Acidity of Oryzanol and Its Contribution to Free Fatty Acids Value in Vegetable Oils

A.G. Gopala Krishna*, K.H. Hemakumar, and Sakina Khatoon

Department of Lipid Science & Traditional Foods, Central Food Technological Research Institute (CFTRI), Mysore-570020, India

ABSTRACT: Model oil systems containing physically refined rice bran oil to which oryzanol was added were examined to determine the effects of oryzanol concentration on FFA values. When oryzanol was added to the model oils at a 0.5% level and FFA was determined, increases in FFA value were 0.28% as determined with phenolphthalein, 0.58% with thymolphthalein, and 0.07% with alkali blue 6B. Oils containing added oryzanol at 0.5–1.5% showed a proportionate increase in FFA values with an average increase of 0.413% per gram of oryzanol. A direct titration of purified oryzanol showed an acidity of 42.5% expressed as FFA. In spectroscopic studies, the phenolic group in the ferulic acid moiety of oryzanol was titrated by sodium hydroxide. Based on these data, indicator correction factors for oryzanol's acidity and a formula for calculating real FFA content of vegetable oils containing oryzanol were developed.

Paper no. J11198 in *JAOCS 83*, 999–1005 (December 2006).

KEY WORDS: Acidity of oryzanol, free fatty acids value, groundnut oil, indicator factor for oryzanol's acidity, oleic acid acidity, oryzanol, real free fatty acids value, rice bran oil, sunflower oil, vegetable oils.

Rice bran oil (RBO) is gaining importance as a health-promoting edible oil because of its unique oryzanol content and high levels of unsaponifiables (1). Oryzanol is a complex mixture of ferulic acid esters of sterols and triterpenols. The unsaponifiables of RBO are known to improve blood cholesterol levels, and oryzanol is an antioxidant, which may delay aging (1,2). However, RBO poses some unique processing challenges because of its high contents of FFA and unsaponifiables.

RBO can be produced by using either chemical and physical refining. During chemical refining with sodium hydroxide, about 90% of the oryzanol present in the crude oil is removed with the soap stock (2). But in physical refining by steam stripping, the oryzanol is mostly retained in the oil (2). The Indian Standards specification for refined oils allows a maximum acid value of 0.5, which is equivalent to 0.25% FFA (3,4). The apparent FFA content of chemically refined RBO is within permitted levels, but the FFA content of physically refined RBO is higher. The acid value of soybean oil increases proportionately with the amount of externally added oryzanol (5,6), so 1% of oryzanol in RBO would be expected to increase its acid value by 1 and its FFA by 0.5 units. But there is no direct evidence to show that the higher FFA value of physically refined RBO is due to the presence of oryzanol or that the FFA value increases proportionately with oryzanol content of the oil. As there is no free carboxylic group in oryzanol, it is not obvious that the observed increase in FFA value can be attributed to oryzanol. We undertook studies to assess the acidity of oryzanol and its effect on the FFA value when present in an oil.

MATERIALS AND METHODS

Materials. Refined sunflower oil (Sundrop®), refined groundnut oil (Nature Fresh®), and refined safflower oil (Saffola®) were procured from a market in Mysore City, India. Phenolphthalein and thymolphthalein were procured from Ranbaxy Laboratories Ltd. (New Delhi, India) and alkali blue 6B from Sigma Chemical Co. (St. Louis, MO). Various brands of physically and chemically refined RBO were supplied by Marico Industries Ltd. (Mumbai, India). Oryzanol (food grade, 99.9% purity) was purchased from Tsuno Rice Fine Chemicals Co., Ltd. (Wakayama, Japan). The composition of the oryzanol used in this study is given in Table 1 and differs slightly from that of oryzanol compositions in RBO reported by others (2). All other chemicals used were of analytical reagent grade.

Methods. (i) FFA content determination and effect of indicator. The AOCS Official Method, Ca 5a-40 (7), was used for the determination of FFA content of oils using 5 ± 0.1 g samples and expressing the results as percent oleic acid. Two other indicators, thymolphthalein (5,6) and alkali blue 6B (5,6), were tested in addition to the indicator (viz., phenolphthalein) specified by the method to see whether the indicator had an effect on the determined value.

(ii) Oryzanol content determination. A spectrophotometric method was used as reported previously (2). The HPLC analysis of the standard oryzanol and of RBO was performed using the procedure of Rogers *et al.* (8) as modified (2).

(iii) Oryzanol effect on the titrimetric results. Groundnut oil and sunflower oil, which do not contain oryzanol, and an RBO containing <0.2% oryzanol were treated with (i) 0.5% standard oryzanol; additionally, (ii) groundnut oil was treated with 0.1, 0.5, 1.0, and 1.5% standard oryzanol. The FFA contents were determined. The formula used for the determination of FFA/g oryzanol added is as follows:

$$\text{FFA/g oryzanol} = V \times N \times 28.2 \times (1/W) \times (1/\text{OZ}) \quad [1]$$

*To whom correspondence should be addressed.
E-mail: aggk_55@yahoo.com

TABLE 1
Ferulate Composition[a] (relative %) of Standard Oryzanol Used in Relation to Rice Bran Oils (RBO) Studied

Ferulate composition	Standard oryzanol	Crude RBO[b]	Chemically refined RBO1[b]	Physically refined RBO2[b,c]	Range (%)
Cycloartanyl ferulate	17.4	8.5	16.5	25.3	8.5–25.3
24-Methylene cycloartanyl ferulate	45.9	54.5	42.1	36.4	36.4–54.5
Campesteryl ferulate	23.1	17.0	29.9	14.8	14.8–29.9
b-Sitosteryl ferulate/cycloartenyl ferulate	9.4	13.8	8.4	15.4	8.4–15.4
Others (unidentified)	4.2	6.2	3.1	8.1	3.1–8.1
Oryzanol content[d] (%)	104	1.8	0.2	1.3	0.2–1.8

[a]Determined in this laboratory.
[b]RBO sample 1 is a chemically refined oil; sample 2 is a physically refined oil; crude RBO and chemically refined RBO are from same factory and the same batch.
[c]Crude RBO used for production of physically refined RBO had the same ferulate composition as that of crude oil used for chemically refined oil production.
[d]By spectrophotometry.

where V = volume of alkali consumed, N = normality of alkali solution used, W = weight of oil sample taken, and OZ = % oryzanol added to the oil.

(iv) Determination of acidity of oryzanol and development of the indicator factor. Standard oryzanol—500, 1000, or 1500 mg—was dissolved in neutralized alcohol, and each oryzanol level was titrated separately with sodium hydroxide using three indicators: phenolphthalein, thymolphthalein, or alkali blue 6B. The acidity was expressed as percent oleic acid using the equation in AOCS method Ca 5a-40 (7). The FFA was divided by 100 to get the acidity value and adjusted for the amount of oryzanol titrated to get the acidity/gram oryzanol for each indicator. This was designated the "indicator factor for oryzanol's acidity."

The acidity of the standard oryzanol also was calculated by the following formula:

$$\text{acidity of oryzanol (as \% NaOH consumed)} = V \times 0.004 \times 100/W \quad [2]$$

where V = volume of 0.1 N sodium hydroxide consumed, 0.004 = factor for converting volume of alkali into weight, and W = weight of oryzanol used for titration, in grams.

(v) Formula development for calculation of the real FFA content. Since both oleic acid and oryzanol showed acidity, the contribution of oryzanol needed to be subtracted from observed FFA content to get the real FFA content ascribable to oleic acid. For this purpose, the following formula was developed:

$$\text{real FFA} = \text{observed FFA (for particular indicator)} - (\%\ \text{oryzanol} \times \text{respective indicator factor for oryzanol's acidity}) \quad [3]$$

The formula was validated through back-calculation of the predicted FFA content of model oils treated with oryzanol by taking into account the initial FFA content of the control model oils and the contribution by oryzanol.

A set of regression equations were obtained by using plots of observed FFA of RBO against the oryzanol content of RBO (presented in Table 2). These equations were also used to obtain the real FFA content of RBO.

(vi) Data analysis. Statistical analyses were carried out by using a Microsoft Windows 98, Excel 2000 program.

(vii) Isolation of the reaction product obtained after acidity determination. Oryzanol in neutralized alcohol was titrated with sodium hydroxide by the method for FFA determination, and the solids were recovered by evaporating the liquid on a steam bath. The residue was washed with distilled water to remove excess alkali and dried on a steam bath for 1 h.

(viii) Characteristics of the isolated reaction product of oryzanol and sodium hydroxide. Solubility, m.p., color, and oryzanol content were determined. The color of a 0.05% solution of the reaction product in ethanol was read in a Lovibond tintometer (Model F) in a 25.4 mm tube (The Tintometer Ltd., Salisbury, United Kingdom). The UV and IR spectra of the isolated reaction product and standard oryzanol were recorded. A dilute solution of the sample in hexane or ethanol was used for obtaining UV-vis absorption spectra in the 190–1100 nm region using a Shimadzu UV-1601 double beam recording spectrophotometer (Shimadzu Corp., Kyoto, Japan). A Nujol mull of the sample was used for recording the IR spectra with a Nicolet 5700 FT-Raman system (Thermo Electron Corporation, Madison, WI).

RESULTS AND DISCUSSION

Effect of indicator on the FFA value. The effect of indicator on the FFA value of commonly available vegetable oils is quite small (Table 2, last row) but for physically refined RBO, differences in FFA levels were highly significant (Table 2) and changed with the indicator.

Effect of adding oryzanol to model vegetable oils on the FFA value. The effect of adding uniform amounts of oryzanol on FFA content of various vegetable oils, viz., groundnut, sunflower, and RBO, is shown in Table 3. This shows that the addition of 0.5% oryzanol to these oils changes the acidity of the oil, measured as FFA content, in a manner that depends on the indicator used for titration. The mean values are 0.28, 0.58, and

TABLE 2
Effect of Indicator on FFA Values of Indian Refined RBO Samples Containing Varied Levels of Natural Oryzanol

Samples	Oryzanol content (g/100 g oil)	% FFA with the indicator[a]		
		Phenolphthalein	Thymolphthalein	Alkali blue 6B
1	1.11	0.40	0.45	0.17
2	1.09	0.47	0.63	0.15
3	1.07	0.48	0.54	0.14
4	1.21	0.46	0.52	0.16
5	1.32	0.49	0.62	0.16
6	0.56	0.43	0.47	0.19
7	0.60	0.35	0.55	0.13
8[b]	0.18	0.16	0.46	0.10
9	0.60	0.43	0.55	0.17
10	0.73	0.44	0.53	0.17
11	0.75	0.47	0.60	0.17
12[b]	0.14	0.14	0.31	0.03
13	0.61	0.29	0.59	0.08
14	1.39	0.55	0.79	0.17
Range[c]	0.14–1.39	0.14–0.55	0.31–0.79	0.03–0.17
Range for Com. Oils[d]	—	0.05–0.09	0.08–0.21	0.02–0.05

[a]Phenolphthalein—colorless to pale pink (pH range: 8.3–10.0). Thymolphthalein—red to blue or bluish green (pH range: 9.3–10.4). Alkali blue 6B—blue to colorless (red color difficult to observe when oil is used) (pH range: 9.0–14.0).
[b]Samples 8 and 12 are chemically refined RBO, and the rest are physically refined RBO. For abbreviation see Table 1.
[c]All determinations were done in triplicate. The CV were within ±2%.
[d]Commercial vegetable oils refer to groundnut, sunflower, and safflower oils

0.07% for phenolphthalein, thymolphthalein and alkali blue 6B, respectively.

To confirm the effect of increasing concentrations of oryzanol on FFA results, oryzanol was incorporated into the model oils, which were then tested with only a phenolphthalein indicator. The results, presented in Table 4, indicate that there is a proportionate increase of FFA value with increase in oryzanol content in the model oil equivalent to 0.413% per gram of oryzanol

Acidity of oryzanol determined using three indicators and expressed as FFA. The acidity of oryzanol was determined by titrating oryzanol in neutralized alcohol using three indicators separately, viz., phenolphthalein, thymolphthalein, and alkali blue 6B, and then the titer obtained was used for calculation of FFA content (expressed as %oleic acid) to show the level of interference when oryzanol and FFA are both present in an oil, such as RBO. The titration data (not shown) found 42.47 ± 1.20% FFA (CV 2.82), which is equivalent to 0.425 g FFA/g oryzanol using phenolphthalein, 48.2 ± 1.73% FFA (CV 3.59), and 0.482 g FFA/g oryzanol for thymolphthalein. But alkali blue 6B indicator gave only 10.22 ± 0.29% FFA (CV 2.83) and 0.102 g FFA/g oryzanol.

Indicator factor for oryzanol's acidity. The acidity of oryzanol should not be mistaken for the acidity expressed as oleic acid, i.e., for real FFA content. Therefore, the acidity of standard oryzanol expressed as FFA was divided by 100 to get FFA/g oryzanol which is termed "the indicator factor for oryzanol's acidity," or 0.425, 0.482, and 0.102 for phenolphthalein, thymolphthalein, and alkali blue 6B indicators, respectively. The indicator factor was also calculated using regression equations developed for oryzanol that is naturally present in RBO, which closely matches that of the indicator factor for standard oryzanol (0.44, 0.425; 0.58, 0.482; and 0.15, 0.102 for phenolphthalein, thymolphthalein, and alkali blue 6B indicators, respectively). This factor is useful for calculation of real FFA content of vegetable oils containing oryzanol.

Contribution by natural oryzanol to FFA in commercial RBO samples. The calculated contribution by oryzanol to FFA for RBO (data not shown) is quite high and has a highly significant correlation ($r = 0.999$) for all three indicators. The FFA predicted based on oryzanol content (Table 2) in RBO using

TABLE 3
Effect of Added Oryzanol (0.5 g/100 g of each sample) to Vegetable Oils on the FFA Content When Using Three Different Indicators

Indicator and oil[a]	%FFA (observed)	%FFA (predicted)
Phenolphthalein		
Groundnut oil	0.23	0.28
Sunflower oil	0.32	0.30
RBO	0.29	0.40
Mean ± SD	0.28 ± 0.04[b]	0.33 ± 0.06[b]
Thymolphthalein		
Groundnut oil	0.53	0.35
Sunflower oil	0.56	0.32
RBO	0.64	0.60
Mean ± SD	0.58 ± 0.06[c]	0.42 ± 0.15[c]
Alkali blue 6B		
Groundnut oil	0.10	0.08
Sunflower oil	0.07	0.09
RBO	0.04	0.10
Mean ± SD	0.07 ± 0.03[d]	0.09 ± 0.01[d]

[a]The FFA values of the initial oils, viz., groundnut, sunflower, and rice bran oils, were 0.07, 0.05, and 0.14%, respectively.
[b,c,d]Mean values in each row for observed and predicted FFA carrying the same superscript letters are not significantly different from each other. For abbreviation see Table 1.

TABLE 4
Effect of Increasing Concentrations of Oryzanol in Vegetable Oils on the Increase in FFA Content When Using Phenolphthalein as Indicator

	% FFA in					
	Groundnut oil		Sunflower oil		RBO	
%Oryzanol added[a]	A[b]	B[c]	A[b]	B[c]	A[b]	B[c]
0.5	0.20	0.40	0.15	0.30	0.23	0.46
1.0	0.40	0.40	0.45	0.45	0.42	0.42
1.5	0.60	0.40	0.68	0.45	0.66	0.44
Mean ± SD for individual oils	0.40 ± 0.00		0.40 ± 0.09		0.44 ± 0.02	
CV[d]	—		22.50		4.55	
All studied oils	Standard oryzanol added in oil[e]		Standard oryzanol[f]		Natural oryzanol present in RBO[g]	
Mean	0.413		0.425		0.44	
SD	0.049		0.012		—	
CV[d]	11.864		2.82		—	

[a]Concentration of 0.1% in the oil showed undetectable changes in titer
[b]Values are expressed as oleic acid.
[c]Values are expressed as FFA/g oryzanol.
[d]Percent error of the mean value.
[e]From data in this table for model oil with added oryzanol.
[f]From data in text for standard oryzanol.
[g]From regression equation in text for commercial physically refined RBO.

regression equations also showed a highly significant correlation ($r = 0.999$) for all three indicators. The regression equation is $y = c + mx$, where y = observed FFA; m = slope; c = intercept; and x = oryzanol content. For phenolphthalein, the specific equation is: $y = 0.15 + 0.29x$; for thymolphthalein, $y = 0.36 + 0.22x$; and for alkali blue 6B, $y = 0.07 + 0.08x$.

Real FFA value calculation. Since oryzanol has acidity and contributes to the measured FFA content when present in an oil, FFA content determined by using phenolphthalein as the indicator (AOCS method for FFA determination) needs to be corrected. The formula for calculating real FFA content is shown below:

$$\text{real FFA} = \text{observed FFA(for phenolphthalein)} - (\%\ \text{oryzanol in the oil}) \times 0.425 \quad [4]$$

$$\text{real FFA} = \text{observed FFA(for thymolphthalein)} - (\%\ \text{oryzanol in the oil}) \times 0.482 \quad [5]$$

$$\text{real FFA} = \text{observed FFA(for alkali blue 6B)} - (\%\ \text{oryzanol in the oil}) \times 0.102 \quad [6]$$

The results presented in Tables 2 and 5 clearly show that observed FFA contents, after correction, varied from –0.07 to 0.1934% but did not exceed the specified limit of 0.25% FFA prescribed by Indian Standards Institution and PFA (Prevention of Food Adulteration Act) specifications (3,4). The real FFA content data (Table 5, column B) that were predicted by using regression equations for natural oryzanol in RBO agree with the real FFA data calculated by using the formula developed for standard oryzanol in the study (Table 5, data in column A for the three indicators).

The titratable acidity of oryzanol. Oryzanol consumes about 15 mL of 0.1 N sodium hydroxide/g. The acidity, expressed as %sodium hydroxide, required to neutralize 100 g of the standard oryzanol is 5.89–6.24 g with a mean value of 6.03 g (Table 6). The FFA determined as oleic acid, which has a M.W. of 282, consumes 40 g of sodium hydroxide/mole of oleic acid, whereas oryzanol, with an average M.W. of about 550, consumes 33.17 g of sodium hydroxide/mole of oryzanol, which is equivalent to 83% of the alkali consumed for oleic acid. Therefore, the acidity contributed by oryzanol is comparable with the acidity contribution by oleic acid, indicating one replaceable hydrogen atom per molecule. Because oryzanol does not have a free carboxylic acid group and is an ester mixture of ferulic acid and phytosterols [cycloartenol, cycloartanol, 24-methylene cycloartanol, campesterol, and b-sitosterol (Table 1)], the question arises of how to explain the acidity of oryzanol.

Chemical reaction between sodium hydroxide and oryzanol. To establish the chemical reaction between oryzanol and sodium hydroxide, standard oryzanol and the reaction product isolated after FFA determination were examined by UV and IR spectroscopic studies (Figs. 1 and 2). The reaction between oryzanol and alkali is expected to be similar to that of the reaction between a phenol and an alkali. Phenols react with alkali to form sodium phenates. Similarly, oryzanol may also react with sodium hydroxide and form sodium phenate of oryzanol which has been designated as "sodium derivative of oryzanol." The probable reaction is given in Scheme 1.

The mechanism for this reaction was confirmed by IR spectra of standard oryzanol and the isolated reaction product, because the absence of the –OH group was indicated in the IR spectrum (Fig. 2).

Physicochemical characteristics of sodium derivative of oryzanol. Characteristics of the sodium derivative of oryzanol are compared with those of oryzanol in Table 6. The sodium derivative of oryzanol is a yellow solid that is insoluble in hexane and has a m.p. of 136–138°C, whereas oryzanol is a white

TABLE 5
Calculated Real FFA Content of Studied Rice Bran Oils

	Phenolphthalein		Thymolphthalein		Alkali blue 6B	
	Real FFA		Real FFA		Real FFA	
Rice bran oil samples	A[b]	B[c]	A[b]	B[c]	A[b]	B[c]
1	–0.07	0.0	–0.08	0.0	0.06	0.01
2	0.01	0.0	0.11	0.03	0.04	0.0
3	0.03	0.02	0.03	0.0	0.03	0.0
4	–0.05	0.04	–0.06	0.0	0.04	0.0
5	–0.07	0.0	–0.02	0.0	0.03	0.0
6	0.19	0.12	0.20	0.0	0.13	0.07
7	0.09	0.02	0.26	0.06	0.07	0.01
8	0.08	0.04	0.37	0.06	0.08	0.01
9	0.16	0.11	0.26	0.06	0.11	0.05
10	0.13	0.08	0.18	0.01	0.10	0.04
11	0.15	0.10	0.24	0.07	0.09	0.04
12[a]	0.08	0.0	0.24	0.0	0.02	0.0
13	0.03	0.04	0.30	0.10	0.02	0.0
14	0.12	0.0	0.12	0.12	0.01	0.0
Range	0.0–0.2	0.0–0.1	0.0–0.4	0.0–0.1	0.0–0.1	0.0–0.1

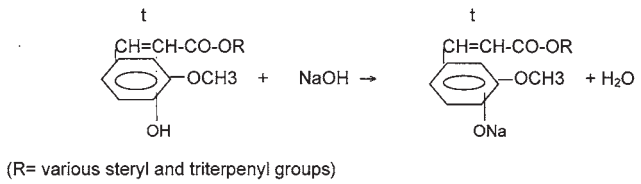
[a]Reference RBO.
[b]Real FFA calculated using the formula provided in text and indicator factor determined by titration of standard oryzanol (Eqs. 4–6).
[c]Real FFA calculated using the formula provided in text for each indicator using the indicator factor generated by regression equation at a normalized oryzanol concentration of 1. For abbreviation see Table 1.

solid that is soluble in hexane but has a similar m.p. point of 136–138°C. The sodium derivative of oryzanol, which as a solid is yellow, appears greenish when dissolved in ethanol at a 0.05% concentration, but the Lovibond color shows a yellow color value of 4 units. On the other hand, a 0.05% ethanolic solution of oryzanol is colorless or off-white. The oryzanol content of the sodium derivative measured in ethanol at 314 nm was 62%, whereas for the starting standard oryzanol it was 104% indicating that the oryzanol content decreases by 40% for the sodium derivative owing to replacement of the acidic hydrogen atom in the phenolic group with a sodium atom.

The UV-vis absorption spectra of oryzanol and the sodium derivative (Fig. 1) show that there is no variation in the spectral pattern in comparison with oryzanol for the sodium derivative, but there is a small shift in λ_{max} from 329 to 328 nm. However, there is a reduction of only 38% in the absorption of the sodium derivative as indicated by the lower $E^{1\%}_{1cm}$. That is, the $E^{1\%}_{1cm}$ value for the sodium derivative is 230.8 at 328 nm, and oryzanol has an $E^{1\%}_{1cm}$ value of 373.3 at 329 nm. The IR spectra presented in Figure 2 for oryzanol, its sodium derivative, and ferulic acid also confirm that there is not much change in the molecular structure of oryzanol during alkali treatment except for the formation of the sodium derivative at the site of the hydroxyl group (around 3550 cm^{-1}) present in the ferulic acid moiety.

TABLE 6
Characteristics of Sodium Derivative of Oryzanol Isolated After Titration with Sodium Hydroxide

Parameters	Sodium derivative of oryzanol	Oryzanol
Color, visual	Yellow	White
Color, Lovibond (5R+Y units)	Red = 0, Yellow = 4, Blue = 0.1, Neutral = 0, (5R + Y = 4)	(0.0)
Melting point (°C)	136–138	136–138
Solubility	Insoluble in hexane, but soluble in ethanol after heating	Soluble in hexane and ethanol (after heating)
Oryzanol (%)	62	104
Acidity as:		
(i) sodium hydroxide consumed (g/100 g)	—	6.03
(ii) FFA (%)	—	42.5
λ_{max}, nm ($E^{1\%}_{1cm}$)	328 (ethanol) (230.8)	314 (hexane) (358.9) 329 (ethanol) (373.3)
Other absorption wavelengths (nm)	237, 219	250, 216 (hexane) 237, 220 (ethanol)



(R= various steryl and triterpenyl groups)

SCHEME 1

Therefore, oryzanol reacts with alkali and contributes significantly to the acidity of the oil expressed as FFA content as shown in Scheme 1. Hence, there is a necessity for correcting the observed FFA of vegetable oils containing naturally present oryzanol, such as in physically refined RBO and its blends with other oils. If oryzanol is incorporated into oils or foods, its acidity should not be mistaken for FFA acidity measured as FFA content, and the formula and regression equations developed here may be used to determine the real FFA content.

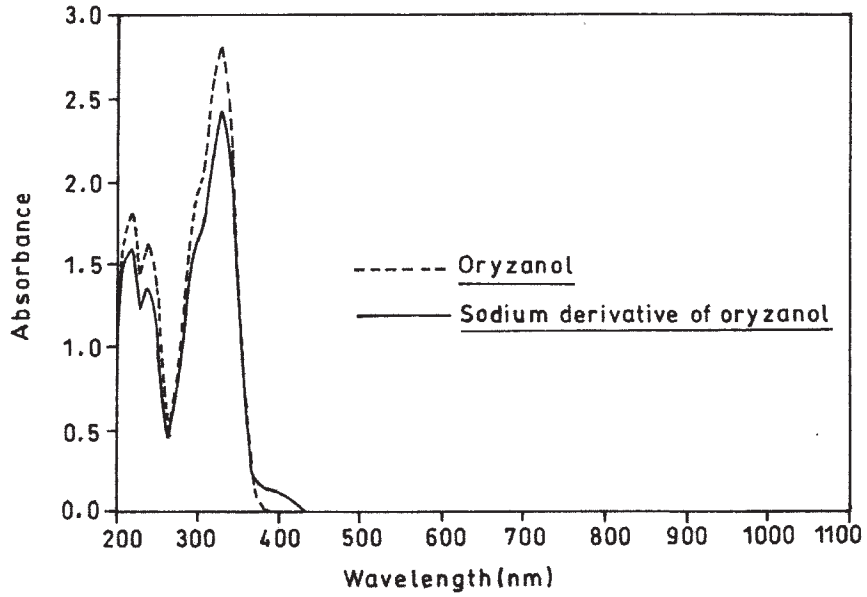


FIG. 1. UV absorption spectra of standard oryzanol and its sodium derivative.

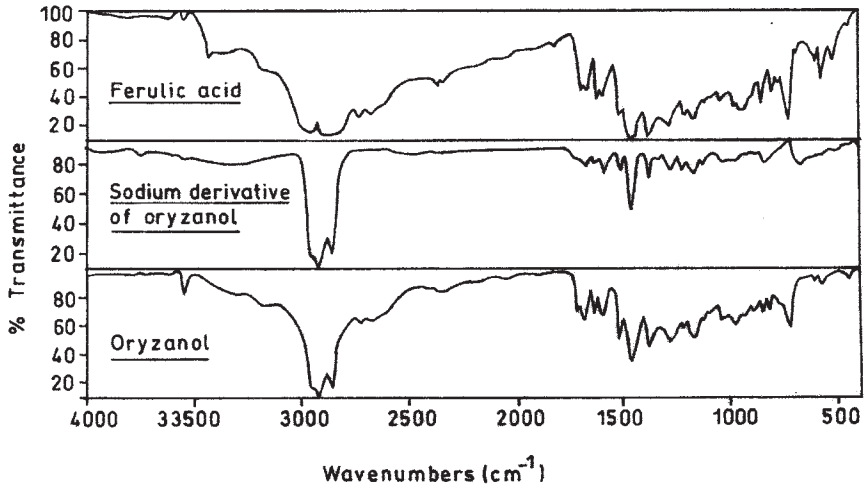


FIG. 2. IR spectra of ferulic acid, standard oryzanol, and the sodium derivative of the latter.

ACKNOWLEDGMENTS

The authors express their thanks to Dr. Visveswaraiah Prakash, Director, CFTRI, Mysore for facilities and encouragement; to Marico Industries Limited (Mumbai, India) for financial assistance; to Leo Gingras (Riceland Foods Inc., Stuttgart, AR), Mukund Sangli, Subham Ray, and Dr. J.I. Lewis from Marico Industries Ltd., Mumbai for useful discussions; B.S. Ramesh and M. Balasaraswathi for statistical analysis, and coordination with industry; and Ms. M. Asha for IR spectra.

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[Received August 1, 2005; accepted August 29, 2006]