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UPLC-Q-Exactive Orbitrap MS Analysis for Identification of Lipophilic Components in Citri Sarcodactylis Fructus from Different Origins in China Using Supercritical CO_2 Fluid Extraction Method

Chengxiao Fu, Mengshi Liu, Yueshan Li, Kanghui Wang, Bo Yang, Lijing Deng, Jingyuan Tian, Guoping Yang,* and Guodong Zheng*

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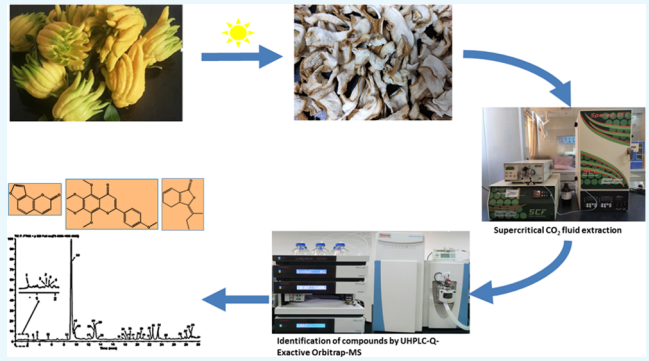
ABSTRACT: To thoroughly evaluate the quality of Citri Sarcodactylis Fructus (CSF) and acquire knowledge of the lipophilic components of CSF from different origins, a simple and efficient approach based on supercritical fluid extraction (SFE) combined with ultraperformance liquid chromatography plus Q-Exactive Orbitrap tandem mass spectrometry (UPLC-Q-Exactive Orbitrap/MS) detection for the discrimination of components from CSF was set up for the first time in this work. Eight batches of CSF samples from five main producing areas were extracted by SFE under optimized conditions, and then SFE extracts were dissected via UPLC-Q-Exactive Orbitrap/MS. The results indicated that 39 lipophilic compounds were successfully separated and unambiguously or tentatively identified, where 4 coumarins, 6 polymethoxyflavones, 3 phthalides, 6 terpenes, and 4 phenolics were not reported formerly. It was illustrated that CSF may be abundant in polymethoxyflavones, as in coumarins. Moreover, there were significant differences in the components of CSF from different origins. Especially, coumarin, dehydrocostus lactone, atractylenolide II, and atractylenolide I were exclusively found in CSF from the Guangdong province; isopsoralen was almost exclusively found in CSF from the Guangxi province; and ferulic acid was exclusively found in CSF from the Zhejiang province. These observations indicated that SFE joint with UPLC-Q-Exactive Orbitrap/MS owing to the potential of characterizing the lipophilic components could be used to promote quality assessment and chemotaxonomic investigation in phytology sciences of CSF.



1. INTRODUCTION

Citri Sarcodactylis Fructus (CSF), the dried fruit of *Citrus medica* L. var. *sarcodactylis* Swingle, which belongs to *Citrus* botany in Rutaceae,[1] is widely cultivated in Guangdong, Guangxi, Zhejiang, Sichuan, and Yunnan provinces of China.[2] It can be utilized as a traditional medicine for the cure of stomach ache, headache, edema, rheumatism, arthritis, and infectious hepatitis[3] and also as a tonic material to make crispy cookies.[4] As it is considered to be one of the homologies in medicine and food, CSF is full of potential for vast development and application prospects.[5] However, the chemical composition of CSF from different origins has not been fully clarified. Therefore, a rapid and efficient method should be built for the systematic analysis and identification of phytochemicals in CSF.

As reported previously, many compounds such as coumarins and limonoids were separated and distinguished in the CSF extracts by use of methanol,[6−8] although it is worth considering that some lipophilic components like nonpolar and low-polarity substances may not be easily obtained by the use of common organic solvents. Up to now, there have been a few studies wherein supercritical fluid CO_2 extraction (SFE) was used to extract the chemical compounds of CSF prior to ingredient analysis. SFE is a low-temperature extraction technology and widely implemented to extract chemical components in plants, due to the potential to extract nonpolar and low-polarity substances.[9,10] In addition, although high-performance liquid chromatography mass spectrometry (HPLC-MS) and gas chromatography mass spectrometry (GC-MS) are usually used for the analysis of plant chemicals, recently, ultraperformance liquid chromatography plus Q-Exactive Orbitrap tandem mass spectrometry (UPLC-Q-

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Exactive Orbitrap/MS) has proved to be a more rapid, efficient, and sensitive implement than HPLC-MS and GC-MS in the field of the analysis of extracts and bioactive constituents from medicine and food plants.[11,12]

Herein, the chemical components of CSF were extracted by SFE and discriminated via UPLC-Q-Exactive Orbitrap/MS technology in eight batches of CSF samples from several main producing areas in China. Notably, 39 lipophilic components were isolated and identified, some of which were first reported, for instance, 4 coumarins, 6 polymethoxyflavones (PMFs), and 3 phthalides. This study could lay the foundation for the quality evaluation and chemotaxonomic exploration of CSF in phytology sciences.

2. RESULTS

2.1. Optimization of SFE and UPLC-Q-Exactive Orbitrap/MS Conditions. The SFE extracts of 8 batches of CSF (seen from Table 1) were analyzed via UPLC-Q-Exactive Orbitrap/MS. To acquire more precise information about compounds of CSF, analytical conditions were determined by optimizing a sequence of parameters like the elution gradient and flow rate of the mobile phase, whereby the components of SFE extracts in CSF were able to be isolated in the positive mode where the detection signal was better than that in the negative-ion mode within 30 min. The application method was efficient, and the mean SFE extraction rate of CSF was 1.42% (1.19−1.73%). Herein, the total ion chromatogram (TIC) of samples and mixed standard substances are displayed in Figure 1. Totally, 39 compounds were separated and identified and labeled 1−39 in accordance with the sequence of the peak time (seen in Table 2). In general, these extraction constituents were divided into several groups consisting of 14 coumarins, 7 PMFs, 3 phthalides, 3 limonoids, 7 terpenes, and 5 phenolics. Among these, 7 compounds were discerned via retention times and fragments by comparison with standard substances and 32 compounds were distinguished according to MS^2 positive-ion fragments and the related information from these studies was reported formerly. All of these chemical structures are displayed in Figure 2.

Table 1. Representative Samples of CSF Investigated in This Study[a]

no.	collecting area (name)	sample source	collection time (yy/mm/dd)
S1	Guangdong province	Lecheng town, Zhaoqing city, Guangdong province	2019/09/25
S2	Guangxi province	Yongfu county, Guilin city, Guangxi province	2019/10/29
S3	Zhejiang province	Luodian town, Jinhua city, Zhejiang province	2019/11/07
S4	Zhejiang province	Chisong town, Jinhua city, Zhejiang province	2019/11/15
S5	Sichuan province	Huidong county, Liangshan prefecture, Sichuan province	2019/10/21
S6	Sichuan province	Peng'an county, Nanchong city, Sichuan province	2019/11/17
S7	Sichuan province[b]	Baiyang town, Wanzhou county, Chongqing city	2019/10/28
S8	Yunnan province	Huaning county, Yuxi city, Yunnan province	2019/10/28

[a]Guangdong province, Guangxi province, Zhejiang province, Sichuan province, and Yunnan province are the main producing areas. [b]Collecting area of Chongqing city was classified into main producing area of the Sichuan province by considering the geography.

2.2. Identification of Coumarins. Coumarins are a kind of natural compound with benzo-α-pyranone parent nucleus, which are the primary constituents in the SFE extracts of CSF. Fourteen coumarins were identified in CSF extracts. In the positive mode, the fragmentation of coumarins from mass spectrometry was characterized by the loss of neutral molecules, such as CO, CO_2, and CH_3, because of high-energy collisions.[13]

Compound **3** produced a protonation ion at m/z 193.0498 and fragment ions at m/z 178.0263 $[M + H - CH_3]^+$, 165.0548 $[M + H - CO]^+$, 150.0313 $[M + H - CH_3 - CO]^+$, and 147.1172 $[M + H - CO_2]^+$. Referring to mass spectrum cracking information from a previous study,[14] compound **3** was identified as isoscopoletin. The precursor ion of compound **8** was m/z 147.0443, which gave daughter ions such as m/z 132.9590, m/z 119.0858, m/z 103.0546, and m/z 91.0548 successively. According to the structure cracking rule of coumarins, compound **8** was detailed to be coumarin as done previously.[15] Meanwhile, compound **10** produced a strong molecular ion at m/z 223.0604 and formed fragments at m/z 208.0370 $[M + H - CH_3]^+$ and 179.0343 $[M + H - CO_2]^+$. Thus, compound **10** was tentatively identified as fraxinol from a previous study.[16] Moreover, compound **11** produced a molecular ion at m/z 177.0547 and formed fragments at m/z 162.0311 $[M + H - CH_3]^+$, 133.0647 $[M + H - CO_2]^+$, and 121.0650 $[M + H - 2CO]^+$. Therefore, compound **11** was identified as 7-methoxycoumarin as before.[17]

Compound **6**, **9**, and **14** were unambiguously identified as scopoletin, scoparone, and 5,7-dimethoxycoumarin, respectively, by referring to the document and the standard substance with the same fragmentation traits.

Compounds **12**, **13**, **15**, **17**, and **19** belong to the linear furanocoumarins that are rich in *Citrus* genus. Compound **12** produced a molecular ion at m/z 187.0392 and formed characteristic fragmentations at m/z 159.0441 $[M + H - CO]^+$, 143.0493 $[M + H - CO_2]^+$, 131.0494 $[M + H - 2CO]^+$, and 115.0546 $[M + H - CO - CO_2]^+$. Thus, compound **12** was identified as isopsoralen from a previous study.[18] Because of an $[M + H]^+$ ion at m/z 305.1020, the fragment ion at m/z 203.0340 indicating loss of the neutral substituent $[M + H - C_5H_{10}O_2]^+$, and sequential cleavage losing the CO radical and generating the typical $[203\text{-}2CO]^+$ fragment at m/z 147.0441, compound **13** was tentatively identified as oxypeucedanin hydrate, which was in accordance with a previous report.[19] Similarly, compound **15** generated a precursor ion m/z 217.0495, while m/z 202.0261, m/z 174.0312, m/z 173.0598, and m/z 146.0361 were characteristic fragment ions that matched with the MS^2 information as reported earlier.[7] Therefore, compound **15** was identified as bergapten (fragmentation pattern is shown in Figure 3A). Compound **17** exhibited a precursor ion at m/z 317.1020 and fragment ions at m/z 299.0896, m/z 273.0750, and m/z 233.0446, while compound **19** exhibited a precursor ion at m/z 287.0913 and fragment ions at m/z 203.0338 and m/z 175.0390. According to the MS^2 information supported by the previous investigation,[20] compounds **17** and **19** were confirmed to be byakangelicol and oxypeucedanin, respectively.

In addition, compound **34** exerted a protonated ion at m/z 193.0497 and fragment ions at m/z 178.0261, m/z 165.0547, m/z 149.0598, and m/z 137.0598. Thus, compound **34** was

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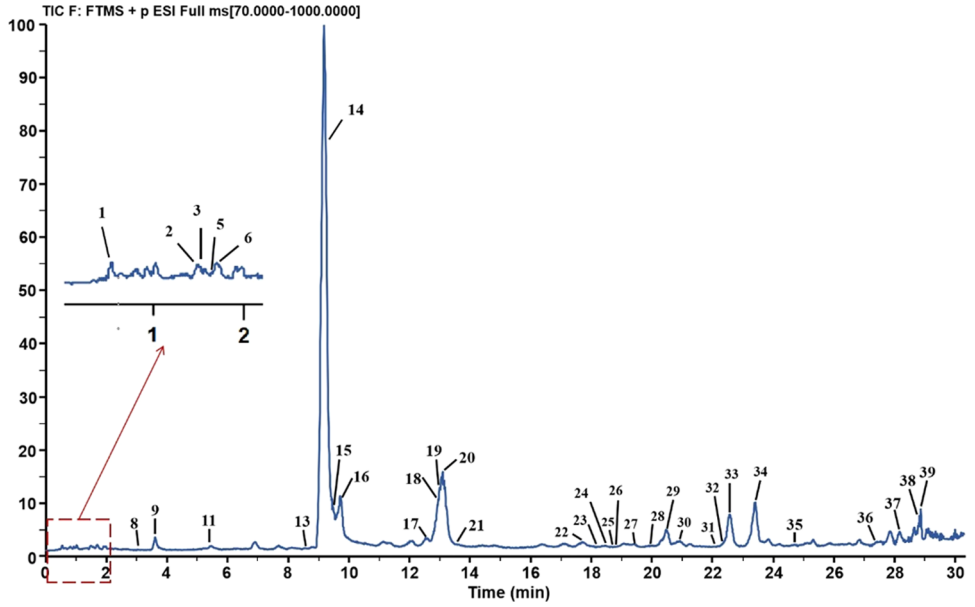

Figure 1. Representative total ion chromatogram of CSF in positive ionization mode. Compound **6**: scopoletin; compound **9**: scoparone; compound **14**: 5,7-dimethoxycoumarin; compound **16**: limonin; compound **20**: nomilin; compound **29**: nobiletin; compound **33**: tangeretin.

confirmed as 5,7-dihydroxy-4-methylcoumarin, as reported previously.[21] Also, compound **38** produced a precursor ion at *m/z* 163.0391 and fragment ions at *m/z* 149.0450, *m/z* 135.0441, *m/z* 119.0493, and *m/z* 107.0494. Following the characteristic fragmentation profiles and a previous study,[22] compound **38** was assigned as 7-hydroxycoumarin.

It was manifested that compounds **9**, **10**, **12**, and **34** were not reported previously. These four coumarins have various functions; for example, compound **12** as isopsoralen has antibacterial, anti-inflammatory, antitumor, and antiosteoporotic properties.[23]

2.3. Identification of PMFs. PMFs are discovered nearly exclusively in *Citrus* species and possess the basic aglycone structure and are different from each other in the location and amount of substituted $-OCH_3$ and/or $-OH$ on the flavonoid mother nucleus. It was believed that characteristic fragment ions were formed by removal of $-CH_3$, CO, and H_2O. After scanning, seven PMFs were identified or tentatively discriminated in CSF.

Because of standard reference, compounds **29** and **33** were determined as nobiletin and tangeretin, respectively. The comprehensive fragmentation pattern of compound **29** is shown in Figure 3B, which included daughter ions at *m/z* 388.1152, *m/z* 373.0919, *m/z* 355.0811, and *m/z* 327.0860.

Compound **23** was assigned to be 5,7,3′-trihydroxy-6,4′,5′-trimethoxyflavone with reference to the literature,[8] which gave a protonated molecular ion at *m/z* 361.0920 and fragment ions at *m/z* 346.0685, *m/z* 331.0449, and *m/z* 303.0501. Besides, compound **25** generated a protonated ion at *m/z* 343.1178 and fragment ions at *m/z* 328.0944, *m/z* 313.0707, and *m/z* 285.0758. Thus, compound **25** was indicated as 6-demethoxytangeretin from reference literature.[24] Compound **28** was assigned as isoflavone for the protonated ion at *m/z* 331.0812 and fragment ions like *m/z* 316.0578, *m/z* 301.0347, *m/z* 285.0393, and *m/z* 273.0393. As a result, compound **28** was assigned as iristectorigenina from the literature reported.[25,26] Compound **30** was plausibly regarded as jaceosidin from reference literature,[27] which exhibited the $[M + H]^+$ at *m/z* 331.0813 and MS^2 ions at *m/z* 316.0597 $[M + H - CH_3]^+$, 301.0344 $[M + H - 2CH_3]^+$, and 288.0629 $[M + H - CH_3 - CO]^+$. The precursor ion of compound **32** was at *m/z* 389.1234, as well as fragment ions, at *m/z* 374.0998, *m/z* 359.0763, *m/z* 341.0659, and *m/z* 331.0818. By comparison with the previous report,[28] compound **32** could be unambiguously identified as 5-demethylnobiletin (fragmentation pattern is shown in Figure 3C).

It was indicated that six PMFs including compounds **25**, **28**, **29**, **30**, **32**, and **33** were not reported previously.

2.4. Identification of Phthalides. Phthalides are also known as γ-hydroxymethyl benzoate lactones, which are characterized by the double ring fusion of the inner ester and benzene. The fragment ions of H_2O, CO, and alkyl chain are easily formed under high-energy collision.[29] Herein, three phthalides were identified.

The protonated ions of compounds **18** and **24** were both at *m/z* 191.1068, with similar second-order fragment ions at *m/z* 173 $[M + H - H_2O]^+$, 149 $[M + H - C_3H_6]^+$, 145 $[M + H - H_2O - CO]^+$, and 130 $[M + H - H_2O - C_3H_6]^+$; they were found to be isomers. Further, the retention times of compound **18** and compound **24** was 12.66 and 18.42 min, respectively; according to the polarity of the compound combined with the above information, compound **18** was finally determined as 3-*n*-butylphthalide and compound **24** (fragmentation pattern shown in Figure 3D), as ligustilide.[30,31] Compound **21** had a precursor ion at *m/z* 193.0498 and secondary fragment ions at *m/z* 175.1120, *m/z* 147.1170, *m/z* 137.0599, and *m/z* 105.0703. Based on previous research,[31] compound **21** was tentatively characterized as senkyunolide A (fragmentation pattern is shown in Figure 3E).

It was clarified that three phthalides including compounds **18**, **21**, and **24** were not reported previously in CSF. Phthalides, especially ligustilide, were effective in improving microcirculation, protecting against cerebral ischemia-reperfusion injury, and treating tumor.[32]

2.5. Identification of Limonoids. Three limonoids, including limonin, nomilin, and obacunone, were identified from the SFE extracts of CSF. Via standard reference, compounds **16** and **20** were identified as limonin and nomilin,

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Table 2. Compounds Identified in CSF by UPLC-Q-Exactive Orbitrap/MS[a]

no.	t_R (min)	theoretical $[M + H]^+$ (m/z)	experimental $[M + H]^+$ (m/z)	major secondary fragment ions (MS/MS)	compound formula	proposed compound	ref
				Coumarins			
3	1.51	193.0495	193.0498	178.0263, 165.0548, 161.0539, 150.0313, 147.1172, 137.0599, 133.0286, 122.0365, 117.0337, 109.0651, 105.0339, 94.0420, 77.0393, 66.0472, 56.9658	$C_{10}H_8O_4$	isoscopoletin	14
6	1.70	193.0495	193.0498	178.0263, 165.0549, 161.0235, 150.0313, 137.0598, 133.0286, 122.0365, 117.0336, 105.0341, 89.0391, 77.0393, 66.0471, 53.0395	$C_{10}H_8O_4$	scopoletin	[c]
8	3.17	147.0440	147.0443	132.9590, 119.0858, 114.9491, 105.0703, 103.0546, 95.0494, 91.0548, 84.9603, 79.0553, 73.9930, 65.0393, 60.9770, 53.0395	$C_9H_6O_2$	coumarin	15
9	3.60	207.0651	207.0654	192.0417, 191.0340, 181.4489, 179.0704, 163.0390, 151.0755, 148.0520, 136.0520, 121.0651, 107.0496, 91.0549, 79.0549	$C_{11}H_{10}O_4$	scoparone[b]	[c]
10	3.96	223.0601	223.0604	208.0370, 190.0263, 179.0343, 162.0314, 153.0105, 149.0964, 134.0364, 125.0154, 106.0418, 91.0547, 78.0472,58.0658	$C_{11}H_{10}O_5$	fraxinol[b]	16
11	5.50	177.0546	177.0547	162.0311, 149.0598, 133.0648, 121.0650, 118.0416, 103.0546, 91.0548, 79.0549, 66.0472, 53.0394	$C_{10}H_8O_3$	7-methoxycoumarin	17
12	6.86	187.0389	187.0392	173.1181, 159.0441, 143.0493, 131.0494, 115.0546, 109.1017, 103.0548, 95.0495, 88.0224, 67.0550, 55.0550	$C_{11}H_6O_3$	isopsoralen[b]	18
13	8.71	305.1019	305.1020	287.1166, 203.0340, 192.0297, 175.0392, 159.0441, 147.0441, 131.0493, 119.0494, 91.0548, 59.0500	$C_{16}H_{16}O_6$	oxypeucedan hydrate	19
14	9.18	207.0651	207.0654	192.0418, 179.0703, 164.0469, 151.0755, 148.0520, 133.0650, 121.0651, 118.0471, 103.0547, 91.0548, 79.0549, 65.0393	$C_{11}H_{10}O_4$	5,7-dimethoxycoumarin[b]	[c]
15	9.57	217.0495	217.0495	202.0261, 174.0312, 173.0598, 167.9620, 161.0596, 146.0361, 131.0491, 115.0545, 105.0704, 91.0548, 79.0549, 53.4664	$C_{12}H_8O_4$	bergapten	7
17	12.98	317.1019	317.1020	299.0896, 273.075, 245.0441, 233.0446, 218.0211, 203.0339, 188.0106, 175.0392, 162.0310, 147.0441, 119.0496, 91.0548, 85.0655, 67.0550, 57.0706	$C_{17}H_{16}O_6$	byakangelicol	20
19	12.99	287.0914	287.0913	203.0338, 175.0390, 174, 159.0441, 147.0440, 131.0492, 119.0491, 91.0546, 85.0653, 67.0550, 59.0500	$C_{16}H_{14}O_2$	oxypeucedanin	20
34	23.40	193.0495	193.0497	178.0261, 165.0547, 149.0598, 137.0598, 134.0364, 121.0650, 109.0652, 106.0418, 91.0549, 79.0549, 67.0186, 53.0394	$C_{10}H_8O_4$	5,7-dihydroxy-4-methylcoumarin[b]	21
38	28.77	163.0389	163.0391	149.0450, 139.9821, 135.0441, 119.0493, 107.0494, 95.0495, 91.0547, 84.9604, 79.0548, 68.9979, 61.6360, 53.0395	$C_9H_6O_3$	7-hydroxycoumarin	22
				Methoxyflavonoids			
23	18.02	361.0917	361.0920	346.0685, 331.0449, 315.0510, 303.0501, 285.0396, 257.0445, 229.0495, 201.0546, 181.0133, 169.0133, 121.0288, 105.0336, 68.9977	$C_{18}H_{16}O_8$	5,7,3′-trihydroxy-6,4′,5′-trimethoxyflavone	8
25	18.72	343.1176	343.1178	328.0944, 313.0707, 285.0758, 270.0524, 257.0806, 242.0570, 211.0751, 199.0237, 181.0132, 153.0183, 135.0442, 125.0235, 107.0126, 85.0287, 69.0339	$C_{19}H_{18}O_6$	6-demethoxytangeretin[b]	24
28	19.93	331.0812	331.0812	316.0578, 301.0347, 285.0393, 273.0393, 257.0445, 242.0571, 214.0622, 186.063, 169.0133, 135.0443, 121.0650, 108.0572, 91.0549, 68.9977	$C_{17}H_{14}O_7$	iristectorigenina[b]	25, 26
29	20.43	403.1387	403.1388	388.1152, 373.0919, 358.0682, 355.0811, 327.0860, 301.0706, 284.0680, 258.0524, 229.0340, 211.0237, 193.0129, 183.0290, 165.0546, 127.0392, 99.0441, 69.0344	$C_{21}H_{22}O_8$	nobiletin[b]	[c]
30	20.92	331.0812	331.0813	316.0579, 301.0344, 288.0629, 273.0395, 257.0448, 245.0444, 229.0493, 199.0389, 169.0133, 148.0520, 135.0442, 121.0651, 108.0574, 86.9453	$C_{17}H_{14}O_7$	jaceosidin[b]	27
32	22.31	389.1230	389.1234	374.0998, 359.0763, 341.0659, 331.0810, 316.0582, 285.0755, 260.0676, 227.0552, 215.0189, 197.0083, 163.0755, 148.0521, 113.0238, 85.0285	$C_{20}H_{20}O_8$	5-demethylnobiletin[b]	28
33	22.51	373.1281	373.1286	358.1050, 343.0816, 328.0579, 297.0762, 285.0401, 271.0603, 254.0570, 229.0322, 211.0240, 183.0292, 135.0443, 127.0392, 99.0441, 69.0339	$C_{20}H_{20}O_7$	tangeretin[b]	[c]
				Phthalides			
18	12.66	191.1066	191.1068	173.0963, 163.1328, 158.0728, 149.0598, 145.1012, 130.0778, 117.0702, 111.0443, 105.0702, 99.0444, 91.0547, 79.0549, 71.0489, 55.0551	$C_{12}H_{14}O_2$	3-*n*-butylphthalide[b]	30, 31
21	13.61	193.1223	193.0498	175.1120, 157.1016, 147.1170, 137.0599, 133.0286, 123.0444, 119.0859, 109.0652, 105.0703, 97.0652, 93.0705, 91.0548, 81.0705, 69.0706, 67.0550, 53.0395	$C_{12}H_{16}O_2$	senkyunolide A[b]	31
24	18.42	191.1066	191.1068	173.0963, 163.1118, 149.0599, 145.1013, 135.0442, 130.0778, 121.0650, 117.0702, 105.0703, 91.0548, 79.0549, 67.0550, 55.0549	$C_{12}H_{14}O_2$	ligustilide[b]	30, 31

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Table 2. continued

no.	t_R (min)	theoretical $[M + H]^+$ (m/z)	experimental $[M + H]^+$ (m/z)	major secondary fragment ions (MS/MS)	compound formula	proposed compound	ref
				Limonoids			
16	9.69	471.2013	471.2013	453.1893, 435.1798, 425.1957, 409.2002, 391.1882, 367.1909, 339.1952, 213.0910, 205.0498, 187.0754, 169.1006, 161.0598, 133.0649, 119.0858, 105.0702, 95.0132, 79.0549, 69.0706	$C_{26}H_{30}O_8$	limonin[b]	[c]
20	13.08	515.2272	515.2272	497.2172, 469.2216, 455.2065, 437.1960, 419.1853, 411.2165, 393.2059, 369.2074, 341.2097, 279.1383, 243.1000, 231.0645, 187.0754, 161.0598, 133.0649, 105.0703, 95.0132, 79.0548, 69.0705	$C_{28}H_{34}O_9$	nomilin[b]	[c]
22	17.73	455.2064	455.2068	437.1958, 419.1862, 409.2012, 391.1910, 377.1368, 359.1272, 349.1434, 331.1335, 303.1391, 273.1281, 235.1120, 209.0965, 187.0757, 161.0599, 133.0650, 119.0860, 105.0703, 95.0133, 81.0341, 67.0551	$C_{26}H_{30}O_7$	obacunone	33
				Terpenoids			
5	1.66	153.1273	153.1274	135.1169, 125.0599, 120.9808, 111.0444, 109.1015, 107.0859, 95.0860, 93.0704, 88.9530, 84.9603, 81.0705, 79.0549, 65.0394, 59.0500, 55.0551	$C_{10}H_{16}O$	camphor	[d]
27	19.59	231.1379	231.1381	213.1277, 203.1432, 195.1168, 189.0915, 185.1328, 175.0752, 163.0391, 157.1013, 145.1015, 143.0857, 131.0858, 119.0859, 105.0702, 91.0548, 79.0549, 69.0707, 55.0550	$C_{15}H_{18}O_2$	dehydrocostus lactone[b]	42
31	22.13	233.1536	233.1539	215.1434, 197.1322, 187.1486, 177.0912, 173.0965, 159.0810, 151.0756, 145.1015, 131.0858, 105.0703, 95.0861, 91.0549, 81.0706, 67.0549, 56.9656	$C_{15}H_{20}O_2$	atractylenolide II[b]	34
35	24.70	231.1379	231.1380	213.1274, 203.1068, 189.0910, 185.1325, 175.0756, 161.1327, 157.1013, 147.0800, 143.0856, 129.0700, 105.0702, 91.0548, 81.0705, 67.0549, 55.0549	$C_{15}H_{18}O_2$	atractylenolide I[b]	35
36	27.43	219.1743	219.1745	203.1433, 187.0761, 173.0234, 163.0390, 145.0284, 131.0857, 119.0858, 107.0859, 95.0860, 91.0547, 81.0705, 79.0549, 69.0706, 67.0550, 55.0551	$C_{15}H_{22}O$	(+)-nootkatone[b]	43
37	28.37	219.1743	219.1745	203.1430, 191.1794, 187.0763, 173.1328, 161.1326, 145.1013, 135.1169, 119.0858, 107.0859, 95.0860, 93.0704, 91.0548, 81.0705, 67.0550, 55.0551	$C_{15}H_{22}O$	α-cyperone[b]	44
39	28.85	219.1743	219.1746	203.1433, 201.1436, 191.1792, 187.0761, 175.1479, 161.1328, 159.1017, 151.1120, 145.1013, 133.1017, 121.1016, 107.0860, 95.0861, 91.0548, 81.0705, 67.0550, 55.0551	$C_{15}H_{22}O$	germacrone[b]	36
				Phenolics			
1	0.48	123.0553	123.0555	106.0292, 96.0448, 95.0498, 90.9482, 81.0706, 80.0501, 79.0549, 72.9379, 67.0550, 60.9389, 56.9430, 53.0395	$C_6H_6N_2O$	nicotinamide[b]	[d]
2	1.49	153.0546	153.0547	135.1170, 125.0599, 111.0444, 107.0859, 93.0340, 84.9602, 81.0706, 79.0548, 71.0500, 65.0394, 59.0500	$C_8H_8O_3$	vanillin[b]	45
4	1.59	183.0651	183.0653	165.0911, 155.0704, 140.0468, 137.0959, 125.0235, 123.0443, 111.0443, 105.0451, 97.0284, 95.0496, 90.9480, 81.0704, 71.0501, 67.0550, 53.0394	$C_9H_{10}O_4$	syringaldehyde[b]	45
7	1.71	195.0651	195.0544	187.4464, 177.0549, 163.0392, 153.0553, 149.0599, 145.0286, 135.0443, 125.0599, 117.0339, 107.095, 95.0494, 89.0392, 84.9606, 79.0549, 63.0237	$C_{10}H_{10}O_4$	ferulic acid	46
26	18.85	179.0702	179.0710	161.0599, 147.0442, 136.0521, 133.0651, 119.0495, 115.0548, 105.0703, 95.0499, 91.0549, 84.9603, 79.0550, 65.0394, 55.0187	$C_{10}H_{10}O_3$	ferulaldehyde[b]	47

[a]*Four* coumarins (compounds **9**, **10**, **12**, and **34**), 6 polymethoxyflavones (compounds **25**, **28**, **29**, **30**, **32**, and **33**), 3 phthalides (compounds **18**, **21**, and **24**), 6 terpenes (compounds **27**, **31**, **35**, **36**, **37**, and **39**), and 4 phenolics (compounds **1**, **2**, **4**, and **26**) were not reported previously. [b]Compounds were not reported in CSF extracts previously. [c]Further confirms the comparison with standard compounds. [d]Refers to the database.

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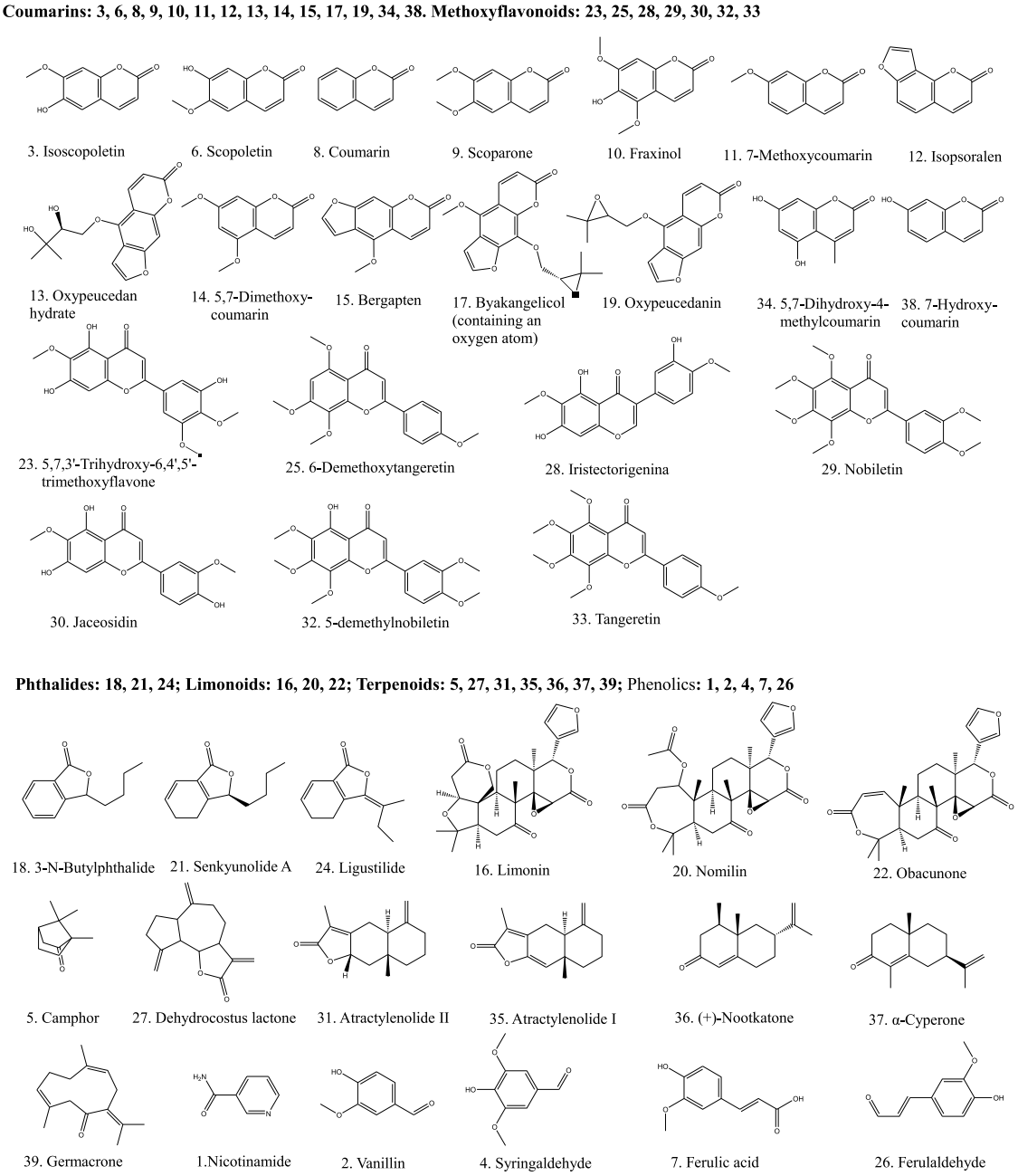

Figure 2. Chemical structure of 39 compounds that were unequivocally and tentatively identified.

respectively. The detailed cracking process of compound **16** is shown in Figure 3F, which included son ions at m/z 425.1957, m/z 409.2002, m/z 367.1909, m/z 339.1952, and m/z 161.0598. In addition, compound **22** had an $[M + H]^+$ ion at m/z 455.2068 and a series of MS^2 ions at m/z 437.1958, m/z 419.1862, and m/z 409.2012; thus, compound **22** was determined to be obacunone, which is also a known ingredient in *Citrus* fruits as reported previously.[33]

2.6. Identification of Terpenoids. In this study, seven terpenoids were identified from CSF where compounds **5**, **27**, **31**, **35**, **36**, **37**, and **39** were unequivocally identified as camphor, dehydrocostus lactone, atractylenolide II, atractylenolide I, (+)-nootkatone, α-cyperone, and germacrone, respectively.

Compound **31** (fragmentation pathway is shown in Figure 3H) exhibited the $[M + H]^+$ ion at m/z 233.1539 in the MS spectrum, and the main fragment ion peaks were at m/z 187.1486, m/z 177.0712, m/z 151.0756, m/z131.0858, m/z 105.0703, and m/z 91.0549. Therefore, compound **31** was confirmed as atractylenolide II from a previous report.[34] Compound **35** produced a molecular ion peak at m/z 231.1380 for $[M + H]^+$ and daughter ions like m/z 213.1274, m/z 185.1325, and m/z 147.0800. On the basis of characteristic fragment profiles, compound **35** was determined to be atractylenolide I, as reported earlier.[35]

In addition, the deprotonated ion of compound **39** appeared at m/z 219.1746, further producing MS^2 fragmentation at m/z 201.1436 and m/z 159.1017. According to the literature,[36] compound **39** was determined to be germacrone.

Note that compounds **27**, **31**, **35**, **36**, **37**, and **39** were not found in previous studies for identification of components in CSF extracts.

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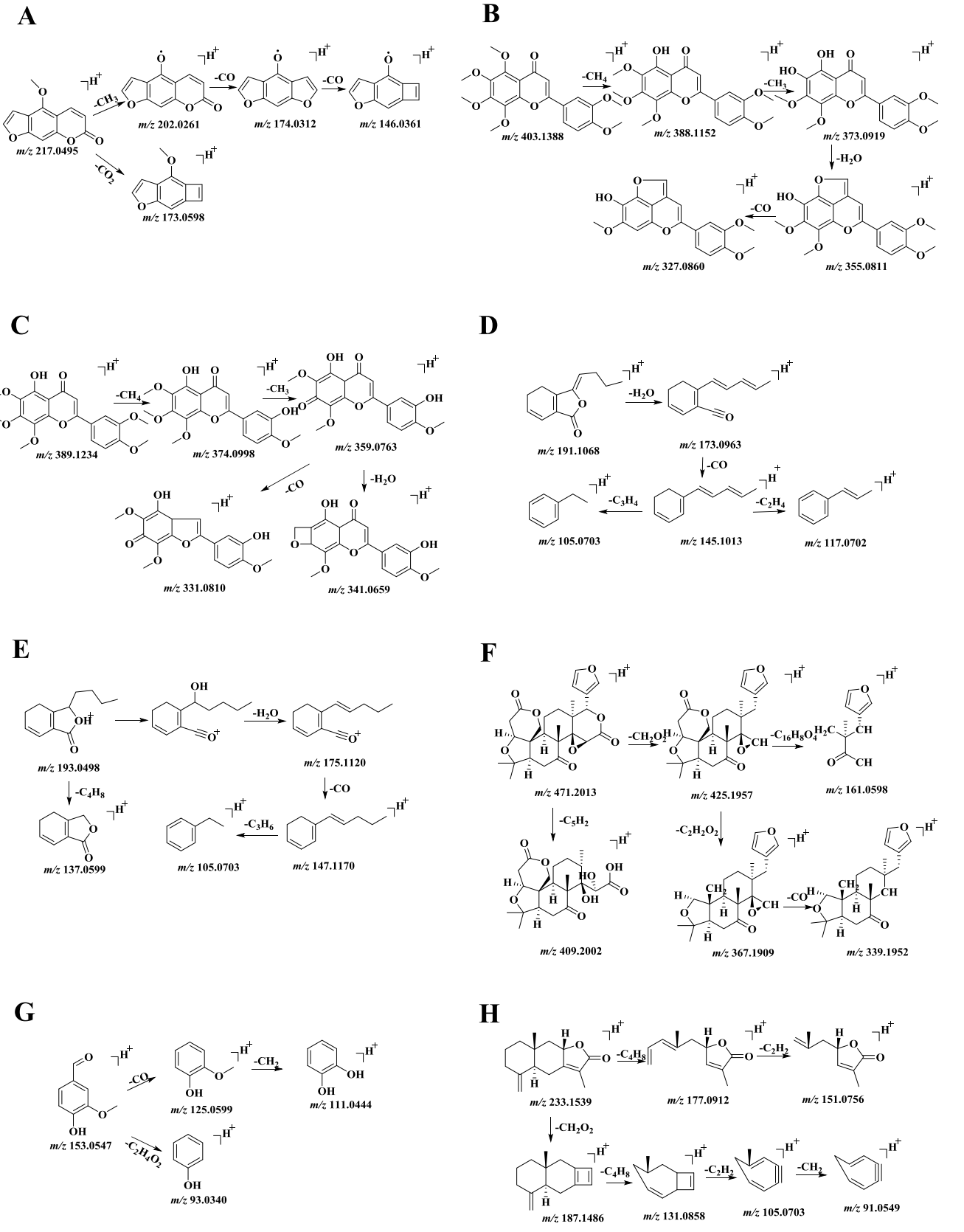

Figure 3. Proposed fragmentation pathways of the main fragment ions in the positive-ion mode. (A) Bergapten; (B) nobiletin; (C) 5-demethylnobiletin; (D) ligustilide; (E) senkyunolide A; (F) limonin; (G) vanillin; and (H) atractylenolide II.

2.7. Identification of Phenolics. In addition, five constituents including compounds **1**, **2**, **4**, **7**, and **26** were unequivocally assigned as nicotinamide, vanillin, syringaldehyde, ferulic acid, and ferulaldehyde, respectively, integrated with the literature and the corresponding formula supplied by MS.

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Taking compound **2** as an example, a precursor ion sign was observed at m/z 153.0547, while in the MS^2 spectrum, the primary fragment ions were at m/z 125.0599 $[M + H - CO]^+$, m/z 111.0444 $[M + H - CO - CH_2]^+$, and m/z 93.0340 $[M + H - C_2H_4O_2]^+$. Compound **2** was identified as vanillin by comparing with a previous study,[37,38] which included a protonated ion at m/z 153.0547 and son ions at m/z 125.0599, m/z 111.0444, and m/z 93.0340. The fragmentation pathways of vanillin are shown in Figure 3G.

Of note, compounds **1**, **2**, **4**, and **26** were not reported previously in CSF extracts.

2.8. Comparison of *Citrus* Samples from Different Origins. The established UPLC-Q-Exactive Orbitrap/MS method was applied to the coinstantaneous determination of the chemical markers in eight batches of CSF samples from several main producing areas, such as the Guangdong province, Guangxi province, Zhejiang province, Sichuan province, and Yunnan province. The results are shown in Table 3 as the mean values of two repeated injections. Twenty-five compounds were found in all of these CSF samples, which showed that they were common in places. Of course, there were differences among these samples. Coumarin, dehydrocostus lactone, atractylenolide II, and atractylenolide I were exclusively found in CSF samples from the Guangdong province by comparison with others. Moreover, compared with others, isopsoralen was exclusively found in CSF samples from the Guangxi province and ferulic acid was exclusively found in CSF samples from the Zhejiang province. In parallel, it should be noted that nicotinamide was not found in CSF samples from the Yunnan province, syringaldehyde was not found in CSF samples from the Guangdong province, and both 3-*n*-butylphthalide and ligustilide were deficient in CSF samples from a region of the Sichuan province (Nanchong city).

3. DISCUSSION

The most widespread technique for isolation of bioactive components from plant materials is the traditional organic solvent extraction. The major drawbacks of this extraction method are high time consumption and relatively poor selectivity. Noticeably, the new generation of extraction techniques are evolving to overcome these shortcomings.[39] SFC has proved to be a powerful separation technique. Because of the low viscosity and high diffusivity of supercritical fluids' mobile phases, high flow rates and thus short analysis time as well as high efficiency could be easily achieved.[40] The SFE approach showed an excellent functioning of extraction of nonpolar and some low-polarity substances. Herein, SFE combined with UPLC-Q-Exactive Orbitrap/MS method was established for identification of components from CSF, whereby 39 lipophilic compounds in SFE extracts were isolated and identified via UPLC-Q-Exactive Orbitrap/MS, among which 23 compounds including 4 coumarins, 6 PMFs, and 3 phthalides were first identified in CSF extracts.

It is noteworthy that it is well known that coumarins are the main components of CSF and that PMFs may not be abundant; however, this study raised the novel interpretation that SFE extracts of CSF may contain a series of PMFs as well as coumarins. Interestingly, seven PMFs composed of 6-demethoxytangeretin, iristectorigenina, nobiletin, jaceosidin, 5-demethylnobiletin, and tangeretin were identified in SFE extracts, illustrating that CSF may contain a series of PMFs. The result was different from that of the study performed by Li et al.,[6] whose investigation demonstrated that PMFs were hardly detected in certain *Citrus* herbs including CSF based on LC-QTOF-MS/MS. The present study was of great significance for a strong supplement to the previous conclusion. In addition, these PMFs have beneficial pharmacodynamics, such as tangeretin exhibiting anti-inflammation, anti-oxidation, and neuroprotection properties.[41]

Moreover, a correlation between their composition and the collection areas was demonstrated. To a great extent, coumarin, dehydrocostus lactone, atractylenolide II, or

Table 3. Differences in Chemical Components Among Different Origins[a]

peak	compound	S1	S2	S3	S4	S5	S6	S7	S8
1	nicotinamide[c]	*	*	*	*	*	*	*	
2	vanillin	*	*	*	*	*	*	*	*
3	isoscopoletin	*	*	*	*	*	*	*	*
4	syringaldehyde[c]		*	*	*	*	*	*	*
5	camphor	*	*	*	*	*	*	*	*
6	scopoletin	*	*	*	*	*	*	*	*
7	ferulic acid[b]			*	*				
8	coumarin[b]	*							
9	scoparone	*	*	*	*	*	*	*	*
10	fraxinol		*	*		*	*		*
11	7-methoxycoumarin	*	*	*	*	*	*	*	*
12	isopsoralen[b]		*						
13	oxypeucedan hydrate	*	*	*	*	*	*	*	*
14	5,7-dimethoxycoumarin	*	*	*	*	*	*	*	*
15	bergapten	*	*	*	*	*	*	*	*
16	limonina	*	*	*	*	*	*	*	*
17	byakangelicol	*	*	*	*	*	*	*	*
18	3-*n*-butylphthalide[c]	*	*	*	*	*		*	*
19	oxypeucedanin	*	*	*	*	*	*	*	*
20	nomilin	*	*	*	*	*	*	*	*
21	senkyunolide A	*	*	*	*				
22	obacunone	*	*	*	*	*	*	*	*
23	5,7,3′-trihydroxy-6,4′,5′-trimethoxyflavone	*	*	*	*	*	*	*	*
24	ligustilide[c]	*	*	*	*	*		*	*
25	6-demethoxytangeretin	*		*		*	*	*	*
26	ferulaldehyde	*				*	*	*	*
27	dehydrocostus lactone[b]	*							
28	iristectorigenina	*	*	*	*	*	*	*	*
29	nobiletin[b]	*	*	*	*	*	*	*	*
30	jaceosidin	*	*	*	*	*	*	*	*
31	atractylenolide II[b]	*							
32	5-demethylnobiletin	*	*	*	*	*	*	*	*
33	tangeretin	*	*	*	*	*	*	*	*
34	5,7-dihydroxy-4-methylcoumarin	*	*	*	*	*	*	*	*
35	atractylenolide I[b]	*							
36	(+)-nootkatone	*	*	*	*	*	*	*	*
37	α-cyperone	*	*	*	*	*	*	*	*
38	7-hydroxycoumarin	*	*	*	*	*	*	*	*
39	germacrone	*	*	*	*	*	*	*	*

[a]S1, CSF samples from the Guangdong province; S2, CSF samples from the Guangxi province; S3 and S4, CSF samples from the Zhejiang province; S5, S6, and S7, CSF samples from the Sichuan province; and S8, CSF samples from the Yunnan province. [b]The compound is exclusively found in the corresponding origins or main producing area. [c]The compound is exclusively deficient in the corresponding origins or main producing area. *The compound is found in the corresponding origins or main producing area.

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atractylenolide I could be the marker compounds used to distinguish CSF of the Guangdong province from that of the other origins, while isopsoralen and ferulic acid may be the marker compounds that are exclusively found in CSF from the Guangxi province and Zhejiang province, respectively.

4. CONCLUSIONS

SFE coupled with UPLC-Q-Exactive Orbitrap MS analysis helped to explore more novel information on lipophilic components in CSF extracts: 23 compounds were not reported in CSF extracts earlier. Meanwhile, it was indicative that PMFs should be abundant in CSF. Moreover, some compounds may be recognized as a marker to distinguish CSF from different origins in China. In brief, the present study can boost the quality of assessment as well as future pharmacodynamics investigation of CSF and provide chemotaxonomic botanical knowledge for this functional food. Further, developing quality control methods is indispensable to ensure the identification of the product from different origins.

5. MATERIALS AND METHODS

5.1. Reagents and Chemicals. Formic acid and methanol of MS grade were purchased from Merck KGaA Company of Germany, Ltd., and ThermoFisher Scientific (China) Co., respectively. Standard compounds 5,7-dimethoxycoumarin (compound **14**), nomilin (compound **20**), scopoletin (compound **6**), and scoparone (compound **9**) were provided by Sichuan Vicky Biotechnology Co. Ltd., and limonin (compound **16**), nobiletin (compound **29**), and tangeretin (compound **33**) were provided by Chengdu Mansite Biotechnology Co. Ltd. The purity of these standard substances was over 98%. There were eight batches of CSF samples (shown in Table 1), which were identified from the dried fruits of *Citrus medica* L. var. *sarcodactoxylis* Swingle in *Rutaceae* by Professor Wu Bo of the School of Pharmacy, Guangzhou Medical University. The samples were kept in the Pharmacognosy Laboratory, School of Pharmacy, Guangzhou Medical University.

5.2. Instrumentation. SFE was operated with an ASI Supercritical Fluid Extraction system (SPE-ed SFE-2) (Applied Separations, Inc., Allentown, PA) equipped with a high-pressure CO_2 ASF-100 pump. Hk-04b swing crusher was produced from Guangzhou Xuyang Machinery Equipment Co. Ltd., and the ME 104 electronic analytical balance was provided by Mettler Toledo Instrument Co. Ltd. The UPLC system mainly consisted of an Ultimate 3000 series ultra-high-performance liquid chromatograph (ThermoFisher Scientific) equipped with an online degasser, a quaternary pump, an autosampler, and a column temperature compartment; it was connected to a Q-Exactive Orbitrap tandem mass spectrometer (ThermoFisher Scientific) via an electrospray ionization interface. Besides, the ZORBAX C_{18} column (2.1 mm × 50 mm, 1.8 μm) was offered by Agilent Technologies.

5.3. SFE Conditions. All SFE extractions were carried out at 50 °C in a dynamic mode with a total CO_2 flow rate of 3 L/min and extractive pressure of 33 MPa. The total process was within 120 min for each extraction.

5.4. Sample Preparation. These reference substances were weighed appropriately and then dissolved in dichloromethane. The CSF samples collected from different origins were cut into smaller pieces and smashed into powder by a swing crusher and sifted through a 40-mesh sieve. The accurately weighed powder (50 g) was directly put into the SFE ax, without any type of support materials or entrainers. SFE extracts were harvested, put into vials, and then stored at 4 °C. Prior to the detection test; the stock solution was diluted with dichloromethane to a suitable concentration. The diluent solution was filtered by a 0.22 μm microporous membrane and then shifted into an autosampler vial for MS analysis.

5.5. Analytical System. The separation was performed on a ZORBAX Rclipse Plus C_{18} column at 35 °C. The mobile phase consisted of 0.1% formic acid aqueous solution (phase A) and methanol (phase B), using a gradient elution of 30% B at 0−3 min; 30−45% B at 3−8 min; 45% B at 8−12 min; 45−50% B at 12−16 min; 50−70% B at 16−25 min; and 70−90% B at 25−30 min. The flow rate was maintained at 0.3 mL/min, and the injected sample volume was 1 μL.

For Q-exactive Orbit-MS analysis, the source settings were as follows: The spray voltage was 3.5 kV. The capillary temperature was 320 °C, and the auxiliary gas heating temperature was 300 °C. Sheath gas, auxiliary gas, and purge gas were set at 30 units, 10 units, and 5 units, respectively. Full scan data collection and dependent scan event data collection were executed from m/z 100 to m/z 1000 in the dd-MS^2 mode with a resolution of 70 000. The system was regulated by Xcalibur software and collected data was processed by Metworks software (ThermoFisher Scientific).

AUTHOR INFORMATION

Corresponding Authors

Guoping Yang − *Center of Clinical Pharmacology, The Third Xiangya Hospital, Central South University, Changsha 410013, Hunan, P. R. China;* orcid.org/0000-0001-5930-586X; Phone: +86-0731-88618931; Email: ygp9880@126.com

Guodong Zheng − *Key Laboratory of Molecular Target & Clinical Pharmacology and the State Key Laboratory of Respiratory Disease, School of Pharmaceutical Sciences & the Fifth Affiliated Hospital, Guangzhou Medical University, Guangzhou 511436, Guangdong, P. R. China;* orcid.org/0000-0003-3708-1193; Phone: +86-020-37103256; Email: gd200237@126.com

Authors

Chengxiao Fu − *Center of Clinical Pharmacology, The Third Xiangya Hospital, Central South University, Changsha 410013, Hunan, P. R. China; Department of Pharmacy, The First Affiliated Hospital of University of South China, Hengyang 421001, Hunan, P. R. China*

Mengshi Liu − *Key Laboratory of Molecular Target & Clinical Pharmacology and the State Key Laboratory of Respiratory Disease, School of Pharmaceutical Sciences & the Fifth Affiliated Hospital, Guangzhou Medical University, Guangzhou 511436, Guangdong, P. R. China*

Yueshan Li − *School of Health and Wellness, Guangzhou Huaxia Technical College, Guangzhou 510935, P. R. China*

Kanghui Wang − *Key Laboratory of Molecular Target & Clinical Pharmacology and the State Key Laboratory of Respiratory Disease, School of Pharmaceutical Sciences & the Fifth Affiliated Hospital, Guangzhou Medical University, Guangzhou 511436, Guangdong, P. R. China*

Bo Yang − *Department of Pharmacy, The First Affiliated Hospital of University of South China, Hengyang 421001, Hunan, P. R. China*

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Lijing Deng − *Department of Pharmacy, The First Affiliated Hospital of University of South China, Hengyang 421001, Hunan, P. R. China*

Jingyuan Tian − *Key Laboratory of Molecular Target & Clinical Pharmacology and the State Key Laboratory of Respiratory Disease, School of Pharmaceutical Sciences & the Fifth Affiliated Hospital, Guangzhou Medical University, Guangzhou 511436, Guangdong, P. R. China*

Complete contact information is available at: https://pubs.acs.org/10.1021/acsomega.0c00854

Author Contributions

Conceptualization: G.Y., C.F., and G.Z.; formal analysis: C.F. and M.L.; investigation: B.Y., G.Z., and G.Y.; methodology: C.F., M.L., K.W., J.T., and L.D.; project administration: M.L.; resources: K.W. and J.T.; writing—original draft: C.F., M.L., and K.W.; writing—review and editing: C.F.

Notes

The authors declare no competing financial interest.

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