Distinctive carbohydrate profiles of black ginseng revealed by IM-MS combined with PMP labeling and multivariate data analysis

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A B S T R A C T
Despite the widely recognized importance of ginseng carbohydrates, their structural analysis is still challenging due to structural complexity. This study presents the first ion mobility-mass spectrometry (IM-MS) combined with 1-phenyl-3-methyl-5-pyrazolone (PMP) labeling and multivariate data analysis for profiling carbohydrates in the seven ginseng products. 11 carbohydrates were tentatively annotated, including 5 first found in ginseng, and three of them were solely detected in black ginseng (BG) samples, speculated as monosaccharides bearing various groups based on MS2 analysis. Furthermore, 3D profiles of carbohydrates in IM-MS of the samples showed good discrimination between the varieties of ginseng which were classified into two groups utilizing carbohydrate contents by PLS-DA. The big difference between BG and the others may be ascribed to the repeated heating and steaming process for preparation of BG products. Our findings may provide insights into the differences in bioactivity of different ginseng varieties for future research and show a valuable methodology for discovering unknown carbohydrates.

1. Introduction

Panax ginseng, a perennial herb native to Korea and China, has been used as a herbal remedy in eastern Asia for thousands of years. It is well known that it has multifunctional properties referring to vitality, immunological function, cancer, cardiovascular diseases, and improvement of cognitive and physical performance (Ahuja et al., 2018; Irfan et al., 2020; Lee et al., 2017; Szliszka et al., 2009). The most widely used commercial ginseng products primarily include fresh ginseng (FG), red ginseng (RG), dried ginseng (DG), and American ginseng (AG).

There are some bioactive ingredients in ginseng, including ginsenosides, polysaccharides, oligosaccharides, monosaccharides, amino acids, volatile oils, alkaloids, aliphatic acids, and mineral elements (Chung et al., 2012; Hua et al., 2020; Luo and Fang, 2008; Qi et al., 2012; Qiu et al., 2008). Undoubtedly, accumulating studies have focused on the biological activities of ginsenosides and various polysaccharides and oligosaccharides among many active substances in ginseng (Fan et al., 2020; Guo et al., 2021; Wan et al., 2012). However, the present studies on ginseng monosaccharides and oligosaccharides are limited. The structures of the carbohydrates are often related to their important biological functions, while structural analysis of carbohydrates is difficult in the past because of the structural complexity, which may limit progress in understanding the structure-activity relationships of carbohydrates (Ji et al., 2011; Pabst and Altmann, 2011; Sastre Torano et al., 2019). The immunoregulatory effects, antitumor activity, and anti-neuroinflammatory activity, etc. of oligosaccharides have gained significant credence (Jiao et al., 2014; Wan et al., 2012; Xu et al., 2016). Therefore, there is considerable interest in detailed structural analysis of ginseng oligosaccharides to establish a structure-activity correlation.

Several analytical methods have been utilized to characterize carbohydrates, such as nuclear magnetic resonance spectroscopy, high-performance liquid chromatography, gas chromatography, and capillary electrophoresis (Agrawal, 1992; Currie and Perry, 2006; Islam et al., 2022; Mantovani et al., 2018; Robinson et al., 2012; Xing et al., 2017). Although these approaches have been used with great success, they suffer from some drawbacks and are often inadequate for many...
challenging problems. For example, high-performance liquid chromatography, gas chromatography, and capillary electrophoresis cannot provide structural information on carbohydrates. Nuclear magnetic resonance spectroscopy can be used to characterize glycosidic linkages, but it requires milligram amounts of materials and cannot detect small amounts of coexisting isomers (Duus et al., 2000). With the emergence of mass spectrometry (MS) technique which has advantages such as high sensitivity and high speed, it has been extensively utilized for structural investigation of carbohydrates (Reale et al., 2004; Wang et al., 2021). Nevertheless, with MS, stereoisomers are especially difficult to analyze, because of their identical atomic composition and mass (Li et al., 2020; Yang et al., 2015). The problem has recently been figured out by combining ion mobility (IM) with MS to separate isomers based on their size and shape (Hofmann et al., 2015; Lanucara et al., 2014). However, IM-MS has not been able to resolve closely related epimers because of their almost identical collision cross sections (CCSs) which are intrinsic properties of given compounds (Im et al., 2016). 1-Phenyl-3-methyl-5-pyrazolone (PMP) was suitable for neutral and acid sugar derivatization with fast, mild, and simple clean-up procedure (Honda et al., 1989). Sensitivity and resolution for detecting carbohydrates can be greatly improved by combination of IM-MS and PMP which contains multiple nitrogen atoms. For example, 3 monosaccharide isomers and 9 disaccharide isomers that differ in composition, linkages, or configuration have been distinguished by PMP labeling in conjunction with IM-MS in our previous report (Yang et al., 2016).

Black ginseng (BG), a new type of manufactured ginseng, is usually produced from white ginseng by nine repeated steaming-drying process, at which point it becomes black (Metwaly et al., 2019). Simultaneously, extensive change has been revealed in the types and amounts of secondary metabolites of BG in comparison with white ginseng, especially for ginsenosides (Metwaly et al., 2019). Up to now, more than 50 ginsenosides have been isolated and characterized from BG (Metwaly et al., 2019; Sun et al., 2009; Zhu et al., 2019), but the present studies of the secondary metabolites of BG in comparison with white ginseng, especially for ginsenosides (Metwaly et al., 2019). Up to now, more than 50 ginsenosides have been isolated and characterized from BG (Metwaly et al., 2019; Sun et al., 2009; Zhu et al., 2019), but the previous studies of carbohydrates from BG are limited.

In this work, we present a strategy combining PMP derivatization, IM-MS, and multivariate data analysis (MDVA) for the exploration and assignment of carbohydrates. The five newly discovered carbohydrates in ginseng were proposed from tandem MS (IM-MS, and multivariate data analysis (MDVA) for the exploration and amounts of coexisting isomers (Duus et al., 2000). With the emergence of tandem MS (IM-MS, and multivariate data analysis (MDVA) for the exploration and amounts of coexisting isomers (Duus et al., 2000). With the emergence of tandem MS (IM-MS, and multivariate data analysis (MDVA) for the exploration and amounts of coexisting isomers (Duus et al., 2000). With the emergence of tandem MS (IM-MS, and multivariate data analysis (MDVA) for the exploration and amounts of coexisting isomers (Duus et al., 2000). With the emergence of tandem MS (IM-MS, and multivariate data analysis (MDVA) for the exploration and amounts of coexisting isomers (Duus et al., 2000). With the emergence of tandem MS (IM-MS, and multivariate data analysis (MDVA) for the exploration and amounts of coexisting isomers (Duus et al., 2000). With the emergence of tandem MS (IM-MS, and multivariate data analysis (MDVA) for the exploration and amounts of coexisting isomers (Duus et al., 2000). With the emergence of tandem MS (IM-MS, and multivariate data analysis (MDVA) for the exploration and amounts of coexisting isomers (Duus et al., 2000).
110.6 Å²; melezitose, 133.5 Å²; maltotriose, 142.9 Å²; isomaltotriose, 142.3 Å²; raffinose, 138.8 Å²; α-cyclodextrin, 200.7 Å²; β-cyclodextrin, 231.4 Å²) (Fenn and McLean, 2011; Huang and Dodds, 2013; Yang et al., 2016). To separate the ions, IMMS data of calibrant ions and analytes were acquired over a range of wave heights at 35, 38, and 40 V and velocities at 500–2500, 550–2500, and 450–2500 m/s. A calibration curve was developed under each condition in order to ascertain the experimental CCSs.

2.5. Data processing and analysis

IM-MS data analysis was performed using MassLynx 4.1 and Drift-Scope 2.9 (Waters Corp., Milford, MA, USA). The [M + H]+, [M + Na]+, and [M + K]+ ions of PMP-derivatized oligosaccharides were extracted from the total ion chromatograms. The chromatographic peaks were integrated to sum the peak areas of PMP-derivatized oligosaccharides. Then, the resulting data set, containing information of oligosaccharides, peak area, and sample code, was generated as an excel file and imported into SIMCA software 14.1 (Umetrics, Umeå, Sweden) to conduct the MDVA. Statistical significance was determined by Student’s t-test using SPSS 21 (IBM Corporation, Chicago, USA).

Fig. 1. The arrival time distributions (ATDs) of protonated or sodiated PMP derivatives of (A) RD, (B) DG, (C) FG, (D) AG, (E) BCFG, (F) BNSDFG, and (G) BCAG. The insets show enlarged graphs at relative low intensities. The bottom graph in each figure demonstrate the relevant 3D map, where the level of variation between light and dark represents the intensity of an ion. The ATDs are from three individual measurements, and deviation is ± 0.01 ms.
3. Results and discussion

3.1. New carbohydrates were detected in BG samples

BCAG and BCFG were prepared by our unique processing method as mentioned above. In an effort to address the difference in carbohydrate components between BG acquired by our method (i.e., curing at low temperature) and other ginseng varieties, the seven ginseng samples involving DG, RG, FG, AG, BCAG, BNSDFG, and BCFG were analyzed simultaneously by PMP derivatization in conjunction with IM-MS. PMP-derivatized carbohydrates extracted from each ginseng sample were profiled by IM-MS, and the extracted ion chromatograms of the derivatized carbohydrate ions were illustrated in Fig. 1. With the goal of confirming some carbohydrates in the ginseng samples, the sixteen carbohydrate standard references (see their structures in Fig. S1) were derivatized by PMP, followed by IM-MS analysis (Fig. 2). Direct comparison of m/z and Iq values in Figs. 2 and 1 revealed the species of carbohydrates in the seven ginseng samples. Among them, there are five common carbohydrates, including fructose, glucose, maltose, maltotriose, and maltotetraose, which is consistent with the previous studies (Li et al., 2020; Zhu et al., 2019).

Note that special compounds were detected in some ginseng samples. Isomaltotetraose, for example, was only found in FG, as shown in Fig. 1C. One reasonable explanation is that FG preserves its original structure without any processing. Also, there exists an uncertain tetrasaccharide in RG, as shown in Fig. 1A, which may result from change in the types and amounts of carbohydrates, especially in BG samples (Metwaly et al., 2019).

The measured mobility of an ion may be used to calculate its collision cross section (CCS) from kinetic theory which is an intrinsic property of an ion and can provide additional information that aids structural assignment (Wei et al., 2020). The average CCSs for PMP-derivatized carbohydrates in the seven ginseng samples are shown by the ions at m/z 383.16 (peak I in Figs. 1) and 385.14 (peak II in Fig. 1) exist in these ginseng varieties except FG. Intriguingly, there are three unknown PMP-derivatized carbohydrate ions only discovered in the three BG samples, involving the sodiated derivatized ions at m/z 391.19, 421.20, and the protonated derivatized carbohydrate ion at m/z 420.21 (Fig. 1E–G). The steaming and drying processes may lead to some changes in the types and amounts of carbohydrates, especially in BG samples (Metwaly et al., 2019).

The overall mobility spectra of the standard references of (A) fructose, (B) glucose, (C) maltose, (D) maltotriose, (E) maltotetraose, (F) isomaltotetraose, (G) laminaribiose, (H) mannos, (I) gentiobiose, (G) cellobiose, (K) isomaltose, (L) lactose, (M) sophorose, (N) isomaltotriose, (O) mannobiose, and (P) mannose are shown by the ions at m/z 673.27, 857.30, and 997.37 and sodiated ions at m/z 695.25, 857.30, 1019.35, respectively. The ions at m/z 511.23 and 510.23 are assigned as protonated glucose and fructose, respectively. The ion at m/z 532.20 corresponds to sodiated glucose. The ATDs were from three individual measurements, and deviation is ± 0.01 ms.

Table 2. The CCS values of PMP-derivatized ions. M1-M6 represent the corresponding unknown carbohydrates.

| Ions                  | m/z     | Ω (Å²) |
|-----------------------|---------|--------|
| [2PMP–Fru + H]⁺       | 510.25  | 153.61 ± 0.27 |
| [2PMP–Glu + H]⁺       | 511.23  | 150.91 ± 0.57 |
| [2PMP–Glu + Na]⁺      | 533.20  | 153.57 ± 0.38 |
| [2PMP–Mal + H]⁺       | 673.27  | 180.10 ± 0.44 |
| [2PMP–Mal + Na]⁺      | 695.25  | 174.98 ± 0.32 |
| [2PMP–Mal + 3H]⁺      | 835.32  | 200.54 ± 0.20 |
| [2PMP–Mal + 3Na]⁺     | 857.32  | 195.32 ± 0.32 |
| [2PMP–Mal + 4H]⁺      | 997.37  | 212.82 ± 0.17 |
| [2PMP–Mal + 4Na]⁺     | 1019.35 | 229.16 ± 0.15 |
| [2PMP–ISOMal 4 + H]⁺  | 997.37  | 233.78 ± 0.39 |
| [2PMP–ISOMal 4 + Na]⁺ | 1019.35 | 216.65 ± 0.22 |
| [PMP–Mal + Na]⁺      | 383.16  | 126.82 ± 0.29 |
| [PMP–Mal + K]⁺       | 399.13  | 129.29 ± 0.20 |
| [PMP–Mal + 2Na]⁺     | 385.14  | 126.78 ± 0.16 |
| [PMP–Mal + 2K]⁺      | 401.11  | 126.73 ± 0.17 |
| [PMP–Mal + Na]⁺       | 391.19  | 129.36 ± 0.19 |
| [PMP–Mal + K]⁺     | 413.17  | 132.61 ± 0.18 |
| [PMP–Mal + 4H]⁺     | 420.21  | 134.41 ± 0.22 |
| [PMP–Mal + 4Na]⁺     | 442.20  | 137.62 ± 0.12 |
| [PMP–Mal + 4+ H]⁺    | 421.20  | 137.79 ± 0.22 |
| [PMP–Mal + 4+ Na]⁺   | 443.18  | 137.32 ± 0.17 |
| [2PMP–M6 + H]⁺      | 997.37  | 224.58 ± 0.24 |
| [2PMP–M6 + Na]⁺      | 1019.35 | 227.56 ± 0.22 |

* The values are expressed as the mean plus or minus the standard error, (n = 9).
carbohydrates as illustrated in Table 2 were calculated, which were in good agreement with the results from ATDs. It was clearly demonstrated that carbohydrate isomers with identical mass but different conformation could be distinguished based on the CCSs of their derivatives.

3.2. The newly discovered carbohydrates were identified by MS²

To identify the newly found compounds, the precursor ions at m/z 383.16, 385.14, 391.19, 420.21, and 421.20 were subjected to MS² analysis, as presented in Fig. 3. From the spectra in Fig. 3, it is obvious that the ion at m/z 175.09 corresponding to [PMP + H]⁺ is detected in each spectrum. Since PMP is a specific derivatization reagent for neutral and acid sugars (Honda et al., 1989), detection of the ion at m/z 175.09 in Fig. 3 indicates that these precursor ions are reducing carbohydrates. Their probable molecular formulas can be speculated according to the fragment ions and “nitrogen rule”.

As illustrated in Fig. 3A and B, the most prominent sodiated ions at m/z 235.09, arose from the loss of C₆H₄O₂ (148.07 Da) from the precursor ion at m/z 383.16 (Fig. 3A) and C₆H₂O₅ (150.05 Da) from the precursor ion at m/z 385.14 (Fig. 3B), respectively. The two protonated fragment ions at m/z 213.11 and 187.09 (Fig. 3A and B) had a mass difference of 26.02 Da, which could be used as a signature to identify the carbon-carbon double bond in MS/MS spectrum (Ma et al., 2016). In addition, the ion at m/z 368.14 was generated from precursor ion at m/z 383.16, corresponding to loss of CH₄. Therefore, the structures of two ions may consist of carbon-carbon double bond and methyl group. Accordingly, the probable molecular formulas and structural formulas of the ions at m/z 383.16 and 385.14 were speculated, as displayed in Fig. 3F and Supplementary Figs. 2A and B, respectively. Regarding the ion at m/z 391.19 in Fig. 3C and 421.20 in Fig. 3D, the loss of 27.99 Da from the fragment ion at m/z 280.12, indicating that there could be a carbonyl group in each structure of the two compounds. Taking this result and other fragments in Fig. 3C and D into consideration, it was speculated that they could potentially be glycoside esters (Rosenthal, 1968). Additionally, the mass difference between the two precursor ions at m/z 391.19 and 421.20 corresponds to CH₂O (30.01 Da), which manifests that the two ions may be pentatomic and hexatomic cyclic carbohydrates, respectively. Hence, the possible structural formulas were drawn in Figs. S2C and D, respectively.

In the case of the ion at m/z 420.21, the loss of H₂O (18.01 Da) and consecutive loss of NH₃ (17.03 Da) from the precursor ion were observed in Fig. 3E, demonstrating that it should be an amino group-containing carbohydrate. Additionally, the loss of CH₂O (30.01 Da) and consecutive loss of C₂H₄O₂ (42.01 Da) from the ion at m/z 247.12 produced [PMP + H]⁺ at m/z 175.09 (Fig. 3E), indicating that the amino group possibly existed at the C-4 position. Consequently, the possible structure was shown in Fig. S2E. These supposed monosaccharide derivatives may be generated by long steaming process of ginseng, thereby causing the changes of carbohydrate structures. To our knowledge, we are the first group to detect the five monosaccharides in ginseng. Carbohydrates containing alkene functional group, esters, or amino group have exhibited diverse biological activities including anti-cancer, anti-microbial, and anti-inflammatory properties (Campana et al., 2019; Chesnokov et al., 2014; Nagao et al., 2011; Snoch et al., 2021). So we expect highly potent activities can be found in these black ginseng varieties in the future research.

3.3. Different ginseng samples revealed distinct 3D graphs

3D plots in IM-MS using m/z ratios, drift time, and intensity as x, y, and z coordinates, respectively, avoid the congestion of the data on either dimension. Besides the m/z information and intensity generated by MS, the extra dimension of separation brought by IM increases the Euclidean distance between the peaks in the dataset and thereby facilitates the discrimination of isomers. In order to compare the differences of carbohydrates in seven ginseng samples more intuitively, a 3D representation of the data set was shown in Fig. 4. All the series were well separated according to their t₀ and m/z values.

![Fig. 3. (A–E) IM-MS/MS spectra of the ions at m/z 383.16, 385.14, 391.19, 420.21, and 420.21, respectively. The detailed information of product ions are shown in the right-hand panels. (F) The supposed molecular formula of the unknown carbohydrates. MW: molecular weight.](image-url)
From Fig. 4, it is clearly seen that maltose (represented as PMP-derivatized maltose at m/z 673.27) contents are significantly higher in RG, DG, AG, and FG than those in the three BG samples. Meanwhile, in contrast, the PMP-labelled carbohydrate ions at m/z 383.16, 385.14, 391.19, 420.21, and 421.20 identified above were in much higher abundance in three BG samples than those in RG, DG, AG, and FG, as illustrated in Fig. 4. Interestingly, a careful review of these 3D plots revealed that the discernible traces of maltotriose and maltotetraose at m/z 857.33 and 1019.39, respectively, were observed in RG, DG, AG, and FG rather than in the three BG samples. It is reasonable to assume that large-size carbohydrates have been converted into smaller ones due to the heating and steaming processes for the preparation of BG products.

3.4. Ginseng products were clustered based on carbohydrate content by MDVA

In the newly created 3D graphs, we found that different carbohydrates were regularly distributed among the seven ginseng varieties. This encouraging result raises the question of whether these ginseng samples can be classified into several categories, in which the ginseng variety can be differentiated by the amount of carbohydrates. MDVA was carried out to address this question.

SIMCA analysis was used to classify samples using models developed from the extracted chromatograms of RG, DG, AG, FG, BCAG, BCFG, and BNSDFG samples, where the training set containing information of m/z on carbohydrates, peak area, and sample code was generated, and a partial least squares-discriminate analysis (PLS-DA) model was constructed using the data from the seven ginseng samples.

Fig. 5A shows the SIMCA classification results that seven ginseng varieties are divided into two groups (for the purpose of this work, named group I and group II). Group I comprised RG, DG, AG, and FG. Group II contained BCAG, BCFG, and BNSDFG. The score plot of the samples was drawn at the 95% confidence level, and the clear separation among seven ginseng varieties displayed differences in the content of carbohydrates in different ginseng samples. As elucidated in Fig. 5B, the variable importance for the projection (VIP) values of carbohydrates summarizes the importance of the x-variables for the model as a whole and molecular features with VIP values larger than 1 point to variables with large importance for the group separation. It was obvious that maltose, the ions at m/z 385.14, 391.19, 420.20, and 420.21, as well as maltotetraose, had higher VIP values than other components (Fig. 5B), which should be the most influential predictors of the model.

Furthermore, the carbohydrates with VIP values larger than 1 were also tested using Student’s t-test to evaluate whether potential markers between the two groups differed statistically (Fig. 5C). It clearly demonstrated that there was significant difference between the two groups for the six components. As illustrated in Fig. 5C, the amounts of maltose and maltotetraose in RG, DG, AG, and FG were higher than those in the three BG samples. Inversely, the peak areas of the ions at m/z 385.14, 391.19, 420.20, and 420.21 were much higher in BG products than those of the other 3 samples. The quantification results in Fig. 5C are in good agreement with the relevant abundances shown in Figs. 1 and 4. The observed phenomena may be caused by long heating and steaming processes for preparation of black ginseng, and involved in the hydrolysis reaction, resulting in large amounts of monosaccharides and a small proportion of oligosaccharides (Metywal et al., 2019).

4. Conclusion

Taken together, IM-MS² combined with PMP labeling and MDVA was first developed for carbohydrate determination in the seven ginseng samples. This strategy enabled measurement of 11 carbohydrates, among which 5 monosaccharides were discovered for the first time in ginseng. Three of them were only detected in BG samples, speculated as monosaccharides bearing alkene functional group, esters, and amino group, respectively, by MS² analysis. The probable reason is that the chemical reactions may have occurred during the repeatedly heating-steaming process for preparation of BG samples. Characteristic 3D profiles of carbohydrates in the seven ginseng products facilitate the discrimination of different kinds of ginseng. In addition, the PLS-DA score plot results showed a clear separation between the four common ginseng samples and the three BG varieties on the basis of their carbohydrate composition. Our study benefits for future efficacy comparison of different kinds of ginseng, and will shed light on the profiling of carbohydrates in foods and beverages, especially on the discovery of new active components.

CRediT authorship contribution statement

Simeng Shao: Investigation, Methodology, Writing – original draft. Weiyin Xu: Investigation, Validation, Data statistic. Zhaoyang Xie: Revision. Mengyuan Li: Revision. Jingli Zhao: Data statistic. Xinxin Yang: Investigation. Peng Yu: Supervision, Writing – review & editing. Hongmei Yang: Methodology, Supervision, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.
Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.crbf.2022.11.007.

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