

Analysis of Monoglycerides, Diglycerides, Sterols, and Free Fatty Acids in Coconut (*Cocos nucifera* L.) Oil by ^{31}P NMR Spectroscopy

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Phosphorus-31 nuclear magnetic resonance spectroscopy (^{31}P NMR) was used to differentiate virgin coconut oil (VCO) from refined, bleached, deodorized coconut oil (RCO). Monoglycerides (MGs), diglycerides (DGs), sterols, and free fatty acids (FFAs) in VCO and RCO were converted into dioxaphospholane derivatives and analyzed by ^{31}P NMR. On the average, 1-MG was found to be higher in VCO (0.027%) than RCO (0.019%). 2-MG was not detected in any of the samples down to a detection limit of 0.014%. On the average, total DGs were lower in VCO (1.55%) than RCO (4.10%). When plotted in terms of the ratio [1,2-DG/total DGs] versus total DGs, VCO and RCO samples grouped separately. Total sterols were higher in VCO (0.096%) compared with RCO (0.032%), and the FFA content was 8 times higher in VCO than RCO (0.127% vs 0.015%). FFA determination by ^{31}P NMR and titration gave comparable results. Principal components analysis shows that the 1,2-DG, 1,3-DG, and FFAs are the most important parameters for differentiating VCO from RCO.

KEYWORDS: ^{31}P NMR spectroscopy; virgin coconut oil; monoglycerides; diglycerides; sterols; free fatty acids; principal components analysis

INTRODUCTION

Coconut oil is a vegetable oil obtained from the nut of the tropical palm tree, *Cocos nucifera* L. Refined coconut oil (RCO) is produced by extraction of the oil from dried copra, followed by chemical refinement, bleaching, and deodorization (1). Virgin coconut oil (VCO), on the other hand, is produced from the fresh mature coconut meat and is processed by mechanical and natural processes (2). There are several methods used to produce VCO, such as natural separation, expeller, and centrifugation (3). VCO has recently gained considerable attention as a health product because of its reputed beneficial effects.

Codex Alimentarius (4) provides standards for composition and quality factors that differentiate coconut oil from other vegetable oils but not to differentiate VCO from RCO. We recently reported that VCO can be differentiated from RCO oil using the following standard tests: % moisture by Karl Fischer, % volatile matter volatile at 120 °C, and peroxide value (5).

The major constituents of plant seed oils are triglycerides (TGs), which account for the majority of the weight of the oil, while the minor constituents can include monoglycerides (MGs), diglycerides (DGs), sterols, and free fatty acids (FFAs). The presence of the minor constituents can vary depending on the processing, storage, and age of the oil.

Various chromatographic and hyphenated mass spectrometric methods have been developed for the analysis of these compounds in vegetable oils (6–11). ^1H and ^{13}C nuclear magnetic resonance (NMR) techniques have been used to quantify and profile refined and virgin olive oils by analysis of TGs, DGs, MGs, and other minor components (12–17).

Since many of the minor components of interest in vegetable oils are either alcohols or carboxylic acids, another strategy is to convert these components into derivatives with an NMR-active nucleus other than ^1H or ^{13}C . MGs, DGs, sterols, and

Table 1. ^{31}P NMR Chemical Shifts of the Phospholane Derivatives of Monoglycerides, Diglycerides, Sterols, and Free Fatty Acids in Virgin Olive Oil^a and in Coconut Oil^b

type of compd	virgin olive oil, from Spyros and Dais (19)		virgin coconut oil, from this work	
	ref compd	^{31}P NMR, ppm	ref compd	^{31}P NMR, ppm
1-monoglyceride	1-monoolein	146.4; 147.6	1-monolaurin	146.8; 148.5
2-monoglyceride	2-monoolein	148.0	2-monolaurin	148.9
1,2-diglyceride	1,2-diolein	148.2	1,2-dipalmitin	149.0
1,3-diglyceride	1,3-diolein	146.7	1,3-dipalmitin	147.5
sterol	β -sitosterol	145.0	cholesterol	145.7
fatty acid	stearic acid	134.8	lauric acid	135.6

^a ^{31}P NMR chemical shifts of samples in virgin olive oil reported by Spyros and Dais (19). ^b ^{31}P NMR chemical shifts of samples in coconut oil obtained from this work.

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Table 2. Detection Limits and Linearity of Calibration for ^{31}P NMR Analysis of Model Compounds for the Analysis of Monoglycerides, Diglycerides, Sterols, and Free Fatty Acids

compd	detection limit, % w/w	coefficient of determination, r^2
1-monolaurin	0.0142	0.988
2-monolaurin	0.0142	0.997
1,2-dipalmitin	0.1932	0.995
1,3-dipalmitin	0.1398	0.902
lauric acid	0.0097	0.999
cholesterol	0.0601	0.994

FFAs have thus been converted into phosphorus-containing dioxaphospholane derivatives and analyzed by ^{31}P NMR (18). Spyros, Fronimaki, and co-workers (19–21) demonstrated that the ^{31}P NMR method is comparable to chromatographic methods for the measurement of total DGs and that it can unambiguously detect and quantify 1-MGs, 2-MGs, 1,2-DGs, 1,3-DGs, sterols, and FFAs in olive oil samples. Dais and co-workers (22) showed that ^{31}P NMR spectroscopy gives comparable results with conventional analytical methods, such as titration, GC, and HPLC, for the measurement of various major and minor constituents in olive oil.

Fronimaki, Spyros, and co-workers (20, 23) showed that the ratio [1,2-DG/total DGs] when plotted against total DGs gives a convenient grouping of virgin olive oils and refined olive oils. Vigli and co-workers (21) used ^1H and ^{31}P NMR and multivariate statistical analysis to classify 13 types of vegetable oil, including RCO, and to detect adulteration in virgin olive oil.

In this paper, we investigate the use of ^{31}P NMR for the analysis of monoglycerides, diglycerides, sterols, and free fatty acids as a method for differentiating VCO from RCO. The analytical data were subjected to principal components analysis (PCA) to determine groupings among the samples and to identify which parameters are most responsible for the grouping.

MATERIALS AND METHODS

Coconut Oil Samples. Twenty-four coconut samples were analyzed: commercial VCO ($n = 16$) and RCO ($n = 8$). The 16 commercial VCO samples are broken down into the following types: expeller process ($n = 4$), centrifuge ($n = 5$), natural separation with heat ($n = 3$), and natural separation, no heat ($n = 4$). In the method of natural separation, the pressed coconut emulsion is allowed to stand for 15–18 h to allow for the separation of the oil and aqueous layers. The resulting oil layer is subjected to mild heating (at 70–100 °C), or it can be allowed to stand for a longer period at room temperature. Samples of VCO were provided by the Virgin Coconut Oil Producers and Marketers Association, Inc. or obtained directly from commercial outlets. Samples of RCO were purchased from supermarkets. The VCO and RCO samples were 6–8 months of age at the time of NMR analysis.

Preparation of Coconut Oil Blank Matrix. A coconut oil blank matrix was prepared as follows: RCO was washed with sodium hydroxide (RG, J.T. Baker, NJ) solution. It was then passed through 60 Å silica gel (Merck, Darmstadt, Germany) and then dried in a vacuum oven at 100 °C and 101 Pa. The blank coconut oil matrix was stored over calcium chloride (CaCl_2) in a desiccator. The relevant characteristics of the blank matrix were determined prior of use as one of the blank runs. This blank matrix was used as the solvent for the calibration solutions.

Chemicals. 1,2-Dipalmitoyl-*rac*-glycerol (99%) and 1,3-dipalmitin (99%) were purchased from Sigma-Aldrich (St. Louis, MO) while 2-monolaurin (95–99% *rac*) was purchased from Larodan AB (Malmö, Sweden). 1-Monolaurin was obtained as a gift from Dr. Jon Kabara; its identity and purity were verified by GCMS, ^1H and ^{13}C NMR, and ^{31}P NMR of its phospholane derivative. It melted sharply at 63.0 °C (lit.: 63.0 °C) (24). The phosphitylating reagent used was 2-chloro-

4,4,5,5-tetramethyl-1,3-dioxaphospholane (2-CITMDOP, Sigma-Aldrich, St. Louis, MO). The NMR solvent used was CDCl_3 (Cambridge Isotope, MA).

Monoglycerides, Diglycerides, Total Sterols, and Free Fatty Acids by ^{31}P NMR. This method is based on the work of Spyros and Dais (19). NMR experiments were performed using a JEOL Lambda (9.4 T magnetic field) using a 10 mm tunable probe. ^{31}P NMR was observed at 162 MHz. FIDs were processed using an exponential window with a matched line broadening factor. ^{31}P NMR chemical shifts were referenced against an external standard of 85% phosphoric acid (0.0 ppm) that was sealed in a capillary tube and inserted into a 10 mm NMR tube and trimethylphosphate (3.6 ppm). The dioxaphospholane derivative of benzoic acid (RG, Merck, Darmstadt, Germany) was used as a secondary internal standard (IS) (136.0 ppm vs 85% phosphoric acid).

A 50.0 mL NMR solvent mixture of pyridine/ CDCl_3 (1.6:1) with 3 mg $\text{Cr}(\text{acac})_3$ spin relaxation agent was prepared. The coconut oil sample (ca. 1.5 g) was dissolved in the NMR solvent mixture in a 10 mm NMR tube and made up to a final volume of 4.0 mL. The IS solution was prepared by dissolving 0.40 g of benzoic acid into 25 mL of CDCl_3 . Benzoic acid IS solution (100 μL) and 80 μL of 2-CITMDOP were injected into the 10 mm NMR tube, shaken, and allowed to react at 25 °C for 30 min. The ^{31}P NMR spectrum was measured at a constant probe temperature of 30 °C. Excess derivitizing agent was used to ensure complete phosphitylization; this was verified by the presence of the peak at ~ 176 ppm due to excess 2-CITMPDOP.

Free Fatty Acids by Titrimetry. Free fatty acids were quantified as percent lauric acid using AOAC Official Method 940.28 (25). Analysis of samples was done in duplicate.

Calibration and Determination of Limit of Detection. The coconut oil blank matrix was used for the calibration solutions. Separate CDCl_3 stock solutions were prepared for each standard compound: 1,2-dipalmitin, 1,3-dipalmitin, 1-monolaurin, 2-monolaurin, lauric acid, and cholesterol. Five calibration concentrations were prepared of each standard compound containing a constant IS concentration.

The limits of detection (LOD) were determined for 1-monolaurin, 2-monolaurin, 1,2-dipalmitin, 1,3-dipalmitin, cholesterol, and lauric acid at 98.3% confidence level using the following formula (26):

$$\text{LOD} = \frac{3s_b}{m} \quad (1)$$

where s_b is the standard deviation of seven determinations of the integration ratio of trimethylphosphate with respect to 85% phosphoric acid and m is the calibration sensitivity.

Principal Components Analysis (PCA). Chemometrics analysis was performed using The Unscrambler (CAMO Process AS, Oslo, Norway). Twenty-four VCO and RCO samples comprised the data table, and the variables were the percent compositions of their MGs, 1,2- and 1,3-DGs, sterols, and FFAs from ^{31}P NMR. The data were first normalized using mean normalization before PCA was carried out.

RESULTS AND DISCUSSION

Calibration Curves and Detection Limits. The coconut oil blank matrix was used as solvent for the determination of the ^{31}P chemical shifts, calibration curves, and detection limits. Chemical shifts of the phospholane derivatives were referenced against the benzoic acid phospholane derivative (136.0 ppm), the chemical shift of which was determined using an 85% phosphoric acid external standard (0.0 ppm). The ^{31}P NMR chemical shifts of the phospholane derivatives of the reference compounds (1-monolaurin, 2-monolaurin, 1,2-dipalmitin, 1,3-dipalmitin, cholesterol, and lauric acid) were determined using the coconut oil blank matrix (Table 1).

A series of calibration solutions was prepared by plotting the mass ratios of the phospholane derivatives versus the corresponding peak area ratio with respect to benzoic acid IS. The calibration coefficients of determination (r^2) were higher than 0.988, except for 1,3-dipalmitin which had a value of 0.902. The limits of detection are reported as percent weight per weight

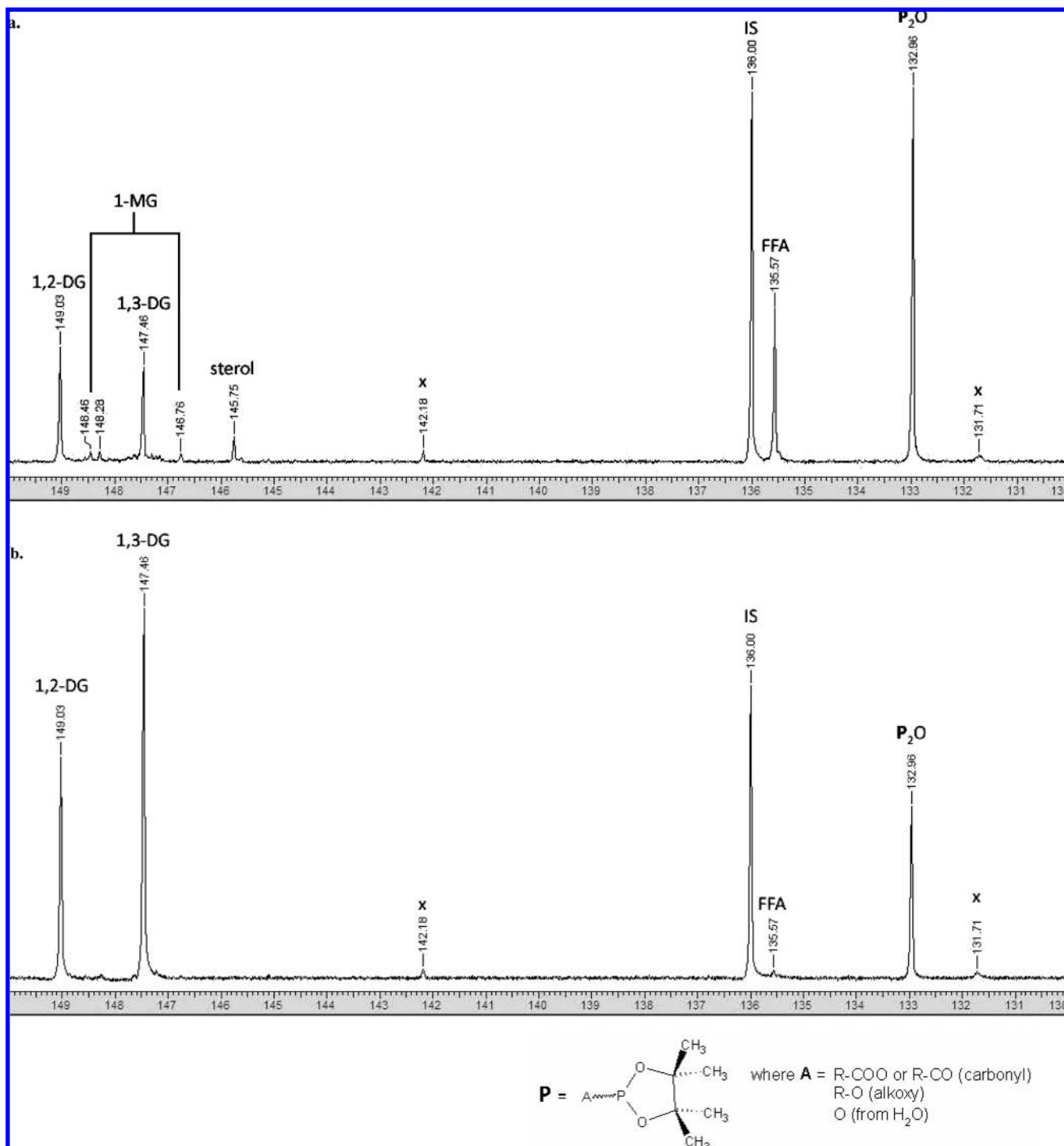


Figure 1. ^{31}P NMR spectra of derivatized MG, DG, sterols, and FFA in: (a) VCO; (b) RCO. The peak at 133.0 ppm is the P_2O peak (where P is the phosphitylizing group). The peak at 131.7 ppm is a contaminant from the phosphitylizing reagent; the peak at 142.2 ppm is unidentified.

for 1-monolaurin, 2-monolaurin, 1,2-dipalmitin, 1,3-dipalmitin, cholesterol, and lauric acid (Table 2).

Aside from predrying of the sample, no separation or other sample preparation was performed. Figure 1 shows a typical ^{31}P NMR spectrum of a VCO and RCO sample. Quantitative ^{31}P NMR data on the MG, DG, sterol, and FFA content are tabulated in Table 3.

Monoglycerides. On the average, VCO had 40% higher 1-MG content than RCO (0.027% compared with 0.019%). 1-MG in VCO samples ranged from 0.022% (for natural separation with heat) to 0.034% (for natural separation without heat). Heating generally means a temperature of 70–100 °C

for several minutes. It appears that the MG content is decreased by exposure to heat. 2-MG was not detected in any of the samples down to a detection limit of 0.014%.

Diglycerides. VCO had a lower average DG content (1.549%) than RCO (4.095%). Among the VCO samples, the lowest total DG content was given by the expeller method (1.285%), while the highest DG content was given by natural separation without heat (1.692%). Vigli and co-workers (21) reported that ^{31}P NMR analysis of 10 RCO samples obtained from European suppliers gave a total DG content mean value of 4.55%. The two sets of results are comparable and reasonable given the nature of the samples analyzed.

Table 3. Average and Range of Values of Monoglycerides, Diglycerides, Sterols, and FFA, % w/w, in VCO and RCO Samples Using ^{31}P NMR Analysis^a

sample	monoglycerides		diglycerides		total sterols	FFA	
	1-MG	1,2-DG	1,3-DG	DG, total		^{31}P NMR	titration
all VCO samples							
average	0.027	1.169	0.374	1.549	0.096	0.127	0.136
range of values	ND–0.052	0.807–1.724	ND–0.892	1.143–1.934	0.068–0.162	ND–0.420	0.018–0.329
centrifuge							
average	0.024	1.290	0.380	1.669	0.087	0.132	0.125
range values	ND–0.052	1.041–1.448	0.173–0.892	1.497–1.934	0.073–0.109	ND–0.420	0.018–0.329
expeller							
average	0.027	1.029	0.232	1.285	0.094	0.081	0.096
range of values	0.020–0.034	0.826–1.221	ND–0.581	1.143–1.407	0.069–0.132	ND–0.163	0.038–0.180
natural separation with heat							
average	0.022	1.176	0.400	1.576	0.088	0.138	0.156
range of values	0.016–0.027	0.807–1.724	0.199–0.532	1.339–1.923	0.068–0.106	0.085–0.176	0.093–0.211
natural separation without heat							
average	0.034	1.153	0.538	1.692	0.117	0.159	0.170
range of values	0.030–0.038	1.024–1.358	0.346–0.638	1.418–1.797	0.081–0.161	0.128–0.178	0.129–0.202
all RCO samples							
average	0.019	2.133	1.962	4.095	0.032	0.015	0.016
range of values	ND–0.038	1.606–3.107	1.473–2.712	3.151–5.819	ND–0.091	ND–0.035	0.008–0.030

^a All data are the average of at least two trials. ND = not detected. See **Table 2** for the respective detection limits. 2-MG was not detected in any of the samples.

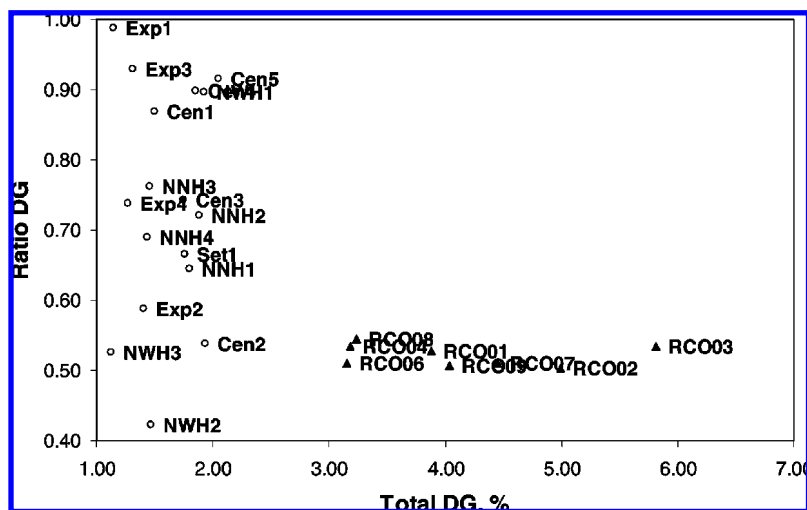


Figure 2. Plot of DG ratio (1,2-DG/total DG) against total DG. Open circles and triangles correspond to VCO and RCO samples, respectively. (VCO samples: Exp, expeller; Cen, centrifuge; NNH, natural separation no heat; NWH, natural separation with heat).

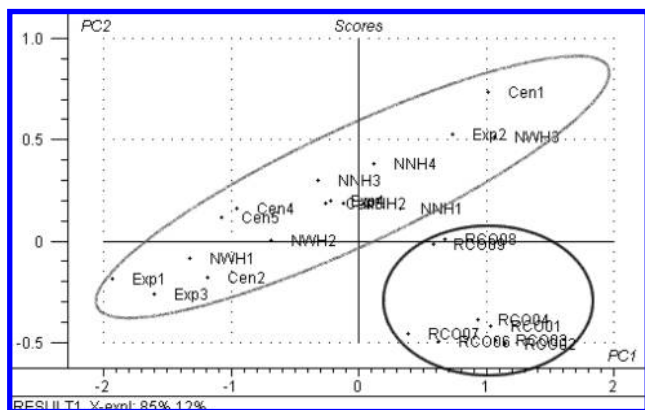


Figure 3. Principal components analysis (PCA) scores plot of VCO and RCO samples using ^{31}P NMR measurements for MG, DG, sterols, and FFA.

The plot of the total DG concentration versus the DG ratio [1,2-DG/total DGs] gave a separate grouping of VCO and RCO (**Figure 2**). This profile is very similar to that observed for refined and virgin olive oils by Fronimaki and co-workers (20).

Sterols. VCO samples gave an average total sterol content of around 0.096%, compared with about 0.032% in RCO samples. These values are comparable to the range of values given for coconut oil in the Codex Alimentarius (0.04–0.12%) (4) and with the values reported by Vigli and co-workers for traded RCO samples (0.070%) (21).

Free Fatty Acids. VCO, on the average, had 8 times as much FFA than RCO (0.127% versus 0.015%). This is expected since RCO undergoes alkaline refinement, while VCO may not be subjected to chemical treatment (2, 4). Among VCO samples, those produced by natural separation without heat yielded the highest FFAs (0.159%), while the VCO produced by other means had similar values. The results of ^{31}P NMR determination of FFAs were comparable to the results obtained using the standard titrimetric method (**Table 3**).

Principal Components Analysis (PCA). The PCA scores plot (**Figure 3**) clearly shows a different grouping for VCO and RCO samples. The wider dispersion of the VCO samples indicates that, as far as the parameters analyzed, VCO samples are more variable. This reflects the variability from the use of different VCO production methods.

The loadings plot (**Figure 4**) indicates that the 1,2-DG, 1,3-DG, and FFA content are the most important parameters which

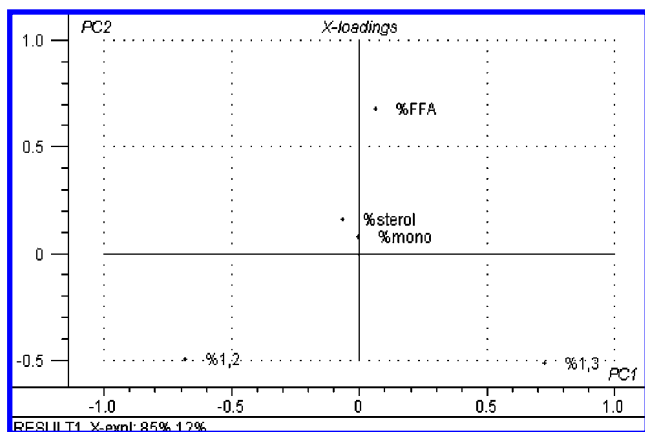


Figure 4. Principal components analysis (PCA) loadings plot of VCO and RCO samples using ^{31}P NMR analysis of MG, DG, sterols, and FFA.

distinguish the VCO from RCO samples. Although 1-MG and sterols were generally higher in VCO than RCO samples, their effect on the loadings plot was smaller than the DG and FFA content. The 1,2- and 1,3-DG content gave comparable loadings values along PC1 and PC2.

^{31}P NMR is an effective method for the analysis of constituents in coconut oil, which contain alcohol and carboxylic acid functional groups, and provides an effective way of differentiating VCO from RCO.

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