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High-Performance Liquid Chromatography Determination of Free Sugars and Mannitol in Mushrooms Using Corona Charged Aerosol Detection

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Abstract

Refractive index detector is usually used in the analysis of sugars in mushrooms, which is characterized by poor sensitivity, reproducibility, and susceptibility to interference from co-eluting sample components. In the current study, identification and determination of free sugars in mushroom samples by high-performance liquid chromatography coupled to corona charged aerosol detector (HPLC-CAD) were presented for the first time. The best chromatographic separation was performed on a Shodex Asahipak NH2P-50 4E 5 μm and mobile phase composed of 75% acetonitrile and 25% water with flow rate was 1 mL/min. The developed method offers good linearity in concentration range 0.001–0.01 or 0.01–0.2 mg/mL for tested compounds with $R^2 > 0.99$. Limit of detection (LOD) for analytes was in the range of 7.1–120.2 ng on column. HPLC-CAD method showed very good reproducibility (RSD < 5.1%). Fructose, mannitol, and glucose were detected in all examined mushroom samples. For white *Agaricus bisporus*, mannitol was the most abundant sugar (7.575 mg/g dw), whereas trehalose for *Pleurotus ostreatus* (3.426 mg/g dw). The developed method was successfully applied for quantification of free sugars and mannitol in mushrooms. The optimized method proved to be sensitive, reproducible, and accurate.

Keywords Free sugars · Polyols · Mushrooms · HPLC · CAD detector

Introduction

Mushrooms have been known to humanity for several thousand years and used for food and medicinal purposes (Wasser 2002). They are valued for their unique taste, aroma, and texture. However, the belief that mushrooms can be valuable and even used as functional foods is relatively new. Interest in edible and medicinal mushrooms is constantly growing, which is also confirmed by numerous scientific publications in the last two decades (Jabłońska-Ryś et al. 2019). Mushrooms are low in fat, rich in protein and fiber, are an important source of many vitamins (B_1, B_2, B_{12}, D_2, C, and E), macro- and microelements, contain all exogenous amino acids, fatty acids, and possess high antioxidant activity (Radzki et al. 2014; Sławińska et al. 2017). For these reasons nowadays, they are part of many food trends, such as diet food, vegetarianism and veganism, the searching for valuable micronutrients or the fifth taste, which is associated with their constantly increasing consumption (Kalač 2009).

The dry matter of mushrooms consists mainly of carbohydrates (from 35 to 70%) (Guillamón et al. 2010). These compounds include polysaccharides such as β-glucans, mono- and disaccharides, sugar alcohols (polyols), glycogen, and chitin (Manzi et al. 2001). The most common representatives of mono-, disaccharides, and polyols in mushrooms are glucose, trehalose, and mannitol (Mocan et al. 2018). The content of glucose and trehalose is usually low; in turn, mannitol, depending on the species, may be present in higher amounts, from 0.2% to nearly 14% of dry matter (Jabłońska-Ryś et al. 2019).

The qualitative and quantitative analysis of sugars and polyols in mushrooms is very important for many reasons. First of all, free and soluble sugars and polyols are important part of chemical composition. What is more, these compounds, apart from free amino acids and 5′-nucleotides, are classified as non-volatile compounds that determine the typical mushroom taste (Phat et al. 2016). According to the authors Beluhan and

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Ranogajec (2011), the high content of sugars and polyols may affect the increase in the feeling of moderate sweet taste of mushrooms. In several scientific papers on mushrooms obtained from natural environments, it was emphasized that the analysis of the chemical composition would allow to create a data base providing the nutritional value and content of non-volatile compounds to improve the characteristics of unique species with high nutritional value and unique flavor (Jedidi et al. 2017).

In addition, the amount of free sugars and sugar alcohols can be an important indicator of storage conditions and preservation. Mushrooms are characterized by high perishability, which is associated with rapid loss of quality after harvest. This creates a problem in their distribution and sale as fresh raw material. Therefore, research is being conducted to extend the shelf life of fresh mushrooms. In this case, the polar compound profile is determined, which includes, in addition to organic acids, sugars and sugar alcohols, which are important indicators of preservation conditions (Cardoso et al. 2019; Fernandes et al. 2016). Determination of sugars in mushrooms at various stages of growth is also important for further processing in the flavors and fragrance industry. Mushrooms, depending on the stage of growth and morphological part (stipes or caps), are characterized by a different composition of sugars and organic acids, which mainly determine their flavor (Chen et al. 2015). The content and profile of sugars and polyols may differ significantly in fruiting bodies of the same species but obtained from different sources (Li et al. 2019; Stojković et al. 2014).

In many regions of the world, the spontaneous lactic fermentation process was traditionally used to preserve the fruiting bodies of wild-growing and cultivated edible mushrooms (Sõukand et al. 2015). Currently, there are no lacto-fermented mushrooms on the European market (Jabłońska-Ryś et al. 2019). However recently, a few publications have been published regarding the process of lactic acid fermentation of this raw material (Jabłońska-Ryś et al. 2016a, b; Khaskheli et al. 2015; Liu et al. 2016), and therefore, the current knowledge in this field needs to be systematized and broaden (Jabłońska-Ryś et al. 2019). *Lactobacillus* species (LAB) use free sugars found in food matrix as a source of energy (Das and Goyal 2012). Moreover, the preferences for simple sugars and polyols are different for LAB isolates used for controlled fermentation of plant material (Ye et al. 2019). Therefore, analysis of the composition of free sugars is important in assessing the suitability of mushroom fruiting bodies as a raw material for lactic acid fermentations. This knowledge will allow to choose the right LAB strains with the desired technological and functional properties.

Therefore, there is a need to develop a quick and simple method that can be used to determine free sugars in the food matrix which are mushrooms. The determination of these compounds is a challenge due to the lack of a chromophore and their polar structure. Various methods are used to determine these compounds. There are many publications describing the use of high-performance liquid chromatography (HPLC) (Agblevor et al. 2007), gas chromatography (GC) (Medeiros and Simoneit 2007), or capillary electrophoresis (CE) (Cao et al. 2004) techniques for sugar analysis. However, gas chromatography requires costly, time-consuming, and problematic sample derivatization prior to analysis. In turn, HPLC and capillary electrophoresis are commonly used analytical tools in combination with UV detection (Asthana et al. 2019). The absence of the sufficient chromophore in sugars and polyols prevents the use of a popular UV detector. For these reasons, the most common choice is the refractive index detector (RID) in sugar analysis. However, the disadvantage of this type of detector is poor sensitivity, reproducibility, and susceptibility to interference from co-eluting sample components (Calull et al. 1992; Grembecka et al. 2014). Several papers describe the use of evaporative light-scattering detector (ELSD) and charged aerosol detector (CAD) for the determination of sugars and sugar alcohols in various food matrices (Grembecka et al. 2014; Ma et al. 2014). Several works confirmed the higher sensitivity and precision of CAD and wider linear response range compared with ELSD (Eom et al. 2010; Jia et al. 2011; Vervoort et al. 2008).

Corona charged aerosol detector is a relatively cheap detection system, easy to use, and requiring only high nitrogen pressure (Ligor and Buszewski 2012). Since 2005, when the commercial use of CAD was first described (Gamache et al. 2005), many papers have been published regarding its application for the determination of many groups of compounds: vitamins, lipids, fatty acids, proteins, polymers, oligosaccharides, and sugars (Ligor and Buszewski 2012; Magnusson et al. 2015). Determination of sugars using CAD detector in food matrices has been described in several works (Gil et al. 2020; Grembecka et al. 2014; Márquez-Sillero et al. 2013); however, to date, no data is available regarding the use of it for mushroom samples. For this purpose, the universal RID detector is mainly used (Cardoso et al. 2019; Jedidi et al. 2017; Li et al. 2018). The application of HPAEC-PAD to determine these compounds in shiitake mushrooms has also been described (Chen et al. 2015).

The aim of the study was to develop and validate a new method using HPLC-CAD to determine free sugars (arabinose, fructose, glucose, sucrose, trehalose) and sugar alcohol (mannitol) in mushrooms. The method was applied to determine the above-mentioned compounds in fruiting bodies of various edible mushrooms with very good results. To our knowledge, this is the first work describing the use of HPLC-CAD for simultaneous determination of sugars and polyols in mushroom samples.

Materials and Methods

Chemicals, Reagents, and Standards

HPLC-grade acetonitrile 99.9% was obtained from Merck (Darmstadt, Germany). All other reagents: (i) ethanol 96%

(analytical grade) was purchased from POCH (Gliwice, Poland); (ii) standards (analytical grade; all contents > 99%): sugars (arabinose, fructose, glucose, sucrose, trehalose) and sugar alcohol (mannitol) were supplied by Sigma-Aldrich (Saint Louis, USA). All aqueous solutions were prepared using ultra pure water from the water purification system Direct-Q 3UV (Merck Millipore, Germany).

Sample Preparation

Mushroom Materials

The three most popular cultivated edible mushrooms in Poland, including white and brown button mushrooms (*Agaricus bisporus* (Lange) Sing.) and oyster mushrooms (*Pleurotus ostreatus* (Jacq.: Fr.) Kumm.) were purchased from local market in Lublin (eastern Poland) in May 2019, in an amount of about 2 kg of each species. The mushrooms were stored at 4 °C and processed within 12 h after purchase.

Extraction Procedure

About 300 g of fresh mushrooms were homogenized (Retsch GM200, Germany). Next suspensions of homogenized mushrooms (2 g) in 8 mL of 80% ethanol were shaken at 150 rpm for 0.5 h at 80 °C. After this time, the mixture was centrifuged for 15 min at 5000*g* (MPV-350R, Poland). The supernatant was filtered and transferred to a 10-mL volumetric flask and made up to 10 mL with 80% ethanol. Then, 1 mL of the resulting solution was taken and 3 mL of acetonitrile was added and put into the freezer for 24 h. Next, the samples were centrifuged for 15 min at 16,000*g* to get clear supernatants, which were used for subsequent analyses by HPLC-CAD. All assays were carried out in triplicate.

Instrumentation and Chromatographic Conditions

A Dionex Ultimate 3000 HPLC system coupled to a Corona Veo RS-charged aerosol detector (Germering, Germany) was used for chromatographic analysis. Nitrogen gas flow rate was regulated automatically and monitored by the CAD device. Gas was supplied by nitrogen generator V350 (F-DGSi, France). The following CAD detector parameters have been set: nitrogen gas pressure: 35 psi; detector response 100 pA; noise filter: high. In addition, UV signal was registered at 210 nm by Dionex Ultimate 3000 RS Diode Array Detector. Separation was carried out using a Shodex Asahipak NH2P-50 4E 5 μm (4.6 × 250 mm) column with precolumn Asahipak NHZP-506 4A. The mobile phase of the optimized method composed of (A) 75% acetonitrile and (B) 25% water. The flow rate was 1 mL/min. The column temperature was maintained at 30 °C. The injection volume was 20 μL for standards and mushroom extracts. The chromatographic separation time was set to 22 min. Chromoleon Dionex Software version 7.2 SR4 was used for data acquisition, instrument control, and data analysis.

Method Validation

For method calibration, series of working solutions with different concentrations of reference standards were prepared from stock solutions (1 mg/mL) by dilution with appropriate volume of water. Linearity was tested by triplicate injections of five different concentrations of the standards. The calibration curve for each sugar was obtained by plotting the area of the respective peaks versus the concentration of a compound. The slope, intercept, and the determination coefficients (R^2) of calibration curves were determined by linear regression analysis.

To evaluate method performance, the inter-day precision and limit of detection (LOD) were determined. The inter-day precision was obtained by duplicate analysis of five concentrations of each analyte on three successive days and is expressed as relative standard deviations (RSD). Limit of detection was determined on the basis of response and slope of each regression equation at a signal to noise ratio (S/N) of 3. The recoveries were tested by triplicate analyses of the lowest and the highest concentrations used in calibration studies.

Determination of Free Sugars in Mushrooms

The identification of sugars and sugar alcohol was performed by comparing the retention times of sample peaks with standards under the same chromatographic conditions described

Table 1 HPLC-CAD method parameters

Carbohydrate	Retention time [min]	Calibration curve	R^2	Calibration range [mg/mL]	RSD [%]	LOD [ng on column]
Arabinose	7.1	$y = 4.5105x–0.0138$	0.997	0.01–0.2	4.2	120.2
Fructose	8.0	$y = 108.2422x–0.0084$	0.999	0.001–0.01	4.6	7.2
Mannitol	9.1	$y = 93.0944x–0.0113$	0.998	0.001–0.01	5.1	7.1
Glucose	10.2	$y = 75.399x–0.0099$	0.999	0.002–0.01	4.4	11.4
Sucrose	14.1	$y = 107.9919x–0.0145$	0.998	0.001–0.01	4.1	9
Trehalose	17.3	$y = 91.1928x–0.0097$	0.999	0.002–0.01	4.8	14.7

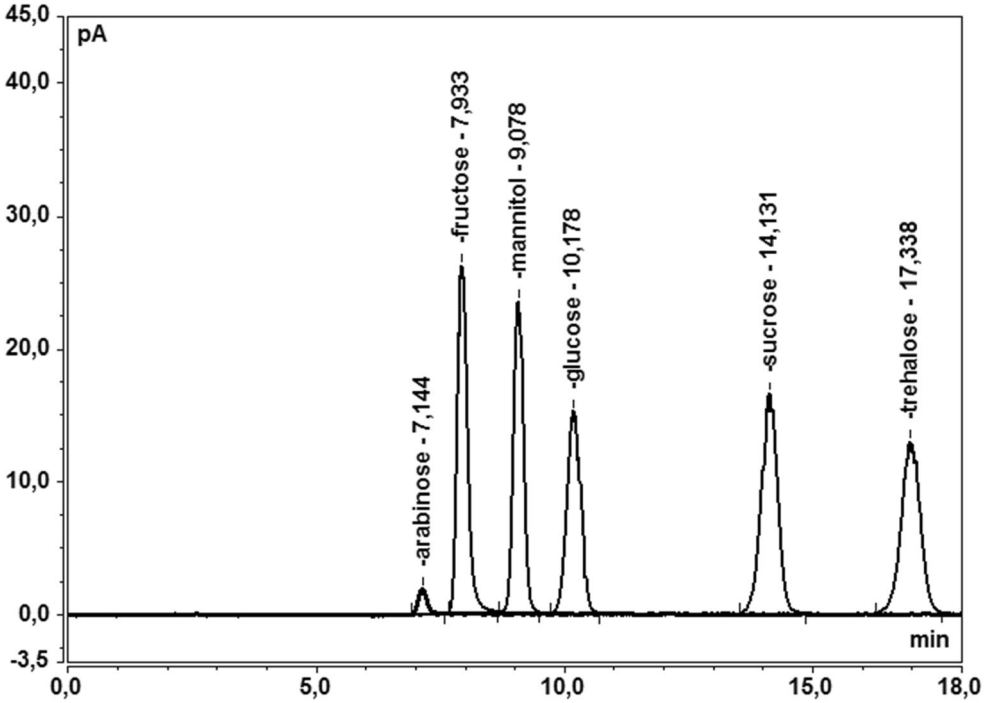

Fig. 1 Overlaid HPLC-CAD chromatograms of arabinose, fructose, mannitol, glucose, sucrose, and trehalose. Column: Shodex Asahipak NH2P-50 4E 5 μm (4.6 × 250 mm), mobile phase: 25% water, 75% acetonitrile, flow 1 mL/min; 1 μg of each saccharide injected on column

in section "Instrumentation and Chromatographic Conditions". The quantification was carried out by the internal standard method. The results were expressed in mg per 1 g of dry weight (dw).

Results and Discussion

Method Validation

Optimized and applied parameters of the chromatographic separation (composition of the mobile phase, flow rate, and column temperature) allowed to obtain negligible baseline noise, indicating complete solvent evaporation, and to quantify the compounds tested. Separation time was initially carried out for 40 min; however, all analyzed compounds were separated in less than 22 min. A similar separation time (19 min) of soluble sugars and mannitol in cocoa was reported by Gil et al. (2020).

The data concerning method validation are summarized in Table 1. An example of an overlaid HPLC-CAD chromatograms of standards (1 μg of each saccharide injected on column) is shown in Fig. 1. All analytes were baseline separated.

Corona CAD response is nonlinear at range of orders of magnitude, but its signal can be close to linear in smaller ranges (Grembecka et al. 2014). The calibration curves of all six analytes showed good linearity ($R^2 > 0.99$). The average of triplicate determinations for each level was used. The LODs ($S/N = 3$) for analyzed compounds were in the range of 7.1–120.2 ng on column. The results indicated highly satisfactory inter-day precision of peak areas (4.1–5.1% RSD).

The recoveries of analytes were tested and were found to be in the range of 96–101%. The relative standard deviation for recoveries did not excide 7%.

One of the latest papers by Pitsch and Weghuber (2019) presents the results of the determination of sugars and polyols in food and beverages using HILIC chromatography coupled with CAD detector. The obtained RDS values were < 5% (ranged from 0.01 to 4.78%), and it was similar to those obtained in our research. The regression coefficients were above 0.99 for all soluble sugars and mannitol in work by Gil et al. (2020). Also, in one of the first works on the determination of sugars and polyols in food using a CAD detector, all standard curves were linear (at range 1–100 μg/mL) except the one of sucrose, which linearity authors obtained in a logarithmic coordinate system. Pitsch and Weghuber (2019) used a different method to calculate linearity than linear calibrations. They obtained good correlation coefficients ($R^2 \geq 0.999$) for nonlinear calibration cures (from 10.0 to 1000 mg/mL), which, as the

Table 2 Carbohydrate content in mushrooms [mg/g dw]

Carbohydrate	*A. bisporus* white	*A. bisporus* brown	*P. ostreatus*
Arabinose	< LOD	< LOD	< LOD
Fructose	0.432	0.213	0.149
Mannitol	7.575	5.409	0.633
Glucose	0.374	0.928	1.366
Sucrose	< LOD	< LOD	< LOD
Trehalose	< LOD	1.032	3.426

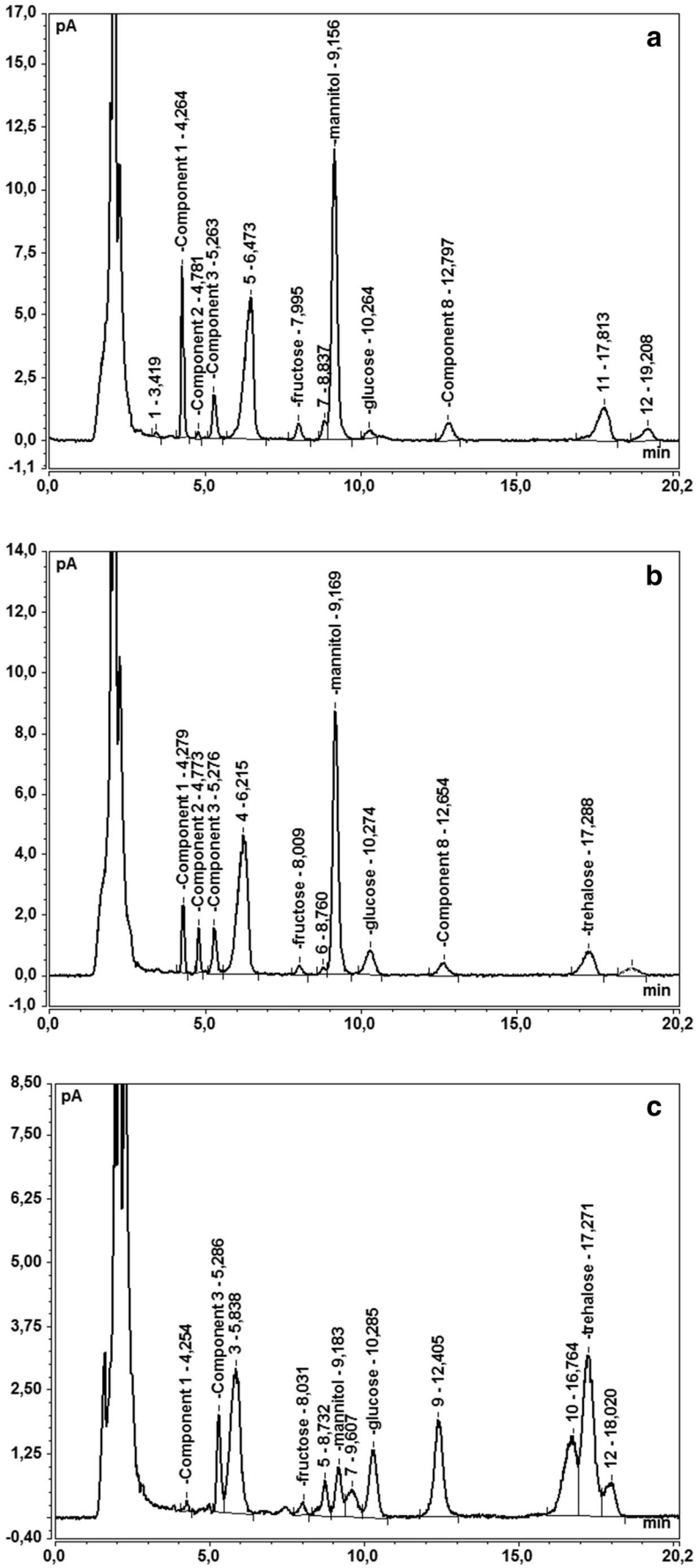

Fig. 2 HPLC-CAD chromatogram of extract from *A. bisporus* white (**a**), *A. bisporus* brown (**b**), and *P. ostreatus* (**c**). Column: Shodex Asahipak NH2P-50 4E 5 μm (4.6 × 250 mm), mobile phase: 25% water, 75% acetonitrile, flow 1 mL/min

authors explain, gives more flexibility in routine analysis. In turn, LOD values presented in the article by Pitsch and Weghuber (2019) were in range of 0.247–2.675 mg/mL, while at work by Gil et al. (2020), LOD values were determined at 0.06–0.15% *w/w* and 0.04–0.19% *w/w* for each analyte estimated in dried and fermented cocoa beans.

Fu et al. in the paper from 2020 presented the results of the separation of sugar isomers and anomers using hydrophilic interaction liquid chromatography (HILIC) with CAD or tandem mass spectrometry. The authors propose this method as useful especially for profiling mono- and disaccharides in the field of glycometabolomics. Their work presents the chromatographic separation of isomeric carbohydrates including isomeric hexoses, pentoses, and disaccharides employing the new commercial HILIC column, by varying the temperature and pH of the chromatographic separation that are important for the separation of sugar isomers. Low temperature (10 or 15 °C) in acidic mobile phase proved to be the best conditions for chromatographic separation.

Quantification of Free Sugars in Edible Mushrooms

The usefulness of the developed analytical method has been confirmed on real samples of the most popular edible cultivated mushrooms in Poland. Figure 2 shows examples of chromatograms for the mushroom samples with described peaks of identified sugars. The other unidentified compounds co-extracted are also visible on the chromatograms, which have been designated as component 1, 2 etc. with the retention times marked. Considering tested sugar compositions (Table 2), fructose, mannitol, and glucose were detected in all the samples. For *A. bisporus*, white and brown mannitol was the most abundant sugar (7.575 and 5.409 mg/g dw, respectively), whereas trehalose for *P. ostreatus* (3.426 mg/g dw). A small amount of trehalose (1.032 mg/g dw) was also found in *A. bisporus* brown, while this disaccharide was observed below LOD in *A. bisporus* white.

The accumulations of the disaccharide trehalose and sugar alcohol mannitol in the fruiting bodies of other species were reported in other research. Among the three compounds analyzed: mannitol, trehalose, and glucose, only mannitol (9.93 g/100 g dw) was found in the fruiting bodies of wild button mushrooms harvested in Tunisia (Jedidi et al. 2017). As reported by Reis et al. (2012), a similar profile of sugars in cultivated mushrooms was obtained as in our study. In white and brown button mushrooms, the highest content of mannitol was detected (5.16 and 4.01 per 100 g fw, respectively), while the main sugar in the fruiting bodies of oyster (4.42 g/100 g fw) and king oyster mushrooms (8.01 g/100 g fw) was trehalose. Except for a small amount of sucrose (0.03 g/100 g fw) in king oyster, this compound was not found in other mushrooms, whereas in all samples, a small amount of fructose was identified (from 0.01 to 0.04 g/100 g fw). Another work confirms that mannitol was found as being the most abundant sugar (36 g/100 g dw) in *A. bisporus* Portobello, and a lower content of fructose (0.6 g/100 g dw) and disaccharide trehalose (1.6 g/100 g dw) was detected (Cardoso et al. 2019).

Conclusions

The HPLC-CAD-developed method is the first of its kind described in the scientific literature that allows simultaneous and accurate determination of six compounds without chromophore groups, sugars (trehalose, glucose, fructose, sucrose, and arabinose) and sugar alcohol—mannitol in mushroom in 22 min. The results obtained from the analyzed real samples showed the possibility of the proposed method to be used for routine analysis of sugars and polyols in mushrooms only with one injection of the sample. The analysis results obtained for examined mushroom samples were in good agreement with those presented by other authors. Furthermore, method validation parameters for the analysis of sugars using CAD demonstrated very good linearity and reproducibility and good limits of detection.

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Compliance with Ethical Standards

Conflict of Interest Aneta Sławińska declares that she has no conflict of interest. Ewa Jabłońska-Ryś declares that she has no conflict of interest. Anna Stachniuk declares that she has no conflict of interest.

Ethical Approval This article does not contain any studies with human participants or animals performed by any of the authors.

Informed Consent Informed consent is not applicable to this study.

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