Comparison of Bio- and Autocatalytic Esterification of Oils Using Mono- and Diglycerides

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ABSTRACT: The purpose of this study was to investigate enzymatic and autocatalytic esterification of FFA in rice bran oil (RBO), palm oil (PO), and palm kernel oil (PKO), using MG and DG as esterifying agents. The reactions were carried out at low pressure (4–6 mm Hg) either in the absence of any added catalyst at high temperature (210–230ºC) or in the presence of *Mucor miehei* lipase at low temperature (60ºC). The reactions were carried out using different concentrations of MG, and the optimal FFA/MG ratio and time were 2:1 (molar) and 6 h, respectively, in both auto- and enzyme-catalyzed processes. With DG as the esterifying agent in the autocatalytic process, the optimal temperature was 220ºC, and the optimal FFA/DG ratio was 1:1.25. For both MG and DG, the enzymatic process was more effective in reducing FFA and produced more favorable levels of unsaponifiable matter and color in the final product. The PV of the final products were also lower (1.8–2.9 mequiv/kg) by using the enzymatic process. To produce edible-grade oil, a single deodorization step would be required after enzymatic esterification; whereas, alkali refining, bleaching, and deodorization would be required after autocatalytic treatment.

Paper no. J10996 in *JAOCS 83*, 443–448 (May 2006).

KEY WORDS: Diglyceride, enzyme, esterification, free fatty acid, monoglyceride, *Mucor miehei*.

Rice bran (*Oryza sativa* L.) oil (RBO), palm (*Elaeis guineensis*) kernel oil (PKO), and palm oil (PO) are all high in FFA content owing to lipase hydrolysis of oil within the seeds. The problems encountered during refining of high acid-containing oils are well documented (1). Refining of these oils is the most critical of all processing steps because it not only removes the undesired components from the oil but also affects the economics of edible oil processing. The conventional refining process involves several steps (e.g., degumming, dewaxing, deacidification, bleaching, and deodorization). Deacidification is responsible for most oil loss.

During the last three to four decades scientists have proposed several physical, chemical, and biochemical methods to solve the problem of high refining loss associated with high FFA-containing oils (1–23). The methods cover a wide spectrum, from the simplest liquid-liquid extraction (2,3), alkali refining (4), miscella refining (3,5,6), and distillative (high-temperature, low-pressure) physical refining (1,7–10), to the latest developments such as membrane deacidification (11,12), chemical esterification (13) and bio-esterification (14–20), autocatalytic esterification (21,22), and supercritical fluid extraction (23). Each process has advantages and disadvantages.

Among the methods just mentioned, the ones attractive to scientists and processors are physical, membrane, and esterification methods, owing to their inherently lower refining loss. Oil loss equal to or greater than the quantity of FFA is inevitable in physical and membrane deacidification. With the esterification process, however, oil yield is increased, not reduced.

Several esterifying agents, such as glycerol (14–18), MG (19,21,22), DG (20), or mono-alcohols, have been investigated to reduce FFA. Glycerol and glyceryl esters are attractive because of their capacities to enhance oil yield by esterification that results in the formation of TG. The esterification process can be carried out either by autocatalytic (without added catalyst: a high temperature-low pressure process) or by chemical or enzymatic means. Esterification is a well-investigated process, and the author has investigated and reported on the autocatalytic process using glycerol and MG (22).

Although a few reports showed the effectiveness of using glycerol, MG, and DG as esterifying agents to reduce the higher FFA level of RBO, PO, and the like, there has been no report comparing the effectiveness of these esterifying agents *vis à vis* different esterification processes. In the present study we compared the effectiveness of MG and DG to esterify the FFA of RBO, PKO, and PO in both autocatalytic and enzymatic (using immobilized *Mucor miehei* lipase) processes.

EXPERIMENTAL PROCEDURES

Materials. Crude RBO sample was supplied by Sethia Oils Ltd. (Burdwan, West Bengal, India). Crude PO and PKO were supplied by Ashwin Vanaspati Industries Ltd. (Samlaya, Vadodara, Gujarat, India). Hexane (b.p. 65–70°C), diethyl ether (b.p. 35–40°C), silica gel (TLC grade), and silicic acid (column chromatographic grade) were purchased from S.D. Fine Chemicals (Boiser, Maharastra, India). Ethyl alcohol was a product of Bengal Chemical Ltd. (Calcutta, India). Immobilized *M. miehei* lipase (Lipozyme RM) was provided by Novo Nordisk A/S (Bagsværd, Denmark). Tonsil earth was provided by Sud Chemie (Jakarta, Indonesia).

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Methods. Prior to esterification, RBO was degummed and dewaxed by a combined single-step degumming-dewaxing process as reported earlier (9). Crude PO and PKO were only degummed by using the conventional water degumming method. For water degumming, the oils were stirred continuously at 65°C for 30 min with 2% (w/w) distilled water, and then the gum was removed by centrifugation at $9000 \times g$ for 10 min. The oil was finally dried at 100°C under a vacuum of 20 mm Hg.

The degummed oil was bleached at 95°C and 20 mm Hg pressure by using 1.5% (w/w) Tonsil earth and 0.5% (w/w) activated carbon. The oil was stirred for 20 min under the conditions above and finally filtered through Whatman filter paper.

Autocatalytic esterification. Degummed, dewaxed, and bleached oil (*ca.* 50 g) was placed into a 150-mL conical flask with a ground glass B-19 joint. A predetermined amount (either stoichiometric or excess) of MG or DG was added to the oil. The oil was then slowly heated to the desired temperature (200–230ºC) at a very low pressure, 2–4 mm Hg, and stirred with a Teflon-coated magnetic stir bar (2.54 cm). Samples were drawn at different time interval (1, 2, 4 h, etc.) until no further appreciable change in FFA was observed. The color and content of unsaponifiable matter (UM) of the reaction products were measured following the standard IUPAC methods (24) for analysis of oils and fats. All reactions and analyses were carried out in triplicate, and the mean values reported.

Enzymatic esterification. For enzymatic esterification, the same procedure as just described for autocatalytic esterification was followed, but the reactions were carried out at 60°C (the optimal reaction temperature of *M. miehei*, as reported by the manufacturer) using 2.0% (w/w) immobilized Lipozyme RM enzyme.

Quantitative determination of MG, DG, and TG. The MG, DG, and TG contents of the crude oil, refined oil, and MG/DG samples were estimated by using the standard column chromatographic method. A glass column (i.d. 1.8 cm; length, 30 cm) was used in which a silicic acid (100–120 mesh size) bed was prepared from a slurry of silicic acid in hexane. MG, DG, and TG were eluted with the standard solvent system (25), and the quantity of each fraction was determined gravimetrically after evaporating the solvent.

MG and DG were prepared by alkali (NaOH)-catalyzed glycerolysis of RBO, PKO, and PO as reported earlier (22). For synthesizing MG, glycerol was taken three times (by mole) to that of oil. For DG synthesis, however, an oil-to-glycerol ratio of 2:1.5 was maintained. High yields of MG and DG were obtained, and these fractions were further purified by alcohol fractionation.

Purification of MG and DG. MG and DG were purified by using the batch alcohol fractionation process. Glycerolysis reaction mixture (100 g) was taken in a 1-L round-bottomed flask and mixed with 500 mL of 90% (v/v) ethanol. After homogenizing, the mixture was allowed to settle, and the immiscible fraction was removed and kept in a constant-temperature water bath for 1 h at 5°C and filtered through Whatman filter paper (No. 40). The solid and liquid fractions were enriched in DG and MG, respectively. The DG fraction was again dissolved in 90% ethanol (1:5, wt/vol) and recrystallized at 5°C for 1 h. The solid fraction was assayed for DG content and used for esterification. The liquid fraction, after distilling off solvent, was rich in MG. The fractionated MG and DG samples were quantified following the methods already mentioned.

Determination of FA composition. FA compositions of the crude oils were determined by GLC of the methyl esters. The procedure and conditions of GLC are detailed by De *et al.* (22).

Color, FFA content, UM, and PV were determined following the standard methods and practices of the American Oil Chemists' Society (26). Saponification and iodine values were determined following standard IUPAC methods (24).

RESULTS AND DISCUSSION

The synthesized MG samples contained 86.1–88.2% (w/w) MG, 7.3–8.5% DG, and 3.1–4.9 % TG, whereas the DG samples contained 7.0–8.8% MG, 85.9–88.8% DG, and 3.1–4.0% TG. All samples contained minor quantities of FFA (0.1–0.3%) and unidentified components (0.1-0.4%).

Table 1 shows the characteristics of oils used for esterification. All three oils had high acidity. Color, PV, and glyceride composition showed that the qualities of the crude oils were inadequate. However, saponification value, UM content, iodine value, and FA composition indicated the authenticity of the samples. Although the samples were authentic, the oils were not suitable for deacidification by conventional alkali refining due to the higher content of FFA, MG, and DG.

Table 2 shows changes in FFA content with time, as well as color and UM, during esterification of RBO, PKO, and PO with MG prepared from the corresponding oil under varying reaction conditions. Reaction conditions were varied by changing the molar ratio of FFA to MG, the reaction temperature, and the catalyst type. For any particular oil, investigation was carried out using three molar ratios (2.5:1, 2:1, and 2:1.5) of FFA present in oil to MG used. In this process the quantity of MG and DG already present in oil was not considered, and the molar ratio of 2:1 was stoichiometric. Although it is known that the use of MG in a quantity less than stoichiometric would not be very effective, the ratio was included since the oil itself contained some MG and DG (Table 1). Temperatures of 210°C for both RBO and PO, and 195°C for PKO were assumed to be optimal for autocatalytic esterification with MG, as reported earler with mowrah fat (MF) and PKO (21). Owing to the similar average MW of FA of RBO, MF, and PO, the optimal esterification temperature was assumed to be the same for all these oils. For enzymatic esterification, 60°C was used as the optimum, as recommended by the manufacturer.

The use of MG at less than the stoichiometric ratio (FFA/MG = 2.5:1) did not give the desired result under any reaction condition. Maximal reduction in FFA was observed when a 50% molar excess of MG over the stoichiometry was used, although the reduction was marginally different from that of the stoichiometric quantity. When stoichiometric or 50% excess MG was used in the autocatalytic process, FFA was re-

TABLE 1
Characteristics of Crude RBO, PKO, and PO[a]

Characteristics	Oils		
	RBO	PKO[b]	PO
FFA (%, w/w)	16.2	12.6	10.4
Color (Lovibond, 1 in. cell)	50Y + 4.8R	46Y + 5.1R	60Y + 10.5R
PV (mequiv/kg)	12.3	14.6	8.9
Saponification value (mg/g)	194.5	240.5	199.0
Unsaponifiable matter (%, w/w)	5.1	0.8	0.8
Iodine value (g/100 g)	103.0	17.5	54.2
Glyceride composition (%, w/w)			
MG	1.6	2.0	2.1
DG	7.0	5.8	6.4
TG	70.1	78.8	80.3
FA composition (%, w/w)			
Palmitic acid (16:0)	16.0	8.5	43.3
Stearic acid (18:0)	2.1	1.6	4.8
Oleic acid (18:1)	49.3	14.2	44.0
Linoleic acid (18:2)	32.6	2.5	7.9

[a]RBO, rice bran oil; PKO, palm kernel oil; PO, palm oil.
[b]PKO also contained the following FA: 8:0, 2.5%; 10:0, 4.5%; 12:0, 50.2%; 14:0, 16.0%.

duced to 1.4–2.8 and 1.3–1.9% (w/w) after 6 and 7 h, respectively. Similarly, in the enzymatic process using the *M. meihei* lipase, the FFA was reduced to 0.9–1.2 and 0.4–0.9% after 6 and 7 h, respectively. Therefore, a 6-h reaction period was judged to be optimal for this reaction as well. The UM content increased for all three oils during the autocatalytic process, as expected. In enzymatically treated oils, however, UM content was not measured since there was hardly any chance for UM content to increase. Color also darkened in the autocatalyzed process and was lighter in enzymatically esterified oils.

Autocatalytic deacidification of RBO, PKO, and PO was investigated using DG as esterifying agent. For RBO the reactions were carried out using three different FFA/DG ratios: stoichiometric (1:1), less than stoichiometric (1.25:1); and 25% excess DG (1:1.25). Table 3 indicates that use of 25% excess was required to bring down the FFA sufficiently. Of

TABLE 2
Deacidification of Different Oils with MG[a]

Oils	Moles FFA/MG	RT (°C)	Catalyst used	FFA content (%, w/w)						UM[b] (%, w/w)	Color[c] (Y,R) in 1-in. cell
				0 h	1 h	2 h	4 h	6 h	7 h		
RBO	2.5:1.0	210	None		14.1	10.6	8.3	5.1	4.5	2.5	33.0, 2.1
RBO	2.0:1.0	210	None		12.1	10.2	6.1	2.8	1.8	2.4	35.2, 2.2
RBO	2.0:1.5	210	None		11.3	9.6	5.0	2.4	1.9	2.4	30.0, 2.3
RBO				16.2							
RBO	2.5:1.0	60	*M. miehei*		13.4	11.2	7.7	4.0	3.1	—	10.1, 1.8
RBO	2.0:1.0	60	*M. miehei*		12.0	8.6	4.0	1.2	0.8	—	11.1, 1.7
RBO	2.0:1.5	60	*M. miehei*		10.1	8.0	5.0	1.2	0.6	—	10.5, 1.8
PKO	2.5:1.0	195	None		10.6	8.8	4.8	3.0	2.4	1.2	28.5, 3.1
PKO	2.0:1.0	195	None		9.6	7.6	4.2	2.1	1.8	1.3	26.0, 3.0
PKO	2.0:1.5	195	None		8.2	5.2	3.0	1.4	1.3	1.2	30.2, 3.3
PKO				12.6							
PKO	2.5:1.0	60	*M. miehei*		10.0	7.6	4.0	1.8	1.4	—	17.1, 1.2
PKO	2.0:1.0	60	*M. miehei*		8.8	6.8	3.0	1.0	0.9	—	18.6, 1.3
PKO	2.0:1.5	60	*M. miehei*		7.6	5.8	2.8	1.0	0.4	—	18.3, 1.2
PO	2.5:1.0	210	None		8.6	6.3	3.9	2.5	2.1	1.1	19.8, 1.5
PO	2.0:1.0	210	None		7.9	5.9	3.1	2.0	1.8	1.1	23.1, 1.3
PO	2.0:1.5	210	None		7.1	4.9	2.9	1.8	1.4	1.0	20.5, 1.5
PO				10.4							
PO	2.5:1.0	60	*M. miehei*		7.9	6.0	3.1	2.0	1.9	—	23.0, 5.3
PO	2.0:1.0	60	*M. miehei*		7.4	5.0	2.9	1.1	0.9	—	25.1, 5.5
PO	2.0:1.5	60	*M. miehei*		6.6	4.9	2.5	0.9	0.5	—	23.9, 5.3

[a]Reactions carried out at 4–6 mm Hg. RT, reaction temperature; UM, unsaponifiable matter; *M. miehei, Mucor miehei;* for other abbreviations see Table 1.
[b]UM contents (%, w/w) in RBO, PKO, and PO were 1.2, 0.3, and 0.2, respectively.
[c]Colors of RBO, PKO, and PO before deacidification were 20.0Y + 1.9R, 23.0Y + 2.1R, and 30.8Y + 7.0R, respectively.

TABLE 3
Effect of Temperature on the Autocatalytic Esterification of RBO Using DG[a]

Oils	Molar ratio of FFA/DG	Temperature (°C)	FFA (%, w/w) 0 h	2 h	4 h	6 h	7 h
RBO	1.25:1	210	16.2	13.2	8.6	5.2	4.8
RBO	1.25:1	220	16.2	11.6	7.5	4.6	3.7
RBO	1.25:1	230	16.2	10.5	6.0	2.5	2.4
RBO	1:1	210	16.2	10.4	5.6	3.2	2.8
RBO	1:1	220	16.2	9.7	5.3	2.6	2.5
RBO	1:1	230	16.2	8.2	5.3	2.0	2.0
RBO	1:1.25	210	16.2	9.0	5.9	2.6	1.9
RBO	1:1.25	220	16.2	9.6	5.8	1.8	1.8
RBO	1:1.25	230	16.2	8.0	6.1	1.8	1.8
PKO	1.25:1	200	12.6	8.1	5.8	3.2	3.0
PKO	1.25:1	210	12.6	7.9	4.3	2.4	2.0
PKO	1.25:1	220	12.6	8.7	5.2	3.3	3.0
PKO	1:1	200	12.6	8.5	5.9	3.3	3.0
PKO	1:1	210	12.6	7.9	4.3	2.4	2.0
PKO	1:1	220	12.6	8.0	4.4	2.2	1.9
PKO	1:1.25	200	12.6	8.4	5.3	2.6	2.4
PKO	1:1.25	210	12.6	7.0	4.0	1.2	0.8
PKO	1:1.25	220	12.6	7.8	4.3	1.2	1.0

[a]Reactions carried out at 4–6 mm Hg. For abbreviations see Table 1.

TABLE 4
Deacidification of Different Oils with DG[a]

Oils	Moles FFA/DG	RT (°C)	Catalyst used	FFA content (%, w/w) 0 h	1 h	2 h	4 h	6 h	7 h	UM[b] (%, w/w)	Color[c] (Y,R) in 1-in. cell
RBO	1.25:1	220	None		13.6	11.6	7.5	4.6	3.7	2.5	30.0, 2.3
RBO	1:1	220	None		12.3	9.7	5.3	2.0	1.7	2.6	33.4, 2.6
RBO	1:1.25	220	None		12.0	9.6	5.8	1.8	1.8	2.5	34.1, 2.5
RBO				16.2							
RBO	1.25:1	60	*M. miehei*		12.1	10.1	6.3	2.8	2.6	—	14.6, 1.7
RBO	1:1	60	*M. miehei*		12.3	8.9	5.0	1.0	0.8	—	13.1, 1.9
RBO	1:1.25	60	*M. miehei*		12.2	9.0	4.1	0.8	0.3	—	13.5, 1.8
PKO	1.25:1	210	None		10.9	8.1	5.8	3.2	3.0	1.1	28.9, 3.2
PKO	1:1	210	None		10.3	7.9	4.3	2.4	2.0	1.0	28.5, 3.5
PKO	1:1.25	210	None		9.8	7.0	4.0	1.2	0.8	1.1	30.5, 2.9
PKO				12.6							
PKO	1.25:1	60	*M. miehei*		9.6	8.1	4.8	3.8	2.6	—	20.1, 1.3
PKO	1:1	60	*M. miehei*		9.5	7.8	4.3	1.4	0.8	—	19.7, 1.2
PKO	1:1.25	60	*M. miehei*		8.7	7.0	4.0	0.6	0.5	—	20.3, 1.3
PO	1.25:1	220	None		8.6	6.8	4.5	3.0	3.0	1.1	22.2, 1.6
PO	1:1	220	None		8.3	6.2	4.0	1.8	1.6	1.1	20.3, 1.4
PO	1:1.25	220	None		8.0	5.8	3.2	1.6	1.5	1.2	23.5, 1.2
PO				10.4							
PO	1.25:1	60	*M. miehei*		7.8	5.7	3.8	2.9	2.7	—	21.3, 5.6
PO	1:1	60	*M. miehei*		8.0	5.7	3.5	0.6	0.4	—	23.1, 5.3
PO	1:1.25	60	*M. miehei*		7.8	5.8	3.0	0.4	0.3	—	26.5, 5.2

[a]Reactions carried out at 4–6 mm Hg.
[b]UM contents (%, w/w) in RBO, PKO, and PO were 1.2, 0.3, and 0.2, respectively.
[c]Colors of RBO, PKO, and PO before deacidification were 20.0Y + 1.9R, 23.0Y + 2.1R, and 30.8Y + 7.0R, respectively. For abbreviations see Tables 1 and 2.

the three temperatures studied, 220°C was the best for RBO. Under this condition the FFA content was reduced to 1.8% after 6 h. Because of the nearly same average MW of FA in RBO and PO, the optimal reaction temperature for PO was assumed to be 220°C. The optimal reaction temperature and FFA/DG molar ratio were determined for esterification of PKO. In this case, the optimal ratio of FFA/DG was also found to be 1:1.25, and the optimal reaction temperature was 210°C. This might be due to the shorter average chain length of the FA in PKO than in RBO and PO.

Finally, deacidification of RBO, PKO, and PO using DG as an esterifying agent was studied (Table 4) both autocatalytically and enzymatically (at 60°C). The use of DG at less than the stoichiometric ratio did not reduce the FFA to an acceptable range as occurred in deacidification using MG (Table 2). In both the enzymatic and the autocatalytic processes, when less than the stoichiometric amount of DG was used, the FFA was reduced to a range of 2.6–3.9% after 7 h. The reduction in FFA was less in the autocatalytic process than in the enzymatic process for any particular time period or reaction temperature. Although the enzymatic reaction rates depend on the amount of enzyme used, the present investigation was carried out by using 2% (w/w) enzyme, since that level had been optimized in previous work (19). When the FFA contents were compared after 6 and 7 h, 6 h seemed to be long enough due to insignificant FFA changes thereafter. When the results of enzymatic reactions were compared with those for the nonenzymatic process, the enzymatic process reduced the FFA content in all three oils at any reaction condition. In the enzymatic process using 25% excess DG, the FFA content was reduced to 0.4–0.8% at 6 h and 0.3–0.8% after 7 h. FFA, however, decreased to 1.2–1.8% at 6 h and to 1.0–1.8% at 7 h in the autocatalytic process. When the stoichiometric amount of DG was used, the FFA was reduced to 0.6–1.4% in the enzymatic process and 1.8–2.2% in the autocatalytic process at 6 h.

The UM contents and the colors of treated oils were also measured when DG was used as the esterifying agent. Again due to treatment at higher temperature in the autocatalytic process, the UM content as well as color increased substantially (Table 4). The color, however, was decreased in the enzymatic process, which may be due to adsorption of pigments onto the surface of the immobilized enzyme.

When comparing deacidification using MG (Table 2) and DG (Table 4), a slightly better FFA reduction was observed when DG was used in the enzymatic reaction. The results also showed that MG and DG performed similarly under the same reaction conditions except that DG was probably slightly more effective owing to its greater solubility in oil.

ACKNOWLEDGMENTS

The author is thankful to his students Vinay Patel, Jignesh Patel, and Tushar Patel for their help in developing the standard method for purifying MG and DG by ethanol fractionation.

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[Received November 21, 2004; accepted January 29, 2006]