Metabolomic Based on Gas Chromatography Mass Spectrometry Analysis Reveals the Differences in Volatile Metabolites of Royal Jelly from Different Honeybee Stocks

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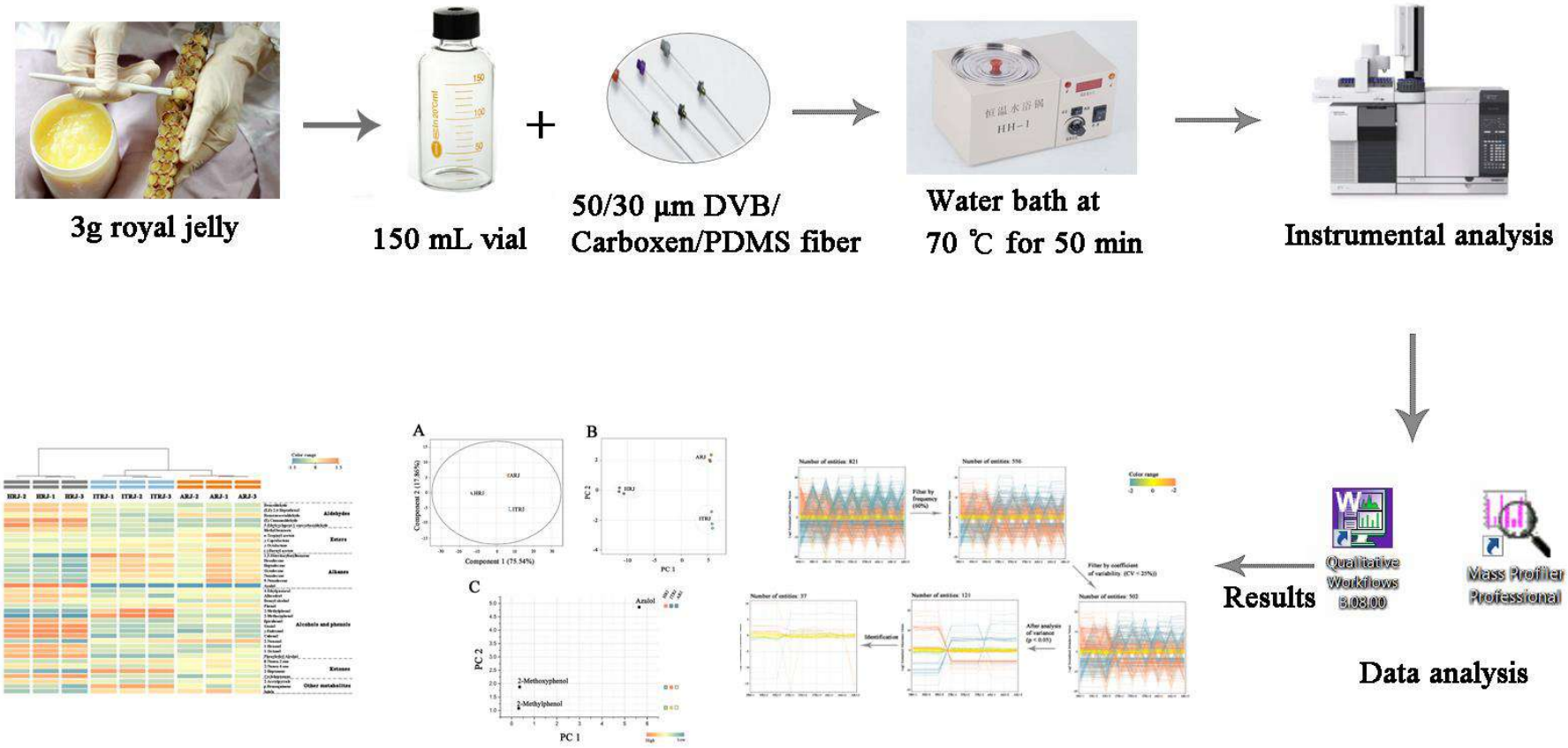
Introduction

- Royal jelly (RJ), secreted by young worker bees, is the decisive factor of sexual determination and longevity of queen bee.
- Royal jelly bees (RJBs) has now been selected, which could produce more than 5 ~ 10 times RJ than that of Italian bees (ITBs).
- Only very limited data is available on volatile metabolites in RJ and the differences between RJ produced from different honeybee stocks.
- Gas-chromatography-mass spectrometry analysis (GC-MS) coupled with chemometric analysis were applied on the study of differences in volatile metabolites of royal jelly from different honeybee stocks.

Method

- Extraction of volatile metabolites in royal jelly was carried out by headspace-solid phase micro-extraction (HS-SPME).
- GC-MS analysis by an Agilent 7890B gas chromatograph with a 7000D triple quadrupole mass spectrometry (GC/MS/MS).
- Data processing was processed by Mass Profiler Professional (MPP) software.



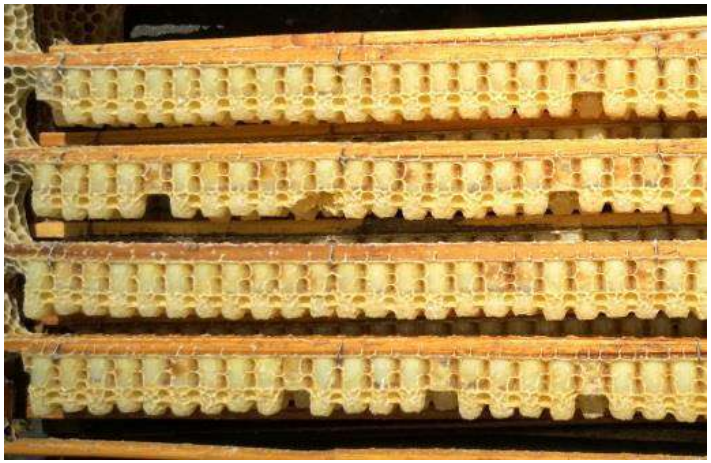
Graphical abstract for this study

Experiment

- **Royal jelly samples**

- Three stocks of honeybees, high RJ bees (RJBs) from China, Italian bees from Italy, and American Italian bees from the USA were used to produce royal jelly.

Experiment



Royal jelly in royal jelly producing frame

Experiment

- **SPME Conditions**

- 3 g of RJ and internal standard were added into a 150 mL vial.

- The vial was sealed immediately and equilibrated at 70 ℃ for 5 min using water bath. Afterward, the SPME fiber was exposed to the royal jelly sample head space for 50 min at 70 ℃.

- Finally, the SPME fiber was desorbed for 4.5 min by maintaining the GC-MS injection port at 270 ℃.

Experiment

- GC Conditions

- GC system: Agilent 7890B;
- Column: DB-5MS (60 m×0.32 mm×0.25 μm);
- Column temperature: 50 ℃ hold 3 min , at 5 ℃ /min to 250 ℃ hold 5 min;
- Carrier gas: Helium; Flow rate: 1.0 mL/min;
- Injection mode: Manual, SPME Fiber
- Injection port temperature: 270 ℃

Experiment

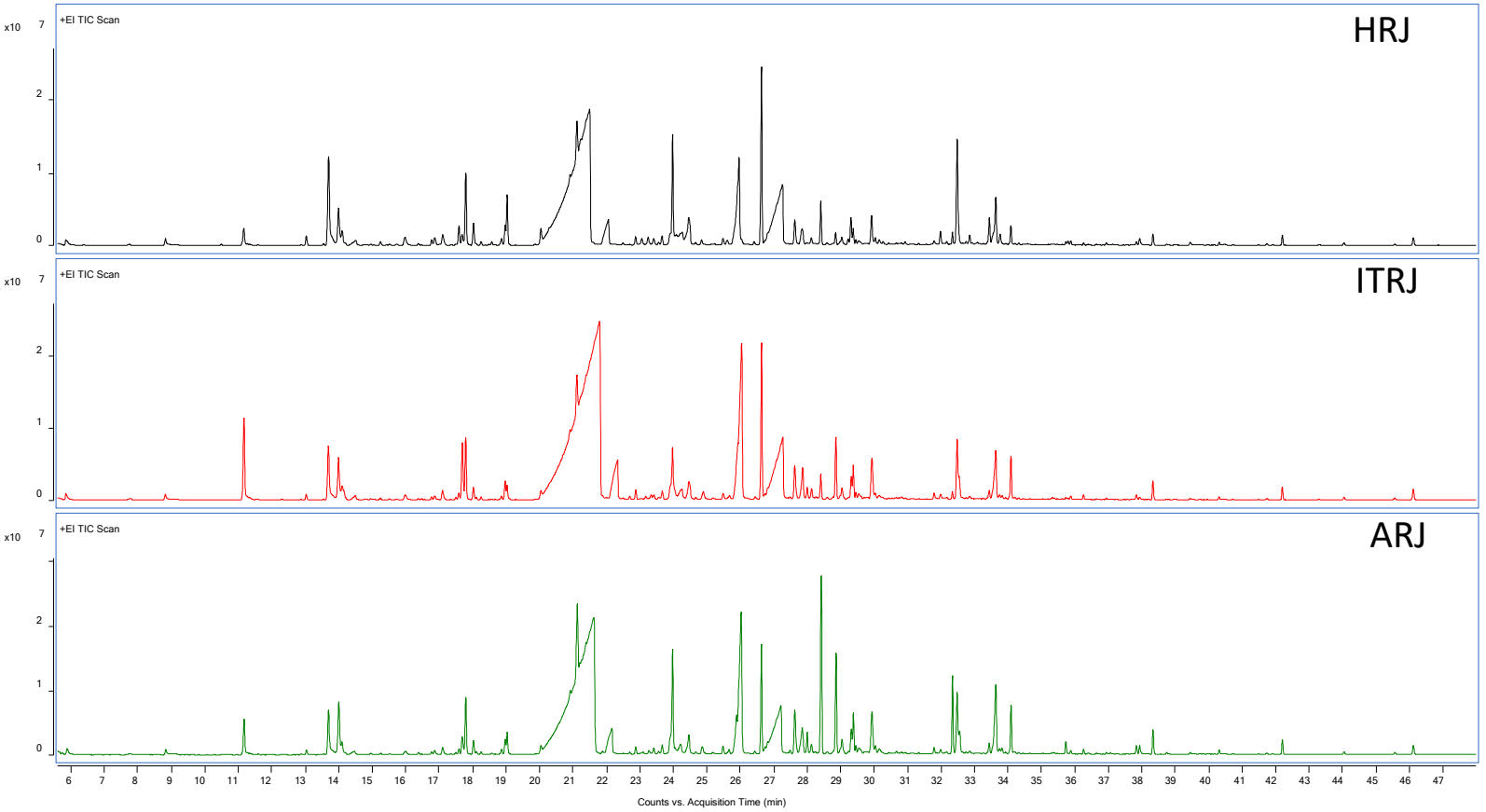
- MS Conditions
- MS system: Agilent 7000D GC/TQ;
- Ion source: EI; Ionization voltage: 70 eV;
- Quadrupole temperature 150 °C
- Ion source temperature: 280℃;
- Scan mode: full scan, 35-500 m/z.

Results and Discussion

- Data extraction

- The total ion chromatograms of three royal jelly samples were shown in the following Figure. Raw data acquired by GC-MS system was first deconvolved by Masshunter Qualitative Analysis software and then exported as .cef documents for further analysis.

Results and Discussion



The total ion chromatograms of three royal jelly samples

Results and Discussion

- **Data Filtering**

- Filtration parameters:
- Frequency filtration (frequency > 60%) ; coefficient of variability (CV < 25%)
- Analysis of variance (ANOVA) was performed to retain entities that displayed significant differences among groups.
- Eventually, 121 differential entities were found out as differential entities.

Results and Discussion

- **Identification of metabolites**

- The differential entities were identified by comparing mass spectra and retention index (RI) with that in NIST 14 Library and available standards.

- In total, 37 entities were identified, including 5 aldehydes, 5 esters, 6 alkanes, 14 alcohols and phenols, 4 ketones and 3 other metabolites.

	Name	Retention time (min)	RI[a]-calculation	RI[b]-NIST 14 Library	RT-STD	RI[c]-STD	HRJ : ITRJ: ARJ Ratio of peak area[e]	
	Aldehydes							
1	Benzaldehyde	13.69	961	962	13.883	966	1 : 0.60 : 0.51	
2	(E,E)-2,4-Heptadienal	15.246	1008	1012	15.45	1014	1: 0.51 : 0.51	
3	Benzeneacetaldehyde	16.375	1042	1045	16.552	1048	1: 0.61 : 1.17	
4	(E)-Cinnamaldehyde	23.469	1274	1270			1 : 0.30 : 0.26	
5	5-Ethylcyclopent-1-enecarboxaldehyde	15.961	1030	1040			1: 0.33 : 0.29	
	Esters							
6	Methyl benzoate	18.024	1093	1094			1 : 0.53 : 0.70	
7	α-Terpinyl acetate	25.478	1346	1350			1 : 0.81 : 1.53	
8	γ-Caprolactone	16.56	1048	1057			1 : 1.24 : 1.47	
9	γ-Octalactone	22.87	1253	1261			1 : 1.21 : 0.94	
10	(-)-Bornyl acetate	23.813	1285	1284			1 : 1.05 : 1.77	

Table of differential metabolites

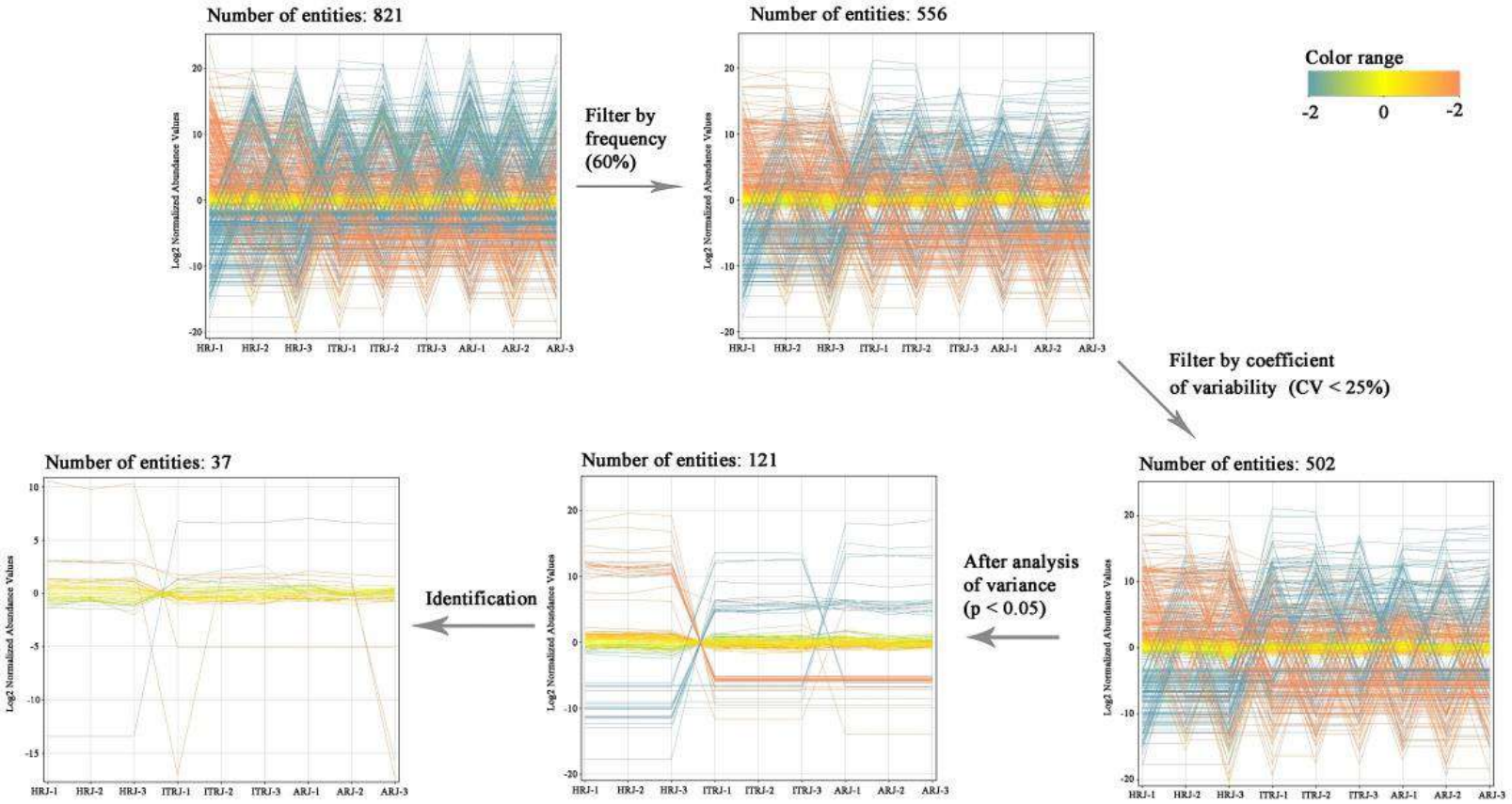
	Name	Retention time (min)	RI[a]-calculation	RI[b]-NIST 14 Library	RT-STD	RI[c]-STD	HRJ : ITRJ: ARJ Ratio of peak area[e]	
	Alkanes							
11	1,3-Ditertiarybutylbenzene	22.697	1247	1247			1 : 4.60 : 2.72	
12	Hexadecane	31.794	1594	1600	31.951	1600	1 : 1.86 : 1.64	
13	Heptadecane	34.087	1693	1700	34.249	1700	1 : 2.59 : 2.19	
14	Octadecane	36.261	1792	1800	36.431	1800	1 : 2.53 : 2.27	
15	Nonadecane	38.333	1892	1900	38.508	1900	1 : 1.95 : 2.01	
16	Guaiazulene	36.057	1783	1775			1 : 0 : 0	
	Alcohols and phenols							
17	4-Ethylguaiacol	23.411	1272	1282	23.278	1277	1 : 0.58 : 0.80	
18	Allocedrol	32.169	1609	1607			1 : 0.43 : 0.64	
19	Benzyl alcohol	15.992	1030	1036	16.135	1035	1 : 0.80 : 0.57	
20	Phenol	13.995	970	980			1 : 1.48 : 1.64	
21	2-Methylphenol	16.478	1045	1054	16.40	1051	1 : 5.12 : 1.79	

Table of differential metabolites

	Name	Retention time (min)	RI[a]-calculation	RI[b]-NIST 14 Library	RT-STD	RI[c]-STD	HRJ : ITRJ: ARJ Ratio of peak area[e]
22	2-Methoxyphenol	17.692	1083	1090			1 : 11.85 : 1.93
23	Epicubenol	32.745	1635	1627			1 : 0.34 : 0.45
24	Guaiol	31.981	1601	1596			1 : 0.27 : 0.27
25	γ-Eudesmol	32.852	1639	1631			1 : 0.26 : 0.23
26	Cubenol	33.096	1650	1642			1 : 0.22 : 0.34
27	2-Nonanol	18.116	1096	1101	17.998	1110	1 : 2.21 : 3.14
28	1-Hexanol	10.49	861	868	10.388	864	1 : 0.42 : 0.37
29	1-Octanol	17.119	1065	1071			1 : 0.45 : 0.39
30	Phenylethyl Alcohol	18.571	1110	1116	18.445	1115	1 : 0.25 : 0.45
	Ketones						
31	8-Nonen-2-one	17.502	1077	1085			1 : 1.74 : 1.53
32	2-Nonen-4-one	18.866	1120	1124			1 : 0.64 : 0.90
33	2-Heptanone	11.17	883	891	11.067	887	1 : 4.34 : 1.91
34	Cycloheptanone	15.429	1013	1015			1 : 0.34 : 0.27
	Other metabolites						
35	2-Acetylpyrrole	16.88	1058	1064			1 : 0.56 : 0.78
36	p-Benzoquinone	12.309	919	912			1 : 3.27 : 1.33
37	Indole	24.001	1292	1295	24.281	1302	0 : 1 : 1.07

Table of differential metabolites

Results and Discussion



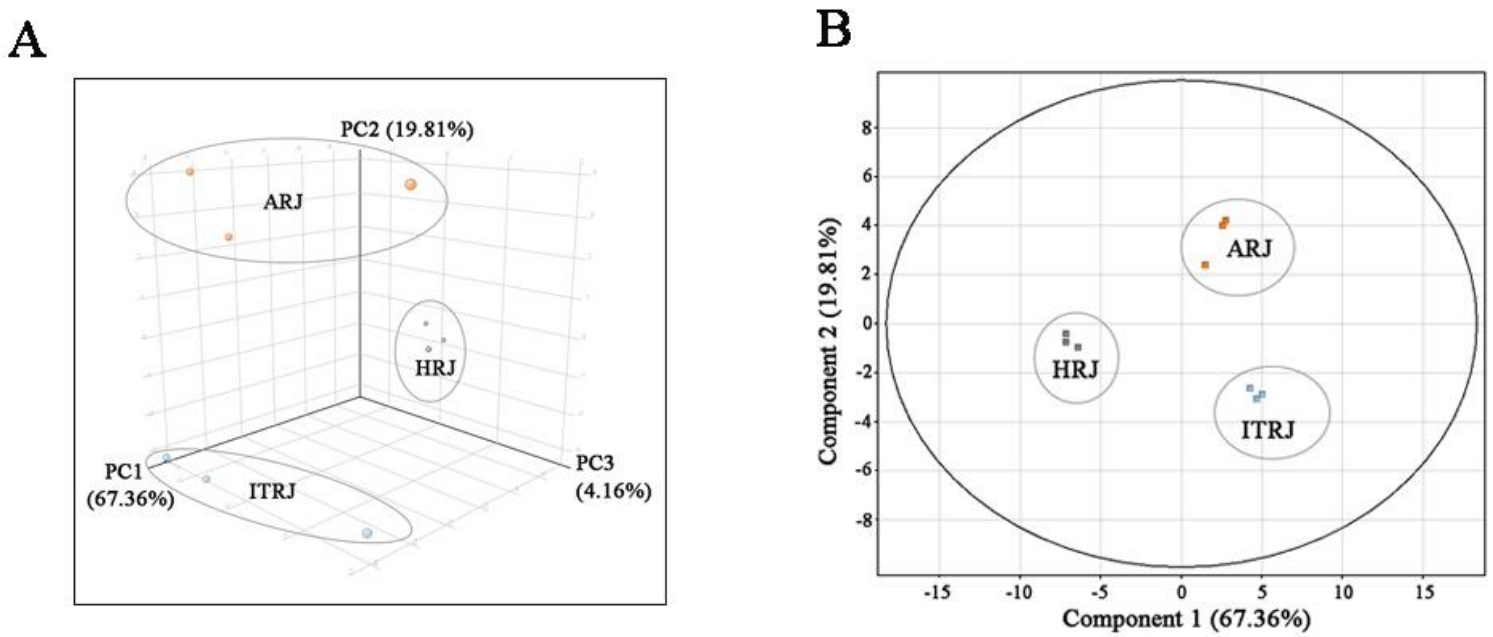
Data filtration to retain metabolites with significant differences in three RJ samples using MPP analysis

Results and Discussion

- Principal Component Analysis (PCA)

- PCA analysis was performed based on 37 differential metabolites using MPP. Excellent separation among three samples from different honeybee stocks were observed. The first three components explained 91.33% of the total variance (67.36%, 19.81% and 4.16%, respectively), indicating significant differences among volatile metabolites in royal jelly from different honeybee stocks.

Results and Discussion



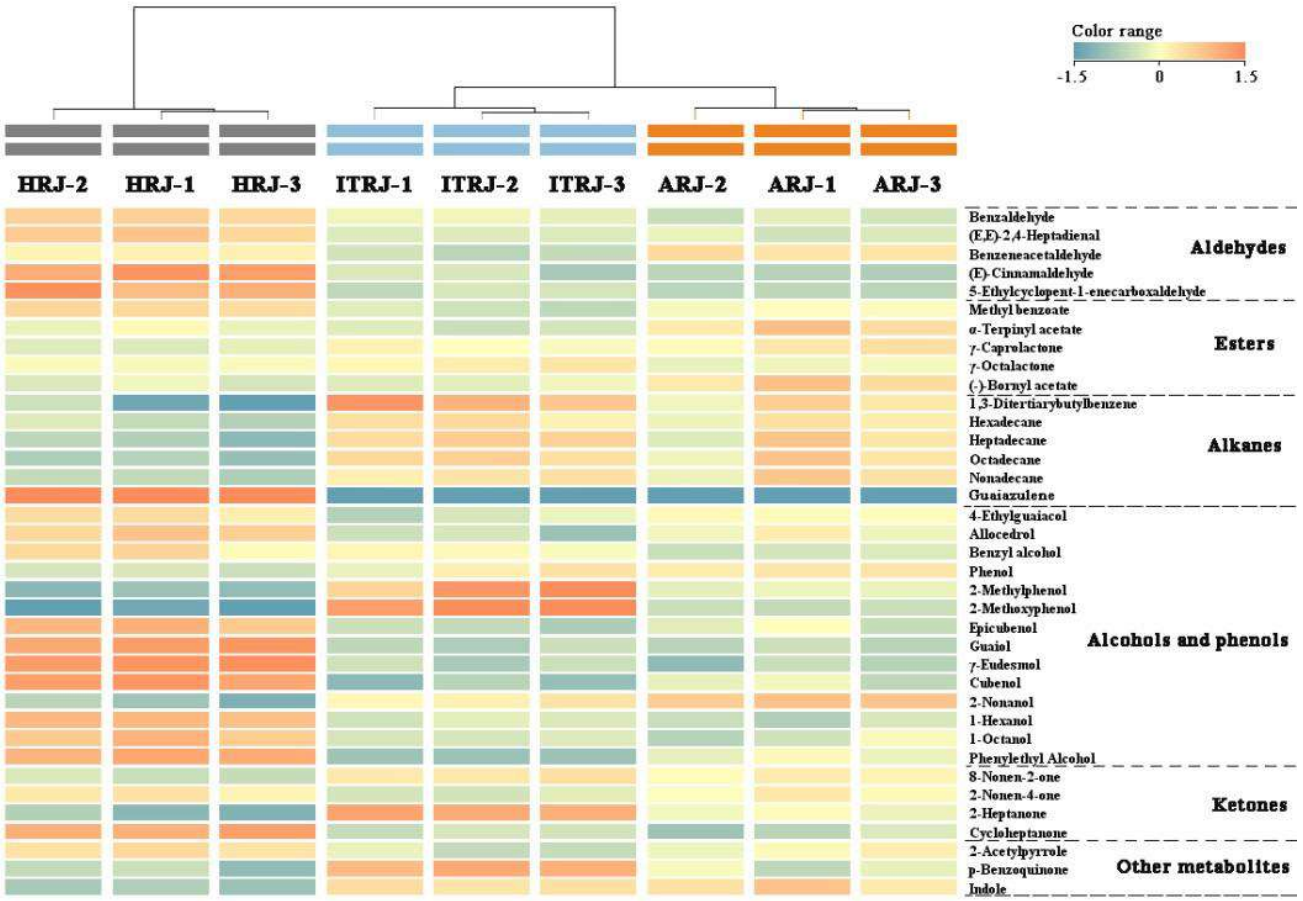
2-D and 3-D principle component analysis(PCA) of three royal jelly samples

Results and Discussion

- **Hierarchical Clustering Analysis (HCA)**

- Three royal jelly samples were divided into two groups, with ITRJ and ARJ in the same group, whereas HRJ was in an isolated one. This suggests that HRJ differ from the other two RJ samples on the level of volatile metabolites. This is in accordance with the PCA analysis.

Results and Discussion



Clustering analysis of differential volatile metabolites in three royal jelly samples

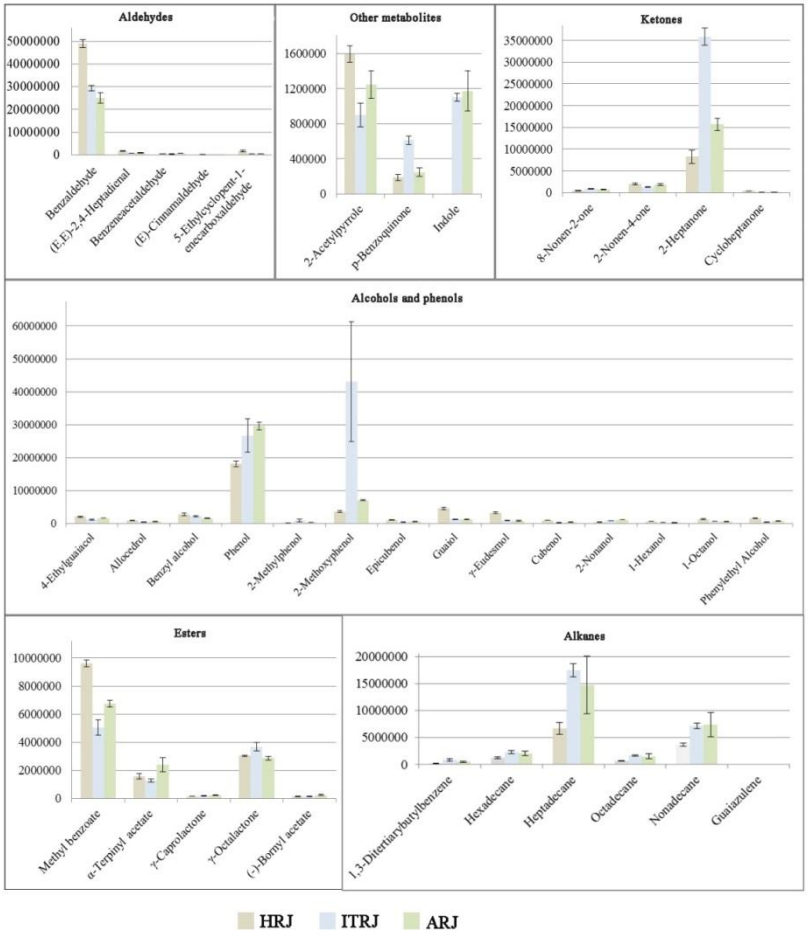
Results and Discussion

- **Histogram**

- The differences of 37 volatile metabolites in three royal jelly samples using histogram. In brief, differences among three royal jelly samples show mostly in content rather than components.

- In particular, guaiazulene was unique to HRJ, and indole was exclusive to ITRJ and ARJ. Phenol-scented 2-methylphenol and sweet 2-methoxyphenol shows extremely high content in ITRJ than HRJ and ARJ.

Results and Discussion



Histogram of 37 differential volatile metabolites in three royal jelly samples

Conclusions

- 1. SPME combined with GC-MS analysis for profiling of volatile metabolites in royal jelly samples has been developed.

- 2. **37 differential volatile metabolites** were identified to distinguish three royal jelly samples produced by different honeybee stocks. It is the first report about the difference among royal jelly samples of high yield and low yield honeybee stocks.

Conclusions

- 3. A lot of volatile metabolites with **unpleasant odor show lower content in HRJ** than ITRJ and ARJ, while metabolites with **sweet, flower, fruit aroma are highly abundant in HRJ** compared with ITRJ and ARJ, suggesting they may contribute a more pleasant aroma of HRJ comparing with the two other RJ samples.

- 4. This findings significantly extend our novel insight into the distinction and potential variation among royal jelly samples secreted by RJBs and unselected Italian bees on the level of volatile metabolites.