically, the traditional Saaz variety contained more frequently oligomers formed by (epi)catechin units only, whereas the varieties Premiant and Agnus produced oligomers consisting of (epi)catechin as well as (epi)gallocatechin units. The relative abundance of proanthocyanidins in studied hop varieties from the two crops, 2011 and 2012, did correspond to each other. In the further perspective, the method may also be used for prediction of qualitative marks or authenticity verification of hops.

1. Introduction

Hops (*Humulus lupulus L.*) were used already during ancient times for their antimicrobial properties. Later, during the 12th century, hops found their use in the brewing process, not only for their bitter taste and flavor, but also for their beneficial influence on the stability of beer foam and their contribution to the microbial stability of beer. We can consider this period as the beginning of the old tradition of hop cultivation in the area of today's Czech Republic [1]. Moreover, hops have also found diverse applications in the cosmetic and pharmaceutical industry and the importance of this plant was supported by the "Medicinal Plant of the Year 2007" award granted by the Study Group for the Historical Development of Medicinal Plant Science at the University of Wuerzburg, Germany or by the selection of hops as the 2008 theme plant by the Garden Walk at the University of Illinois at Chicago, USA. Likely, the increasing number of scientific publications on the beneficial activities of hops is the main reason for such recognition [2]. The most important components of hops are hop resins, causing the bitterness of beer, essential oils and polyphenols, having both positive and negative effects in terms of beer technology and the resulting quality of beer. Even though, prooxidant effects of some polyphenols are also known [3], a great number of papers describing antioxidant properties of these substances have been published [4]. Antioxidant properties of polyphenols are caused by their ability to serve as radical scavengers: they prevent the formation of hydroxyl radicals during oxidation of fatty acids (Fenton cycle) [5]. Accordingly, polyphenols are considered biologically active substances with significant health benefits including possible preventive effects to cardiovascular diseases, certain types of cancer and age-related diseases [6–8].

An important group of compounds belonging to polyphenols are proanthocyanidins, better known as condensed tannins, which are defined as flavan-3-ol oligomers and polymers and which give anthocyanidins upon acid depolymerization reactions. Proanthocyanidins exhibit general toxicity towards fungi, yeast and bacteria [6]. Furthermore, they have antioxidant properties (protection against LDL oxidation), which can induce cardioprotective effects and inhibit the platelet activity and vasodilatation [9,10].

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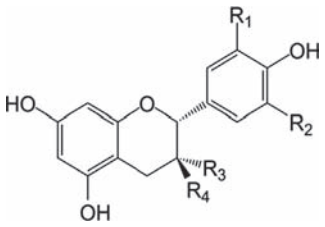
Moreover, hop proanthocyanidins can help prevent nitric-oxide-related disorders such as Alzheimer's and Parkinson's diseases [4]. The biological activity of proanthocyanidins is dependent on their chemical structure and concentration [11]. For example, procyanidins (proanthocyanidins consisting of catechin and epicatechin units only) of higher molecular weight are more effective than shorter-chain procyanidins against liposome oxidation [12]. By contrast to other beer constituents, the brewing value of hop proanthocyanidins is not yet well-understood. However, it is known that these compounds have a tendency to make complexes with proteins and contribute to non-biological haze formation [13,14]; but on the other hand, they can also stabilize the organoleptic properties and color and contribute to the astringency and bitterness [15,16]. Proanthocyanidins constitute about 20% of the total plant polyphenols [17].

The basic building blocks of proanthocyanidins are single monomeric units (flavan-3-ols) called afzelechin, epiafzelechin, catechin, epicatechin, gallocatechin and epigallocatechin (see Fig. 1). Proanthocyanidins composed of two to seven monomer units are called "oligomeric", while proanthocyanidins with more than seven subunits are called "polymeric" [15]. Proanthocyanidins can be divided, according to the linkage of the monomers, into two groups, A and B. The B-type group is represented by oligo (poly)mers linked mainly through C4–C8 or occasionally C4–C6 atoms. If an additional ether linkage is formed between C2 and C7, i.e. C2–O–C7, the compounds belong to the A-type group.

A summary of more than 70 methods for analysis of proanthocyanidins in different plant and non-plant matrices were summarized in an extensive review [18]. A broad spectrum of preparation and analytical liquid chromatography techniques, coupled usually with mass spectroscopy (MS) have been applied for analysis of various proanthocyanidins in hops [5,19–21], barley [11,22] or beer [23]. Taylor et al. [19] extracted proanthocyanidins from hops by Sephadex LH-20 column chromatography using a step gradient of methanol, water, and acetone. The resulting fractions were assayed by two-dimensional thin layer and gel permeation chromatography as well as electrospray ionization (ESI) and matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry (MS). Magalhães et al. [20] developed a method based on the sample purification by adsorption of phenolic compounds from the matrix to polyvinylpolypyrrolidone and subsequent desorption of the adsorbed polyphenols with acetone/water (70:30, v/v). Subsequently, the extract was analyzed by high-performance liquid chromatography (HPLC) coupled with a diode array detector and by HPLC–ESI–MS/MS (quadropole and ion trap mass analyzers). Li et al. [21] used HPLC hyphenated with atmospheric pressure chemical ionization tandem MS (triple-quadrupole mass analyzer) and HPLC hyphenated with ESI tandem MS (triple-quadrupole mass analyzer) for identification of newly isolated proanthocyanidins from hops. Whittle et al. [11] elucidated isolated proanthocyanidins using HPLC–ESI–MS (quadrupole mass analyzer) and surveyed the contents of these compounds in several barley varieties by HPLC with electrochemical detection. Dvorakova et al. [22] used HPLC–ESI–MS (ion trap mass analyzer) for identifications of monomeric and oligomeric flavan-3-ols in 10 barley varieties and corresponding malts. Lastly, Callemien et al. [23] elucidated structures of three procyanidins (B1, B3, and B4) and one prodelphinidin (B3) by HPLC–ESI–MS/MS in beer. In summary, all these methods are based on the classical HPLC methods usually coupled with a low-resolution mass analyzer. Therefore, the application of the ultra-high-performance liquid chromatography (UHPLC) technique and ESI-MS with a reflectron TOF high-resolution mass analyzer represents an important novelty for this application. In addition to that, the published methods are aimed at target analysis and structure elucidation. Statistical evaluation using a target analysis was performed only by Whittle et al. To our best knowledge, a non-target profiling method of proanthocyanidins followed by the statistical analysis has not been described yet.



Flavan-3-ol monomer	Substitution pattern			
	R_1	R_2	R_3	R_4
Catechin	OH	H	H	OH
Epicatechin	OH	H	OH	H
Gallocatechin	OH	H	H	OH
Epigallocatechin	OH	OH	OH	H
Afzelechin	H	OH	H	OH
Epiafzelechin	H	H	OH	H

Fig. 1. Chemical structure of proanthocyanidins.

The relative proportion of the various proanthocyanidins in barley and hops depends upon the variety and climatic conditions, as shown in several studies. Whittle et al. [11] elucidated more than 50 proanthocyanidin structures; they correlated their content using principal component analysis (PCA) with barley varieties and beer types. However, the sample preparation using Sephadex LH-20 and subsequent 150 min long HPLC analysis make the method extremely time consuming. In 2006, Li et al. [21] published a comprehensive study about structural identification and distribution of proanthocyanidins in 13 different hops. They established the correlation between the profiles and the geographic origin of hops; however, the work focused more on laborious isolation and structure elucidation of the compounds. Forster et al. [24] carried out a comparison study of 11 hop varieties in an effort to determine whether the relative amounts of certain components can be used to identify the hop variety. They concluded that low molecular-weight polyphenols may help to differentiate hop varieties and also proved the dependence of polyphenol profiles on the growing region.

The aim of this study was to develop a fast and simple profiling method based on UHPLC-TOF MS analysis of proanthocyanidins in hops and to determine the correlation between the type and amount of proanthocyanidins and both, the hop variety and the growing locality. In principle, we obtained the UHPLC-TOF MS profiles (chromatograms) of proanthocyanidins without their individual identification. Each profile provided a "chemical picture" of these compounds in the hop samples. Subsequently, we correlated these profiles with the earlier mentioned hop characteristics. In addition to that, the application of UHPLC, the current state-of-the-art fast liquid chromatography technique, facilitated the method high-throughput.

2. Experimental

2.1. Chemical and reagents

Acetonitrile (LC-MS, Biosolve, Netherlands), purified water (Direct-Q UV system, Millipore, Germany), formic acid (98–99%, Merck, Germany), leucine-enkephalin acetate hydrate (Sigma-Aldrich, Germany) were used for the UHPLC-TOF MS analyses. Standards of catechin and epicatechin were purchased from Fluka, Buchs SG, Switzerland. Acetone (p.a., LACH NER, Czech Republic) was used for proanthocyanidin extraction from hops together with re-distilled water (Merck Millipore, Germany). Paper filter (density 84 g m^2, Munktell & Filtrak, DE) was used for filtration of the acetone extract.

2.2. Sample selection

Analyzed samples included four varieties Saaz (SA), Sladek (SL), Preminat (PR) and Agnus (AGN) from different Czech growing localities (24 localities, A–Z). Moreover, samples of both standard

(ST) and virus-free (VF) seed types of Saaz variety were tested. Eleven hop samples were analyzed in 2011 (SA, SL, PR and AGN varieties in quantities of 4, 3, 3, and 1 sample, respectively) and 40 hop samples were analyzed in 2012 (SA, SL, PR and AGN varieties in the number of 18, 9, 9, and 4 samples, respectively). Hop samples were obtained from all three hop growing regions in the Czech Republic so that they cover the whole crop area of hops (4336 ha in 2012) in the country.

2.3. Sample pre-treatment

The air-dried and homogenized samples of hop cones were fine milled, the powder (0.8 g) was suspended in 250 mL of acetone/water (70:30 v/v), shaken for 40 min at 140 rpm under nitrogen atmosphere, filtered, 10 mL of the filtrate was evaporated to dryness, reconstituted in 2 mL of methanol and diluted with 2 mL of water.

2.4. UHPLC–TOF MS analysis

The prepared sample was analyzed on the Acquity UPLC system with LCT premier XE TOF MS (Waters). The LC column Acquity UPLC BEH Shield RP18 (50 mm × 2.1 mm I.D., particle size 1.7 μm, Waters) was used for proanthocyanidins separation using two-component mobile phase. The mobile phase A and B consisted of 0.1% formic acid in water and 0.1% formic acid in acetonitrile, respectively. The analyses were performed under a linear gradient program (min/%B) 0/10, 1.0/10, 6.5/26 followed by a 1.5 min column clean-up (100% B) and a 1 min equilibration (10% B). The total analysis time was 9 min. The column temperature was set at 35 °C, flow rate at 0.4 mL min^{-1} and the injection volume was 1 μL. The mass spectrometer operated in the "V" negative mode with capillary voltage set at −1900 V, cone voltage −85 V, desolvation gas temperature, 350 °C; ion source block temperature, 120 °C; cone gas flow, 50 L h^{-1}; desolvation gas flow, 650 L h^{-1}; ion guide 1 and 2 RFs, 200 and 400 V, respectively; hexapole RF, 150 V. The signal was acquired with the scan time, 0.1 s; interscan delay was 0.01 s (0.1 s for lock spray). The mass accuracy was kept below 5 ppm using lock spray technology with leucine enkephalin as the reference compound (2 ng μL^{-1}, 5 μL min^{-1}). The data were processed by MassLynx V4.1 software (Waters). The chromatograms were extracted at the *m*/*z* corresponding to the compound theoretical mass; the tolerance window was ±0.01 Da. The peaks were subsequently smoothed using the mean method (two interactions, smoothing width 1). The retention time and the area under the peak were used for statistical evaluation.

2.5. Statistical evaluation

The obtained profiles were evaluated by PCA and cluster analysis using Statgraphics in order to reveal the relations between the objects (hop variety and growing locality) and the variables (proanthocyanidin profiles).

3. Results and discussion

3.1. UHPLC-TOF MS method development

The mobile phase composition, chromatographic column, chromatographic column temperature and elution by isocratic as well as gradient programs were considered using one real sample (hop extract prepared as described earlier). The chromatograms extracted at 289.07, 577.14, 865.20, 1153.26, and 1441.23 corresponding to proanthocyanidin mono-, di-, tri-, tetra-, and pentamers, respectively, consisting of (epi)catechin units were assessed by the peak resolution.

Acetonitrile and methanol have been used as organic modifiers of the mobile phase in previous works concerned with proanthocyanidin analysis [21,22]. In our case, acetonitrile provided better ionization than methanol and, in addition to that, acetonitrile as a stronger elution agent provided significantly faster analysis with resolution of the peaks of interest comparable with the resolution obtained when using methanol. Therefore, acetonitrile was used with respect to higher sensitivity as well as shorter analysis time. For the aqueous part of the mobile phase, previously reported 5 mM ammonium acetate, pH 3.0, was tested and found suitable [25]. However, 0.1% formic acid provided very similar analyses and was, therefore, chosen considering its simplicity of preparation. Trifluoroacetic acid (0.1%) presumably suppressed the ionization because it significantly decreased the signal when it was used as a part of the mobile phase. Six UHPLC columns were tested for proanthocyanidins separation:

- BEH C18: Acquity UPLC BEH C18 (50 mm × 2.1 mm I.D., particle size 1.7 μm, Waters)
- BEH Shield: Acquity UPLC BEH Shield RP18 (50 mm × 2.1 mm I.D., particle size 1.7 μm, Waters)

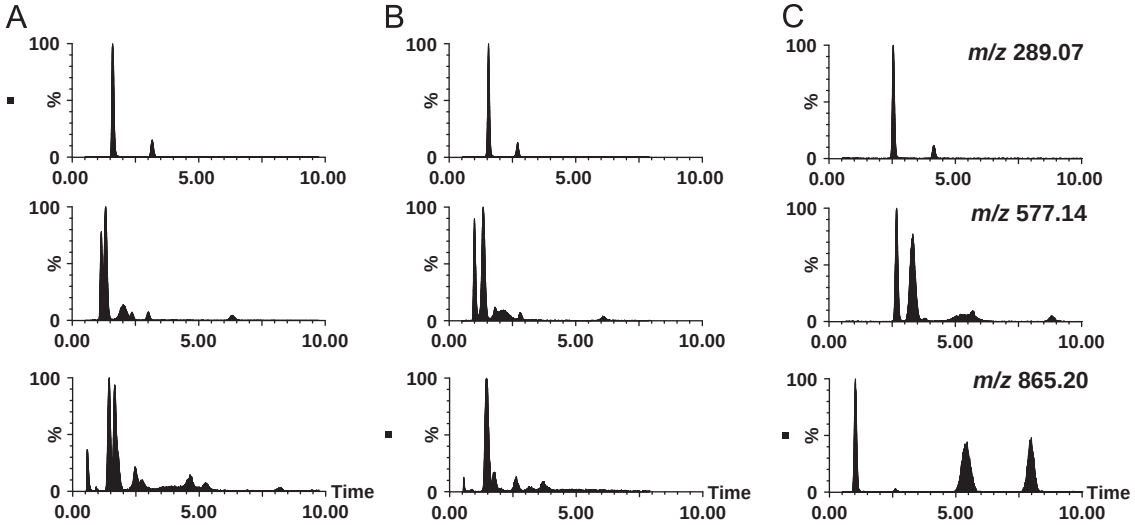


Fig. 2. Comparison of LC columns for separation of proanthocyanidins (A) BEH C18, (B) Kinetex PFP, (C) BEH Shield. For the column specification, see the text. Chromatographic conditions: mobile phase A – 0.1% HCOOH and B – acetonitrile; isocratic elution: 8% B; column temperature, 30 °C; flow rate, 0.4 mL min^{-1}, injection volume, 1 μL.

- BEH Phenyl: Acquity UPLC BEH Phenyl (50 mm × 2.1 mm I.D., particle size 1.7 μm, Waters)
- CSH C18: Acquity UPLC CSH C18 (50 mm × 2.1 mm I.D., particle size 1.7 μm, Waters)
- Kinetex C18: Kinetex C18 (50 mm × 2.1 mm I.D., particle size 2.6 μm, Phenomenex)
- Kinetex PFP: Kinetex Pentafluorophenyl (50 mm × 2.1 mm I.D., particle size 2.6 μm, Phenomenex)

The best separation of proanthocyanidins with identical mass, assessed as resolution of the monitored peaks, was achieved using BEH C18, BEH Shield, and Kinetex PFP columns. In Fig. 2, one can compare the performance of these three columns under isocratic elution (8% B). Evidently, the best resolution of the major peaks in the *m/z* 577.14 and 865.20 extracted chromatograms was achieved with the BEH Shield column (Fig. 2C). The isocratic elution was then modified to a linear gradient program in order to reduce the analysis time down to 9 min, which included the column clean-up and equilibration. For comparison, Li et al. [21] used the Synergi Hydro-RP-80A (250 mm × 4.6 mm I.D., particle size 4 μm, Phenomenex) HPLC column with 1% formic acid and methanol as the mobile phase and a linear gradient program from 5% to 50% methanol over 50 min at the flow rate of 0.8 mL/min. Whittle et al. [11] used the Zorbax SB-C18 (150 mm × 4.6 mm I.D., particle size 5 pm, Agilent) HPLC column with 0.1% acetic acid in water and in 0.1% acetic acid in acetonitrile and a linear gradient program from 2.5% to 100% acetonitrile over 105 min followed by a 35 min column equilibration step. Lastly, Dvorakova et al. [22] analyzed the target compounds on LiChroCart RP-18 (125 mm × 3.0 mm I.D., particle size 3 μm, Merck Millipore) HPLC column with a flow rate of 0.2 mL/min; the mobile phase consisted of 0.1% formic acid (A) and methanol (B) and was used in the gradient program of 10% B in 0 min, from 10% to 40% B over 75 min, followed by a column equilibration step of 15 min. The analysis time of our new UHPLC method is significantly lower compared to the previously described methods with analysis times of 50 min [21], 140 min [11] or 90 min [22] reported.

Both negative [22] as well as positive [11,21] modes were applied for proanthocyanidins mass spectrometry detection. Under our conditions, a significantly higher signal was obtained when negative mode was applied. Further, the optimal capillary voltage and cone voltage in negative mode were found in order to enhance the detector response and thus the method sensitivity. The capillary voltage was tested in the range from −900 to −2900 V (100 V steps). A negligible influence of the capillary voltage on the detector response was observed for *m/z* 577.14 and 1441.32 ions. A slight, gradual increase of signal was observed for *m/z* 865.20 and 1153.26 ions with increasing capillary voltage. On the other hand, the signal significantly decreased for *m/z* 289.07 ions with increasing capillary voltage. Therefore, the capillary voltage of −1900 V was chosen as a compromise. The dependence of the detector response on the cone voltage is depicted in Fig. 3. Interestingly, the increasing cone voltage improved the sensitivity in the range from 10 up to 70–90 V for the *m/z* 289.07 ions and less pronounced for the *m/z* 577.14 ions. The sensitivity of the other monitored ions appeared independent on cone voltage. This observation does not comply with the general phenomenon (higher cone voltage usually results in lower signal); however, similar findings have already been described [26], suggesting that the optimal cone voltage should be determined experimentally. The value of −85 V was chosen as optimal with respect to all monitored ions. Under the developed MS conditions,

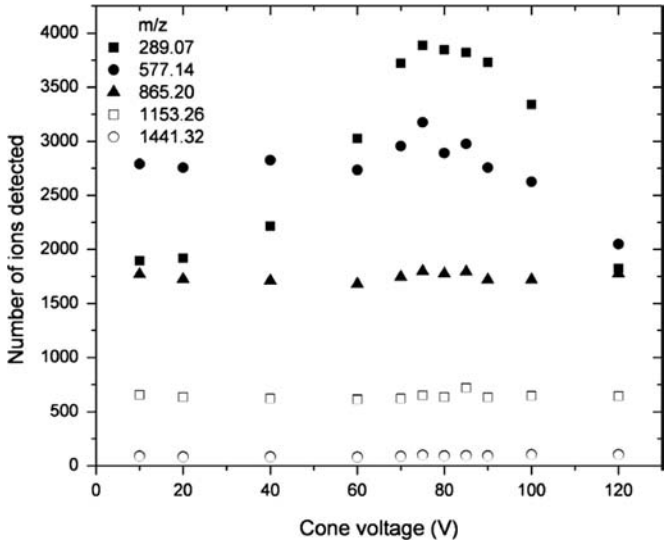


Fig. 3. Dependence of MS response on cone voltage for selected ions. MS conditions: "V" negative mode; capillary voltage, −1900 V; desolvation gas temperature, 350 °C; ion source block temperature, 120 °C; cone gas flow, 50 L h^{-1}; desolvation gas flow, 650 L h^{-1}; ion guide 1 and 2 RFs, 200 and 400 V, respectively; hexapole RF, 150 V; scan time, 0.1 s; interscan delay, 0.01 s (0.1 s for lock spray).

Table 1
List of proanthocyanidins considered for statistical evaluation.

No.	Number of units	Type of units	Retention time (min)	Theoretical mass $[M-H]^-$	Intra-day repeatability RSD (%) (peak area, $n=6$)
1	1	C	1.61	289.0712	8.09
2	1	E	2.16	289.0712	6.63
3	1	(E)G I	0.87	305.0661	9.10
4	1	(E)G II	1.18	305.0661	6.58
5	1	(E)G III	1.53	305.0661	9.51
6	2	(E)C-(E)C I	1.52	577.1346	5.76
7	2	(E)C-(E)C II	1.86	577.1346	7.36
8	2	(E)C-(E)C III	2.46	577.1346	4.75
9	2	(E)C-(E)C IV	3.10	577.1346	6.96
10	2	(E)C-(E)C V	3.56	577.1346	5.65
11	2	(E)C-(E)G I	0.84	593.1295	6.02
12	2	(E)C-(E)G II	0.97	593.1295	7.09
13	2	(E)C-(E)G III	1.16	593.1295	9.96
14	2	(E)C-(E)G IV	2.51	593.1295	6.82
15	2	(E)C-(E)G V	4.96	593.1295	2.73
16	2	(E)C-(E)G VI	5.27	593.1295	1.81
17	2	(E)C-(E)A	2.41	561.1397	8.79
18	2	(E)G-(E)G I	0.79	609.1244	9.10
19	2	(E)G-(E)G II	4.42	609.1244	2.12
20	2	(E)G-(E)G III	4.59	609.1244	2.43
21	3	(E)C-(E)C-(E)C I	0.74	865.1980	6.35
22	3	(E)C-(E)C-(E)C II	1.49	865.1980	8.41
23	3	(E)C-(E)C-(E)C III	2.37	865.1980	6.91
24	3	(E)C-(E)C-(E)C IV	2.67	865.1980	5.33
25	3	(E)C-(E)C-(E)C V	3.03	865.1980	6.41
26	3	(E)C-(E)C-(E)C VI	3.21	865.1980	4.41
27	3	(E)C-(E)C-(E)C VII	3.43	865.1980	6.66
28	3	(E)C-(E)C-(E)C VIII	3.84	865.1980	5.84
29	3	(E)C-(E)C-(E)G I	0.62	881.1929	9.89
30	3	(E)C-(E)C-(E)G II	1.69	881.1929	7.07
31	3	(E)C-(E)C-(E)G III	1.83	881.1929	8.49
32	3	(E)C-(E)C-(E)G IV	2.37	881.1929	5.66
33	3	(E)C-(E)G-(E)G I	0.56	897.1879	5.36
34	3	(E)C-(E)G-(E)G II	1.17	897.1879	5.28
35	4	(E)C-(E)C-(E)C-(E)C I	2.10	1153.269	9.00
36	4	(E)C-(E)C-(E)C-(E)C II	2.54	1153.269	9.78
37	4	(E)C-(E)C-(E)C-(E)C III	2.95	1153.269	4.49
38	4	(E)C-(E)C-(E)C-(E)C IV	3.21	1153.269	3.77
39	4	(E)C-(E)C-(E)C-(E)C V	3.78	1153.269	2.95
40	5	(E)C-(E)C-(E)C-(E)C-(E)C	3.50	1441.325	*n/p*

C – catechin, E – epicatechin, (E)C – (epi)catechin, (E)G – (epi)gallocatechin, (E)A – (epi)afzelechin; *n/p* – not present.

proanthocyanidins were detected almost exclusively as pseudomolecular $[M–H]^-$ ions and no significant fragmentation was observed. Additionally, we have not detected any possible interfering fragments or adducts of the analytes that could correspond to any proanthocyanidins consisting of either a lower or higher number of units compared to the analyte.

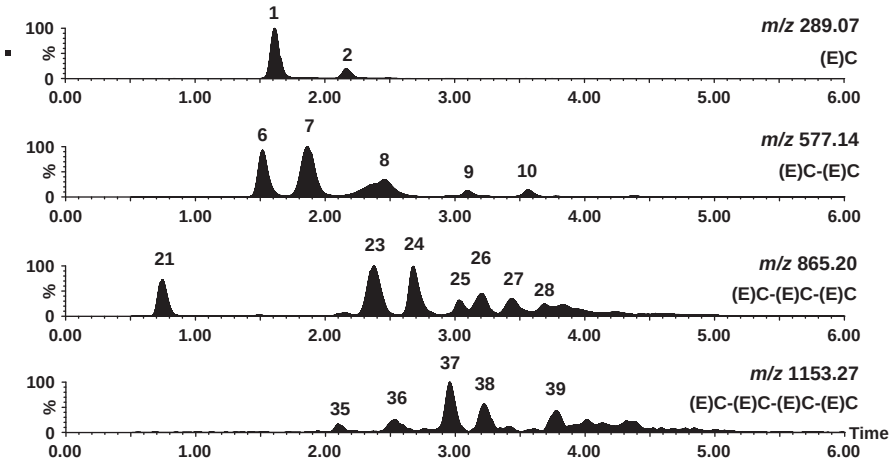


Fig. 4. Hops sample profile of proanthocyanidins consisting of only (E)C units. Variety – Premiant, locality – Zatec, for chromatohgraphic conditions see Section 2. The peak annotations correspond to the compound Nos. in Table 1.

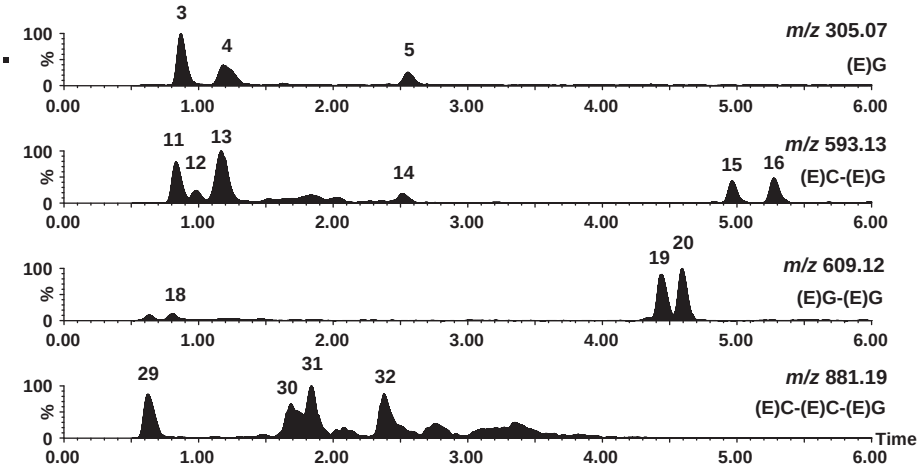


Fig. 5. Hop sample profile of proanthocyanidins consisting of at least one (E)G unit. Variety – Premiant, locality – Zatec, for chromatohgraphic conditions see Section 2. The peak annotations correspond to the compound Nos. in Table 1.

Table 2
Relative abundance of proanthocyanidins in studied hop varieties in 2011 and 2012 crop seasons.

Number of units	Type of units	Relative abundance of proanthocyanidins (%)							
		2011				2012			
		Saaz	Sladek	Premiant	Agnus	Saaz	Sladek	Premiant	Agnus
1	C	19.4	18.1	16.5	6.8	18.2	15.4	17.1	7.9
1	E	2.8	2.9	3.2	11.4	3.3	3.4	3.5	9.0
1	(E)G	0.5	1.3	1.7	0.8	0.6	1.0	1.5	1.0
2	(E)C-(E)C	36.5	33.4	32.9	33.5	38.2	36.2	35.7	35.7
2	(E)C-(E)G	4.1	7.1	7.9	7.8	5.3	6.9	8.6	8.0
2	(E)A-(E)C	< 0.5	< 0.5	< 0.5	< 0.5	0.5	< 0.5	< 0.5	< 0.5
2	(E)G-(E)G	3.6	6.3	6.0	9.9	4.6	6.7	6.1	9.5
3	(E)C-(E)C-(E)C	26.6	21.8	21.9	21.2	23.0	22.9	19.6	20.8
3	(E)C-(E)C-(E)G	2.0	3.8	4.8	3.2	2.0	3.0	4.5	3.1
3	(E)C-(E)G-(E)G	< 0.5	0.7	1.0	0.5	< 0.5	< 0.5	< 0.5	< 0.5
4	(E)C-(E)C-(E)C-(E)C	4.0	4.2	4.0	4.6	4.2	4.3	3.3	4.7
5	(E)C-(E)C-(E)C-(E)C-(E)C	< 0.5	< 0.5	< 0.5	< 0.5	< 0.5	< 0.5	< 0.5	< 0.5

C – catechin, E – epicatechin, (E)C – (epi)catechin, (E)G – (epi)gallocatechin, (E)A – (epi)afzelechin.

3.2. Partial validation – intra-day repeatability

In terms of the method validation, intra-day repeatability of the UHPLC-TOF MS method was investigated. Retention times and the areas under the peaks corresponding to the compounds that were considered in the method application were evaluated. Relative standard deviation (RSD) values obtained from six replicates revealed that the retention time intra-day repeatability was within 1.39% for all the compounds. In terms of the area under the peak, intra-day repeatability was also obtained from six replicates, and the RSD values were within 9.89% for all the compounds assessed (see Table 1). These results confirm that the validation of the method assessed by the intra-day repeatability is sufficient and the method is suitable for its purpose. However, it would be mandatory to include an internal standard to assure the MS detection reproducibility if more samples were analyzed or inter-day repeatability was required.

3.3. Method application and statistical evaluation

The presented method was designed for separation of proanthocyanidins, a large group of hop secondary metabolites. The method provided profiles of proanthocyanidins, whose partial identification was based on accurate mass (<5 ppm) of their pseudomolecular $[M–H]^-$ ions. This approach could not provide an exact compound structure; however, it was possible to establish the type of monomer units, from which the proanthocyanidin oligomers were composed. In this way, (epi)catechin, (epi)gallocatechin and (epi)afzelechin differing in mass can be distinguished from each other, whereas isomer pairs such as catechin and epicatechin or gallocatechin and epigallocatechin provide identical MS signals, but they differ in their retention times. The identification of catechin and epicatechin monomers was performed based on comparisons with their authentic standards. Two examples of proanthocyanidin profiles are presented in Fig. 4 (proanthocyanidins consisting of only (epi)catechin units) and in Fig. 5 (proanthocyanidins consisting of at least one (epi)gallotechin unit). The complete list of 40 proanthocyanidins we considered for statistical evaluation is given in Table 1. The sample inter-comparing by statistics using the accurate mass of the pseudomolecular ion defining the type and number of monomer units composing the proanthocyanidin, and the peak area and the retention time, as the input data, revealed the following results.

The relative abundance of flavan-3-ol monomers and proanthocyanidin oligomers, expressed as the percentage of the total abundance of all detected mono- and oligomers, revealed significant differences between the varieties as shown in Table 2. These results obtained in 2011 and 2012 corresponded to each other. The results from 2012 are specified in the following paragraph.

Catechin was the most abundant flavan-3-ol monomer in Saaz, Sladek and Premiant hops (15.4–18.2%) by contrast to the Agnus variety, where the major monomer was epicatechin (9.0%). As for the oligomers, the main contribution of the detected substances belongs to the (epi)catechin dimers (35.7–38.2%) and trimers (19.6–23.0%). Saaz exhibited slightly higher relative abundance of these dimers and trimers in comparison to Agnus, Sladek and Premiant. Further, the oligomers formed by at least one unit of (epi)gallocatechin were at a lower abundance in Saaz when compared to all the other varieties, e.g. the abundance of E(C)–E(G) was 5.3% in Saaz, while it was ranging from 6.9% to 8.6% in the other varieties; the abundance of E(G)–E(G) in Saaz was 4.6%, while it was ranging from 6.1% to 9.5% in the other varieties. The relative abundance of (E)C–(E)G–(E)G trimers and (E)C pentamers was very low in all varieties (lower than 0.5%). Moreover, we have found a low amount of (epi)afzelechin incorporated in a proanthocyanidin, which is in agreement with the results of Li et al. [21]. Interestingly, (epi)afzelechin was only identified in the dimer E(C)–E(A) in both our investigations (2011 and 2012) and those published earlier [21,23]. This dimer corresponds to propelargonidin B or its isomer.

A more comprehensive interpretation of the profile similarities and differences was performed using multivariate statistical methods PCA and cluster analysis. Fig. 6A shows the PCA of observations (i.e. individual hop samples), and Fig. 6B shows the PCA of variables (i.e. proanthocyanidin monomers and oligomers); both figures describe the 2012 data. The closer the points in the graph (Fig. 6A) are, the more similar the relative abundance of proanthocyanidins in the corresponding samples is. Accordingly, it is possible to separate the four tested varieties into four segments based on the proanthocyanidin profile: Saaz (left part of the graph), Sladek (right part), Premiant (upper part) and Agnus (in the middle). Similar results were obtained by processing the data from 2011 crop (data not shown). As follows from Fig. 6A, hop samples were grouped into the “varietal clouds”. The “clouds” are slightly broad and some samples are close to the cloud borders.

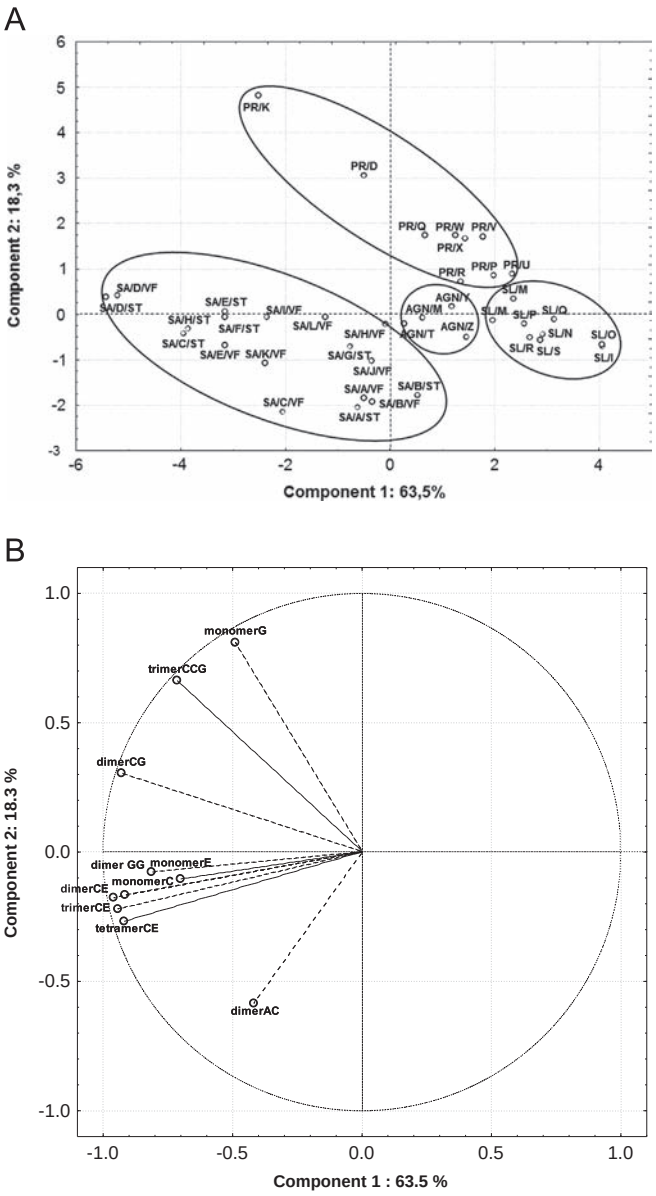


Fig. 6. A. Principal components analysis (scatterplot of hop samples). Results of 2012 crop. SA – Saaz, SL – Sladek, PR – Preminat, AGN – Agnus, A, B – Z growing localities The Saaz variety: ST – standard, VF – virus-free. B. Principal components analysis (scatterplot of oligomers). Results of 2012 crop. For the legend see Fig. 6A.

This scattering can be explained by the general phenomenon that the content and composition of hop secondary metabolites (hop resins, essential oils, polyphenols) does not depend only on the variety, but also on the climatic conditions, time of the crop and the age of the hop plant [27]. For example, the content of α-bitter acids fluctuates in the range of several tens of percent (Saaz, 2012, RSD=21.1%, n=95) [28].

When Fig. 6A and B is virtually overlaid, it is evident that oligomers consisting of catechin, epicatechin and afzelechin regardless of the number of monomeric units were present more frequently in the traditional Czech aroma hop variety Saaz, while more oligomers formed by (epi)catechin and (epi)gallocatechin were present in the hop variety Premiant and Agnus. Sladek variety samples contained the lowest amount of all detected compounds. PCA showed that the hop cultivars differed in the content and proportions of oligomeric proanthocyanidins. In addition to this, considerable influence of the above mentioned non-varietal behaviors summarized in the term growing locality was observed.

The result of the cluster analysis (Fig. 7) illustrates the hierarchical structure of the objects (hop variety and growing locality) in the cluster. Except of two Premiant hop samples (PR/K and PR/D), the hops are again clearly grouped depending on their variety. The greatest distance was observed between the traditional Saaz fine aroma variety and the other three tested hybrid varieties. Within these three varieties, Sladek and Premiant were closer to each other, whereas Agnus clustered in a separate clade. These results comply with the genetic relatedness of the varieties except for the two Premiant samples (PR/K and PR/D), which are out of their cluster (Fig. 7) and also they are distant from the rest of the Premiant samples in the PCA analysis (see Fig. 6A). These two samples actually exhibit higher similarity to the Saaz variety.

In general, the tested hop varieties have been grouped according to genetic relatedness: Agnus was originally selected from hybrid progenies of Sladek, Bor, Saaz, Norther Brewer and Fuggle and its genome is similar to Euro-American bitter hops Nugget, Magnum and Horizon. Premiant and Sladek, both have Northern Brewer and Saaz in their pedigree [27], Saaz is related with German varieties Spalt and Tettneng [29]. In 2011, the largest difference was found between the Agnus sample and the three aroma varieties (Saaz, Sladek and Premiant), variety Sladek and Premiant were not differentiated (data not shown). This could be caused by the fact that a small set of samples in the 2011 experiment was tested.

Within the Saaz hop variety, the standard and virus-free seed types were tested. Proanthocyanidins have a protective function in plants; therefore their lower amounts in the virus-free planting material would be expected. Differences between the two seed types in the cluster analysis were not found, so the hypothesis of significantly different spectrum of the studied compounds cannot be confirmed.

In the context of papers published earlier, the present study is unprecedented in terms of the number of analyzed samples and the growing area coverage. In a similar but smaller study, where 13 samples were included (11 cultivars, only the Villamette cultivar originated from three different localities), Li et al. [21] investigated the composition of proanthocyanidin oligomers in hops. Authors presented differences in proanthocyanidin content in relation to both, variety and growing area. Their and our studies are in accordance in terms of the established dependence of proanthocyanidin profiles on the variety and growing locality. A more detailed comparison of these studies is difficult with respect to different methodology, mainly the statistical data processing and evaluation, which was applied in each case. By contrast to Li et al. [21], we have processed data from two crops (11 samples in 2011 and 40 samples in 2012), which facilitated to confirm a strong varietal dependence.

The achieved results of the proanthocyanidin profile study in hops can be summarized in the sense that the flavan-3-ol monomers and proanthocyanidin composition appears to be distinct varietally, i.e. we have observed the genetically determined dependence. But also the influence of the environment, soil and climatic conditions during the hop growth and hop cones maturation has to be considered.

4. Conclusion

The newly developed method for proanthocyanidins profiling is, in comparison with the previously published methods [11,21], less laborious and thus more high-throughput. Particularly, sample pre-treatment involves only extraction of hops with organic solvent followed by direct injection to LC–MS and the analysis is only 9 min long including column clean-up and equilibration. We have concluded that the content and composition of oligomers strongly depended on the variety, but other factors that are summarized under the term growing locality also contributed to

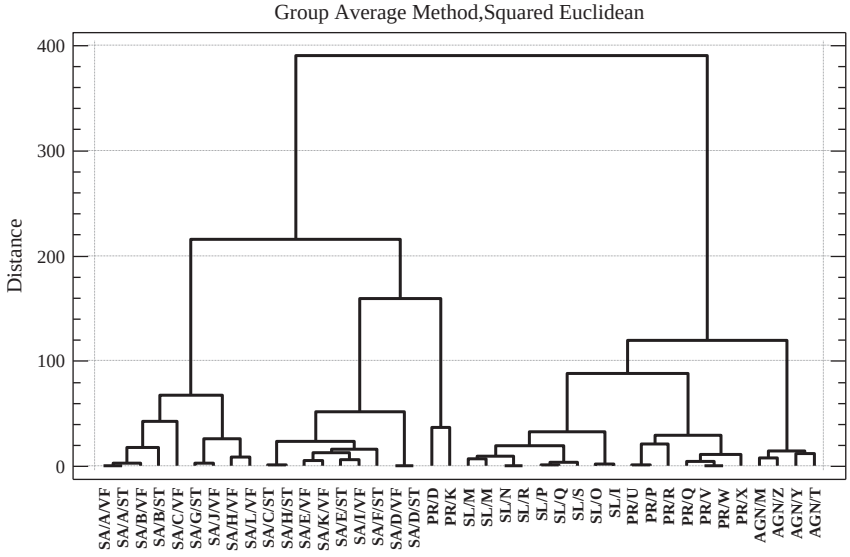


Fig. 7. Cluster analysis (dendrogram). Results of 2012 crop. For the legend see Fig. 6A.

the proanthocyanidin profiles. These results are consistent with previously published findings [20], where the dependence of proanthocyanidins composition on genetic relatedness was formulated. The agreement between the results of two consecutive years indicates that the method is reliable and reproducible. The method will subsequently be used for the prediction of qualitative marks of hops produced in the Czech Republic and for the development of a tool for hops authenticity verification. Besides that, the method will be used for the study and monitoring of bioactive polyphenol compounds with proved or potential health benefits.

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