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Effects of Extraction Methods on Volatile Oil Profiles of *Cinnamomi ramulus–Zingiberis rhizoma recens* Couplet Medicines

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Abstract: *Cinnamomi ramulus* (CR, Guizhi in Chinese) and *Zingiberis rhizoma recens* (ZRR, Shengjiang in Chinese) are couplet medicines (drug pairs or drug combinations) in traditional Chinese medicine prescriptions. They contain a high amount of volatile oils which endow them with unique flavors and pharmacological activities. Extraction methods have a great influence on the extraction efficiency and composition of volatile oils. Firstly, the volatile oils of CR and ZRR were extracted by steam distillation (SD) and analyzed by GC-MS to obtain their chemical profiles. In total, 35 and 55 compounds were identified in the volatile oils of CR and ZRR, respectively. In order to find a suitable extraction method for the couplet medicine CR-ZRR, subsequently, steam distillation (SD), azeotropic distillation (AD) and supercritical fluid extraction (SFE) were applied to extract the volatile oils from CR-ZRR. The average extraction yields by SD, AD and SFE were 0.573%, 0.62% and 2.135%, respectively. The chemical composition of the volatile oils was then analyzed by GC-MS. In total, 73, 59 and 71 compounds were identified from the extracts obtained by SD, AD and SFE, respectively. Principal component analysis (PCA) and OPLS-DA showed that citral, (E)-cinnamaldehyde dimethyl acetal, zingiberene, cinnamaldehyde and β-sesquiphellandrene were the main contributors to distinguish the volatile oils that were obtained by different processes. Considering the chemical diversity and the total content of the main bioactive components of the volatiles oils, SD was more suitable for CR-ZRR volatile oil extraction. This study provides a basis for elucidating the chemical composition and suitable extraction method for the volatile oils of CR-ZRR.

Keywords: *Cinnamomi ramulus*; *Zingiberis rhizoma recens*; volatile oil; steam distillation; azeotropic distillation; supercritical fluid extraction; HS-GC-MS

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1. Introduction

Couplet medicines are those medicines that normally are used together in prescriptions according to the guidance of traditional Chinese medicine. Couplet medicines are assembled based on their tropism of taste and compatibility. The study of the compatibility of couplet medicines is important for the elucidation of the scientific connotation

of prescriptions. *Cinnamomi ramulus* (CR, Guizhi in Chinese) and *Zingiberis rhizoma recens* (ZRR, Shengjiang in Chinese) are classic couplet medicines in traditional Chinese medicine prescriptions [1,2]. For instance, 80 and 75 prescriptions in the "Treatise on Cold Pathogenic and Miscellaneous Diseases" included CR and ZRR, and they were used as couplet medicines over 40 times [3–5].

CR and ZRR have a distinct impact on organs in the human body, such as the heart, lung, spleen, stomach and others. The function of CR is to eliminate colds and alleviate problems in the body. It can stimulate the meridians and promote the circulation of Qi and blood [6]. ZRR has shown effects of relieving colds, warming the stomach, relieving vomiting and coughing and reducing the toxicity of seafood [7]. Chemical and pharmacology studies have indicated CR and ZRR contain a large amount of volatile oils which show considerable bioactivity and are contributors to their clinical efficacies [8–10].

Volatile oil is a large class of active ingredients in CR and ZRR [11]. Natural volatile oils extracted from herbs are commonly used as natural antibacterial agents, and in the near future, volatile oils may become a class of drugs for treating diseases [12–14].

Currently, there are multiple methods for extracting volatile components, such as steam distillation (SD), azeotropic distillation (AD) and supercritical fluid extraction (SFE) [15–18]. SD is a simple and convenient method for volatile oil extraction. However, it only involves one gas–liquid mass transfer and equilibrium process [19]. Based on the traditional steam extraction of volatile oil devices, a distillation column is added to achieve a multistage gas–liquid equilibrium and multistage gas–liquid mass transfer effect. The higher the number of stages (theoretical plates) of the distillation apparatus, the higher the concentration of volatile components enriched in the gas phase, and therefore the higher the extraction efficiency of the volatile oil [20,21]. Compared to SD and AD, SFE with carbon dioxide as the extraction medium is a rapid and solvent-free sample extraction technique [22,23]. SFE has been used widely in the plant-extraction and pharmaceutical industry.

Couplet medicines work as a bridge connecting single herbs and complex prescriptions. Starting from the drug combinations of CR and ZRR, the best extraction method for the volatile oils was determined. The extraction rates and chemical profiles of the volatile oils extracted by SD, AD and SFE from CR-ZRR were investigated. HS-GC-MS was applied for the qualitative and quantitative analysis of the volatile components. The obtained volatile oil profiles were compared and investigated with multivariate statistical analysis methods.

2. Materials and Methods

2.1. Chemicals and Reagents

HPLC grade methanol was purchased from Fisher Scientific (Pittsburgh, PA, USA). Ultrapure water was prepared by the Synergy water purification system (Millipore, Billerica, MA, USA). Chemical reagent n-hexane (GC grade) was obtained from Shanghai Aladdin Biochemical Technology Co., Ltd. (Shanghai, China). Other chemicals and reagents were analytical grade.

2.2. Materials

CR (origin: Guangxi, China. Lot: 25623003) and ZRR (origin: Hebei, China. Batch number: 2001588) were purchased from Hebei Renxin Pharmaceutical Co., Ltd. (Anguo, China) on 15 April 2023 and air-dried at room temperature (25 °C). Both of them were identified by Professor Fangjie Hou, Department of Traditional Chinese Medicine Identification and Processing, Hebei University of Chinese Medicine, China. CR-ZRR couplet medicines were prepared by mixing CR and ZRR in a 1:1 ratio according to the prescription. The voucher specimens were preserved in the medicinal plant herbarium, Hebei University of Chinese Medicine, China.

2.3. Methods

2.3.1. Extraction of Volatile Oils by Steam Distillation (SD)

In total, 100 g of medicinal herb materials (CR, ZRR or CR-ZRR) was soaked in 1 L water for 30 min. Then, steam distillation (Clevenger-type device) was performed for 6 h until the volatile oils were completely extracted. Five replicates were performed under the same conditions.

2.3.2. Extraction of Volatile Oils by Azeotropic Distillation (AD)

An azeotropic distillation column was equipped on a steam distillation device. In total, 100 g of CR-ZRR was distilled with water for 6 h until the volatile oils were completely extracted. Five replicates were performed under the same conditions.

2.3.3. Extraction of Volatile Oils by Supercritical Fluid Extraction (SFE)

A total of 2500 g of CR-ZRR was mixed with 2 mm glass beads and loaded into a 5 L SFX220 supercritical fluid extractor (Lincoln, NE, USA). The extraction temperature was set to 45 °C and the pressure to 25 MPa. The pressures of the first- and second-stage separation kettle were set to 8 MPa and 6 MPa, respectively. The frequency of the pump was set at 18 Hz. Then, the herbs were dynamically extracted with CO_2 for 2 h at a flow rate of 50 L/h. The extracts of the different stages were collected and combined as a total extract. Five replicates were performed under the same conditions.

2.4. Analysis of Volatile Oils by HS-GC-MS

An Agilent 7697A GC-MS (Agilent, Santa Clara, CA, USA) and HP-5MS capillary column (30 m × 0.25 mm, 0.25 mm film thickness, Agilent, Santa Clara, CA, USA) were used for GC-MS analysis. Helium (≥99.999%) was used as the carrier gas at a constant flow rate of 1.0 mL·min^{-1}.

The extracted volatile oils were dried with N_2 gas and anhydrous sodium sulfate, then diluted 50 times with HPLC grade methanol (Fisher Scientific, Pittsburgh, PA, USA). Then, 50 μL of the diluted volatile oils was taken into a 20 mL headspace sample vial and balanced at 50 °C for 15 min. Then, the evaporated sample was introduced into the GC injector in a split mode at a temperature of 50 °C, and the split ratio was set to 25:1. The quantitative ring was 1 mL with its temperature set at 110 °C. The transmission line was regulated to 120 °C. The oven temperature program was initially set at 60 °C and held for 2 min, then raised to 70 °C at a rate of 2 °C·min^{-1}, followed by raising the temperature to 76 °C at a rate of 1 °C·min^{-1}. Subsequently, it was raised to 84 °C at a rate of 2 °C·min^{-1} and then increased to 114 °C at a rate of 1 °C·min^{-1}. Finally, the temperature was held constantly at 114 °C for 10 min. The ionization voltage of the electron-impact (EI) ion source was 70 eV. The mass spectrometer operates in a full scan mode with a scan range of 50–550 m/z. n-Alkane standard solution (C8–C20, 40 mg·L^{-1}, Sigma-Aldrich, Buchs, Switzerland) was analyzed under the same conditions for retention index (RI) calculation.

2.5. Data Processing and Statistical Analysis

The yields of the volatile oils were calculated based on the volume (mL) of the extracted volatile oils from 100 g of material. Five baches of extractions were processed as replicates with their average yields and standard deviations were calculated with Excel. For qualitative analysis, the spectrums of the corresponding metabolites detected by GC-MS with similarities more than 80% to those spectrums of standard compounds in the NIST17 standard library were identified using the Agilent MassHunter analysis program (Agilent, Santa Clara, CA, USA). For quantitative analysis, the peak areas of identified metabolites were extracted and considered as variables for analysis. The transfer rates of the chemical components were calculated by using the corresponding peak area of CR-ZRR divided by half of the sum of the peak areas of CR and ZRR and then multiplied by 100%. SIMCA P13 software (Umetrics, Umea, Sweden) was used for principal component analysis (PCA). The cluster analysis (CA) and heatmap were performed with Origin Pro 2020 (OriginLab

Corporation, Northampton, MA, USA) software. The significant levels of the candidates were presented with lower case letters according to results of the Tamheini test, which was performed with SPSS Statistics 23.0 (IBM, Armonk, NY, USA) software.

3. Results

3.1. Extraction of Volatile Oils from CR, ZRR and CR-ZRR by SD

3.1.1. Yields of Volatile Oils from CR, ZRR and CR-ZRR by SD

SD is a frequently used method for the extraction of volatile oils due to its affordability and accessibility in comparison to AD and SFE. Therefore, we first extracted the volatile oils of CR, ZRR and CR-ZRR by SD to compare the volatile oil profile of CR-ZRR with the profiles of the two single herbs.

The volatile oil yields of CR, ZRR and CR-ZRR by SD were 0.940% ± 0.004%, 0.182% ± 0.006% and 0.573% ± 0.009% (X^{-} ± standard deviation, n = 5), respectively (Figure 1). The yield of the CR-ZRR volatile oil was approximately equal to half of the sum of the oil yield of CR and ZRR.

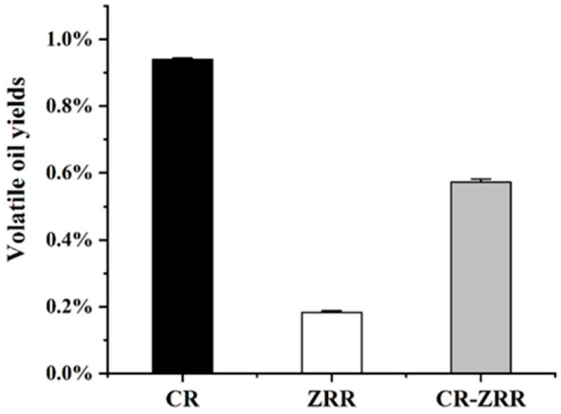

Figure 1. The yield of volatile oils of CR, ZRR and CR-ZRR obtained by SD.

3.1.2. Chemical Composition of CR, ZRR and CR-ZRR Volatile Oils

The profiles of volatile oils were obtained by HS-GC-MS. In total, 80 compounds were identified and relatively quantified (Figures 2 and 3, Table 1). Fifteen of them were present in all the extracted volatile oils. In total, 34 compounds were identified in the volatile oil of CR. Cinnamaldehyde (74.56%) and (Z)-cinnamaldehyde dimethyl acetal (20.28%) were the most abundant constituents. It was reported that cinnamaldehyde served as the primary source of CR's pungent flavor [24,25] At the same time, a small amount of estragole (0.29%) was detected in CR, which was absent in the volatile oils of ZRR and CR-ZRR (Table 1).

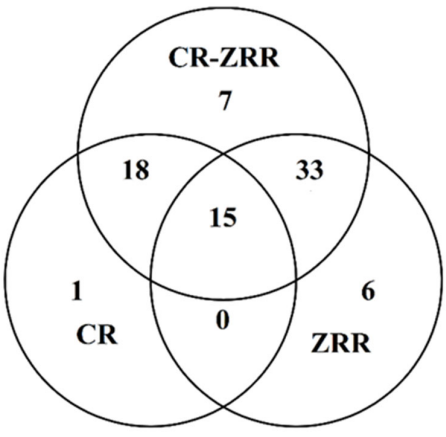

Figure 2. Venn diagram showing the overlap and distinctions of the chemical compositions of CR, ZRR and CR-ZRR obtained by SD.

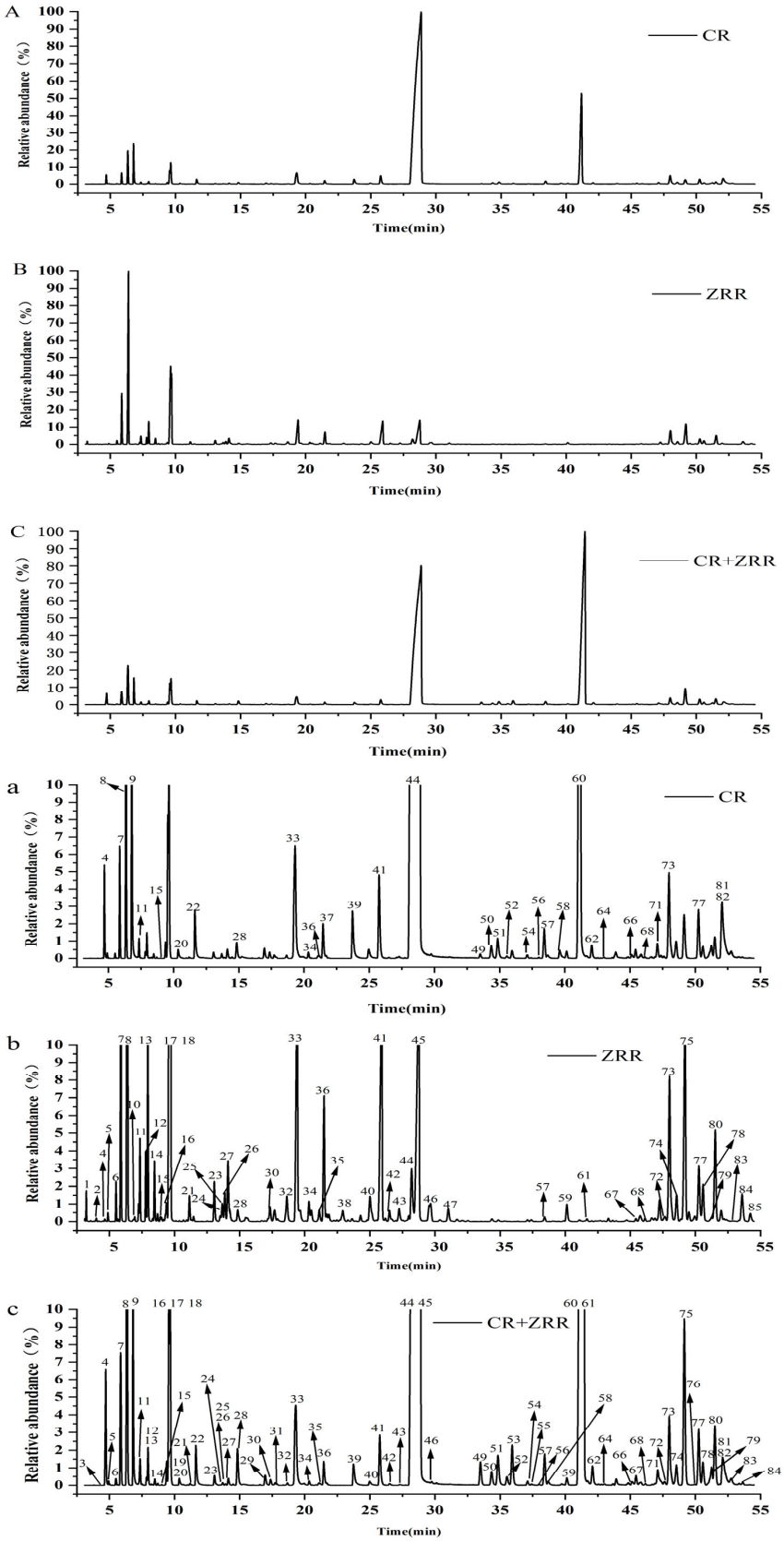

Figure 3. The total ion chromatograms (TICs) of the volatile oils extracted by SD from (**A**) CR, (**B**) ZRR and (**C**) CR-ZRR analyzed by HS-GC-MS. (**a**–**c**) are enlarged figures of (**A**–**C**), respectively.

Table 1. The information of the volatile compounds of CR, ZRR and CR-ZRR analyzed by GC-MS.

Peak No.	RT /min	RI	Compounds	Molecular Formula	Molecular Weight	CR —SD (%)	ZRR —SD (%)	CR+ZRR — SD (%)	CR+ZRR —AD (%)	CR+ZRR —SFE (%)
1	3.223	982	2-(pentyloxy)tetrahydro-2H-pyran	$C_{10}H_{20}O_2$	172.27	-	0.25	-	-	-
2	3.990	991	4-Methyloctane	C_9H_{20}	128.26	-	0.04	-	-	-
3	4.197	993	2-Propenal	C_3H_4O	56.00	-	-	1.50×10^{-5}	1.31×10^{-5}	-
4	4.685	998	Styrene	C_8H_8	104.15	0.02	0.04	0.44	0.03	-
5	4.909	1001	2-Heptanol	$C_7H_{16}O$	116.10	-	0.10	0.01	0.05	-
6	5.500	1007	Tricyclene	$C_{10}H_{16}$	136.23	-	0.43	0.44	0.03	-
7	5.866	1011	3-Carene	$C_{10}H_{16}$	136.23	0.05	5.93	0.61	0.50	0.05
8	6.342	1016	(+)-Camphene	$C_{10}H_{16}$	136.23	0.04	24.03	1.86	2.14	0.14
9	6.783	1021	Benzaldehyde	C_7H_6O	106.12	1.63	-	0.85	1.35	0.06
10	6.945	1023	Cosmene	$C_{10}H_{14}$	134.23	-	0.10	-	-	-
11	7.346	1027	Sabinen	$C_{10}H_{16}$	136.23	0.01	1.02	0.11	0.08	0.01
12	7.828	1032	Sulcatone	$C_8H_{14}O$	126.20	-	0.87	0.02	0.10	-
13	7.946	1034	(-)-β-Pinene	$C_{10}H_{16}$	136.23	-	2.93	0.07	0.26	0.02
14	8.471	1039	α-Phellandrene	$C_{10}H_{16}$	136.23	-	0.83	0.02	0.04	0.03
15	8.911	1044	2,2,4,4,6,8,8-Heptamethylnonane	$C_{10}H_{16}$	136.23	3.02×10^{-5}	0.07	2.07×10^{-5}	-	-
16	9.373	1049	*p*-Cymene	$C_{10}H_{14}$	134.23	-	0.40	0.09	0.06	0.02
17	9.553	1051	Pseudolimonene	$C_{10}H_{16}$	136.23	-	0.75	0.86	1.10	1.29
18	9.700	1052	Eucalyptol	$C_{10}H_{18}O$	154.23	-	7.16	1.09	2.09	-
19	10.343	1059	Salicylaldehyde	$C_7H_6O_2$	122.12	-	-	0.04	0.02	1.34×10^{-5}
20	10.421	1060	Benzyl 4-hydroxyphenyl ketone	$C_{14}H_{12}O_2$	212.24	0.03	-	2.33×10^{-5}	-	-
21	11.176	1068	4,5-Dimethylnonane	$C_{11}H_{24}$	156.31	-	0.48	0.01	0.01	0.06
22	11.624	1073	Acetophenone	C_8H_8O	120.15	0.06	-	0.26	0.06	4.72×10^{-7}
23	13.057	1089	α-Terpinene	$C_{10}H_{16}$	136.23	-	0.79	0.05	0.04	0.02
24	13.495	1094	2-Nonanone	$C_9H_{18}O$	136.23	-	0.04	1.18×10^{-5}	-	-
25	13.685	1096	cis-Chrysanthenol	$C_{10}H_{16}O$	152.22	-	0.24	0.01	0.04	0.03
26	13.870	1098	Rosefuran	$C_{10}H_{14}O$	150.22	-	0.36	1.58×10^{-5}	0.01	3.46×10^{-5}
27	14.129	1100	Linalool	$C_{10}H_{18}O$	154.25	-	0.95	0.04	0.16	0.06
28	14.841	1100	Benzaldehyde dimethyl acetal	$C_9H_{12}O_2$	152.15	0.11	0.31	0.30	0.58	1.18×10^{-5}
29	16.968	1100	4-Methoxystyrene	$C_9H_{10}O$	134.15	-	-	0.07	2.43×10^{-4}	1.73×10^{-5}
30	17.365	1100	(-)-Camphor	$C_{10}H_{16}O$	152.23	-	0.43	0.04	0.21	1.69×10^{-5}
31	17.707	1100	Camphene hydrate	$C_{10}H_{18}O$	154.10	-	-	0.01	0.04	0.02
32	18.662	1101	Citronellal	$C_{10}H_{18}O$	154.25	-	0.75	0.01	0.05	-
33	19.315	1101	Isoborneol	$C_{10}H_{18}O$	154.23	0.75	6.83	0.76	0.93	0.58
34	20.334	1101	4-Terpineol	$C_{10}H_{18}O$	154.23	0.01	0.44	0.03	0.05	0.03
35	21.133	1101	α-Terpineol	$C_{10}H_{18}O$	154.23	-	0.30	0.01	0.05	9.82×10^{-6}
36	21.463	1101	(-)-α-Terpineol	$C_{10}H_{18}O$	154.25	0.04	2.77	0.15	0.24	0.19
37	22.162	1101	Estragole	$C_{10}H_{12}O$	148.20	0.29	-	-	2.70×10^{-4}	0.02
38	22.940	1205	Decanal	$C_{10}H_{20}O$	156.27	-	0.35	-	3.17×10^{-4}	1.87×10^{-4}
39	23.723	1213	Cinnamic aldehyde	C_9H_8O	132.16	0.86	-	0.15	0.22	1.45×10^{-4}
40	24.985	1227	Benzenepropanol	$C_9H_{12}O$	136.15	-	0.88	4.26×10^{-4}	-	0.16
41	25.753	1236	(E)-verbenol	$C_{10}H_{16}O$	152.23	0.08	7.58	0.35	0.93	0.50

Table 1. *Cont.*

Peak No.	RT /min	RI	Compounds	Molecular Formula	Molecular Weight	CR —SD (%)	ZRR —SD (%)	CR+ZRR — SD (%)	CR+ZRR —AD (%)	CR+ZRR —SFE (%)
42	26.505	1244	(1R,4S)-1-Methyl-4-(prop-1-en-2-yl)cyclohex-2-enol	$C_{10}H_{16}O$	153.23	-	0.30	1.29×10^{-4}	0.04	0.03
43	27.242	1252	Geraniol	$C_{10}H_{18}O$	154.25	-	0.34	1.52×10^{-4}	-	0.09
44	28.212	1262	Cinnamaldehyde	C_9H_8O	132.16	74.56	1.64	35.15	47.77	38.77
45	28.622	1267	Citral	$C_{10}H_{16}O$	152.23	-	9.89	30.86	1.44×10^{-4}	0.80
46	29.652	1278	Heptadecane	$C_{21}H_{44}$	296.57	-	0.45	6.59×10^{-4}	0.04	0.07
47	31.056	1293	Methyl nonyl ketone	$C_{11}H_{22}O$	170.29	-	0.40	-	2.53×10^{-4}	0.10
48	32.237	1306	Cinnamyl Alcohol	$C_9H_{10}O$	134.10	-	-	-	3.91×10^{-4}	0.08
49	33.481	1318	Neral dimethyl acetal	$C_{12}H_{22}O_2$	198.20	4.60×10^{-5}	-	0.17	0.56	0.32
50	34.364	1327	(3,3-dimethoxypropyl)-Benzene	$C_{11}H_{16}O_2$	180.21	2.90×10^{-5}	-	0.12	0.08	1.24×10^{-5}
51	34.824	1332	(Z)-Cinnamaldehyde dimethyl acetal	$C_{11}H_{14}O_2$	178.10	5.81×10^{-5}	-	0.27	0.52	1.36×10^{-5}
52	35.542	1339	1H-Pyrrolo[3,2-d]pyrimidine-2,4(3H,5H)-dione	$C_6H_5N_3O_2$	151.12	1.16×10^{-5}	-	0.05	0.03	1.70×10^{-5}
53	35.952	1343	Geranial dimethyl acetal	$C_{12}H_{22}O_2$	198.20	-	-	0.26	0.83	0.53
54	37.143	1355	Cubenene	$C_{15}H_{24}$	204.35	8.22×10^{-5}	-	0.03	1.60×10^{-5}	0.11
55	37.398	1358	β-Humulene	$C_{15}H_{24}$	204.20	-	-	4.02×10^{-3}	3.06×10^{-5}	0.12
56	37.982	1364	Ylangene	$C_{15}H_{24}$	204.20	1.03×10^{-4}	-	0.01	3.35×10^{-5}	0.16
57	38.421	1368	(-)-α-Copaene	$C_{15}H_{24}$	204.35	0.01	0.12	0.23	0.01	0.44
58	39.611	1380	Methyl Cinnamate	$C_{10}H_{10}O_2$	162.18	0.14	-	0.05	0.07	0.01
59	40.132	1385	1,5-Cyclodecadiene	$C_{15}H_{24}$	204.35	-	0.48	0.05	-	0.55
60	41.181	1396	(E)-Cinnamaldehyde dimethyl acetal	$C_{11}H_{14}O_2$	178.21	20.28	-	21.53	38.47	8.20
61	41.663	1401	Sesquithujene	$C_{15}H_{24}$	204.35	-	3.49×10^{-5}	0.02	5.50×10^{-5}	0.10
62	42.086	1406	Caryophyllene	$C_{15}H_{24}$	204.20	1.27×10^{-4}	-	0.13	-	0.13
63	42.499	1411	(-)-Isosativene	$C_{15}H_{24}$	204.20	-	-	-	3.48×10^{-5}	7.93×10^{-6}
64	42.981	1417	Cis-β-Copaene	$C_{15}H_{24}$	204.20	3.40×10^{-4}	-	0.01	-	0.04
65	43.590	1424	Coumarin	$C_9H_6O_2$	146.00	-	-	-	-	0.56
66	45.126	1442	(E)-Cinnamyl acetate	$C_{11}H_{12}O_2$	176.21	0.25	-	0.02	-	3.05×10^{-5}
67	45.418	1445	Alloaromadendrene	$C_{15}H_{24}$	204.35	-	0.06	0.05	-	0.16
68	46.139	1454	β-Springene	$C_{20}H_{32}$	272.47	0.08	0.11	0.01	-	0.17
69	46.649	1460	γ-Gurjunene	$C_{15}H_{24}$	204.20	-	-	-	-	0.06
70	46.913	1463	Selina-4(15),7(11)-diene	$C_{15}H_{24}$	204.20	-	-	-	-	0.04
71	47.094	1465	γ-Cadinene	$C_{15}H_{24}$	204.35	0.03	-	0.09	-	1.09
72	47.638	1472	δ-Bisabolene	$C_{15}H_{24}$	204.35	-	0.17	0.01	-	0.08
73	47.992	1476	α-Curcumene	$C_{15}H_{22}$	202.35	0.06	4.30	0.46	-	3.72
74	48.549	1483	(+)-Calarene	$C_{15}H_{24}$	204.35	-	0.78	0.13	0.01	1.41
75	49.152	1490	Zingiberene	$C_{15}H_{24}$	204.35	-	6.04	0.28	0.05	22.45
76	49.929	1499	1,2,3,4,4a,5,6,7-octahydro-4-methyl-7-methylene-1-(1-methylethyl)-, (1S,4S,4aR)-Naphthalene	$C_{15}H_{24}$	204.20	-	-	1.50×10^{-4}	0.05	0.15
77	50.252	1503	β-Bisabolene	$C_{15}H_{24}$	204.35	0.03	1.61	0.36	-	3.75

Table 1. *Cont.*

Peak No.	RT /min	RI	Compounds	Molecular Formula	Molecular Weight	CR —SD (%)	ZRR —SD (%)	CR+ZRR — SD (%)	CR+ZRR —AD (%)	CR+ZRR —SFE (%)
78	50.594	1507	α-Farnesene	$C_{15}H_{24}$	204.35	-	1.06	0.14	3.72×10^{-5}	3.35
79	51.250	1515	(+)-δ-Cadinene	$C_{15}H_{24}$	204.35	-	0.13	0.12	-	1.26×10^{-4}
80	51.505	1518	β-Sesquiphellandrene	$C_{15}H_{24}$	204.35	-	2.57	0.37	-	6.93
81	52.065	1524	2-Methoxycinnamaldehyde	$C_{10}H_{10}O_2$	162.18	0.58	-	0.26	4.35×10^{-5}	1.71
82	52.385	1528	(Z)-2-Methoxycinnamaldehyde	$C_{10}H_{10}O_2$	162.10	1.15×10^{-4}	-	1.00×10^{-3}	-	1.63×10^{-4}
83	52.764	1532	α-Calacorene	$C_{15}H_{20}$	200.20	-	3.85×10^{-5}	1.21×10^{-3}	4.24×10^{-5}	-
84	53.591	1542	α-Elemol	$C_{15}H_{26}O$	222.37	-	0.88	0.01	-	0.42
85	54.197	1549	(Z)-Sesquisabinene hydrate	$C_{15}H_{26}O$	222.37	-	0.24	-	-	1.42×10^{-4}

Note: '-' indicates that the component was not detected.

In total, 54 components were characterized in the volatile oil of ZRR. The main constituents were (+)-camphene (24.03%), citral (9.89%), (E)-verbenol (7.58%), eucalyptol (7.16%), isoborneol (6.83%) and zingiberene (6.04%). They accounted for over 60% of the total volatile oil in ZRR. At the same time, a small amount of 2-(pentyloxy) tetrahydro-2H-pyran (0.25%), 4-methyloctane (0.04%), cosmene (0.10%), decanal (0.35%), methyl nonyl ketone (0.40%) and (Z)-sesquisabinene hydrate (0.24%) were also detected, which were not detected in the volatile oils of CR and CR-ZRR.

The main components of CR-ZRR volatile oil included cinnamaldehyde (35.15%), citral (30.86%) and (E)-cinnamaldehyde dimethyl acetal (21.53%). Although no new compounds were detected in CR-ZRR, major compounds in CR and ZRR were also detected at relatively high levels in CR-ZRR. The chemical profile of CR-ZRR was a comprehensive combination of CR and ZRR profiles but not a simple average profile of them. Such a result indicated interactions between CR and ZRR during extraction. In addition, seven chemical constituents were detected exclusively in CR-ZRR, including 2-propenal, salicylaldehyde (0.04%), 4-methoxystyrene (0.07%), camphene hydrate (0.01%), geranial dimethyl acetal (0.26%), β-humulene and 1,2,3,4,4a,5,6,7-octahydro-4-methyl-methylene-1-(1-methylethyl)-(1S,4S,4a R)-Naphthalene.

3.1.3. The Component Transfer Rate from CR and ZRR to Couplet Medicines

Further, the transfer rates of the detected components were calculated by comparing their peak areas in CR-ZRR with the half peak areas in CR and ZRR. The results (Table 2) indicated the extraction efficiencies of some components were enhanced in CR-ZRR. For instance, the transfer rates of styrene (peak 4), acetophenone (peak 22), citral and γ-cadinene were 3414.18%, 2142.07%, 1185.01% and 1572.11%, respectively. The changes in component transfer rates might be due to the promotion effects of low-boiling-point compounds in CR on the extraction of high-boiling-point components in ZRR.

Table 2. Transfer rates of the detected components.

No.	Compounds	Peak Area			Transfer Rates (%)
		CR	ZRR	CR-ZRR	
4	Styrene	14691	39598	926761	3414.18
5	2-Heptanol	-	112327	20940	37.28
6	Tricyclene	-	481270	926761	385.13
7	3-Carene	42254	6629112	1292984	38.76
8	(+)-Camphene	29636	26853528	3958503	29.45
9	Benzaldehyde	1350684	-	1810546	268.09
11	Sabinen	4930	1141339	223776	39.04
16	*p*-Cymene	-	451937	188831	83.57
17	Pseudolimonene	-	834509	1818212	435.76
18	Eucalyptol	-	8007077	2323724	58.04
22	Acetophenone	50856	-	544685	2142.07
23	α-Terpinene	-	878417	97914	22.29
25	cis-Chrysanthenol	-	269432	29053	21.57
27	Linalool	-	1064598	90822	17.06
28	Benzaldehyde dimethyl acetal	90889	348585	631424	287.35
30	(-)-Camphor	-	481313	77278	32.11
33	Isoborneol	624216	7628668	1611529	39.05
34	4-Terpineol	5554	492279	53678	21.56
36	(-)-α-Terpineol	36921	3091945	313114	20.01
39	Cinnamic aldehyde	711804	-	318950	89.62
41	(E)-verbenol	63296	8466831	745173	17.47
44	Cinnamaldehyde	61651195	1837030	74628567	235.09
45	Citral	-	11056141	65508050	1185.01

Table 2. *Cont.*

No.	Compounds	Peak Area			Transfer Rates (%)
		CR	ZRR	CR-ZRR	
57	(-)-α-Copaene	7144	130396	480610	698.87
58	Methyl Cinnamate	114634	-	104134	181.68
59	1,5-Cyclodecadiene	-	538380	98747	36.68
60	(E)-Cinnamaldehyde dimethyl acetal	16766672	-	45702592	545.16
66	(E)-Cinnamyl acetate	208628	-	45470	43.59
67	Alloaromadendrene	-	65881	111250	337.73
68	β-Springene	64871	121349	17654	18.96
71	γ-Cadinene	23786	-	186971	1572.11
72	δ-Bisabolene	-	190706	27706	29.06
73	α-Curcumene	50342	4803278	984293	40.56
74	(+)-Calarene	-	869494	286210	65.83
77	β-Bisabolene	28633	1801059	773487	84.55
78	α-Farnesene	-	1188897	300444	50.54
79	(+)-δ-Cadinene	-	146588	244839	334.05
80	β-Sesquiphellandrene	-	2877551	777295	54.02
81	2-Methoxycinnamaldehyde	478059	-	556297	232.73

3.2. Effects of Different Extraction Methods on the Chemical Composition of Volatile Oil from CR-ZRR

3.2.1. Yields of Volatile Oil from CR-ZRR by Different Extraction Methods

To scrutinize the effects of different extraction methods, SD, AD and SFE were applied for the extraction of volatile oils from CR-ZRR. The volatile oil yield by SD was 0.573 ± 0.009%, by AD was 0.62 ± 0.003% and by SFE was 2.135 ± 0.002% (X^{-} ± SD, n = 5) (Figure 4). The volatile oil yield by SFE was significantly higher than by SD and AD. There is no significant difference in the volatile oil yield between the SD and AD group.

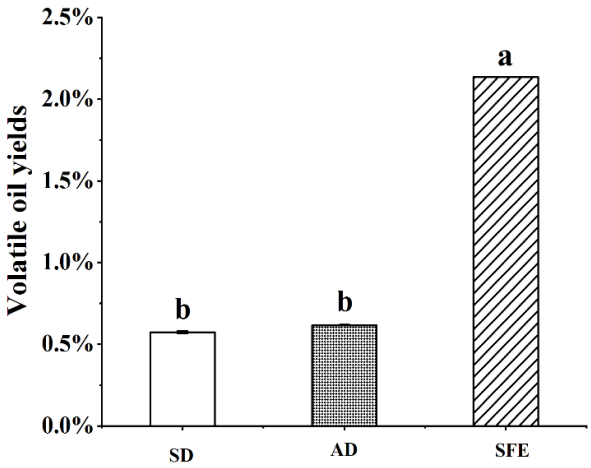

Figure 4. The yields of volatile oil from CR-ZRR obtained by SD, AD or SFE (X^{-} ± SD, n = 5). "a" and "b" represents the significant levels ($p < 0.05$) according to results of the Tamheini test.

3.2.2. Comparison of the Chemical Profiles of Volatile Oils from CR-ZRR by Different Extraction Methods

In order to compare profiles of the volatile oils extracted from CR-ZRR by SD, AD and SFE (Figure 4), the peaks were identified by comparing their mass spectrums with that of the standards in NIST (17.0). The peak areas of the identified chemical components were extracted and analyzed for relative contents by Quant Analysis (B.09.00) software. A total of 82 compounds were identified in the volatile oils of CR-ZRR. Further, 73, 59 and 71 compounds were detected in the volatile oils extracted by SD, AD and SEF, re-

spectively (Figures 5 and 6, Table 1). Three components were detected exclusively in the volatile oils extracted by SD, which were 2,2,4,6,8,8-heptamethylnonane (peak 15), benzyl 4-hydroxyphenyl ketone (peak 20) and 2-nonanone (peak 24), as shown in Table 1.

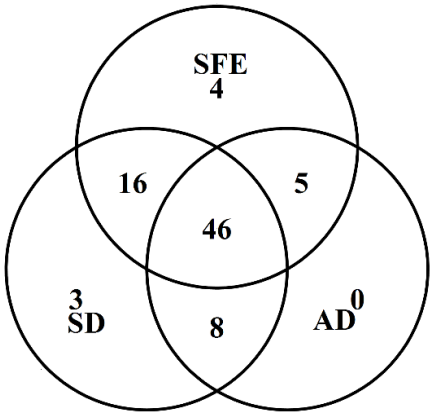

Figure 5. Venn diagram showing the overlap and distinctions of the chemical composition of CR-ZRR obtained by SD, AD or SFE.

The main components in CR-ZRR volatile oils extracted by SD and AD were cinnamaldehyde (peak 44) and (E)-cinnamaldehyde dimethyl acetal (peak 60), which accounted for over 85% of the total volatile oils.

Besides cinnamaldehyde (38.77%) and (E)-cinnamaldehyde dimethyl acetal (8.20%), the main components of CR-ZRR volatile oil extracted by SFE also contained a high amount of zingiberene (22.45%) (peak 75). They accounted for 69.42% of the total volatile oil. In addition, four components, including coumarin, γ-gurjunene, selina-4(15), 7(11)-diene and (Z)-sesquisabinene hydrate, were unique in the volatile oils extracted by SFE (Figure 5). This might be due to the wide dissolution range of supercritical extraction fluid, which is different from the extraction principle of steam distillation and azeotropic distillation.

The above results revealed variations in the chemical compositions of CR-ZRR volatile oils extracted by different extraction processes. SD mainly collects the low-boiling-point components of the mixed volatile oil, which are mainly derived from CR, and the relatively high content of (+)-citral from ZRR. SFE collects mainly the components with higher boiling points, mostly from ZRR, while AD combines the characteristics of SFE and SD, with different degrees of the collection of components from low to high boiling points. AD is more suitable for the extraction of mixed volatile oils from CR and ZRR.

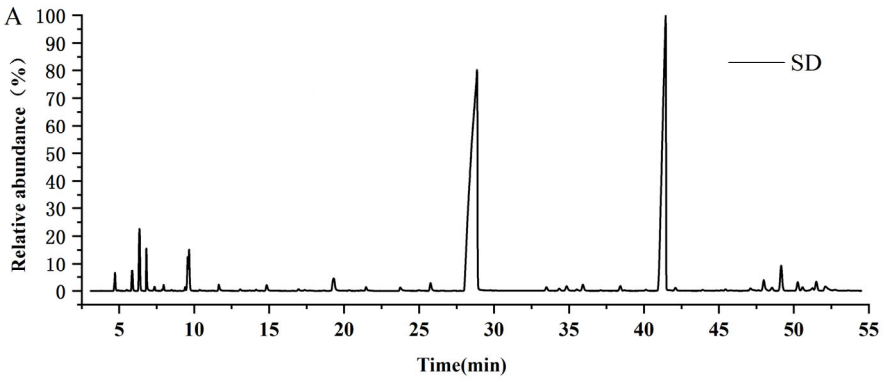

Figure 6. *Cont.*

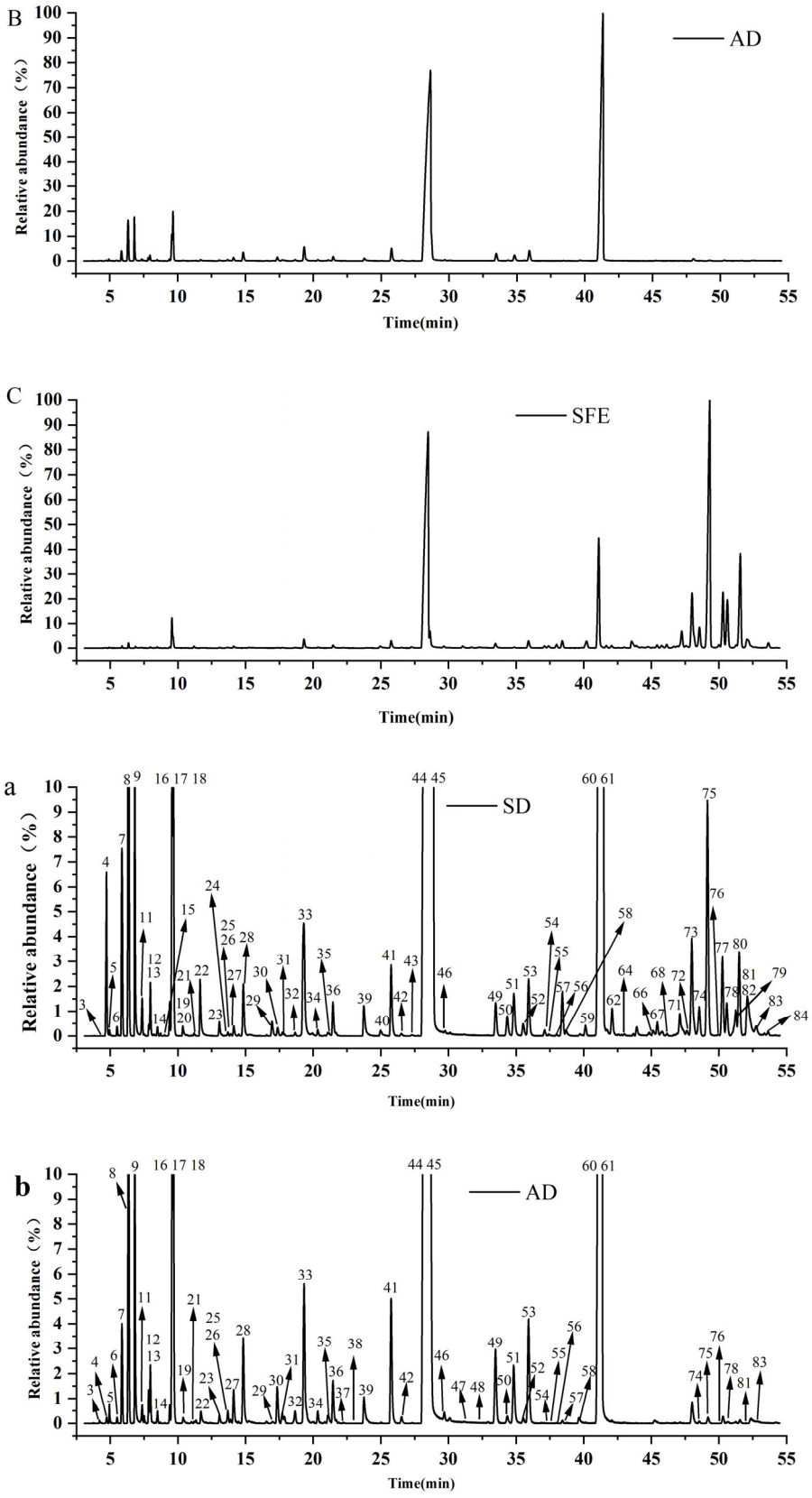

Figure 6. *Cont*.

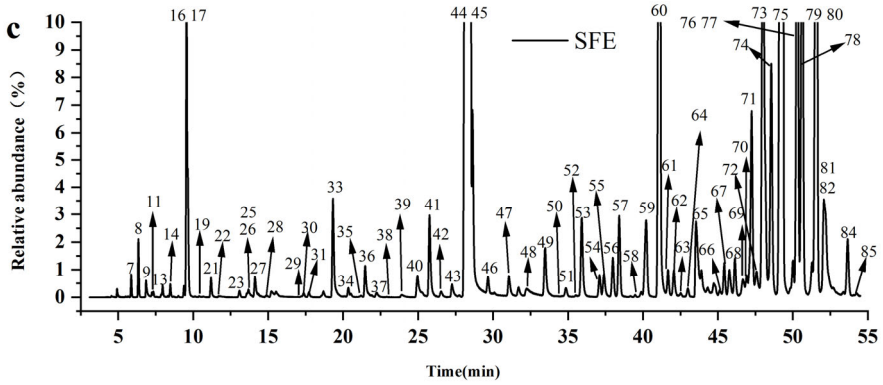

Figure 6. The total ion chromatograms (TICs) of volatile oils extracted by (**A**) SD, (**B**) AD and (**C**) SFE from CR-ZRR analyzed by HS-GC-MS. (**a**–**c**) are enlarged figures of (**A**–**C**), respectively.

3.2.3. PCA and OPLS-DA

Unsupervised principal component analysis (PCA) was performed by importing the normalized peak areas of the compounds into SIMCA 14.1 software. As shown in the PCA score plot (Figure 7), the samples extracted by SD, AD and SFE were obviously dispersed among three regions, indicating discrepancies in their volatile profiles.

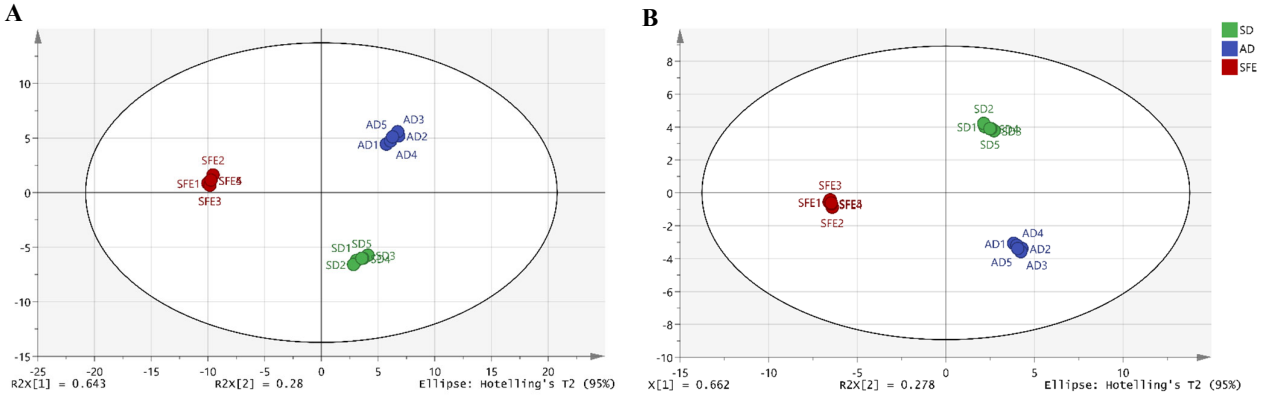

Figure 7. (**A**) PCA (PC1 64.3%, PC2 28%) and (**B**) OPLS-DA (PC1 66.2%, PC2 27.8%) score plots of CR-ZRR volatile oils obtained by different extraction processes.

Then, supervised orthogonal partial least squares discriminant analysis (OPLS-DA) was performed for the screening of differential components. A permutation test (n = 200) was performed to confirm the validity of the OPLS-DA model (Figure 7B). The results of R2Y = 0.988 and Q2 = 0.978 suggested the good stability and predictability of the established mode, and there was no overfitting phenomenon. As shown in the OPLS-DA score plot, the volatile oils of CR-ZRR obtained from different extraction processes can be clearly grouped in three regions, similar to the results of PCA. Furthermore, the variable importance in projection (VIP) predictive value of each component in the OPLS-DA model was used to screen the differential compounds. In the OPLS-DA model, compounds with VIP > 2 had a meaningful impact on the classification of the three groups (Supplementary Figure S1). Based on this foundation, five differential compounds were screened out, which were citral (VIP = 4.78824), (E)-cinnamaldehyde dimethyl acetal (VIP = 3.87469), zingiberene (VIP = 3.56402), cinnamaldehyde (VIP = 2.99252) and β-sesquiphellandrene (VIP = 2.00189).

Cinnamaldehyde and (E)-cinnamaldehyde dimethyl acetal were the main and effective components of CR, while zingiberene was the representative component of ZRR. Thus, these components play an important role in evaluating the quality of CR-ZRR volatile oils. There was no significant difference in the content of cinnamaldehyde (Figure 8). The content of (E)-cinnamaldehyde dimethyl acetal in the SD and AD groups were significantly

higher than those in the SFE group (Figure 8). While the concentrations of zingiberene and β-sesquiphellandrene in the SFE group were significantly higher than those in the SD and AD group (Figure 8). The content of citral in the SD group was significantly higher than that in the AD and SFE groups. To sum up, the total peak area of these five principal components was the highest in the SD group, followed by the SFE group, and the AD group showed the lowest peak areas (Figure 8).

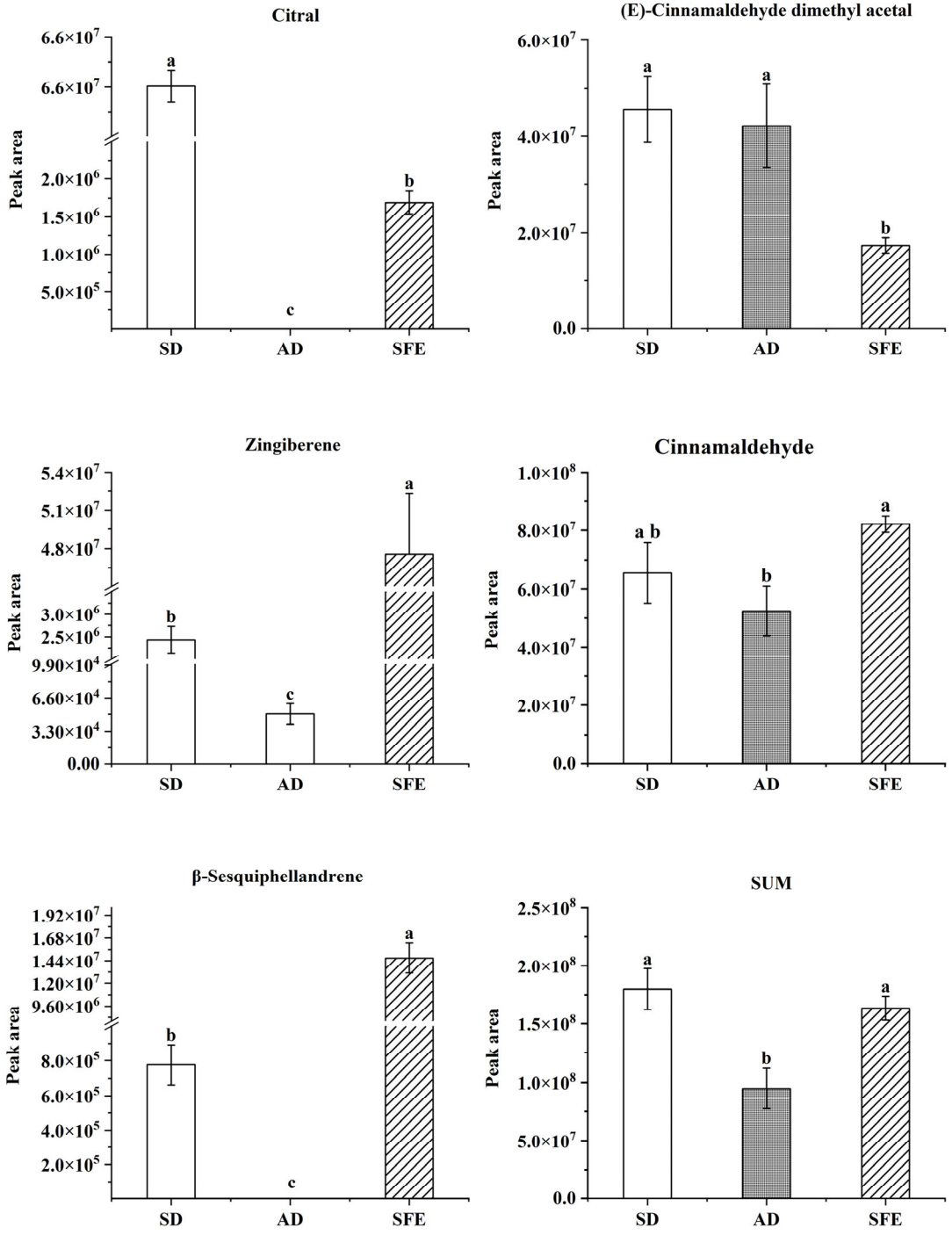

Figure 8. Comparison of the relative contents of the differential compounds extracted by different methods. "a", "b" and "c" represents the significant levels ($p < 0.05$) according to results of the Tamheini test.

3.2.4. Clustering Analysis

Clustering analysis (CA) was performed, and the result was demonstrated with a heatmap (Figure 9). The volatile oil samples obtained by SD, AD and SFE were grouped into three groups, indicating differences in their composition. Samples extracted by SD and AD comprised a sub-branch which was separated from the branch containing samples extracted by SFE. Such a result indicated a greater similarity between samples extracted by SD and AD and a greater discrepancy with samples extracted by SFE.

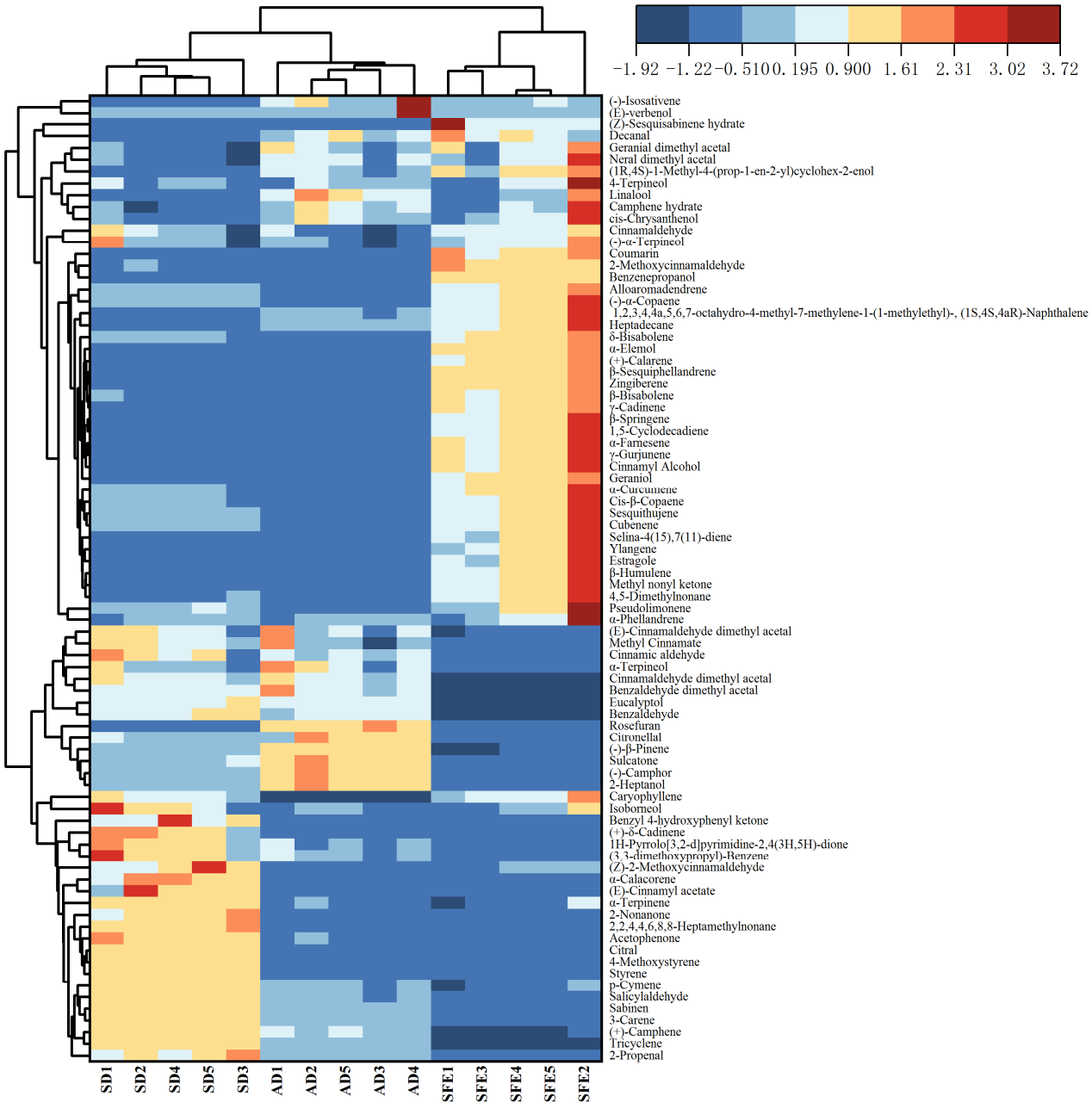

Figure 9. Heatmap of the components of the mixed volatile oil samples from CR and ZRR obtained by different extraction processes.

The heatmap visually displays the relative content of the volatile components in the samples obtained through different processes, which is convenient for screening the suitable extraction process of volatile oils from CR and ZRR. The results showed that the relative contents of citral, cinnamaldehyde and (E)-cinnamaldehyde dimethyl acetal have a

relatively higher concentration in SD, while zingiberene and β-sesquiphellandrene were higher in SFE.

4. Discussion

Chinese couplet medicines are composed of selected traditional Chinese medicinal herbals that have been widely used clinically for thousands of years. Couplet medicines are assembled based on their tropism of taste and compatibility according to the guidance of traditional Chinese medicine. Modern research has revealed that the chemical composition of couplet medicine extracts are not simply a summing up of the chemical compositions of single medicines [26]. Such variation indicates interactions between herbs during extraction. Physical changes as well as chemical reactions might occur during the extraction process [27,28].

In terms of chemical composition, the volatile composition of CR-ZRR was not simply an average profile of CR and ZRR. The transfer rates of the different components varied significantly. For instance, the transfer rates of styrene, acetophenone, citral and γ-cadinene were 3414.18%, 2142.07%, 1185.01% and 1572.11%, respectively, while the transfer rates of linalool, (E)-verbenol and β-springene were less than 20%. The changes in component transfer rates might be due to the promotion effects of low-boiling-point compounds in CR on the extraction of high-boiling-point components in ZRR. Chemical reactions such as oxidation, reduction, condensation and hydrolysis [27,28] might have happened during the extraction process, which resulted in the decrease in the extraction rates of some components, especially for herbal medicines such as CR and ZRR that are rich in highly active components such as alkenes, aldehydes, ketones and alcohols.

It is foreseeable that further research work is still necessary to uncover the mechanisms of the interactions of couplet medicines. And the evaluation of the pharmacological activity of the volatile oils of single medicines and couplet medicines extracted with different methods would also be helpful for revealing the formulation and compatibility of couplet medicines.

5. Conclusions

The volatile oil profiles of CR, ZRR and CR-ZRR by SD revealed the synergistic effects of some components. Analysis of the volatile oils of CR-ZRR by SD, AD and SFE revealed the most chemically diverse volatile oils were obtained by SD. Five differential components including cinnamaldehyde, (E)-cinnamaldehyde dimethyl acetal, zingiberene, citral and β-sesquiphellandrene were screened out by multivariate statistical methods. Considering the total contents of them, it was concluded that SD was more suitable for extracting volatile oils from CR-ZRR compared to AD and SPF.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/separations11070206/s1, Figure S1: VIP value diagram.

Author Contributions: X.X.: data curation, formal analysis, writing—original draft; X.L.: data curation, formal analysis, writing—original draft; J.C.: data curation, formal analysis, methodology; C.S.: formal analysis, methodology; X.S.: data curation, formal analysis; L.W.: funding acquisition, writing—review and editing; C.L.: conceptualization, funding acquisition, resource, supervision, writing—review and editing. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement: The original contributions presented in the study are included in the article and Supplementary Materials, further inquiries can be directed to the corresponding authors.

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Conflicts of Interest: The authors declared no conflicts of interest.

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