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Original Article

Comprehensive quality evaluation and comparison of *Angelica sinensis* radix and *Angelica acutiloba* radix by integrated metabolomics and glycomics

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ABSTRACT

Angelica radix (*Danggui* in Chinese) used in China and Japan is derived from two species of *Angelica*, namely *Angelica sinensis* and *Angelica acutiloba*, respectively. The differences in quality between *A. sinensis* radix (ASR) and *A. acutiloba* radix (AAR) should be therefore investigated to guide the medicinal and dietary applications of these two species. Secondary metabolites and carbohydrates have been demonstrated to be the two major kinds of bioactive components of *Danggui*. However, previously, quality comparison between ASR and AAR intensively concerned secondary metabolites but largely overlooked carbohydrates, thus failing to include or take into consideration an important aspect of the holistic quality of *Danggui*. In this study, untargeted/targeted metabolomics and glycomics were integrated by multiple chromatography-based analytical techniques for qualitative and quantitative characterization of secondary metabolites and carbohydrates in *Danggui* so as to comprehensively evaluate and compare the quality of ASR and AAR. The results revealed that not only secondary metabolites but also carbohydrates in ASR and AAR were different in type and amount, which should collectively contribute to their quality difference. By providing more comprehensive chemical information, the research results highlighted the need to assess characteristics of both carbohydrates and secondary metabolites for overall quality evaluation and comparison of ASR and AAR.

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1. Introduction

Angelica radix (*Danggui* in Chinese) is one of the most common traditional herbal medicines used in Asian countries, especially in China and Japan. According to classical document records, 70 herbal formulae in China and 56 herbal formulae in Japan contain *Danggui*. *Danggui* has been traditionally prescribed in the treatment of gynecological diseases due to its ability to "replenish and invigorate the blood", in traditional Chinese medical terms [1]. Besides its medicinal usage, *Danggui* is also used by women worldwide as a health food supplement. *Danggui* used in China and Japan is derived from two species of the genus *Angelica*, namely *Angelica sinensis* and *Angelica acutiloba*, respectively. If and how *A. sinensis* radix (ASR) and *A. acutiloba* radix (AAR) differ in quality is a topic of intense interest. The answers would be helpful to guide their medicinal and dietary applications, such as whether they can be used alternatively or they should be employed for treating different diseases. To achieve this, systematic chemical comparison between ASR and AAR is essential.

Thus far, many studies have been performed comparing the chemical components of ASR and AAR in order to characterize their quality differences [2–4]. However, these studies focused on secondary metabolites and largely overlooked carbohydrates. It is well-recognized that secondary metabolites and carbohydrates are the two major kinds of bioactive chemicals in medicinal/dietary herbs, and *Danggui* is no exception [5–9]. Accumulated phytochemical and biological experiments have demonstrated that, in addition to such secondary metabolites as phthalides and organic acids [10,11], *Danggui* also contains abundant carbohydrates (poly-/oligo-/mono-saccharides) with multifaceted bioactivities including anti-oxidant, anti-tumor, and immune-regulatory effects [12–14]. Thus, in order to characterize and compare the holistic quality of ASR and ARR, carbohydrates should be adequately taken into account for overall chemical profiling.

Metabolomics aims to collectively and dynamically characterize a set of small biomolecules (metabolome) in an organism, and is being widely employed for the "overall" chemical characterization of herbal medicines [15,16]. Hyphenated liquid chromatography and mass spectrometry (LC-MS)-based metabolomics is a particularly powerful method to provide global profiles of complex (up to hundreds of) secondary metabolites by determining their presence, amount and sometimes their structures [17,18]. In contrast to secondary metabolites, the chemical characterization of carbohydrates is a challenge due to their different chemical properties, for example, macromolecular mass and intricate multidimensional structures of polysaccharides [19]. The recently-coined glycomics seeks to explore the qualitative and quantitative information of a certain glycome (the entire carbohydrate components), for which the combined deployment of various analytical approaches to determine multiple chemical parameters of different carbohydrates is essential [20,21]. For example, chemical modifications such as hydrolysis and/or derivative formation are always needed prior to chromatographic or mass spectrometric analysis of carbohydrates to decompose their advanced structures and thereby improve their analytical adaptability. Similar with metabolomics, glycomics is a promising approach for delineating carbohydrate components in herbal medicines from a holistic perspective.

In this study, metabolomics and glycomics were integrated to comprehensively characterize the chemical components of ASR and AAR so as to holistically compare the quality of the two *Angelica* cultivars. The experimental procedure was designed as follows. First, batches of ASR and AAR samples were collected. Since ASR is mainly grown in China but AAR is grown in Japan, Taiwan and China, ASR samples were collected only from China while AAR samples were collected from the three regions to additionally investigate the effects of cultivation regions on AAR quality. Then, untargeted/targeted metabolomics approaches by ultra-performance liquid chromatography-quadrupole time-of-flight mass spectrometry (UPLC-QTOF-MS/MS) and ultra-performance liquid chromatography-triple quadrupole mass spectrometry (UPLC-TQ-MS/MS) were developed to qualitatively and quantitatively determine secondary metabolites in the samples. Meanwhile, targeted glycomics that combined analytical techniques including UPLC-TQ-MS/MS, high performance gel permeation chromatography coupled with evaporative light scattering detector (HPGPC-ELSD) and high performance liquid chromatography coupled with evaporative light scattering detector (HPLC-ELSD) were applied to characterize polysaccharides, oligosaccharides and monosaccharides in the samples. Finally, the obtained data were integrated and processed by multivariable statistical analysis for holistic quality comparison of all ASR and AAR samples.

2. Materials and methods

2.1. Chemicals and materials

MS-grade acetonitrile, formic acid, ammonium acetate and methanol were purchased from Merck (Darmstadt, Germany). Trifluoroacetic acid (TFA) used for acid hydrolysis of polysaccharides was from Riedel-de Haën (Seelze, Germany). 1-Phenyl-3-methyl-5-pyrazolone (PMP) for monosaccharide and oligosaccharide derivatization was bought from Sigma (St. Louis, USA). Ultra-pure water was produced by a Milli-Q water purification system (Millipore, Bedford, USA).

The reference substances of dextrans with known molecular sizes (1–670 kDa), together with D-galacturonic acid monohydrate (GalA), D-glucuronic acid (GlcA), L-arabinose (Ara), D-mannose (Man), D-galactose (Gal), D-glucose (Glc), L-rhamnose monohydrate (Rha), D-fucose (Fuc), D-fructose (Fru), maltose (Mal), maltotriose (Tri), maltopentaose (Pen), maltohexaose (Hex), maltoheptaose (Hep), cellobiose (Cel), melibiose (Meli) and sucrose (Suc) were purchased from Sigma. The reference substances of coniferyl ferulate (CF), senkyunolide H (SH) and senkyunolide I (SI) were provided by Phytomarker Ltd (Tianjin, China); ferulic acid (FA), senkyunolide A (SA), Z-ligustilide (Z-lig), butylphthalide (BP) and levistolide A (LA) were obtained from Chengdu Must Bio-Technology Co., Ltd (Chengdu, China); Z-butylidenephthalide (BDP) was bought from Sigma. The purity of these references was higher than 95.0% as indicated by HPLC analysis.

Details of the collected ASR and AAR samples are summarized in Supplementary Table 1. All the *Danggui* samples were authenticated by Prof. H.-B. Chen according to the monographs on *Danggui* documented in China Pharmacopoeia (2015 Version) and Japanese Pharmacopoeia (2016 Version). The voucher specimens were deposited at the School of Chinese Medicine, Hong Kong Baptist University, Hong Kong.

2.2. Sample preparation for secondary metabolites analysis

Dried powder of sample (0.1 g) was ultrasonic-extracted with 5 mL 70% methanol at room temperature for 30 min. The extracted solutions were then centrifuged at 4500 rpm for 10 min. The supernatant was centrifuged again (14000 rpm for 10 min) for UPLC-TQ-MS/MS and UPLC-QTOF-MS/MS analysis, respectively.

2.3. Sample preparation for polysaccharides analysis

Dried powder of sample (0.25 g) was extracted with water at 100 °C (5 mL × 1 h × 2 times). The extracts were centrifuged at 4500 rpm for 10 min, and the supernatants were then collected and combined. A portion of the supernatant (2.5 mL) was precipitated by adding ethanol to make a final concentration of 95% (v/v), and left overnight (12 h) under 4 °C. After centrifugation (4500 rpm) for 10 min, the precipitate was washed with ethanol three times and dried in a water bath at 60 °C to remove residual ethanol. The dried extract was then re-dissolved in 1 mL hot water (60 °C) for further analysis. For the determination of molecular weight distribution, the polysaccharide solution was directly injected into HPGPC-ELSD; for the analysis of monosaccharide composition, the solution was subjected to acid hydrolysis and then PMP derivatization.

2.4. Sample pretreatment for analysis of monosaccharides and oligosaccharides

The supernatant of ethanol precipitation prepared as described above was collected and evaporated at 55 °C on a rotary evaporator until dry. Then 2.5 mL water was added to dissolve the residue. The solution was centrifuged at 14000 rpm for 10 min, and the supernatant was employed for further analysis. For non-reducing monosaccharide and oligosaccharide analysis, the obtained supernatant was analyzed by HPLC-ELSD; for reducing monosaccharide and oligosaccharide determination, PMP derivatization was performed prior to UPLC-TQ-MS/MS analysis.

2.5. Acid hydrolysis of polysaccharides

Acid hydrolysis of polysaccharides was performed as described in our previous research [22]. Specifically, 0.5 mL crude polysaccharide solution was mixed with 2.5 mL of 2 M TFA solution, and then hydrolyzed for 2 h at 120 °C. After cooling, the hydrolysate was evaporated at 55 °C on a rotary evaporator until dry. Then 1.0 mL water was added to dissolve the hydrolysate, and the precipitate was removed after centrifugation (14000 rpm, 5 min). Finally, the supernatant was subjected to PMP derivatization.

2.6. PMP derivatization of reducing monosaccharides and oligosaccharides

0.1 mL acid hydrolysate was mixed with 0.1 mL NH_3 solution and 0.2 mL 0.5 M PMP methanolic solution. The mixture was allowed to react at 70 °C for 30 min and then was cooled to room temperature. Afterwards, 0.1 mL glacial acetic acid and 0.5 mL chloroform were successively added to neutralize the reaction solution and remove the excess PMP reagents, respectively. After vigorous shaking followed by centrifugation at 14000 rpm for 5 min, the organic phase was discarded. The operation was performed five times, and finally the aqueous layer was diluted and centrifuged at 14000 rpm for 10 min before UPLC-TQ-MS/MS analysis. The standard solution was also treated in this way.

2.7. Secondary metabolites analysis

2.7.1. UPLC-TQ-MS/MS analysis

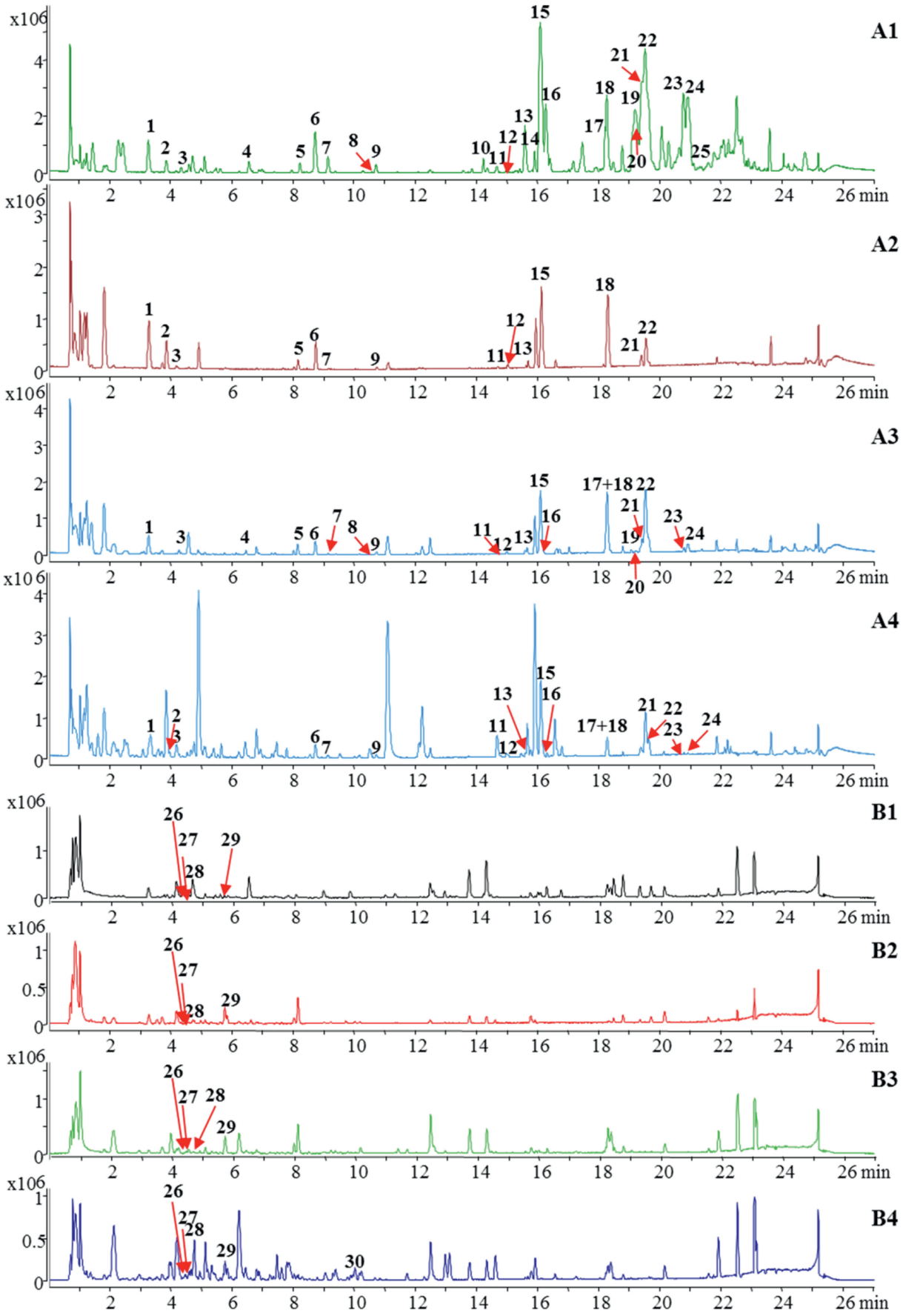
UPLC was performed on an Agilent system (Agilent Technologies, Palo Alto, USA) equipped with a binary solvent delivery system and auto-sampler. The chromatographic separation was performed with a Waters ACQUITY HSS C18 (2.1 mm × 100 mm, i.d. 1.8 μm). The mobile phase consisted of (A) water (0.1% formic acid) and (B) acetonitrile (0.1% formic acid). The elution condition was optimized as follows: 20–35% B (0–2 min), 35–75% B (2–12 min), 75–100% B (13–15 min). The flow rate was 0.35 mL/min. The column was maintained at 35 °C. The injection volume was 2 μL.

Mass spectrometry was performed on an Agilent 6460 TQ/MS system equipped with electrospray ionization (ESI) source. The conditions of the ESI source were as follows: drying gas (N_2) flow rate, 10.0 L/min; drying gas temperature, 300 °C; nebulizer pressure, 45 psi; capillary voltage, 3500 V. The analysis was performed using MRM mode, and the mass range was set at 100–1700 Da in the positive mode. Agilent Mass Hunter Quantitative Analysis Software B.04.00 was used to collect and process mass data.

2.7.2. UPLC-QTOF-MS/MS analysis

UPLC was performed on an Agilent system equipped with a binary solvent delivery system and auto-sampler. The chromatographic separation was performed with a Waters ACQUITY HSS C18 (2.1 mm × 100 mm, i.d. 1.8 μm). The mobile phase consisted of (A) water (0.1% formic acid) and (B) acetonitrile (0.1% formic acid). The elution condition was optimized as follows: 5% B (0–1 min), 5%–33% B (1–10 min), 33–75% B (10–20 min), 75–100% B (20–22 min). The flow rate was 0.35 mL/min. The column was maintained at 40 °C. The injection volume was 2 μL.

Mass spectrometry was performed on an Agilent 6540 QTOF/MS system equipped with electrospray ionization (ESI) source. The conditions of the ESI source were as follows: drying gas (N_2) flow rate, 8.0 L/min; drying gas temperature, 300 °C; nebulizer pressure, 40 psi; capillary voltage, 3500 V; fragmentor voltage, 150 V. The analysis was performed using full scan mode, and the mass range was set at 100–1700 Da in both negative and positive modes. The secondary collision


A1
A2
A3
A4
B1
B2
B3
B4
x10 6
26 min

energy was set between 25 and 45 V. Agilent MassHunter Workstation Software Version B.06.00 process was used to collect and process mass data.

2.8. Carbohydrates analysis

2.8.1. UPLC-TQ-MS/MS for analysis of reducing monosaccharides and oligosaccharides

UPLC was performed on an Agilent 1200 system equipped with a binary solvent delivery system and auto-sampler. The chromatographic separation was achieved with a Waters ACQUITY HSS C18 (2.1 mm × 100 mm, i.d. 1.8 μm). The mobile phase consisted of (A) 20 mM ammonium acetate in water and (B) acetonitrile. The elution condition was optimized as follows: 19% B (0–9 min), 19%–30% B (9–14 min). The flow rate was 0.3 mL/min. The column was maintained at 35 °C. The injection volume was 2 μL.

Mass spectrometry was performed on an Agilent 6460 TQ/MS system equipped with electrospray ionization (ESI) source. The conditions of the ESI source were as follows: drying gas (N_2) flow rate, 10.0 L/min; drying gas temperature, 300 °C; nebulizer pressure, 45 psi; capillary voltage, 3500 V. The analysis was performed using MRM mode, and the mass range was set at 100–1700 Da in the positive mode. Agilent Mass Hunter Quantitative Analysis Software B.04.00 was used to collect and process mass data.

2.8.2. HPLC-ELSD for analysis of non-reducing monosaccharides and oligosaccharides

The analysis was performed on an Agilent 1100 series HPLC-DAD system coupled with evaporative light scattering detector (ELSD). The separation was achieved on a Shodex Asahipak NH_2P-50 4E column (250 mm × 4.6 mm, i.d. 5 μm) system operated at 40 °C. The mobile phase consisted of (A) water and (B) acetonitrile. The elution condition was optimized as follows: 65% B (0–7 min), 65%–62% B (7–9 min), 62%–60% B (9–15 min). The flow rate was 1.0 mL/min. The signal from ELSD was transmitted to Agilent Chemstation for processing through an Agilent 35900E interface. The parameters of ELSD were set as follows: drift tube temperature, 110 °C; nebulizer nitrogen gas flow rate, 3.2 L/min; impact mode, off. An aliquot of 20 μL solution was injected for analysis.

2.8.3. HPGPC-ELSD for polysaccharide analysis

The analysis was performed on an Agilent 1100 series HPLC-DAD system coupled with evaporative light scattering detector (ELSD). The separation was achieved on a two tandem TSK $GMPW_{XL}$ column (300 mm × 7.8 mm, i.d. 10 μm) system operated at 40 °C. 20 mM Ammonium acetate aqueous solution was used as mobile phase at a flow rate of 0.6 mL/min. The signal from ELSD was transmitted to Agilent Chemstation for processing through an Agilent 35900E interface. The parameters of ELSD were set as follows: drift tube temperature, 120 °C; nebulizer nitrogen gas flow rate, 3.2 L/min; impact mode, off. An aliquot of 20 μL solution was injected for analysis.

Aqueous stock solutions of dextrans with known molecular weights (1 kDa, 5 kDa, 12 kDa, 25 kDa, 50 kDa, 80 kDa, 150 kDa, 270 kDa, 410 kDa, 670 kDa) were injected into the HPGPC using the same conditions for the construction of the molecular weight-retention time calibration curve by plotting the logarithm of the molecular weight versus the retention time of each analyte.

2.9. Method validation

The quantitative method was validated in terms of linearity, sensitivity, precision, accuracy and stability.

Working solutions of the mixed reference compounds were diluted to appropriate concentrations for the construction of calibration curves. Six different concentrations of each compound were analyzed in triplicate, and then the calibration curves were constructed by plotting the peak areas versus the concentrations of analytes. The limits of detection (LODs) and limits of quantification (LOQs) under the present conditions were determined at an S/N (signal to noise) of about 3 and 10, respectively.

Intra- and inter-day variations were chosen to assess the precision of the developed assay. For intra-day variability test, the ASR sample was extracted and analyzed in six replicates within one day. For inter-day variability tests, the same sample was analyzed in duplicate for three consecutive days. Variations were expressed by the RSDs of the data.

The spike recovery test was conducted to evaluate the accuracy of the method. The ASR sample with known contents of the target analytes were weighed and different amounts (high, middle and low level) of reference were spiked, then extracted and analyzed in triplicate. The spike recoveries were calculated from the following equation: spike recovery (%) = (total amount detected-amount original) × 100%/amount spiked.

The stability test was performed by analyzing the ASR sample extract over periods of 0 h, 2 h, 4 h, 6 h, 8 h, 10 h, 12 h and 24 h. The RSDs of the peak areas of each analyte were taken as the measures of stability.

2.10. Data analysis

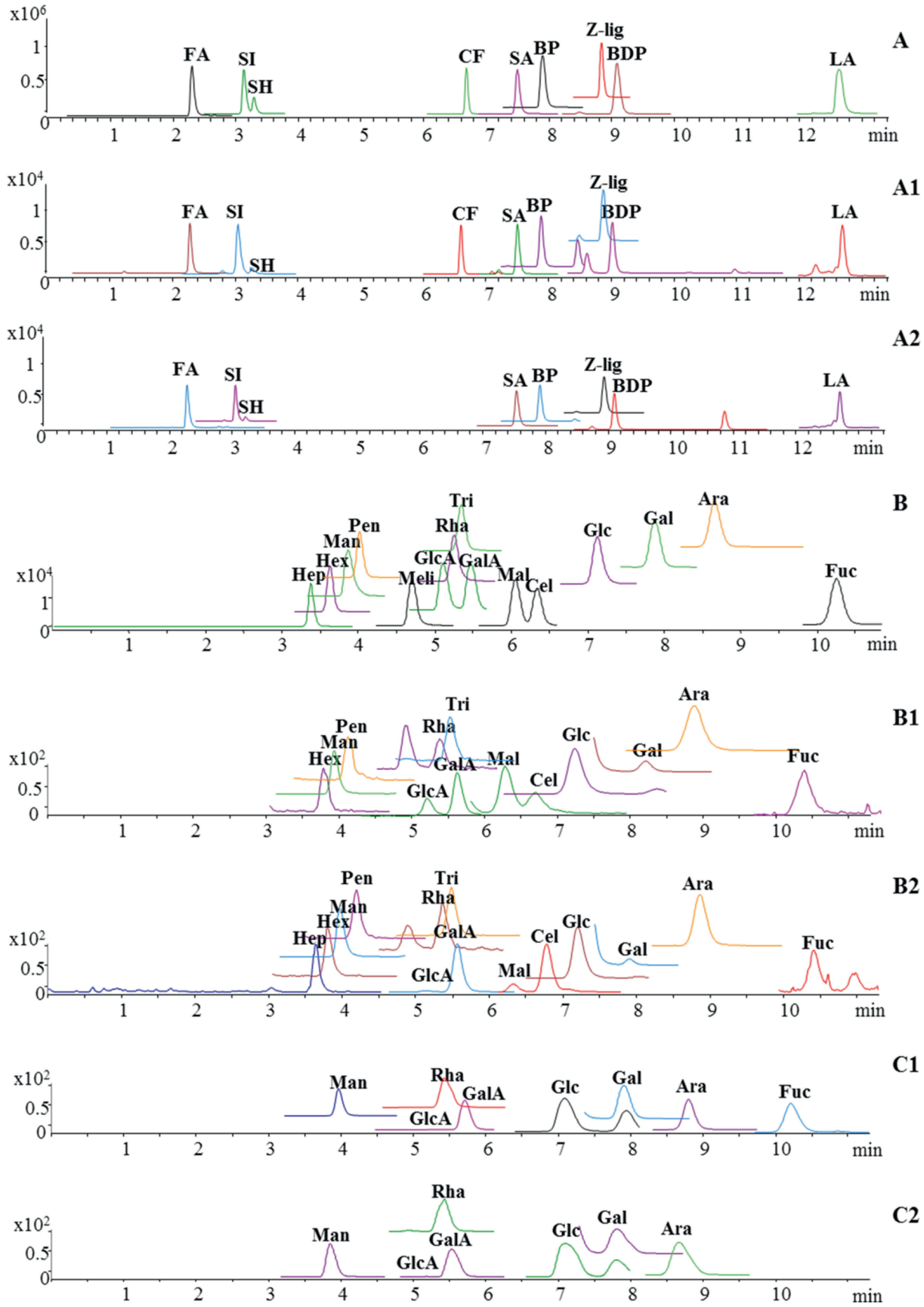
The obtained qualitative and quantitative data of secondary metabolites and carbohydrates were respectively processed by principle component analysis (PCA) using Agilent Mass Profiler Professional 2.2 and SIMCA 13.0 (Umetrics, Sweden). Differences between two groups were assessed with unpaired Student's *t*-test by Prism software (GraphPad, USA). Data were presented as mean ± SD of the determinations. Significant difference is indicated in the figures by * ($p < 0.05$).

3. Results and discussion

3.1. Optimization of sample preparation and analysis

The extraction conditions for different analytes in the *Danggui* samples were optimized. Two repeated extractions under the current conditions followed by quantitative analysis indicated

Fig. 1 – Typical UPLC-QTOF-MS/MS chromatograms of *Danggui* samples detected in positive (A1-A4) and negative mode (B1-B4). A1, B1: ASR; A2, B2: CAAR; A3, B3: JAAR; A4, B4: TAAR.


A
x10^6
FA
SI
SH
CF
SA
BP
Z-lig
BDP
LA
min
A1
x10^4
A2
B
Tri
Pen
Rha
Man
Hex
Hep
GlcA
GalA
Meli
Mal
Cel
Glc
Gal
Ara
Fuc
B1
x10^2
B2
C1
C2

that more than 95% of secondary metabolites in the ASR and AAR materials were extracted by one-time extraction. The carbohydrates in the samples could be completely extracted at 100 °C after two extractions (1 h each time) because no saccharide was detected by sulfuric acid-phenol method in the subsequent third extraction. The acid hydrolysis of polysaccharides and the PMP derivatization of acid hydrolysates and/or reducing carbohydrates were accordingly performed, in which the conditions for acid hydrolysis were selected as follows: 2 M TFA, 120 °C and 2 h based on our previous study on ASR [22].

Several types of tandem mass spectrometry coupled with liquid chromatography are being currently employed for chemical analysis of medicinal herbs [23]. While UPLC-QTOF-MS/MS provides high mass resolution, accurate mass measurement as well as abundant fragment ions information and is therefore adept at qualitative elucidation [24,25], UPLC-TQ-MS/MS is competent for quantitative determination due to its excellent sensitivity [26]. Hence, in this study, UPLC-QTOF-MS/MS-based untargeted metabolomics and UPLC-TQ-MS/MS-based targeted metabolomics were developed for comprehensive qualitative and quantitative characterization of secondary metabolites in the *Danggui* samples. In the UPLC-QTOF-MS/MS analysis, both positive and negative ion modes were employed for qualitative identification (Fig. 1). In the UPLC-TQ-MS/MS analysis, multiple reaction monitoring (MRM) mode was adopted by selectively screening one ion pair for each analyte for improving the quantitative performance (Fig. 2A). Positive ion mode was selected based on the responses of the analyte ions. Then, collision energy was individually optimized for each analyte (Table 1). Under the optimized conditions, all analytes presented abundant parent ions with protonated molecular ions $[M + H]^+$ except for adduct ion $[2M + Na]^+$ for CF. Their abundant daughter ions were characteristic, namely $[M + H\text{-}H_2O]^+$ for FA, SI and SH, $[M + H\text{-}HCOOH]^+$ for BP, $[M + H\text{-}C_{12}H_{14}O_2]^+$ for LA, $[M\text{-}C_{10}H_{10}O_4{+}Na]^+$ for CF, $[M\text{-}C_7H_6O_2{+}Na]^+$ for Z-lig, $[M\text{-}C_7H_8O_2{+}Na]^+$ for SA and $[M + H\text{-}C_2H_5O_2]^+$ for BDP (Table 1). The potential fragmentation pathways to generate the daughter ions were showed in Fig. 3. These parent and daughter ions were used for the quantification of nine secondary metabolites in the *Danggui* samples by UPLC-TQ-MS/MS under the MRM mode.

The qualitative and quantitative analysis of carbohydrates in the *Danggui* samples was performed using the glycomics approach we previously established [27]. In brief, the molecular weight distribution of the polysaccharides was characterized using HPGPC-ELSD (Fig. 4A). The monosaccharide compositions of the polysaccharides together with the free reducing oligosaccharides and monosaccharides were quantified by pre-column derivatization and then UPLC-TQ-MS/MS under MRM mode. For the latter, protonated molecular ions $[M{+}H]^+$ and PMP protonated molecular ions $[PMP + H]^+$ were selected as the ion pair for each analyte (Fig. 2B and C). Non-reducing oligosaccharides and monosaccharides were directly determined by HPLC-ELSD (Fig. 4B).

3.2. Method validation

The linearity, sensitivity, precision, accuracy and stability data of the UPLC-TQ-MS/MS quantitative assay of secondary metabolites were summarized in Table 1. The coefficients of determination (R^2) were all above 0.9936, indicating good linearity within the linear ranges. The LODs and LOQs were, respectively, 0.11 ng/mL and 0.38 ng/mL. The RSDs of intra- and inter-day precisions were lower than 8.55% and 10.76%, respectively. The developed method also provided acceptable accuracy with a spike recovery ranging from 95.25% to 109.44%. In the stability test, the RSD within 24 h was less than 9.41%. The results indicated that the developed UPLC-TQ-MS/MS method was linear, sensitive, precise, accurate and stable enough for simultaneous quantification of nine chemicals in the *Danggui* samples. The targeted glycomics assay has been previously validated [27].

3.3. Secondary metabolites in ASR and AAR

The collected ASR and AAR samples were first characterized by UPLC-QTOF-MS/MS-based untargeted metabolomics (Fig. 1). Totally 30 chromatographic peaks were identified by chemical standards or tentatively identified by comparing their *quasi*-molecular ions, empirical molecular formulas, and/or fragment ions with those of known chemicals [5,28,29]. These chemicals were from different types including phthalides, organic acids, amino acids, glycosides, esters and coumarin (Fig. 1 and Table 2), and their mass fragmentation pathways were structurally characteristic. For example, the loss of H_2O (*e.g.* SI), CO (*e.g.* BP), C_4H_8 (*e.g.* SA) or 1/2M (*e.g.* LA) were mainly involved in the fragmentation of phthalides; organic acids easily produced fragments of $[M + H\text{-}H_2O]^+$ (*e.g.* FA) or $[MCO_2]^-$ (*e.g.* phthalic acid); the major fragment pathways of amino acids, coumarin, esters and glycosides respectively corresponded to the loss of NH_3 (*e.g.* tryptophan), the cleavage of CH_3 (*e.g.* schinicoumarin), hydrolysis (*e.g.* chlorogenic acid), deglycosylation (*e.g.* 4-glucopyranosyloxy-ferulic acid). As shown in Fig. 1, the chromatograms clearly indicated that the chemical profiles in the collected samples were qualitatively different. We then constructed a Venn diagram, which presents all possible relations between a finite collection of different sets by depicting elements as points in some closed curves [30], to better understand the differences (Fig. 5A). According to the diagram, compounds 1–29 occurred in all ASR samples, in which compounds 10 (CF), 14 (BDP isomer), and 25 (ligustilide dimer) were found exclusively in ASR samples and never in AAR samples. As to the AAR samples from Japan, Taiwan and China, their secondary metabolites profiles varied. The majority of the chemicals in most of the AAR samples were the same, while compounds 4 (FA), 8

Fig. 2 – Typical UPLC-TQ-MS/MS chromatograms of 9 secondary metabolites (A), 15 reducing carbohydrates (B), and monosaccharide compositions of polysaccharides (C). A, B: mixed references; A1, B1: ASR; A2, B2: JAAR; C1: monosaccharide compositions of ASR polysaccharides; C2: monosaccharide compositions of JAAR polysaccharides.

Table 1 – MRM conditions in UPLC-TQ-MS/MS analysis and method validation for quantitative determination of secondary metabolites in *Danggui* samples.

Analyte	MRM	Collision Voltage (eV)	Calibration curve			Sensitivity (ng/mL)		(RSD, %)						Stability (n = 8)
								Precision (n = 6)		Spike Recovery (n = 3)				
			Range (μg/mL)	Equation	R^2	LODs	LOQs	Intra-day	Inter-day	High	Middle	Low		
FA	195.0 → 177.1	9	0.21–3.30	y = 75.75x+1815.81	0.9995	0.97	3.22	3.17	9.65	106.04 (4.52)	102.56 (5.70)	106.51 (5.25)		3.99
SI	225.1 → 207.1	3	0.21–3.35	y = 1008.31x+13554.63	0.9993	0.47	1.56	4.38	8.87	100.69 (3.07)	99.03 (1.23)	99.29 (3.58)		7.63
SH	225.1 → 207.1	3	0.22–7.19	y = 287.19x+445.53	0.9997	0.73	2.42	3.04	10.76	101.43 (2.74)	102.55 (1.84)	101.17 (3.77)		7.56
CF	735.3 → 185.1	25	0.21–6.75	y = 6.51x-3014.92	0.9933	7.59	25.30	6.23	8.51	105.95 (7.48)	102.01 (4.31)	95.25 (7.03)		7.34
SA	193.1 → 91.1	27	0.23–7.38	y = 105.19x+1209.97	0.9998	1.48	4.93	1.98	9.46	102.28 (5.20)	103.54 (1.24)	102.24 (1.81)		8.41
BP	191.1 → 145.1	11	0.21–3.41	y = 789.88x-246.29	0.9999	0.73	2.43	2.28	9.26	102.42 (5.51)	100.81 (5.16)	102.14 (1.54)		8.78
Z-lig	191.1 → 91.1	24	0.94–30.00	y = 2.12x+453.37	0.9998	0.65	2.17	3.07	9.15	103.85 (8.62)	99.57 (0.73)	102.78 (3.31)		9.41
BDP	189.1 → 128.1	27	0.21–1.71	y = 1943.71x+2031.93	0.9995	0.23	0.75	1.51	7.91	98.59 (0.78)	98.90 (1.57)	96.81 (2.59)		8.68
LA	381.2 → 191.1	11	0.05–1.73	y = 553.16x+5521.60	0.9925	0.11	0.38	8.55	8.31	109.44 (5.97)	100.70 (1.87)	96.89 (5.30)		8.98

(senkyunolide B or C), 19 (ligustilide dimer) and 20 (ligustilide dimer) in JAAR, compound 2 (4-glucopyranosyloxy-ferulic acid) in CAAR, and compound 30 (schinicoumarin or isomer) in TAAR were detected and were characteristic of their respective samples. Different bioactivities of these chemicals have been demonstrated. For example, CF (in ASR) possesses antineoplastic activity [31], and schinicoumarin (in AAR) is reported to inhibit platelet aggregation [32]. Therefore, these differences in secondary metabolites suggested that both the two *Danggui* cultivars and the AAR samples from different regions differed in chemical quality and clinical efficacy.

The entire set of untargeted metabolomics data was further processed by PCA. The score plots of PCA illustrated that all *Danggui* samples clustered into four groups: i.e. ASR, CAAR, JAAR and TAAR (Fig. 5B). Moreover, the AAR samples were found to be gathered close to one another, especially CAAR and JAAR, but far away from ASR. Based on the dispersion degree of score plots in each group, it was deduced that the secondary metabolites in TAAR were more varied than those in other groups. The PCA results stated that both cultivars and cultivation regions could significantly contribute to the differences in secondary metabolites of *Danggui*, and that the cultivars should be more decisive.

In order to further explore the differences at the quantitative level, nine typical bioactive secondary metabolites in the *Danggui* samples belonging to the categories of organic acid, ester and phthalide were then investigated for quantitative determination by the UPLC-TQ-MS/MS-based targeted metabolomics method. As shown in Fig. 6A and Supplementary Table 2, (a) the total contents of the nine secondary metabolites in ASR (67.19 mg/g) were significantly higher than those in AAR ($p < 0.05$), and (b) the contents of these metabolites in AAR samples were relatively consistent with regard to cultivation region, namely 8.56 mg/g in CAAR, 9.43 mg/g in JAAR, 7.24 mg/g in TAAR. Currently, some of these chemicals, such as FA, BDP and Z-lig, are commonly used as the chemical markers for the quality assessment of *Danggui* due to their favorable bioactivities [3,33,34]. Here we have demonstrated that they occurred more abundantly in ASR than AAR, for example, FA (0.89 mg/g in ASR and 0.02 mg/g in JAAR), BDP (0.03 mg/g in ASR and 0.01 mg/g in CAAR) and Z-lig (65.09 mg/g in ASR and 6.84 mg/g in TAAR) ($p < 0.05$). Thus, on the side of secondary metabolites, the qualitative and quantitative characterization by untargeted/targeted metabolomics suggested the quality of ASR should be better than AAR.

3.4. *Carbohydrates in ASR and AAR*

Then, the glycomes in the *Danggui* samples were qualitatively and quantitatively analyzed by targeted glycomics. First, the molecular weight distributions of polysaccharides were calculated by HPGPC-ELSD using the established molecular weight-retention time calibration curve ($y = -0.316x+13.58$, $R^2 = 0.994$), and the monosaccharide compositions of polysaccharides were also determined by UPLC-TQ-MS/MS (Figs. 2C and 6B). The HPGPC chromatograms showed that ASR polysaccharides possessed narrower molecular weight ranges (1–514 kDa) than AAR polysaccharides (1-more than 670 kDa), among which CAAR polysaccharides exhibited the widest

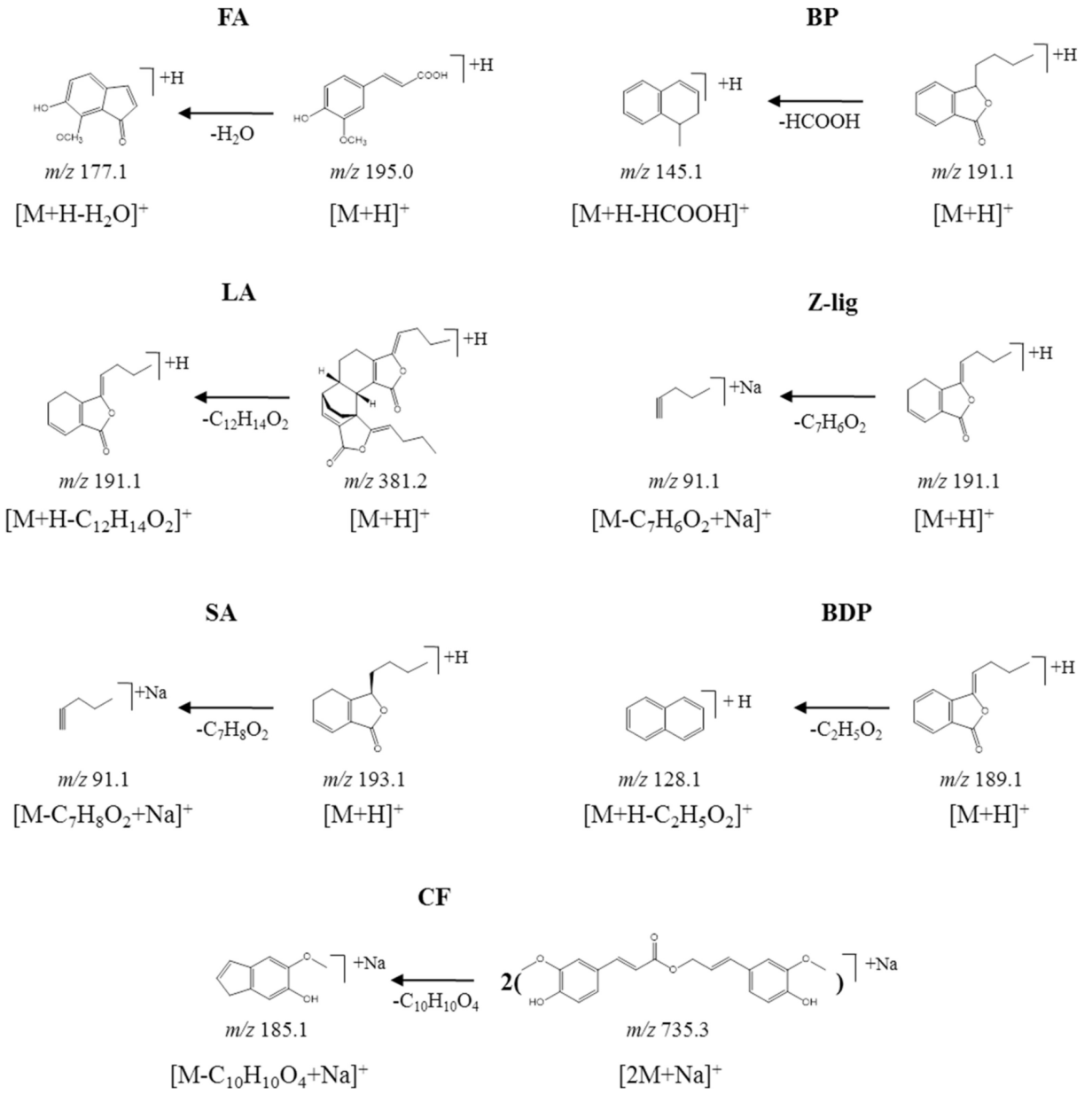


Fig. 3 – Selected ion pair for quantification of representative secondary metabolites references monitored with MRM mode.

molecular weight distribution (Fig. 4A). The monosaccharide composition analysis indicated that the polysaccharides in all *Danggui* samples shared seven monosaccharides, *i.e.* Glc, Gal, Ara, GlcA, GalA, Man and Rha, while ASR and TAAR polysaccharides additionally comprised Fuc. Besides, the mole ratios of monosaccharides in the four kinds of *Danggui* samples were also different, although Glc (45.78% in ASR, 65.15% in CAAR, 67.88% in JAAR, 68.15% in TAAR) was detected as the major compositional monosaccharide in all samples. Calculated by the total amounts of compositional monosaccharides, the polysaccharide contents in AAR (24.17 mg/g in CAAR, 18.79 mg/g in JAAR, 39.31 mg/g in TAAR) were higher than those in ASR (17.65 mg/g). Moreover, unlike the quantified secondary metabolites, the polysaccharide contents in the three batches of AAR samples differed greatly.

Then, the free oligosaccharides and monosaccharides were quantitatively characterized by UPLC-TQ-MS/MS and HPLC-ELSD (Figs. 2B and 4B). Totally seven oligosaccharides were detected in the ASR and AAR samples, namely Hep, Hex, Pen, Tri, Mal, Cel and Suc. Suc is among the most abundant in all the samples (176.20 mg/g in ASR, 138.78 mg/g in CAAR, 130.00 mg/g in JAAR, 53.83 mg/g in TAAR), which was consistent with a previous report that AAR cultivated in China contains a high amount of Suc [35]. The total contents of seven oligosaccharides in the *Danggui* samples could be ranked, in descending order, as follows: 176.32 mg/g in ASR, 144.33 mg/g

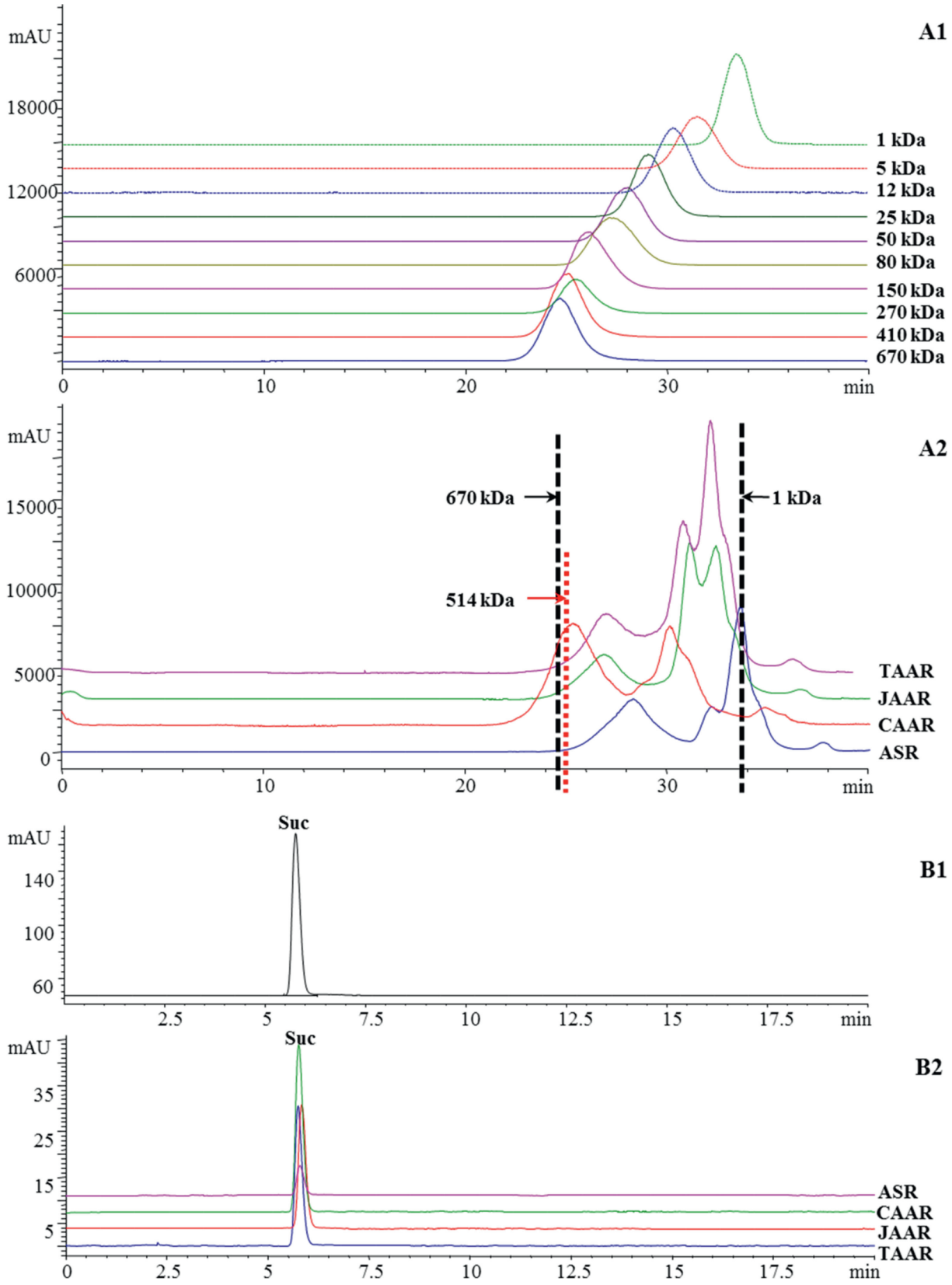


Fig. 4 – Typical chromatograms of HPGPC-ELSD (A) and HPLC-ELSD (B). A1, B1: sugar references; A2, B2: *Danggui* samples.

Table 2 – Secondary metabolites identified in the *Danggui* samples by untargeted metabolomics.

No.	t_R (min)	Identity	Molecular formula	Mean measured mass (Da)	Theoretical exact Mass (Da)	Mass accuracy (ppm)	Related fragment and/or adduct ions (mass accuracy, ppm)	Classification
1	3.31	Tryptophan	$C_{11}H_{12}N_2O_2$	205.0972	205.0977	−2.4	188.0706 $[M + H\text{-}NH_3]^+$ (−3.2)	Amino acid
2	3.90	4-glucopyranosyloxy-ferulic acid	$C_{16}H_{20}O_9$	357.1178	357.1185	−2.0	379.0996 $[M + Na]^+$ (−2.4) 177.0552 $[M + H\text{-}C_6H_{12}O_6]^+$ (0.0)	Glycoside
3	4.20	Chlorogenic acid	$C_{16}H_{18}O_9$	355.1026	355.1029	−0.8	377.0845 $[M + Na]^+$ (−1.1) 163.0390 $[M + H\text{-}C_7H_{12}O_6]^+$ (−3.1)	Ester
4	6.58	FA	$C_{10}H_{10}O_4$	195.0655	195.0657	−1.0	177.0549 $[M + H\text{-}H_2O]^+$ (−1.7) 149.0598 $[M + H\text{-}HCOOH]^+$ (−3.4)	Organic acid
5	8.23	(E)-Butylidene-4,5,6,7-tetrahydro-6,7-dihydroxyphthalide	$C_{12}H_{16}O_4$	225.1125	225.1127	−0.9	247.0943 $[M + Na]^+$ (−1.2) 207.1018 $[M + H\text{-}H_2O]^+$ (−1.4)	Phthalide
6	8.73	SI	$C_{12}H_{16}O_4$	225.1124	225.1127	−1.3	247.0940 $[M + Na]^+$ (−2.4) 207.1015 $[M + H\text{-}H_2O]^+$ (−2.9)	Phthalide
7	9.18	SH	$C_{12}H_{16}O_4$	225.1121	225.1127	−2.7	247.0943 $[M + Na]^+$ (−1.2) 207.1018 $[M + H\text{-}H_2O]^+$ (−1.4)	Phthalide
8	10.61	Senkyunolide B or C	$C_{12}H_{12}O_3$	205.0864	205.0865	−0.5	227.0676 $[M + Na]^+$ (−3.5) 187.0750 $[M + H\text{-}H_2O]^+$ (−4.8)	Phthalide
9	10.73	4-hydroxy-3-butylphthalide	$C_{12}H_{14}O_3$	207.1012	207.1021	−4.3	229.0836 $[M + Na]^+$ (−2.2) 189.0913 $[M + H\text{-}H_2O]^+$ (−1.6) 161.0962 $[M + H\text{-}H_2O\text{-}CO]^+$ (−2.5)	Phthalide
10	14.27	CF	$C_{20}H_{20}O_6$	357.1320	357.1338	−5.0	735.2412 $[2M + Na]^+$ (−0.4) 379.1150 $[M+Na]^+$ (−2.1)	Ester
11	14.69	SA	$C_{12}H_{16}O_2$	193.1224	193.1229	−2.6	215.1044 $[M + Na]^+$ (−1.9) 175.1121 $[M + H\text{-}H_2O]^+$ (−1.1) 137.0597 $[M + H\text{-}C_4H_8]^+$ (−4.4)	Phthalide
12	15.03	BP	$C_{12}H_{14}O_2$	191.1065	191.1072	−3.7	213.0887 $[M + Na]^+$ (−1.9) 173.0964 $[M + H\text{-}H_2O]^+$ (−1.2) 145.1011 $[M + H\text{-}H_2O\text{-}CO]^+$ (−4.1)	Phthalide
13	15.61	E-ligustilide	$C_{12}H_{14}O_2$	191.1068	191.1072	−2.1	213.0888 $[M + Na]^+$ (−1.4) 173.0959 $[M + H\text{-}H_2O]^+$ (−4.0) 145.1015 $[M + H\text{-}H_2O\text{-}CO]^+$ (−1.4)	Phthalide
14	15.82	BDP isomer	$C_{12}H_{12}O_2$	189.0909	189.0916	−3.7	211.0730 $[M + Na]^+$ (−2.4) 171.0806 $[M + H\text{-}H_2O]^+$ (−2.3)	Phthalide
15	16.12	Z-lig	$C_{12}H_{14}O_2$	191.1077	191.1072	2.6	213.0889 $[M + Na]^+$ (−0.9) 173.0965 $[M + H\text{-}H_2O]^+$ (−0.6) 145.1011 $[M + H\text{-}H_2O\text{-}CO]^+$ (−4.1)	Phthalide
16	16.26	BDP	$C_{12}H_{12}O_2$	189.0908	189.09101	−4.2	211.0725 $[M + Na]^+$ (−4.7) 171.0802 $[M + H\text{-}H_2O]^+$ (−4.7)	Phthalide
17	18.25	Ligustilide dimer	$C_{24}H_{28}O_4$	381.2066	381.2066	0.0	403.1889 $[M + Na]^+$ (1.0) 191.1066 $[M + H\text{-}C_{12}H_{14}O_2]^+$ (−3.1)	Phthalide
18	18.30	Ansaspirolide	$C_{24}H_{26}O_4$	379.1890	379.1909	−5.0	401.1731 $[M + Na]^+$ (0.5) 189.0916 $[M + H\text{-}C_{12}H_{14}O_2]^+$ (0.0)	Phthalide
19	19.18	Ligustilide dimer	$C_{24}H_{26}O_4$	381.2066	381.2066	0.0	403.1889 $[M + Na]^+$ (1.0) 363.1964 $[M + H\text{-}H_2O]^+$ (1.1) 191.1072 $[M + H\text{-}C_{12}H_{14}O_2]^+$ (0.0)	Phthalide
20	19.27	Ligustilide dimer	$C_{24}H_{26}O_4$	381.2061	381.2066	−1.3	403.1892 $[M + Na]^+$ (1.7) 191.1071 $[M + H\text{-}C_{12}H_{14}O_2]^+$ (−0.5)	Phthalide

21	19.54	Riligustilide	$C_{24}H_{26}O_4$	381.2076	381.2066	2.6	403.1904 $[M + Na]^+$ (4.7) 363.1959 $[M + H\text{-}H_2O]^+$ (−0.3) 191.1071 $[M + H\text{-}C_{12}H_{14}O_2]^+$ (−0.5)	Phthalide
22	19.65	LA	$C_{24}H_{26}O_4$	381.2072	381.2066	1.6	403.1893 $[M + Na]^+$ (2.0) 363.1959 $[M + H\text{-}H_2O]^+$ (−0.3) 191.1069 $[M + H\text{-}C_{12}H_{14}O_2]^+$ (−1.6)	Phthalide
23	20.79	Ligustilide dimer	$C_{24}H_{26}O_4$	381.2061	381.2066	−1.3	403.1892 $[M + Na]^+$ (1.7) 191.1071 $[M + H\text{-}C_{12}H_{14}O_2]^+$ (−0.5)	Phthalide
24	20.94	Ligustilide dimer	$C_{24}H_{26}O_4$	381.2066	381.2066	0.0	403.1895 $[M + Na]^+$ (2.5) 191.1068 $[M + H\text{-}C_{12}H_{14}O_2]^+$ (−2.1)	Phthalide
25	21.28	Ligustilide dimer	$C_{24}H_{26}O_4$	381.2067	381.2066	0.3	403.1886 $[M + Na]^+$ (0.2) 191.1071 $[M + H\text{-}C_{12}H_{14}O_2]^+$ (−0.5)	Phthalide
26	4.51	Phthalic acid	$C_8H_6O_4$	165.0196	165.0188	4.8	121.0295 $[M\text{-}H\text{-}CO_2]^-$ (4.1)	Organic acid
27	4.59	Caffeic acid	$C_9H_8O_4$	179.0353	179.0344	5.0	215.0116 $[M+Cl]^-$ (2.3)	Organic acid
28	4.71	Vanillic acid	$C_8H_8O_4$	167.0352	167.0344	4.8	135.0452 $[M\text{-}H\text{-}CO_2]^-$ (4.4) 123.0451 $[M\text{-}H\text{-}CO_2]^-$ (4.1)	Organic acid
29	5.77	3-O-feruloylquic acid	$C_{17}H_{20}O_9$	367.1052	367.1029	3.5	403.0815 $[M + Cl]^-$ (4.7)	Organic acid
30	10.01	Schinicoumarin or isomer	$C_{12}H_{12}O_5$	235.0617	235.0606	4.7	220.0375 $[M\text{-}H\text{-}CH_3]^-$ (1.4)	Coumarin

in CAAR, 130.32 mg/g in JAAR and 54.40 mg/g in TAAR) ($p < 0.05$) (Fig. 6C). As to monosaccharides (Fig. 6D), Glc was of the highest contents in the *Danggui* samples, varying from 24.17 mg/g in CAAR to 1.68 mg/g in ASR ($p < 0.05$), and this contributed to the much greater amounts of total monosaccharides in AAR (25.75 mg/g in CAAR, 4.98 mg/g in JAAR, 10.52 mg/g in TAAR) than in ASR (1.91 mg/g).

A Venn diagram for the glycome was also constructed to visualize the characteristic carbohydrate components in different *Danggui* samples (Fig. 5A). Due to the different qualitative and quantitative properties, polysaccharides were selected as the characteristic carbohydrate in each kind of *Danggui* sample. In addition, Hep was detected in CAAR, JAAR and TAAR but not ASR, suggesting that Hep could be used as a chemical maker to discriminate between AAR and ASR. Furthermore, the AAR samples from different regions also had characteristic carbohydrates. For example, Cel, Gal and Fuc that occurred in JAAR and TAAR were not found in CAAR. The entire set of glycome data was then processed by PCA. The score plot of carbohydrates also showed four clusters, corresponding to ASR, CAAR, JAAR and TAAR (Fig. 5C). Notably, the ASR and AAR clusters were very close, and CAAR exhibited the most dispersed score plots among the four clusters. The PCA pattern is different from that of metabolomics. It could be therefore concluded that, although the cultivar type and cultivation region significantly contributed to the differences in both secondary metabolites and carbohydrates of *Danggui*, such impacts were different for the two kinds of chemicals.

The integrated metabolomics and glycomics demonstrated that not only secondary metabolites but also carbohydrates in ASR and AAR were qualitatively and quantitatively different. The results indicated that both cultivar and cultivation region could affect secondary metabolome and glycome in *Danggui*. More interestingly, the effects presented chemical specificity. For example, ASR contained higher contents of secondary metabolites and oligosaccharides than AAR, but AAR had more polysaccharides and monosaccharides; as to AAR samples, CAAR possessed higher amounts of oligosaccharides and monosaccharides, while TAAR had more polysaccharides. In addition, the results also highlight the importance of simultaneous characterization of secondary metabolites and carbohydrates for comprehensive chemical and biological comparison of ASR and AAR. For example, as mentioned above, the more kinds and higher contents of secondary metabolites supported that the quality of ASR should be better than AAR. However, the conclusion might be one-sided or even false if the holistic chemical profiling were considered. In recent years, metabolomics has been often employed to characterize and compare the quality of herbal medicines in several cases including cultivars, cultivation regions, growth years and post-harvest processing [36–39]. However, these studies have focused extensively on secondary metabolites but have largely overlooked carbohydrates, from which the obtained conclusion may be therefore not convincing. The results presented in this study aim to draw scientists' attention to the issue that carbohydrates should be valued for comprehensive quality control of herbal medicines.

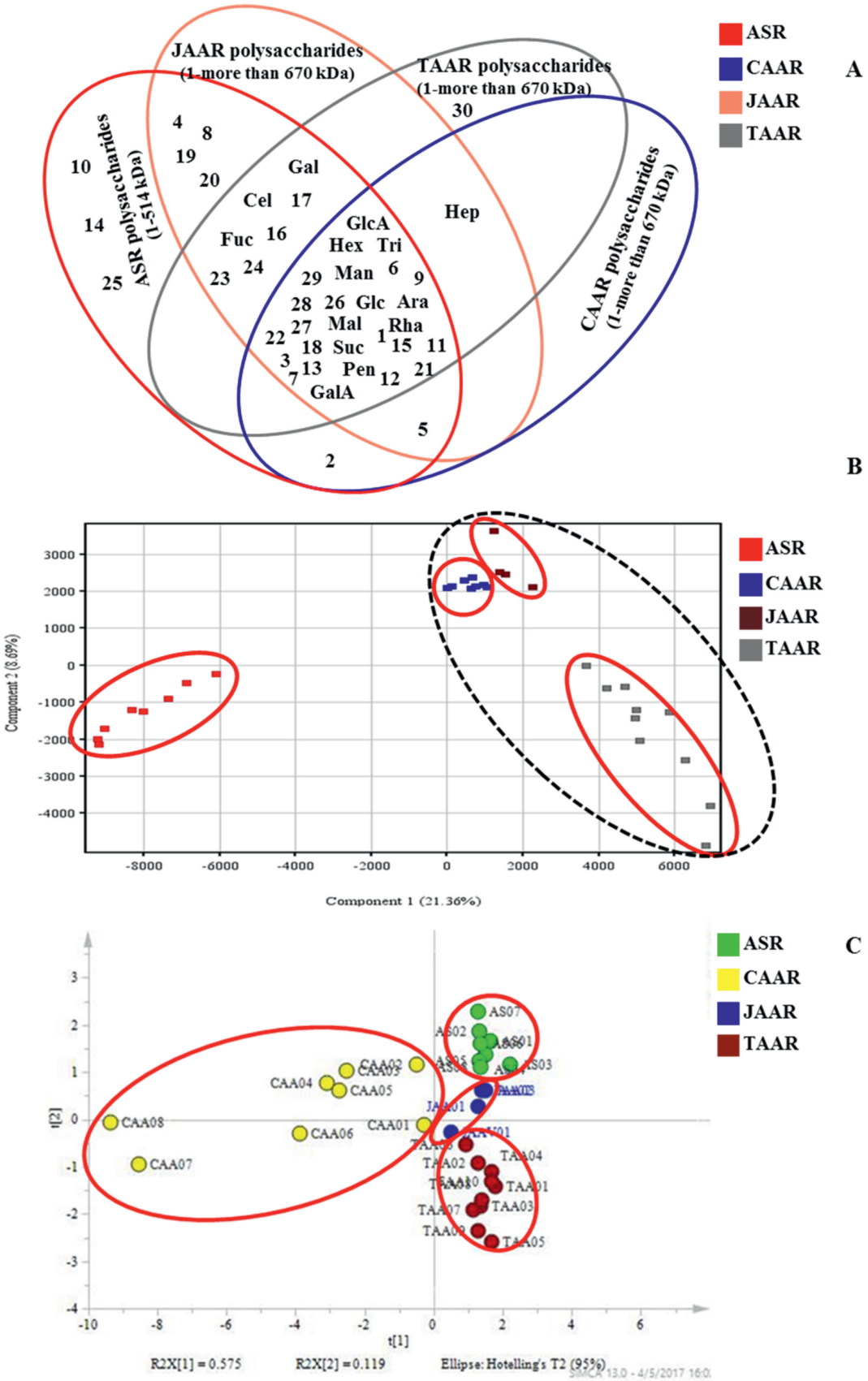


Fig. 5 – Venn diagram based secondary metabolites and carbohydrates (A), PCA score plots based secondary metabolites (B) and carbohydrate contents (C) for qualitative and quantitative chemical comparison between ASR and AAR. A: "polysaccharides" in different locations respectively represented the total polysaccharides in ASR, CAAR, JAAR and TAAR with specific molecular weight distribution and monosaccharide composition.

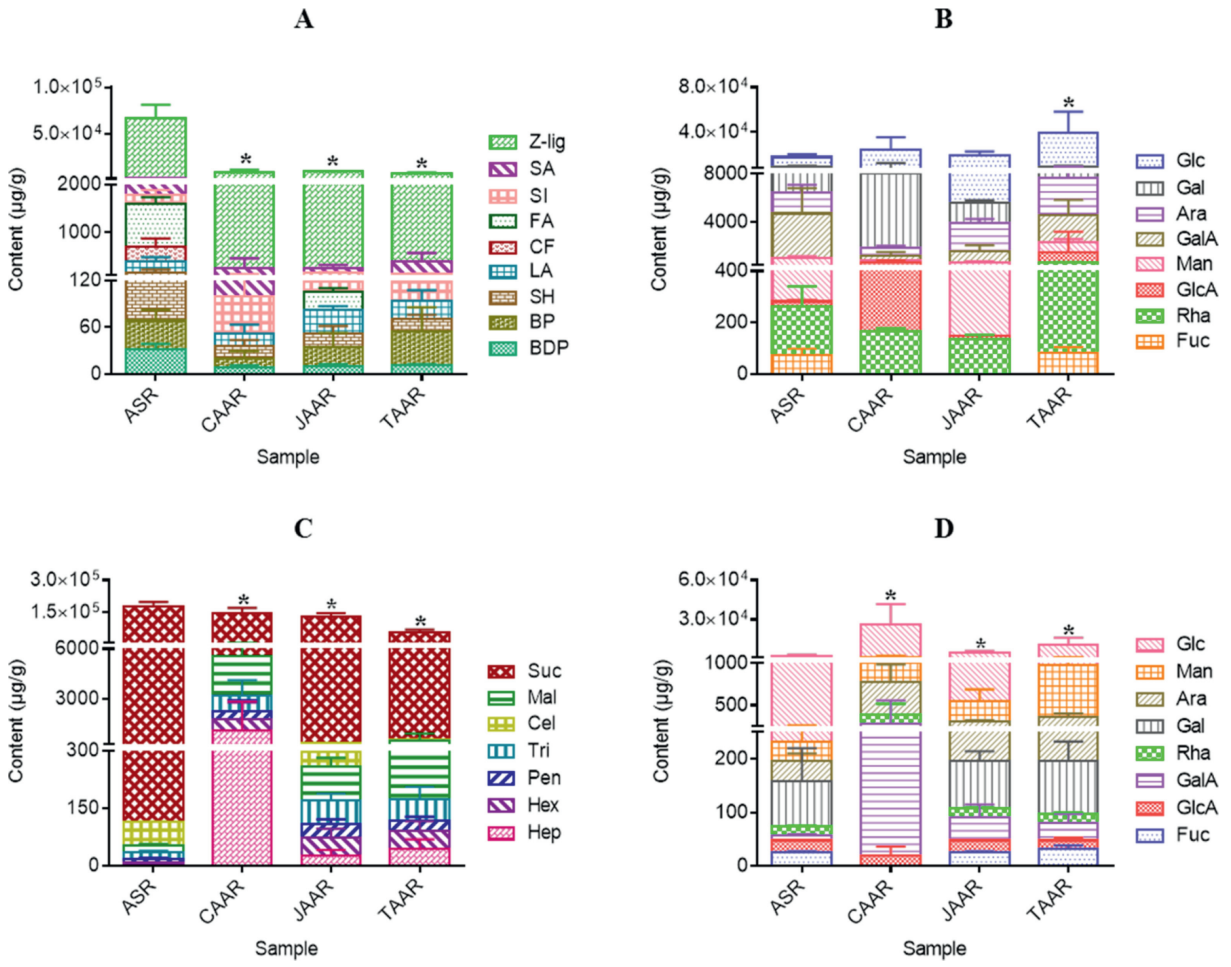


Fig. 6 – Contents differences between ASR and AAR. (*: $p < 0.05$, compared with the ASR sample, evaluated by unpaired Student's t-test). A: secondary metabolites; B: polysaccharides; C: oligosaccharides; D: monosaccharides.

4. Conclusion

In this study, untargeted/targeted metabolomics and glycomics were integrated by multiple chromatography-based analytical techniques for qualitative and quantitative characterization of secondary metabolites and carbohydrates in *Danggui* so as to comprehensively evaluate and compare the quality of ASR and AAR. The results revealed that ASR and AAR possessed different secondary metabolites and carbohydrates at both qualitative and quantitative levels. The chemical profiles of AAR from different cultivation regions (China, Taiwan, Japan) differed. By providing more comprehensive chemical information, the research deliverable highlighted that the carbohydrates should be investigated as thoroughly as secondary metabolites for overall quality evaluation and comparison of ASR and AAR.

Conflicts of interest

All authors declare no competing financial interest.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.jfda.2018.01.015.

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