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GC-MS based metabolomics to evaluate three commercial products of *Chrysanthemun morifolium Hang-ju* in different flowering and processing stages

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Abstract:

Hang-ju was one of five officinal varieties of Flos chrysanthemum for its edible and potable usage. Besides Flos Chrysanthemum (FL), there were also Bud Chrysanthemum (BC) and Fetal Chrysanthemum (FC) at the early and late stage of buds, respectively, in the consumption market of *Hang-ju* with higher prices. Whether the quality and efficiency of BC and FC was superior to FL or merely consumption misunderstandings? Three commercial products of Hongxinju, a representive cultivar of *Hang-ju* were studied with a GC-MS based metabolomics approach, complemented with morphology, contents of moisture and protein and the anti-oxidant activity, to reveal the metabolic alterations of violate components in Hongxinju in different flowering stage and at different processing periods. It revealed that most of the violate components were increased from fresh FC to FL, and the low-boiling fractions, inflammatory methyl arachidonate and air-polluting component of ethylbenzene were declined while the representative components with pungent flavor and cool nature of α-curcumene and (Z,Z,Z)-9,12,15-octadecatrienoic acid, vision improving carotenol of rhodopin and high-boiling fractions were elevated after processed in final FL compared with that in BC and/or FC. Though the content of protein and anti-oxidative capacity of final BC and FC were nearly equal to those of FL, in comprehensive consideration of the representative components related with the efficiency in heat cooling and vision improving, as well as the representative components related with inflammation and air-pollution, final FL was recommended other than BC and FC in the practice of medicine with the yield and quality integrated into account.

Keyword:

Flos chrysanthemum; Bud Chrysanthemum; Fetal Chrysanthemum; Hongxinju; GC-MS based metabolomics; violate components

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1. Introduction

Flos chrysanthemum (FL) is the dried capitulum of *Chrysanthemum morifolium* Ramat. with the efficiency of relieving heat and expelling wind, clearing liver and improving vision, and cooling the internal heat and detoxifying the body, and usually used as an antiphlogistic and against cold, headache, vertigo, and conjunctivitis. Native to China, *C. morifolium* has been cultivated for thousands of years. As a well-known folk medicine, it has been used as a remedy for fever, dizzy, headache, conjunctive congestion with swelling and pain, and so on [1]. Rich in a variety of chemical constituents, with volatile oil [2], flavonoids [3], phenolic acids [4], phytosterols [5], sequitepene lactone [5], polysaccharide [6, 7] included, *C. morifolium* and its main chemical components have been reported to have many beneficial functions, such as antibacterial [8], antiviral [3], anti-inflammatory [9], anti-oxiditive [6], hypotensive [10], hypoglycemic [11, 12], hypolipidemic [13], dormitive [14], cardiovascular protective [15], neuroprotective [4], anti-angiogenic [7], etc. Flos chrysanthemum was classified as a “top grade” drug that was supposed to promote health and prolong life in the Divine Husbandman's Classic of Materia Medica (ShenNong Ben Cao Jing) which was thought to be the earliest material medica in China compiled in the late Eastern Han Dynasty. Due to the different origins and processing methods, a number of medicinal varieties of Flos chrysanthemum have been formed. Varieties of *C. morifolium* plants have been cultivated in China with a long history, with production place and processing method alters, eight mainstream cultivars have been formed, among which five officinal varieties have also been listed in the 2015 edition of Chinese Pharmacopeia including *Bo-ju*, *Chu-ju*, *Gong-ju*, *Hang-ju* and *Huai-ju*. *Hang-ju* was the most popular one for its edible and potable usage, which was originated from Tongxiang City, Zhejiang Province. At present, Yangma Town, Sheyang County, Jiangsu Province have far surpassed Tongxiang in the planting area and yield of *Hang-ju*, becoming the largest planting region of *Hang-ju* in China.

In the traditional application of *Hang-ju*, the inflorescence was in full bloom as it was recorded in Chinese Materia Medica that the harvesting period of Flos Chrysanthemum was in early November when the petals of inflorescence were flat, with the color from yellow to white, and the exposed tubular flower in inflorescence was slightly yellowish[16]. It was said that the *Hang-ju* was suitable for harvesting from late October to early November when the petals were unfolded with 80% of the tubular flower in inflorescence uncovered in Chinese Medicine Dictionary[17]. A similar description of *Hang-ju* was also recorded as butterfly-shaped or oblate-shaped with a diameter of 2.5-4.0 cm in appearance, flat or slightly folded ligulate flower adhered to each other usually without glandular spots in white or yellow, and mostly of tubular flower exposed in the Chinese Pharmacopoeia.

However, the commonly used commercial product of Flos Chrysanthemum (blooming anthodium of *C. morifolium*) in the consumption market of *Hang-ju*, there were also Bud Chrysanthemum (BC) at early stage of buds with intact sepals and unstretched petals and Fetal

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Chrysanthemum (FC) with unstretched petals just broken out of the bracts at the late stage of buds with higher prices, respectively. The acquisition prices of BC and FC were 41-42 RMB and 43-44 RMB per kilogram, higher than that of FL, 30 RMB per kilogram in 2018. As a result of domestic market-promotion and consumption-orientation, quite an amount of the cultivars of *Hang-ju* in the flower bud period were put on the market after being harvested and processed as BC and FC. It was reported that 1 kilogram of BC would grow into 5 kilograms of FL. The large scale of picking of BC was bound to have a certain impact a certain impact on the market of FL, resulting in a decline in the production of FL and affecting the market of FL. It was reported that 1 kilogram of fresh BC would produce 1.2-1.3 kilograms of FC or 2.2-2.5 kilograms of FL, and the large consumption of BC and FC led to reduced production of FL and caused serious waste of the resource of *C. morifolium*. Whether the quality and efficiency of BC and FC was superior to traditional FL or merely consumption misunderstandings?

As it's known, volatile oil was one of the main active ingredients of medicinal Flos Chrysanthemum with various bioactivities of anti-microbial[18], anti-oxidant[19], anti-hyperuricemia[20] and so on. It revealed that the components in volatile oil of Flos Chrysanthemum were mainly monoterpenes and sesquiterpenes with camphor, chrysanthenone, α-pinene, myrcene, eucalyptol, verbenone, β-phellandrene and camphene included[21]. Gas chromatography-mass spectrometry (GC-MS) is the most popular method for the qualitative and quantitative determination of volatile oil. Enormous studies had been undertaken to analyze the volatile oil of Flos Chrysanthemum based on GC-MS, such as headspace solid-phase microextraction coupled with GC-MS[22] and dynamic headspace collection complemented with auto thermal desorber-GC-MS[19], and so on. Here in this manuscript, three commercial products of Hongxinju, a representive cultivar of *Hang-ju* in Sheyang of Jiangsu Province, were studied with a GC-MS based metabolomics approach, complemented with morphology, contents of moisture and protein and the anti-oxidant activity, to reveal the metabolic alterations of Hongxinju in different flowering stage and in different processing periods. So far as we know, there was no study on metabolomics profiles of BC, FC and FL of Hongxinju, and this study would provide scientific reference for the commercial normalization of *Hang-ju*.

2. Results

2.1 Morphological features of three commercial products of Hongxinju

Referring to those description in People's Republic of China Pharmacopoeia 2015 Edition, vol Ⅰ, and complemented with the botany characteristics were recorded[23], with plant morphological indices of the three commercial products of Hongxinju including capitula length and diameter, shape and color of torus, layer and color of calyx, shape, layer and color of bract, morphology of florets ligulate and tubiform floret (Table 1). Capitula length and diameter was measured with digital calipers, with the length from the point of attachment among the torus to the tip. The floret size of

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diameter, height of inflorescence, and morphology of florets ligulate and tubiform floret showed a significant difference among BC, FC, and FL (Fig. 1).

Table 1 Morphological features of final commercial Hongxinju

Morphological features	Bud Chrysanthemum	Fetal Chrysanthemum	Flos Chrysanthemum
Overall appearance of capitula	oblate-shaped with hidden petals	Turbination or cylinder-shaped	butterfly-shaped
Capitula diameter (cm)	0.80-0.90	1.30-1.50	2.25-2.50
Capitula length (cm)	0.45-0.55	1.50-1.60	0.80-1.40
Shape of torus	Flat, slightly bend inward	Flat, slightly bend inward	Flat, slightly bend inward
Color of torus	Olive green or brown	Olive green or brown	Olive green or brown
Color of calyx	Dark green in the base and brown in the tip	Dark green in the base and brown in the tip	Dark green in the base and brown in the tip
Layer of calyx	Three	Three	Three
Shape of bract	ovate to oblong ovate	ovate to oblong ovate	ovate to oblong ovate
Layer of bract	Monolayer	Monolayer	Monolayer
Color of bract	Dark green	Dark green	Dark green
Morphology of florets ligulate	Winding inward of ray florets	Uplifting of outer florets and winding inward of inner florets	Flat or slightly fold, attachment in the base, without glandular dots
Color of florets ligulate	Off-white or yellow	Off-white, earthy yellow or golden	Off-white or light yellow
Feature of tubiform floret	Underdeveloped	Majority, covered by ligulate flower	Majority, exposure
Color of tubiform floret	Underdeveloped	Majority, covered by ligulate flower	Luminous yellow

Figure 1. Appearance of three commercial products of Bud Chrysanthemum (A), Fetal Chrysanthemum (B) and Flos Chrysanthemum (C)

2.2 Content of moisture in different Hongxinju

There were no significant differences in the moisture contents of fresh Hongxinju with range from 84.79% to 86.02%. After the first drying, the moisture was highest in semi-dried BC of 36.48% and lowest of 19.72% in semi-dried FL, while for the final samples, the moisture was highest in FL and lowest in BC (Table 2).

Table 2 Moisture contents of Hongxinju at three flowering and processing stages (n=6)

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Moisture (%)	Bud Chrysanthemum	Fetal Chrysanthemum	Flos Chrysanthemum
Fresh	84.79±1.02	85.08±1.34	86.02±1.75
Semi-dried	36.48±0.83**a	28.55±0.52**a	19.72±0.57
Final	10.73±0.27*a	11.05±0.13	11.44±0.33

a * p<0.05 versus. Flos Chrysanthemum, ** p<0.01 versus. Flos Chrysanthemum.

2.3 Content of protein in different Hongxinju

Contents of protein in fresh Hongxinju were increased and then decreased from BC to FL, and peaked in FC. After the first drying process, the protein content was significantly increased in semi-dried BC, slightly decreased in semi-dried FL, and constant in semi-dried FC. However, there were no obvious differences in the protein contents among the three final Hongxinju (Table 3).

Table 3 Contents of protein in Hongxinju at three flowering and processing stages (n=6)

Protein content (%)	Bud Chrysanthemum	Fetal Chrysanthemum	Flos Chrysanthemum
Fresh	18.34±1.93	25.49±2.14**	19.74±1.70
Semi-dried	30.20±2.68**	25.36±2.13**	16.12±1.08
Final	15.70±1.22	15.30±1.46	15.44±0.33

a * p<0.05 versus. Flos Chrysanthemum, ** p<0.01 versus. Flos Chrysanthemum.

2.4 Comparison of anti-oxidant assays of Hongxinju

It showed that the samples had different antioxidant activity dependent of the assay used. In the DPPH assay, most of the samples showed good radical scavenging activity. The EC_{50} values for BC were slightly higher than those of FC and FL, revealing the weaker anti-oxidant activity, but no significant difference was found ($p>0.05$). In the FRAP assay, the antioxidant activity of fresh BC and FC were significantly stronger compared with that of FC ($p<0.01$, $p<0.05$). For the semi-dried samples, the anti-oxidation of the FC was slightly and notably ($p<0.05$) higher than that of BC and FL, respectively. There were no significant differences in the anti-oxidative activity between final FC and final FL, slightly lower than that of BC (Table 5).

Table 4 Antioxidant activity values of Hongxinju by DPPH assay (n=6)

EC_{50} (mg/mL)	Bud Chrysanthemum	Fetal Chrysanthemum	Flos Chrysanthemum
Fresh	6.94±0.63	2.70±0.72	4.25±0.84
Semi-dried	0.52±0.08	0.21±0.05	0.39±0.09
Final	0.87±0.26	0.79±0.31	0.74±0.17

Flos Chrysanthemum. The EC_{50} for vitamin C was 0.018 mg/mL.

Table 5 Antioxidant activity values of Hongxinju by FRAP assay (n=6)

C (Fe^{2+}, mmol/g)	Bud Chrysanthemum	Fetal Chrysanthemum	Flos Chrysanthemum
Fresh	5.15±0.26**	2.97±0.16*	1.78±0.12
Semi-dried	8.09±0.42	8.23±0.63*	7.62±0.47
Final	6.18±0.22	5.60±0.35	5.59±0.41

Antioxidant activity expressed as mmol $FeSO_4$ equivalents per 1 g sample. 4.8 mmol $FeSO_4$ equivalents per 25 mg of vitamin C.
a * p<0.05 versus. Flos Chrysanthemum, ** p<0.01 versus. Flos Chrysanthemum.

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2.5 Assignment of the violate components in the fresh, semi-dried and final Hongxinju

Referred to the standard compounds, and aided by those previously reported and those stored in the NIST and WILLEY Standard Mass Spectrometry Library, a total of 71 violate metabolites were assigned and were mainly categorized as: monoterpenoid, semi-terpenoid, aromatic derivate, alcohols, and hydrocarbon compounds, with terpenoids and hydrocarbons predominated and the other compounds typically present in smaller amounts (Table 6). Camphor, ethylbenzene, o-xylene, and nonane were the richest components and were present at particularly high levels.

Table 6. The assignment of violate components in the fresh, semi-dried and final Bud Chrysanthemum, Fetal Chrysanthemum and Flos Chrysanthemum.

No.	Retention time (min)	Metabolite	Molecular formula	Molecular weight	MS fragment peaks
1	3.46	Ethylbenzene	C8H10	106.0783	91, 106, 51, 92, 65, 77, 79, 105, 39, 79
2	3.56	α,β-dimethyl-benzeneethanol	C10H14O	150.1045	91, 106, 105, 77, 78, 79, 103, 43, 107
3	3.86	Styrene	C8H8	104.0626	104, 103, 78, 51, 77, 105, 50, 52, 39, 102
4	3.9	o-Xylene	C8H10	106.0783	39, 51, 65, 75, 91, 103, 109
5	4.47	4-Thujanol	C10H18O	154.1358	93,121, 91, 77,92, 39, 41, 43, 27, 79
6	4.76	1,5,5-Trimethyl-6-methylene-cyclohexene	C10H16	136.1252	121, 93, 136, 79, 41, 91, 107, 77, 105, 39
7	5.09	3-Carene	C10H16	136.1252	93, 91, 79, 77, 92, 121, 80, 94, 105, 136
8	5.25	exo-2-Hydroxycineole	C10H18O2	170.1307	43, 108, 71, 126, 69, 93, 41, 111, 55, 83
9	6.11	trans-β-Ocimene	C10H16	136.1252	27, 41, 53, 67, 79, 93, 105, 121, 136
10	6.54	2,6,10-trimethyl-Tetradecane	C17H36	240.2817	57, 43, 71, 85, 41, 55, 56, 84, 69, 155
11	7.35	7-Methyl-Z-tetradecen-1-ol acetate	C17H32O2	268.2402	43, 55, 41, 81, 57, 85, 67, 69, 99, 83
12	7.45	(E)-10-Heptadecen-8-ynoic acid methyl ester	C18H30O2	278.2246	79, 80, 57, 41, 43, 55, 91, 93, 67, 29
13	7.71	5,5-dimethyl-1-ethyl-1,3-Cyclopentadiene	C9H14	122.1096	107, 91, 122, 79, 77, 105, 108, 93, 92, 39
14	8.09	Methyl arachidonate	C21H34O2	318.2559	41, 43, 79, 67, 55, 29, 81, 91, 80, 44
15	8.24	Camphor	C10H16O	152.1201	95, 81, 41, 108, 69, 152, 109, 55, 83, 67
16	8.46	Arachidonic acid methyl ester	C21H34O2	318.2559	41, 43, 79, 67, 55, 29, 81, 91, 80, 44
17	8.62	endo-Borneol	C10H18O	154.1358	95, 41, 110, 93, 55, 67, 139, 121, 96, 69
18	9.59	verbenyl acetate	C12H18O2	194.1307	119, 43, 109, 134, 91, 93, 41, 59, 81, 77
19	10.23	Limonen-6-ol, pivalate	C15H24O2	236.1776	57, 41, 43, 93, 55, 107, 109, 91, 85, 119
20	10.47	2,6,10-Trimethyltetradecane	C17H36	240.2817	57, 43, 71, 85, 41, 55, 56, 84, 69, 155
21	10.68	Bornyl acetate	C12H20O2	196.1463	95, 93, 121, 43, 136, 41, 108, 80, 92, 110
22	12.06	(E)-10-Heptadecen-8-ynoic acid, methyl ester	C18H30O2	278.2246	79, 80, 57, 41, 43, 55, 91, 93, 67, 29
23	12.65	7-Methyl-Z-tetradecen-1-ol acetate	C17H32O2	268.2402	43, 55, 41, 81, 57, 85, 67, 69, 99, 83

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No.	Retention time (min)	Metabolite	Molecular formula	Molecular weight	MS fragment peaks
24	12.98	4,8,8-Trimethyl-2-methylene-4-vinylbicyclo[5.2.0]nonane	C15H24	204.1878	93, 133, 91, 105, 79, 69, 107, 41, 81, 119
25	13.4	Caryophyllene	C15H24	204.1878	41, 69, 93, 133, 79, 91, 55, 81, 107, 105
26	13.54	Limonen-6-ol, pivalate	C15H24O2	236.1776	57, 41, 43, 93, 55, 107, 109, 91, 85, 119
27	13.76	α-curcumene	C15H22	202.1722	132, 119, 41, 105, 91, 202, 131, 55, 117, 145
28	13.92	β-Cubebene	C15H24	204.1878	161, 105, 91, 120, 41, 119, 81, 79, 93, 55
29	13.98	(-)-Zingiberene	C15H24	204.1878	93, 119, 41, 69, 77, 91, 56, 55, 92, 105
30	14.43	cubedol	C15H26O	222.1983	161, 105, 119, 43, 91, 41, 207, 81, 93, 55
31	14.77	β-Cedrene	C15H24	204.1878	161, 69, 41, 204, 93, 105, 120, 91, 92, 57
32	16.51	Eudesm-7(11)-en-4-ol	C15H26O	222.1983	161, 204, 189, 43, 41, 81, 105, 135, 107, 93
33	17.42	trans-β-Terpinyl pentanoate	C15H26O2	238.1932	41, 93, 29, 81, 57, 68, 94, 136, 55, 43
34	17.75	Glyceryl linolenate	C21H36O4	352.2613	79, 41, 67, 43, 29, 81, 95, 57, 93, 69
35	18.09	(Z,Z,Z)-9,12,15-Octadecatrienoic acid, 2,3-dihydroxypropyl ester	C21H36O4	352.2614	79, 41, 67, 43, 29, 81, 95, 57, 93, 69
36	18.19	(E,Z,Z)-9,12,15-Octadecatrienoic acid, 2,3-dihydroxypropyl ester	C21H36O4	352.2614	79, 41, 67, 43, 29, 81, 95, 57, 93, 69
37	18.26	1-Heptatriacotanol	C37H76O	536.5896	43, 55, 69, 41, 81, 57, 95, 67, 82, 91
38	18.45	9,12,15-Octadecatrienoic acid, 2,3-dihydroxypropyl ester, (Z,Z,Z)-	C21H36O4	352.2614	79, 41, 67, 43, 29, 81, 95, 57, 93, 69
39	18.76	Phytol	C20H40O	296.3079	81, 82, 43, 95, 123, 55, 41, 57, 71, 68
40	18.85	1-Heptatriacotanol	C37H76O	536.5896	43, 55, 69, 41, 81, 57, 95, 67, 82, 91
41	19.07	β-Santanol acetate	C17H26O2	262.1932	94, 121, 93, 43, 122, 202, 107, 119, 95, 79
42	19.26	Spiro[4.5]dec-6-en-8-one, 1,7-dimethyl-4-(1-methylethyl)-	C15H24O	220.1827	109, 82, 41, 69, 93, 135, 55, 79, 81, 108
43	19.39	1-Heptatriacotanol	C37H76O	536.5896	43, 55, 69, 41, 81, 57, 95, 67, 82, 91
44	19.52	Glyceryl 1-linolenate	C21H36O4	352.2614	79, 41, 67, 43, 29, 81, 95, 57, 93, 69, 57, 95, 67, 82, 91
45	19.8	Geranyl isovalerate	C15H26O2	238.1933	85, 43, 57, 41, 69, 71, 121, 81, 68, 55
46	19.92	1-Methoxybicyclo[2,2,2]oct-5-en-2-yl methyl ketone	C11H16O2	180.1150	110, 109, 43, 137, 95, 77, 94, 111, 79, 180
47	20.24	Ascorbic acid dipalmitate	C38H68O8	652.4914	57, 73, 43, 60, 71, 55, 69, 41, 129, 85

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No.	Retention time (min)	Metabolite	Molecular formula	Molecular weight	MS fragment peaks
48	20.54	7-Isopropyl-1,4-dimethyl-2-azulenol	C15H18O	214.1358	214, 199, 128, 171, 143, 185, 197, 215, 141, 115
49	21.24	5,8,11,14-Eicosatetraynoic acid	C20H24O2	296.1776	165, 41, 167, 115, 179, 152, 153, 91, 55, 29
50	21.32	9,19-Cyclolanost-24-en-3-ol acetate	C33H54O3	498.4073	57, 43, 55, 41, 81, 95, 83, 109, 97, 85
51	21.6	7-Methyl-Z-tetradecen-1-ol acetate	C17H32O2	268.2402	43, 55, 41, 81, 57, 85, 67, 69, 99, 83
52	21.82	Heptacosane	C27H56	380.4382	57, 43, 71, 85, 55, 41, 69, 99, 56, 29
53	22.01	E-8-Methyl-9-tetradecen-1-ol acetate	C17H32O2	268.2402	43, 55, 71, 81, 69, 97, 57, 67, 41, 85
54	23.34	Pentacosane	C25H52	352.4069	57, 71, 43, 85, 55, 99, 41, 113, 69, 83
55	24.01	3-Ethyl-5-(2-ethylbutyl)octadecane	C26H54	366.4225	43, 57, 71, 85, 55, 41, 69, 281, 83, 97
56	24.18	Barrigenol R1	C30H50O6	506.3607	249, 280, 207, 190, 189, 231, 262, 175, 135, 298
57	25.04	Nonacosane	C29H60	408.4695	57, 43, 71, 85, 55, 41, 99, 69, 83, 113
58	25.21	3,4,3',4'-Tetrahydrospirilloxanthin	C42H64O2	600.4906	73, 69, 91, 105, 81, 55, 145, 119, 41, 93
59	25.82	Clocortolone pivalate	C27H36ClFO5	494.2235	57, 139, 85, 69, 95, 109, 159, 121
60	25.91	Glyceryl 1,2-dipalmitate	C35H68O5	568.5067	43, 57, 55, 41, 98, 73, 60, 71, 69, 239
61	25.99	Tetratriacontane	C34H70	478.5478	57, 71, 43, 85, 55, 113, 41, 69
62	26.19	Rhodopin	C40H58O	554.4488	69, 41, 81, 55, 105, 95, 119, 91
63	26.31	Vitamin E	C29H50O2	430.3810	165, 43, 57, 164, 430, 71, 55, 41, 85, 166
64	26.94	Nonacosane	C29H60	408.4695	57, 43, 71, 85, 55, 41, 99, 69, 83, 113
65	27.09	Betulin	C30H50O2	442.3810	189, 95, 135, 81, 203, 55, 93, 121, 107, 41
66	27.17	Monolinolenin TMS	C27H52O4Si2	496.3404	75, 149,155, 207, 117, 131, 129, 57, 223, 281
67	27.29	17-Pentatriacontene	C35H70	490.5478	57, 43, 97, 83, 55, 69, 71, 41, 111, 85
68	27.87	Hexatriacontane	C36H74	506.5791	57, 71, 43, 85, 99, 55, 113, 41, 83, 69
69	28.5	Ingenol 3-octoate	C28H42O6	474.2981	122, 57, 43, 123, 41, 135, 121, 93, 55, 151
70	29.1	17-Pentatriacontene	C35H70	490.5477	57, 43, 97, 83, 55, 69, 71, 41, 111, 85
71	29.86	Hexatriacontane	C36H74	506.5791	57, 71, 43, 85, 99, 55, 113, 41, 83, 69

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2.6 The influence of flowering developments in metabolic profiles

Unsupervised PCA was used to perform the metabolic differences of Hongxinju in different flowering development of BC, FC and FL at the same processing stage which revealed first main component PC1 could not distinguish the metabolic alteration among BC, FC and FL in the score plot (Fig 2A-2C). To remove the noise and interference with group-independent variables, a supervised OSC-PLS-DA (OSC=1) was further performed, presenting a clear clustering of the three groups with well goodness of fit and statistical significance in the score plot (Fig 2D-2F). It showed that FrBC, FrFC and FrFL were separated with each other, with FrBC in the middle, near to FrFC in the score plot, revealing time-independent metabolic alteration of the fresh Hongxinju from BC to FL (Fig 2D). For the semi-dried and final samples, the clusters of BC, FC and FL were mainly separated in PC1, with SdFC located between SdBC and SdFL, nearing to SdBC (Fig 2E), and FnFC in the middle of FnBC and FnFl, nearer to FnFL (Fig 2F), respectively. It showed that the volatile components in Hongxinju were greatly influenced by the flowering development.

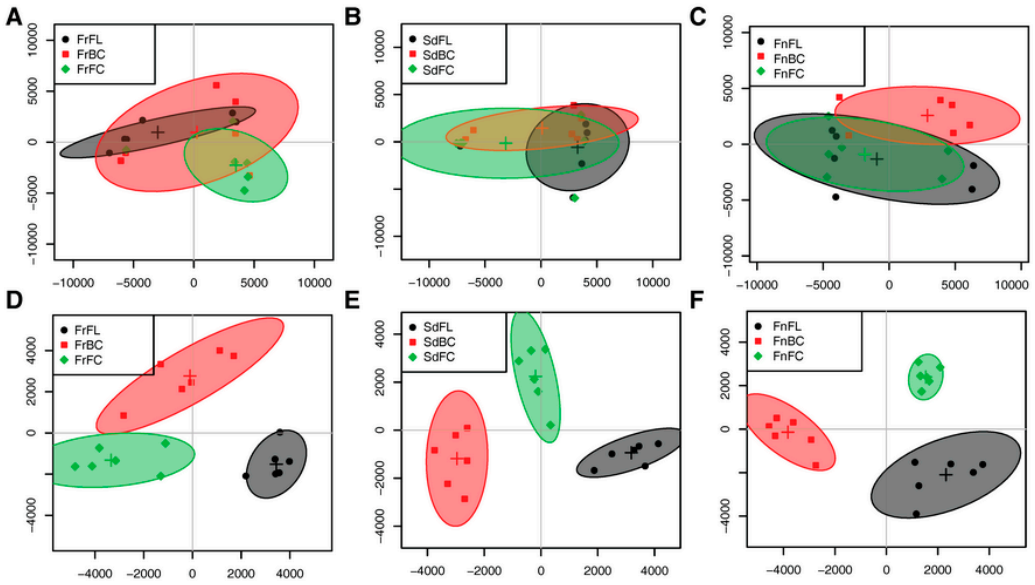

Figure 2. Score plots of PCA and OSC-PLS-DA (OSC=1), revealing the flowering period dependent metabolic alteration in Hongxinju at fresh (2A/2D), semi-dried (2B/2E) and final processing stage (2C/2F). (2A-2C: PCA; 2D-2F: OSC-PLS-DA).

The loading plot of OSC-PLS-DA with the chromatographic retention time marked was color-encoded according to the correlation coefficients (R^2) and visualized in a covariance-based pseudo-spectrum, with the color from red to blue indicating correlation from strong to weak (Fig. 3A, 3B and 3C for fresh, semi-dried and final samples, respectively). For fresh Hongxinju, most of the violate components were increased in FL compared to FC, with significantly increased levels of ethylbenzene, 4-thujanol, 3-carene, verbenyl acetate, α-curcumene, (-)-zingiberene, clocortolone pivalate, glyceryl 1,2-dipalmitate and vitamin E, and notably decreased levels of 5,5-dimethyl-1-ethyl-1,3-cyclopentadiene, (E)-10-heptadecen-8-ynoic acid methyl ester, β-cubebene, phytol, 1-Heptatriacotanol and botulin.

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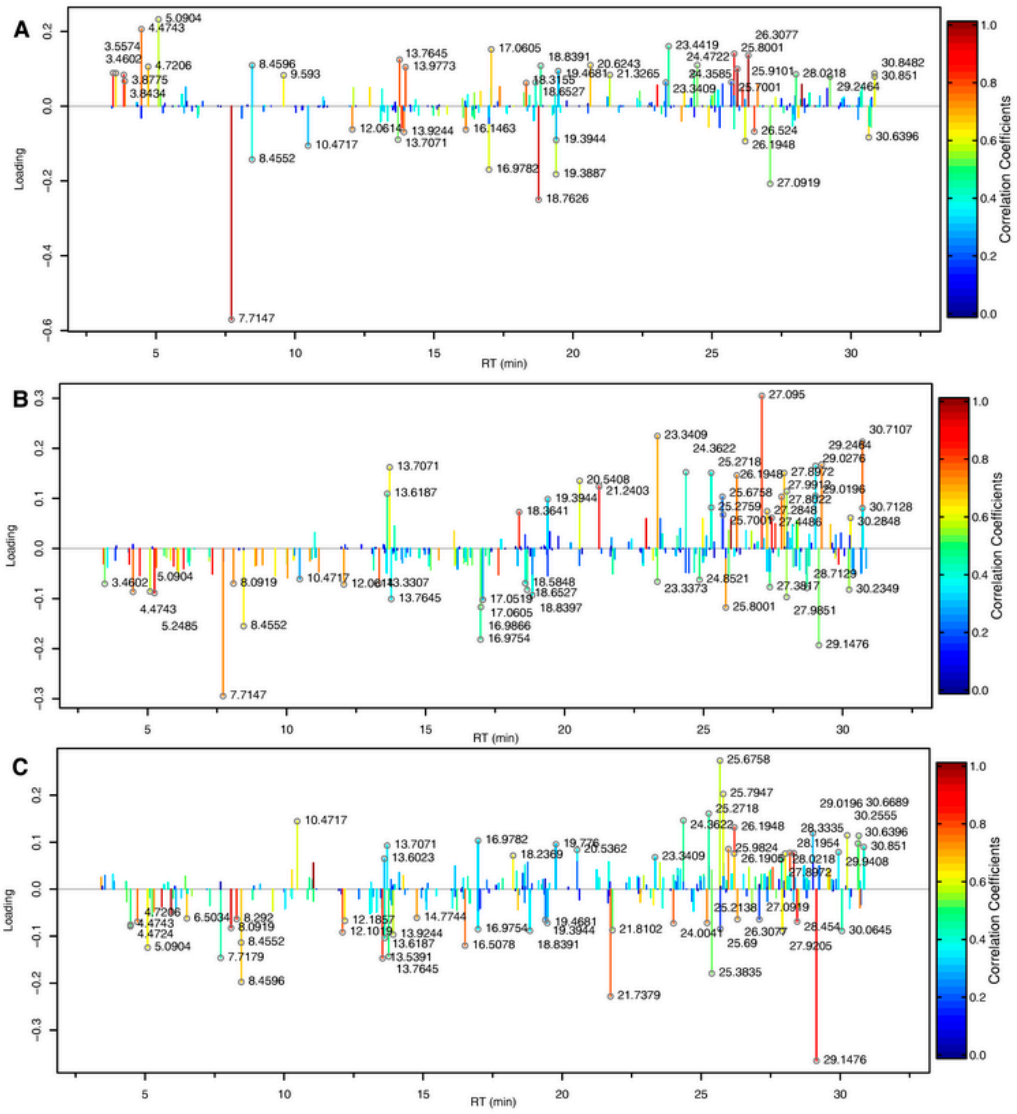

Figure 3. Color encoded loading plots of OSC-PLS-DA (OSC=1), revealing the flowering period dependent differential metabolites in Hongxinju at fresh (A), semi-dried (B) and final processing stage (C).

For semi-dried samples, compared with SdBC, most of the low-boiling fractions were declined while the high-boiling compounds were elevated, with remarkably reduced levels of ethylbenzene, 4-thujanol, 3-carene, exo-2-hydroxycineole, 5,5-dimethyl-1-ethyl-1,3-cyclopentadiene, methyl arachidonate, arachidonic acid methyl ester, 2,6,10-trimethyltetradecane and clocortolone pivalate, and escalated content of α-curcumene, 1-heptatriacotanol, 7-isopropyl-1,4-dimethyl-2-azulenol, 5,8,11,14-eicosatetraynoic acid, pentacosane, rhodopin, betulin, hexatriacontane and 17-pentatriacontene in the SdFL.

In comparison with FnBC, noticeably high levels of the 2,6,10-trimethyltetradecane, (E,Z,Z)-9,12,15-Octadecatrienoic acid, 2,3-dihydroxypropyl ester, clocortolone pivalate and rhodopin, and greatly low levels of 4-thujanol, 3-carene, 2,6,10-trimethyl-tetradecane, methyl arachidonate, camphor, arachidonic acid methyl ester, (E)-10-heptadecen-8-ynoic acid methyl ester, limonen-6-ol,

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pivalate, β-cedrene, eudesm-7(11)-en-4-ol, heptacosane, 3-ethyl-5-(2-ethylbutyl) octadecane, 3,4,3',4'-tetrahydrospirilloxanthin, ingenol 3-octoate and 17-pentatriacontene were in FnFL. And most of the significantly differential metabolites in the OSC-PLS-DA were in accord with those in the univariate analysis (Fig 4).

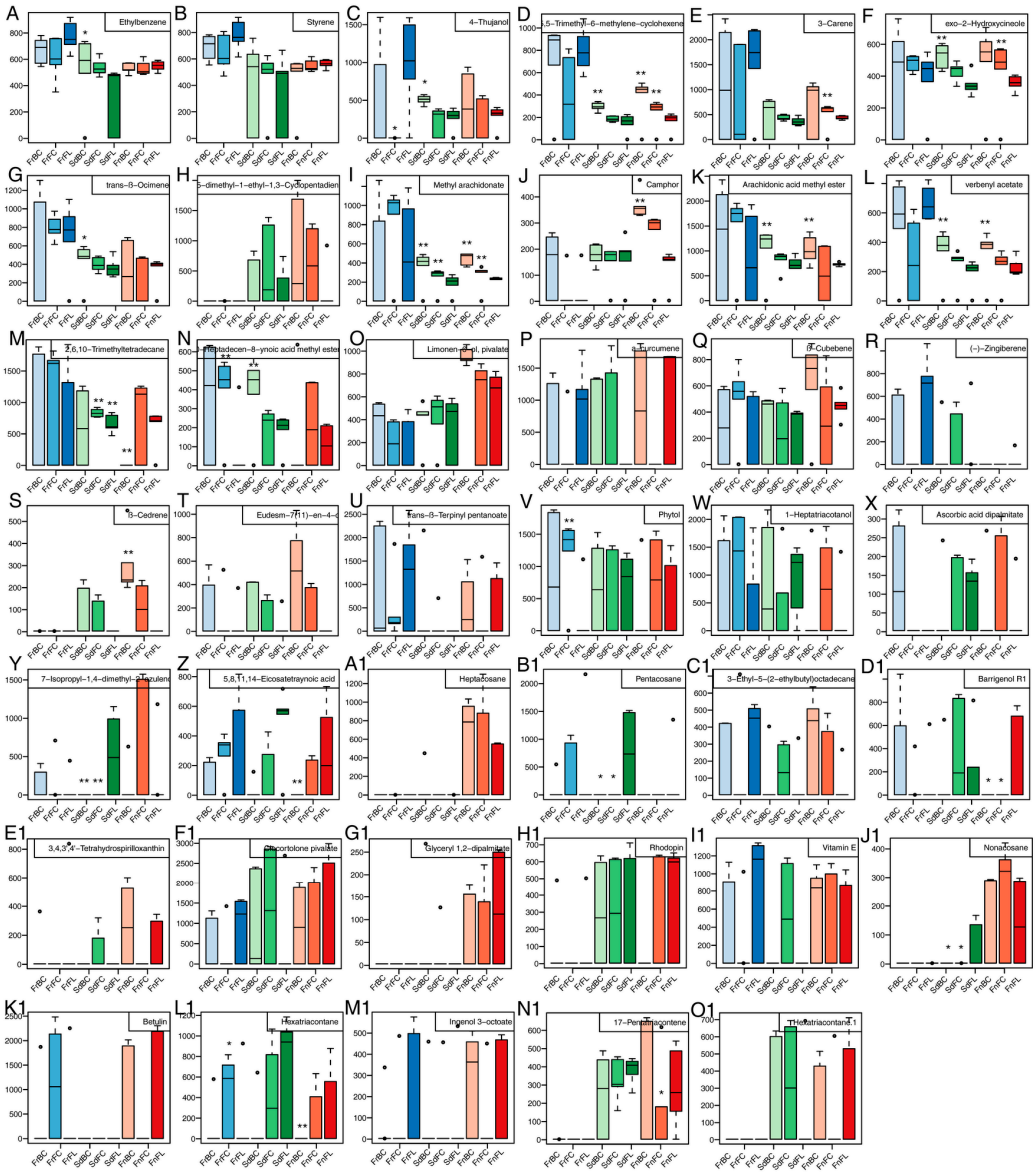

Figure 4. Boxplots of significantly changed violate components in the OSC-PLS-DA analysis. The boxes covered 25% quartile and 75% quartile of the data. The line in the box represented the median value. The extended whiskers show the extent of the rest of the data. Outliers were shown as open circle. * $p<0.05$ versus. FL, ** $p<0.01$ versus. FL.

2.7 The influence of processing stage on the metabolic profiles

PCA analysis revealed that fresh, semi-dried and final Hongxinju were severely overlapped with each other, especially for semi-dried and final samples, on the first component in score plots (Fig 5A, 5B and 5C for BC, FC and FL, respectively). OSC-PLS-DA analysis exhibited that the

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three groups were well separated with each other, with semi-dried samples of SdBC, SdFC and SdFL in the middle between the fresh and the final Hongxinju, nearer to final ones of FnBC, FnFC and FnFL, respectively. It showed that the metabolic profiles of BC were greatly affected by the processing of drying and the dryness degree, the more the dryness degree, the more significant metabolic alterations (Fig 5D). For FC and FL, the fresh samples in the negative area were obviously distinguished with the semi-dried and final ones in the positive religion of the score plot (5E and 5F for FC and FL, respectively), which revealed that the influence of dryness degree on the metabolic profile of FC and FL was less than those of BC.

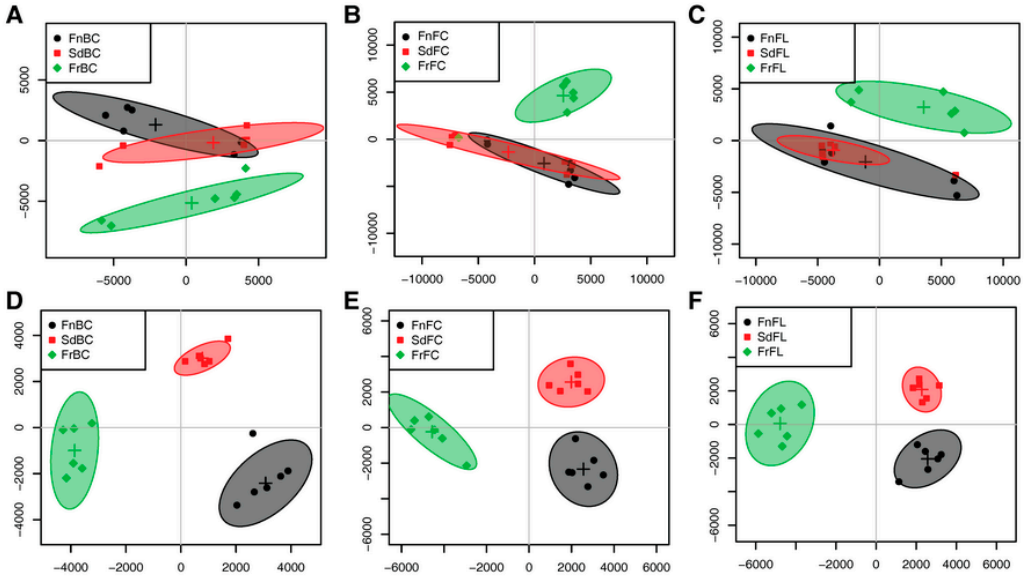

Figure 5. Score plots of PCA and OSC-PLS-DA (OSC=1), revealing the processing period dependent metabolic alteration in Bud Chrysanthemum (A/D), Fetal Chrysanthemum (B/E) and Flos Chrysanthemum (C/F). (4A-4C: PCA; 4D-4F: OSC-PLS-DA).

In comparison with FrBC, notably increased levels of 5,5-dimethyl-1-ethyl-1,3-cyclopentadiene, limonen-6-ol, pivalate, phytol, heptacosane, clocortolone pivalate, nonacosane and 17-pentatriacontene, and obviously decreased levels of ethylbenzene, styrene, 1,5,5-trimethyl-6-methylene-cyclohexene, 3-carene, exo-2-hydroxycineole, trans-β-ocimene, arachidonic acid methyl ester, verbenyl acetate and 1-heptatriacotanol were in FnBC in the loading plot (Fig. 5A).

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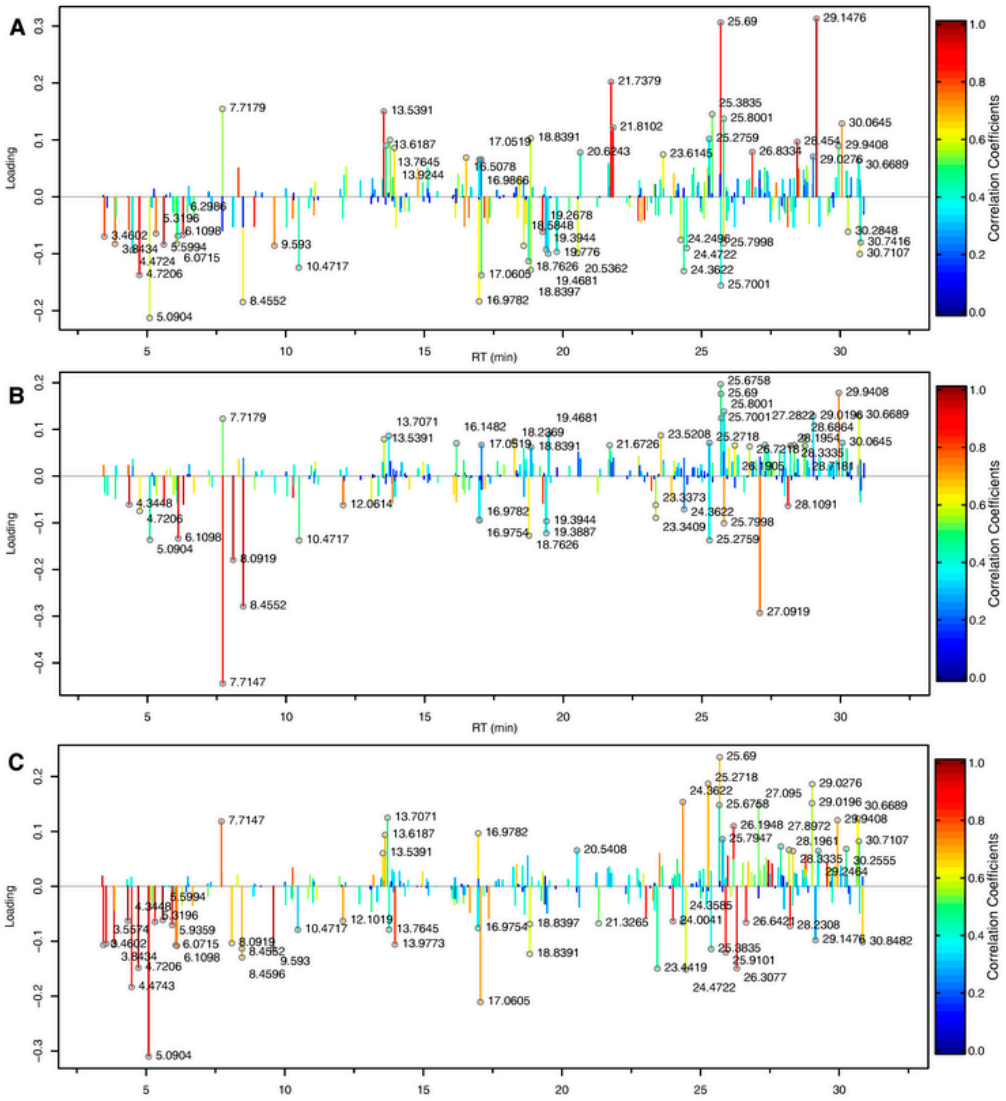

Figure 5. Color encoded loading plots of OSC-PLS-DA (OSC=1), revealing the processing period dependent differential metabolites in Bud Chrysanthemum (A), Fetal Chrysanthemum (B) and Flos Chrysanthemum (C).

Compared with FrFC, most of the violate compounds were decreased in FnFC, especially with significantly elevated levels of hexatriacontane, and the obviously declined levels of 4-thujanol, 1,5,5-trimethyl-6-methylene-cyclohexene, trans-β-ocimene, 5,5-dimethyl-1-ethyl-1,3-cyclopentadiene, methyl arachidonate, arachidonic acid methyl ester, 2,6,10-trimethyltetradecane, (E)-10-heptadecen-8-ynoic acid methyl ester, clocortolone pivalate and botulin (Fig. 5B).

It revealed that notably raised levels of 5,5-dimethyl-1-ethyl-1,3-cyclopentadiene, limonen-6-ol, pivalate, α-curcumene, barrigenol R1, 3,4,3',4'-Tetrahydrospirilloxanthin, clocortolone pivalate, rhodopin and hexatriacontane, and notably reduced levels of ethylbenzene, α,β-dimethyl-benzeneethanol, styrene, 4-thujanol, 1,5,5-trimethyl-6-methylene-cyclohexene, 3-carene, exo-2-hydroxycineole, trans-β-ocimene, methyl arachidonate, arachidonic acid methyl ester, verbenyl acetate, (E)-10-heptadecen-8-ynoic acid methyl ester, (-)-zingiberene, trans-β-Terpinyl pentanoate,

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glyceryl 1,2-dipalmitate, vitamin E, nonacosane and ingenol 3-octoate were in FnFL compared with those in FrFL (Fig. 5C).

3. Discussion

As the bud and fetal of *C. morifolium*, there were significant morphological differences between BC, FC and FL, mainly in the length and diameter of inflorescence, and the shape of torus, florets ligulate and tubiform floret. Besides the differences in morphology, obvious distinction was found in the moisture, content of protein, anti-oxidation and the violate metabolic profiles of BC, FC and FL at different flowing development and processing stage.

In general, it was hardly for inner moisture to be removed when most external moisture had been driven off during the first constant drying, especially for the compact bud in which most of the bracts and florets ligulate were folded, avoiding the inner moisture to be out. It was reasonable for the semi-dried samples disposed still at room temperature to allow the inner moisture diffused to the external before the second drying process. For fresh and final samples, the moisture content was less influenced by flowering development, while for the semi-dried samples, the moisture content was affected greatly by flowering development. It provided a reference for drying method study of some compact biomass like bud and fetal.

For Bud Chrysanthemum, the protein content measured by BCA kits was obviously increased during the first drying process, and then significantly decreased in the final products. The changes in the content of protein might attributed to the alteration in the amounts of free amino groups or the contents of arginine and/or aromatic amino acids during drying process due to some chemical reaction, such as the Maillard reaction between protein and flavonoid. It was worthy to further investigated to uncover the reasons of the changes in the protein during processing.

The anti-oxidation tests exhibited an assay depending, different result between DPPH and FRAP methods, revealing the complication of the anti-oxidative activity in vitro, and therefore multiply methods needed to be integrated to afford a considerate result. For the fresh samples, there were notable differences in the anti-oxidation among BC, FC and FL, however, the anti-oxidant activities of final BC, FC and FL were nearly equal to each other after two steps of drying either by DPPH or FRAP methods. Consequently, FL was likely replaced by BC and FC in the field of anti-oxidation.

Elevated level of α-curcumene (Fig. 4P) in fresh FL comparing with FC, and increased level of (Z,Z,Z)-9,12,15-octadecatrienoic acid were found in final FL in comparison with those in BC. In the previous reports, α-curcumene and (Z,Z,Z)-9,12,15-octadecatrienoic acid were deemed as representative components in the essential oil of herbal medicine with pungent flavor and cool nature according to the theory of TCM, the higher contents of these two compounds in FL may indicate the superior efficacy of FL in expelling wind and cooling the internal heat than FC and BC[24]. Raised content of rhodopin (Fig. 4H1) and reduced levels of methyl arachidonate (Fig. 4I)

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were in both the semi-dried and final FL compared with those in BC, indicating possible unique commercial feature and medical efficiency of FL distinguished from that of BC. Rhodopin, a kind of carotenol, its higher level in final FL revealed better vision-improving effect of FnFL compared with that of FnBC, which was in accordance with the supplementation with vitamins C and E and beta carotene retarding age-related macular degeneration and vision loss[25]. Lower content of methyl arachidonate in final FL possibly implied less inflammatory and better safety of FL superior to BC. 5,5-Dimethyl-1-ethyl-1,3-cyclopentadiene was found in Hangju cultivated in Tongxiang at high level[26], however, low content of 5,5-dimethyl-1-ethyl-1,3-cyclopentadiene (Fig. 4H) was in the final FL, revealing the influence of planting religion on the herbal medicine. The level of ethylbenzene (Fig. 4A) was increased in fresh FL, but were decreased in semi-dried FL compared with those in FC and BC, revealing its thermal instability and variability during the drying processing. Ethylbenzene, one of the common compound in air pollution, the lower level in the final FL than in fresh FL revealed the reasonability of processing to improve the safety.

4. Material and methods

4.1 Materials and chemicals

2, 2-diphenyl-1-picrylhydrazyl (DPPH), tripyridyl-triazine (TPTZ) and vitamin C were bought from Sigma (St. Louis, MO, USA). Cyclohexane and methanol of LC-MS grade used in this study were supplied by Merck (Darmstadt, Germany). Ultra-pure distilled water was prepared from a Milli-Q purification system in the lab. BCA kit was purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China)

4.2 Plant collection and identification

The samples of BC, FC and FL of Hongxinju used in this study was collected from Sheyang Ma Town, the main producing area of *Hang-ju* in October 20th 2017 when harvest (Jiangsu Hexiang Juhai Modern Agricultural Industrial Park Development Co., Ltd., Yancheng, China). Cultivar of Hongxinju selected from '*Hang-ju*' (Nanjing university of agriculture, Nanjing, China) were kindly provided by Prof. Fadi Chen (Nanjing university of agriculture, Nanjing, China). The specimens were authenticated by Professor Jin-ao Duan as *C. morifolium* Ramat. cv. Hongxinju (Nanjing University of Chinese Medicine, Nanjing, China), and were disposed in the herbarium of Jiangsu Collaborative Innovation Center of Chinese Medicinal Resources Industrialization. All of the study complied with the Convention on Biological Diversity and the Convention on the Trade in Endangered Species of Wild Fauna and Flora.

4.3 The processing of BC, FC and FL

The processing of the flowers was in three stages. Firstly, fresh flowers were fumigated with 170-180 °C distilled high pressure steam in 10 s to inactivate the intrinsic oxidative enzymes avoiding been brown, and then the fumigated flowers were dried in 60 °C in hot-dry oven for 4 h to remove the external moisture. Secondly, the semi-dried samples disposed at room temperature for

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24 h to allow the inner moisture diffused to the surface were dried for another 4 h to remove the internal moisture, thereby forming the final product. All of fresh, semi-dried and final products of Hongxinju samples were immediately stored in a -80 °C refrigerator before analysis as follow: fresh Bud Chrysanthemum (FrBC), fresh FC (FrFC), fresh FL (FrFL), semi-dried Bud Chrysanthemum (SdBC), semi-dried FC (SdFC), semi-dried FL (SdFL), final Bud Chrysanthemum (FnBC), final FC (FnFC), and final FL (FnFL).

4.4 Determination of moisture

About 2 g of FrBC, FrFC, FrFL, SdBC, SdFC, SdFL, FnBC, FnFC, and FnFL were cut into pieces and the moisture content values were determined using the oven drying method at the standard oven drying temperature of 110±5° C[27]. An analytical balance, accurate to 0.1 mg was in the drying oven. Remove the sample from the oven dishes and allow them to cool to room temperature in a desiccator before the next measurement.

4.5 Protein content test

A hundred mg of the samples for the protein assay kits were weighed, added with 1.0 ml of pre-cooled phosphate buffered saline (pH 7.4, 0.05 M, m/v=1:10) and a stainless steel ball, shaken, left to stand for extraction. Homogenates were filtered and centrifuged by using a refrigerated centrifuge at 3,500 × g at 4 °C to afford stock solutions, which were kept in a refrigerator at 4 °C and in dark situation. Then, these supernatants were used for the determination of total protein concentration by the bicinchoninic acid assay at 562 nm according to the operation instructions of BCA kit.

4.6 Anti-oxidant activity measurement

In vitro DPPH radical scavenging assay and ferric reducing antioxidant power (FRAP) were routinely practiced for the assessment of antiradical properties of different compounds and extracts[28, 29]. The above supernatants of Hongxinju were diluted with ultra-pure water, and DPPH assay was performed according to previously reported[30]. In brief, 100 μL of Hongxinju extract solution was mixed with 100 μL solution of 200 μM DPPH solution in methanol or buffered methanol to start the reaction, with vitamin C as the positive control. The reaction mixture was kept at 30 °C for 30 min and the absorbance was measured at 517 nm at room temperature using a BioRad Model 550 plate reader.

The FRAP assay measured the ability of the antioxidants in Hongxinju extracts to reduce the Fe^{3+}-TPTZ complex to the blue-colored ferrous form (Fe^{2+}), which absorbs light at 595 nm. Subsequently, 20 μL of each sample of Hongxinju was mixed with 180 μL of FRAP reagent in each well of a 96-well plate. The plate was inserted in the plate reader and was read at 595 nm after 30 min. The effective concentration (EC_{50}) value was determined for the antioxidants.

4.7 Sample preparation for GC-MS analysis

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To minimize the influence of drying, powdering and other mechanical wounding, frozen tissue of FrBC, FrFC, FrFL, SdBC, SdFC, SdFL, FnBC, FnFC, and FnFL were cut with a sharp scalpel blade. And ca. 0.050 g of the samples were weighed accurately with an analytical balance (Sartorius, BT 125D, German) after stabilizing under constant temperature (20 °C) and placed in a 2.0 ml round bottom centrifuge tube with 1.00 ml of pre-cooled cyclohexane (m/v=1:20, chromatographically grade, German Merck KGaA) and a stainless steel ball with a diameter of 3 mm. The samples were placed in a frozen tissue homogenizer for 25 s to homogenate for 30 s, vortex for 10 s, shake at room temperature for 5 min to fully extract volatile components and to sedimentate protein therein. The above samples were centrifuged at 12 000 rpm for 10 min at 4 °C using a microcentrifuge (Microfuge 22R Centrifuge, Beckman Coulter, Fullerton, CA, USA), and the supernatant was filtered through an organic filter of 0.22 μm and was stored in a refrigerator at -80 °C for GC-MS analysis.

4.8 GC-MS analysis

Before GC-MS analysis, samples were equilibrated in a 4 °C refrigerator. GC-MS data was collected on a PerkinElmer Clarus 680 with a PerkinElmer Axion iQT MS/MS. Compounds were then separated on a 30 m HP-5MS quartz capillary column with an internal diameter of 0.25 mm and a film thickness of 0.25 μm (Agilent, USA). The injection volume was 0.5 μL, and the split ratio was 10:1. High-purity helium (99.999 %) was used as the carrier gas at a flow rate of 1.0 mL/min. The GC oven temperature was then programmed as follows: initial temperature 70 °C for 2 min, 8 °C/min to 280 °C, 280 °C for 3 min. The interface temperature was 280 °C, and the quadrupole temperature was set to 150 °C. The mass spectrometer was fitted with an electron ionization (EI) source, in cation mode, operated at 70 eV with a source temperature of 250 °C, and mass spectra were recorded in the range of m/z 40 to 400 amu in full-scan acquisition mode.

4.9 Preprocessing of the GC-MS data

All of the raw spectra data obtained from the ChemStation was converted into a netCDF format file by AxION eCipher software, and then was subjected to a series of standardized pretreatment, with area standardization, probability normalization, mean centering and pareto scaling included.

4.10 Assignment of the volatile compounds

AxION eCipher software was used to evaluate chromatograms and spectra, and the volatile components were assigned based on the retention indices and by comparison of their mass spectral fragmentation patterns with those reported in the literature and stored in the NIST Standard Mass Spectrometry Library (2011 edition) purchased from the National Institute of Standards and Technology and the WILLEY Standard Mass Spectrometry Library bought from John Wiley & Sons, USA.

4.11 Multivariate analysis

The preprocessed data was firstly subjected to a standard unsupervised statistical technique of

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principal component analysis (PCA) for dimensionality reduction to find holistic representations which had been applied to a broad class of complicated systematic biological problems. And then a supervised statistical method of orthogonal signal correction partial least squares discriminant analysis (OSC-PLS-DA) with OSC filter enabling variable filtration, without losing the spectral structure of the loadings, and consequently facilitating interpretation through identification of the metabolites which were the basis of the discrimination, was further performed to remove the information unrelated to the target variables and to screen the potential biomarkers[31].

4.12 Statistical analysis

Univariate (Student's t-test) and multivariate statistical analyses including PCA and OSC-PLS-DA were conducted by a suite of scripts developed in-house running in the R software (http://cran.r-project.org/, version 2.14.2), with detailed analysis processes demonstrated as previously reported[32].

5. Conclusion

The content of protein in the fresh FC was higher than that in fresh BC and FC, however, they were almost the same in the final BC, FC and FL, a litter lower than that in fresh samples. Independent of the assay used, it revealed similar antioxidant activities of the final BC, FC and FL, and a litter stronger anti-oxidative capacity of the final samples than the fresh ones. The volatile components in Hongxinju were greatly influenced by the flowering development and processing stage. Most of the violate components were increased from fresh FC to FL, and the low-boiling fractions of camphor and β-cedrene, inflammatory methyl arachidonate and air-polluting component of ethylbenzene were declined while the representative components with pungent flavor and cool nature of α-curcumene and (Z,Z,Z)-9,12,15-octadecatrienoic acid, vision improving carotenol of rhodopin and high-boiling fractions were elevated after processed in final FL compared with that in BC and/or FC. It revealed fresh Hongxinju superior than the process samples for collection of the low-boiling violate compounds. Though the content of protein and anti-oxidative capacity of final BC and FC were nearly equal to those of FL, in comprehensive consideration of the representative components related with the efficiency in heat cooling and vision improving, as well as the representative components related with inflammation and air-pollution, final FL was recommended other than BC and FC in the practice of medicine with the yield and quality integrated into account.

Conflicts of interest

We confirmed that this manuscript has neither been published elsewhere nor under consideration by another journal. All authors had approved the manuscript and agreed with its submission to your journal. The authors had no conflicts of interest to declare.

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