CHAPTER IV

RESULTS AND DISCUSSION

4.1 Extraction of Crude Rice Bran Oil

In this study, crude rice bran oil was first extracted by MeOH yielding the crude MeOH 36.93, 46.50, 64.59 and 40.95 g (18.47, 23.25, 23.92 and 20.48 % yield in rice bran) from Pathumthani 1, Kao Dok Mali 105, Sunpatong 1, and Go Ko Chai Nat 1 rice bran, respectively (Table 4.1).

Most researches focusing on oryzanol extracted the oil from mixed rice varieties, whereas, our research extracted rice bran oil from each four rice cultivars comprising Pathumthani 1, Kao Dok Mali 105, Sunpatong 1, and Go Ko Chai Nat 1. Freshly milled rice bran was transported from several sources and stored at -20 $^{\circ}$C until analyzed. An endogenous lipase activity is activated by milling, resulting in rapid deterioration of the oil and rendering it unsuitable for human consumption. Thus, some researches inactivated lipase by heating the rice bran at 130-140 $^{\circ}$C for 3-90 seconds, yielding stabilized rice bran (Fang *et al.*, 2003; McCaskill and Orthoefer, 1994; Qureshi *et al.*, 2002; Randall *et al.*, 1985). Miller *et al.*, (2004) demonstrated that lipase preparations from different sources can not hydrolyze steryl ferulate of oryzanol, so we did not use stabilized rice bran in this study. Although, there was no document compared oryzanol content obtained from freshly milled and stabilized rice bran.

Hexane is commonly used as a solvent to extract oil from rice bran (Johnson and Lusas, 1983; Xu and Godber, 1999). Norton (1994, 1995), Seitz (1989) and Moreau *et al.*, (1996) have reported that a hexane extract from corn bran contains high levels of ferulate ester, similar in composition to oryzanol found in rice bran and rice bran oil. As all components of oryzanol contain the hydroxyl groups in the benzene ring of the ferulate portion, the more polar solvents, short-chain alcohols especially ethanol, MeOH and isopropanol, have been proposed as alternative extraction solvents (Chen and Bergman, 2005). Isopropanol or isopropanol-hexane (1:1 v/v) extracted more oryzanol from rice bran than did hexane at elevated temperature, even though hexane recovered more rice bran oil (Hu *et al.*, 1996; Xu and Godber, 2000).

Table 4.1 Amount (% yield in rice bran) of oil in different extraction fractions extracted from various cultivars.

Extracted fractions	% Yield in rice bran			
	Pathumthani 1	Kao Dok Mali 105	Sunpatong 1	Go Ko Chai Nat 1
Crude MeOH	18.47	23.25	23.92	20.48
Crude rice bran oil	10.29	15.65	11.93	13.19
MeOH extract	8.18	7.59	11.08	6.79

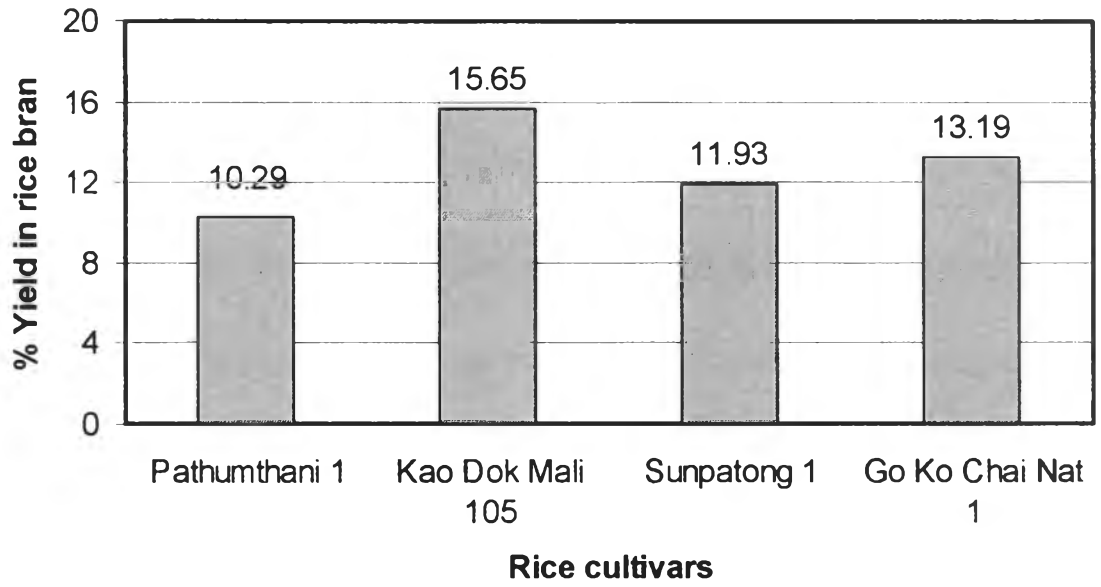


Figure 4.1 Comparison of the amount (% yield in rice bran) of crude oil extracted from various cultivars

Methanol, a more polar solvent than isopropanol and hexane, has been used on a direct solvent extraction method for the rapid analysis of oryzanol in rice bran. Chen and Bergman (2005) demonstrated that MeOH and isopropanol are suitable for extracting oryzanol from rice bran at ambient temperature. They found that oryzanol was readily extracted by ambient hexane and had 94-96% recovery compared to the hexane at 60 °C, while MeOH extracted 10% more oryzanol than hexane at 60 °C. A mixture of chloroform-MeOH and dichloromethane-MeOH were also used for extraction of oryzanol from rice material (Folch *et al.*, 1957; Miller *et al.*, 2003).

From the Yasukawa *et al.* (1998) extraction method with some modifications, the crude rice bran oil was obtained from the partition of crude MeOH in hexane-MeOH-H_2O (19:19:2 v/v/v) (Table 4.1). As shown in Figure 4.1, the crude rice bran oil contents extracted from our condition were less than 18 %yield in rice bran. Typically, commercial rice bran contains 18-22% oil (Cicero and Gaddi, 2001; Qureshi *et al.*, 2002). As for the rice bran previously extracted with MeOH, alcohol tends to extract more nonglyceride materials and contain more phosphatide and unsaponifiable compounds than hexane, due to their greater polarity (Hu *et al.*, 1996; Johnson and Lusas, 1983). Moreover, Qureshi and colleagues (2000) suggested that hexane-soluble fraction, prepared from partition with MeOH-soluble fraction, should be left at 0 °C overnight before filtering and drying under vacuum to remove some of the sterols and triacylglycerides as precipitates.

Generally, crude rice bran oil was extracted using direct solvent extraction method or solvent extraction method with saponification. Saponification has been used to remove interfering triacylglycerides, neutral lipids and fatty acid; even so, saponification may hydrolyze the ester bond between phytosterol and ferulic acid of oryzanol (Diack and Saska, 1994; Xu and Godber, 2000). In the present study, we extracted the crude oil without saponification to avoid the loss in saponification probably related to degradation in alkaline media and possibly to trapping of the oryzanol in the precipitates of the fatty acid salts generated during this process (Lang *et al.*, 1992). On the other hand, Hakala and coworkers (2002) modified saponification technique to wash neutral lipids from the acetone extracted samples. During method development, they claimed the hexane washing of the alkaline methanolic extract did not remove steryl

ferulates. With respect to avoid oxidation in rice bran, some documents were used small amount of ascorbic acid combination with organic solvent extraction (Diack and Saska, 1994; Shin *et al.*, 1997; Xu and Godber, 1999).

4.2 Estimation of Oryzanol

The total oryzanol content of each solvent extracted fraction from each rice variety was estimated spectrophotometrically by measuring their optical densities in EtOAc at 321 nm. Standard oryzanol, possessed wavelength of maxima at 315 nm, was used as a reference compound. The specific extinction coefficient, ε_{1cm}, of standard oryzanol dissolved in EtOAc was 21226 $cm^{-1}M^{-1}$. The total oryzanol contents and the percentage of estimated oryzanol in rice bran were calculated and presented in Table 4.2 and Figure 4.2, respectively.

The most commonly used techniques for the determination of oryzanol in rice bran oil are HPLC and UV spectrophotometry. Since the absorption spectra of the individual components of oryzanol are almost identical (Xu and Godber, 1999), UV spectrophotometry is preferred to determine its total content (De and Bhattacharyya, 2000; Gong and Yao, 2001; Qiu and Tang, 1998; Tsuchiya *et al.*, 1957).

As shown in Figure 4.2, crude MeOH and crude rice bran oil from various rice varieties were expected to contain approximately 0.21-0.27 and 0.18-0.23 % oryzanol in rice bran, respectively. In the previous studies, the content of oryzanol in rice bran can range from 0.18-0.60 % (Qureshi *et al.*, 2002; Rao *et al.*, 2002; Xu *et al.*, 2001) depending on the rice varieties and analytical methods. Thus, our extraction methods exhibited appreciate oryzanol content. However, there was a loss of oryzanol in MeOH extract, 0.030-0.046 % oryzanol in rice bran. These MeOH extracts possibly contain not only oryzanol, but also ferulic acid, since ferulic acid is more polar than oryzanol and less partitioned in hexane portion, These estimated oryzanol contents in MeOH extract might influenced by ferulic acid, as ferulic acid was also absorb UV at 321 nm, λ_{max} of ferulic acid = 325 nm (Xu and Godber, 1999; Robbins, 2003). From H^1-NMR information Figure 1 in Appendix A), MeOH extracts mostly contain carbohydrate, e.g. mono-, di- and polysaccharide, thus the MeOH extracts were not further purified in this research.

Table 4.2 The estimated content of oryzanol from various cultivars

Rice varieties	Oryzanol contents (mg)		
	Crude MeOH	Crude rice bran oil	MeOH extract
Pathumthani 1	430	343	91
Kao Dok Mali 105	503	449	79
Sunpatong 1	568	497	81
Go Ko Chai Nat 1	543	465	72

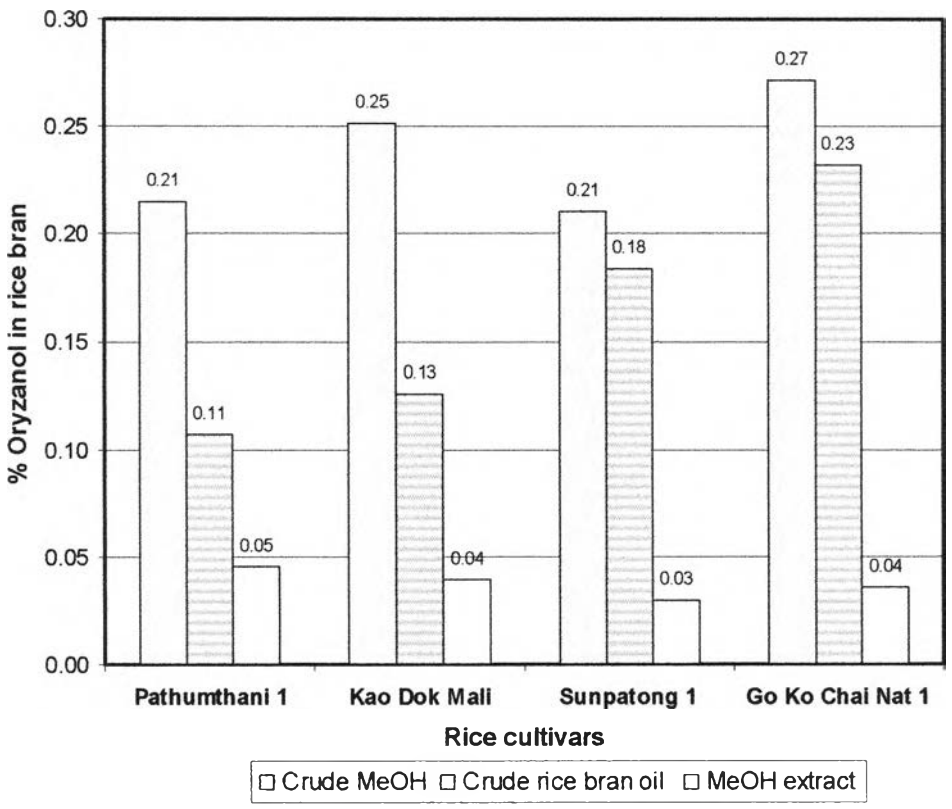


Figure 4.2 The percentage of estimated oryzanol in various rice cultivars

4.3 Identification of Individual Oryzanol

Identification of individual oryzanol extracted from each rice variety was undertaken by MS/MS technique. Chemical structures of these compounds were determined by analyzes of spectroscopic data, including IR, NMR and Mass spectra, as well as by comparison their spectral data with those of published values.

4.3.1 Identification of Individual Oryzanol from Pathumthani 1 Rice Bran

4.3.1.1 Identification of Individual Oryzanol in Oryzanol Mixture PF1

Oryzanol mixture PF1 was obtained from crystallization as the white powder (4.2 mg, 0.02% yield of crude rice bran oil). The structure of oryzanol mixture PF1 was elucidated by using spectroscopic techniques.

The IR spectrum of oryzanol mixture PF1 is shown in Appendix A Figure 2 and the absorption peaks were assigned as Table 4.3. Its IR spectrum indicated important absorption band at 3439 cm^{-1}(O-H stretching), 1704 cm^{-1} (aliphatic ester C=O stretching), 1640 cm^{-1} (C=C in aromatic ring) and 1558 cm^{-1} (C=C in unsaturated part).

The ^{1}H-NMR spectrums (Figure 3 in Appendix A) of oryzanol mixture PF1 possessed a hydroxyl proton of ferulate portion at 5.85 ppm, a methoxy proton of ferulate portion at 3.94 ppm and 3 aromatic protons of ferulate portion at 6.91, 7.04 and 7.08 ppm and protons of sterol portion at 0 - 2 ppm. The ^{1}H-NMR information of oryzanol mixture PF1 was listed in Table 4.4 comparison with 24-methylenecycloartanyl ferulate from the previous investigation. (Yasukawa *et al.*, 1998).

In negative-ion mode mass spectra, the deprotonated molecular ions of oryzanol mixture PF1 showed the peak at *m/z* 589, 615, 617 and 663 Figure 4 in Appendix A). These ions were further deprotonated and yielded $[M\text{-}H\text{-}CH_3]^-$ ion (Figure 5-8 in Appendix A). All MS/MS spectra of oryzanol mixture PF1 showed base peak of $[M\text{-}H\text{-}CH_3]^-$ ion resulting from the loss of a methyl group in ferulate moiety.

Ion mass of *m/z* 600 and 177 were observed from *m/z* 615 precursor as expected for 24-methylenecycloartanyl ferulate (Figure 4.3 and Figure 6 in Appendix A). The other ions of deprotonated molecular ions at *m/z* 598, 617 and 633 were identified as sitosteryl ferulate, 24-cycloart-25-ene-3β,24-diol-3β ferulate and 24-hydroxy-24-methylcycloartanyl ferulate, respectively. The mechanism proposed for formation of

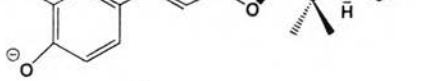
product ions was shown in Figure 4.4-4.6. Moreover, anion, $[M-H-2CH_3]^-$, was yielded only by sitosteryl ferulate from oryzanol mixture PF1. The structures of these four oryzanol components were previously identified in rice bran oil (Akihisa *et al.*, 2000; Fang *et al.*, 2003; Rogers *et al.*, 1993; Xu and Godber, 1999).

Table 4.3 The IR absorption band assignment of oryzanol mixture PF1

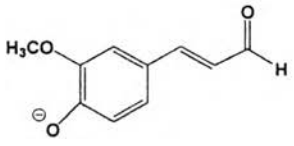
Wavenumber (cm^{-1})	Assignment
3439	O-H stretching
2920	aromatic C-H stretching
2853	hydrocarbon C-H stretching
1704	C=O stretching in ester
1640	C=C stretching in aromatic ring
1558	C=C stretching
1412	$-CH_2$ and $-CH_3$ bending
1015	C-O bending
805	C-H bending out of plane

Table 4.4 ^{1}H-NMR spectral data of 24-methylenecycloartanyl ferulate from oryzanol mixture PF1 and the literature in $CDCl_3$

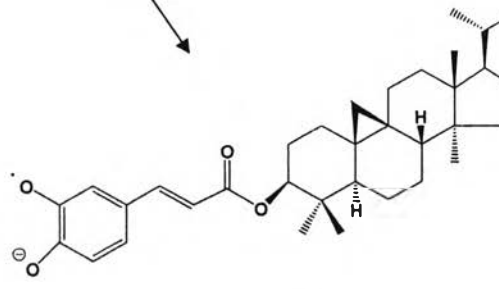
H position	^{1}H-NMR chemical shifts (δ/ppm, 400 MHz, $CDCl_3$)	
	Oryzanol mixture PF1	24-Methylenecycloartanyl ferulate
3	4.70 (1H, m)	4.71 (1H, m)
18	0.97 (3H, s)	0.98 (3H, s)
19	0.37 (d, *J* = 4.0 Hz, exo) 0.60 (d, *J* = 3.6 Hz, endo)	0.37 (d, *J* = 4.0 Hz, exo) 0.60 (d, *J* = 4.0 Hz, endo)
21	0.91 (3H, d, *J* = 5.5 Hz)	0.90 (3H, d, *J* = 5.5 Hz)
24	4.66 (br s) 4.72 (br s)	4.67 (br s) 4.72 (br s)
25	2.24 (1H, sept, *J* = 6.8 Hz)	2.24 (1H, sept, *J* = 7.0 Hz)
26	1.02 (3H, d, *J* = 6.8 Hz)	1.03 (3H, d, *J* = 6.6 Hz)
27	1.03 (3H, d, *J* = 6.8 Hz)	1.04 (3H, d, *J* = 7.0 Hz)
28	0.91 (3H, s)	0.90 (3H, s)
29	0.97 (3H, s)	0.98 (3H, s)
30	0.91 (3H, s)	0.92 (3H, s)
2'	6.29 (1H, d, *J* = 15.6 Hz)	6.30 (1H, d, *J* = 16.1 Hz)
3'	7.59 (1H, d, *J* = 16.0 Hz)	7.60 (1H, d, *J* = 15.8 Hz)
5'	7.04 (1H, d, *J* = 1.6 Hz)	7.04 (1H, d, *J* = 1.8 Hz)
8'	6.91 (1H, d, *J* = 8.4 Hz)	6.92 (1H, d, *J* = 8.4 Hz)
9'	7.08 (1H, dd, *J* = 1.6, 8.4 Hz)	7.08 (1H, dd, *J* = 1.8, 8.4 Hz)
6'-OCH_3	3.94 (3H, s)	3.94 (3H, s)
7'-OH	5.85 (1H, br s)	5.85 (1H, br s)



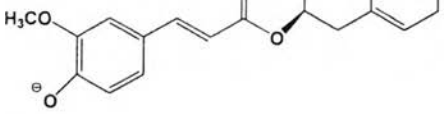
$[M-H]^-$ at *m/z* 615



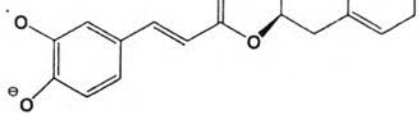
ion at *m/z* 177



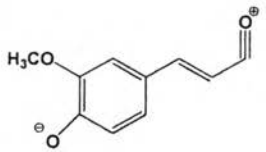
$[M-H-CH_3]^-$ at *m/z* 600

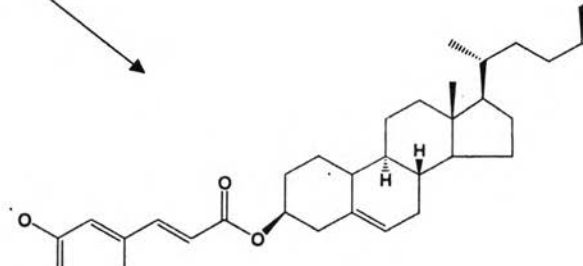


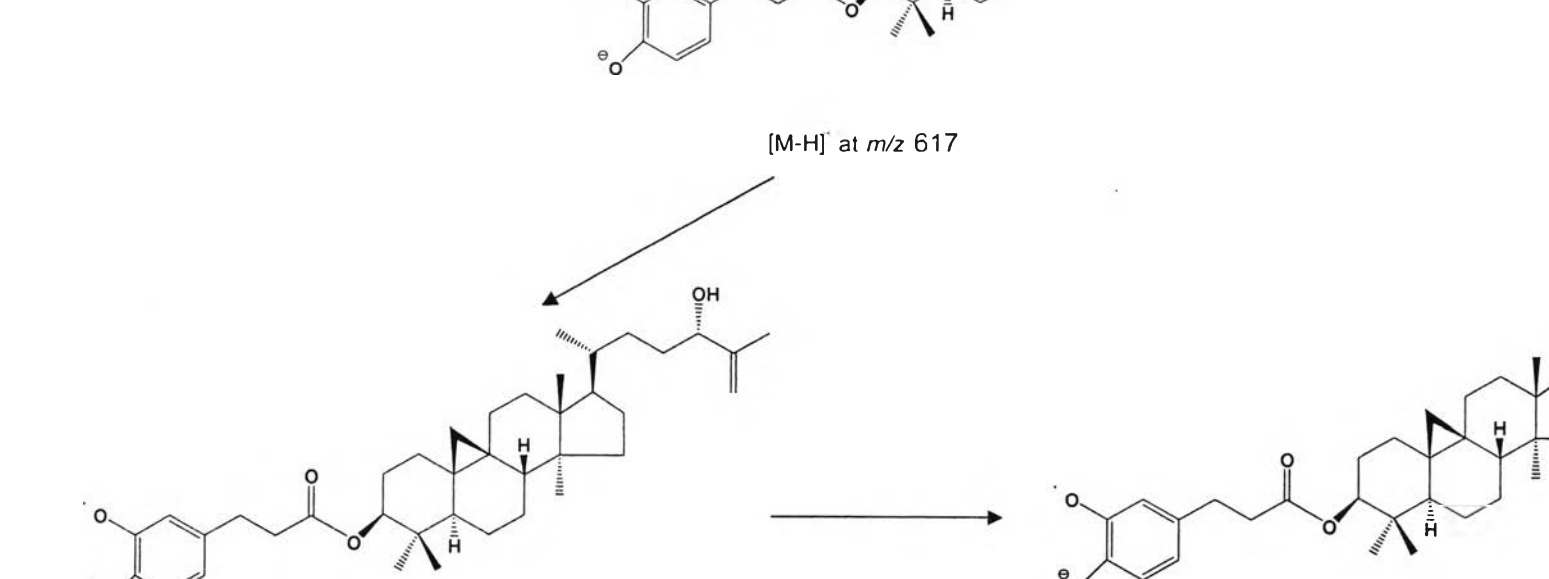
[M-H]$^{\cdot}$ at *m/z* 589



[M-H-CH_3]$^{\cdot}$ at *m/z* 574

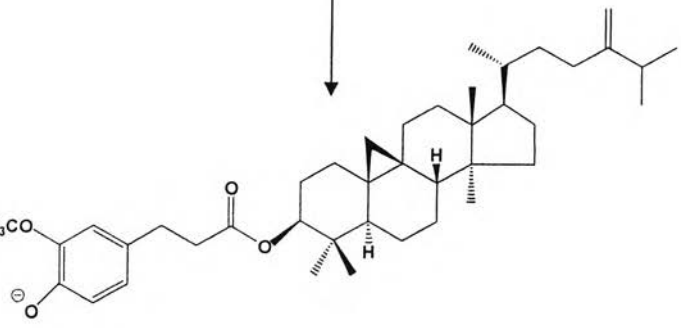


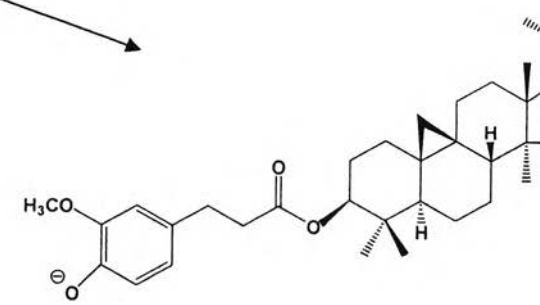



[M-H]⁻ at m/z 617

[M-H]$^{-}$ at *m/z* 633

[M-H-CH$_3$]$^{-}$ at *m/z* 618





4.3.1.2 Identification of Individual Oryzanol in Oryzanol mixture PF2

Oryzanol mixture PF2 was the slightly yellowish-white powder (58.6 mg, 0.29% yield of crude rice bran oil), melting point 126-127 °C. The structure of oryzanol mixture PF2 was elucidated by using spectroscopic techniques.

The IR spectrum of oryzanol mixture PF2 (the significant region is reported in Appendix A Figure 9, Table 4.5) shows several bands, 3433 cm^{-1}(O-H stretching), 1707 cm^{-1} (aliphatic ester C=O stretching), 1646 cm^{-1} (C=C in aromatic ring) and 1561 cm^{-1} (C=C in unsaturated part).

The ^{1}H-NMR spectrum (Figure 10 in Appendix C) of oryzanol mixture PF2 possessed a hydroxyl proton of ferulate portion at 5.85 ppm, a methoxy proton of ferulate portion at 3.93 ppm and 3 aromatic protons of ferulate portion at 6.91, 7.04 and 7.09 ppm and protons of sterol portion at 0 - 2 ppm. The ^{1}H-NMR information of oryzanol mixture PF2 was listed in Table 4.6-4.7 comparison with 24-methylenecycloartanyl ferulate and cycloartenyl ferulate from the previous investigation. (Yasukawa *et al.*, 1998).

Using negative-ion mode, MS and MS/MS spectra of oryzanol mixture PF2 were shown and listed in Appendix A Figure 11-19 and Table 4.8, respectively.

From all spectroscopic information, at least 8 components of oryzanol from oryzanol mixture PF2 were identified as 24-methylenecycloartanyl ferulate, cycloartenyl ferulate, campesteryl and/or Δ^{7}-campesteryl ferulate, campestanyl ferulate, campesteryl caffeate, sitosteryl and/or Δ^{7}-sitosteryl ferulate, stigmastanyl ferulate and 24-methylenecholesteryl ferulate (Akihisa *et al.*, 2000; Fang *et al.*, 2003; Iwatsuki *et al.*, 2003; Rogers *et al.*, 1993; Xu and Godber, 1999; Yasukawa *et al.*, 1998). The structures of these oryzanols are depicted in Figure 4.7.

In summary, at least 10 constituents of oryzanol were found in Pathumthani 1 rice bran, i.e., 24-methylenecycloartanyl ferulate, cycloartenyl ferulate, campesteryl and/or Δ^{7}-campesteryl ferulate, campestanyl ferulate, campesteryl caffeate, sitosteryl and/or Δ^{7}-sitosteryl ferulate, stigmastanyl ferulate, 24-methylenecholesteryl ferulate, 24-hydroxy-24-methylcycloartanol ferulate and 24-cycloart-25-ene-3β,24-diol-3β ferulate (Figure 4.7).

Table 4.5 The IR absorption band assignment of oryzanol mixture PF2

Wavenumber (cm^{-1})	Assignment
3433	O-H stretching
2960	aromatic C-H stretching
2859	hydrocarbon C-H stretching
1707	C=O stretching in ester
1646	C=C stretching in aromatic ring
1561	C=C stretching
1408	$-CH_2$ and $-CH_3$ bending
1018	C-O bending
805	C-H bending out of plane

Table 4.6 ^{1}H-NMR spectral data of 24-methylenecycloartanyl ferulate from oryzanol mixture PF2 and the literature in $CDCl_3$

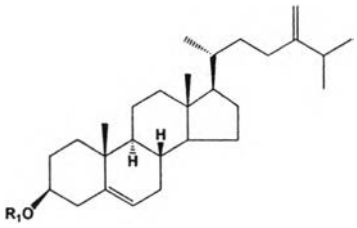
H position	^{1}H-NMR chemical shifts (δ/ppm, 400 MHz, $CDCl_3$)	
	Oryzanol mixture PF2	24-Methylenecycloartanyl ferulate
3	4.72 (1H, m)	4.71 (1H, m)
18	0.97 (3H, s)	0.98 (3H, s)
19	0.34 (d, *J* = 3.6 Hz, exo) 0.60 (d, *J* = 4.0 Hz, endo)	0.37 (d, *J* = 4.0 Hz, exo) 0.60 (d, *J* = 4.0 Hz, endo)
21	0.89 (3H, d, *J* = 5.7 Hz)	0.90 (3H, d, *J* = 5.5 Hz)
24	4.67 (br s) 4.72 (br s)	4.67 (br s) 4.72 (br s)
25	2.24 (1H, sept, *J* = 7.2 Hz)	2.24 (1H, sept, *J* = 7.0 Hz)
26	1.02 (3H, d, *J* = 6.8 Hz)	1.03 (3H, d, *J* = 6.6 Hz)
27	1.03 (3H, d, *J* = 7.0 Hz)	1.04 (3H, d, *J* = 7.0 Hz)
28	0.91 (3H, s)	0.90 (3H, s)
29	0.97 (3H, s)	0.98 (3H, s)
30	0.93 (3H, s)	0.92 (3H, s)
2'	6.30 (1H, d, *J* = 16.0 Hz)	6.30 (1H, d, *J* = 16.1 Hz)
3'	7.60 (1H, d, *J* = 16.0 Hz)	7.60 (1H, d, *J* = 15.8 Hz)
5'	7.04 (1H, d, *J* = 1.8 Hz)	7.04 (1H, d, *J* = 1.8 Hz)
8'	6.91 (1H, d, *J* = 8.0 Hz)	6.92 (1H, d, *J* = 8.4 Hz)
9'	7.09 (1H, dd, *J* = 1.8, 8.0 Hz)	7.08 (1H, dd, *J* = 1.8, 8.4 Hz)
6'-OCH_3	3.93 (3H, s)	3.94 (3H, s)
7'-OH	5.85 (1H, br s)	5.85 (1H, br s)

Table 4.7 ^{1}H-NMR spectral data of cycloartenyl ferulate from oryzanol mixture PF2 and the literature in $CDCl_3$

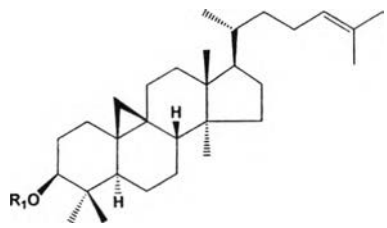
H position	^{1}H-NMR chemical shifts (δ/ppm, 400 MHz, $CDCl_3$)	
	Oryzanol mixture PF2	Cycloartenyl ferulate
3	4.72 (1H, m)	4.71 (1H, m)
18	0.97 (3H, s)	0.97 (3H, s)
19	0.34 (d, *J* = 3.6 Hz, exo) 0.60 (d, *J* = 4.0 Hz, endo)	0.37 (d, *J* = 4.4 Hz, exo) 0.60 (d, *J* = 4.0 Hz, endo)
21	0.89 (3H, d, *J* = 5.7 Hz)	0.89 (3H, d, *J* = 6.2 Hz)
24	5.10 (1H, br t, *J* = 7.0 Hz)	5.11 (1H, br t, *J* = 7.0 Hz)
26	1.68 (3H, s)	1.69 (3H, s)
27	1.61 (3H, s)	1.61 (3H, s)
28	0.91 (3H, s)	0.90 (3H, s)
29	0.97 (3H, s)	0.98 (3H, s)
30	0.93 (3H, s)	0.91 (3H, s)
2'	6.30 (1H, d, *J* = 16.0 Hz)	6.30 (1H, d, *J* = 16.1 Hz)
3'	7.60 (1H, d, *J* = 16.0 Hz)	7.60 (1H, d, *J* = 15.8 Hz)
5'	7.04 (1H, d, *J* = 1.8 Hz)	7.04 (1H, d, *J* = 1.8 Hz)
8'	6.91 (1H, d, *J* = 8.0 Hz)	6.92 (1H, d, *J* = 8.1 Hz)
9'	7.09 (1H, dd, *J* = 1.8, 8.0 Hz)	7.08 (1H, dd, *J* = 1.8, 8.1 Hz)
6'-OCH_3	3.93 (3H, s)	3.94 (3H, s)
7'-OH	5.85 (1H, br s)	5.85 (1H, br s)

Table 4.8 MS/MS data for oryzanols in oryzanol mixture PF2

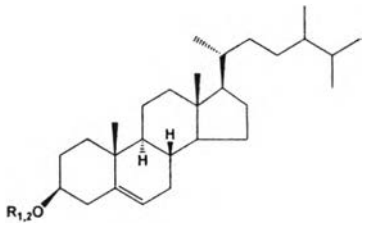
Oryzanols	M.W.	Negative MS/MS spectra (*m/z*)				
		$[M-H]^-$	$[M-H-CH_3]^-$	$[M-H-2CH_3]^-$	$[feruloyl]^-$	ion from freuloyl part
Campesteryl caffeate	562	561	546			
24-Methylenecholestery ferulate	574	573	558		193	
Campesteryl and/or Δ^7-campesteryl ferulate	576	575	560		193	177
Campestanyl ferulate	578	577	562		193	178
Sitosteryl and/or Δ^7-sitosteryl ferulate	590	589	574			
Stigmastanyl ferulate	592	591	576			178
Cycloartenyl ferulate	602	601	586		192	177
24-Methylenecyclortanyl ferulate	616	615	600	585		



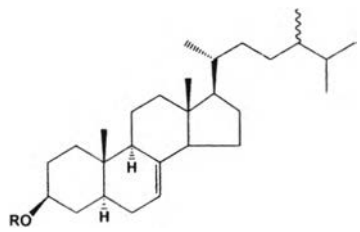
24-Methylenecholesteryl ferulate



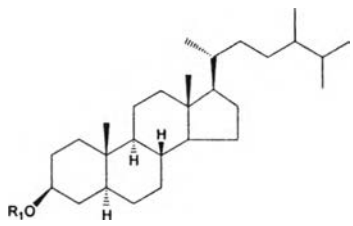
Cycloartenyl ferulate



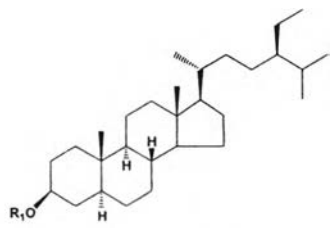
Campesteryl caffeate and ferulate



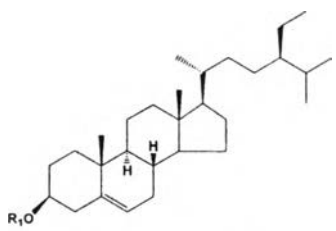
Δ^7-Campesteryl ferulate



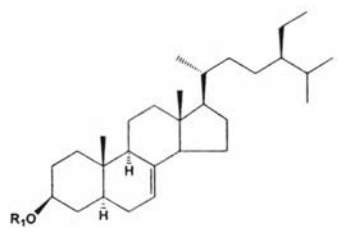
Campestanyl ferulate



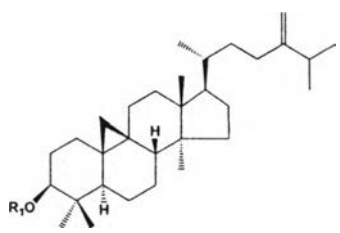
Stigmastanyl ferulate



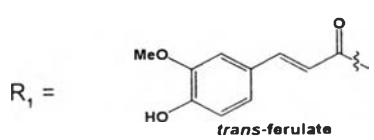
Sitosteryl ferulate



Δ^7-Sitosteryl ferulate



24-Methylenecycloartanyl ferulate



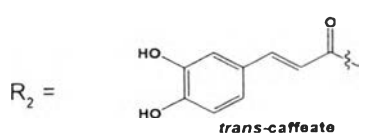


Figure 4.7 Oryzanol structures from Pathumthani 1 rice bran

4.3.2 Identification of Individual Oryzanol from Kao Dok Mali 105 Rice Bran

4.3.2.1 Identification of Individual Oryzanol in Oryzanol Mixture WF1

Oryzanol mixture WF1 was obtained from preparative TLC as the white powder (29.0 mg, 0.09% yield of crude rice bran oil), melting point 138-139 °C. The structure of oryzanol mixture WF1 was elucidated by using spectroscopic techniques.

The IR spectrum of oryzanol mixture WF1 is shown in Appendix A Figure 20 and the absorption peaks were assigned as Table 4.9. Its IR spectrum indicated important absorption band at 3439 cm^{-1}(O-H stretching), 1707 cm^{-1} (aliphatic ester C=O stretching), 1661 cm^{-1} (C=C in aromatic ring) and 1561 cm^{-1} (C=C in unsaturated part).

The ^{1}H-NMR spectrum (Figure 21 in Appendix A) of oryzanol mixture PF1 possessed a hydroxyl proton of ferulate portion at 5.85 ppm, a methoxy proton of ferulate portion at 3.93 ppm and 3 aromatic protons of ferulate portion at 6.92, 7.04 and 7.07 ppm and protons of sterol portion at 0 - 2 ppm. The ^{1}H-NMR information of oryzanol mixture WF1 was listed in Table 4.10-4.11 comparison with 24-methylenecycloartanyl ferulate and cycloartenyl ferulate from the previous investigation. (Yasukawa *et al.*, 1998).

Molecular ion and major fragmented ions in the mass spectrum oryzanol each individual precursor ion, $[M-H]^{-}$, were listed in Table 4.12 (Appendix A Figure 22-29). Compare with the literatures, at least 7 components of oryzanol mixture WF1 were identified as 24-methylenecycloartanyl ferulate, cycloartenyl ferulate, cycloartenyl caffeate, campestanyl ferulate, sitosteryl and/or Δ^{7}-sitosteryl ferulate, campesteryl and/or Δ^{7}-campesteryl ferulate and 24-methylenecholesteryl ferulate (Akihisa *et al.*, 2000; Fang *et al.*, 2003; Iwatsuki *et al.*, 2003; Rogers *et al.*, 1993; Xu and Godber, 1999; Yasukawa *et al.*, 1998). The structures of these oryzanols are depicted in Figure 4.8.

Table 4.9 The IR absorption band assignment of oryzanol mixture WF1

Wavenumber (cm^{-1})	Assignment
3439	O-H stretching
2923	aromatic C-H stretching
2850	hydrocarbon C-H stretching
1707	C=O stretching in ester
1661	C=C stretching in aromatic ring
1561	C=C stretching
1421	$-CH_2$ and $-CH_3$ bending
1024	C-O bending
653	C-H bending out of plane

Table 4.10 ^{1}H-NMR spectral data of 24-methylenecycloartanyl ferulate from oryzanol mixture WF1 and the literature in $CDCl_3$

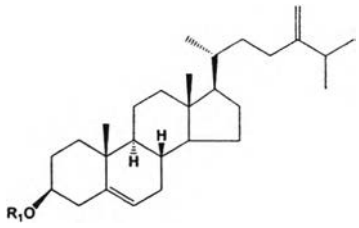
H position	^{1}H-NMR chemical shifts (δ/ppm, 400 MHz, $CDCl_3$)	
	Oryzanol mixture WF1	24-Methylenecycloartanyl ferulate
3	4.69 (1H, m)	4.71 (1H, m)
18	0.97 (3H, s)	0.98 (3H, s)
19	0.36 (d, *J* = 4.4 Hz, exo) 0.59 (d, *J* = 4.0 Hz, endo)	0.37 (d, *J* = 4.0 Hz, exo) 0.60 (d, *J* = 4.0 Hz, endo)
21	0.88 (3H, d, *J* = 6.0 Hz)	0.90 (3H, d, *J* = 5.5 Hz)
24	4.66 (br s) 4.72 (br s)	4.67 (br s) 4.72 (br s)
25	2.24 (1H, sept, *J* = 6.8 Hz)	2.24 (1H, sept, *J* = 7.0 Hz)
26	1.02 (3H, d, *J* = 6.4 Hz)	1.03 (3H, d, *J* = 6.6 Hz)
27	1.04 (3H, d, *J* = 7.0 Hz)	1.04 (3H, d, *J* = 7.0 Hz)
28	0.91 (3H, s)	0.90 (3H, s)
29	0.97 (3H, s)	0.98 (3H, s)
30	0.93 (3H, s)	0.92 (3H, s)
2'	6.30 (1H, d, *J* = 16.0 Hz)	6.30 (1H, d, *J* = 16.1 Hz)
3'	7.60 (1H, d, *J* = 16.0 Hz)	7.60 (1H, d, *J* = 15.8 Hz)
5'	7.04 (1H, d, *J* = 1.8 Hz)	7.04 (1H, d, *J* = 1.8 Hz)
8'	6.92 (1H, d, *J* = 8.4 Hz)	6.92 (1H, d, *J* = 8.4 Hz)
9'	7.07 (1H, dd, *J* = 1.6, 8.0 Hz)	7.08 (1H, dd, *J* = 1.8, 8.4 Hz)
6'-OCH_3	3.93 (3H, s)	3.94 (3H, s)
7'-OH	5.85 (1H, br s)	5.85 (1H, br s)

Table 4.11 ^{1}H-NMR spectral data of cycloartenyl ferulate from oryzanol mixture WF1 and the literature in $CDCl_3$

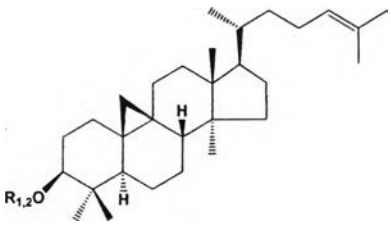
H position	^{1}H-NMR chemical shifts (δ/ppm, 400 MHz, $CDCl_3$)	
	Oryzanol mixture WF1	Cycloartenyl ferulate
3	4.69 (1H, m)	4.71 (1H, m)
18	0.97 (3H, s)	0.97 (3H, s)
19	0.36 (d, *J* = 4.4 Hz, exo) 0.59 (d, *J* = 4.0 Hz, endo)	0.37 (d, *J* = 4.4 Hz, exo) 0.60 (d, *J* = 4.0 Hz, endo)
21	0.88 (3H, d, *J* = 6.0 Hz)	0.89 (3H, d, *J* = 6.2 Hz)
24	5.10 (1H, br t, *J* = 7.0 Hz)	5.11 (1H, br t, *J* = 7.0 Hz)
26	1.68 (3H, s)	1.69 (3H, s)
27	1.63 (3H, s)	1.61 (3H, s)
28	0.91 (3H, s)	0.90 (3H, s)
29	0.97 (3H, s)	0.98 (3H, s)
30	0.93 (3H, s)	0.91 (3H, s)
2'	6.30 (1H, d, *J* = 16.0 Hz)	6.30 (1H, d, *J* = 16.1 Hz)
3'	7.60 (1H, d, *J* = 16.0 Hz)	7.60 (1H, d, *J* = 15.8 Hz)
5'	7.04 (1H, d, *J* = 1.8 Hz)	7.04 (1H, d, *J* = 1.8 Hz)
8'	6.92 (1H, d, *J* = 8.4 Hz)	6.92 (1H, d, *J* = 8.1 Hz)
9'	7.07 (1H, dd, *J* = 1.6, 8.0 Hz)	7.08 (1H, dd, *J* = 1.8, 8.1 Hz)
6'-OCH_3	3.93 (3H, s)	3.94 (3H, s)
7'-OH	5.85 (1H, br s)	5.85 (1H, br s)

Table 4.12 MS/MS data for oryzanols in oryzanol mixture WF1

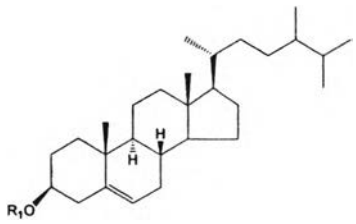
Oryzanols	M.W.	Negative MS/MS spectra (*m/z*)	
		$[M-H]^-$	$[M-H-CH_3]^-$
24-Methylenecholesteryl ferulate	574	573	558
Campesteryl and/or Δ^7-campesteryl ferulate	576	575	560
Campestanyl ferulate	578	577	562
Cycloartenyl caffeate	588	587	572
Sitosteryl and/or Δ^7-sitosteryl ferulate	590	589	574
Cycloartenyl ferulate	602	601	586
24-Methylenecycloartanyl ferulate	616	615	600



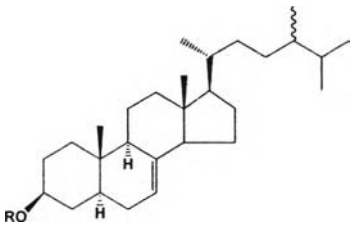
24-Methylenecholesteryl ferulate



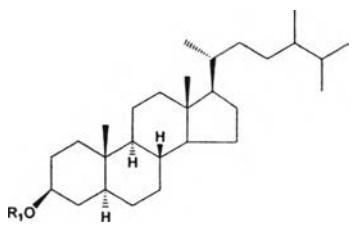
Cycloartenyl caffeate and ferulate



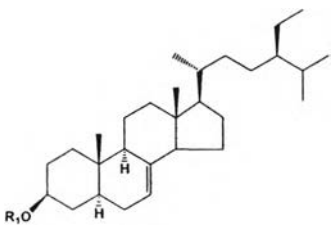
Campesteryl ferulate



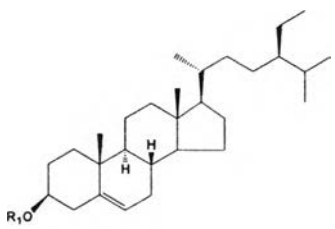
Δ^7-Campesteryl ferulate



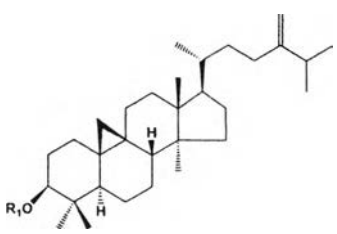
Campestanyl ferulate



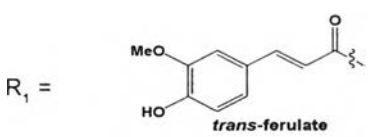
Δ^7-Sitosteryl ferulate



Sitosteryl ferulate



24-Methylenecycloartanyl ferulate



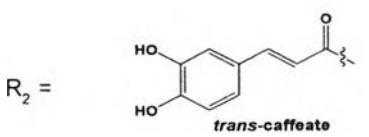


Figure 4.8 Oryzanol structures from Kao Dok Mali 105 rice bran

4.3.3 Identification of Individual Oryzanol from Sunpatong 1 Rice Bran

4.3.3.1 Identification of Individual Oryzanol in Oryzanol Mixture SF1

Oryzanol mixture SF1 was obtained from preparative TLC as the yellow solid (17.2 mg, 0.05% yield of crude rice bran oil), melting point 101-102 °C. The structure of oryzanol mixture SF1 was elucidated by using spectroscopic techniques.

The IR spectrum of oryzanol mixture SF1 is shown in Appendix A Figure 30 and the absorption peaks were assigned as Table 4.13. Its IR spectrum indicated important absorption band at 3429 cm^{-1}(O-H stretching), 1704 cm^{-1} (aliphatic ester C=O stretching), 1643 cm^{-1} (C=C in aromatic ring) and 1555 cm^{-1} (C=C in unsaturated part).

The ^{1}H-NMR spectrum (Figure 31 in Appendix A) of oryzanol mixture SF1 possessed a hydroxyl proton of ferulate portion at 5.94 ppm, a methoxy proton of ferulate portion at 3.93 ppm and 3 aromatic protons of ferulate portion at 6.91, 7.04 and 7.08 ppm and protons of sterol portion at 0 - 2 ppm. The ^{1}H-NMR information of oryzanol mixture SF1 was listed in Table 4.14-4.15 comparison with 24-methylenecycloartanyl ferulate and cycloartenyl ferulate from the previous investigation. (Yasukawa *et al.*, 1998).

Characterization of oryzanol from oryzanol mixture SF1 was undertaken by specteral MS/MS comparison with the literature data for the known oryzanol constituents (Appendix A Figure 32-37 and Table 4.16).

From all spectroscopic information, at least 5 components of oryzanol from oryzanol mixture SF1 were identified as 24-methylenecycloartanyl ferulate, cycloartenyl ferulate, campesteryl and/or Δ^{7}-campesteryl ferulate, sitosteryl and/or Δ^{7}-sitosteryl ferulate and stigmastanyl ferulate (Akihisa *et al.*, 2000; Fang *et al.*, 2003; Iwatsuki *et al.*, 2003; Rogers *et al.*, 1993; Xu and Godber, 1999; Yasukawa *et al.*, 1998). The structures of these oryzanols are depicted in Figure 4.9.

Table 4.13 The IR absorption band assignment of oryzanol mixture SF1

Wavenumber (cm^{-1})	Assignment
3429	O-H stretching
2936	aromatic C-H stretching
2850	hydrocarbon C-H stretching
1704	C=O stretching in ester
1643	C=C stretching in aromatic ring
1555	C=C stretching
1415	$-CH_2$ and $-CH_3$ bending
1021	C-O bending
811	C-H bending out of plane

Table 4.14 ^{1}H-NMR spectral data of 24-methylenecycloartanyl ferulate from oryzanol mixture SF1 and the literature in $CDCl_3$

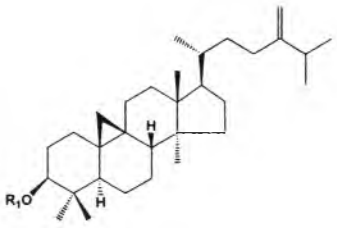
H position	^{1}H-NMR chemical shifts (δ/ppm, 400 MHz, $CDCl_3$)	
	Oryzanol mixture SF1	24-Methylenecycloartanyl ferulate
3	4.71 (1H, m)	4.71 (1H, m)
18	0.97 (3H, s)	0.98 (3H, s)
19	0.38 (d, *J* = 4.0 Hz, exo) 0.61 (d, *J* = 4.0 Hz, endo)	0.37 (d, *J* = 4.0 Hz, exo) 0.60 (d, *J* = 4.0 Hz, endo)
21	0.89 (3H, d, *J* = 6.2 Hz)	0.90 (3H, d, *J* = 5.5 Hz)
24	4.66 (br s) 4.71 (br s)	4.67 (br s) 4.72 (br s)
25	2.23 (1H, sept, *J* = 6.8 Hz)	2.24 (1H, sept, *J* = 7.0 Hz)
26	1.03 (3H, d, *J* = 6.4 Hz)	1.03 (3H, d, *J* = 6.6 Hz)
27	1.04 (3H, d, *J* = 7.0 Hz)	1.04 (3H, d, *J* = 7.0 Hz)
28	0.89 (3H, s)	0.90 (3H, s)
29	0.97 (3H, s)	0.98 (3H, s)
30	0.92 (3H, s)	0.92 (3H, s)
2'	6.31 (1H, d, *J* = 16.0 Hz)	6.30 (1H, d, *J* = 16.1 Hz)
3'	7.60 (1H, d, *J* = 16.0 Hz)	7.60 (1H, d, *J* = 15.8 Hz)
5'	7.04 (1H, d, *J* = 1.6 Hz)	7.04 (1H, d, *J* = 1.8 Hz)
8'	6.91 (1H, d, *J* = 8.0 Hz)	6.92 (1H, d, *J* = 8.4 Hz)
9'	7.08 (1H, dd, *J* = 1.6, 8.4 Hz)	7.08 (1H, dd, *J* = 1.8, 8.4 Hz)
6'-OCH_3	3.93 (3H, s)	3.94 (3H, s)
7'-OH	5.94 (1H, br s)	5.85 (1H, br s)

Table 4.15 ^{1}H-NMR spectral data of cycloartenyl ferulate from oryzanol mixture SF1 and the literature in $CDCl_3$

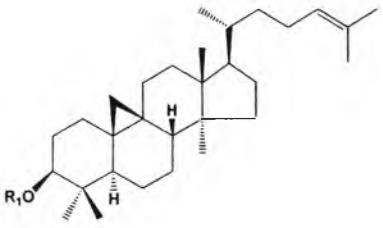
H position	^{1}H-NMR chemical shifts (δ/ppm, 400 MHz, $CDCl_3$)	
	Oryzanol mixture SF1	Cycloartenyl ferulate
3	4.71 (1H, m)	4.71 (1H, m)
18	0.97 (3H, s)	0.97 (3H, s)
19	0.38 (d, *J* = 4.0 Hz, exo) 0.61 (d, *J* = 4.0 Hz, endo)	0.37 (d, *J* = 4.4 Hz, exo) 0.60 (d, *J* = 4.0 Hz, endo)
21	0.89 (3H, d, *J* = 6.2 Hz)	0.89 (3H, d, *J* = 6.2 Hz)
24	5.11 (1H, br t, *J* = 7.0 Hz)	5.11 (1H, br t, *J* = 7.0 Hz)
26	1.69 (3H, s)	1.69 (3H, s)
27	1.61 (3H, s)	1.61 (3H, s)
28	0.89 (3H, s)	0.90 (3H, s)
29	0.97 (3H, s)	0.98 (3H, s)
30	0.92 (3H, s)	0.91 (3H, s)
2'	6.31 (1H, d, *J* = 16.0 Hz)	6.30 (1H, d, *J* = 16.1 Hz)
3'	7.60 (1H, d, *J* = 16.0 Hz)	7.60 (1H, d, *J* = 15.8 Hz)
5'	7.04 (1H, d, *J* = 1.6 Hz)	7.04 (1H, d, *J* = 1.8 Hz)
8'	6.91 (1H, d, *J* = 8.0 Hz)	6.92 (1H, d, *J* = 8.1 Hz)
9'	7.08 (1H, dd, *J* = 1.6, 8.4 Hz)	7.08 (1H, dd, *J* = 1.8, 8.1 Hz)
6'-OCH_3	3.93 (3H, s)	3.94 (3H, s)
7'-OH	5.94 (1H, br s)	5.85 (1H, br s)

Table 4.16 MS/MS data for oryzanols in oryzanol mixture SF1

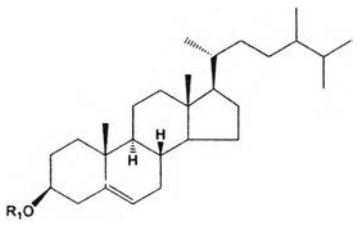
Oryzanols	M.W.	Negative MS/MS spectra (*m/z*)		
		$[M-H]^-$	$[M-H-CH_3]^-$	$[M-H-2CH_3]^-$
Campesteryl and/or Δ^7-campesteryl ferulate	576	575	560	
Sitosteryl and/or Δ^7-sitosteryl ferulate	590	589	574	
Stigmastanyl ferulate	592	591	576	561
Cycloartenyl ferulate	602	601	586	
24-Methylenecycloartanyl ferulate	616	615	600	



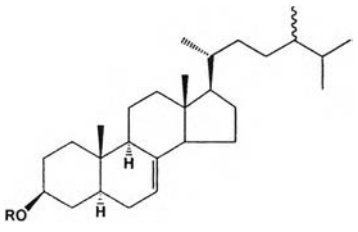
24-Methylenecycloartanyl ferulate



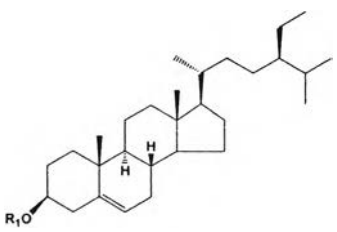
Cycloartenyl ferulate



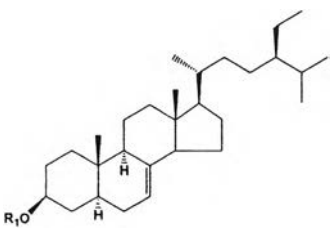
Campesteryl ferulate



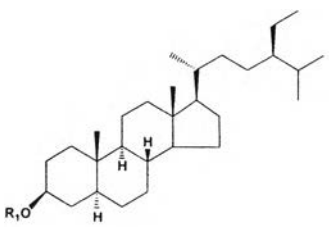
Δ^7-Campesteryl ferulate



Sitosteryl ferulate



Δ^7-Sitosteryl ferulate



Stigmastanyl ferulate

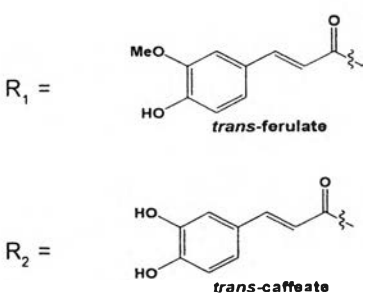


Figure 4.9 Oryzanol structures from Sunpatong 1 rice bran

4.3.4 Identification of Individual Oryzanol from Go Ko Chai Nat 1 Rice Bran

4.3.4.1 Identification of Individual Oryzanol in Oryzanol Mixture CF1

Oryzanol mixture CF1 was obtained from crystallization as the white crystal (45.8 mg, 0.17% yield of crude rice bran oil), melting point 174.5-175.0 ^{o}C. The structure of oryzanol mixture CF1 was elucidated by using spectroscopic techniques.

The IR spectrum of oryzanol mixture CF1 is shown in Appendix A Figure 38 and the absorption peaks were assigned as Table 4.17. Its IR spectrum indicated important absorption band at 3442 cm^{-1}(O-H stretching), 1668 cm^{-1} (C=C in aromatic ring) and 1558 cm^{-1} (C=C in unsaturated part).

The ^{1}H-NMR spectrum (Figure 39 in Appendix A) of oryzanol mixture CF1 possessed a hydroxyl proton of ferulate portion at 5.86 ppm, a methoxy proton of ferulate portion at 3.94 ppm and 3 aromatic protons of ferulate portion at 6.91, 7.04 and 7.08 ppm and protons of sterol portion at 0 - 2 ppm. The ^{1}H-NMR information of oryzanol mixture CF1 was listed in Table 4.18-4.19 comparison with 24-methylenecycloartanyl ferulate and cycloartenyl ferulate from the previous investigation. (Yasukawa *et al.*, 1998).

Identification of 2 oryzanol components, 24-methylenecycloartanyl ferulate and cycloartenyl ferulate, was performed by MS/MS comparison with the previously reports (Appendix A Figure 40-42 and Table 4.20; Akihisa *et al.*, 2000; Fang *et al.*, 2003; Iwatsuki *et al.*, 2003; Rogers *et al.*, 1993; Xu and Godber, 1999; Yasukawa *et al.*, 1998). The structures of these oryzanols are depicted in Figure 4.10.

Table 4.17 The IR absorption band assignment of oryzanol mixture CF1

Wavenumber (cm^{-1})	Assignment
3442	O-H stretching
2954	aromatic C-H stretching
2863	hydrocarbon C-H stretching
1668	C=C stretching in aromatic ring
1558	C=C stretching
1418	$-CH_2$ and $-CH_3$ bending
1024	C-O bending
808	C-H bending out of plane

Table 4.18 ^{1}H-NMR spectral data of 24-methylenecycloartanyl ferulate from oryzanol mixture CF1 and the literature in $CDCl_3$

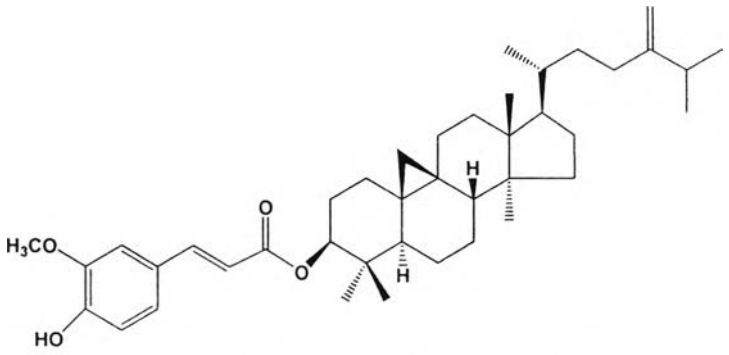
H position	^{1}H-NMR chemical shifts (δ/ppm, 400 MHz, $CDCl_3$)	
	Oryzanol mixture CF1	24-Methylenecycloartanyl ferulate
3	4.70 (1H, m)	4.71 (1H, m)
18	0.97 (3H, s)	0.98 (3H, s)
19	0.37 (d, *J* = 4.0 Hz, exo) 0.60 (d, *J* = 4.0 Hz, endo)	0.37 (d, *J* = 4.0 Hz, exo) 0.60 (d, *J* = 4.0 Hz, endo)
21	0.90 (3H, d, *J* = 5.2 Hz)	0.90 (3H, d, *J* = 5.5 Hz)
24	4.67 (br s) 4.71 (br s)	4.67 (br s) 4.72 (br s)
25	2.24 (1H, sept, *J* = 7.6 Hz)	2.24 (1H, sept, *J* = 7.0 Hz)
26	1.02 (3H, d, *J* = 6.8 Hz)	1.03 (3H, d, *J* = 6.6 Hz)
27	1.03 (3H, d, *J* = 7.2 Hz)	1.04 (3H, d, *J* = 7.0 Hz)
28	0.91 (3H, s)	0.90 (3H, s)
29	0.97 (3H, s)	0.98 (3H, s)
30	0.91 (3H, s)	0.92 (3H, s)
2'	6.30 (1H, d, *J* = 16.0 Hz)	6.30 (1H, d, *J* = 16.1 Hz)
3'	7.59 (1H, d, *J* = 16.0 Hz)	7.60 (1H, d, *J* = 15.8 Hz)
5'	7.04 (1H, d, *J* = 1.6 Hz)	7.04 (1H, d, *J* = 1.8 Hz)
8'	6.91 (1H, d, *J* = 8.0 Hz)	6.92 (1H, d, *J* = 8.4 Hz)
9'	7.08 (1H, dd, *J* = 1.6, 8.0 Hz)	7.08 (1H, dd, *J* = 1.8, 8.4 Hz)
6'-OCH_3	3.94 (3H, s)	3.94 (3H, s)
7'-OH	5.86 (1H, br s)	5.85 (1H, br s)

Table 4.19 ^{1}H-NMR spectral data of cycloartenyl ferulate from oryzanol mixture CF1 and the literature in $CDCl_3$

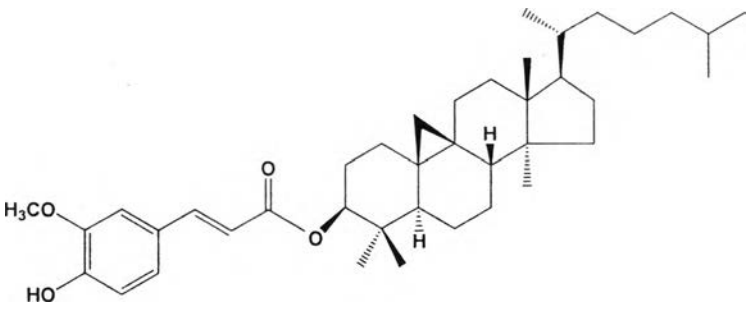
H position	^{1}H-NMR chemical shifts (δ/ppm, 400 MHz, $CDCl_3$)	
	Oryzanol mixture CF1	Cycloartenyl ferulate
3	4.70 (1H, m)	4.71 (1H, m)
18	0.97 (3H, s)	0.97 (3H, s)
19	0.37 (d, *J* = 4.0 Hz, exo) 0.60 (d, *J* = 4.0 Hz, endo)	0.37 (d, *J* = 4.4 Hz, exo) 0.60 (d, *J* = 4.0 Hz, endo)
21	0.90 (3H, d, *J* = 5.2 Hz)	0.89 (3H, d, *J* = 6.2 Hz)
24	5.10 (1H, br t, *J* = 7.0 Hz)	5.11 (1H, br t, *J* = 7.0 Hz)
26	1.68 (3H, s)	1.69 (3H, s)
27	1.63 (3H, s)	1.61 (3H, s)
28	0.91 (3H, s)	0.90 (3H, s)
29	0.97 (3H, s)	0.98 (3H, s)
30	0.91 (3H, s)	0.91 (3H, s)
2'	6.30 (1H, d, *J* = 16.0 Hz)	6.30 (1H, d, *J* = 16.1 Hz)
3'	7.59 (1H, d, *J* = 16.0 Hz)	7.60 (1H, d, *J* = 15.8 Hz)
5'	7.04 (1H, d, *J* = 1.6 Hz)	7.04 (1H, d, *J* = 1.8 Hz)
8'	6.91 (1H, d, *J* = 8.0 Hz)	6.92 (1H, d, *J* = 8.1 Hz)
9'	7.08 (1H, dd, *J* = 1.6, 8.0 Hz)	7.08 (1H, dd, *J* = 1.8, 8.1 Hz)
6'-OCH_3	3.94 (3H, s)	3.94 (3H, s)
7'-OH	5.86 (1H, br s)	5.85 (1H, br s)

Table 4.20 MS/MS data for oryzanols in oryzanol mixture CF1

Oryzanols	M.W.	Negative MS/MS spectra (*m/z*)	
		$[M-H]^-$	$[M-H-CH_3]^-$
Cycloartenyl ferulate	602	601	586
24-Methylenecycloartanyl ferulate	616	615	600



24-Methylenecycloartanyl Ferulate



Cycloartenyl ferulate

Figure 4.10 Oryzanol structures from Go Ko Chai Nat 1 rice bran

Oryzanol constituents of various rice bran, Pathumthani 1, Kao Dok Mali, Sunpatong 1 and Go Ko Chai Nat 1 were investigated in this study. Results from ^{1}H-NMR spectra providing additional evidence for oryzanol compositions represented expected ferulate resonance. 24-Methylenecycloartanyl ferulate and cycloartenyl ferulate, possessed the sharp signals in ^{1}H-NMR spectra, were found in all varieties. This finding confirmed that these two steryl ferulates are the major components of oryzanol in rice bran (Evershed *et al.*, 1988; Norton, 1995; Roger *et al.*, 1993; Xu and Godber, 1999).

To confirm the present of constituents of oryzanol in rice bran as indicated by IR and ^{1}H-NMR, the oryzanol structures were mostly constructed on the basis of the information from MS/MS, at least 11 components of oryzanol identified in this study. The MS/MS technique is very useful for identification of oryzanol components from the mixture, since it required less time consuming to characterize the sample with high sensitivity. On the other hand, identification of novel or all oryzanol components in sample was limited. MS/MS alone may not be used for quantitative method; MS/MS coupled with chromatography, e.g., HPLC might be the better method to investigate oryzanol mixture. Fang and colleagues (2003) successfully characterized 24 oryzanol components in rice bran using LC-MS/MS. Also, some oryzanol components possessed the same molecular weight can not be identified in this study. For example, molecular ion at *m/z* 589 was possibly yielded from sitosteryl and/or Δ^7-sitosteryl ferulate. Moreover, some sterols, i.e., cycloartenol, citrostadienol, cycloeucalenol, also possessed the same molecular weight (Akihisa *et al.*, 2000). As cycloartenyl ferulate was one of the major components oryzanol in rice bran oil and cycloartenyl caffeate was previously reported (Fang *et al.*, 2003; Iwatsuki *et al.*, 2003; Rogers *et al.*, 1993; Xu and Godber,1999), only cycloartenol was proposed in this study. These data demonstrate the need for appropriate analytical methods to differentiate the individual constituent of oryzanol.

The conventional methods for analysis of oryzanol content in rice bran are carried out by two procedures: separation by multiple chromatographic steps and identification of individual oryzanol component by positive chemical ionization- mass spectrometry (CI-MS) and NMR or analysis of TMS derivatives by gas chromatography-

electron impact mass spectrometry (GC-EI-MS) after saponification of oryzanol (Akihisa *et al.*, 2000; Diack and Saska, 1994; Rogers *et al.*, 1993; Xu and Godber, 1999; Yasukawa *et al.*, 1998); and direct analysis of oryzanol constituents by reverse-phase LC-MS/MS (Fang *et al.*, 2003) and normal phase LC-GC/MS (Miller *et al.*, 2003). The advantages of reverse- over normal-phase systems for separating complex mixtures are undisputable, quick column equilibration and better reproducibility are usually obtained; however, it has been proven that normal-phase chromatography on silica presents better selectivity for fat-soluble vitamins and oryzanol (Diack and Saska, 1994). Analytical precision and sensitivity of the various techniques for sterol assays seems to follow the order gas chromatography> high-performance liquid chromatography> supercritical fluid chromatography> capillary electrochromatography. The sensitivity order may vary depending on sterol structures, e.g. fluorescent labels, and detectors employed (Abidi, 2004).

4.3.5 Identification of standard oryzanol

The IR spectrum of standard oryzanol is also shown in Appendix A Figure 43 and the absorption peaks were assigned as Table 4.21. Its IR spectrum indicated important absorption band at 3433 cm^{-1}(O-H stretching), 1683 cm^{-1} (aliphatic ester C=O stretching), 1637 cm^{-1} (C=C in aromatic ring) and 1567 cm^{-1} (C=C in unsaturated part).

The ^{1}H-NMR spectrum (Figure 44 in Appendix A) of standard oryzanol possessed a hydroxyl proton of ferulate portion at 5.93 ppm, a methoxy proton of ferulate portion at 3.93 ppm and 3 aromatic protons of ferulate portion at 6.91, 7.04 and 7.07 ppm and protons of sterol portion at 0 - 2 ppm. The ^{1}H-NMR information of standard oryzanol was listed in Table 4.22-4.25 comparison with 24-methylenecycloartanyl ferulate, cycloartenyl ferulate, sitosteryl ferulate and campesteryl ferulate from the previous investigation. (Yasukawa *et al.*, 1998). It was assumed that this standard oryzanol contains 24-methylenecycloartanyl ferulate, cycloartenyl ferulate, sitosteryl ferulate and campesteryl ferulate.

Table 4.21 The IR absorption band assignment of standard oryzanol

Wavenumber (cm^{-1})	Assignment
3433	O-H stretching
2951	aromatic C-H stretching
2863	hydrocarbon C-H stretching
1683	C=O stretching in ester
1637	C=C stretching in aromatic ring
1567	C=C stretching
1418	$-CH_2$ and $-CH_3$ bending
1027	C-O bending
802	C-H bending out of plane

Table 4.22 ^{1}H-NMR spectral data of 24-methylenecycloartanyl ferulate from standard oryzanol and the literature in $CDCl_3$

H position	^{1}H-NMR chemical shifts (δ/ppm, 400 MHz, $CDCl_3$)	
	Standard oryzanol	24-Methylenecycloartanyl ferulate
3	4.71 (1H, m)	4.71 (1H, m)
18	0.97 (3H, s)	0.98 (3H, s)
19	0.36 (d, *J* = 4.4 Hz, exo) 0.59 (d, *J* = 4.0 Hz, endo)	0.37 (d, *J* = 4.0 Hz, exo) 0.60 (d, *J* = 4.0 Hz, endo)
21	0.88 (3H, d, *J* = 6.0 Hz)	0.90 (3H, d, *J* = 5.5 Hz)
24	4.66 (br s) 4.71 (br s)	4.67 (br s) 4.72 (br s)
25	2.23 (1H, sept, *J* = 6.8 Hz)	2.24 (1H, sept, *J* = 7.0 Hz)
26	1.02 (3H, d, *J* = 6.8 Hz)	1.03 (3H, d, *J* = 6.6 Hz)
27	1.03 (3H, d, *J* = 6.8 Hz)	1.04 (3H, d, *J* = 7.0 Hz)
28	0.91 (3H, s)	0.90 (3H, s)
29	0.97 (3H, s)	0.98 (3H, s)
30	0.93 (3H, s)	0.92 (3H, s)
2'	6.30 (1H, d, *J* = 16.0 Hz)	6.30 (1H, d, *J* = 16.1 Hz)
3'	7.59 (1H, d, *J* = 16.0 Hz)	7.60 (1H, d, *J* = 15.8 Hz)
5'	7.04 (1H, d, *J* = 1.6 Hz)	7.04 (1H, d, *J* = 1.8 Hz)
8'	6.91 (1H, d, *J* = 8.0 Hz)	6.92 (1H, d, *J* = 8.4 Hz)
9'	7.07 (1H, dd, *J* = 1.6, 8.0 Hz)	7.08 (1H, dd, *J* = 1.8, 8.4 Hz)
6'-OCH_3	3.93 (3H, s)	3.94 (3H, s)
7'-OH	5.93 (1H, br s)	5.85 (1H, br s)

Table 4.23 ^{1}H-NMR spectral data of cycloartenyl ferulate from standard oryzanol and the literature in $CDCl_3$

H position	^{1}H-NMR chemical shifts (δ/ppm, 400 MHz, $CDCl_3$)	
	Standard oryzanol	Cycloartenyl ferulate
3	4.71 (1H, m)	4.71 (1H, m)
18	0.97 (3H, s)	0.97 (3H, s)
19	0.36 (d, *J* = 4.4 Hz, exo) 0.59 (d, *J* = 4.0 Hz, endo)	0.37 (d, *J* = 4.4 Hz, exo) 0.60 (d, *J* = 4.0 Hz, endo)
21	0.88 (3H, d, *J* = 6.0 Hz)	0.89 (3H, d, *J* = 6.2 Hz)
24	5.10 (1H, br t, *J* = 6.8 Hz)	5.11 (1H, br t, *J* = 7.0 Hz)
26	1.70 (3H, s)	1.69 (3H, s)
27	1.60 (3H, s)	1.61 (3H, s)
28	0.91 (3H, s)	0.90 (3H, s)
29	0.97 (3H, s)	0.98 (3H, s)
30	0.93 (3H, s)	0.91 (3H, s)
2'	6.30 (1H, d, *J* = 16.0 Hz)	6.30 (1H, d, *J* = 16.1 Hz)
3'	7.59 (1H, d, *J* = 16.0 Hz)	7.60 (1H, d, *J* = 15.8 Hz)
5'	7.04 (1H, d, *J* = 1.6 Hz)	7.04 (1H, d, *J* = 1.8 Hz)
8'	6.91 (1H, d, *J* = 8.0 Hz)	6.92 (1H, d, *J* = 8.1 Hz)
9'	7.07 (1H, dd, *J* = 1.6, 8.0 Hz)	7.08 (1H, dd, *J* = 1.8, 8.1 Hz)
6'-OCH_3	3.93 (3H, s)	3.94 (3H, s)
7'-OH	5.93 (1H, br s)	5.85 (1H, br s)

Table 4.24 ^{1}H-NMR spectral data of sitosteryl ferulate from standard oryzanol and the literature in $CDCl_3$

H position	^{1}H-NMR chemical shifts (δ/ppm, 400 MHz, $CDCl_3$)	
	Standard oryzanol	Sitosteryl ferulate
3	4.71 (1H, m)	4.75 (1H, m)
4	2.38 (br s) 2.40 (br s)	2.39 (br s) 2.41 (br s)
6	5.40 (1H, m)	5.41 (1H, m)
18	0.68 (3H, s)	0.69 (3H, s)
19	1.04 (3H, s)	1.05 (3H, s)
21	0.88 (3H, d, *J* = 6.0 Hz)	0.89 (3H, d, *J* = 6.2 Hz)
24	0.84 (3H, t, *J* = 6.8 Hz)	0.85 (3H, t, *J* = 7.0 Hz)
26	0.78 (3H, d, *J* = 7.6 Hz)	0.82 (3H, d, *J* = 7.6 Hz)
27	0.80 (3H, d, *J* = 7.2 Hz)	0.84 (3H, d, *J* = 7.0 Hz)
2'	6.30 (1H, d, *J* = 16.0 Hz)	6.30 (1H, d, *J* = 16.1 Hz)
3'	7.59 (1H, d, *J* = 16.0 Hz)	7.60 (1H, d, *J* = 15.8 Hz)
5'	7.04 (1H, d, *J* = 1.6 Hz)	7.04 (1H, d, *J* = 1.8 Hz)
8'	6.91 (1H, d, *J* = 8.0 Hz)	6.92 (1H, d, *J* = 8.1 Hz)
9'	7.07 (1H, dd, *J* = 1.6, 8.0 Hz)	7.08 (1H, dd, *J* = 1.8, 8.1 Hz)
6'-OCH_3	3.93 (3H, s)	3.94 (3H, s)
7'-OH	5.93 (1H, br s)	5.85 (1H, br s)

Table 4.25 ^{1}H-NMR spectral data of campesteryl ferulate from standard oryzanol and the literature in $CDCl_3$

H position	^{1}H-NMR chemical shifts (δ/ppm, 400 MHz, $CDCl_3$)	
	Standard oryzanol	Campesteryl ferulate
3	4.71 (1H, m)	4.71 (1H, m)
4	2.38 (br s) 2.40 (br s)	2.39 (br s) 2.41 (br s)
6	5.40 (1H, m)	5.41 (1H, m)
18	0.68 (3H, s)	0.69 (3H, s)
19	1.04 (3H, s)	1.05 (3H, s)
21	0.88 (3H, d, *J* = 6.0 Hz)	0.89 (3H, d, *J* = 6.2 Hz)
24	0.77 (3H, t, *J* = 6.4 Hz)	0.78 (3H, d, *J* = 6.6 Hz)
26	0.84 (3H, d, *J* = 6.8 Hz)	0.85 (3H, d, *J* = 6.2 Hz)
27	0.82 (3H, d, *J* = 6.4 Hz)	0.81 (3H, d, *J* = 6.8 Hz)
2'	6.30 (1H, d, *J* = 16.0 Hz)	6.30 (1H, d, *J* = 16.1 Hz)
3'	7.59 (1H, d, *J* = 16.0 Hz)	7.60 (1H, d, *J* = 15.8 Hz)
5'	7.04 (1H, d, *J* = 1.6 Hz)	7.04 (1H, d, *J* = 1.8 Hz)
8'	6.91 (1H, d, *J* = 8.0 Hz)	6.92 (1H, d, *J* = 8.1 Hz)
9'	7.07 (1H, dd, *J* = 1.6, 8.0 Hz)	7.08 (1H, dd, *J* = 1.8, 8.1 Hz)
6'-OCH_3	3.93 (3H, s)	3.94 (3H, s)
7'-OH	5.93 (1H, br s)	5.85 (1H, br s)

4.4 Antioxidant Activity of Oryzanol Mixture on DPPH Radicals

DPPH radicals are commonly used for assessment of antioxidant activity *in vitro* and are foreign to biological systems. It is generally accepted that antioxidant ability of chemical substances on free radical scavenging inhibits lipid oxidation. DPPH assay is one of the short methods for investigation of the hydrogen donating potency (Blois, 1958; Brand-Willams *et al.*, 1995; Kikuzaki *et al.*, 2002).

Scavenging ability of all tested compounds, i.e., standard oryzanol, α-tocopherol (positive control), oryzanol mixture PF1, PF2, WF1, SF1, and CF1, increased with concentration in the range of 0-200 μg/ml (data shown in Appendix B). The reason for using various concentrations of an antioxidant is that, although a positive dose-response relationship usually is present; such relationships may be different for various antioxidants. Examining the dose-response relationships is important so the optimal antioxidant concentration can be determined. In this study, activity of standard oryzanol and all oryzanol mixtures increased gradually with an increase in concentration, while α-tocopherol activity increased sharply with an initial increase in concentration and tended to level off at higher concentration.

The concentration required to cause a 50% inhibition (IC_{50}) for each sample was shown in Table 4.26, which was expressed as micrograms/milliliter, μg/ml. Each value is the mean of triplicate measurements. Most of oryzanol mixtures performed IC_{50} about 25-40 μg/ml and there were slight different activities among tested rice varieties. All the rice varieties exhibited appreciable scavenging activity against DPPH radicals.

The antioxidant activity decreased in the order of α-tocopherol > oryzanol mixture SF1 > standard oryzanol > oryzanol mixture CF1 > oryzanol mixture PF1 > oryzanol mixture WF1 > oryzanol mixture PF2. As described above, the oryzanol mixture extracted from Sunpatong 1 rice bran (oryzanol mixture SF1) possessed the highest antioxidant activity among all rice varieties. The key difference that could be thought to make a difference in the antioxidant activity is the sterol composition. The extracted steryl ferulates from each rice cultivar are comprised of numerous sterols with similar constituents but different in content of each oryzanol composition. Moreover, we also found significant antioxidative differences within rice cultivar, Pathumthani 1, which may causes by differences in oryzanol components.

Over the past decade, there were a number of well-established antioxidant activities of oryzanol in several models. Campesteryl ferulate was shown to inhibit linoleic acid oxidation caused by UV irradiation (Yagi and Ohishi, 1979). Xu and co-workers studied the antioxidative properties of rice bran oryzanol components and showed that cycloartenyl, 24-methylenecycloartanyl and campesteryl ferulate inhibited cholesterol oxidation induced by 2,2'-azobis-(2-methylpropanimidamide)-dihydrochloride (AAPH) even more than vitamin E components that are well-known for their antioxidant properties (Xu *et al.*, 2001). Further, another study of the same authors, oryzanol (the same compositions) was shown to inhibit also linoleic acid oxidation (Xu and Godber, 2001). Oryzanol also inhibited cholesterol autoxidation in an aqueous model system (Kim *et al.*, 2001). Oryzanol components, namely, cycloartenyl ferulate and 24-methylenecycloartanyl ferulate, were shown to act as antioxidants in methyl linoleate bulk and multiphase lipid systems and as radical scavengers (Kikuzaki *et al.*, 2002). Cycloartenyl and 24-methylenecycloartany ferulate scavenged 21.8 and 20.4 % DPPH radical, respectively. Antioxidant activity of oryzanol mixture was also undertaken in that study; oryzanol mixture scavenged about 21.2 % DPPH radical. However, we did not compare our results with that information because it was performed in different condition. Kikuzaki and co-workers (2002) investigated DPPH assay with 100 μM DPPH ethanolic solution and 20 μM of tested compounds incubated at 25 °C, whereas our condition was undertaken at 200 μM DPPH solution, 0-332 μM of oryzanol mixture and 37 °C incubation.

Additionally, oryzanol extracts from wheat and rye bran were also studies for their capability to inhibit hydroperoxide formation in bulk methyl linoleate and methyl linoleate emulsion and to scavenge DPPH radical (Nyström *et al.*, 2005). Nonrice cereal extracts of oryzanols, sitosteryl and cholesteryl ferulats, exhibited good antioxidant activity even more than rice oryzanol, especially in the bulk lipid system. The sterol portion in these cereals is principally desmethylsterols that have no methyl groups. Similar result was seen in the study by Wang *et al.* (2002), in which sitostanyl ferulate was a more effective antioxidant than oryzanol. However, this is in contrast with the study of Xu *et al.* (2001) who demonstrated that in an accelerated diphase system 24-methylenecycloartenyl ferulate (dimethylsterol) was a better antioxidant than

campesteryl ferulate desmethylsterol). It may be possible that the ring structure of 4,4'-dimethylsterols and 4-desmethylsterols affects, for example, the solubility of the compound in a way that the desmethylsteryl ferulates are better antioxidants in nonpolar environments, whereas dimethylsterols are more active in diphasic systems.

An antioxidative function of oryzanol has been previously reported (Kikuzaki *et al.*, 2002; Kim *et al.*, 2001; Nyström *et al.*, 2005; Okada and Yamaguchi, 1983; Wang *et al.*, 2002; Xu *et al.*, 2001; Xu and Godber, 2001). Since radical scavenging activity was similar to that of nonesterified ferulic acid, it has been suggested that the phenolic hydroxyl group in the ferulate esters of oryzanol might be responsible for its antioxidative function. Actually, Ferulic acid was a slightly more active than its sterol esters (Kikuzaki *et al.*, 2002; Kim *et al.*, 2001; Nyström *et al.*, 2005; Wang *et al.*, 2002). On the other hand, polar compounds such as ferulic acid have a limited solubility in very nonpolar lipid matrix. Kikuzaki and colleagues (2002) indicated that oryzanol had better affinity to the hydrophobic phase than ferulic acid. Therefore, it is necessary to study antioxidant capability of a compound in different surrounding environments. Generally, phenolic antioxidants inhibit lipid oxidation by trapping the peroxy radical to yield the hydroperoxide there by preventing the peroxy radical from reacting to produce a lipid radical and propagate a free-radical chain reaction.

Our results indicated that oryzanol mixtures from various cultivars are poorer antioxidants than α-tocopherol in DPPH assay, as about 2-fold concentration is needed to accomplish equal activity, these results were confirmed the findings in previous studies (Kikuzaki *et al.*, 2002; Nyström *et al.*, 2005). The fact that the antioxidant activity of steryl ferulates from cereals was less than that of tocopherol does not however reduce their importance as natural antioxidants and health-promoting compounds because the content of steryl ferulates in rice and wheat bran is over 10-fold the content of tocopherols (Nicolosi *et al.*, 1994; Piironen *et al.*, 1986; Xu *et al.*, 2001). Although, a significant quantity of vitamin E (tocopherol) and oryzanol was estimated from five rice varieties in Pakistan, indicating that oryzanol contents were over 2 times higher than that of tocopherol and these contents varied substantially according of the origin of rice bran (Iqbat *et al.*, 2005).

Table 4.26 Antioxidative efficiency of the oryzanol mixture

Oryzanol Fraction	IC_{50} (μg/ml)
Standard oryzanol	33.58
Oryzanol mixture PF1	35.07
Oryzanol mixture PF2	59.78
Oryzanol mixture WF1	40.12
Oryzanol mixture SF2	25.00
Oryzanol mixture CF1	33.88
α-Tocopherol	11.66

Future studies should focus on developing appropriate technique for investigating the constituents of oryzanol in rice bran oil for commercial purpose. Characterization and quantification of oryzanol should be performed in other Thai rice varieties and other cereals.