Optimization of SPME-GC-MS and Characterization of Floral Scents from *Aquilegia Japonica* and *A. Amurensis* Flowers

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Abstract

The floral scent of plants plays a key role in plant reproduction through the communication between plants and pollinators. *Aquilegia* as a model species for studying evolution, however, there have been few studies on the floral scents and relationships between floral scents and pollination for *Aquilegia* taxa. In this study, three types of solid-phase micro-extraction (SPME) fiber coatings (DVB/PDMS, CAR/PDMS, DVB/CAR/PDMS) were evaluated for their performance in extracting volatile organic compounds (VOCs) from flowers of *Aquilegia amurensis*, which can to contribute to the future studies of elucidating the role of floral scents in the pollination process. In total, 55 VOCs were identified, and among them, 50, 47 and 45 VOCs were extracted by the DVB/CAR/PDMS fiber, CAR/PDMS fiber and DVB/PDMS fibers, respectively. Only 30 VOCs were detected in *A. japonica* taxa. Furthermore, the relative contents of 8 VOCs were significant different (VIP > 1 and $p < 0.05$) between the *A. amurensis* and *A. japonica*. Therefore, the results can be applied in new studies of the relationships between the chemical composition of floral scents and the processes of attraction of pollinator. It may provide new ideas for rapid evolution and frequent interspecific hybridization of *Aquilegia*

Introduction

Volatile organic compounds (VOCs), emitted by plant organs such as leaves, flowers and fruits, has serve multiple biological functions, including defense against pathogens, parasites, and herbivores [1]. In particular, floral aromas are important in the reproductive processes of many plants by attracting pollinators. Traits with a large effect on pollinator preference could play an important role in the evolution of plant reproductive isolation and speciation [2-4]. In addition, it has been reported that diversification of the North American clade of *Aquilegia* (Columbines) was associated mainly with the difference in pollinators [5]. Researchers have studied the relationships between floral morphologies and pollinators. For example, the changes in nectar spur length and flower orientation are highly correlated with the shifts of pollinators from bee to hummingbird, and from hummingbird to hawkmoth [6]. Moreover, most attempts to classify interactions between insects and flowers have focused on floral odors [7]. For instance, *Mimulus lewisii* with three monoterpene volatiles can attract bumblebee pollinators, but due to the lack of three terpenoids in its sister species *M. cardinalis*, the pollinator is not bumblebee, so the reproductive isolation between the two sister species can be maintained [4]. Similarly, a single volatile compound (indole) present in flowers of *Ipomosis.tenuituba* but not its sister species *I. aggregata*, which can attract hawkmoths to flowers [8]. This information says little, however, about the relationships between floral scents and pollination, evolution, and phylogeny of *Aquilegia* taxa. Until now, approximately 1700 chemical compounds identified in floral scent have been isolated from more than 90 plant families [9]. Among these compounds, the monoterpenes limonene, (E)-β-ocimene, myrcene, linalool, α- and β-pinene, and the benzenoids benzaldehyde, methyl 2-hydroxybenzoate (methyl salicylate), benzyl alcohol, and 2-phenyl ethanol are most common [10].

In our study, using headspace solid-phase micro extraction coupled with gas chromatography–mass spectrometry (SPME-GC–MS), which is common method in the detection of VOCs, the floral scent characteristics of *Aquilegia japonica* and *A. amurensis* were evaluated. *A. japonica* individuals are distributed in Northeast of China, North Korea, South Korea and Japan, while *A. amurensis* is restricted to the northern Greater Khingan Mountains of China, Siberia and Mongolia. *A.japonica* and *A. amurensis* are sister species, both of two species with different distribution areas are difficult to identify in nature because of their highly similar shape morphology traits. Therefore, Floral of China holds that both of two species are one species [11]. However, the analysis based on genome showed that the differentiation of the two species was obvious (unpublished). Thus, research focusing on the distribution and combinations of floral scent compounds at species and subspecies levels may be of the utmost importance for understanding the molecules responsible for attracting pollinators and promoting adaptations and evolutionary processes in angiosperms.

In the analysis of the VOCs, the SPME technique is characterized by its simplicity, speed and sensitivity. It is a convenient sample preparation technique that can be followed by thermal desorption directly in an analytical instrument [12, 13]. Recently, several types of SPME fiber coatings have become available for the extraction of analytes, such as nonpolar polydimethylsiloxane (PDMS) fibers, carboxen-polydimethylsiloxane (CAR-PDMS) fibers, polydimethylsiloxane/divinylbenzene (PDMS/DVB) fibers and divinylbenzene/ carboxen/polydimethyl siloxane (DVB/CAR/PDMS) fibers. Furthermore, due to the different compounds that make up the floral scents of different plant taxa, researchers use different types of fiber to study them, for example, Fan et al. (2018) used PDMS/DVB fibers for *Malus* plants [14], Gao et al. (2018) used CAR-PDMS fibers for *Freesia* x *hybrid* [15] and Mohammed et al. (2019) used DVB/CAR/PDMS fibers for *Rose* [16]. Silva et al. (2018) found that PDMS fiber in melon flowers has poor adsorption for polar compounds [17]. In addition, previous studies have observed polar molecules such as protoanemonin, nonanal, dimethoxytoluene, 2-phenyl ethanol and phenyl acetaldehyde in *Aquilegia*'s floral scents [18]. Therefore, in the present study, SPME fibers coated with PDMS/DVB (65 μm), CAR/PDMS (75 μm) and DVB/CAR/PDMS (μm) were used to identify fibers suitable for measuring the floral scents in *Aquilegia*. Consequently, our study has not only assessed the performance of different fibers in extracting the VOCs of *Aquilegia* flowers, but also evaluated the main differences in compounds among the two taxa and provided fundamental information for the scent traits of *Aquilegia*.

Experimental

Plant Material

The *A. japonica* and *A. amurensis* were cultivated in a garden from 2017 at Changchun, Jilin, China. Fully expanded flowers of the same size, approximately 0.6 g, were collected at around between 9 a.m. and 10 a.m. (). After sampling, the flowers were cut and sealed into 20 mL solid-phase micro extraction (SPME) vials (Agilent Technologies, Germany) immediately for further analysis. Additionally, six samples of *A. japonica* flowers at the full-flowering stage were collected to discriminate different scent intensities of *Aquilegia* taxa. In addition, an admixture of a certain number of accurately weighted n-alkanes (C7-C30) diluted with hexane (w = 5%) was used as a standard.

Gas chromatography-mass spectrometry experiments

To select an efficient type of fiber coating to extract volatile compounds from the flowers, SPME fibers with coatings of three different polarities were used: 65 μm DVB/PDMS (divinylbenzene/ polydimethylsiloxane), 75 μm CAR/PDMS (carboxen/polydimethylsiloxane) and 50/30 μm DVB/CAR/PDMS (divinylbenzene/carboxen/polydimethylsiloxane) (Supelco, Bellefonte, PA, USA). Prior to the analyses, fibers were conditioned for 1 h according to the temperature recommended by the manufacturer. After 10 min equilibration between the flower and the headspace, the SPME fiber was exposed to the headspace of the capped vial to absorb volatile compounds of each sample under heating at 60 °C for 30 min and for 10 min at room temperature. After extraction, the fiber was removed from the flask and immediately inserted into the gas chromatograph injector (GC–MS) for 3 min for thermal desorption at 240 °C. Three replicates were tested for each fiber and taxon.

The flower samples were analyzed and identified using a GC-MS Agilent 7890b gas chromatograph coupled with a 5977b mass spectrometer. Chromatographic separation (GC) was performed using a DB-5MS capillary column (30 m x 0.25 mm x 0.25 μm film thickness, Agilent Technologies, Wilmington, DE, USA). The analytical conditions used were as follows: spitless injection at 240 °C; helium as the carrier gas at a flow rate of 1.0 ml/min; and GC column temperature program of GC was initially set at 40 °C for 2 min, then heated to 150 °C for 3 °C/min, maintained for 5 min, and finally increased to 250 °C at 20 °C/min and maintained for 8 min. For MS detection, an electron ionization (EI) system was used at 70 eV; the temperature of the transfer line and ionization source was 150 and 230 °C, respectively; and full-scan acquisition mode was performed with a mass range of 20–550 Da. Constituents were identified by comparing mass spectra with the National Institute of Standards and Technology (NIST) 14 library (similarity > 75%) and with published data (NIST, http://webbook.nist.gov/chemistry/; PubChem, http://pubchem.ncbi.nlm.nih.gov/). Moreover, the retention time of various compounds in the standard was measured according to the above experimental conditions. According to the retention time of compounds in the floral scents and n-alkanes in the standard, the retention index (RI) was calculated, and compared with the RI in the literature to further determine the components in the floral scents. In addition, relative amounts of compounds were calculated in relation to the total area of the chromatogram by normalizing the peak area (Chemstation B.07.05).

Comparison of compound extraction sensitivity

$$AVk = [Ak\ (PDMS/DVB) + Ak\ (CAR/PDMS) + Ak\ (DVB/CAR/PDMS)]/3;$$

$$NAk(X) = Ak\ (X)/AVk;$$

$$CAk(X) = \sum_{n=1}^{\infty} NAn(X).$$

In the equations: AVk is the average peak area of compound K measured by the three SPME fibers; Ak(X) is the absolute peak area of compound K extracted by the X SPME fiber, where X is any of the PDMS/DVB, CAR/PDMS and DVB/CAR/PDMS SPME fibers; NAk(X) is the standardized value of peak area of compound K extracted by the X fiber; and CAk(X) is the cumulative area normalization value of one to more compounds extracted by the X fiber. At the same retention time, when the CANV is larger, the sensitivity of the SPME fiber is considered to be higher.

Characterization of VOCs from *A. japonica* and *A. amurensis* Flowers

One-way analysis of variance (ANOVA) using R software was performed to investigate the significant differences ($p<0.05$) in the relative amounts of compounds between the two taxa. The GC-MS dataset was imported to SIMCA-P 14.1 software for statistical analysis. Principal component analysis (PCA) and partial least squares-discriminant analysis (PLS-DA) were used to differentiate the samples and identify marker metabolites. Afterwards, the variable influence on projection (VIP), which summarizes the importance of the X-variables in the PLS-DA model with many components, was used to illustrate the variables that contributed to the separation.

Results

Fiber performance

Three kinds of SPME fibers were used for the SPME-GC/MS full scan analysis of *A. amurensis* samples. The total ion chromatogram is shown in Figure 1 and clear ion spectrum was obtained. In our study, three types of fiber coatings (DVB/PDMS, CAR/PDMS, DVB/CAR/PDMS) were evaluated for their performance in absorbing VOCs, which was determined based on the number of chromatographic peaks that they detected, from flowers of columbines. In total, 55 volatile compounds were identified, belonging to the following different chemical classes: fatty acid derivatives (10), benzenoids (2), monoterpenoids (24) and sesquiterpenoids (20) (Table S1). Among them, 50 volatile compounds were extracted by DVB/CAR/PDMS fiber, 47 volatile compounds were extracted by CAR/PDMS fiber and 45 volatile compounds were extracted by the DVB/PDMS fiber. The correlation between the three repetitions of each fiber in the detection of compounds was shown in Table S1. The CANV of DVB/CAR/PDMS, CAR/PDMS and DVB/PDMS fibers was 85.12, 56.16 and 29.72, respectively. Therefore, the DVB/CAR/PDMS fiber showed the best efficiency and was used to extract volatile compounds in *A. japonica*.

In addition, 39 compounds were common to the three types of fiber used, and the most abundant compounds were D-limonene (47.65%), 1R-α-pinene (11.23%), γ-muurolene (8.00%), (-)-β-pinene (7.85%) and 1-hexanol (6.63%), accounting for approximately 81% of the total GC peak area. However, a few of scarce compounds were adsorbed only by one type of fiber. Specifically, the CAR/PDMS fiber exclusively extracted 4 compounds (longifolene-(V4), α-farnesene, 1-methyl-4-(6-methylhept-5-en-2-yl)cyclohexa-1,3-diene and β-sesquiphellandrene), while 2 compounds (viridiflorene, 2-isopropenyl-5-methylhex-4-enal) were extracted only by the DVB/CAR/PDMS fiber. Additionally, m-cymene was detected only when using the DVB/ PDMS fiber. Furthermore, there were 5 compounds that just the CAR/PDMS fiber did not extracted, (-)-terpinen-4-ol, verbenone, benzene, 1-methoxy-4-methyl-2-(1-methylethyl)-, myrtenyl acetate and viridiflorol. In addition, there were another 6 compounds that just the DVB/PDMS fiber did not extracted: decanal, pentanoic acid 2,2,4-trimethyl-3-carboxyisopropyl isobutyl ester, benzoic acid-ethyl ester and β-bisabolene.

Discrimination of the different taxa

The identified compounds and their relative contents (%) in *A. japonica* flowers were analyzed using DVB/CAR/PDMS-coated SPME fiber because this type of fiber was more efficient for the extraction of compounds. In order to ensure the accuracy, 6 repetitions were set. A total of 30 volatile compounds were putatively identified in this taxon, including fatty acid derivatives (15), benzenoids (2) and monoterpenoids (13) (Table 1). The correlation between the six replicates was shown in Table S2. The relative contents of 8 volatile compounds were significantly different (VIP > 1, $p < 0.05$) between the two different taxa, including 3-Hexen-1-ol, (E)-, 3-Hexen-1-ol, acetate, (Z)-, Methyl decanoate, 1R-α-pinene, (-)-β-pinene, 3-Carene, o-cymene, γ-muurolene and α-muurolene, constituting 29.78% and 15.17% of the total content in *A. amurensis* and *A. japonica*, respectively. Furthermore, 12 analytes were not detected in *A. amurensis* taxa (15.75% of the total content in *A. japonica*), and 32 volatile compounds were not detected in *A. japonica* taxa (18.49% of the total content in *A. amurensis*).

In addition, the main floral scents in *A. amurensis* were d-limonene and 1R-α-pinene (47.65% and 11.23% of the total content, respectively) while the primary volatile components in *A. japonica* included d-limonene and 1-hexanol (constituting 58.19% and 9.61% of the total, respectively) (Table 1). The relative contents of the different chemical classes (fatty acid derivatives, benzenoids, monoterpenoids and sesquiterpenoids) between the two taxa were calculated and compared (Figure S1). The kinds of terpenes were more in *A. amurensis* than in *A. japonica*, and sesquiterpenoids were not detected in *A. japonica*. However, the kinds of fatty acid derivatives in *A. japonica* was more than that in another taxon (Figure S1).

Moreover, PCA, an unbiased statistical approach, was used to evaluate the separation of the different taxa (Figure 1a). The two taxa were clearly separated and were located in the positive and negative axes of PC1. However, the model described 48.5% of the variation (R2X (cum) = 0.918). Then, a supervised method, PLS-DA, was applied, and the PLS-DA score plot showed a good separation (R2X(cum) = 0.852, R2Y(cum) = 1, Q2(cum) = 0.952) (Figure 2b). Furthermore, variables with VIP > 1 were considered important for the discrimination of samples in the PLS-DA score plot. This result indicated that the compounds (E)-3-hexen-1-ol, (Z)-3-hexen-1-ol acetate, Methyl decanoate, 1R-α-pinene, (-)-β-pinene, 3-carene, γ-murolene and α-muurolene compounds were probably responsible for the observed separation (VIP > 1, $p < 0.05$) (Table 1).

Table 1 Volatile compounds identified in the flowers of two *Aquilegia* taxa extracted by the fibers DVB/CAR/PDMS

Compounds		RT.	Mean Relative Content (%)		RI		VIP
			A. amurensis	*A. japonica*	Measurements value	Reference value	
Fatty acid derivatives							
C6H12O	Hexanal	5.429	0.1605	0.6845	817	803	0.698914**
C6H10O	3-Hexenal, (Z)-	5.548	ND	0.2180	820	814	0.43607
C6H12O	3-Hexen-1-ol, (E)-	5.68	ND	4.8236	824	842	1.98148*
C6H12O	Cyclobutanol, 2-ethyl-	5.796	0.1260	ND	827	828	0.316328**
C6H14O	1-Hexanol	6.435	6.6324	9.6073	843	838	1.75657
C7H14O	Heptanal	8.01	ND	0.0418	883	899	0.180242
C8H16O	Octanal	13.22	0.4728	1.4104	996	1005	0.927995
C8H14O2	3-Hexen-1-ol, acetate, (Z)-	13.34	ND	5.1579	998	1025	2.05217*
C8H18O	1-Octanol	16.931	3.3207	4.1932	1071	1069	0.884593
C11H24	Undecane	18.32	ND	0.3648	1098	1100	0.51752
C9H18O	1-Nonanal	18.573	0.4056	0.4892	1104	1105	0.343943
C9H18O2	Octanoic acid, methyl ester	19.45	ND	0.1359	1122	1128	0.329421*
C10H20O	Decanal	23.57	0.2624	0.3637	1207	1208	0.310842
C11H22O2	Methyl decanoate	28.91	ND	2.0242	1323	1325	1.32765**
C14H20	Bicyclo[4.1.0]heptane, 7-bicyclo[4.1.0]hept-7-ylidene-	31.929	0.0574	ND	1392	1427	0.205755
C16H30O4	Pentanoic acid, 2,2,4-trimethyl-3-carboxyisopropyl, isobutyl ester	39.84	0.0371	ND	1584	1581	0.14884
C16H30O4	2,2,4-Trimethyl-1,3-pentanediol diisobutyrate	39.939	0.5203	1.2692	1586	1588	0.788247**
C17H34O2	Methyl palmitate	48.07	ND	0.7717	1929	1905	0.822098**
Benzenoids							
C7H6O	Benzaldehyde	10.87	ND	0.2345	946	954	0.454126
C9H10O2	Benzoic acid, ethyl ester	21.47	0.1364	ND	1163	1170	0.255591
C8H8O3	Methyl salicylate	22.731	0.1401	0.1259	1189	1190	0.184799
Monoterpenoids							
C10H16	α-Thujene	9.176	0.2126	ND	911	931	0.435131**
C10H16	1R-α-Pinene	9.495	11.2283	2.4221	918	922	2.77679**
C10H16	Bicyclo[3.1.1]heptane, 6,6-dimethyl-2-methylene-, (1S)-	11.56	0.5131	0.9536	961	978.6	0.590684**
C10H16	(-)-β-Pinene	11.765	7.8487	0.7496	965	979	2.51042**
C10H16	β-Myrcene	12.47	0.4926	2.7938	980	991	1.37679
C10H16	3-Carene	13.431	1.3132	ND	1000	1021	1.01581*
C10H14	Cycloheptane, 1,3,5-tris(methylene)-	14.16	ND	0.4180	1015	1039	0.62015
C10H14	O-Cymene	14.33	0.3795	0.0621	1019	1006	0.508208*
C10H16	D-Limonene	14.65	47.6526	58.1922	1025	1033	3.16367
C10H16	trans-Ocimene	15.35	0.0309	ND	1039	1049	0.127914*
C10H16	cis-β-Ocimene	15.591	0.1276	ND	1044	1038	0.327404**

Compounds		RT.	Mean Relative Content (%)		RI		VIP
			A. amurensis	*A. japonica*	Measurements value	Reference value	
C10H16	γ-Terpinene	16.074	0.3200	0.0648	1053	1061	0.465106**
C10H16	Terpinolen	17.462	0.2289	ND	1081	1087	0.453098**
C10H18O	Linalool	18.22	0.4935	0.0371	1096	1098	0.626081**
C10H14	p-Mentha-1,5,8-triene	18.73	ND	0.1059	1107	1097	0.315963
C10H16	(E,Z)-2,6-Dimethylocta-2,4,6-triene	19.84	0.1551	ND	1130	1129	0.288125*
C10H16O	(+)-(E)-Limonene oxide	20.16	0.1970	0.3550	1136	1146	0.385326
C10H16O	2-Isopropenyl-5-methylhex-4-enal	22.164	0.2050	ND	1178	1198	0.339786
C10H18O	(-)-Terpinen-4-ol	22.236	0.0562	ND	1179	1175	0.183238
C10H18O	α-Terpineol	22.96	0.2684	ND	1194	1194	0.490675**
C10H16O	2-Cyclohexen-1-ol,2-methyl-5-(1-methylethenyl)-,cis	23.08	ND	1.4500	1196	1207	1.17303
C10H14O	Verbenone	23.45	0.0858	ND	1204	1204	0.226339
C11H16O	Thymol methyl ether	24.62	0.0767	ND	1229	1162	0.254136**
C10H14O	2-cyclohexene-1-one,3-Methyl-6-(1-methylethenyl)-, (S)-	26.347	0.4664	0.4798	1267	1279	0.350405
C12H18O2	Myrtenyl acetate	28.682	0.2245	ND	1320	1306	0.439773**
Sesquiterpenoids							
C15H24	1H-Cycloprop[e]azulene, decahydro-1,1,7-trimethyl-4-methylene-	30.831	0.0564	ND	1367	1386	0.203899
C15H24	Copaene	31.163	1.0909	ND	1375	1388	0.966962**
C15H24	Zingiberene	31.736	0.0996	ND	1388	1412	0.271292*
C15H24	1H-Cycloprop[e]azulene, 1a,2,3,4,4a,5,6,7b-octahydro-1,1,4,7-tetramethyl-, [1aR-(1aα,4α,4aβ,7bα)]-	32.388	0.2504	ND	1403	1419	0.428622*
C15H24	Caryophyllene	32.992	0.0206	ND	1417	1424	0.12333
C15H24	1,5,9,9-Tetramethyl-1,4,7-cycloundecatriene	34.494	1.4732	ND	1454	1476	1.03835
C15H24	(-)-Alloaromadendrene	34.669	0.7734	ND	1458	1435	0.815822**
C15H24	γ-Muurolene	35.394	7.9972	ND	1475	1475	2.63621**
C15H24	α-Curcumene	35.629	0.3330	ND	1481	1483	0.494602*
C15H24	Viridiflorene	35.979	0.0715	ND	1490	1484	0.246633*
C15H24	α-Muurolene	36.311	1.3965	ND	1498	1501	1.1025**
C15H24	1-Methyl-4-(6-methylhept-5-en-2-yl)cyclohexa-1,3-diene	36.715	0.1433	ND	1507	1506	0.326625*
C15H24	Cadina-1(10),4-diene	37.096	0.1616	ND	1517	1531	0.370244*
C15H24O	α-Copaen-11-ol	37.989	0.4202	ND	1538	1537	0.595396*

Compounds		RT.	Mean Relative Content (%)		RI		VIP
			A. amurensis	*A. japonica*	Measurements value	Reference value	
C15H26O	Viridiflorol	40.107	0.1509	ND	1589	1580	0.336428
	C15H26O	α-Bisabolol	44.6280	0.7130	ND	1690	1680	0.727594*

* represents significant differences between different taxa 0.01 < p < 0.05;

** represents significant differences between different taxa p < 0.01;

RT — retention time; ND — not detected; RI — retention index; VIP — variable importance in projection.

Discussion

Fiber Selection

The choice of the most appropriate fibers is made to can cover as many metabolites as possible. To select the most efficient fiber coating for the extraction of VOCs in the *Aquilegia* taxa, three SPME fibers (DVB/CAR/PDMS, DVB/PDMS, CAR/PDM) were used. In our study, the DVB/CAR/PDMS fiber exhibited better extraction efficiency than the DVB/PDMS and CAR/PDMS fibers, presenting the highest CANV (85.12) compared to the other fibers (56.16 and 29.72, respectively). The affinity of the fiber for an analyte depends on the principle of 'like dissolves like'. Previous studies have demonstrated that many polar molecules in the *Aquilegia*'s flora scents [18] as well as the DVB/CAR/PDMS fiber have an intermediate polarity and some studies also confirmed its efficiency [20, 21]. The high efficiency may be because the coating with three different components improves the ability to adsorb compounds [22]. The DVB/PDMS fiber is preferred for the extraction of analytes with higher molecular weights (MW 50-300), such as volatiles, amines, and nitroaromatic compounds. Specifically, 8 fatty acid derivatives, 1 benzenoids and 36 terpenes were identified using the DVB/PDMS fiber, fewer than those detected by the other two fiber types. However, the CAR/PDMS fiber is more efficient for the extraction of gasses and low molecular weight compounds (MW 30-225) [23]. Among the 5 compounds that just the CAR/PDMS fiber did not extracted, most have intermediate and higher molecular weights, which is consistent with the results of the Silva et al. [17].

Moreover, these adsorbent type coatings, carried out by sorption of analytes in internal pores, are formed by porous solids. Therefore, saturation of the surface available for adsorption occurs because of the limited thickness. Competition between compounds was more intense when used the CAR/PDMS fiber than used the DVB/CAR/PDMS. When considering repeatability, the CAR/PDMS fiber is better than the 50/30 m DVB/CAR/PDMS fiber, and Kataoka et al. also reported this result [24]. However, when considering sensitivity, the DVB/CAR/PDMS fiber show higher performance than that of CAR/PDMS and DVB/PDMS. Thus, the DVB/CAR/PDMS fiber has been selected for use in the measurement of the floral scents of *Aquilegia* and six replicate *A. japonica* flowers were evaluated when using the DVB/CAR/PDMS fiber.

Scent composition in relation to the pollinators of the two *Aquilegia* taxa

Speciation in radiating flowering plants is often accompanied by diversification of animal pollinators [24-26]. Perhaps the most well-known signal in *Aquilegia* is floral color, orientation and the structure of spurs [6, 27]. Meanwhile, the roles of floral scents have been investigated in other systems [28-30], showing that the floral scents are important signals for communication between plants and pollinators, representing an important cue for pollinators [31, 32]. Therefore, a prezygotic reproductive barrier is expected when the composition of the floral scent is different. For example, Huber et al. (2005) proposed that flowers of two *Gymnadenia* species with different floral odors, as well as other floral traits such as color and spur length, attracted different pollinators, enhancing prezygotic isolation [33].

The variability of floral scents among entomophilous plants has been reported to depend on the reliance on different pollinator groups with different olfactory preferences [34]. For example, the high relative content of the most volatile monoterpene alkenes (e.g. limonene) in the floral scent of *Silene gallica* and *S. coeli-rosa* pollinated by bees has suggested that these compounds are used as attractants of bees [35]. Jürgens and Dötterl investigated floral scents of four *Aquilegia* taxa, *A. vulgaris*, *A. canadensis*, *A. chrysantha* and *A. glandulosa* [18]. They found that the dominant compound of these four *Aquilegia* species was octanal (29.5%-42%). In contrast, high relative amounts of the monoterpene d-limonene, 47.65% for *A. amurensis* and 58.19% for *A. japonica* were detected. The individuals were selected for the experiment that produced much less octanal, 0.47% and 1.41% for *A. amurensis* and *A. japonica*, respectively. There may be two reasons for this difference: one is that Jürgens A et al. did not use SPME to detect the VOCs *Aquilegia*. Different detection methods lead to different compounds of the floral scent's compounds of *Aquilegia* in different regions. In future research, we should increase the species of samples and use the same method to measure the VOCs of *Aquilegia*; the other reason is that they are located in order to adapt to different pollinators, Aquilegia in different regions have different VOCs.

Our study has identified that the floral scents of the two taxa are dominated by the same one compound (d-limonene), suggesting an adaptation to the same pollinator. Nevertheless, the low-abundance scent components may be effective specific attractants of potential pollinators and cannot be ignored [29]. For instance, the main floral scent compound of the floral four *Aquilegia* species that Jürgens and Dötterl studied was octanal (29.5%-42%), but the pollinators for these species were varied. The visitation of *A. chrysantha* was visited by hawk moths may correlate with relatively high amount of 2-phenyl ethanol (13.5%) compared to that of the other three *Aquilegia* species [29]. Therefore, the fact that the two taxa share the same main floral scent components may be attributed to their closer phylogenetic relationship.

The notable differences between the taxa were the increase in the relative amounts of fatty acid derivatives and the decrease in the relative amounts of monoterpenoids in *A. japonica* and the detection of various sesquiterpenes only in *A. amurensis*. Among the fatty acid derivatives, the relative proportions of (Z)-3-hexen-1-ol acetate, (E)-3-hexen-1-ol and Methyl decanoate (VIP > 1, $p < 0.05$) were significantly different between the two species, representing nearly 12% of the total floral scents of *A. japonica* but not detected in *A. amurensis*. However, (Z)-3-hexen-1-ol acetate is often released from vegetation rapidly after damage [36]. It can be hypothesized that this compound may have a defense function. The large number of low-abundance sesquiterpenoids in *A. amurensis* may represent biosynthetic byproducts, as the monoterpenes and sesquiterpenes are derived from the mevalonic acid pathway via farnesyl pyrophosphate [37]. Further experiments are necessary to draw conclusions regarding whether these sesquiterpenes are by-products or serve critical functions in plant pollinator relationships, further experiments are necessary to draw conclusions.

Conclusion

In this study, by evaluating the properties of different coatings of SPME fibers, the method of extracting and identifying the VOCs of *Aquilegia* flowers can be optimized. The DVB/CAR/PDMS fiber had the good performance, including sensitivity and repeatability, which is suitable for the subsequent detection of *Aquilegia* floral scents' compounds. In the flowers of two sister species of *A. japonica* and *A. amurensis*, there were significant differences in the type and contents of VOCs: in addition to sesquiterpenes not detected in *A. japonica*, there were also significant differences in the contents of eight compounds. The result provides important information for the future studies involving the VOCs of *Aquilegia* flowers and can be applied to the new study of relationship between the chemical components of floral scents and the attraction process of pollinators.

Declarations

Author Contributions: X. HX. designed the study and evaluated the results. W. HY. and Z. W. prepared the manuscript. W. HY. and D. JH. analyzed the results. In addition, Z. W. and W. H. were responsible for the entire experiment. W. YH revised the manuscript. All authors both read and approved the manuscript.

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References

1. Holopainen JK, Gershenzon J: **Multiple stress factors and the emission of plant VOCs**. *Trends Plant Sci* 2010, **15**(3):176-184.
2. Bradshaw H, Otto KG, Frewen BE, McKay JK, Schemske DW: **Quantitative trait loci affecting differences in floral morphology between two species of monkeyflower (Mimulus)**. *Genetics* 1998, **149**(1):367-382.
3. Schemske DW, Bradshaw H: **Pollinator preference and the evolution of floral traits in monkeyflowers (Mimulus)**. *Proceedings of the National Academy of Sciences* 1999, **96**(21):11910-11915.
4. Bradshaw Jr H, Schemske DW: **Allele substitution at a flower colour locus produces a pollinator shift in monkeyflowers**. *Nature* 2003, **426**(6963):176.
5. Hodges SA, Derieg NJ: **Adaptive radiations: From field to genomic studies**. *Proceedings of the National Academy of Sciences* 2009, **106**(Supplement 1):9947-9954.
6. Whittall JB, Hodges SA: **Pollinator shifts drive increasingly long nectar spurs in columbine flowers**. *Nature* 2007, **447**(7145):706.
7. Cordeiro G, Pinheiro M, Dötterl S, Alves-dos-Santos I: **Pollination of Campomanesia phaea (Myrtaceae) by night-active bees: a new nocturnal pollination system mediated by floral scent**. *Plant Biology* 2017, **19**(2):132-139.
8. Bischoff M, Raguso RA, Jürgens A, Campbell DR: **Context-dependent reproductive isolation mediated by floral scent and color**. *Evolution* 2015, **69**(1):1-13.
9. Knudsen JT, Eriksson R, Gershenzon J, St?hl B: **Diversity and Distribution of Floral Scent || Diversity and Distribution of Floral Scent**. *Botanical Review*, **72**(1):1-120.
10. Dunkel M, Schmidt U, Struck S, Berger L, Gruening B, Hossbach J, Jaeger IS, Effmert U, Piechulla B, Eriksson R: **SuperScent—a database of flavors and scents**. *Nucleic acids research* 2008, **37**(suppl_1):D291-D294.
11. Yang Z, Nielsen R, Hasegawa M: **Models of amino acid substitution and applications to mitochondrial protein evolution**. *Molecular biology and evolution* 1998, **15**(12):1600-1611.
12. Zhu F, Xu J, Ke Y, Huang S, Zeng F, Luan T, Ouyang G: **Applications of in vivo and in vitro solid-phase microextraction techniques in plant analysis: a review**. *Analytica chimica acta* 2013, **794**:1-14.
13. Miguel MG: **Antioxidant and anti-inflammatory activities of essential oils: a short review**. *Molecules* 2010, **15**(12):9252-9287.
14. Fan J, Zhang W, Zhou T, Zhang D, Zhang D, Zhang L, Wang G, Cao F: **Discrimination of Malus Taxa with Different Scent Intensities Using Electronic Nose and Gas Chromatography–Mass Spectrometry**. *Sensors* 2018, **18**(10):3429.
15. Gao F, Liu B, Li M, Gao X, Fang Q, Liu C, Ding H, Wang L, Gao X: **Identification and characterization of terpene synthase genes accounting for volatile terpene emissions in flowers of Freesia x hybrida**. *Journal of experimental botany* 2018, **69**(18):4249-4265.
16. Ibrahim M, Agarwal M, Yang JO, Abdulhussein M, Du X, Hardy G, Ren Y: **Plant Growth Regulators Improve the Production of Volatile Organic Compounds in Two Rose Varieties**. *Plants* 2019, **8**(2):35.
17. Silva FAN, da Silva AA, de Sousa Fernandes N, Rodrigues THS, Canuto KM, do Nascimento RF, de Brito ES, de Aragão FAS, Freitas BM, Zocolo GJ: **Evaluation of Headspace Solid-Phase Microextraction Gas Chromatography–Mass Spectrometry for the Characterization of Volatile Organic Compounds from Melon (Cucumis melo L.) Flowers**. *Chromatographia* 2018, **81**(8):1231-1239.

18. Jürgens A, Dötterl S: **Chemical composition of anther volatiles in Ranunculaceae: genera-specific profiles in Anemone, Aquilegia, Caltha, Pulsatilla, Ranunculus, and Trollius species**. *American Journal of Botany* 2004, **91**.
19. Tat L, Comuzzo P, Stolfo I, Battistutta F: **Optimization of wine headspace analysis by solid-phase microextraction capillary gas chromatography with mass spectrometric and flame ionization detection**. *Food Chemistry* 2005, **93**(2):361-369.
20. Rocha SM, Caldeira M, Carrola J, Santos M, Cruz N, Duarte IF: **Exploring the human urine metabolomic potentialities by comprehensive two-dimensional gas chromatography coupled to time of flight mass spectrometry**. *Journal of Chromatography A* 2012, **1252**:155-163.
21. Araújo AM, Moreira N, Lima AR, de Lourdes Bastos M, Carvalho F, Carvalho M, de Pinho PG: **Analysis of extracellular metabolome by HS-SPME/GC–MS: optimization and application in a pilot study to evaluate galactosamine-induced hepatotoxicity**. *Toxicology letters* 2018, **295**:22-31.
22. Zhang M, Pan Q, Yan G, Duan C: **Using headspace solid phase micro-extraction for analysis of aromatic compounds during alcoholic fermentation of red wine**. *Food Chemistry* 2011, **125**(2):743-749.
23. Risticevic S, Lord H, Gorecki T, Arthur CL, Pawliszyn J: **Protocol for solid-phase microextraction method development**. *Nature protocols* 2010, **5**(1):122.
24. Kataoka H, Lord HL, Pawliszyn J: **Applications of solid-phase microextraction in food analysis**. *Journal of chromatography A* 2000, **880**(1-2):35-62.
25. Grant V: **Pollination systems as isolating mechanisms in angiosperms**. *Evolution* 1949, **3**(1):82-97.
26. Stebbins GL: **Adaptive radiation of reproductive characteristics in angiosperms, I: pollination mechanisms**. *Annual review of ecology and systematics* 1970, **1**(1):307-326.
27. Miller RB, Willard CL: **The pollination ecology of Aquilegia micrantha (Ranunculaceae) in Colorado**. *The Southwestern Naturalist* 1983:157-164.
28. Harder LD, Johnson SD: **Darwin's beautiful contrivances: evolutionary and functional evidence for floral adaptation**. *New Phytologist* 2009, **183**(3):530-545.
29. Schiestl FP: **On the success of a swindle: pollination by deception in orchids**. *Naturwissenschaften* 2005, **92**(6):255-264.
30. Yuan Y-W, Byers KJ, Bradshaw Jr H: **The genetic control of flower–pollinator specificity**. *Current opinion in plant biology* 2013, **16**(4):422-428.
31. Knudsen JT: **Variation in floral scent composition within and between populations of Geonoma macrostachys (Arecaceae) in the western Amazon**. *American Journal of Botany* 2002, **89**(11):1772-1778.
32. Plepys D, Ibarra F, Löfstedt C: **Volatiles from flowers of Platanthera bifolia (Orchidaceae) attractive to the silver Y moth, Autographa gamma (Lepidoptera: Noctuidae)**. *Oikos* 2002, **99**(1):69-74.
33. Huber FK, Kaiser R, Sauter W, Schiestl FP: **Floral scent emission and pollinator attraction in two species of Gymnadenia (Orchidaceae)**. *Oecologia* 2005, **142**(4):564-575.
34. Dobson HE: **Relationship between floral fragrance composition and type of pollinator**. In: *Biology of floral scent*. CRC press; 2006: 161-212.
35. Jürgens A: **Flower scent composition in diurnal Silene species (Caryophyllaceae): phylogenetic constraints or adaption to flower visitors?** *Biochemical Systematics and Ecology* 2004, **32**(10):841-859.
36. Kessler A, Baldwin IT: **Defensive function of herbivore-induced plant volatile emissions in nature**. *Science* 2001, **291**(5511):2141-2144.
37. Kaiser R, Müller P, Lamparsky D: **Perfumes: Art, Science and Technology**. In.: London: Elsevier Applied Science; 1991.

Figures

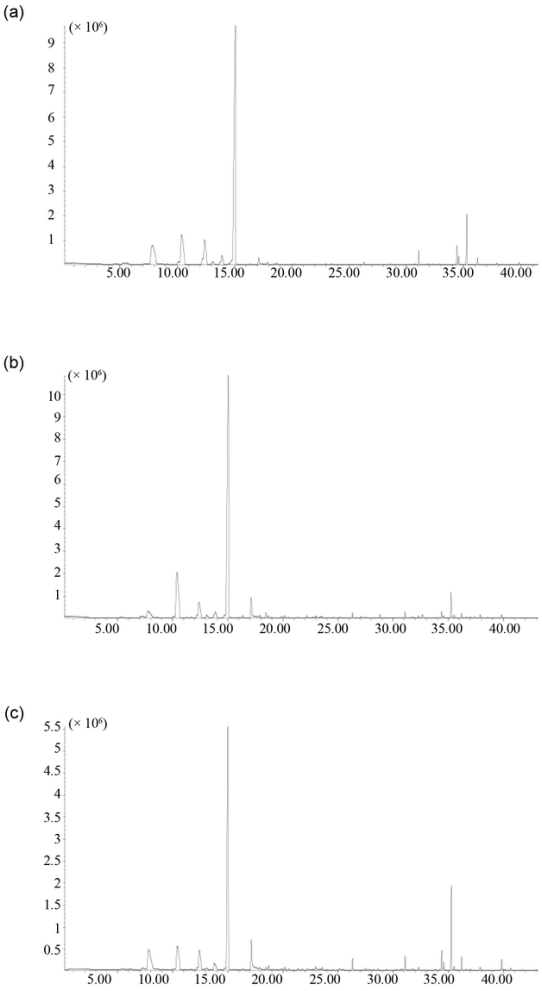

Figure 1

Total ion chromatogram. The x axis represents retention time (min) and the y axis represents relative abundance.

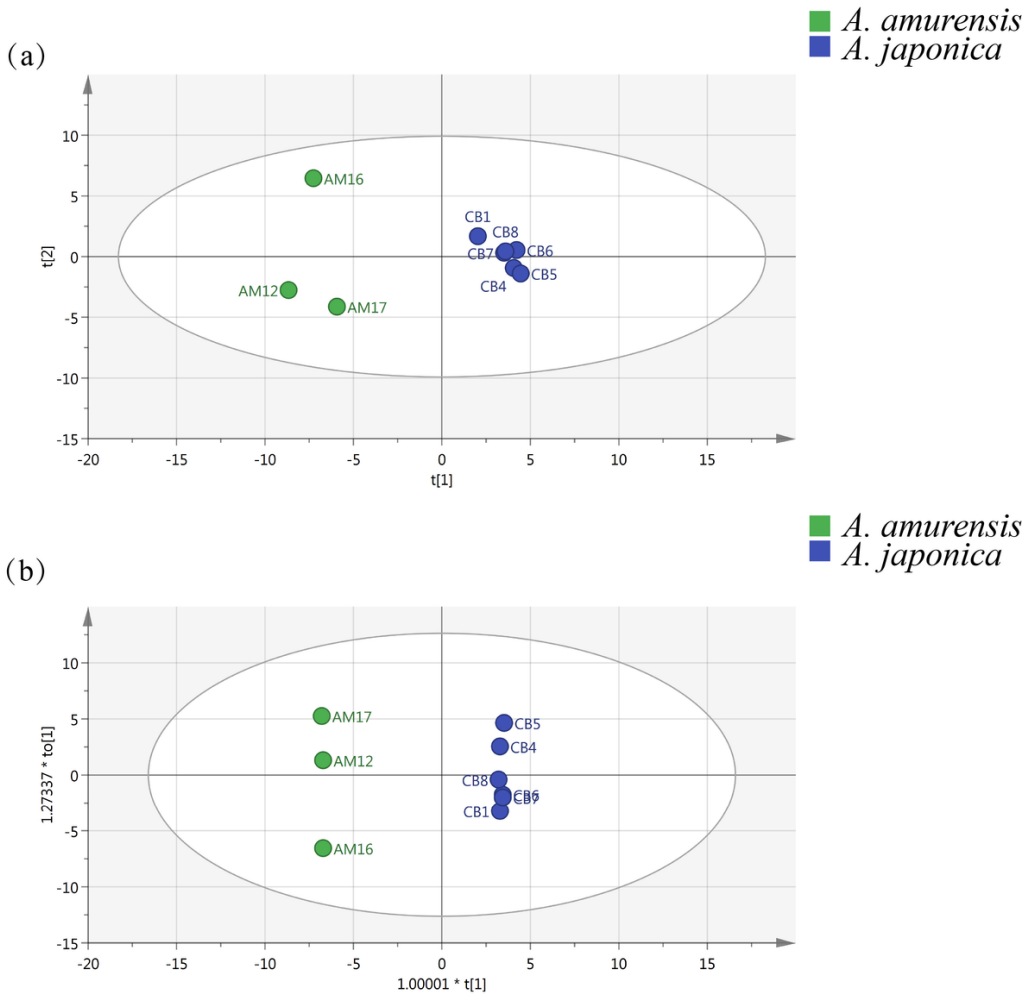

Figure 2

The PCA score plots (a) and PLS-DA score plots (b) for datasets of GC-MS from the two taxa.

Supplementary Files

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