CHAPTER III

MATERIALS AND METHODS

สำนักงานวิทยทรัพยากร จุฬาลงกรณ์มหาวิทยาลัย

3.1 Instruments and Equipments

3.1.1 Fourier Transform Infrared Spectrophotometer (FT-IR)

The FT-IR spectra were recorded on a Nicolet Impact 410 Fourier Transform Infrared Spectrophotometer. Solid samples were generally examined by incorporating the sample with potassium bromide (KBr) to form a pellet. Spectra of liquid samples were recorded as thin film on a sodium chloride (NaCl) cell.

3.1.2 Nuclear Magnetic Resonance Spectrometer (NMR)

The ^{1}H-NMR, ^{13}C-NMR, COSY, NOESY, HSQC, and HMBC spectra were recorded on a Varian Mercury Spectrometer operated at 400 MHz for ^{1}H nuclei and at 100 MHz for ^{13}C nuclei. Deuterated solvent; chloroform-*d* ($CDCl_3$), was used in NMR experiments. The chemical shift was assigned in ppm unit and internally referenced with the residual protonated chloroform at δ 7.26 ppm (^{1}H) and 77.1 (t) ppm (^{13}C).

3.1.3 Mass Spectrometer (MS)

The mass spectra were recorded on an Esquire HCT Mass Spectrometer (MS/MS), Bruker Daltonics, Inc., Billerica, USA.

3.1.4 UV-Vis Spectrophotometer

The UV-VIS spectra were recorded on a Varian Cary 50 Probe UV-Visible spectrophotometer.

3.1.5 Electrothermal Apparatus

Melting points were examined using an Electrothermal 9100 apparatus.

3.1.6 HPLC

Semi-preparative normal-phase HPLC were carried out on a hyperprep HS silica column (Hypersil silica column, 250 x 10 mm i.d., particle size 20 μm; Thermo

Electron Corporation, USA) with a 100 µL sample loop. The HPLC system consisted of a Varian (Walnut Creek, CA) ProStar 230 solvent delivery module, 330 photodiode array detector. UV detection set at 320 ± 10 nm (Fang et al., 2003) and the spectrum was measured between 220 – 400 nm. A chromatography workstation version 6.30 was used to record the chromatogram and calculate concentrations. The initial mobile phase was hexane in ethyl acetate 88%, which was programmed to 100% ethyl acetate from 27 - 30 min. This final mobile phase was maintained for 20 min with flow rate 2.0 mL/min.

3.2 Chemicals

3.2.1 Solvents

The organic solvents used for isolating oryzanol in this research such as acetone, chloroform ($CHCl_3$), ethyl acetate (EtOAc), hexane, methanol (MeOH) and acetic acid (AcOH) were prepared by distilling commercial grade solvents. HPLC grade acetone, $CHCl_3$, EtOAC, hexane, MeOH, acetronitrile (ACN) and 2-propanol purchased from Merck (Darmstadt, Germany) and water purified by Milli-Q Plus (Molsheim, France) were used as received for HPLC analysis. All other laboratory grade solvents and deuterated solvent, $CDCl_3$, used were purchased from Merck (Darmstadt, Germany.)

3.2.2 Other Chemicals

3.2.2.1 Merck's silica gel 60 Art. 1.07734.1000 (70-230 mesh ASTM) was used as adsorbent for low-pressure column chromatography.

3.2.2.2 Merck's silica gel 60 Art. 1.09385.1000 (230-400 mesh ASTM) was used as adsorbent for low-pressure column chromatography.

3.2.2.3 Merck's TLC aluminum sheets, silica gel $^{60}F_{254}$ precoated 25 sheets, 20x20 cm^2, layer thickness 0.2 mm were used as adsorbent for TLC analysis.

3.3 Samples

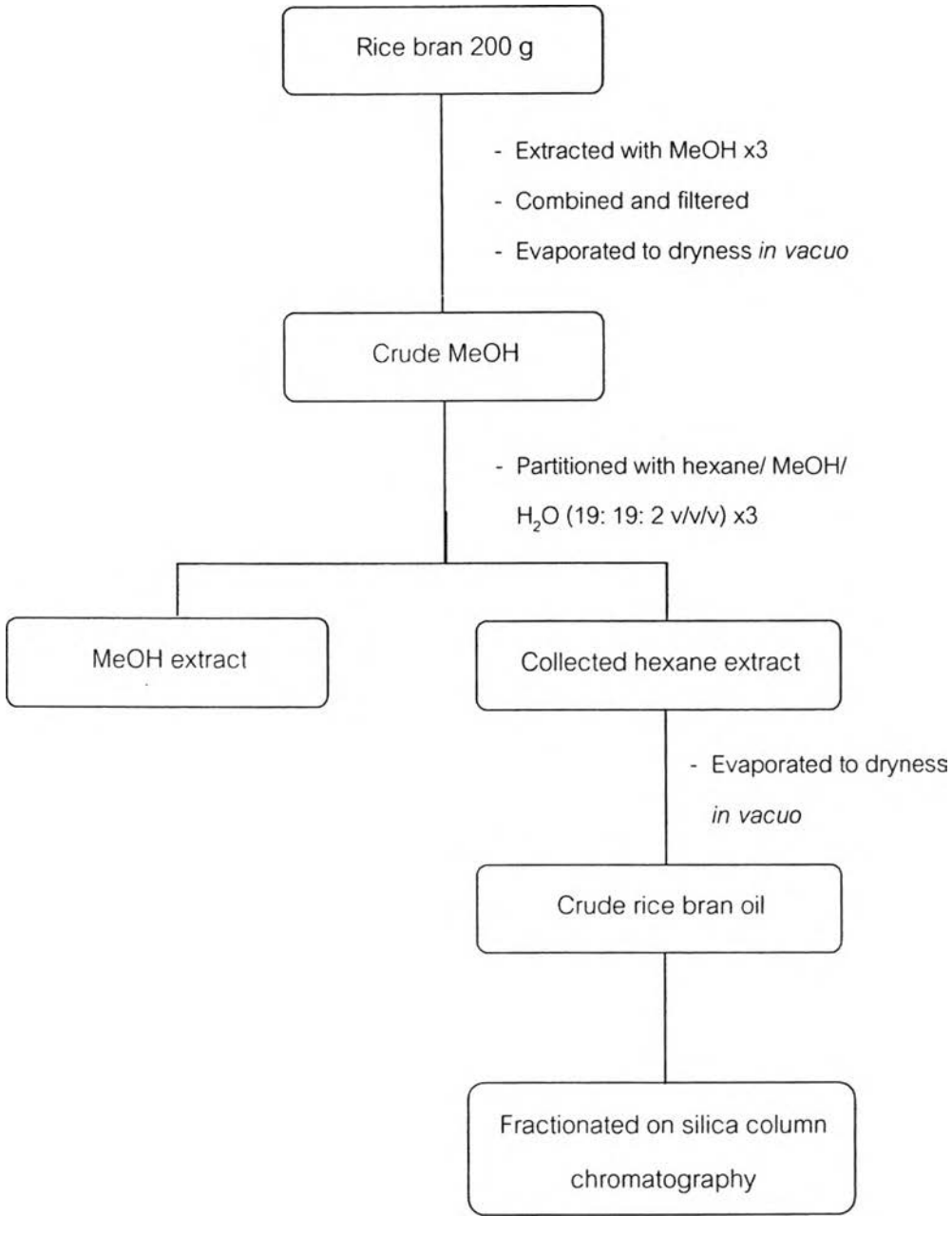
Rice Bran samples were obtained from different local mills, i.e., Sunpatong 1 from Chiang Mai; Pathumthani 1, Go Ko Chai Nat 1 , Kao Dok Mali 105 from Suphanburi. The bran was received and stored at -20 $^{\circ}$C until processing. There were two kinds of rice in this study; for instance, glutinous rice (or waxy rice) and nonglutinous rice. Sunpatong 1 is glutinous rice but the others are nonglutinous rice. Unlike Go Ko Chai Nat 1 which is ordinary rice, Pathumthani 1, Kao Dok Mali 105 are jasmine rice.

Standard oryzanol was used as reference compound. It was kindly provided by Mr. Pravit Santiwattana (Thai Edible Oil Co., Ltd.)

3.4 Extraction Procedure of Crude Rice Bran Oil

Rice bran (200g) was treated with MeOH at 1:10 w/v bran-to-solvent ratio in Flasks, capped, and shaking horizontally (GFL shaker; Ges ellschaft Für Labortechnik mbH, Burgwedel, Germany) for 1 day at room temperature, a total of three times. Extracts were filtered through a Whatman No. 93 paper filter and MeOH was evaporated in a rotary evaporator. The crude MeOH extract was obtained as a mixture of brown solid and dark brown viscous liquid.

This extract was partitioned three times between hexane/ MeOH/ H_2O (19: 19: 2 v/v/v) according to the method of Yasukawa and his colleague (Yasukawa *et al.*, 1998) with some modifications. Vacuum evaporation (Buchi Rotavapor R-124; Buchi Labortechnik, Flawil, Switzerland) of hexane yielded crude rice bran oil. Extraction procedure of rice bran oil is shown in Scheme 3.1.



Scheme 3.1 Diagram for extraction of rice bran oil.

3.5 Determination of Crude Rice Bran Oil Profile

A few milligrams of crude bran oil in the section 3.4 were dissolved with hexane for determination of the profile of the extracts by using TLC technique that is described in later.

Analytical Thin-Layer Chromatography (TLC)

Technique	: one dimension ascending
Adsorbent	: silica gel F_{254} coated on aluminium sheet (Merck)
Layer thickness	: 250 μm
Distance	: 5 cm
Temperature	: laboratory temperature (25-30°C)
Detection	: 1. Visual detection under ultraviolet light at wavelengths of 254 and 365 nm
	2. Visual detection in iodine vapor
	3. Visual detection after spraying with vanillin reagent

TLC spots were visualized under ultraviolet light at wavelengths of 254 and 365 nm, in iodine vapor, and under daylight after spraying with vanillin reagent (Dissolve 0.5 g vanillin in 95 ml ethanol and add 4.5 ml concentrated sulfuric acid) and heating until the colors developed.

3.6 Estimation of oryzanol

The oryzanol content of the crude MeOH, crude rice bran oil and MeOH extracts was estimated by UV spectrophotometry, using the specific extinction coefficient calculated from standard oryzanol.

3.7 Purification of Oryzanol

The crude oil was subjected to column chromatography on silica gel using an hexane - EtOAc gradient of 1:0 - 0:1, which yielded a mixture containing oryzanol. Oryzanol mixture was further purified by flash column chromatography using silica gel for preparative layer chromatography as the stationary phase. Hexane/ EtOAc solvent system was used as the mobile phase. Each peak was collected manually and mobile phase was evaporated with rotary evaporator. The oryzanol was purified by crystallized

in MeOH-acetone (1:1).

3.7.1 Isolation of Oryzanol in Pathumthani 1 Crude Oil

Pathumthani 1 rice bran (200 g) was extracted three times at room temperature for 1 day each with MeOH to give an extract (36.93 g). The MeOH extract was partitioned between hexane/ MeOH/ H_2O (19: 19: 2 v/v/v) and yielded MeOH portion (16.36 g) and Pathumthani crude oil (Scheme 3.2).

The crude oil (20.57 g) was subjected to column chromatography on silica gel (255 g) using a hexane-EtOAc gradient of 1:0-0:1. Each fraction (100 ml) was collected and examined. The fractions were combined by silica gel TLC. Fractions with the same TLC pattern were combined, scanned for UV spectrum, and dried.

The results from the separation and purification of Pathumthani 1 crude oil are shown in Scheme 3.3 and Table 3.1.

3.7.1.1 Isolation of Oryzanol Mixture PF1

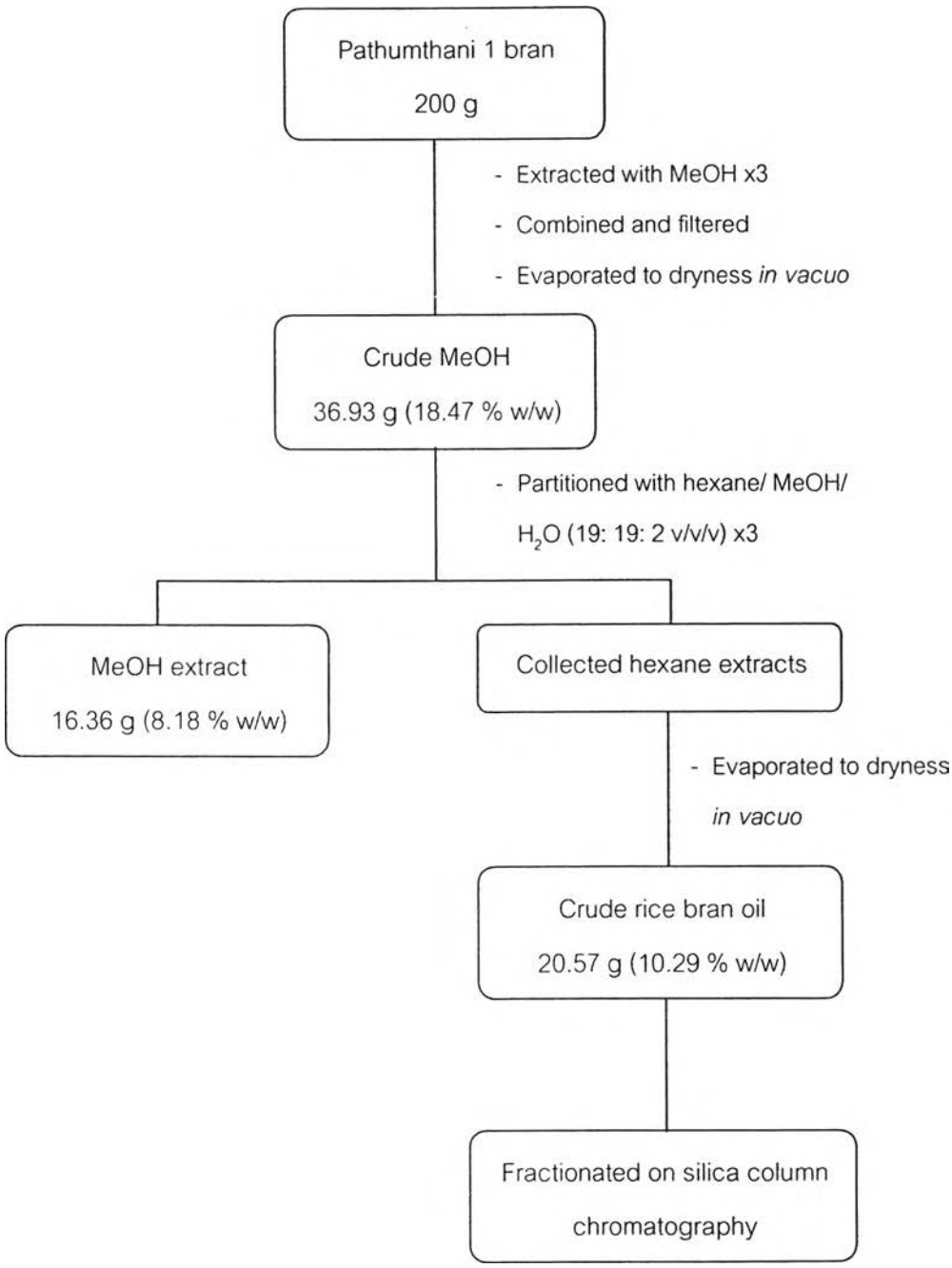
Combined fraction P08 was obtained from the elution of silica gel chromatography with 15% EtOAc in hexane and was further purified by re-column chromatography and flash column chromatography using silica gel for column chromatography and preparative layer chromatography, respectively, as the stationary phase. Hexane/ EtOAc solvent system was used as the mobile phase. Re-crystallization with MeOH and acetone yielded oryzanol mixture PF1, white powder (4.2 mg).

Identification of individual steryl ferulate in mixture PF1 was performed by spectroscopic technique (MS/MS).

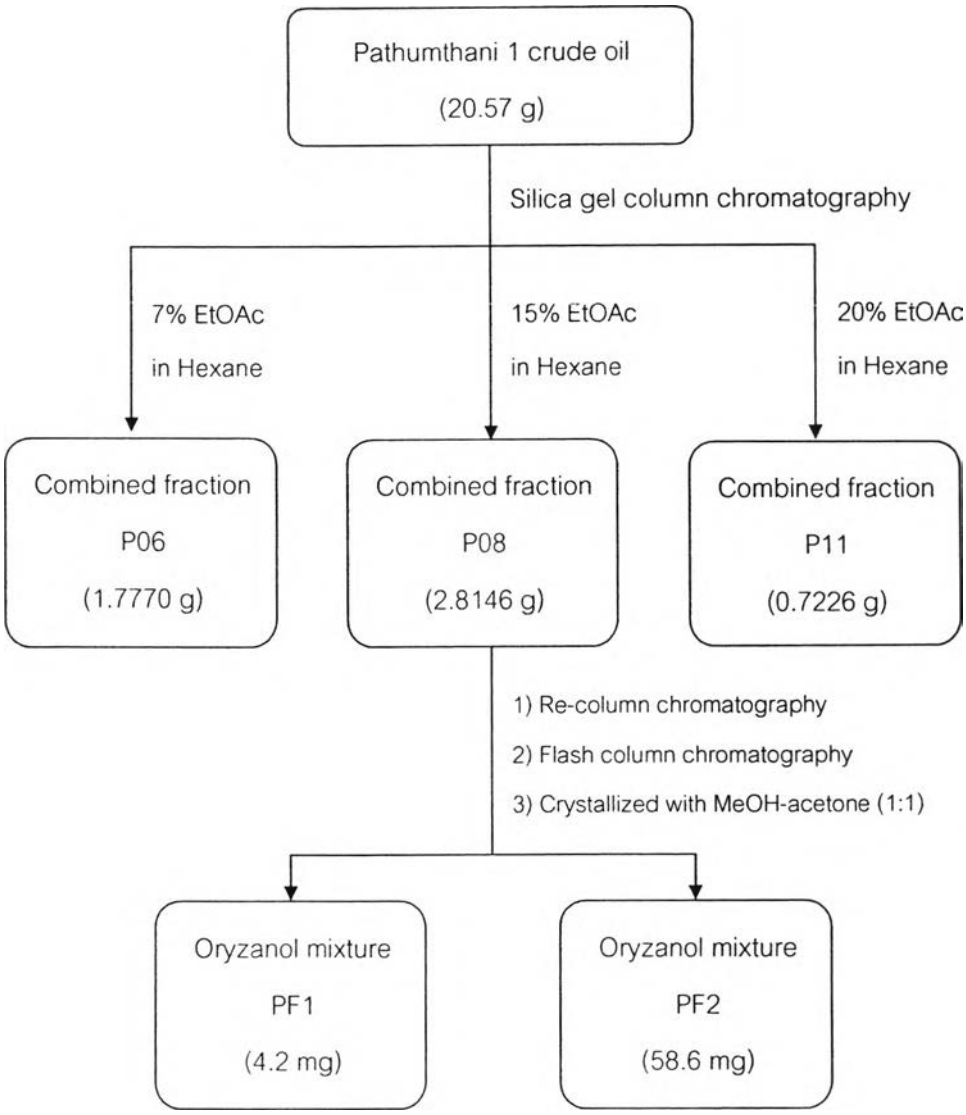
3.7.1.2 Isolation of Oryzanol Mixture PF2

Similarly perform as in 3.7.1.1, oryzanol mixture PF2, slightly yellowish-white powder (58.6 mg), was obtained from re-crystallization with MeOH and acetone.

Oryzanol constituents in mixture PF2 were identified by MS/MS.



Scheme 3.2 Diagram for extraction of Pathumthani 1 oil.



Scheme 3.3 Diagram of method for oryzanol isolation from Pathumthani 1 Crude Oil.

Table 3.1 Characteristics of separation fraction from Pathumthani 1 crude oil

Combined fractions	Fraction No.	Eluents	Appearance	Weight (g)	% w/w of oil
P01	1-25	100% Hexane	Colourless viscous liquid	0.0069	0.07
P02	26-35	5% EtOAc in Hexane	Yellow viscous liquid	1.2253	11.91
P03	36-43	5% EtOAc in Hexane	White solid with yellow viscous liquid	1.9140	18.60
P04	44-51	5% EtOAc in Hexane	White solid	2.2015	21.39
P05	52-54	5% EtOAc in Hexane	White solid	1.5572	15.13
P06	55-81	7% EtOAc in Hexane	Yellow viscous liquid with white solid	1.7770	17.27
P07	82-91	10% EtOAc in Hexane	Yellow viscous liquid with white solid	1.1568	11.24
P08	92-103	15% EtOAc in Hexane	Yellow viscous liquid with white solid and colorless crystal	2.8146	27.35
P09	104-114	20% EtOAc in Hexane	Colorless crystal with yellow viscous liquid	1.3015	12.65
P10	115-125	20% EtOAc in Hexane	Yellow viscous liquid	0.8753	8.51
P11	126-138	20% EtOAc in Hexane	Yellowish green viscous liquid	0.7226	7.02
P12	139-144	20% EtOAc in Hexane	Yellow viscous liquid	0.8572	8.33
P13	145-149	20% EtOAc in Hexane	White solid	0.6776	6.59
P14	150-154	25% EtOAc in Hexane	Yellow viscous liquid	0.7022	6.82
P15	155-167	30% EtOAc in Hexane	Yellow solid	0.7447	7.24
P16	168-190	40% EtOAc in Hexane	Yellow viscous liquid	0.0042	0.04
P17	191-220	50% EtOAc in Hexane	Yellow viscous liquid	0.0484	0.47
P18	221-248	60% EtOAc in Hexane	Yellow viscous liquid	0.0173	0.17
P19	249-273	70% EtOAc in Hexane	Yellow viscous liquid	0.3514	3.41
P20	274-281	80% EtOAc in Hexane	Yellow liquid	0.4057	3.94
P21	282-291	90% EtOAc in Hexane	Light brown liquid	0.6496	6.31
P22	292-300	100% EtOAc	Brown liquid	0.3887	3.78

3.7.2 Isolation of Oryzanol in Kao Dok Mali 105 Crude Oil

Kao Dok Mali 105 rice bran (200 g) was extracted three times at room temperature for 1 day each with MeOH to give an extract (46.50 g). The MeOH extract was partitioned between hexane/ MeOH/ H_2O (19: 19: 2 v/v/v) and yielded MeOH portion (15.17 g) and Kao Dok Mali 105 crude oil (Scheme 3.4).

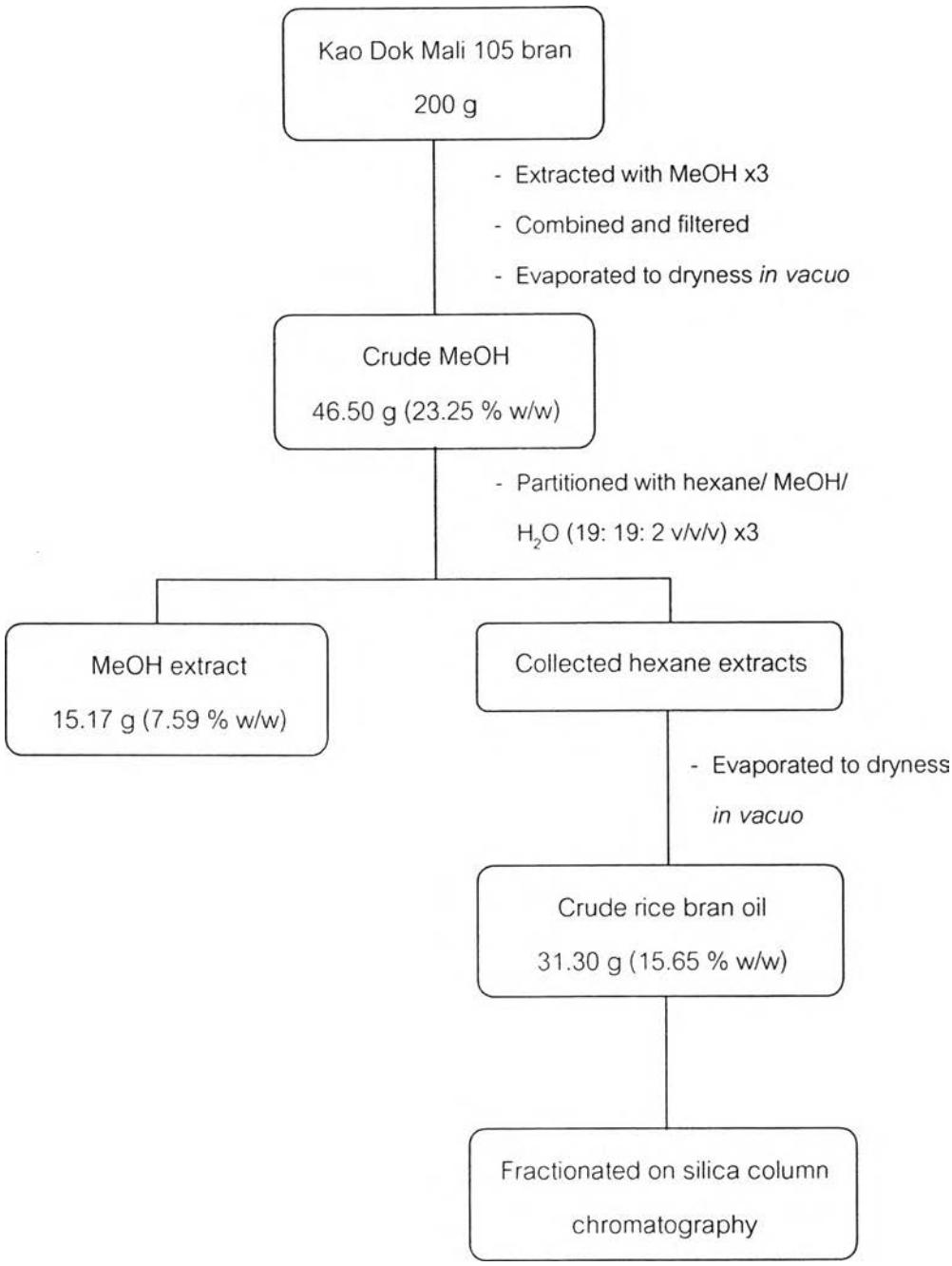
The crude oil from rice bran (31.30 g of wet weight) was fractionated by chromatography on a silica gel column (silica gel 290 g). Elution systems were Hexane, Hexane-EtOAc gradients, and EtOAc. Fractions (100 ml each) were collected and examined. Fraction combination was by TLC on Silica gel plates with hexane, hexane and EtOAc mixtures, and EtOAc as the developing solvent. Fractions with the same TLC pattern were pooled, scanned for UV spectrum, and dried.

The results from the isolation of Kao Dok Mali 105 crude oil are shown in Scheme 3.5 and Table 3.2.

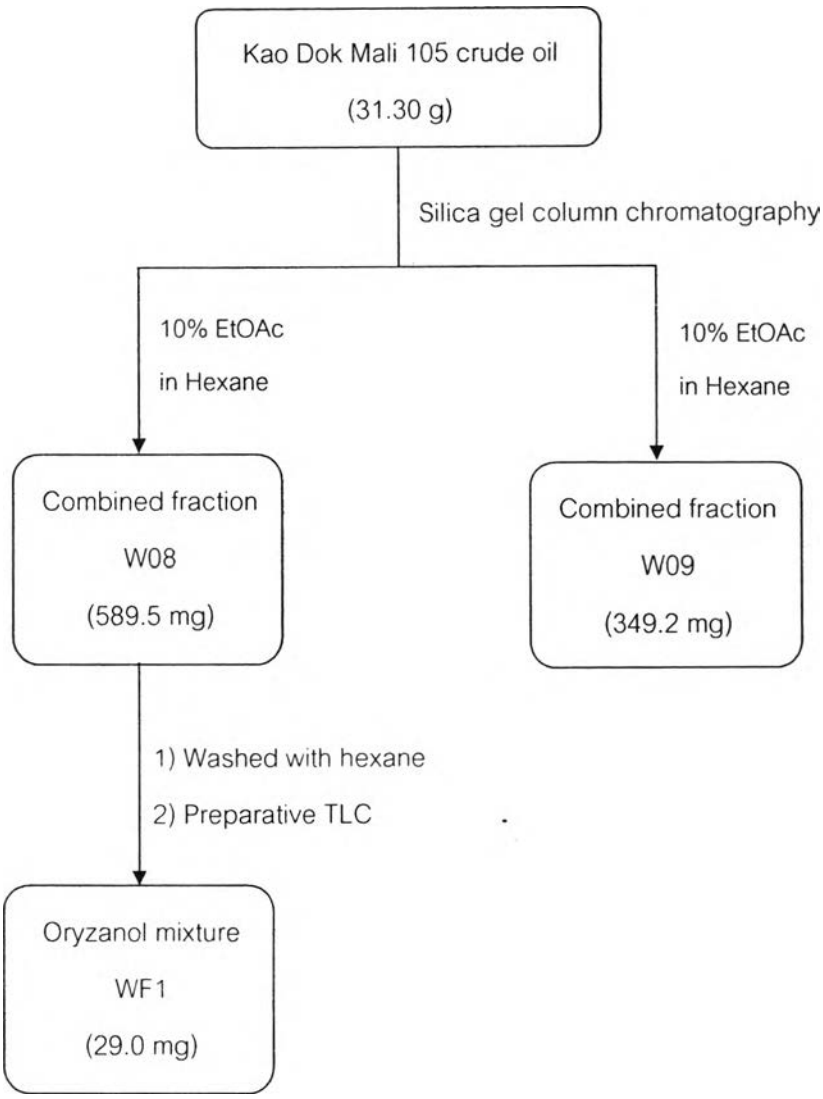
3.7.2.1 Isolation of Oryzanol Mixture WF1

Combined fraction W08 was obtained from the elution of silica gel chromatography with 10% EtOAc in hexane and was purified by washing with hexane to remove oil in this fraction. A portion (87.7 mg) of the fraction was further purified by preparative TLC on silica gel ($^{60}F_{254}$ precoated, Merck; 0.2 mm thick; 20 x20 cm). Preparative TLC was develop using 20% EtOAc in hexane and yielded oryzanol mixture WF1, yellowish-white powder (29.0 mg).

Identification of individual oryzanol in mixture WF1 was performed by MS/MS.



Scheme 3.4 Diagram for extraction of Kao Dok Mali 105 crude oil.



Scheme 3.5 Isolation procedure of Kao Dok Mali 105 crude oil.

Table 3.2 Characteristics of separation fraction from Kao Dok Mali 105 crude oil

Combined fractions	Fraction No.	Eluents	Appearance	Weight (g)	% w/w of oil
W01	1-8	100% Hexane	Colourless viscous liquid	0.0752	0.24
W02	9-14	3% EtOAc in Hexane	Yellow solid	1.2679	4.05
W03	15-17	5% EtOAc in Hexane	White solid with colorless viscous liquid	3.9067	12.48
W04	18-20	5% EtOAc in Hexane	Yellow viscous liquid	3.3296	10.64
W05	21-32	7% EtOAc in Hexane	Yellow solid	3.9839	12.73
W06	33-40	7% EtOAc in Hexane	White solid with yellow viscous liquid	2.6977	8.62
W07	41-43	7% EtOAc in Hexane	White solid	0.9309	2.97
W08	44-48	10% EtOAc in Hexane	White solid with orange liquid	0.5895	1.88
W09	49-50	10% EtOAc in Hexane	White solid with yellow viscous liquid	0.3492	1.12
W10	51-52	10% EtOAc in Hexane	Colorless crystal with yellow viscous liquid	0.1542	0.49
W11	53-62	10% EtOAc in Hexane	White solid with yellow viscous liquid	1.9944	6.37
W12	63-76	10% EtOAc in Hexane	Yellow viscous liquid	1.0855	3.47
W13	77-81	15% EtOAc in Hexane	White solid	0.4320	1.38
W14	82-117	20% EtOAc in Hexane	Yellow viscous liquid	0.5931	1.89
W15	118-150	30-40% EtOAc in Hexane	White solid with yellow viscous liquid	0.3424	1.09
W16	151-163	50% EtOAc in Hexane	Yellow viscous liquid	0.8125	2.60
W17	164-176	60% EtOAc in Hexane	Yellow solid	1.7284	5.52
W18	177-194	70-90% EtOAc in Hexane	Yellow viscous liquid	1.7612	5.63
W19	195-201	100% EtOAc	Yellow solid	1.6726	5.34
W20	201-205	100% EtOAc	Yellow viscous liquid	0.7750	2.48

3.7.3 Isolation of Oryzanol in Sunpatong 1 Crude Oil

Sunpatong 1 rice bran (270 g) was extracted three times at room temperature for 1 day each with MeOH to give an extract (64.59 g). The MeOH extract was partitioned between hexane/ MeOH/ H_2O (19: 19: 2 v/v/v) and yielded MeOH portion (29.92 g) and Sunpatong 1 crude oil (Scheme 3.6).

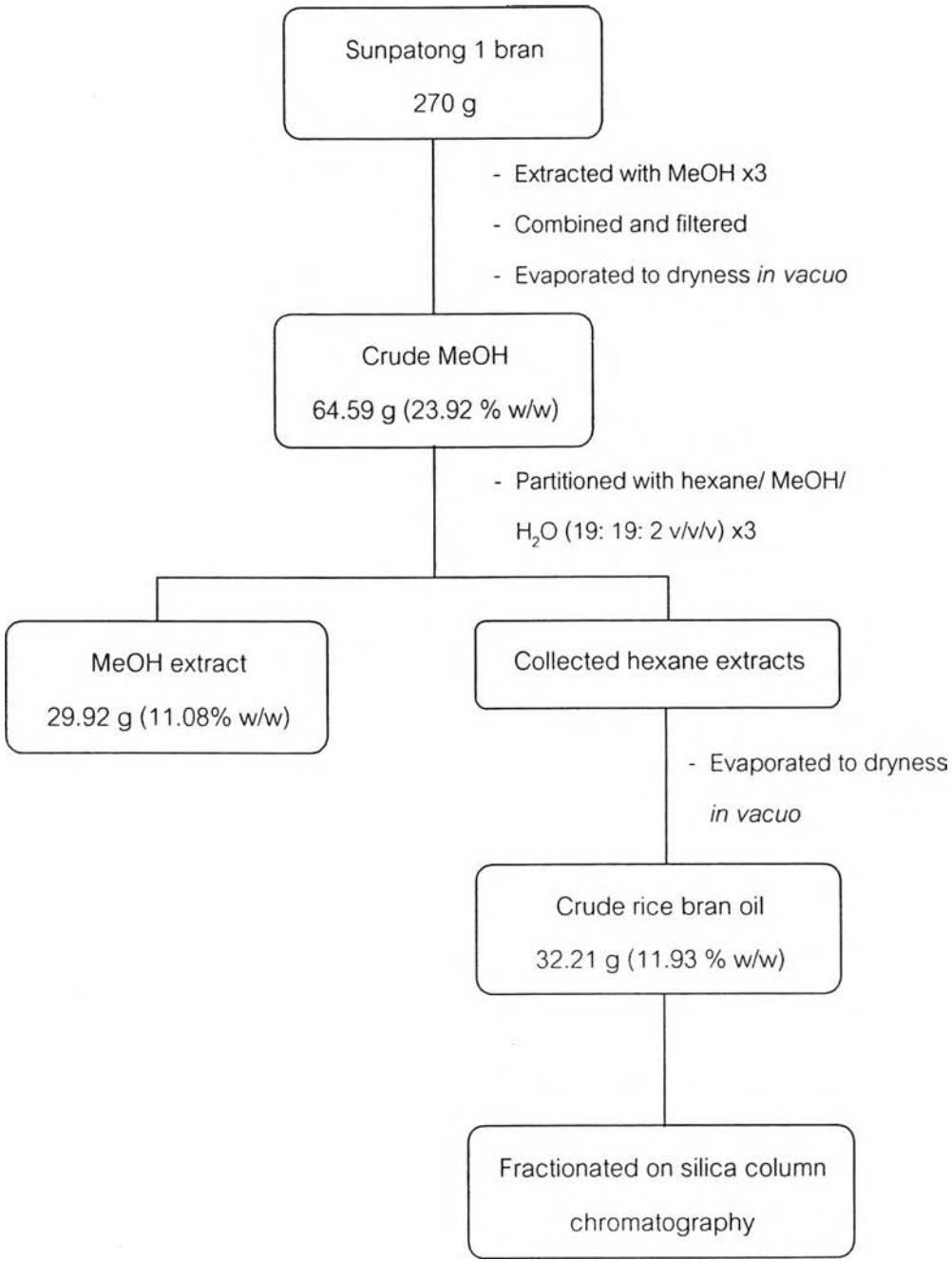
The Sunpatong 1 crude oil (32.21 g of wet weight) was subjected to column chromatography (silica gel, 255 g) using eluents of increasing polarity from hexane to MeOH. Fractions (100 ml each) were collected. Silica gel TLC analysis was used for monitoring each fraction. Fractions with the same TLC pattern were combined, evaporated, and scanned for UV spectrum (250-350 nm) compared with standard oryzanol.

The extraction of crude oil is shown in Scheme 3.7. The results from purification of Sunpatong 1 crude oil are presented in Table 3.3.

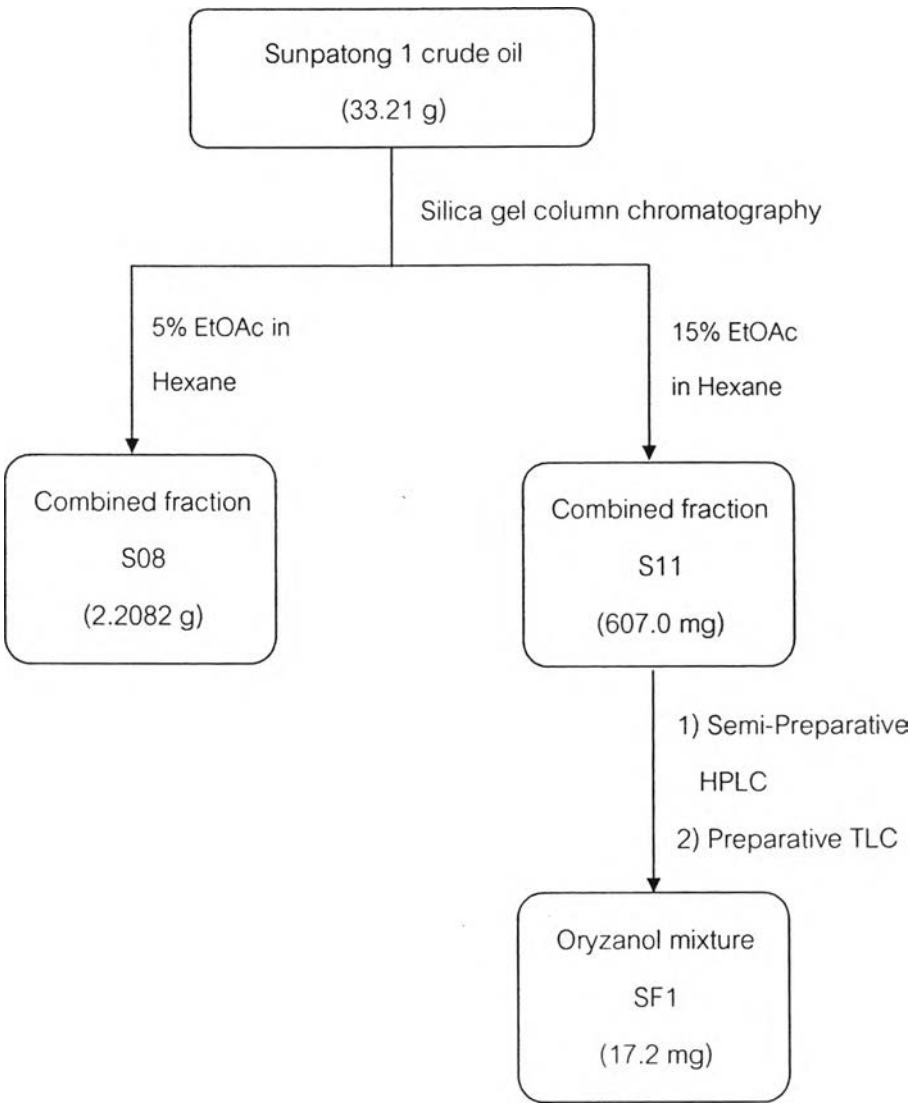
3.7.3.1 Isolation of Oryzanol Mixture SF1

Combined fraction S11 was obtained from the elution of silica gel chromatography with 15% EtOAc in hexane. A portion (100 mg) of this fraction was further purified by semi-preparative HPLC and preparative TLC on silica gel ($^{60}F_{254}$ precoated, Merck; 0.2 mm thick; 20 x20 cm). Preparative TLC was develop using 20% EtOAc in hexane and yielded oryzanol mixture SF1, yellow crystalline solid (17.2 mg).

Identification of individual oryzanol constituent in mixture SF1 was performed by MS/MS.



Scheme 3.6 Diagram for extraction of Sunpatong 1 crude oil.



Scheme 3.7 Isolation procedure of Sunpatong 1 crude oil.

Table 3.3 Characteristics of separation fraction from Sunpatong 1 crude oil

Combined fractions	Fraction No.	Eluents	Appearance	Weight (g)	% w/w of oil
S01	1-9	100% Hexane	Yellow viscous liquid	0.0164	0.05
S02	10-29	2% EtOAc in Hexane	White solid	1.1484	3.57
S03	30-31	2% EtOAc in Hexane	White solid	1.6378	5.08
S04	32-35	2% EtOAc in Hexane	White solid	3.1909	9.91
S05	36-38	2% EtOAc in Hexane	White solid	3.9715	12.33
S06	39-58	5% EtOAc in Hexane	White solid with yellow viscous liquid	3.0309	9.41
S07	59-61	5% EtOAc in Hexane	White solid	2.3768	7.38
S08	62-74	5% EtOAc in Hexane	White solid with yellow liquid	2.2082	6.86
S09	75-82	7% EtOAc in Hexane	White solid	0.6284	1.95
S10	83-97	10-13% EtOAc in Hexane	White solid with yellow viscous liquid	1.0222	3.17
S11	98-104	15% EtOAc in Hexane	White solid with yellow viscous liquid	0.6070	1.88
S12	105-114	15% EtOAc in Hexane	Yellowish green viscous liquid	2.5237	7.84
S13	115-121	15-20% EtOAc in Hexane	Yellowish green viscous liquid	1.7314	5.38
S14	122-138	20-30% EtOAc in Hexane	Yellow solid	0.8840	2.74
S15	139-146	30-35% EtOAc in Hexane	Yellow solid	0.8544	2.65
S16	147-158	40% EtOAc in Hexane	Yellow viscous liquid	0.8150	2.53
S17	159-195	45-60% EtOAc in Hexane	Yellow solid	0.6203	1.93
S18	196-202	70-90% EtOAc in Hexane	Yellow viscous liquid	0.0276	0.09
S19	203-265	100% EtOAc-15% MeOH in EtOAc	Brown solid with yellow viscous liquid	1.6726	5.19

3.7.4 Isolation of Oryzanol in Go Ko Chai Nat 1 Crude Oil

Go Ko Chai Nat 1 rice bran (200 g) was extracted three times at room temperature for 1 day each with MeOH to give an extract (40.95 g). The MeOH extract was partitioned between hexane/ MeOH/ H_2O (19: 19: 2 v/v/v) and yielded MeOH portion (13.58 g) and Go Ko Chai Nat crude oil (**Scheme 3.8**).

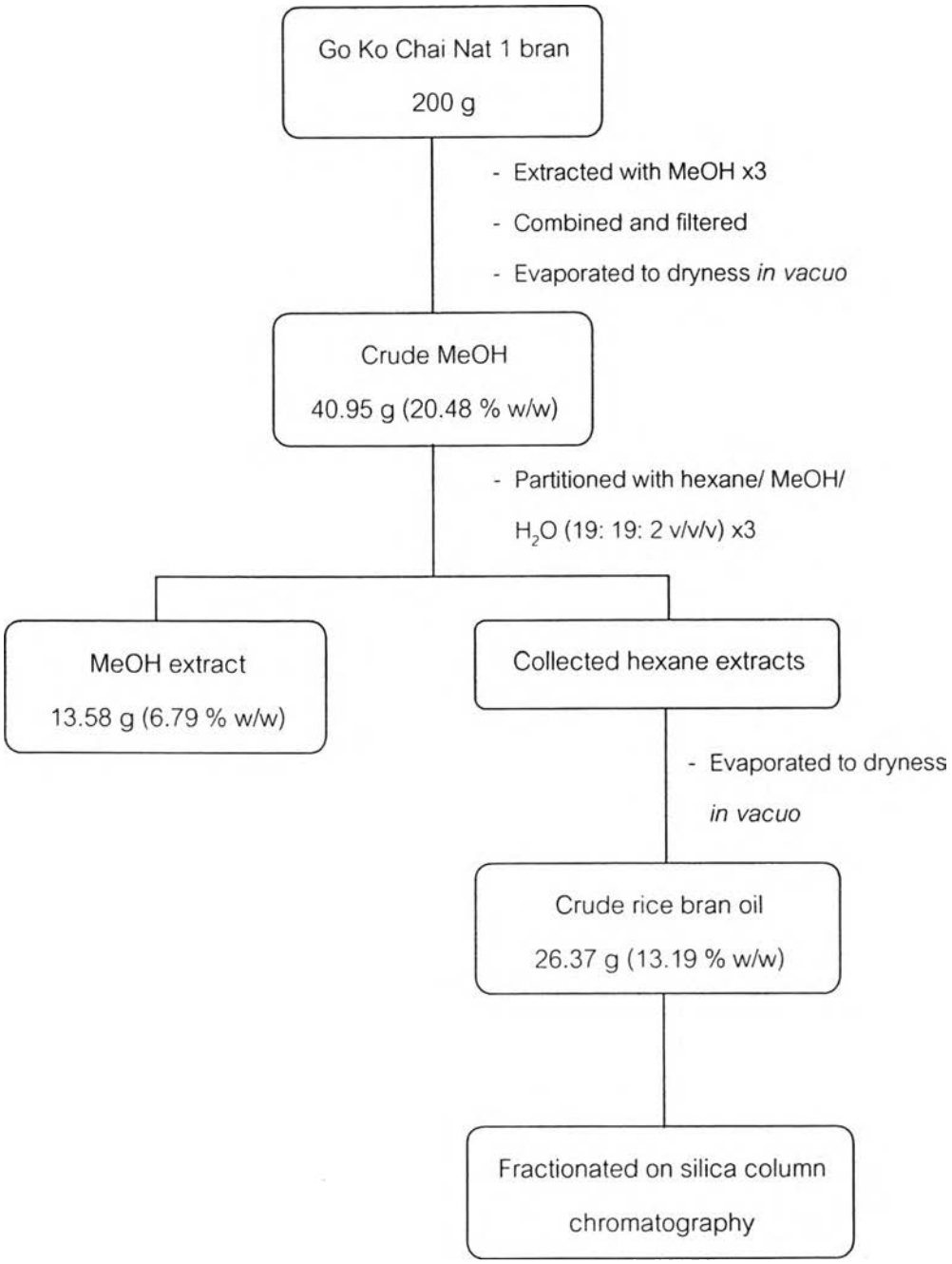
The Go Ko Chai Nat 1 crude oil (26.37 g of wet weight) was purified by using silica gel column chromatography (silica gel, 300 g) with Hexane to EtOAc gradient as the solvent system. The pooled fractions were scanned for UV spectrum.

The results from the purification of Go Ko Chai Nat 1 crude oil was presented in **Scheme 3.9** and **Table 3.4**.

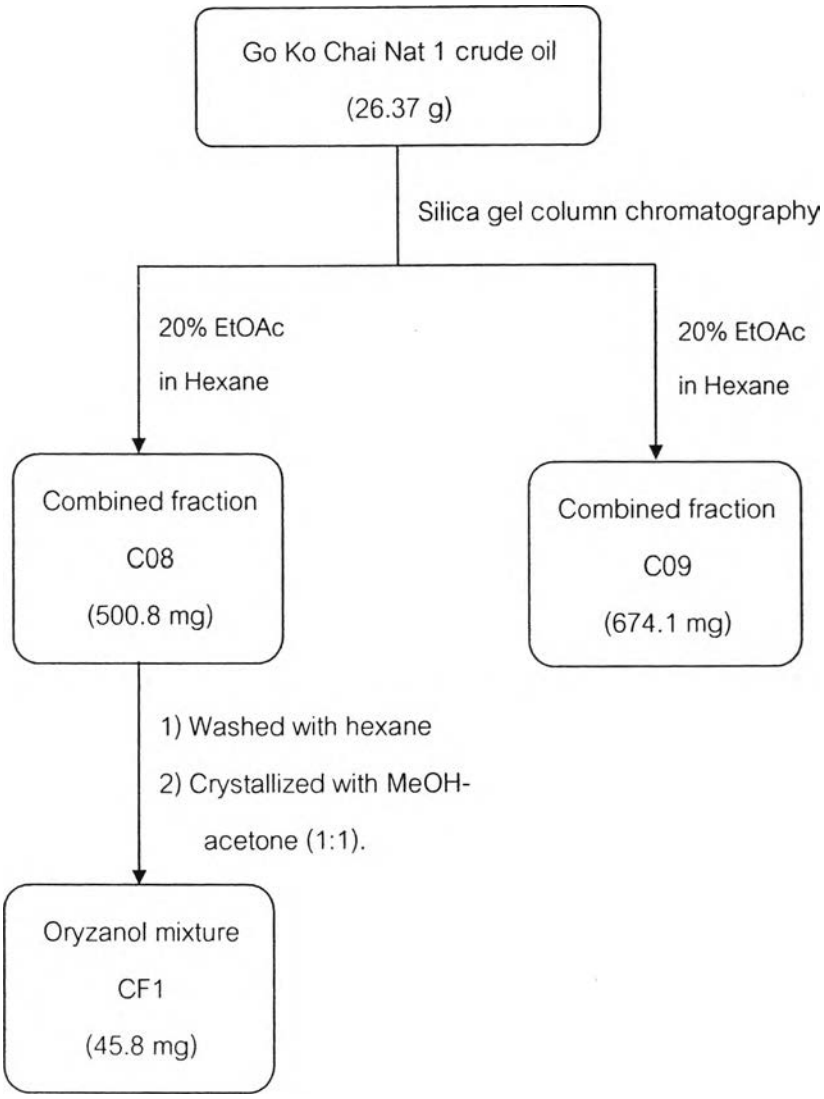
3.7.4.1 Isolation of Oryzanol Mixture CF1

Combined fraction C08 was obtained from the elution of silica gel chromatography with 20% EtOAc in hexane. Fraction C08 was further purified by washing with hexane and re-crystallization with MeOH and acetone to obtain oryzanol mixture CF1, white crystal (45.8 mg).

Identification of individual oryzanol in mixture CF1 was performed by MS/MS.



Scheme 3.8 Diagram for extraction of Go Ko Chai Nat 1 crude oil.



Scheme 3.9 Isolation procedure of Go Ko Chai Nat 1 crude oil.

Table 3.4 Characteristics of separation fraction from Go Ko Chai Nat 1 crude oil

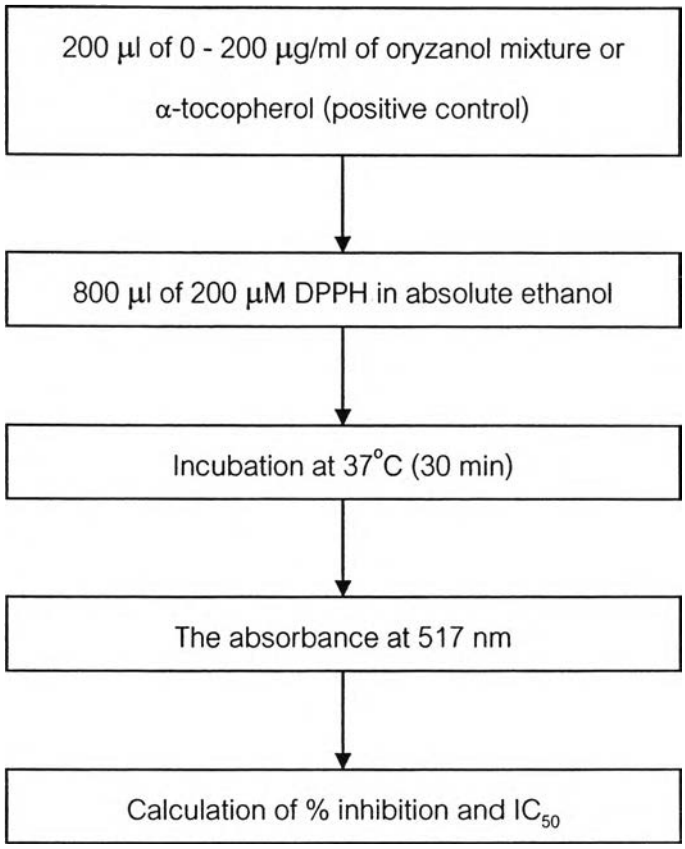
Combined fractions	Fraction No.	Eluents	Appearance	Weight (g)	% w/w of oil
C01	1-25	0-3% EtOAc in Hexane	Colourless viscous liquid	0.0078	0.03
C02	25-33	5-8% EtOAc in Hexane	White solid with colorless viscous liquid	14.7025	55.75
C03	34-38	8% EtOAc in Hexane	Yellow viscous liquid	2.0550	7.79
C04	39-47	10% EtOAc in Hexane	Yellow viscous liquid	2.3235	8.81
C05	48-50	15% EtOAc in Hexane	White solid with colorless viscous liquid	0.9209	3.49
C06	51	15% EtOAc in Hexane	White solid with colorless viscous liquid	0.1597	0.61
C07	52-53	15-20% EtOAc in Hexane	Colourless viscous liquid	0.4110	1.56
C08	54	20% EtOAc in Hexane	White solid with yellow viscous liquid	0.5008	1.90
C09	55	20% EtOAc in Hexane	Colorless crystal with Colourless viscous liquid	0.6741	2.56
C10	56	20% EtOAc in Hexane	White solid with colorless viscous liquid	0.4492	1.70
C11	57	20% EtOAc in Hexane	Yellow viscous liquid	0.3524	1.34
C12	58-62	20% EtOAc in Hexane	White solid with yellow viscous liquid	1.2801	4.85
C13	63-70	30% EtOAc in Hexane	Yellow viscous liquid	0.6542	2.48
C14	71-77	40% EtOAc in Hexane	Yellow viscous liquid	0.2852	1.08
C15	78-81	50% EtOAc in Hexane	White solid with yellow viscous liquid	0.0686	0.26
C16	82-83	50% EtOAc in Hexane	Colorless solid with yellow viscous liquid	0.0631	0.24
C17	84-85	60% EtOAc in Hexane	Colorless solid with colourless viscous liquid	0.1528	0.58
C18	86-93	70-90% EtOAc in Hexane	Yellow viscous liquid	0.3685	1.40
C19	94-95	100% EtOAc	Colourless viscous liquid	0.0160	0.06

3.8 Identification and Characterization of Individual Oryzanol

Identification of individual steryl ferulate was performed by MS/MS. Characterization of oryzanol mixture was also performed by spectroscopic (UV, ^{1}H-NMR and FT-IR).

3.9 Antioxidant Activity Determination by DPPH$^{\cdot}$ Assay

Free radical scavenging activities of oryzanol mixture was determined by using a stable 2,2'-diphenyl-1-picrylhydrazyl radical (DPPH$^{\cdot}$). Oryzanol mixture and α-tocopherol (positive control) were prepared in absolute ethanol (EtOH) as stock solutions (1 mg/ml). Oryzanol mixtures and α-tocopherol were pipetted in to each tube (covered tube with aluminium foil). The appropriate concentration of sample was added in vary concentration such as 200, 100, 80, 60, 40, 20, 0 μg/ml and added 800 μl of freshly prepared DPPH solution (200 μM in absolute EtOH; kept in dark by covered with aluminium foil until used). Thus it has about 1 ml final volume. The mixture was shaken vigorously and incubated at 37°C for 30 min. The absorbance of the remaining DPPH was determined colormetrically at 517 nm and the absorbance of 200 μl DPPH solution was also measured at 517 nm (**Scheme 3.10**). The remaining concentration of DPPH in the reaction mixture was calculated and expressed as a percentage of inhibition (Nyström *et al.*, 2005) and concentration required to cause a 50% inhibition (IC_{50}) was also determined from a standard calibration curve. All analyses were carried out in triplicate.



Scheme 3.10 Flowchart for antioxidant activity determination.