REPUBLIC OF SOUTH AFRICA

REPUBLIEK VAN SUID AFRIKA

PATENTS ACT, 1978

CERTIFICATE

In accordance with section 44 (1) of the Patents Act, No. 57 of 1978, it is hereby certified that:

ZHEJIANG UNIVERSITY

Has been granted a patent in respect of an invention described and claimed in complete specification deposited at the Patent Office under the number

2021/10928

A copy of the complete specification is annexed, together with the relevant Form P2.

imony thereof, the seal of the Patent Office has been affixed at Pretoria with effect from the **30th** day of **March 2022**

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Registrar of Patents

REPUBLIC OF SOUTH AFRICA FORM P2
PATENTS ACT, 1978
REGISTER OF PATENTS

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ZHEJIANG UNIVERSITY						
71	Applicant(s) substituted:				Date registrered	
71	Assignee(s):				Date registrered	
72	Full name(s) of inventor(s):					
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Priority claimed:		Country		Number		Date
54	Title of invention					
METHOD OF EFFECTIVELY SEPARATING AND PURIFYING SEVEN FLAVONOID COMPOUNDS FROM OUGAN FLAVEDO						
Address of applicant(s)/patentee(s):						
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REPUBLIC OF SOUTH AFRICA FORM P7

PATENTS ACT, 1978

COMPLETE SPECIFICATION

[Section 30(1) – Regulation 28]

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FULL NAME(S) OF APPLICANT(S)

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TITLE OF INVENTION

54	METHOD OF EFFECTIVELY SEPARATING AND PURIFYING SEVEN FLAVONOID COMPOUNDS FROM OUGAN FLAVEDO

METHOD OF EFFECTIVELY SEPARATING AND PURIFYING SEVEN FLAVONOID COMPOUNDS FROM OUGAN FLAVEDO

TECHNICAL FIELD

[01] The present invention belongs to the technical fields of extraction, separation, and purification of active ingredients in natural plants, and relates to a method of concurrently separating and purifying seven flavonoid compounds from Ougan (*Citrus reticulata* cv. *Suavissima*) flavedo by a combined use of solvent extraction, Sephadex gel filtration and high-speed counter-current chromatography (HSCCC) using Ougan flavedo as a raw material.

BACKGROUND ART

[02] Ougan (*Citrus reticulata cv. Suavissima*) is a cultivar of the Citrus species of the the family Rutaceae, which is believed to have curative effects of anti-cancer, fever cooling and temperature reduction, removing stasis and resolving cough, resolving toxin with clearing coolness, improving blood circulation, reducing blood pressure, and the like. These curative effects may be associated with the flavonoid compounds rich in the fruits. In the prior art, the extraction and separation of Citrus flavonoids are mainly achieved by organic solvent extraction in combination with crystallization, which, however, has low process efficiency, requires long production cycle, results in severe pollution, and is not conducive to mass production. Sephadex gel columns can be used to conduct a separation of natural products relying on a comprehensive use of the principles of gel filtration and reversed phase partition. HSCCC, based on the principle of liquid-liquid partition, resolves drawbacks of sample adsorption, denaturation, and contamination that are caused by solid supports or carriers. The combined use of solvent extraction, gel filtration and HSCCC can effectively and rapidly separate and purify Citrus flavonoids.

SUMMARY

[03] The objective of the present invention is to provide a method of separating and

purifying seven flavonoid compounds from Ougan flavedo, which is achieved by the following steps:

[04] (1) solvent extraction: conducting an ultrasonic extraction on a preset mass of powder of Ougan flavedo with 80% ethanol (at a solid-liquid ratio of 1:20) 3 times, 30 min each time, subjecting to a vacuum filtration, combining supernatants, evaporating to dryness using a rotary evaporator until no ethanol phase exists, dissolving in deionized water, extracting twice respectively with n-hexane, chloroform, and n-butanol in sequence according to the solvent polarity, and combining extracts after the two extractions to obtain an n-hexane extract, a chloroform extract, and an n-butanol extract;

[05] (2) Sephadex LH-20 gel filtration: dissolving the n-hexane extract and the chloroform extract after being evaporated to dryness in 40% methanol, and loading on a Sephadex LH-20 gel column with a sample flow rate of 2 mL/min, eluting the Sephadex LH-20 gel column loaded with sample using methanol having volume ratios of 40%, 60%, 80%, and 100% in sequence, with 4 bed volumes for each gradient and an elution flow rate of 2 mL/min, performing a high-performance liquid chromatography (HPLC) on the eluent, combining the eluent rich in flavonoid compounds, and evaporating to dryness using a rotary evaporator at 50°C to obtain a crude extract of flavonoids with pigment removed;

[06] (3) HSCCC separation and purification: preparing different HSCCC solvent systems, shaking well and staying overnight to obtain an upper phase as a stationary phase and a lower phase as a mobile phase, pumping the stationary phase first into HSCCC at a flow rate of 20 mL/min, after being stable, turning on a counter-current chromatograph and adjusting the speed to 800 rpm, and passing the mobile phase through HSCCC at a speed of 2 mL/min, after the effluent is layered, dissolving the pigment-removed n-hexane and chloroform extracts in the lower phase of n-hexane-ethyl acetate-methanol-water (having a volume ratio of 1:08:1:1), dissolving the n-butanol extract in the lower phase of ethyl acetate-n-butanol-water (having a volume ratio of 4:1:5), collecting each component based on the spectrum collected by chromatograph, determining the composition of each component by HPLC, combining test tubes containing a single flavonoid compound, and evaporating to dryness using a

rotary evaporator at 50°C to obtain a total of seven flavonoid compounds, namely, naringin, hesperidin, neohesperidin, sinensetin, nobiletin, tangeretin, and 5-hydroxy-6,7,8,3',4'-pentamethoxyflavone (5HPMF), each with a purity of above 97%. The seven flavonoid compounds purified and obtained in the present invention are identified by HPLC and mass spectrometry (MS) to be consistent with those reported in literatures.

[07] The material where the flavonoid compounds of the present invention are extracted is selected from Ougan flavedo. n-hexane, chloroform, and n-butanol are used in a segmented extraction of the flavonoid compounds from the Ougan flavedo. Sephadex LH-20 gel column is used to remove pigment molecules in the n-hexane and chloroform extracts. When performing an HSCCC purification, the n-hexane and chloroform extracts are purified using the solvent system of n-hexane-ethyl acetate-methanol-water (1:0.8:1:1) to obtain sinensetin, nobiletin, tangeretin, and 5HPMF, and the n-butanol extract is purified using the solvent system of ethyl acetate-n-butanol-water (4:1:5) to obtain naringin, hesperidin, and neohesperidin. The purities of the seven flavonoid compounds obtained by purification are all above 97%.

[08] The present invention adopts the combined technology of solvent extraction, Sephadex gel filtration and HSCCC, and uses dry powder of Ougan flavedo as a raw material to undergo simultaneous separation and purification to yield seven flavonoid compounds with high purity. Compared with the prior processes, the present invention has the following advantages: (1) the synthetic process is simple without the need of high temperature, high pressure or inert gas protection; (2) the solvent used in HSCCC can be recycled and reused, thus being cost-effective; (3) the method can be used for separating flavonoid compounds with very similar properties, showing wide applicability; (4) the production cycle is short, only requiring a few hours to complete the entire purification process; (5) the purified product has a high recovery rate and a high purity; (6) the entire purification process is precisely quantified by HPLC with high accuracy.

BRIEFT DESCRIPTION OF THE DRAWINGS

[09] FIG. 1 is a flow chart showing the process of the present invention.

[10] FIG. 2 is a diagram showing structures of the seven flavonoid compounds in Ougan flavedo.

[11] FIG. 3 is an HSCCC spectrum of the n-hexane extract, where 1 represents nobiletin, 2 represents tangeretin, and 3 represents 5HPMF.

[12] FIG. 4 is an HSCCC spectrum of the chloroform extract, where 1 represents sinensetin, 2 represents nobiletin, and 3 represents tangeretin.

DETAILED DESCRIPTION OF THE EMBODIMENTS

[13] The present invention is further illustrated in conjunction with the drawings and embodiments.

[14] Embodiment 1 The present invention screens and determines influence factors through the following tests

[15] 1. Selection of material for purification

[16] Fresh Ougan (*Citrus reticulata* cv. *Suavissima*) fruit includes four parts: flavedo, albedo, segment membrane, and juice sac. After being fully freeze-dried in liquid nitrogen, the fresh Ougan fruit is dried in a freeze dryer at -80 °C for 72 h to reach a constant weight, and the dry sample is ground with a grinder for use. A certain amount of dry powders of the four different parts of Ougan is accurately weighed, and subjected to an ultrasonic extraction with 80% ethanol (at a solid-liquid ratio of 1:20) for 30 min. The extract is centrifuged on a centrifuge at 4000 rpm for 10 min. Such operations are repeated twice, and supernatants are combined for an HPLC analysis of flavonoid compounds in the four different parts of Ougan fruit. Conditions of the HPLC analysis are as follows: water (solution A) and chromatographic acetonitrile (solution B) are used as mobile phases, and gradient elution is adopted. The gradient of elution is as follows: elution with 20% solution B for 0^{th}-15^{th} min, elution with solution B from 20% to 60% for 15^{th}-35^{th} min, elution with solution B from 60% to 100% for 35^{th}-40^{th} min, and then elution with 100% solution B for 2 min; elution with solution B from 100% to 20% for 42^{th}-45^{th} min; elution with 20% solution B for 5 min to end a cycle. The detection wavelengths are 280 nm (flavanones) and 330 nm (polymethoxyflavones), the

temperature is 25°C, and the injection volume is 10 μL. The results show that in the four different parts of Ougan fruit, the flavedo contains the most types of flavonoid compounds, which can be used as the material for purification (see Table 1).

[17] Table 1

Flavonoid compounds	Content (mg/g DW)			
	Flavedo	Albedo	Segment membrane	Juice sac
Naringin	0.44 ± 0.05	4.55 ± 0.59	5.30 ± 0.16	0.51 ± 0.01
Hesperidin	2.45 ± 0.23	3.87 ± 0.54	3.35 ± 0.12	0.33 ± 0.01
Neohesperidin	38.19 ± 3.06	69.51 ± 8.67	32.97 ± 1.09	10.01 ± 0.14
Sinensetin	0.83 ± 0.09	n.d.	n.d.	n.d.
Nobiletin	9.10 ± 0.42	n.d.	n.d.	n.d.
Tangeretin	8.43 ± 0.50	n.d.	n.d.	n.d.
5HPMF	0.76 ± 0.10	n.d.	n.d.	n.d.

[18] n.d. indicates the compound is not detected.

[19] 2. Selection of solvent for extraction

[20] According to the solvent polarity, n-hexane, chloroform and n-butanol are successively used to extract the flavonoid compounds from an aqueous solution of Ougan flavedo extracts. The results are shown in Table 2. Different flavonoid compounds are extracted using different solvents. The n-hexane extract contains most of tangeretin and parts of nobiletin and 5HPMF; the chloroform extract includes most of nobiletin and parts of sinensetin and tangeretin; the n-butanol extract includes naringin, hesperidin and neohesperidin, the remaining water extract only contains a low content of flavonoid compounds.

[21] Table 2

Flavonoid compounds	Content (mg/g DW)			
	n-hexane extract	Chloroform extract	n-butanol extract	Water extract
Naringin	n.d.	n.d.	0.15 ± 0.01	n.d.
Hesperidin	n.d.	n.d.	0.54 ± 0.04	0.03 ± 0.01
Neohesperidin	n.d.	n.d.	5.61 ± 0.62	0.25 ± 0.03
Sinensetin	n.d.	0.10 ± 0.01	n.d.	n.d.
Nobiletin	0.11 ± 0.01	1.15 ± 0.13	n.d.	n.d.
Tangeretin	0.77 ± 0.06	0.34 ± 0.02	n.d.	n.d.
5HPMF	0.05 ± 0.01	0.04 ± 0.01	n.d.	n.d.

[22] n.d. indicates the compound is not detected.

[23] 3. Sephadex LH-20 gel filtration to remove pigment

[24] Sephadex LH-20 is used to separate flavonoid compounds and pigments in the n-hexane and chloroform extracts. The elution results show that the pigments are first eluted through the gel column due to their large molecular weight, and the flavonoid compounds are then eluted due to their small molecular weight. Most of polymethoxyflavones are present in 40%-60% methanol eluate, and the eluate is combined and evaporated to dryness on a rotary evaporator at 50°C to obtain a crude extract of flavonoids with pigment removed. Pigment molecules can be effectively removed by a one-step purification.

[25] 4. Identification of purified products

[26] Ten flavonoid compounds purified and obtained from the Ougan flavedo are identified by MS. The liquid chromatography-mass spectrometry (LC-MS) experiment is carried out with Agilent 1290-G6640 triple quadrupole system. The analysis process is performed using total ion chromatography and electrospray ionization (ESI). The identification of flavanones is conducted in a negative ion mode ($[M-H]^-$), the identification of polymethoxyflavones is performed in a positive ion mode $[M+H]^+$). Operating conditions are as follows: the capillary pressure is 3000 v for positive ions and 3500 v for negative ions, the atomizer is 45 psi, the flow rate of dry gas is 5 L/min, and the temperature is 325°C. The results are shown in Table 3.

Flavanone	Rt (min)	λmax	$[M-H]^-$(m/z)	MS^2 (m/z)
Naringin	15.17	282.9, 328.2	579	151, 271, 313
Hesperidin	16.79	282.9, 327	609	286, 301
Neohesperidin	20.16	284.1	609	286, 301
Polymethoxyflavone	Rt (min)	λmax	$[M+H]^+$(m/z)	MS^2 (m/z)
Sinensetin	32.72	240.2, 329.4	373	312, 343, 357
Nobiletin	34.96	269.9, 333.0	403	342, 373, 388
Tangeretin	37.56	371.1, 322.2	373	297， 325
5HPMF	38.61	281.7, 338.8	389	374, 359,341

[27] Embodiment 2

[28] The method of separating and purifying flavonoid compounds from Ougan flavedo of the present invention is achieved by the following three steps, referring to FIG. 1.

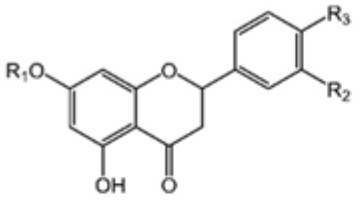
[29] (1) Solvent extraction: 20 g of dry powder of Ougan fruit flavedo is weighed and subjected to an ultrasonic extraction with 400 mL of 80% ethanol (at a solid-liquid ratio of 1:20) for 40 min (at an ultrasonic frequency of 60 kHz and a power of 30 W), the resulting extract is centrifuged at 4000 rpm for 10 min on a centrifuge. The operations are repeated twice, and supernatants are combined and evaporated to dryness using a rotary evaporator until no ethanol phase exists. The resulting mixture is dissolved in 200 mL of water, and extracted twice respectively with n-hexane, chloroform, and n-butanol in sequence (200 mL each time, and 4 h each time) according to the solvent polarity. Extracts after the two extractions are combined to obtain an n-hexane extract, a chloroform extract, and an n-butanol extract. Each extract is subjected to a rotary evaporation and then dissolved in methanol, and compositions of flavonoids in each extract are determined by HPLC.

[30] (2) Sephadex LH-20 gel filtration: Sephadex LH-20 gel is dissolved and swelled in water for 3 h at room temperature, and the dissolved and swelled gel is continuously poured into a chromatographic column (×500 mm BV=20 mL) while stirring, followed by free setting in the column. After balancing with water for 2-3 h, 0.5 mL of a sample solution (n-hexane extract or chloroform extract is evaporated to dryness and then dissolved into 5 mL of 40% methanol) from the top of the column. The Sephadex LH-20 gel column loaded with sample are eluted with 40%, 60%, 80%, and 100% methanol in sequence, with 4 bed volumes (BV) for each gradient and an elution flow rate of 2 mL/min. The eluent is detected by HPLC, followed by combining the eluent rich in flavonoid compounds, and evaporating to dryness using a rotary evaporator at 50°C to obtain a crude extract of flavonoids with pigment removed.

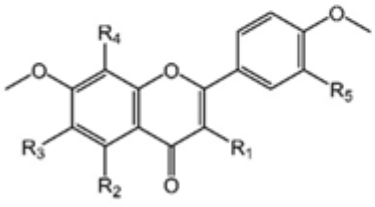
[31] (3) HSCCC separation and purification: two different HSCCC solvent systems, i.e., ethyl acetate-n-butanol-water (4:1:5, v/v) and n-hexane-ethyl acetate-methanol-water (1:08:1:1, v/v), are respectively used to purify seven flavonoid compounds in Ougan flavedo. The solvent system is shaken well after preparation and stays overnight to be layered to obtain an upper phase as a stationary phase and a lower phase as a mobile phase. The upper phase is first pumped into and fills in the HSCCC column at a flow rate of 20 mL/min, then the speed of the instrument is adjusted to 800

rpm, and the lower phase is pumped into the column at a flow rate of 2 mL/min. When the lower phase steadily flows out of the outlet, two-phase equilibrium is established in the column. The pigment-removed n-hexane extract, chloroform extract and n-butanol extract are respectively dissolved in lower phases of 5 mL of corresponding solvent systems. The sample is injected from the sample loop, the eluent is detected by a UV detector, and the effluent is collected at 2 mL/tube and analyzed by HPLC. The HSCCC spectra are shown in FIGS. 2-4. The eluent components are combined separately, vacuum dried to obtain powder, and weighed. In this purification process, 16 mg of nobiletin, 51 mg of tangeretin, and 18 mg of 5HPMF are obtained from the n-hexane extract; 12 mg of sinensetin, 38 mg of nobiletin, and 17 mg of tangeretin are obtained from the chloroform extract; 1.2 mg of naringin, 22 mg of hesperidin, and 67 mg of neohesperidin are obtained from the n-butanol extract. The seven flavonoid compounds purified and obtained in the present invention are identified by HPLC and MS to be consistent with those reported in literatures. The purities of the products are all above 97%.

[32] The structures are as follows:



	R1	R2	R3
naringin	*O*-Neohesperidose	OH	H
hesperidin	*O*-Rutinose	OH	OCH_3
neohesperidin	*O*-Neohesperidose	OH	OCH_3



	R1	R2	R3	R4	R5
sinensetin	H	OCH_3	H	H	OCH_3
nobiletin	H	OCH_3	OCH_3	OCH_3	OCH_3
tangeretin	H	OCH_3	OCH_3	OCH_3	H
5HPMF	H	OH	OCH_3	OCH_3	OCH_3

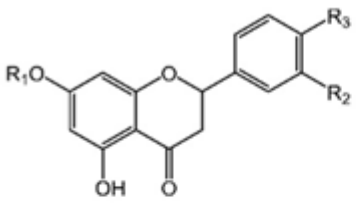
[33] Embodiment 3

[34] The method of separating and purifying flavonoid compounds from Ougan flavedo of the present invention is achieved by the following three steps, referring to

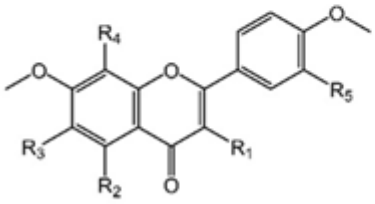
FIG. 1.

[35] Solvent extraction: 100 g of dry powder of Ougan fruit flavedo is weighed, and other conditions are identical to those in Embodiment 2. Finally, 86 mg of nobiletin, 28.5 mg of tangeretin, and 10.5 mg of 5HPMF are obtained from the n-hexane extract; 7.5 mg of sinensetin, 17.8 mg of nobiletin, and 9.6 mg of tangeretin are obtained from the chloroform extract; 6.8 mg of naringin, 11.4 mg of hesperidin, and 29.6 mg of neohesperidin are obtained from the n-butanol extract. The seven flavonoid compounds purified and obtained in the present invention are identified by HPLC and MS to be consistent with those reported in literatures. The purities of the products are all above 97%.

[36] The structures are as follows:



	R1	R2	R3
naringin	*O*-Neohesperidose	OH	H
hesperidin	*O*-Rutinose	OH	OCH_3
neohesperidin	*O*-Neohesperidose	OH	OCH_3



	R1	R2	R3	R4	R5
sinensetin	H	OCH_3	H	H	OCH_3
nobiletin	H	OCH_3	OCH_3	OCH_3	OCH_3
tangeretin	H	OCH_3	OCH_3	OCH_3	H
5HPMF	H	OH	OCH_3	OCH_3	OCH_3

WHAT IS CLAIMED IS:

1. A method of separating and purifying seven flavonoid compounds from Ougan (*Citrus reticulata* cv. *Suavissima*) flavedo, comprising the following steps:

(1) solvent extraction: conducting an ultrasonic extraction on Ougan flavedo powder with 80% ethanol 3 times, wherein a solid-liquid ratio is 1:20, and each time of the ultrasonic extraction lasts 30 min, subjecting to a vacuum filtration, combining supernatants, evaporating to dryness using a rotary evaporator until no ethanol phase exists, dissolving in deionized water, extracting twice respectively with n-hexane, chloroform, and n-butanol in sequence, and combining extracts after the two extractions to obtain an n-hexane extract, a chloroform extract, and an n-butanol extract;

(2) Sephadex LH-20 gel filtration: dissolving the n-hexane extract and the chloroform extract after being evaporated to dryness in 40% methanol, and loading on a Sephadex LH-20 gel column with a sample flow rate of 2 mL/min, eluting the Sephadex LH-20 gel column loaded with sample using 40%, 60%, 80%, and 100% methanol in sequence, with 4 bed volumes for each gradient and an elution flow rate of 2 mL/min, performing a high-performance liquid chromatography (HPLC) on the eluent, combining the eluent rich in flavonoid compounds, and evaporating to dryness using the rotary evaporator at 50°C to obtain a crude extract of flavonoids with pigment removed;

(3) high-speed counter-current chromatography (HSCCC) separation and purification: preparing different HSCCC solvent systems, shaking well and staying overnight to obtain an upper phase as a stationary phase and a lower phase as a mobile phase, pumping the stationary phase first into HSCCC at a flow rate of 20 mL/min, after being stable, turning on a counter-current chromatograph and adjusting the speed to 800 rpm, and passing the mobile phase through HSCCC at a speed of 2 mL/min, after the effluent is layered, dissolving the pigment-removed n-hexane and chloroform extracts in the lower phase of n-hexane-ethyl acetate-methanol-water having a volume ratio of 1:08:1:1, dissolving the n-butanol extract in the lower phase of ethyl acetate-n-butanol-water having a volume ratio of 4:1:5, collecting each component based on the spectrum collected by a chromatograph detector, determining the composition of each component

by HPLC, combining test tubes containing a single flavonoid compound, and evaporating to dryness using the rotary evaporator at 50°C to obtain a total of seven flavonoid compounds, namely, naringin, hesperidin, neohesperidin, sinensetin, nobiletin, tangeretin, and 5-hydroxy-6,7,8,3',4'-pentamethoxyflavone (5HPMF).

2. The method of separating and purifying seven flavonoid compounds from Ougan (*Citrus reticulata* cv. *Suavissima*) flavedo according to claim 1, wherein a material for the extraction of the flavonoid compounds is selected from Ougan flavedo.

3. The method of separating and purifying seven flavonoid compounds from Ougan (*Citrus reticulata* cv. *Suavissima*) flavedo according to claim 1, wherein n-hexane, chloroform, and n-butanol are used in a segmented extraction of the flavonoid compounds in the Ougan flavedo.

4. The method of separating and purifying seven flavonoid compounds from Ougan (*Citrus reticulata* cv. *Suavissima*) flavedo according to claim 1, wherein the Sephadex LH-20 gel column is used to remove pigment molecules in the n-hexane and chloroform extracts.

5. The method of separating and purifying seven flavonoid compounds from Ougan (*Citrus reticulata* cv. *Suavissima*) flavedo according to claim 1, wherein when performing an HSCCC purification, the n-hexane and chloroform extracts are purified using the solvent system of n-hexane-ethyl acetate-methanol-water having the volume ratio of 1:0.8:1:1 to obtain sinensetin, nobiletin, tangeretin, and 5HPMF, and the n-butanol extract is purified using the solvent system of ethyl acetate-n-butanol-water having the volume ratio of 4:1:5 to obtain naringin, hesperidin, and neohesperidin, purities of the seven flavonoid compounds obtained by purification are all above 97%.

DA van Zantwijk
Sibanda & Zantwijk Patent Attorneys

Drawings

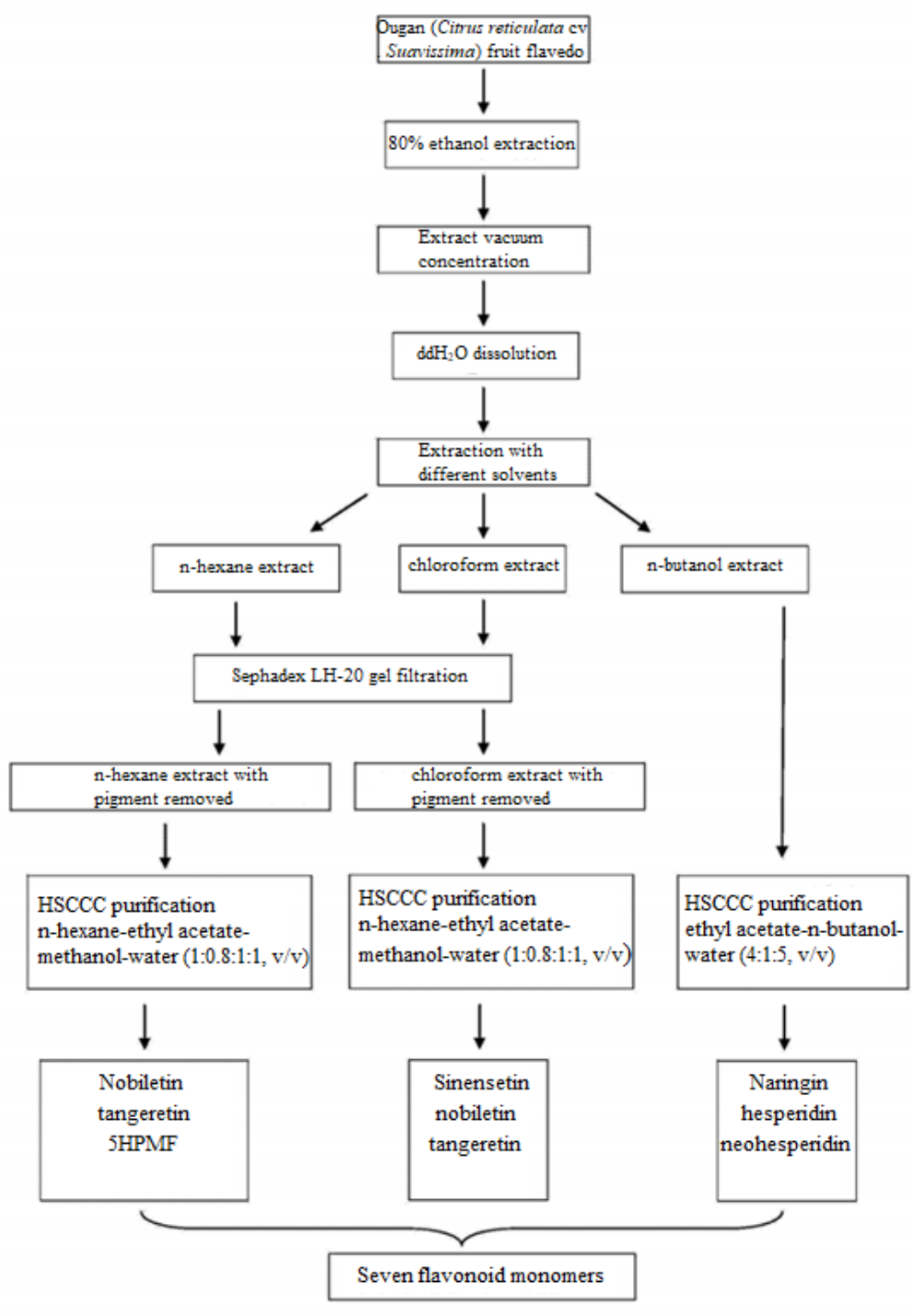


FIG. 1

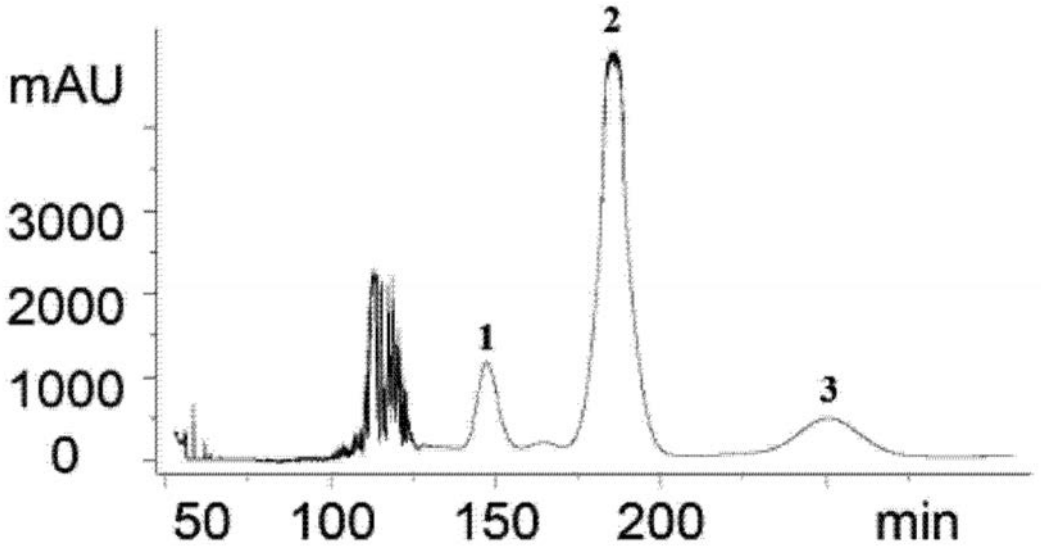


FIG. 2

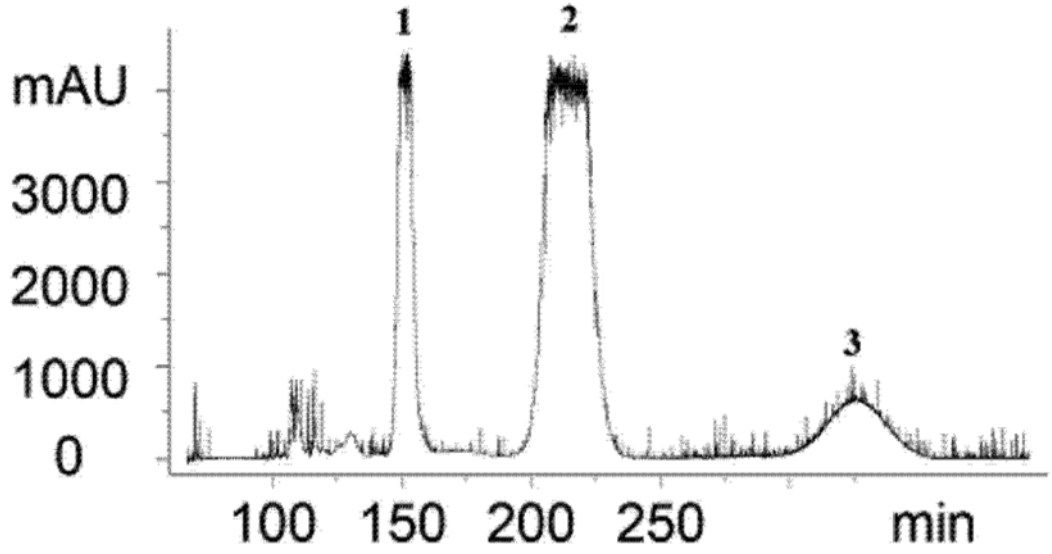

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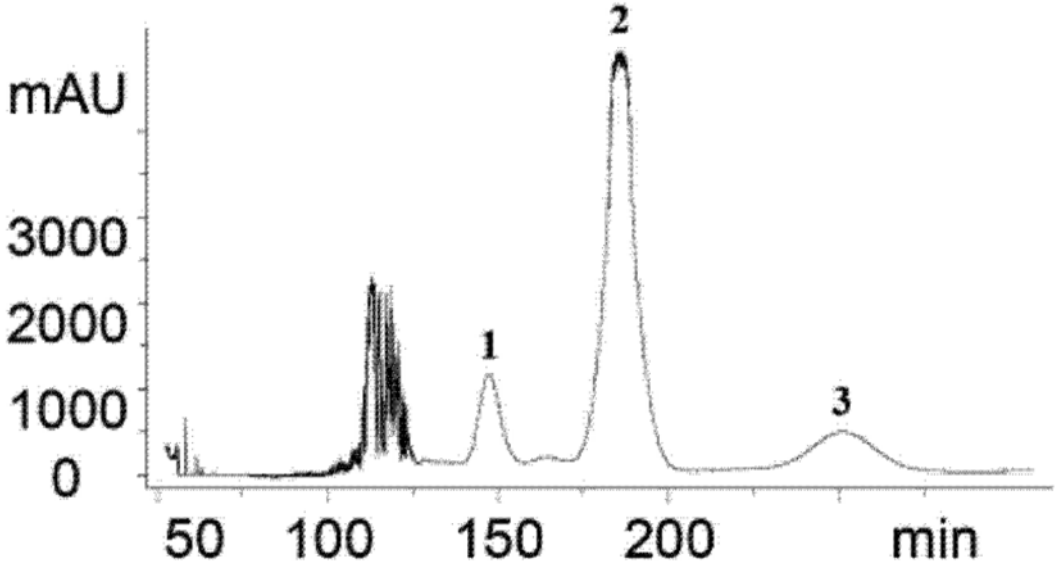
FIG. 3


2
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min

FIG. 4

ABSTRACT OF THE DISCLOSURE

The present invention provides a method of effectively separating and purifying seven flavonoid compounds from Ougan (*Citrus reticulata* cv. *Suavissima*) flavedo, including three steps of solvent extraction, Sephadex LH-20 gel filtration, and high-speed counter-current chromatography (HSCCC) purification. Ougan fruit flavedo is used as a raw material for purification to finally obtain seven flavonoid compounds, each of which has a purify of above 97%. The method of the present invention has simple operations and short production cycle, requires low sample consumption and equipment investment, and is performed under conditions that are stable and easily controlled. The seven flavonoid compounds include naringin, hesperidin, neohesperidin, sinensetin, nobiletin, tangeretin, and 5-hydroxy-6,7,8,3',4'-pentamethoxyflavone (5HPMF), and the present invention is suitable for industrial production and scientific research.

ABSTRACT DRAWING

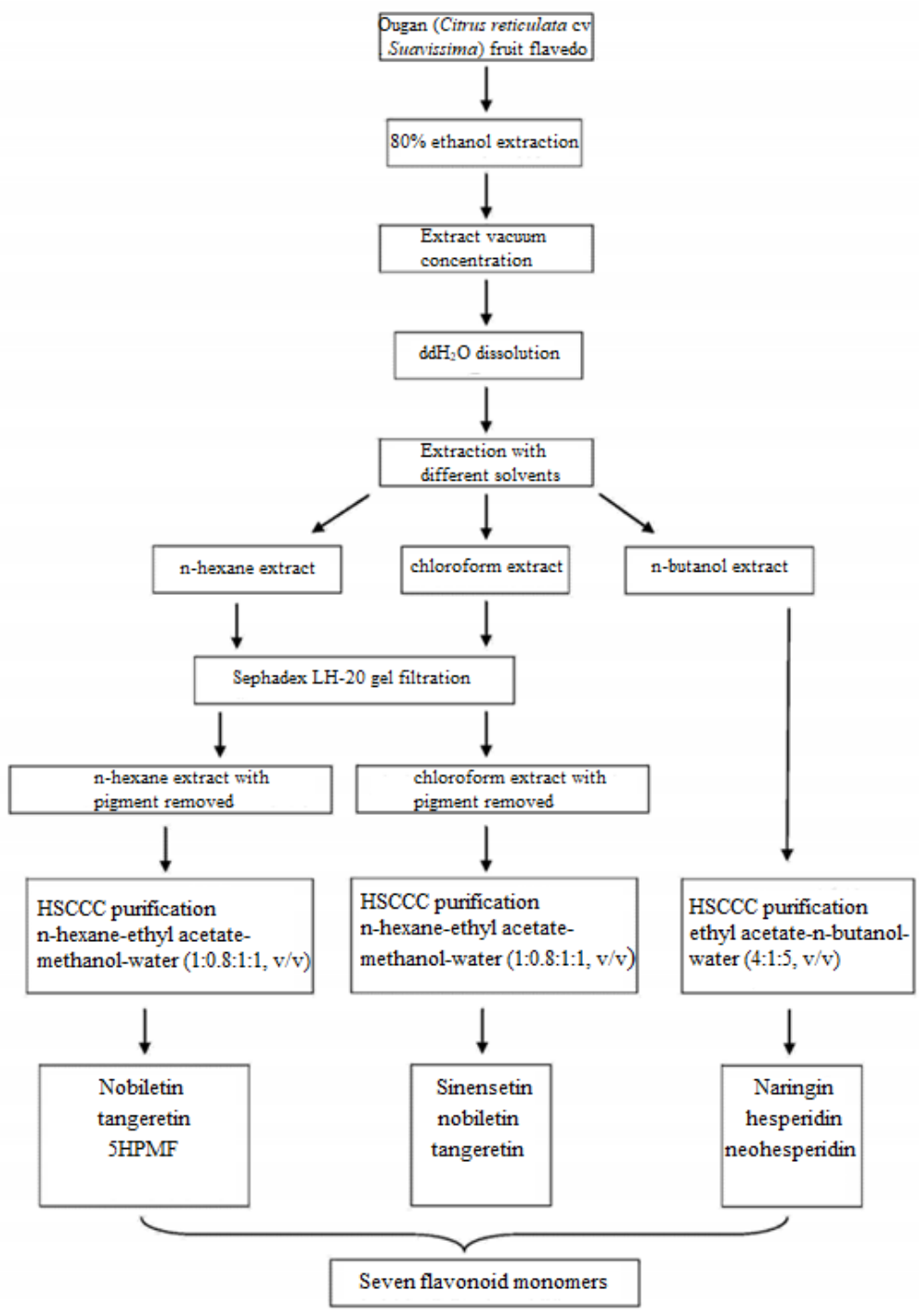
Ougan (Citrus reticulata cv Suavissima) fruit flavedo
80% ethanol extraction
Extract vacuum concentration
ddH2O dissolution
Extraction with different solvents
n-hexane extract
chloroform extract
n-butanol extract
Sephadex LH-20 gel filtration
n-hexane extract with pigment removed
chloroform extract with pigment removed
HSCCC purification n-hexane-ethyl acetate-methanol-water (1:0.8:1:1, v/v)
HSCCC purification n-hexane-ethyl acetate-methanol-water (1:0.8:1:1, v/v)
HSCCC purification ethyl acetate-n-butanol-water (4:1:5, v/v)
Nobiletin tangeretin 5HPMF
Sinensetin nobiletin tangeretin
Naringin hesperidin neohesperidin
Seven flavonoid monomers

FIG. 1