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Determination of *D*-limonene content of volatile oil clathrates by HPLC

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Abstract: *Aim The method of D-limonene of Fructus Aurantii Immaturus volatile oil clathrates (clathrates) was established and the solubility of clathrates was determined. Methods: The detection method of clathrates was HPLC, and the solubility of clathrates was determined by equilibrium method. Results Welch UItimate XB-C18 chromatographic column (4.6 mm×250 mm, 5 μm); The detection wavelength: 205 nm; column temperature: 35℃; sample size: 10 μL; mobile phase: methyl cyanide (A)-water (B); current speed: 1 mL/min, gradient elution program: 0~10 min 65%~75% (A); 10~28 min 75%~78% (A); 28~32 min 78%~65% (A); 32~42 min 65% (A). The solubility of the clathrates in water is 12.75 times that of the volatile oil. Conclusion HPLC method of D-limonene content and inclusion rate is accurate and reliable, it can be used as the method to measuring the rate of clathrates. And this experimental method is stable and reliable, can prepare the clathrates with good solubility and stable properties.*

Keywords: *D-limonene; hydroxypropyl-β-cyclodextrin; clathrates; HPLC; solubility*

1. Introduction

Fructus Aurantii Immaturus is a plant lime of the rutaceae family and its cultivated varieties or dried young fruits of sweet oranges, with the effect of breaking qi and dissipation, dissolving phlegm and eliminating ruffles, often used to treat stasis and internal arrest, full swelling pain, stool obstruction and other symptoms[1], volatile oil is one of the main active ingredients of Fructus Aurantii Immaturus, studies have shown that volatile oil is an important component of regulating qi and activating blood, activating blood circulation and removing stasis[2], the main components are *D*-limonene and linalool[3], but because it is difficult to dissolve in water, the properties are unstable, thus limiting the application[4]. The volatile oil is prepared into hydroxypropyl-β-cyclodextrin (HP-β-CD) clathrate, which has excellent cladding effect, can improve the stability of the coated substance, increase the solubility of poorly soluble drugs, reduce the toxic side effects of drugs and improve the bioavailability of drugs[5], the test uses HPLC method to determine the content of *D*-limonene in volatile oils, calculate volatile oil clathrates, and provide efficient, simple and accurate methods for the determination of volatile oil cladding rates of citrus husks, in order to provide an efficient, simple and accurate method for further related research.

2. Material

2.1. Instruments

KDM temperature-controlled electric heating sleeve (Shanghai Kuntian Laboratory Instrument Co.,LTD.); Type JJ224BC electronic analytical balance (Shanghai Precision Instrument Co., Ltd.); ZNCL-GS intelligent magnetic agitator (Shanghai Sinovac Instrument Co., Ltd.); FD-1B-50 vacuum freeze dryer (Shanghai Yuming Instrument Co., Ltd.); LC-20AD high-performance liquid chromatograph and SPD-20A detector (Shimadzu Corporation, Japan); ZQP-75D desktop incubator (Tianjin Leiport Instrument and Equipment Co., LTD.).

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2.2. Reagents and drugs

The Fructus Aurantii Immaturus volatile oil (homemade); HP-β-CD (Hubei Whitzepu Pharmaceutical Technology Co., Ltd.), absolute ethanol, petroleum ether (30~60) (analytical pure) were purchased from Tianjin Yongda Chemical Reagent Co., Ltd., *D*-limonene and linalool control (Shanghai Yuanyuan Biotechnology Co., Ltd., 98% purity, batch number: FY40027; B20387, specification: 20mg).

2.3. Herbs

The herbs was purchased from Nanjing Haiyuan Chinese Medicine Co., LTD. (batch No.: 201008), and identified as Citrus aurantium L. by researcher Dong Zibo of School of Pharmacy, Jiangsu Ocean University.

3. Methods and results

3.1. Optimization of the extraction process produced by water vapor distillation

3.1.1. Unifactor test

3.1.1.1. Soaking times

Take 50 g Fructus Aurantii Immaturus in 1000 mL round bottom flask, fixed extraction time is 4 h, ultrasonic times is 30 min, liquid ratio of 1:8, 0.5, 1, 1.5, 2, 2.5 h respectively. The results are shown in Table 1. As the soaking time increased within the test range, the volatile oil extraction increased slowly first, and the increase trend flattened out after not obvious, so the fixed soaking time was 2 h for subsequent tests. According to the extraction rate of orange oil according to the method of 2020 edition of Chinese Pharmacopoeia, the formula is the extraction rate = (quality of volatile oil / quality of Fructus Aurantii Immaturus) ×100%.

3.1.1.2. Material-liquid ratios

The fixed soaking time was 2 h, the extraction time was 4 h, and the ultrasonic times n was 30 min, the material-liquid ratios were 1:6, 1:7, 1:8, 1:9 and 1:10, respectively. The results are shown in Table 1.C hoose 1:8, 1:9, 1:10 for follow-up investigation.

3.1.1.3. Ultrasound times

The fixed immersion time was 2 h, the extraction time was 4 h, the material-liquid ratio was 1:9, and the ultrasound was investigated for 20, 30, 40, 50 and 60 min, respectively. The results are shown in Table 1. 35, 40, 45 min were selected for follow-up investigation.

3.1.1.4. Extraction times

Table 1: Results of univariate experiments

factors	levels	extraction percentage (%)
Soaking times(t/h)	0.5	0.1152
	1	0.1210
	1.5	0.1342
	2	0.1523
	2.5	0.1581
Material-liquid ratios(g/mL)	1:6	0.1320
	1:7	0.1468
	1:8	0.1832
	1:9	0.1872
	1:10	0.1607
Ultrasound times(t/min)	20	0.1511
	30	0.1878
	40	0.2066
	50	0.1405
	60	0.1485
Extraction times(t/h)	3	0.1700
	4	0.2060
	5	0.2235
	6	0.2385
	7	0.2401

The fixed immersion time was 2 h, the material-liquid ratio was 1:9, the ultrasonic time was 4 h,

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and the continuous extraction was 7 h, the results were shown in Table 1, so 4, 5 and 6 h were selected for follow-up investigation.

3.1.2. Orthogonal tests

On the basis of the univariate test, the orthogonal test was carried out according to the $L_9(3^4)$ table, the factor levels are shown in Table 2, and the results are shown in Table 3. By comparing the ranges of the three factors, it can be seen that the size of the factors affecting the yield of citrus oil is $R_C>R_B>R_A$, that is, extraction times> ultrasonic times> material-liquid ratios, Therefore, the best extraction process of volatile oil is A1B3C3, that is, the material-liquid ratio is 1:8 g/mL, the ultrasonic time is 40min, and the extraction time is 6 h.

Table 2: Level of univariate test factors

levels	Material-liquid ratios(g/mL)	Ultrasound times(t/min)	Extraction times(t/h)
1	8	35	4
2	9	40	5
3	10	45	6

Table 3: Orthogonal test design and results

test numbers	A	B	C	D(blanks)	extraction percentage (%)(%)
1	1	1	1	1	0.1722
2	1	2	2	2	0.17
3	1	3	3	3	0.2886
4	2	1	2	3	0.1683
5	2	2	3	1	0.2638
6	2	3	1	2	0.1788
7	3	1	3	2	0.234
8	3	2	1	3	0.1742
9	3	3	2	1	0.1746
k_1	0.2102	0.1915	0.1750	0.1750	-
k_2	0.2036	0.2026	0.1709	0.1942	-
k_3	0.1942	0.214	0.2241	0.1475	-
R	0.016	0.0225	0.0531	0.0467	-

3.2. Preparation of clathrates

Weigh HP-β-CD: volatile oil (g:ml) as 21:1, add purified water to make HP-β-CD concentration of 33.5%, put it in a magnetic stirrer, heat 60 °C, dissolve it at 600 r/min speed and then cool it to 45 °C, slowly drop into HP-β-CD solution with a volatile ratio of 1:1 volatile oil and absolute ethanol, seal and stir for a specified time, petroleum ether extraction to remove free citrus volatile oil (10 mL each time, extracted twice), Freeze-dried for 24 h (freezing temperature -50°C) to obtain the clathrate.

3.3. HPLC measured the content of D-limonene

3.3.1. Selection of measurement wavelength

An appropriate amount of volatile oil and HP-β-CD solution were prepared with absolute ethanol, absolute ethanol was used as a blank control, and two solutions were scanned in the wavelength range of 190~400 nm by ultraviolet-visible spectrophotometry. The results showed that the volatile oil had the maximum absorption at 205 nm, while HP-β-CD had no absorption at this wavelength, so the determination wavelength of the volatile oil was determined to be 205 nm.

3.3.2. Chromatographic conditions

Welch UItimate XB-C18 Column (4.6 mm×250 mm, 5 μm); Detection wavelength: 205 nm; Column temperature: 35°C; Injection volume: 10 μL; Mobile phase: acetonitrile (A)-water (B); Flow rate: 1 mL/min, gradient elution procedure: 0~10 min 65%~75% (A); 10~28 min 75%~78% (A); 28~32 min 78%~65% (A); 32~42 min 65% (A). The results are shown in Figure 1.

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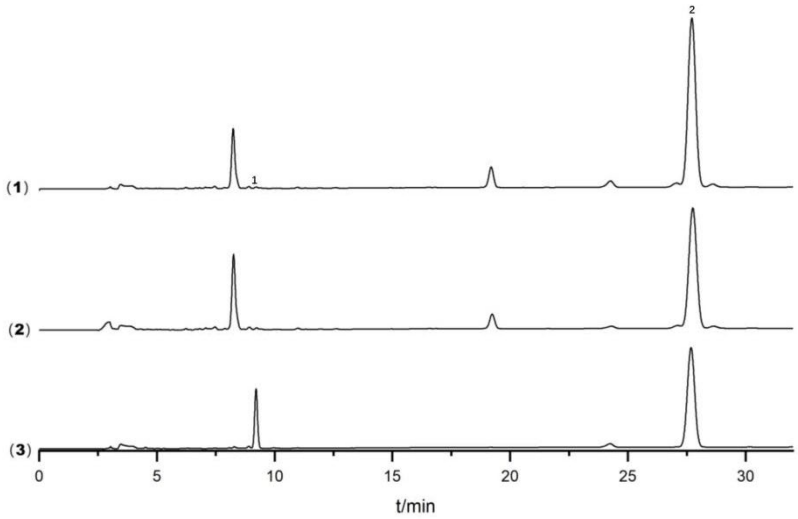

Notes: (1) volatile oil; (2) clathrates; (3) mixed control products: 1 linalool, 2 D-limonene

Figure 1: HPLC chromatogram mapjuhy

3.3.3. Configuration of the solution

3.3.3.1. Preparation of volatile oil samples of Citrus aurantium

Precision pipette 0.1 mL of volatile oil, placed in a 50 mL volumetric flask, methanol dissolved, fixed, shaken, filtered, and obtained.

3.3.3.2. Preparation of test products

Weigh 0.1 g of the clathrate, put it in a 25 mL volumetric flask, add 20 ml of methanol, sonicate for 10 min, take out, place it at room temperature, set the volume of methanol, shake well, filter, and obtain.

3.4. Methodological

3.4.1. Investigation of linear relationships

Precision weighing 2.76 mg of *D*-limonene standard was placed in a 25mL measuring flask, and methanol was set to the scale to prepare a *D*-limonene standard solution with a concentration of 110.32 μg/mL. take an appropriate amount of *D*-limonene standard solution, dilute it with methanol, and prepare a *D*-limonene standard series solution with concentration gradients of 110.32, 88.26, 66.19, 44.13 and 22.06; 10μL was injected into HPLC, measured under "3.3.2" chromatographic conditions, and the standard curve of *D*-limonene was plotted with concentration as abscissa and peak area as ordinate, and the regression equation of *D*-limonene reference was y=137005x+513947, R^2=0.9991. The linear relationship is good between 22.06 mg/mL~110.32 μg/mL.

3.4.2. Precision test

Take 110.32μg/mL of reference solution, perform precision test, operate according to "3.3.2" chromatographic conditions, continuously inject 6 times, determine the peak area and calculate the RSD value. The calculated peak area RSD value was 0.97%, indicating that the test precision was good.

3.4.3. Stability test

The test solution prepared under the item "2.3.3.2" was injected at 0, 2, 4, 6, 8, 10, 15, 20, 25 h, and the RSD value was calculated by measuring the peak area. By calculating, the RSD value of the peak area of the limonene peak of the test solution within 25 h was 1.01%, indicating that the test solution was stable within 25 h.

3.4.4. Reproducibility tests

Take 6 parts of the same batch of volatile oil complexes of citrus husk, each part of 0.1g, precision weighing, prepare the test solution according to the "3.3.3.2" item, inject and determine according to the "3.3.2" chromatographic conditions, and calculate the RSD value by measuring the peak area. The

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RSD of the *D*-limonene peak area was calculated to be 0.78%, indicating that the test treatment process and analysis method had good reproducibility.

4. Determination of clathrate solubility

The mixture of clathrate (1.05 g), HP-β-CD (1.02 g) and volatile oil (35 μL) prepared under optimal process conditions, and the volatile oil (35 μL) were placed in three 10 mL plugged test tubes, distilled water was added to the scale, and filtered after oscillation for 24 h under small amplitude conditions in a (25.0±0.5) °C benchtop constant temperature oscillation box, the detection wavelength was 205 nm, and the obtained peak area was brought into the standard curve.

It can be seen from the measurement results that the solubility of volatile oil, physical mixture and clathrate of volatile oil is 0.20 mg/mL, 0.22 mg/mL and 2.54 mg/mL, respectively, and the solubility of the clathrate is 12.7 times that of volatile oil, indicating that the water solubility of volatile oil is improved after HP-β-CD coating.

5. Conclusion

A HPLC was established for the determination of Fructus Aurantii Immaturus volatile oil-HP-β-CD. The method of *D*-limonene content in the clathrates was used to calculate the inclusion rate, and the method was verified by methodology. Among them, the other linalool component was qualitatively analyzed in order to contribute to the follow-up research. The solubility of inclusion complex is 12.75 times that of volatile oil. In conclusion, it is proved that this test method is simple and fast, and the results obtained are accurate and reliable. The solubility of the prepared inclusion complex was improved, and the bioavailability of the volatile oil was mentioned.

Acknowledgments

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