

Alkanes and Chemical Markers Identified in the Essential Oil from Pericarp of Nanfengmiju (*Citrus kinokuni* Hort. ex Tanaka)

Jian Wang^{1,2*}

¹Department of Chinese materia medica, Chongqing College of Traditional Chinese medicine, Chongqing, China.

²Department of Chemistry and Analyses of Chinese Materia Medica, College of Chinese Medicine, Chongqing Medical University, Chongqing, China.

*Corresponding author: Jian Wang, email: wj_2000_abc@cqmu.edu.cn; Phone number: 0086-18523909839.

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Abstract. Based on the previously researches, this manuscript comprehensively analysed the chemicals in essential oil (EO) from pericarp of Nanfengmiju, a variety of *Citrus kinokuni* Hort. ex Tanaka. The isolated crystals from the EO were mainly composed of a series of alkanes. In total, 33 alkanes were identified, in which 14 ones were firstly reported in the peels EOs from *Citrus* L., to the best of my knowledge. Previously, alkanes were neglected and never thought as the chemical markers of peels EOs from *Citrus*. In fact, some of them can be chosen as the markers such as tricosane and pentacosane. Eight compounds including limonene, γ -terpinene, α -terpineol, α -farnesene, linalool, thymol, α -sinensal, and methyl-N-methyl anthranilate had already been identified as the markers of peels EOs from *Citrus reticulata* Blanco. α -Sinensal and methyl-N-methyl anthranilate were undetected in this study. At the same time, another 2 compounds β -terpineol and δ -cadinene were first selected, and 2 compounds such as spathulenol and isospathulenol were identified previously as the markers of peel EO from Nanfengmiju. In total, twelve markers were identified for peels EO from Nanfengmiju. **Keywords:** Alkanes; chemical markers; tricosane; essential oils; Nanfengmiju (*Citrus kinokuni* Hort. ex Tanaka).

Resumen. Con base en investigaciones previas, en este trabajo se analizan exhaustivamente los productos químicos presentes en el aceite esencial (EO) del pericarpio de Nanfengmiju, una variedad de *Citrus kinokuni* Hort. ex Tanaka. Los cristales aislados del EO estaban compuestos principalmente por una serie de alcanos. En total, se identificaron 33, entre los cuales, de acuerdo con lo que sabemos, 14 se informaron por primera vez en los aceites esenciales de cáscaras de Citrus L. En este trabajo se proponen algunos alcanos como marcadores químicos de los aceites esenciales de las cáscaras de cítricos, como el tricosano y el pentacosano. Ocho compuestos, incluidos limoneno, γ -terpineno, α -terpineol, α -farneseno, linalol, timol, α -sinensal y antranilato de metil-N-metilo, ya se han identificado como marcadores de AE de cáscaras de *Citrus reticulata* Blanco. En este estudio no se detectaron ni el α -sinensal ni el antranilato de metil-N-metilo. Al mismo tiempo, se seleccionaron otros 2 nuevos marcadores: β -terpineol y δ -cadineno, y compuestos como el espatulenol y el isospatulenol se identificaron previamente como marcadores del EO de la cáscara de Nanfengmiju. En total, se identificaron doce marcadores para EO de Nanfengmiju.

Palabras clave: Alcanos; marcadores químicos; tricosano; aceite esencial; Nanfengmiju (*Citrus kinokuni* Hort. ex Tanaka).

Introduction

Citrus reticulata Blanco, also called mandarin, belongs to *Citrus* L. of Rutaceae, which are planted in the world with many varieties such as *Citrus kinokuni* Hort. ex Tanaka, *Citrus reticulata* 'Dahongpao', *Citrus chachiensis* Hort. in China [1]. Nanfengmiju should be the origin of *C. kinokuni* which mainly planted in Nanfeng county, Fuzhou city, Jiangxi province, China for more than 1300 years [1]. The pericarp collected in different growth stages from some varieties such as *C. reticulata* 'Dahongpao', *C. chachiensis*, can be used as two kinds of Chinese materia medica (CMM). In generally, the peel gathered from November to December and stored for at least three years are called Citri Pericarpium Reticulate (CPR), while the peel collected from April to October are named as Citri Pericarpium Viride (CPRV), in which CPRV is collected from August to October, Fructus Citri Immaturus (FCI) gathered from April to July. CPR and CPRV are often used in Chinese medicine as the Qi-regulated CMM due to their special pharmacological activities and low-cost [2]. Peels of *C. reticulata* are rich in essential oil (EO). Up to now, more than 1000 compounds were identified in EO from different parts of *Citrus* such as peels, flowers, leaves, branches, majority from the peels [3]. Usually, alkanes are minor or undetected [3]. Previously, a total of 24 alkanes were reliable identified in the peels EOs from nine species of *Citrus* to the best of my knowledge. Among them, fifteen were detected in *C. reticulata* including 9 *n*-alkanes, 1 cycloalkane, and 5 branched (seen in Table 1) [4-21]. The peels EOs of mandarin are characterized by limonene (usually 60-95 %), followed by γ -terpinene (sometimes above 15 %) [3,22]. The oxygenated components are present at low extent such as the linalool (can reach up to 10.7 %) [3,23], α -terpineol (can reach up to 4.5 % in this study) [3], thymol (can reach up to 2.3 %) [3,17], and α -sinensal (0.1-0.7 %). The α -farnesene (can reach up to 1.5 % in this study) is an important hydrocarbon sesquiterpenes (HS) [3,17,20,23]. However, the characteristic component in mandarin peels EOs is the presence of an unusual nitrogen derivative, methyl-N-methyl anthranilate (can reach up to 2.0 % in peel EO of Shima-mikan, a rare variety of *C. kinokuni* grown locally at Azuma-cho, Kagoshima, Japan [24]), which, notwithstanding its low amount, confers the typical mandarin fragrance, is a chemical marker in peels EO of *C. chachiensis* [3, 22,25]. These eight compounds can be selected as the markers of peels EOs from *C. reticulata* [3,22,25]. Based on the previously researched [17,20], this study analyzed the chemicals in peels EOs of Nanfengmiju further through a series of separation processes such as freezing crystallization, vacuum distillation in programmed increased temperature, preparative thin-layer chromatography (PTLC), and column chromatography (CC). As a result, the separated parts and crystals were gotten. The crystals were mainly composed of a series of alkanes. It is the first time to report 33 alkanes in peels EO just from one variety such as Nanfengmiju of *Citrus*, in which 14 ones were firstly identified in the peels EOs from *Citrus* to the best of my knowledge. Some of them can be selected as the markers such as tricosane and pentacosane. In addition, two new markers such as β -terpineol and δ -cadinene, were identified in the peels EO of Nanfengmiju.

Experimental

Materials and methods

Sample collection

Peels of Nanfengmiju were collected from April to May in 2016 from the plants growing in the same garden in Nacheng town, Fuzhou city, Jiangxi province of China, which corresponded to FCI weighted as 5753.4 g. The peels were identified by Chunfang Huang, the owner of the garden.

Chemicals

n-Hexane with high-performance liquid chromatography (HPLC) grade, linalool (98 %+) was purchased from Adamas Reagent Company, Limited (Ltd.). *d*-Limonene (96 %) was purchased from Acros organics, USA. Thymol was purchased from Shanghai Titan Chemistry Company, Ltd., China. Carvacrol was purchased from Tokyo Chemical Industry, Japan. *n*-Alkanes standard solution of C10-C25 purchased from Dr. Ehrenstorfer incorporated company, Germany, was used to determine LRIs. Octacosane (99 %) was purchased from Aldrich.

Extraction and separation

Since all the peels were dried, hydrodistillation was used. The peels of FCI were divided into about 0.4 cm × 0.4 cm sections weighed 5564.8 g, which were extracted step by step. About 1000 g peels were swollen with about 6-7 times volume (V, mL) of pure water in a 10000 mL round-bottomed flask, then soaked for 0.5 h at 40 °C. The EO were extracted by Clevenger-type apparatus for 3-4 h each time, when the extraction rate (Volume of EO mL/Weight of peels g) was less than 0.03 %, the extraction process should be stopped. Then, the other peels should be continued as this process, until all the peels were extracted. The collected EO was removed the remained water by anhydro Na₂SO₄. As a result, a total of 115 mL EO was gotten, weighted 96.7 g, with the density as 0.8, the extracted rate as 2.1 %. The remained EO 114 mL, weighted 95.9 g, was stored in a refrigerator at 4 °C for the following experiment after 1 mL EO weighted 0.8 g was kept as the sample.

Crystals weighted 4.5 g were gotten from the EO after it was stored in -80 °C. The EO removed crystals in -80 °C weighted 91.5 g was then separated by vacuum distillation in gradient temperature such as 100, 130, 160, 190, 220, and final 250 °C. As a result, the distillations in different temperature and the residue after this process were gotten, respectively. Then, they were separated further, respectively.

The distillation in 100 °C was subjected to vacuum distillation in gradient temperature such as 90, 130, 140, 150 °C secondly.

The distillation in 130 °C was subjected to vacuum distillation in gradient temperature such as 120, 150, 160 °C secondly. The second distillations in 150 and 160 °C were separated further, respectively.

The second distillation in 150 °C, weighted 6.8 g, volume 7.5 mL, density 0.9, was separated by PTLC. The second distillation in 160 °C, volume 6.6 mL, was separated by PTLC either. The specification of plate was (0.9-1.0)×200×200 mm of GF254.

For the process of PTLC, 1.5 or less than 1.5 mL distilled EO was spotted on the beginning of each plate, then it was developed by n-hexane: EtOAc 9: 1 (V: V, the same for the following), respectively. As a result, the distilled EO was separated into 7 parts from the origin to the frontier, which were named as 0, 1, 2, 3, 4, 5, 6, respectively. The corresponding silica gels were scratched from the plate, and the scratched silica gels were dissolved by n-hexane and chloroform in sequence, then the solutions of hexane and chloroform were recovered solvent, respectively. Then, the solute was dissolved in hexane, respectively, and the two kinds of solution were combined. In the end, the samples 0, 1, 2, 3, 4, 5, 6 of 130-150 °C and 130-160 °C dissolved in n-hexane, respectively, were gotten.

In addition, distillation of 130-150 °C weighted 12.8 g was separated by CC through using 50 fold (w/w) 200-300 mesh silica gels weighted 639.0 g. The column was packed with n-hexane. Then, the column was eluted by 5 gradients such as n-hexane, n-hexane: EtOA with the ratio as 97:3, 93:7, 88:12, and 8:2, in sequence. In the end, 38 fractions were gotten. All the fractions were dissolved in n-hexane except for fraction 21 which was dissolved in chloroform. The crystals were gotten from 35 after storage in -18 °C, were gotten from 1, 4, 7, 11, 21, 22, 29, 30, 31, 33, 36, 37 after storage in -80 °C, respectively.

The distillation in 160 °C was subjected to vacuum distillation in gradient temperature such as 150, 160, 170-190 °C secondly. The distillation in 190 °C was subjected to vacuum distillation in gradient temperature such as 180-190 °C secondly. The distillation in 220 °C was subjected to vacuum distillation in gradient temperature such as 210-250 °C secondly.

The residue of EO after the distillation (containing some n-hexane), 9.4 mL, density 0.7, was weighted 7.0 g. The 6.9 g remained residue of EO after 74 mg was kept as the sample, was separated by CC through using 60 times 100-200 mesh silica gels weighted 413 g. The column was packed with n-hexane. The column was eluted by 5 gradients such as n-hexane, n-hexane: EtOAc in different ratio as 97:3, 9:1, 8:2, respectively, and EtOAc final. As a result, 16 fractions were obtained. Crystals were gotten from 1, 15, 16 after storage in -18 °C, respectively.

Preparation of samples

The samples of 130-150, 130-160 °C, were firstly diluted as V_{distilled EO}: V_{n-hexane (HPLC)} 1: 50 (1.96 %), then some diluted samples were diluted as this ratio secondly, V_{diluted samples}: V_{n-hexane (HPLC)} 1: 50 (0.04 %). Other samples were diluted as V_{EO or distilled EO}: V_{n-hexane (HPLC)} 1: 50 (1.96 %).

GC-FID detection

A GC-2010 (Shimadzu, Japan) was used for GC-FID detection. An Agilent fused silica capillary column - DB-5 (30 m×0.25 mm i.d., 0.25 μm film thickness) was used. The oven temperature was programmed from 60 °C (3-min hold) to 250 °C at 2.5 °C min⁻¹, and then held for 2 min. The carrier gas was Nitrogen with total flow as 16.1 mL min⁻¹. The injector and detector were maintained at 250 °C and 250 °C, respectively. The splitting ratio was from 5: 1 to 50: 1, injection volume was 1 μL.

GC-MS detection

A GCMS-TQ8040 (Shimadzu, Japan) matched with a NIST 14 MS database, and an Agilent 7890B-5977A GC-MS (USA) matched with a NIST 17 MS database was used for GC-MS analyses, respectively. A column DB-5 (30 m×0.25 mm i.d., 0.25 μm film thickness) and a column free fatty acid phase (FFAP) (30 m×0.32 mm×0.5 μm) were used for GCMS-TQ8040 detection. DB-5 was also used for 7890B-5977A GC-MS detection. The oven temperature for DB-5 used in GCMS-TQ8040 detection was programmed from 60 °C (3-min hold) to 280 °C at 2.5 °C min⁻¹, and then held for 2 min; for DB-5 used in 7890B-5977A GC-MS detection was programmed from 60 °C (3-min hold) to 250 °C at 2.5 °C min⁻¹, and then held for 3 min; for FFAP used in GCMS-TQ8040 detection was programmed from 60 °C (3-min hold) to 230 °C at 2.5 °C min⁻¹, and then held for 2 min. The carrier gas was Helium at a constant flow of 1 mL min⁻¹. The splitting ratio was from 20: 1 to 100: 1. The solvent delay was 2.5 min for FFAP and DB-5 used in GCMS-TQ8040 detection, was 3 min for DB-5 used in 7890B-5977A GC-MS detection. The injector, ion-source, and interface were maintained at 250, 200, and 250 °C, respectively. Electron impact mass spectra were taken at 70 eV. Scan from m/z 25~450 amu. and the injection volume was 1 μL.

Component identification

The peaks in total ion chromatograms (TICs) obtained by GC-MS were identified by probability based matching (PBM) first. Since the overlapped or even embedded peaks extensively exist in the TICs, the identification result may be incorrect for these peaks. In such condition, these peaks should be purified by AMDIS (Automated Mass spectral Deconvolution & Identification System) compiled in the Agilent 7890B-5977A GC-MS Chemstation if possible. After deconvolution, the purified mass spectra of these peaks were compared with the NIST 17 or 14 database, or the mass spectra of standards, respectively. The LRIs of detected compounds were calculated relatively to the retention time of a homolog series of n-alkanes (C10-C25) and then compared with the LRIs of the corresponding chemicals provided by NIST 17 database, literatures or standards. The peaks in chromatograms obtained by GC-FID were identified by comparison with those of the corresponding TICs and LRIs.

Quantitation

In each chromatogram, usually the major 20 peaks were quantitative. The peak area normalization was used to calculate the relative area percentage of each compound.

Results and discussion

The chromatograms of EO-1, PTLC-1-distillation in 150 °C of distillation in 130 °C-3, and Crystals in -80 °C from elution 1-2 can be seen in Fig. 1. The mass spectra of 10 chemical markers can be seen in Fig. 2. As can be seen in the supplementary Table S1, a total of 95 compounds were qualitative and quantified in 72 detections of 64 samples.

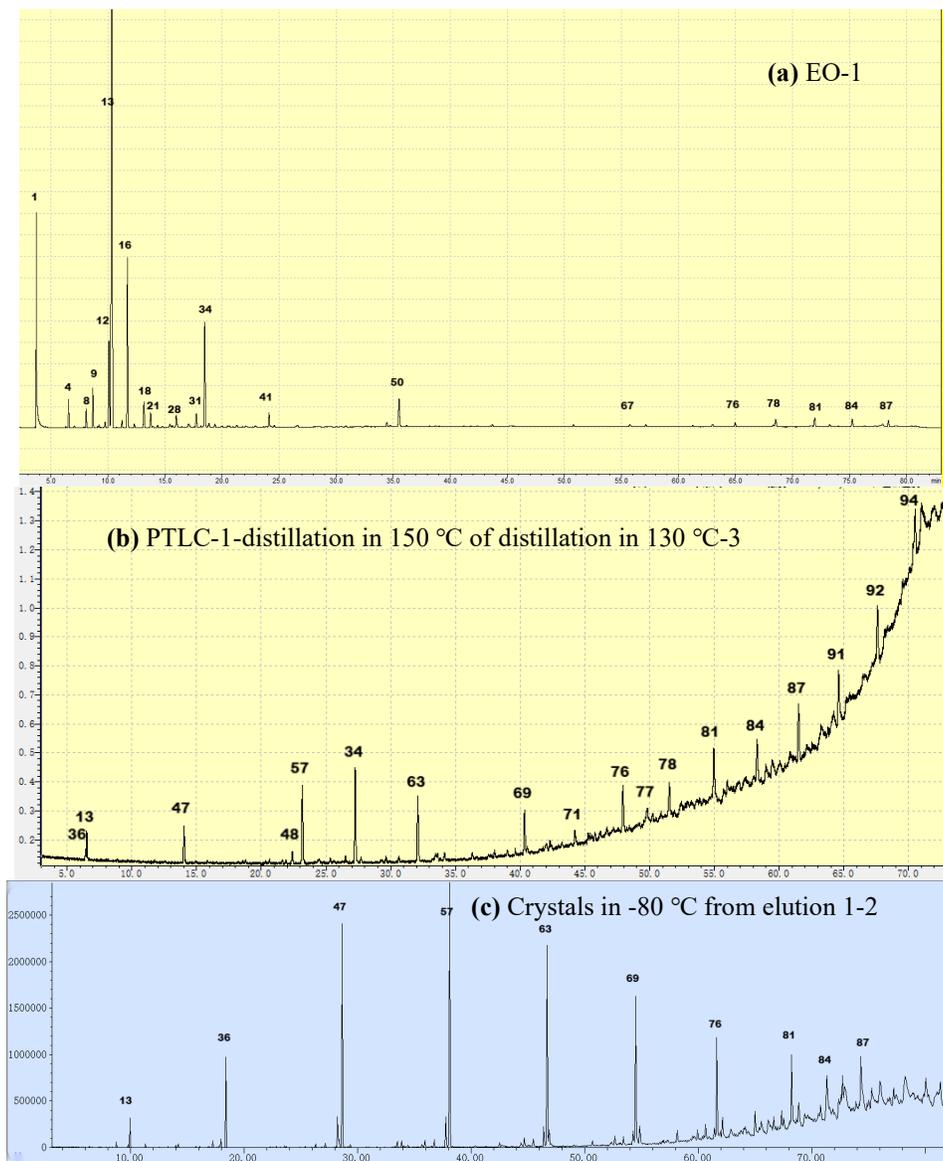
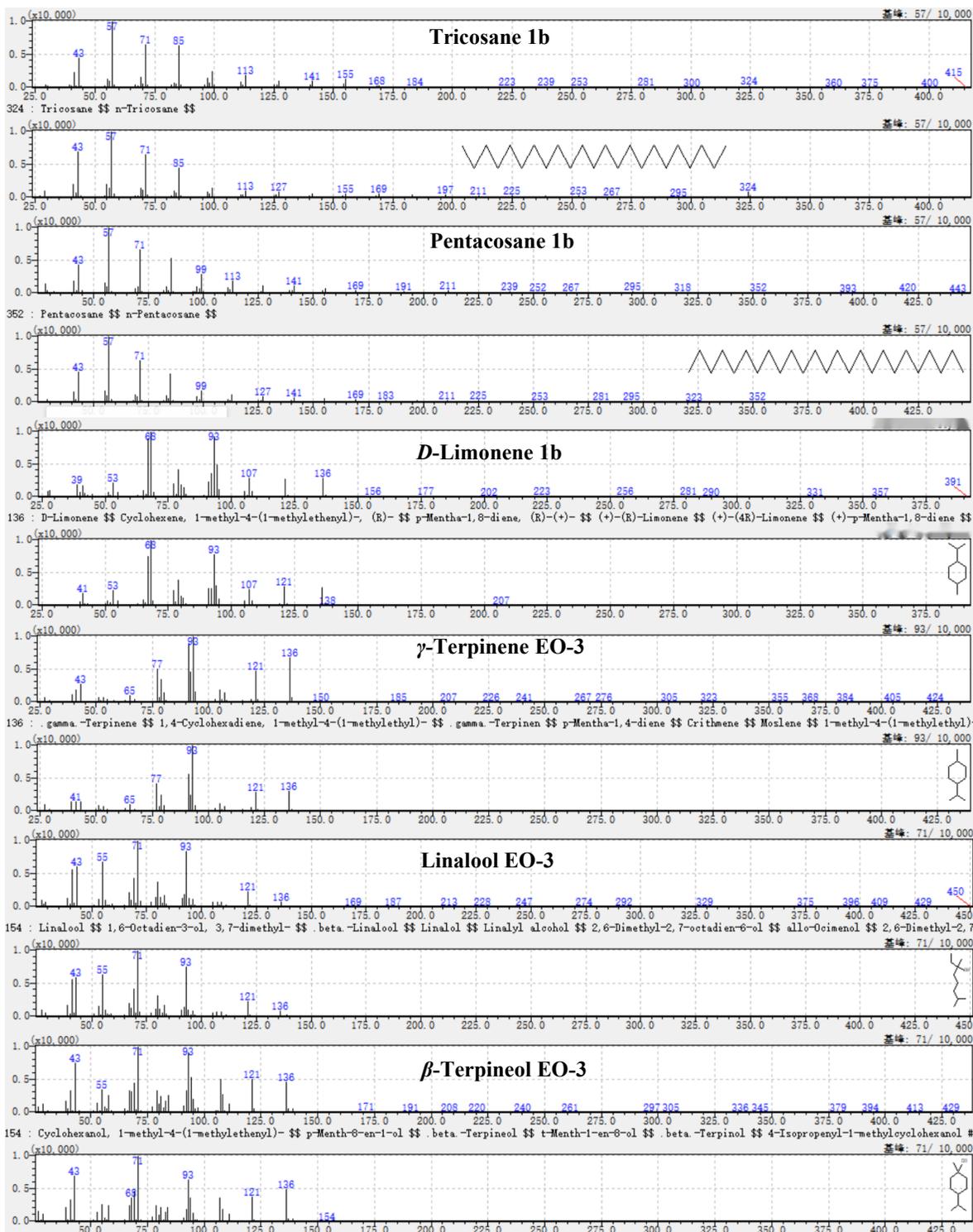


Fig. 1. Chromatograms of EO and its separated parts. 1, 4, 8, 9, 12, 13, 16, 18, 21, 28, 31, 34, 36, 41, 47, 48, 50, 57, 63, 67, 69, 71, 76, 77, 78, 81, 84, 87, 91, 92, 94 corresponding to Furfural, α -Pinene, β -Pinene, β -Myrcene, *p*-Cymene, Limonene, γ -Terpinene, Isoterpinolene, Linalool, β -Terpineol, Terpinen-4-ol, α -Terpineol, Dodecane, Thymol, Tetradecane, β -Caryophyllene, α -Farnesene, Hexadecane, Octadecane, *n*-Hexadecanoic acid, Eicosane, Heneicosane, Docosane, 2-Methyldocosane, Tricosane, Tetracosane, Pentacosane, Hexacosane, Heptacosane, Octacosane, and Nonacosane, respectively.



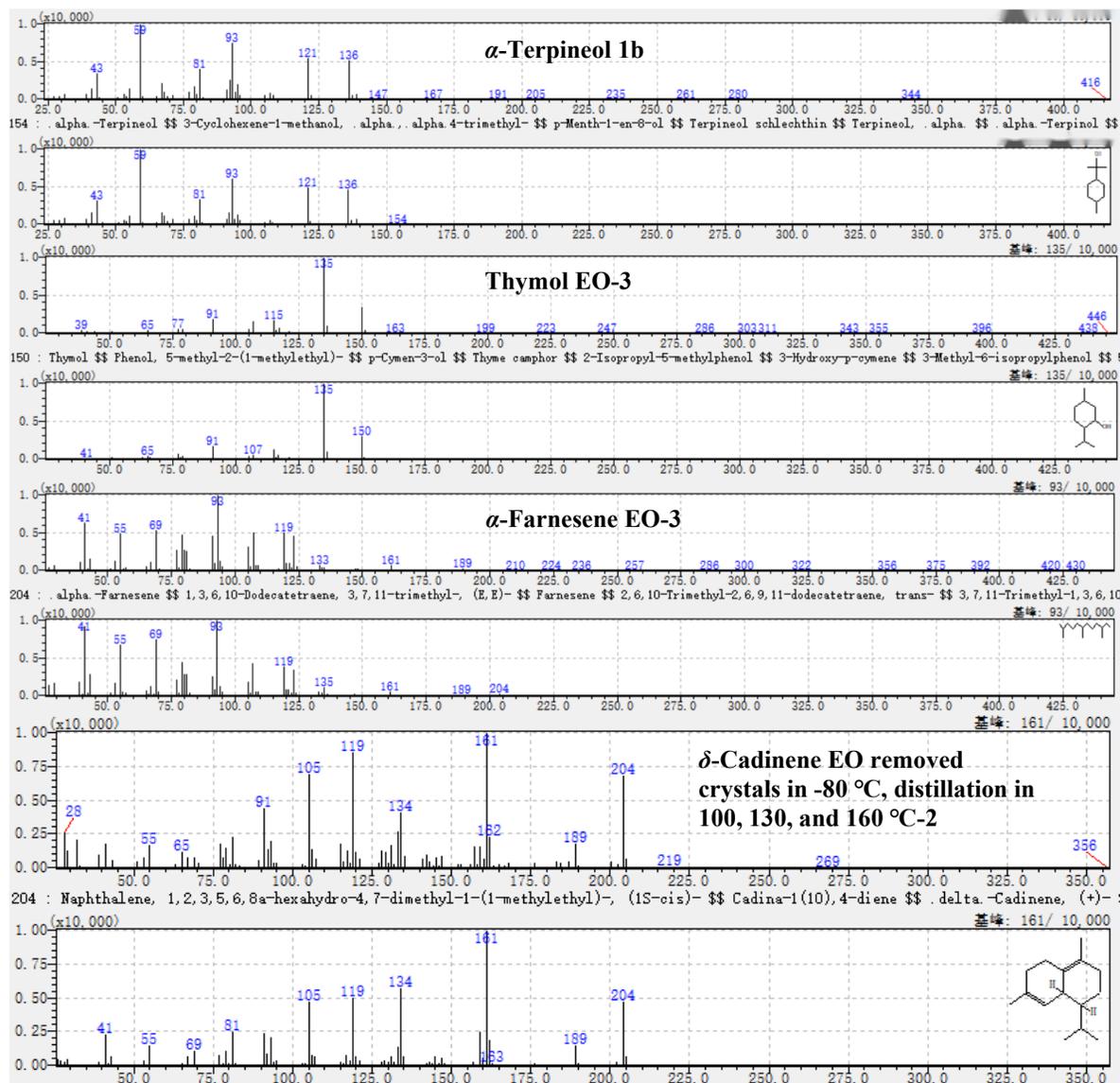


Fig. 2. The detected mass spectra (Upper) and their corresponding mass spectra (Down) from NIST 14, respectively. 1b means the mass spectrum was gotten from the (b) in Fig. 1

The alkanes and other prominent compounds

Thirty-three alkanes were identified and quantified in 53 detections of 47 samples including 21 *n*-alkanes and 12 branched alkanes, in which 14 ones such as 2,6,10,14-tetramethylpentadecane, nonadecane, eicosane, 2-methyltricosane, 3-methyltetracosane, 3-methylpentacosane, hexacosane, squalane, 2-methylhexacosane, 3-methylhexacosane, octacosane, 2-methyloctacosane, nonacosane, 2-methylnonacosane, were firstly identified in *Citrus*, to the best of my knowledge [3-21].

As for EO, the prominent compounds were **furfural**, **β -myrcene**, ***p*-cymene**, **limonene**, **γ -terpinene**, **α -terpineol**, **α -farnesene**, **tricosane**, **tetracosane**, and **pentacosane**. The identification and quantitation results were basically in agreement with the previously reports on peels EO from Nanfengmiju [17,20]. Previously,

furfural was undetected [17,20], which was seldom detected in peel EO of *Citrus*, was reported as 1.9 % in EO from Album Citri Reticulatae (ACR, a kind of mesocarp of *C. reticulata*) [8]. Limonene usually accounts for 60.7-95.7 % in peels EOs of mandarin [3, 26-27], while it can be down as 45.6 % in previously research [20]. α -Terpineol usually was not more than 3.0 % in previous reports on peels EOs of *Citrus* [3,27], α -farnesene was already ascertained as an important marker of peels EO of Nanfengmiju [17,20], which can also reach up to 1.5 % in another research [3,23]. α -Sinensal and methyl-N-methyl anthranilate were undetected in this study. Previously, α -sinensal (0-tr in EO, 0-4.4% in separated parts) was detected, methyl-N-methyl anthranilate was also undetected [17,20]. Spathulenol (0.1-0.6 %) and isospathulenol (0.1-0.5 %, identified as (-)-spathulenol [17]), were selected as two markers of peels EO of Nanfengmiju previously [17,20]. However, spathulenol and isospathulenol was inconspicuous in this study which should be due to the early collected time of the fruits [17,20]. Spathulenol was seldom detected in the peel EOs of *Citrus* and usually not more than 0.2 %, but it can reach 2.5% in peel EO from Tunisian mandarin [28], to the best of my knowledge. Isospathulenol was only reported on the previously reserach on Nanfengmiju [17,20] to the best of my knowledge [3,23]. **Tricosane** and **pentacosane** were detected with trace amount, tetracosane was undetected, 2,6,10-trimethylpentadecane was detected in the separated parts previously [17,20]. However, there is no related information of 2,6,10-trimethylpentadecane in this study.

In crystals in -80 °C, distillation in 130 °C of crystals in -80 °C, and residue of crystals in -80 °C, the prominent components were limonene, α -terpineol, and α -farnesene.

In EO removed crystals in -80 °C, the main compounds were β -myrcene, *p*-cymene, limonene, γ -terpinene, ***p*-cymenene**, α -terpineol, and α -farnesene. *p*-Cymenene was rarely detected and usually with tr content in peels EOs of mandarin, to the best of my knowledge [3,29].

In distillation in 100 °C of EO removed crystals in -80 °C, its second distillation parts in 90, 130, 140, 150 °C, respectively, and residue of distillation in 100 °C, the prominent compounds were furfural, *p*-cymene, limonene, and γ -terpinene.

In EO removed crystals in -80 °C and distillation in 100 °C, the prominent were β -myrcene, *p*-cymene, limonene, γ -terpinene, *p*-cymenene, α -terpineol, **thymol**, α -farnesene, and tetracosane. Thymol was seldom detected in the peels EOs of *Citrus* and usually not more than 0.5 % in peels EOs of mandarin [3,30], it accounted for 0.6% and 0.6-2.3 % in the EO presently and previously [17,20], which demonstrated that thymol is an important marker to differentiate Nanfengmiju from other varieties of mandarin, and other species of *Citrus*.

As for distillation in 130 °C of remained EO, and its second distillation parts in 120, 150, 160 °C, residue of distillation in 130 °C,, the main compounds were β -myrcene, *p*-cymene, limonene, and γ -terpinene.

In PTLC parts 0, 1, 2, 3, 4, 5, 6, from distillation in 150 °C of distillation in 130 °C, the main compounds were limonene. A series alkanes were first presented as the main components in these parts.

As for crystals from fractions as 1, 4, 7, 11, 21, 22, 29, 30, 31, 33, 35, 36, 37, from distillation in 150 °C of distillation in 130 °C, the prominent compounds were hexadecane, octadecane, eicosane, docosane, tetracosane, and hexacosane. These alkanes were undetected in previously researches on Nanfengmiju [17,20].

For PTLC parts 0, 1, 2, 3, 4, 5, 6, from distillation in 160 °C of distillation in 130 °C, the major compounds were limonene.

In EO removed crystals in -80 °C, distillation in 100 and 130 °C, the major compounds were *p*-cymene, limonene, γ -terpinene, *p*-cymenene, linalool, **β -terpineol**, terpinen-4-ol, α -terpineol, γ -terpineol, thymol, α -farnesene, and *n*-hexadecanoic acid. Linalool was detected usually 0-10.7 % in the peels EOs of mandarin [3,23]. β -Terpineol (0.6 %) was detected with relative high content in the EO presently, was also detected with content 0-0.1% in the EO previously, was undetected in peels EOs from other varieties of mandarin, to the best of my knowledge [3,17,20], which demonstrated β -terpineol is an important marker to differentiate Nanfengmiju from other varieties of mandarin. Terpinen-4-ol was detected with relative high content 0.6% in the EO presently, was also detected with content 0.5-1.0 % in the EO previously, was frequently detected in peels EOs from other varieties of mandarin usually not more than 0.5% [3,17,20,26]. γ -Terpineol was detected in the EO (0.2%) presently, undetected in previously studies [17,20], also undetected in other varieties of mandarin, to the best of my knowledge [3]. *n*-Hexadecanoic acid was detected in the EO (0.1 %) presently, also detected in previously the EO (0-0.4 %) previously, seldom detected in other varieties of mandarin and usually with tr, once reported as 0.6% [3,17,20,31].

In distillation in 160 °C of remained EO, its second distillation in 150, 160, and 170-190 °C, and residue of distillation in 160 °C, the major compounds were *p*-cymene, limonene, γ -terpinene, *p*-cymenene, linalool, and α -terpineol.

In EO removed crystals in -80 °C, distillation in 100, 130, and 160 °C, the major compounds were limonene, γ -terpinene, *p*-cymenene, linalool, β -terpineol, terpinen-4-ol, α -terpineol, γ -terpineol, thymol, α -farnesene, **δ -cadinene**, *n*-hexadecanoic acid, and tricosane. **δ -Cadinene** was undetected in the EO presently, was tr-0.1 in the EO previously [17,20], which was seldom detected in peels EOs of *Citrus*, usually not more than 0.1% in peel EO of mandarin [3,23], which demonstrate it should also be an important biomarker of Nanfengmiju.

In distillation in 190 °C of remained EO, its second distillation in 180-190 °C, and residue of distillation in 190 °C, the major compounds were *p*-cymene, limonene, linalool, β -terpineol, terpinen-4-ol, α -terpineol, γ -terpineol, thymol, and α -farnesene.

In EO removed crystals in -80 °C, distillation in 100, 130, 160, and 190 °C, the major compounds were limonene, *p*-cymenene, β -terpineol, terpinen-4-ol, α -terpineol, thymol, α -farnesene, δ -cadinene, *n*-hexadecanoic acid, phytol, 2-methyldocosane, tricosane, 3-methyltricosane, and pentacosane. Phytol was undetected in previously studies [17,20]. was rarely detected in peels EOs of *Citrus*, was only detected once in peels EOs of mandarin with tr-0.2 % [8].

In distillation in 220 °C of remained EO, its second distillation in 210-250 °C, and residue of distillation in 220 °C, the major compounds were *p*-cymene, limonene, β -terpineol, α -terpineol, thymol.

In EO removed crystals in -80 °C, distillation in 100, 130, 160, 190, and 220 °C, the major compounds were α -terpineol, thymol, α -farnesene, *n*-hexadecanoic acid, 2-methyldocosane, tricosane, 3-methyltricosane, pentacosane.

In distillation in 250 °C of remained EO, the major compounds were *p*-cymene, limonene, linalool, β -terpineol, terpinen-4-ol, α -terpineol, **carvone**, thymol, carvacrol, α -farnesene, and *n*-hexadecanoic acid. Carvone was detected in the EO (0.1 %) presently, was also detected in the EO (tr-0.2 %) previously [17, 20], was frequently detected usually with tr-0.1% in peel EO of mandarin [3]. Carvacrol was detected in the EO (0.1-0.4 %) previously [17,20], was rarely detected in peels EOs of *Citrus*, usually (0-0.3 %) in peels EOs of mandarin [3,28,30].

For remained EO, the main compounds were limonene, α -terpineol, thymol, and *n*-hexadecanoic acid.

For crystals from hexane 1, hexane: EtOAc (8: 2)-2, and the last elution, the major compounds were pentacosane, hexacosane, heptacosane, octacosane.

In addition, β -Pinene (2.1-6.4 %) was prominent in the EO previously [17,20], *trans*-Geraniol (1.4-15.4 %), limonene glycol (17.1-38.0 %), and *trans*-nerolidol (0.4-1.2 %) in separated parts were prominent previously [17]. β -pinene was frequently detected in peel EO of mandarin and can reach up to 14.2% [3,23]. *trans*-Geraniol was minor in this study, sometimes detected in the peels EOs of mandarin with tr content [3]. Limonene glycol was undetected in the EO presently, which was rarely detected in the peels EOs of *Citrus*, was only detected once in the peel EO of mandarin with 6.6 % [3,18]. *trans*-Nerolidol was undetected in the EO presently, was usually tr in peels EOs of mandarin [3].

Four alkenes including **1-octadecene**, **1-eicosene**, **1-docosene**, **1-hexacosene**, were unreported as the chemicals in EOs of *Citrus* except one report on the unreliable detection of **1-hexacosene** in EO from flowers of *C. grandis* previously, to the best of my knowledge [3,32].

Three phthalate including **dibutyl phthalate**, **phthalic acid**, **di(2-propylpentyl) ester**, and **bis (2-ethylhexyl) phthalate** detected in the EO or separated parts presently, undetected in previous study [17,20], should be contaminants from outside [33-34]. Benzene, 1-ethyl-2,4-dimethyl- detected in Crystals in -80 °C from elution 1-2 and Crystals in -80 °C from elution 4-2 should be a contaminant from the solution. Benzoic acid was unreported in the peels EOs of *Citrus* to the best of my knowledge, which may be a contaminant from solvent [3]. 2,4-Di-tert-butylphenol was undetected in peel EO from Nanfengmiju presently and previously [17, 20], was rarely detected in peel EO of *Citrus*, just once in ACR with 0.3 % [8], which also may be from the solvent.

In total, 30 compounds were prominent including twelve alkanes.

Conclusion

Alkanes were never considered to be the chemical markers of *Citrus* previously. However, in this study, through a series separation and purification process, those unimpressive compounds were highlighted. **Tricosane** and **pentacosane** can be selected as two markers of peel EO from Nanfengmiju. Besides, three alkenes including **1-octadecene**, **1-eicosene**, **1-docosene**, were first reported in the EO of *Citrus* to the best of my knowledge. In addition, two compounds such as, **β -terpineol** and **δ -cadinene** were identified as the new markers of peels EO of Nanfengmiju. Including limonene, γ -terpinene, linalool, α -terpineol, thymol, α -farnesene, spathulenol, and isospathulenol, twelve compounds were identified as the markers of Nanfengmiju. This study has promoted the advancement on the markers identified in peels EOs from *C. reticulata* and even *Citrus*.

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Table 1. The alkanes identified and quantified in peels EOs from nine species of *Citrus* [3].

No.	Alkanes	LRIs ^a , LRIs ^b	<i>C. reticulata</i> (15)	<i>C. grandis</i> (2)	<i>C. paradisi</i> (4)	<i>C. aurantifolia</i> (5)	<i>C. sinensis</i> (6)	<i>C. aurantium</i> (2)	<i>C. bergamia</i> (1)	<i>C. junos</i> (3)	<i>C. limon</i> (9)
			Abundance (%)								
1	Heptane	700, 700									tr [4]
2	Octane	800, 800									tr [4]
3	Cyclooctane	927, 1023	0.1 [5]					0.2 [5]			
4	Nonane	900, 900				0-tr [6]					tr [4], 0.1% [7]
5	5-(2-Methylpropyl) nonane	-, -	0-0.3 [8]								
6	Decane	1000, 1000				0-tr [6]					
7	Undecane	1100, 1100					0.1-0.2 [9]			0.8 [9]	tr [4]
8	Dodecane	1200, 1200			0-0.8 [10]	0-tr [6]			tr [11]		tr [4, 12]
9	Tridecane	1300, 1300	tr [13]			tr [13]	tr-0.1 [9]			tr [9]	tr [4, 12]
10	Tetradecane	1400, 1400	0.1 [13]	tr [13]		tr [13]	tr [9, 13], 0-tr [14]	tr-0.2 [6]		tr [9], 0-tr [15]	tr [4], 0-tr [12]
11	Pentadecane	1500, 1500									0.1 [4]
12	Hexadecane	1600, 1600			0-0.3 [16]						tr [4, 12]
13	2,6,10-Trimethylpentadecane	1633, -	0-14.5 in separated parts of EO [17]								
14	Heptadecane	1700, 1700	0.3 [18]		0-0.6 [16]						
15	Octadecane	1800, 1800	tr [13]	tr [13]			tr [13], 0 or tr [14]				
16	Heneicosane	2100, 2100	2.4 [18]		4.5 [18]		1.8-3.8 [8]				
17	Docosane	2200, 2200	0-0.5 [19]								
18	2-Methyldocosane	2263, -	0-0.5 [19]								
19	Tricosane	2300, 2300	0-1.0 [19], tr-0.5 [20]								
20	3-Methyltricosane	2372, -	0-0.3 [19]								
21	Tetracosane	2400, 2400	0-0.4 [19]								
22	2-Methyltetracosane	2462, -	0-0.4 [19]								
23	Pentacosane	2500, 2500	0-0.6 [19], 0-0.4 [20]								
24	Heptacosane	2700, 2700					0-2.0 [21]				

Note: LRIs^a (Linear retention indices) were acquired by semi-standard apolar column, LRIs^b were acquired by polar column in NIST (National Institute of Standards and technology) 17 MS (Mass spectral) database. The “-” denoted there was no corresponding value. tr, trace, if the area percentage is less than 0.05%. If the data just reported in one species, the corresponding cell would be full with orange. The number in brackets after the species denotes the number of detected Alkane