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ORIGINAL PAPER

Utilization of Olive-Pomace Oil for Enzymatic Production of Cocoa Butter-like Fat

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Abstract Refined olive-pomace oil (ROPO) was utilized as a source for the production of a cocoa butter (CB)-like fat. Immobilized *sn*-1,3 specific lipase-catalyzed acidolysis of ROPO with palmitic (PA) and stearic (SA) acids was performed at various substrate mole ratios (ROPO:PA:SA) to produce major triacylglycerols (TAGs) of CB. Products obtained for various substrate mole ratios were compared to a commercial CB in terms of TAG content, melting profile, solid fat content (SFC) and microstructure. The fat produced at a substrate mole ratio of 1:2:6 was the most similar to CB. The product contained 11% POP, 21.8% POS, 15.7% SOS while commercial CB contained 18.9% POP, 33.1% POS and 24.7% SOS. The product had a melting peak of 29.9 °C while CB had one of 28.5 °C. Polarized light microscope (PLM) images showed that fat crystal network microstructures of this product and CB were very similar.

Keywords Olive-pomace oil · Cocoa butter-like fat · Acidolysis · Thermal characteristics · Microstructure

Introduction

The commercial value and functional properties of fats and oils depend not only on the fatty acid composition, but also on the distribution of fatty acids on the glycerol backbone [1]. Lipase-catalyzed acidolysis reactions are widely used for lipid modifications to improve functional properties of fats and oils, e.g., conversion of cheap commercial oils to high value added products [2]. Production of cocoa butter (CB)-like fats by lipase-catalyzed acidolysis of fats and oils is a good way of producing value added products.

CB is a highly valued ingredient primarily used in the chocolate industry. Due to its unique composition, CB gives desirable physical properties to the manufactured product, e.g. gloss, snap, melting properties, etc. [3]. CB is composed of three main TAGs: 1,3-dipalmitoyl-2-oleoyl-glycerol (POP); 1(3)-palmitoyl-3(1)stearoyl-2-oleoyl-glycerol (POS); 1,3-distearoyl-2-oleoyl-glycerol (SOS); with oleic acid at the *sn*-2 position of glycerol backbone [4]. Because of the high cost and fluctuations in the supply and demand, industry used alternatives (i.e., cocoa butter equivalents, CBEs) with similar TAG composition instead of CB.

Production of CBEs by enzymatic acidolysis can be done by using *sn*-1,3 specific lipases that catalyze the incorporation of palmitic acid (PA) and stearic acid (SA) at the *sn*-1,3 positions of a source oil containing oleic acid at the *sn*-2 position until a similar composition of CB is obtained.

There are many studies reporting the production of CBEs from different sources such as lard, tea seed oil, palm oil midfraction, sal fats, mango fat, illipe fat, kokum fat and shea oil [5–7]. The chemical composition of refined olive-pomace oil (ROPO) does not differ from refined olive oil. So, olive-pomace oil can also be considered as a good potential source for CB-like fat production because of its high *sn*-2 position oleic acid content and low cost. Olive-pomace is a by-product of virgin olive oil processing and is of varying importance to all of the countries of the Mediterranean basin where olives are grown [8]. The oil extracted from olive-pomace by means of a solvent is called olive-pomace oil. After refining, this oil may be used for food applications.

The objective of this study was to observe the effect of varying substrate mole ratios on the characteristics of

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products obtained by enzymatic acidolysis to produce CB-like fat from ROPO.

Materials and Methods

Materials

ROPO was kindly provided by Bilginoglu Marbil Yağ San. ve Tic. AŞ., İzmir, Turkey. TAG standards (triolein (OOO), POP, POS and SOS) and fatty acid standards were obtained from Sigma Chemical Co. (St. Louis, MO). PA (≥98% purity) and SA (≥97% purity), porcine pancreatic lipase (type II, crude) (EC 3.1.1.3), silica gel (SG 60, 70–230 mesh), thin layer chromatography (TLC) plates (Kieselgel G) were obtained from Merck (Darmstadt, Germany). Immobilized *sn*-1,3 specific lipase (Lipozyme IM, immobilized from *Mucor miehei*, 140 U/g) was purchased from Fluka Chemie GmbH. Acetone, acetonitrile and *n*-hexane were purchased from Sigma-Aldrich. All solvents used were of HPLC grade. All other reagents and solvents were of analytical or chromatographic grade.

Enzymatic Acidolysis

Acidolysis reactions of ROPO with PA and SA were performed at varying mole ratios of PA and SA, keeping the mole ratio of ROPO constant, (ROPO:PA:SA; 1:1:3, 1:2:3, 1:3:3, 1:3:1, 1:3:2, 1:2:6, 1:3:9, 1:4:12), 20% enzyme load (based on weight of substrates), 45 °C temperature and time up to 8 h. Selection of substrate mole ratios, enzyme load and reaction temperature were based on our previous studies [9]. Trials have shown that better results are obtained in the absence of water (data not shown). For this reason, the reactions were carried out in the absence of water. The weight of the substrates refers to the sum of the weights of ROPO, PA and SA in a reaction mixture. The weights of PA and SA were adjusted according to the initial amount of ROPO. The molecular weight of ROPO was considered to be same as that of OOO. The reaction mixtures were dissolved in 50 mL *n*-hexane in 100-mL Erlenmeyer flasks and incubated in a rotary incubator (New Brunswick Scientific, Nova 40, USA) at 200 rpm. Aliquots of 50 μL were withdrawn at specific time intervals from the reaction mixtures into glass vials and stored at −20 °C prior to analysis. All reactions were performed in duplicate.

Isolation of TAGs

The TAGs (CB-like fat) that were produced were isolated in two steps. First the mixture obtained from the reaction was neutralized to remove free fatty acids, and then it was purified by silica gel column chromatography. Neutralization was done in a similar manner to the method of Lee and Akoh [10] with minor modifications. The reaction mixture (6–8 g) was mixed with 150 mL hexane, 1 mL phenolphthalein solution and a required amount of 0.5 N KOH in 20% (v) ethanol (B mL) in a separatory funnel. The separatory funnel was shaken, and the upper phase was collected. The lower phase was washed again with 50 mL hexane and the upper phase was again collected. Then, 2.66 × B mL of 0.5 N KOH in 20% (v) ethanol and 1.33 × B mL of saturated NaCl solution was added to collected upper phase. After being shaken, the upper phase was collected and hexane was evaporated to obtain the neutralized product containing TAGs, diacylglycerols (DAGs) and monoacylglycerols (MAGs). The neutralized product was centrifuged in case soap traces were present.

Then, TAGs of the neutralized product were separated from MAGs and DAGs by column chromatography on silica gel (SG 60, 70–230 mesh, Merck). 1.6–2.0 g of the neutralized product was dissolved in 20 mL petroleum ether:diethylether (87:13, v/v) and eluted through the silica column with petroleum ether:diethylether (87:13, v/v). Then, the solvent was evaporated and the purified reaction product (PRP) was obtained [11].

sn-2 Fatty Acid Analysis

The fatty acid composition at the *sn*-2 position of ROPO was determined using the method developed by Brockerhoff [12]. The oil was hydrolyzed with porcine pancreatic lipase, a lipase selective for *sn*-1,3 positions of TAGs. The products of lipolysis were separated by TLC plates that were developed with petroleum ether:diethyl ether:acetic acid (70:30:1, v/v/v). The *sn*-2 MAG band was scraped off and was extracted with diethyl ether. The extracted lipid was analyzed by GC as described below.

GC Analysis

sn-2 MAGs of ROPO were converted to fatty acid methyl esters (FAME) and then analyzed by an Agilent 6,890 series gas chromatograph equipped with an HP88, 100 m × 0.250 mm × 0.25 μm capillary column (Agilent Technologies Inc., CA, USA). One microliter of the FAME mixture was injected into the GC system with a split/splitless injector and a flame ionization detector (FID). The inlet temperature was 250 °C and the split ratio was 50:1. The carrier gas was hydrogen at 2.0 mL/min constant flow. The oven temperature was programmed at an initial 120 °C, held for 1 min, followed by an increase of 10 °C/min up to 175 °C, held for 10 min, followed by an increase of 5 °C/min up to 210 °C, held for 5 min, followed by an increase of 5 °C/min up to 230 °C and held for 5 min. The

detector was set at 280 °C with 450 mL/min airflow, 40 mL/min hydrogen flow, and 30 mL/min helium makeup flow. Fatty acid standards were used to identify the peaks.

HPLC Analyses

The time course of the acidolysis reactions was followed by analysis of the reaction mixtures for their POP, POS and SOS contents by a reversed phase HPLC. HPLC system consisting of quadratic pump (model LC-10ADVP; Shimadzu, Japan) equipped with a column (Sphereclone 5 μm ODS (2), 250 × 4.6 mm; Phenomenex, USA) with an accompanying guard column (40 × 3-mm id) of the same phase and an ultraviolet (UV) detector (Hewlett Packard Series 1100). Elution was monitored by UV absorbance at 215 nm. The mobile phase consisted of acetone and acetonitrile (50:50, v/v) with a flow rate of 1.0 mL/min. The column temperature was set at 50 °C with a column heater (Eppendorf CH-30 column heater). All TAG contents were given in percentage area.

Thermal Characteristics

Melting profile and SFC% of the purified products were analyzed by DSC (Perkin Elmer DSC-6, Norwalk, CN, USA). DSC procedure of Siew and Faridah [13] was followed. The samples were completely melted at 80 °C before being weighed. A 10 ± 0.5-mg of the molten sample was hermetically sealed in an aluminium pan, with an empty pan as reference. Samples were initially heated to 80 °C and held at this temperature for 10 min in the DSC instrument to erase the previous thermal history. The samples were then cooled to −60 °C at 40 °C/min. At the end of the cooling, the samples were heated from −60 to 80 °C at 10 °C/min. The SFC at various temperatures was calculated from the data of the DSC heating thermograms by partial integration according to Tieko and Aparecida [14]. The partial areas were obtained directly from DSC software (Pyris version 7.0) calculation.

Microstructure

The crystal network microstructure of the purified products was examined by a polarized light microscope (PLM) (Olympus BX51, Olympus Optical Co., Ltd., Tokyo, Japan) equipped with a Pixera color video camera (model PVC 100C, Los Gatos, CA, USA). A static crystallization method similar to the method of Narine and Marangoni [15] was used. Samples were melted and held at 80 °C for 10 min in order to erase the crystal memory and 20 μL of the melt placed on a glass microscope slide which was heated to the same temperature. A glass coverslip at the same temperature as the sample was placed on top of the samples. Samples were then allowed to crystallize for 48 h at room temperature (21–23 °C). A 40× lens was used to image the grayscale photograph of the samples.

Statistical Analysis

Statistical analysis of the obtained data was carried out by using the SPSS (version 10.0) package program at a 95% confidence interval [16].

Results and Discussion

CB-Like Fat Production

Although the content of major TAGs of CB in ROPO was very low, the *sn*-2 oleic acid content of TAGs in ROPO was quite high (85%). This makes ROPO a suitable source for CB-like fat production using Lipozyme IM, *sn*-1,3 specific lipase, which incorporates PA and SA to the 1 and 3 positions of the TAGs of ROPO to produce a fat rich in POP, POS and SOS. Firstly, acidolysis reactions were carried out at various substrate mole ratios to obtain the best empirical substrate mole ratio giving a TAG composition similar to CB. The commercial CB used in this study contained 18.9% POP, 33.1% POS and 24.7% SOS.

Figures 1a, b, c show the change in POP, POS and SOS contents with time at different substrate mole ratios. It has been observed that there is a similar trend of product formation for all TAGs. Contents of target TAGs (POP, POS and SOS) increased with increasing substrate mole ratio and reaction time. POP, POS and SOS contents increased up to 6 h and remained constant after this time. Among the studied substrate mole ratios (1:1:3, 1:2:3, 1:3:3, 1:3:1, 1:3:2), 1:1:3 gave the most similar TAG content of CB. At 1:1:3 substrate mole ratio and 6 h, product contained 8.8% POP, 14.4% POS and 12.2% SOS. Similar to the results of Liu et al. [4], it has been observed that SA is a poor acyl donor for this system compared to PA.

As shown in Fig. 2, doubling the substrate mole ratio from 1:1:3 to 1:2:6 had a significant effect on the yield ($p < 0.05$). The product contained 11% POP, 21.8% POS, 15.7% SOS at the 1:2:6 substrate mole ratio. Further increases in the substrate mole ratio (1:3:9, 1:4:12) did not have any significant effect on the yield ($P > 0.05$). High substrate mole ratios are not feasible economically. Purification of the products obtained from 1:3:9 and 1:4:12 caused a large amount of neutral oil loss. This means an extra process and cost. Therefore, 1:2:6 was selected as the best substrate mole ratio. Approximately 50% of the fat produced has major TAGs of CB. Abigor et al. [17] obtained a yield of 45.6% for the production of CB-like fat from palm oil and hydrogenated soybean oil. Chang et al. [18] reported a yield

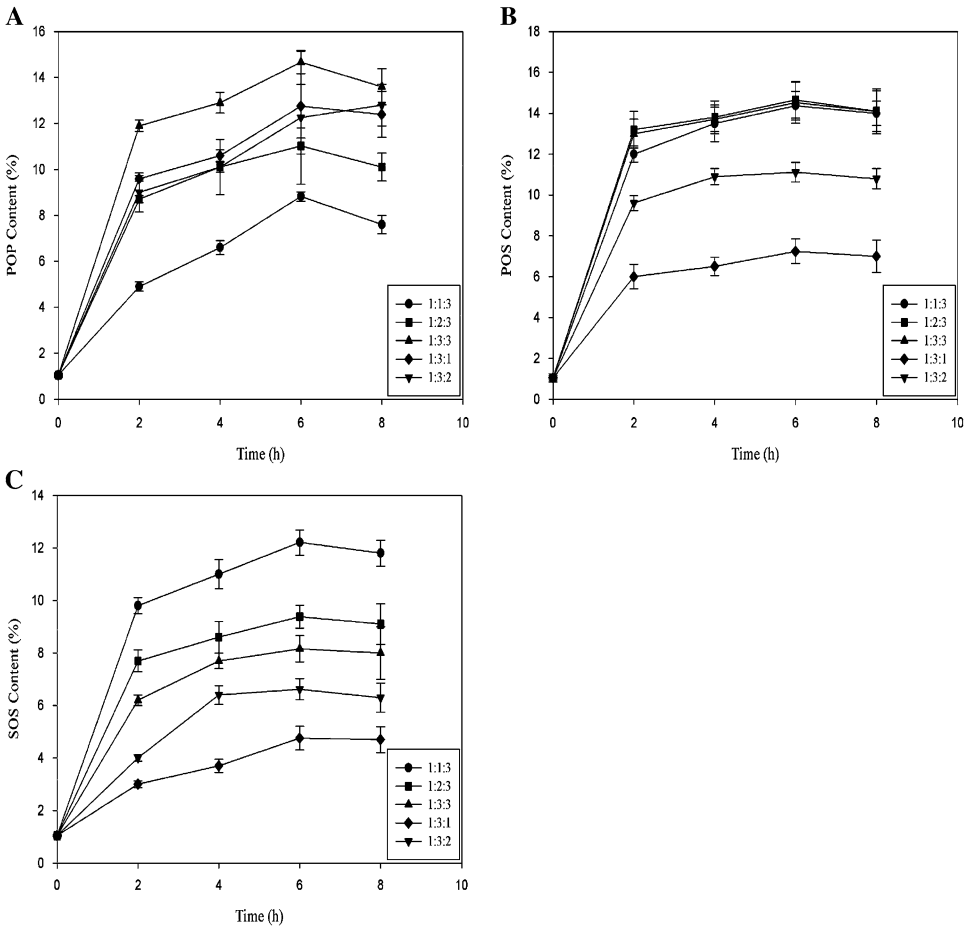

Fig. 1 Effect of substrate mole ratio on production of POP (**a**), POS (**b**), and SOS (**c**) as a function of time

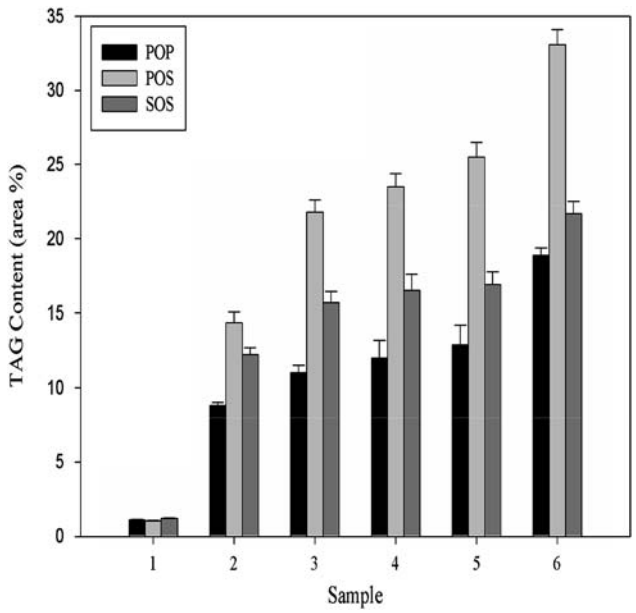

Fig. 2 Content of target TAGs of ROPO, CB and the 1:1:3 substrate mole ratio and multiples thereof. (1) ROPO, (2) 1:1:3, (3) 1:2:6, (4) 1:3:9, (5) 1:4:12 and (6) CB

of CB-like fat of 19% when fully hydrogenated cottonseed oil was interesterified with olive oil.

Melting Characteristics

Figure 3 shows the melting profiles of the products from the mole ratio study. All samples exhibited more than one endothermic peak, indicating that the samples contained different melting components. The major melting peak of each sample was labeled A. The peaks from the lower-melting polymorphs seemed to change gradually toward higher melting ranges as the number of moles of PA and SA in the substrate ratio increased. The increase in the content of major TAGs has the effect of increasing the melting peak. As seen from Fig. 3, 1:1:3 gave the best melting property among all empirical substrate mole ratios (1:1:3, 1:2:3, 1:3:3, 1:3:1, 1:3:2).

Increasing the substrate mole ratio from 1:1:3 to 1:2:6 shifted the melting peak from 17.7 to 29.9 °C, and made it sharper and more pronounced. Our product gave its major peak at 29.9 °C while CB gave its at 28.5 °C. As seen on graph, a slight shoulder is seen on the peak of CB, similar

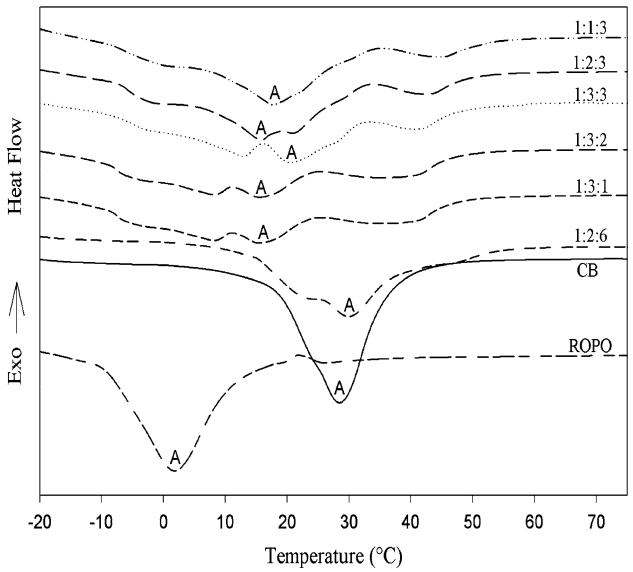

Fig. 3 DSC melting profiles of CB and products of the mole ratio study

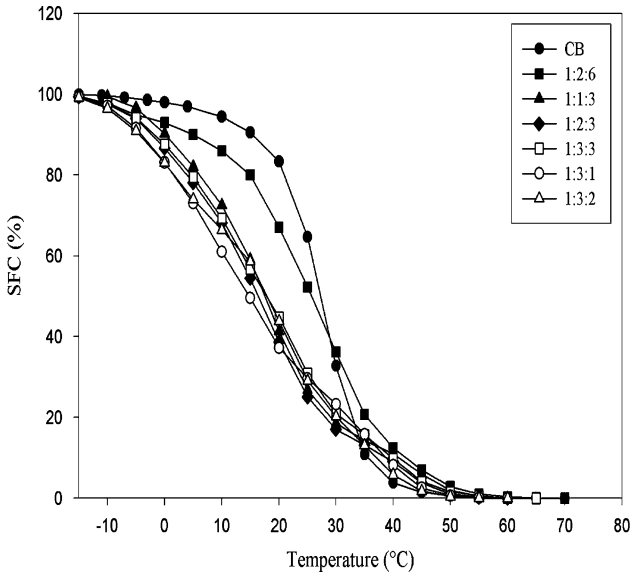

Fig. 4 Changes in the SFC of CB and the products of the mole ratio study as a function of temperature

to the one on our product's major melting peak. The big shoulder and the broad peak of the product of 1:2:6 substrate mole ratio is due to the presence of some lower-melting point TAGs of ROPO which were not converted to target TAGs. CB samples may have different compositions depending on the origin, so, they may have different melting behaviors. Solis-Fuentes and Duran-de-Bazua [19] reported the thermogram of CB similar to our product with a low melting point fraction, with a maximum temperature of 11.64 °C. Undurraga et al. [5] reported a CB melting profile with two minor low melting point peaks between 5 and 15 °C, and the other between 15 and 23 °C.

Solid Fat Content

For the characterization of fats, rather than the melting peak temperature, melting behavior is important. Melting behavior must have a sharp and narrow range of melting for a CB-like fat. Figure 4 shows the change in the SFC profiles of the fat samples from the mole ratio study as a function of temperature. Products of all substrate mole ratios exhibited lower SFC than that of CB at all temperatures. Among all the SFC profiles, the SFC profile of 1:2:6 was similar to that of CB. The difference between SFC profiles of CB and the product of 1:2:6 was due to unconverted TAGs of ROPO, as they caused a broader melting peak on the DSC thermogram. All samples and CB became completely liquid at 55 °C.

Microstructure

The microstructure of fat crystal networks has a great effect on macroscopic properties [20]. Fats having similar thermal properties may have different rheological properties if their microstructures are different. So, microstructure must also be considered to determine the macroscopic properties of a fat crystal network [15]. Grayscale PLM photographs of CB and samples are shown in Fig. 5. The solid phase appears white or gray while the liquid phase appears black. Because under polarization, the anisotropic solid phase of the network does not refract light in the same way as the isotropic liquid phase does [15]. As seen from Fig. 5, the product obtained from the substrate mole ratio of 1:2:6 had the crystal network characteristics most similar to those of CB. As can be seen from the images, CB has a well-defined microstructural network. This well-defined network is due to the strong and defined interactions between microstructural elements leading to a three-dimensional arrangement with similar intermicrostructural distances [15]. Narine and Marangoni [15] stated that the highly ordered nature of TAGs in CB results in this regular network. We can conclude that the most similar TAG content to CB was obtained from the mole ratio of 1:2.6 even only by visual inspection of the PLM images. Images obtained for other fats have an irregular, random spatial distribution of crystals showing the presence of asymmetry in the molecular structure of TAGs. The irregular nature of the crystals shows the presence of weak interactions between them.

Conclusion

The highest yield (11% POP, 21.8% POS, 15.7% SOS) was obtained at 1:2:6 substrate mole ratio, 20% enzyme load,

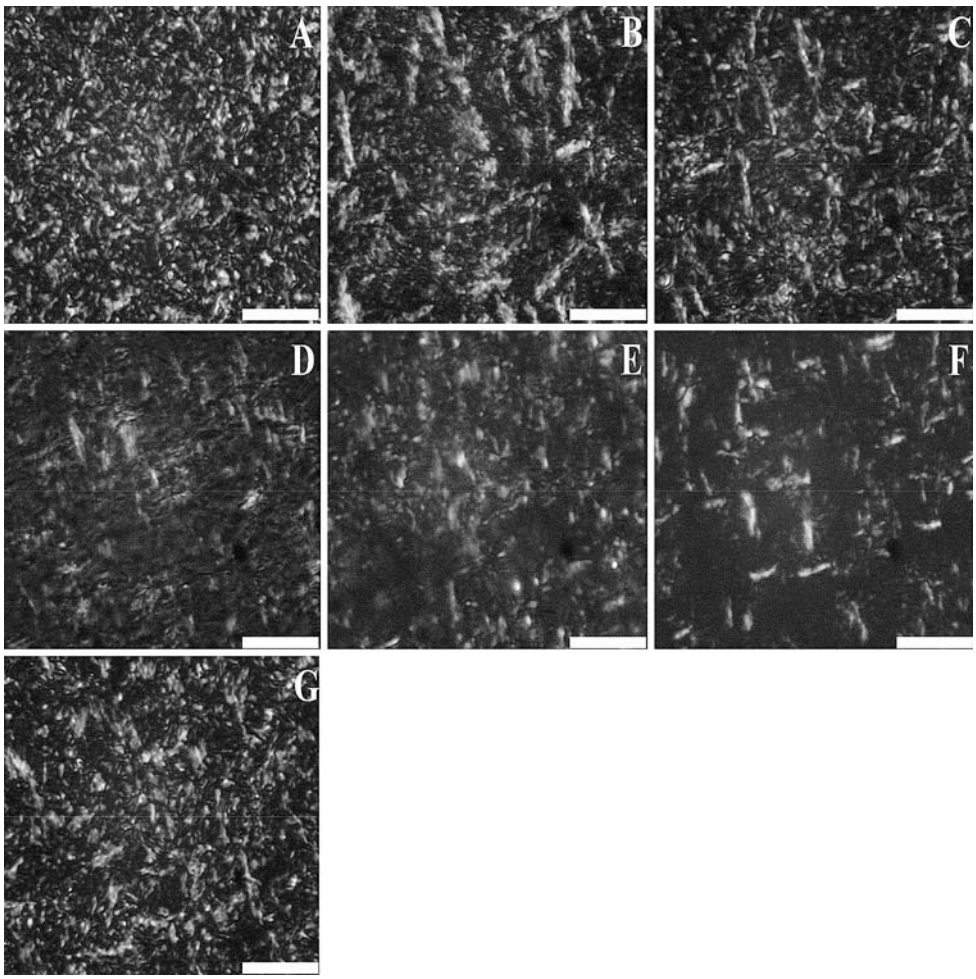

Fig. 5 Polarized light micrograph grayscale images of CB and products of the mole ratio study. **a** CB, **b** 1:1:3, **c** 1:2:3, **d** 1:3:3, **e** 1:3:1, **f** 1:3:2 and **g** 1:2:6. The horizontal length of the inset bar represents 50 μm

6 h reaction time and 45 °C reaction temperature. All methods used for product characterization confirmed each other. The product produced here has the potential to add value to a by-product of the olive oil industry. Enzymatically modified ROPO may be useful in the confectionary industry as a partial CB replacement. Replacement of CB up to a certain level with this product may reduce the costs of confectionary manufacturers.

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