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Physico-Chemical and Microbiological Parameters in the Deterioration of Virgin Coconut Oil

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The deterioration of virgin coconut oil (VCO) due to physico-chemical oxidation and hydrolysis and microbiological processes was studied. The physico-chemical oxidation of VCO in the air at room temperature was negligible. Oxidation of VCO was observed only in the presence of air, UV radiation, ferric ion (Fe^{3+}), and high free fatty acid (FFA) content. Chemical hydrolysis was performed at varying moisture levels and temperatures. The rate of hydrolysis to produce FFAs was measured using ^{31}P NMR under conditions of saturated water (0.22%) and 80°C was found to be 0.066 µmol/g-hr (expressed as lauric acid). At 0.084% moisture and 80°C, the rate of FFA formation was found to be 0.008 µmol/g-hr.

The microbial decomposition of VCO was determined after four days of incubation at 37°C. At low moisture levels (<0.06%), VCO was stable to microbial decomposition. However, at higher moisture levels, there was an increase in the formation of organic acids, in particular, lactic acid, dodecanoic acid, succinic acid, acetic acid, and fumaric acid, indicating that microbial action had occurred. The most important conditions that influence the physico-chemical and microbial degradation of VCO are moisture, temperature, and the presence of microorganisms. These degradation processes can be minimized if the moisture level is maintained below 0.06%.

Key Words: hydrolysis, microbial deterioration, oxidation, virgin coconut oil

INTRODUCTION

Virgin coconut oil (VCO) is defined as coconut oil that is extracted from fresh coconut meat and processed using only physical and other natural methods (PNS/BAFPS 2004, APCC 2006). These methods can include fermentation, centrifugation and use of an expeller.

The problem of rancidity of coconut oil has been the subject of studies dating back to the early 1900s. Walker (1906, 1908) suggested that molds, air and light had significant effects on oil quality. Brill & Parker (1917) measured the formation of aldehydes while Perkins (1919) observed that high acidity was obtained in samples under conditions of high moisture content, exposure to air and light, and presence of fat-soluble enzymes. Mañalac & Soliven (1969) and Banzon & Velasco (1982) concluded that rancidity was due to exposure to air, although coconut oil was still found to be more stable than other vegetable oils which have a higher content of unsaturated fatty acids.

The deterioration of vegetable oils can occur due to chemical hydrolysis, chemical oxidation and/or microbial action (Ho & Chen 1994) which can be promoted by enzymes, metals, heat, light and air (Sherwin 1978). However, the presence of minor

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components or additives in the oil, such as phenols, can increase the stability of the oil.

Chemical oxidation in vegetable oils generally occurs when dioxygen (O_2) in the air, in the presence of heat, radiation and/or catalyst, reacts with unsaturated fatty acids to form hydroxyperoxides which are the primary products formed in the initiation step (Ho & Chen 1994; Yang & Min 1994; Shih & Hu 1999). The hydroperoxide intermediates are subsequently converted to aldehydes, epoxides, acids, alcohols, and other hydrocarbons (Shahidi & Wanasundara 1996). Of these, aldehydes have attracted the most attention because they are significant determinants of the rancid odor and taste (Clausen et al. 2008). Hexanal, which is formed from the oxidation of linoleic acid, is a commonly-used indicator for oxidation of vegetable oils (Gray 1978).

Transition metals, such as ferric (Fe^{3+}) and cupric (Cu^{2+}) ions, catalyze the oxidative decomposition of the intermediate hydroperoxides (Gray 1978) and their effects can be enhanced by metal complexation with free fatty acids (Nalini & Balasubramanian 1993). Chlorophyll, which absorbs visible light at around 663 nm, was suggested as a possible sensitizing agent in the photo-oxidation of VCO (Rukmini & Raharjo 2010).

There are several standard methods for monitoring the extent of oxidation in vegetable oils (Shahidi & Wanasundara 1996). The peroxide value (PV) test, the most commonly used method, measures the amount of hydroperoxides. PV is expressed in mmol of peroxide per kg of oil sample or mmol of O_2 per 2 kg of oil. However, PV does not give a complete assessment of the extent of oxidation of the oil since hydroperoxides can further decompose to aldehydes (Kaynak et al. 2004).

Aldehydes can be measured using the anisidine value (AV) test. The AOCS (1995) gives a procedural definition for AV which is expressed as a dimensionless number.

In order to account for the presence of both peroxides and aldehydes, the total oxidation value, TOTOX, was defined as the sum of AV plus twice PV to account for the stoichiometric formation of 2 aldehyde moieties for every peroxide: TOTOX = AV + 2PV (Sherwin 1978). However, since the standard methods express PV in terms of mmol/kg and AV as a dimensionless number, these values cannot be directly added together. Shahidi and Wanasundara (1996) used an alternative test for aldehydes using TBARS (2-thiobarbituric acid reactive substances) and defined $TOTOX_{TBA}$ as equal to (2PV + TBARS). However, there are many compounds that cause interference in the TBARS test, including ketones, acids, sugars and proteins (Devasagayam et al. 2003). Wai and co-workers (2009) developed a method based on the sequential determination of peroxides and aldehydes using a common triiodide potentiometric system which is designed for automated analysis.

The oxidative degradation of VCO at high temperature can be studied using thermogravimetric analysis in the presence of O_2. Thermogravimetric analysis measures the change in the mass of the sample wherein a gain in mass indicates oxidation of the sample, while a decrease in mass is taken as the loss of volatile oxidation products. This method has been used to study the oxidative degradation of olive oil (Gennaro et al. 1998). Naturally occurring or artificially added antioxidants, such as phenols, have been shown to retard the oxidative degradation of vegetable oils (Papadopoulos & Boskou 1991; Chen & Ahn 1998; Rastrelli et al. 2002), while tocopherols have shown poor correlation with oxidative protection (Baldioli 1996).

Hydrolysis of the oil occurs when acids or bases in the presence of water reacts with glyceryl esters of a tri-, di-, or mono-acylglyceride releasing free fatty acids (FFA) (Moreira et al. 1999). The accumulation of FFA contributes to the sour smell and taste of vegetable oils. Heat and moisture accelerate hydrolysis (Naz et al. 2005; Shimizu et al. 2004). Among vegetable oils, coconut oil appears to be particularly susceptible to hydrolysis when it is subjected to heat (Kritchevsky et al. 1962).

The standard method for determining the extent of hydrolysis is by titrimetry. At lower levels (< 0.01%) where titrimetry is unable to give accurate results, FFA can be measured using GC (Wan & Dowd 2007) and ^{31}P NMR (Fronimaki et al. 2002; Dayrit et al. 2008). Beyond its role in the stoichiometry of hydrolysis, water is essential to the activity of enzymes and microorganisms (Brown 1976).

Microorganisms play an important role in the deterioration of coconut oil. Among the major products which have been identified from the fermentation of coconut oil are organic acids and methyl ketones (Brill & Parker 1917; Stokoe 1928; Hoover et al. 1973; Kinderlerer 1987). These decomposition compounds result in rancid odor or taste.

The objective of this paper is to describe the physico-chemical and microbiological processes that lead to the oxidation, hydrolysis and microbial deterioration of VCO. In the case of physico-chemical oxidation, the effects of temperature, O_2, UV radiation, and metal (Fe^{3+}) were investigated. The volatile organic compounds (VOC) associated with the oxidation of vegetable oils were identified and quantified by solid

phase microextraction-gas chromatography mass spectrometry (SPME-GCMS). Titrimetry and ^{31}P NMR were used to quantify FFA. The effect of microorganisms in the deterioration of VCO was studied by identifying and quantifying the organic acids which were formed in VCO using GC and GC-MS methods.

MATERIALS AND METHODS

All analyses were done in duplicate. The standard deviation was estimated from the range using the formula: $s = R / d_2$, where d_2 is an estimator at a given *n* number of replicates. For duplicate measurements (n=2), the factor 1.128 was used (Mullins 2003). The d_2 method in estimating the standard deviation gives better results than the sum of squares when n is small (Woodall & Montgomery 2000).

Coconut oil and other vegetable oil samples

The VCO samples used in this study were provided by the Virgin Coconut Oil Producers and Traders Association of the Philippines, Inc. (VCO Phil). Five representative VCO samples that were used in this study were prepared by fermentation without heat, fermentation with heat, centrifuge, and expeller methods. Refined, bleached and deodorized coconut oil (RCO) samples were provided by Minola Corp. Virgin olive oil (Marca Señorita) and refined soybean oil (Pure Wesson) were purchased from the supermarket.

Reagents and chemicals

All reagents and solvents were ACS grade. From JT Baker: acetone (HPLC grade), chloroform (100%), ethanol (99.9%), 2,2,4-trimethylpentane (99.9%), sublimed iodine (99.9%), potassium iodide (99.1%), sodium chloride (100.7%), sodium thiosulfate pentahydrate (100.0%). From Merck: potassium dichromate (99.96%), sodium hydroxide (99%), fumaric acid (100%,), propionic acid (99%), pyridine (100%). From Sigma-Aldrich: *para*-anisidine (99%), 2-octanol (97%), boron trifluoride in methanol (14%), decanoic acid (98%), hippuric acid (98%), dodecanoic acid (98%), butyric acid (99%), hexanoic acid (99%), octanoic acid (99%), 2-chloro-4,4,5,5-tetramethyl-1,3,2-dioxaphospholane (95%). From Mallinckrodt: acetic acid (99.7%). From Fluka: bromine (99.5%), 2,4,6-Tri(2-pyridyl)-s-triazin (99%). From Trimedia: ferric chloride hexahydrate (99%) and nutrient agar. From LabScan: hydrochloric acid (36.46%). From Riedel-de Haen: pyruvic acid (100%), succinic acid (100%), phenolphthalein. From Fisher Scientific: formic acid (100%). From Univar: lactic acid (85%). From Cambridge Isotope: deuterated chloroform (99.8%).

Standard analytical methods

The fatty acid profile of the VCO samples was determined by methylation using boron trifluoride in methanol, followed by GC analysis (AOAC 1995; Dayrit et al. 2007).

Free fatty acid (FFA) was determined by titrimetry. The method was standardized using lauric acid as reference and the result was expressed as % free lauric acid (AOAC 1995; Dayrit et al. 2007). The lowest detectable level of FFA by titrimetry was determined to be 0.01%.

The moisture content was determined using a KEM MKS-500 Karl Fischer titrator (AOAC 1995). Ten milliliters of oil sample were weighed and dissolved in the titration vessel and the solution was autotitrated to the endpoint. This method gave a recovery of 102%.

The iodine value was determined using the Hanus Method (AOAC 1995; Dayrit et al. 2007) and was validated by spiking linoleic acid standard.

The total oxidation (TOTOX) value was obtained by separately determining the peroxide value (PV) (AOAC 1995) and the anisidine value (AV) (Papadopoulou & Roussis 2007). In the anisidine value determination, the resulting color change was measured at 350 nm. The limit of detection (LOD) for PV and AV were 0.2 mmol/kg and 0.05, respectively.

The presence of chlorophyll and other colored compounds in VCO was measured from 400 to 800 nm as a neat sample and in a 1:1 mixture with UV-grade acetone (Shimadzu UV-2401PC).

Protein percentage was measured by the Philippine Institute of Pure and Applied Chemistry (PIPAC) using the Kjeldahl method (AOAC 1995). The lowest detectable level was 0.01 %.

Carbohydrate percentage was measured using the anthrone assay (Dulikgurgen 2006). The LOD was 0.001%. The recovery percentage of the oil samples spiked with glucose standard was 98%.

ICP-AES analysis

VCO and RCO samples were analyzed by PIPAC using inductively coupled plasma-atomic emission spectrophotometry (ICP-AES) (Shimadzu ICPS 7510). The oil samples were charred, ashed at 525°C and digested using nitric acid. The digests were collected in a 25.00 ml volumetric flask and analyzed.

Determination of Anisidine Value-aldehyde equivalent (AV_{AE})

Aliquots (0.4 g) from a VCO sample were spiked with increasing amounts of hexanal. Five milliliters of the solution were reacted with 1 mL *p*-anisidine reagent for

10 minutes and the absorbance of the solution was read at 350 nm. The increase in AV was plotted versus the amount of hexanal added (mmol/kg). The equation of the line was then obtained using the linear regression function (Figure 1). The regression coefficient was 0.994. The minimum detectable level for AV_{AE} was 0.5 mmol/kg.

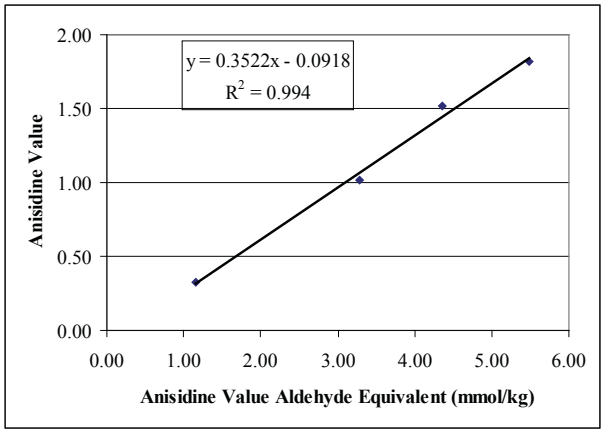

Figure 1. Determination of anisidine value-aldehyde equivalent, AV_{AE}. This plot relates the dimensionless AV to a proposed AV-aldehyde equivalent (AV_{AE}).

Total phenolics by ferric reducing antioxidant power (FRAP) assay

A 3 g aliquot of the oil was extracted with 3 mL methanol. The FRAP reagent was prepared by mixing 80 mL pH 3.6 acetate buffer, 8 mL 10 mM 2,4,6-Tri(2-pyridyl)-s-triazin solution (in 400 mM HCl) and 8 mL 20 mM ferric chloride ($FeCl_3$). A 5.00 mL portion FRAP reagent was added to the 0.10 mL methanol extract and the reaction was allowed to proceed for eight minutes at 25°C and the absorbance was read at 593 nm (Shimadzu UV-2401PC). Total polar antioxidants were measured against gallic acid standard solution (Valavanidis et al. 2004; Ou et al. 2002; Benzie & Strain 1999). The recovery percentage was 107% and the LOD was 3.0 mg gallic acid equivalent (GAE) per 100 g oil.

Analysis of α-tocopherol

The analysis of α-tocopherol was done by PIPAC. The oil samples were first saponified using KOH solution. The liberated natural tocopherols and from hydrolyzed tocopherol esters were then extracted using petroleum ether. The extracts were then dried, reconstituted with *n*-hexane, and injected into a high performance liquid chromatograph. LOD was determined to be 2.5 mg/kg.

Thermogravimetric analysis

Thermogravimetric analysis was performed using the following temperature program: the temperature was raised to 120°C at 15°C/min, held for 10 minutes to release all the volatile components, then was raised to 150°C at 10° C/min, then to 320°C at 4°C/min, and finally to 800° C at 15°C/min (Shimadzu TA 501 Thermal Analyzer). The intersection of the tangent lines in the thermogram gave the induction temperature, which is indicative of the stability of the oil.

The effect of air on the induction temperature was compared with dinitrogen (N_2) atmosphere as control. The effect of Fe^{3+} on the induction temperature was determined by spiking the VCO sample with a known amount of ethanolic Fe^{3+} solution (final concentration: 35 µg Fe^{3+}/g oil) and heating under N_2 atmosphere and in air.

Raw data were subjected to statistical analysis using the Microsoft Excel™ 2007 Analysis ToolPak. Two-way Analysis of Variance (ANOVA) with interaction was used to test if the differences in the induction temperature between oils and between treatments were significant. One-tailed, paired *t*-test was used to determine whether the presence of Fe^{3+} significantly decreases the induction temperature. The α-value was set at 0.05.

Effect of UV radiation

Fifteen g aliquots of VCO were placed in ceramic evaporating dishes and were exposed to UV light (λ=254 nm) inside a black box with 3 UV light bulbs (HAICAO F10T8 BLB-05). PV and AV were determined to measure the extent of oxidation (TOTOX). To determine the effect of Fe^{3+} on UV oxidation, VCO samples were prepared containing about 30 µg Fe^{3+}/g oil. Exposure times were 1, 2, and 3 hours.

Solid Phase Microextraction-Gas Chromatography Mass Spectrometry (SPME-GCMS)

An aliquot of the sample was mixed with internal standard solution (2-octanol) in a 15 mL vial capped with PTFE/ Silicone septum. The headspace was sampled at 40°C for 30 min using a 1 cm 50/30 µm divinylbenzene/carboxen/ polydimethylsiloxane StableFlex™ fiber (Supelco) (Vichi et al. 2003). GCMS analysis was done using a HP 5890 Series II gas chromatograph with a DB-1 column (J&W Scientific, 60 m length, 0.250 mm ID, 0.25 µm thickness) interfaced to a MAT95 mass spectrometer (Bremen, Germany). The volatile compounds adsorbed on the fiber were desorbed at 260°C in the gas chromatograph injector port. The column temperature was held at 40°C for 10 min before ramping to 200°C at 3°C/min. MS analysis was carried out by electron ionization at 70 eV, scanning from m/z 40 to 240.

The effect of UV radiation, Fe^{3+} and added FFA (as lauric acid) on the formation of volatile oxidation products was determined. Solution 1 was prepared by dissolving 0.038 g $FeCl_3 \cdot 6H_2O$ in 0.75 g ethanol. Solution 2 was prepared

by dissolving 0.038 g $FeCl_3 \cdot 6H_2O$ and 0.120 g lauric acid in 0.75 g ethanol. Solutions 1 and 2 were spiked into two different 15 g VCO aliquots giving a final concentration of 35 µg Fe^{3+}/g oil. The samples were exposed to UV radiation for 2 hr and were analyzed by SPME-GCMS.

Accelerated hydrolysis

Two grams of VCO with varying moisture content (0.063%, 0.084% and saturated water [approx. 0.22% w/w]) were heated in 36 separate sealed glass vials at 60°, 70°, and 80°C for one and two weeks. FFA percentage was measured by ^{31}P NMR analysis (see below).

^{31}P Nuclear Magnetic Resonance (NMR) analysis

The FFA in the VCO were converted into phosphorous derivatives and analyzed by ^{31}P NMR at 161 MHz on a JEOL Lambda NMR spectrometer (Dais et al. 2002; Dayrit et al. 2008). The NMR solvent was a 1.6:1.0 v/v Pyridine:$CDCl_3$ mixture, with $Cr(AcAc)_3$ added as relaxation agent. One and a half gram of oil sample was dissolved in the NMR solvent in a 10 mm NMR tube up to a final volume of 4.0 mL. The internal standard (IS) solution was prepared by dissolving 2.0 g benzoic acid in 25.00 mL deuterated chloroform. Fifty µL IS solution and 100 µL 2-CITMDOP derivatizing agent were added to the sample into a sealed NMR tube. The mixture was allowed to react at 30°C for 30 min. The ^{31}P NMR spectrum was measured using an inverse-gated pulse sequence with ^{1}H decoupling at 30°C.

Microbial degradation of VCO

VCO was produced in the laboratory using the fermentation process (Bawalan 2005). VCO samples of different moisture content (0.027% to 0.22%, saturated) were prepared by addition of non-sterile coconut milk. The moisture content was measured using the Karl Fisher method. The VCO samples were incubated for 4 days at 37°C. Then the organic acids were extracted, derivatized and analyzed by GC and GC-MS (see below) and %FFA was determined by titrimetry.

Analysis of organic acids

The organic acids which were formed during the fermentation process were extracted using acid-base/ether extraction. Five milliliters VCO sample were spiked with cyclohexanecarboxylic acid as the internal standard (250 µg/mL). The organic acids were derivatized using the BF_3-butanol method (Supelco 1997). One microliter of the ester extract was injected into a Shimadzu GC-14B gas chromatograph equipped with a flame ionization detector and packed with a DB-1 capillary column (J&W Scientific, polydimethylsiloxane, 60m×0.25mm i.d.; 0.25µm film thickness). The column temperature was held at 80°C for 8 min; then, the temperature was increased to 230° C at 20° C/min finally, the temperature was held for 15 min at 230°C. The injector and detector temperatures were set at 230°C and 250°C, respectively. The relative response factor for each identified organic acid in the samples was obtained versus the IS by taking the average response factor calculated from five determinations for each organic acid.

For identification, the derivatized standard organic acids were injected into a Shimadzu QP2000A Gas Chromatograph/Mass Spectrometer with an OV-1 column using similar GC oven conditions.

RESULTS

Chemical characteristics of VCO and RCO samples

The chemical characteristics of the commercial VCO samples which were used in this study are summarized in Table 1. In general, the chemical characteristics and experimental results using the five VCO samples were comparable and the ranges are given in the Table along with the standards set in PNS/BAFPS 22:2004. The FFA and volatile matter values were slightly higher in one sample; otherwise, the samples were within the specifications.

Samples of VCO, RCO, virgin olive oil, and refined soybean oil were analyzed for linoleic acid content, iodine value and total antioxidant (Table 2A). The results were comparable with standard values (Codex 2008).

The analyses for the presence of proteins in commercial VCO gave values from ND to 0.07% (LOD = 0.01%). This result is comparable to that of Dia et al. (2005) who reported 0.07% to 0.12% protein content in commercial VCO. On the other hand, the presence of carbohydrates could not be detected (LOD = 0.001%).

The presence of antioxidants and α-tocopherol in the VCO samples was determined. The FRAP assay for total antioxidants revealed that the VCO and RCO samples contained from ND to 6.1 mg GAE/100 g oil (LOD= 3 mg GAE/100 g oil). In comparison, using the method of Folin & Ciocalteu (1927), Dia et al. (2005) reported values of 23–92 mg/kg phenols as catechin, while Marina et al. (2009) obtained values in the range of 7.78–29.18 mg GAE/100 g oil. In order to arrive at a comparison, these results can be recalculated as mmol antioxidant/100 g oil: this work: ND to 0.036 (LOD=0.017); Dia et al. (2005): 0.008 to 0.032; and Marina et al. (2009): 0.045 to 0.170. These values may reflect differences in coconut varieties and the inclusion of testa in the extraction.

Table 1. **A**. The fatty acid composition of the VCO samples are compared with PNS/BAFPS 22:2004. **B**. Other quality parameters of the VCO samples were also determined and compared with the standard. "ND": not detectable; LOD values are given in (parenthesis).

Parameter	PNS/BAFPS 22:2004	VCO samples (range of values)
A. Fatty acid, %		
Caproic acid, C6:0	ND (<0.1) - 0.7	0.1 – 0.7
Caprylic acid, C8:0	4.6 -10.0	6.3 – 10.9
Capric acid, C10:0	5.0 -8.0	6.2 – 7.8
Lauric acid, C12:0	45.1 -53.2	48.5 – 51.0
Myristic acid, C14:0	16.8 -21.0	15.6 – 19.3
Palmitic acid, C16:0	7.5 -10.2	6.9 – 8.5
Palmitoleic acid, C16:1	ND (<0.1)	ND (<0.1)
Stearic acid, C18:0	2.0 -4.0	1.9 – 3.0
Oleic acid, C18:1	5.0 -10.0	4.5 – 5.4
Linoleic acid, C18:2	1.0 -2.5	0.7 – 1.3
Linolenic acid, C18:3	ND (<0.1) -0.2	ND (<0.1)
B. Other Quality Parameters		
Free fatty acids (max), % as C12	0.10	0.03 – 0.14
Peroxide value (max), meq/kg oil	3.0	ND (<0.2) – 1.00
Iodine value, g I_2/100g oil	4.1 – 11.00	6.5 - 8.8
α-Tocopherol, mg/kg	No specification	ND (2.5)
Matter volatile at 120°C (max), %	0.20	0.10 – 0.14
Moisture (max), % by Karl Fischer	0.10	0.08 - 0.10
Protein, %	No specification	ND (<0.01%) – 0.07
Carbohydrate, %	No specification	ND (<0.001)
Heavy metals mg/kg (max.)		
Iron (Fe)	5.0	0.8 – 2.1
Copper (Cu)	0.4	ND (<0.1)
Lead (Pb)	0.1	ND (<0.1)
Arsenic (As)	0.1	ND (<0.1)

Table 2. **A**. Chemical parameters of VCO, RCO, virgin olive oil, and refined soybean oil samples. The reference values for RCO and refined soybean oil are taken from Codex (2008) while those for virgin olive oil are from Codex (2001). **B**. The induction temperatures from the thermogravimetric analysis give comparative tendencies for the vegetable oil samples to undergo thermal degradation. The value in (parenthesis) is the standard deviation of duplicate runs.

Parameter	Vegetable Oil			
	VCO	RCO	Virgin Olive Oil	Refined Soybean Oil
A. Chemical Parameters				
Linoleic Acid, %	1.1 (±0.1)	1.3 (±0.0)	6.5 (±0.1)	55.8 (±0.8)
Reference Values		1.0-2.5	3.5-21.0	48.0-59.0
Iodine Value, g I_2/100g oil	6.8 (±0.7)	8.8 (±0.2)	81.1 (±0.7)	125.2 (±0.0)
Reference Values		6.3-10.6	75-94	124-139
Total Antioxidants, mg GAE/100g	6.1 (±1)	4 (±1)		
B. Thermogravimetric Induction Temperature (°C)				
Heated under N_2	292 (±0)	291 (±10)	276 (±3)	276 (±3)
Heated under N_2 with 35 μg/g Fe^{3+} added	290 (±0)	283 (±4)	273 (±1)	276 (±2)
Heated under air	270 (±0)	268 (±3)	257 (±1)	252 (±2)
Heated under air with 35 μg/g Fe^{3+} added	268 (±5)	265 (±4)	251 (±1)	253 (±4)

Analysis of α-tocopherol in the VCO samples by HPLC gave non-detectable levels (LOD=2.5 mg/kg). This result is similar to the results of Dia et al. (2005) who reported non-detectable amounts of α-tocopherol (LOD not specified). PNS/BAFPS 22:2004 does not specify a standard for VCO but Codex (2008) lists the α-tocopherol content for RCO as ND to 17 mg/kg.

The visible spectrum of VCO did not show any absorbance in the entire range from 400 to 800 nm. Thus, the presence of chlorophyll could not be detected by UV-visible spectroscopy. This method has an estimated LOD of 3 mg/kg (Ward et al. 1994).

Total Oxidation-Aldehyde Equivalent ($TOTOX_{AE}$)

A relatively simple approach for the determination of TOTOX expressed as mmol/kg was developed. This proposed method adopts the same procedure for the measurement of PV but uses a modified expression of AV using hexanal as standard. A series of hexanal concentrations was prepared using VCO as the matrix and their anisidine values (AV) were determined and plotted (Figure 1). From this plot, the AV was extrapolated to its hexanal concentration, which is defined as AV_{AE} (mmol/kg). Total oxidation-aldehyde equivalent ($TOTOX_{AE}$) can then be calculated using the formula: $TOTOX_{AE} = 2PV + AV_{AE}$, where PV, and AV_{AE} have the same unit of mmol/kg. $TOTOX_{AE}$ was used to determine the extent of oxidation by UV and Fe^{3+} (see below).

Presence of metals in VCO

The five VCO samples were analyzed for the presence of metals using ICP-AES. Only iron (0.8 to 2.1 µg/g) and zinc (0.1 µg/g) were detected. Because iron was the metal found in highest amount and because of its possible role in oxidation (Mellican et al. 2003), the effect of Fe^{3+} on the oxidation of vegetable oils was investigated (see below).

Thermogravimetric Analyses

The oil samples were subjected to accelerated oxidation in a thermogravimetric analyzer under varying conditions (Table 2B). A higher induction temperature (Figure 2) indicates higher stability of the oil against thermal degradation.

Statistical analysis (two-way ANOVA) of the data shows that independent of the conditions of the experiment, the induction temperatures of different oils are significantly different (p-value = 3.0 x 10^{-9}). The conclusion is that VCO and RCO have higher induction temperatures than refined soybean oil and virgin olive oil, and thus have higher resistance to thermal oxidation. The contribution of the type of oil – treatment interaction is not significant (p-value = 0.66). Regardless of type of oil, the induction temperatures of the oils under different treatments are significantly different (p-value = 5.1 x 10^{-12}).

The induction temperature of VCO decreased by 20°C in air compared to N_2; this may be taken as the oxidizing effect of O_2. Upon addition of Fe^{3+} (35 µg Fe^{3+}/g oil), the induction temperature of VCO under N_2 atmosphere and in air decreased by 2°C. One-tailed, paired t-test shows that the decrease is significant (p-value = 0.014). The results indicate that the presence of Fe^{3+} contributes to the oxidation of VCO but this is to a lesser extent compared to the effect of the presence of oxygen.

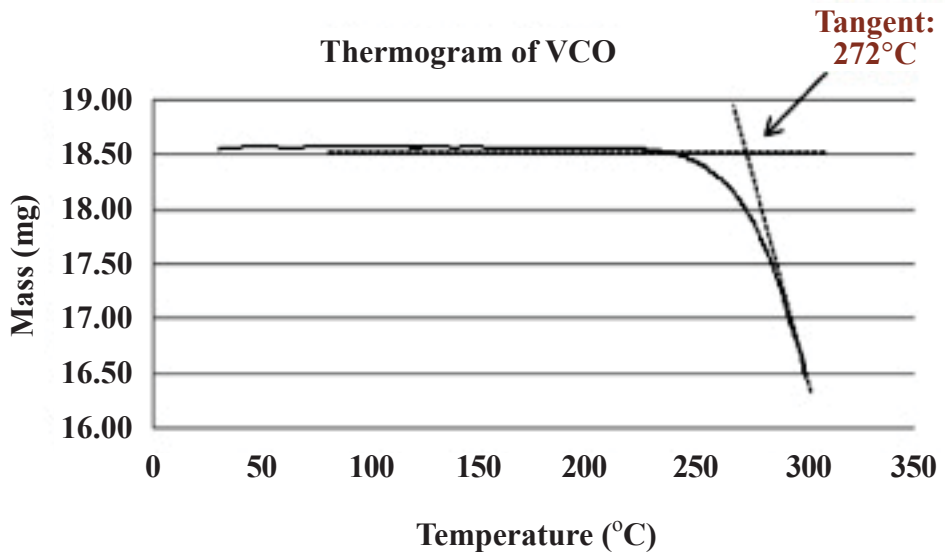

Figure 2. Thermogram of a VCO sample. The induction temperature is extrapolated from the intersection of the tangent lines. A higher induction temperature indicates higher physico-chemical stability.

Oxidation by exposure to UV radiation

The samples were exposed to UV light (λ=254 nm) for one to three hours and the extent of oxidation was determined by $TOTOX_{AE}$ (Figure 3). However, the $TOTOX_{AE}$ value remained virtually unchanged at 5 mmol/kg. To further accelerate the UV-oxidation of VCO, Fe^{3+} (30 µg/g oil) was added to the oil. The Fe^{+3}-spiked VCO sample exhibited an increase of about 15 mmol/kg $TOTOX_{AE}$ value after 3 hr of exposure to UV, which was due mainly to the increase in the amount of aldehydes. This indicates that both UV-radiation and Fe^{+3} are needed to oxidize VCO.

Volatile Organic Compounds (VOCs) from VCO exposed to UV radiation

The volatile degradation products from various UV- and Fe^{+3}-initiated oxidation processes were sampled by SPME and analyzed by GCMS (Table 3 and Figure 4). The baseline experiment was performed in the presence of air and UV radiation.

The addition of Fe^{+3} caused a large increase for hexanal from 1.02 to 20 µg/g and smaller increases for other aldehydes, in particular, pentanal, heptanal, octanal and nonanal. Addition of lauric acid (2.52 µmol/g oil; 4 mole-equivalents relative to Fe^{3+}) resulted in an increase of hexanal from 20 to 27 µg/g. No other significant changes among the other VOCs were noted. This indicates that FFA, may further promote chemical oxidation by solubilizing the ferric ions. This suggests that VCO, in the presence of air, UV radiation, Fe^{3+} and high FFA content, becomes more susceptible to chemical oxidation.

Except for the small but inconsistent increase noted for 2-heptanone, the concentrations of methyl ketones remained generally constant, indicating that methyl ketones are not formed via physico-chemical oxidation. This is consistent with the report that methyl ketones are formed by bacteria and fungi (Stokoe 1928; Kinderlerer 1987).

Determination of Chemical Hydrolysis of VCO by ^{31}P NMR

Preliminary experiments to determine the rate of hydrolytic degradation of VCO indicated that the hydrolysis rate was slow at room temperature and that titrimetry was not sensitive enough to detect changes in

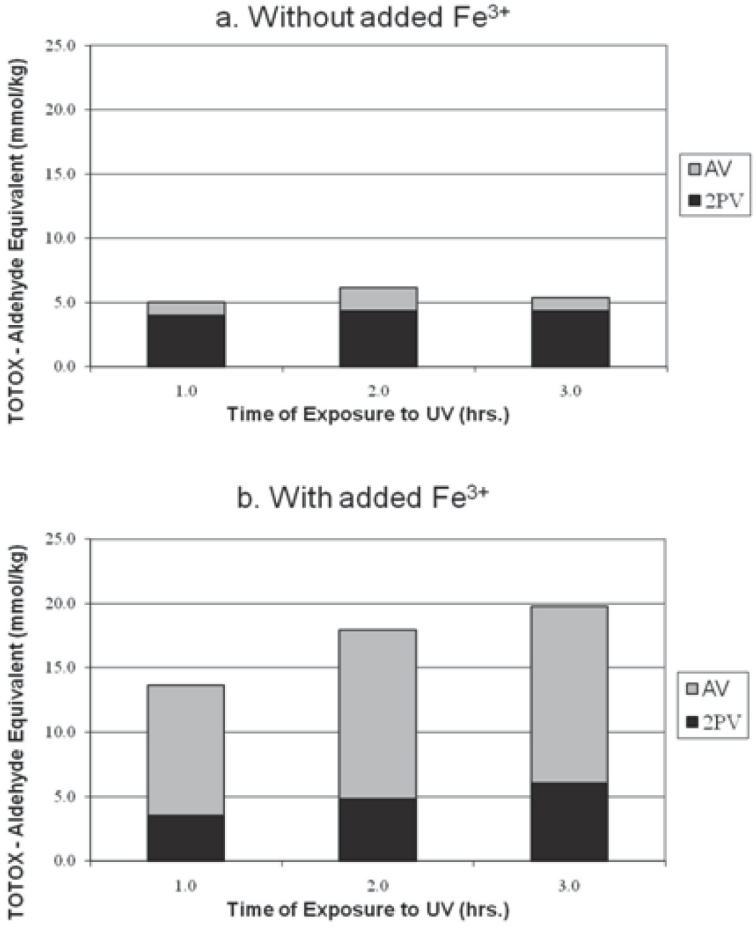

Figure 3. $TOTOX_{AE}$ values of VCO exposed to UV radiation for 1, 2 and 3 hours, with and without added Fe^{3+}. The amount of Fe^{3+} added was about 30 µg/g.

Table 3. Volatile organic compounds (VOCs) found in VCO exposed to air and UV radiation at room temperature. The effects of the addition of Fe^{+3} (35 µg/g oil) and lauric acid (200 µg/g oil) were also studied. The value in parenthesis is the standard deviation of duplicate runs.

Volatile Organic Compound	Amount present when exposed to air and UV at room temperature (µg/g)		
	VCO only	VCO + $FeCl_3$	VCO + $FeCl_3$ + lauric acid
2-Pentanone	0.16 (±0.03)	ND*	0.15 (±0.02)
Pentanal	ND*	2.9 (±0.7)	3.3 (±0,7)
Hexanal	1 (±0)	20 (±4)	27 (±6)
2-Heptanone	7 (±0)	13 (±1)	7 (±1)
Heptanal	ND	0.7 (±0.1)	ND
2-Octanone	23 (±1)	24 (±1)	22 (±7)
Octanal	ND*	1.6 (±0.2)	ND*
2-Nonanone	1.8 (±0.0)	2.1 (±0.2)	2.0 (±0.2)
Nonanal	ND*	0.6 (±0.1)	ND*
Octanoic ethyl ester	1.1 (±0.1)	0.8 (±0.2)	1.1 (±0.1)
δ-Nonalactone	11 (±0)	10 (±1)	11 (±1)
2-Undecanone	0.50 (±0.05)	0.30 (±0.10)	ND*

*LOD values (µg/g): 2-Pentanone: 0.09; Nonanal: 0.3

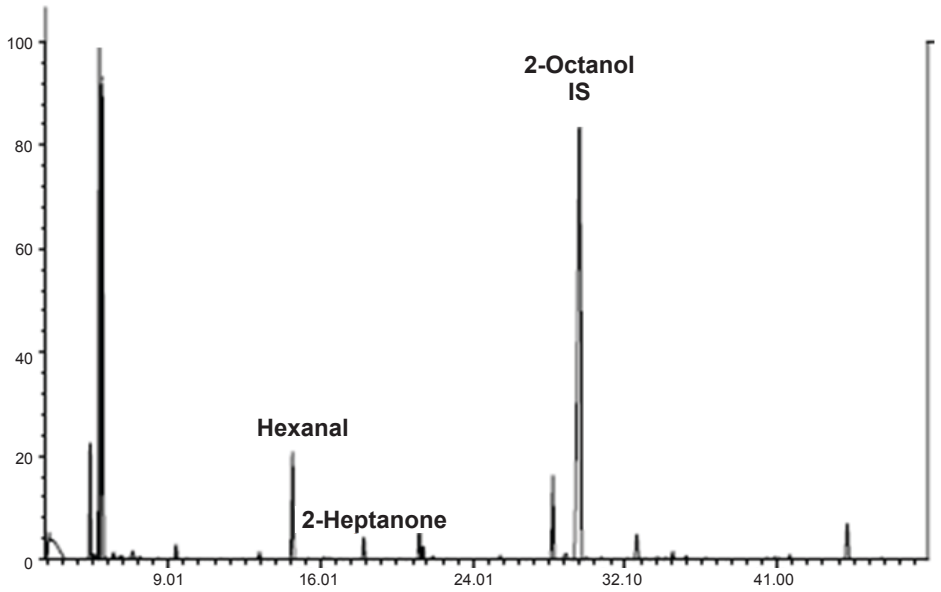

Figure 4. Gas chromatogram of volatile organic compounds (VOC) from VCO which had been exposed to UV light in the presence of 35 µg/g Fe^{3+}. The VOCs were sampled using solid phase microextraction.

FFA concentration lower than 0.01%. Therefore, the more sensitive method of ^{31}P NMR was utilized to measure FFA. This involves derivatization of FFA with 2-CITMDOP to form the phosphityl followed by ^{31}P NMR analysis (Dayrit et al. 2008). The ^{31}P NMR analysis yields quantitative signals for FFA and the various alcohols that are present, such as monoglycerides and diglycerides (Figure 5). By measuring the increase in FFA concentration over time, it is possible to obtain the instantaneous rate of formation of the FFA as: $+d[FFA]/dt = k[FFA]$.

The rate of the formation of FFA in VCO was studied at 60°, 70°, and 80°C over a period of two weeks at varying levels of water content: 0.063%, 0.084%, and 0.22% moisture (saturated). The trends in the rate of FFA formation are shown in Figure 6 and are summarized

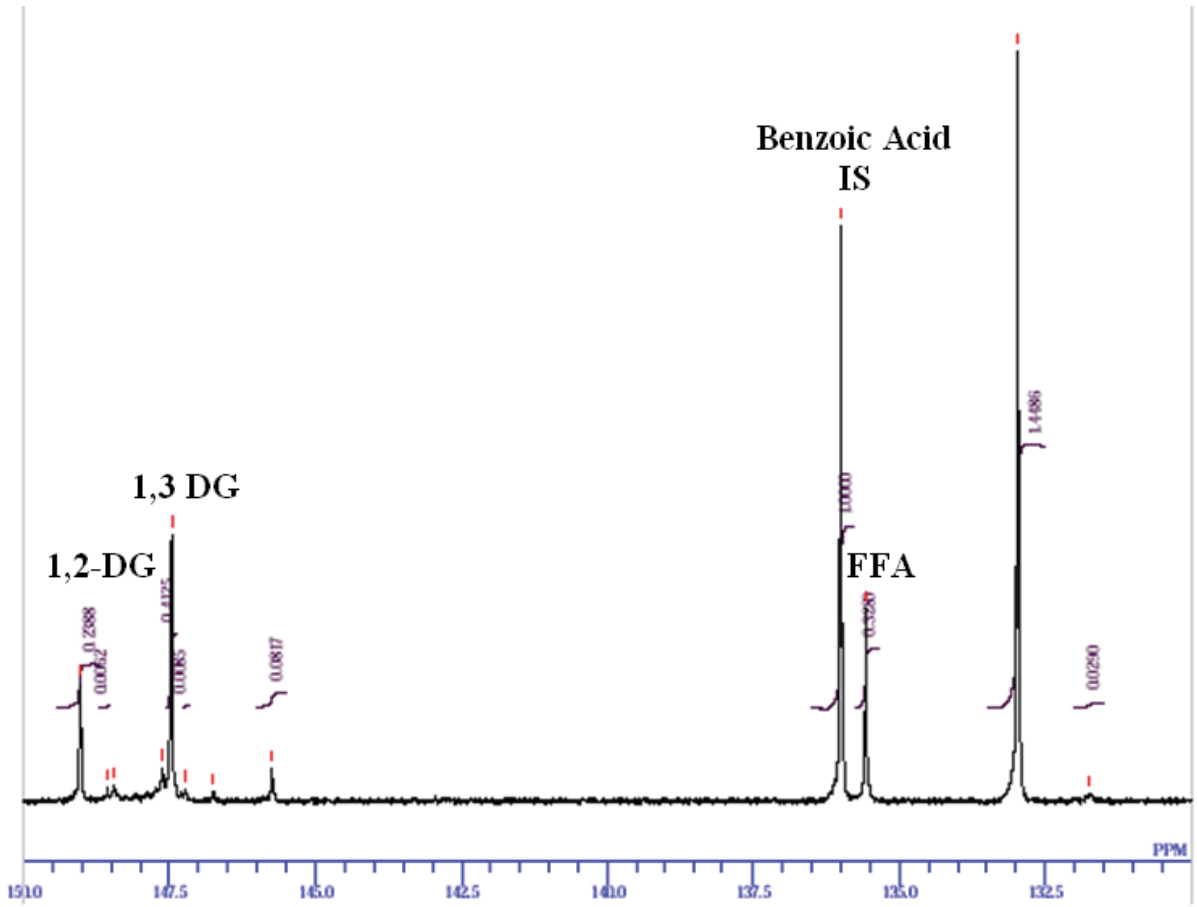

Figure 5. ^{31}P NMR spectrum of VCO to measure hydrolysis. This VCO sample was heated to 80° C for 72 hrs in the presence of 0.22% moisture (saturated) in a sealed tube.

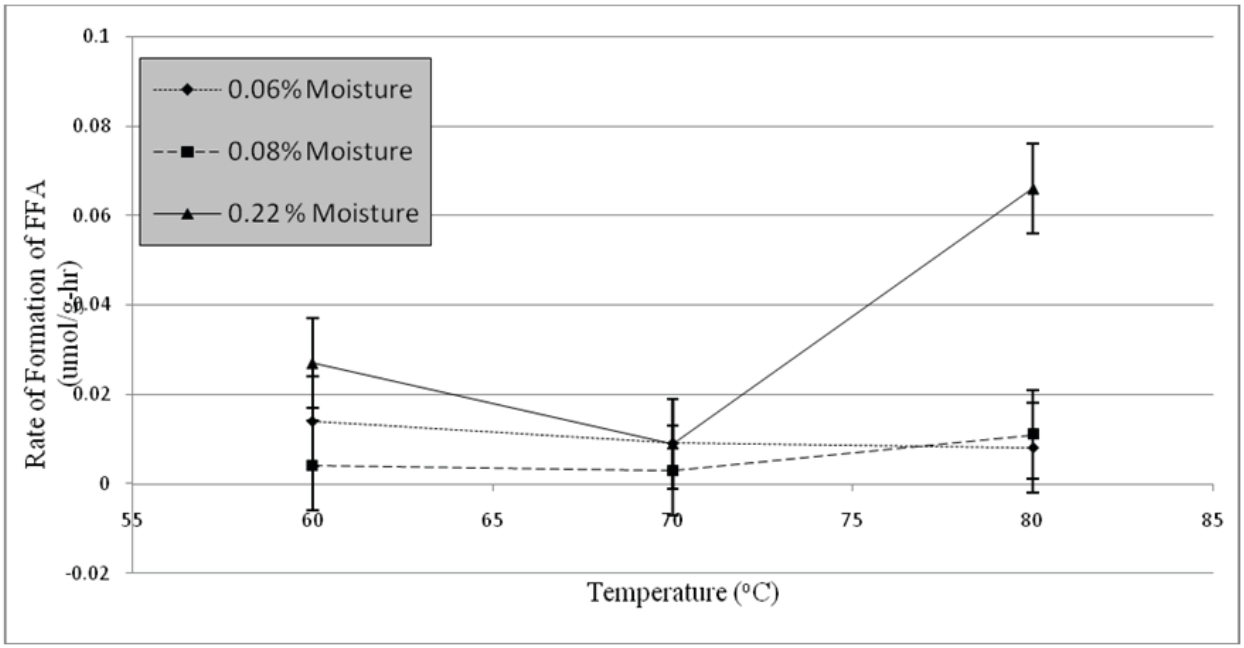

Figure 6. Rates of formation of free fatty acids (FFA) (μmol/g-hr) in VCO at different moisture levels and temperatures, in a closed system. The standard deviation was calculated from the range of data with n = 2 using the d_2 estimator (Mullins 2003). The error bar represents the average expanded uncertainty at 95% confidence.

on Table 4. At room temperature (30°C) and 0.063% moisture, the rate of FFA formation was found to be less than 0.001 µmol/g-hr (expressed as lauric acid). In this case, it will take over 197 days to reach 0.10% FFA. On the other hand, FFA formed fastest in VCO in the presence of 0.22% moisture (saturated mixture) at 80°C at a rate of 0.066 µmol/g-hr. In this case, it will take about three days to reach 0.10% FFA from the hydrolysis of VCO. It is clear from these results that higher temperatures and moisture lead to faster rates of hydrolysis of VCO.

Table 4. Hydrolysis reaction rates from ^{31}P NMR experiments. The hydrolysis reaction rate was obtained by measuring the change in concentration of FFA in VCO over one week at various temperatures in the presence of moisture (see Figure 6).

% Moisture	Temperature, °C	Rate of FFA formation, µmol/g-hr, as lauric acid	Time to form 0.1% FFA, days
0.063	30	<0.001	>197
0.063	80	0.008	26
0.084	80	0.011	19
0.22 (satd)	80	0.066	3

Microbial Oxidation of VCO

Microorganisms were collected from commercial VCO samples and inoculated and grown in nutrient agar at 37°C. Gram-positive and gram-negative bacteria were isolated and these were spiked into sterile VCO samples containing various amounts of moisture. The spiked samples were incubated for four days at 37°C.

The peroxide value of the samples spiked with microorganisms was measured after four days. There was no difference in the PV of the samples with and without microorganisms at 0.10% and 0.22% moisture level. Since no increase in PV was detected, it can be concluded that microbial oxidation of VCO is insignificant.

Microbial Hydrolysis of VCO

The hydrolysis of VCO in the presence of inoculated microorganisms was measured at 0.10% and 0.22% moisture levels (Table 5). The change in titratable acidity in the VCO samples four days after inoculation with microorganisms was determined. Only the VCO which had 0.22% moisture and gram-positive bacteria exhibited a measurable increase in titratable acidity of 0.017.

The presence of gram-positive bacteria, in particular *Bacillus*, *Lactobacillus* and *Streptococcus* species, has been reported in various VCO samples (Belandres & Sanchez 1994). Interestingly, VCO spiked with gram-negative bacteria did not show a measurable increase in titratable acidity.

Table 5. Change in titratable acidity in VCO samples four days after inoculation with coconut milk and incubation at 37°C. Titratable acidity is expressed as % lauric acid. "ncd": no change detected, difference in titratable acidity <0.005.

Moisture content	Titratable Acidity		
	VCO Only	VCO with gram negative bacteria	VCO with gram positive bacteria
0.10%	ncd	ncd	ncd
0.22%	ncd	ncd	0.017

Formation of Organic Acids from Microbial Fermentation of VCO

In order to determine the effect of bacteria and moisture on the quality of VCO, small aliquots of non-sterile coconut milk were spiked into four flasks containing VCO producing four solutions with different moisture contents (0.027%, 0.045%, 0.059% and 0.097%). The VCO solutions were then incubated at 37°C for four days. Eleven organic acids were identified by GC-MS, confirmed by comparison with standards and quantified. These were identified as: acetic acid, butyric acid, decanoic acid, dodecanoic acid, fumaric acid, hexanoic acid, hippuric acid, lactic acid, octanoic acid, pyruvic acid, and succinic acid (Table 6 and Figure 7).

Lactic acid was the predominant organic acid. It was measured at 1.5, 1.7, 1.1, 3.8 mg/mL, as the moisture level increased from 0.027%, 0.045%, 0.059%, and 0.097%, respectively. Pyruvic acid was measured at a comparable concentration of 1.4 mg/mL at 0.027% moisture, but its concentration did not increase significantly from this level at higher moisture levels. Dodecanoic acid increased from 0.73 mg/mL at 0.027% moisture to 1.3 mg/mL at 0.097%.

The most notable pattern that can be seen from Figure 7 is that up to 0.059% moisture level, the concentrations of the organic acids were either constant or increasing slowly, but that above 0.059% moisture, there was a marked increase, particularly for lactic, dodecanoic, succinic, fumaric and acetic acids, with smaller increases for octanoic and decanoic acids. The organic acids that remained generally unchanged in concentration were hippuric, butyric and hexanoic acids.

The FFAs – hexanoic, octanoic, decanoic and dodecanoic acids – which accounted for about 20% of the total acidity were presumably formed from hydrolysis of triglycerides. However, the most significant changes, in particular above 0.059% moisture level, were the organic acids which can be attributed to the action of microorganisms; these acids accounted for about 80% of titratable acidity, in particular lactic, succinic, fumaric, and acetic acids.

Table 6. GC analysis of organic acids in VCO showing the effect of bacteria and moisture content after 4 days of incubation at 37°C. **A**. The value in parenthesis is the % relative standard deviation of duplicate runs. The data are plotted in Figure 7. **B**. Comparison of organic acids from FFA (dodecanoic, decanoic, octanoic and hexanoic acids) and organic acids produced from microbial action (lactic, succinic, pyruvic, fumaric, acetic, hippuric and butyric acids).

Organic acid	Moisture Level			
	0.027%	0.045%	0.059%	0.097%
A	**Concentration of organic acids in VCO after 4 days of incubation at 37°C (µg/mL)**			
Lactic	1484 (±21%)	1736 (±12%)	1072 (±20%)	3787 (±13%)
Dodecanoic	731 (±4%)	576 (±2%)	686 (±14%)	1280 (±1%)
Succinic	388 (±3%)	403 (±35%)	282 (±11%)	1070 (±4%)
Pyruvic	1352 (±24%)	629 (±22%)	770 (±17%)	985 (±21%)
Fumaric	369 (±8%)	359 (±24%)	343 (±2%)	881 (±1%)
Acetic	457 (±19%)	417 (±23%)	305 (±11%)	843 (±7%)
Decanoic	103 (±26%)	131 (±26%)	30 (±50%)	305 (±30%)
Octanoic	140 (±3%)	153 (±17%)	118 (±3%)	298 (±8%)
Hippuric	253 (±4%)	302 (±11%)	231 (±8%)	264 (±9%)
Butyric	51 (±61%)	37 (±23%)	30 (±18%)	41 (±10%)
Hexanoic	ND	ND	11 (±20%)	23 (±32%)
B	Proportion of titratable acids			
Organic acids from microbial action	81.7%	81.9%	78.2%	80.5%
FFA	18.3%	18.1%	21.8%	19.5%

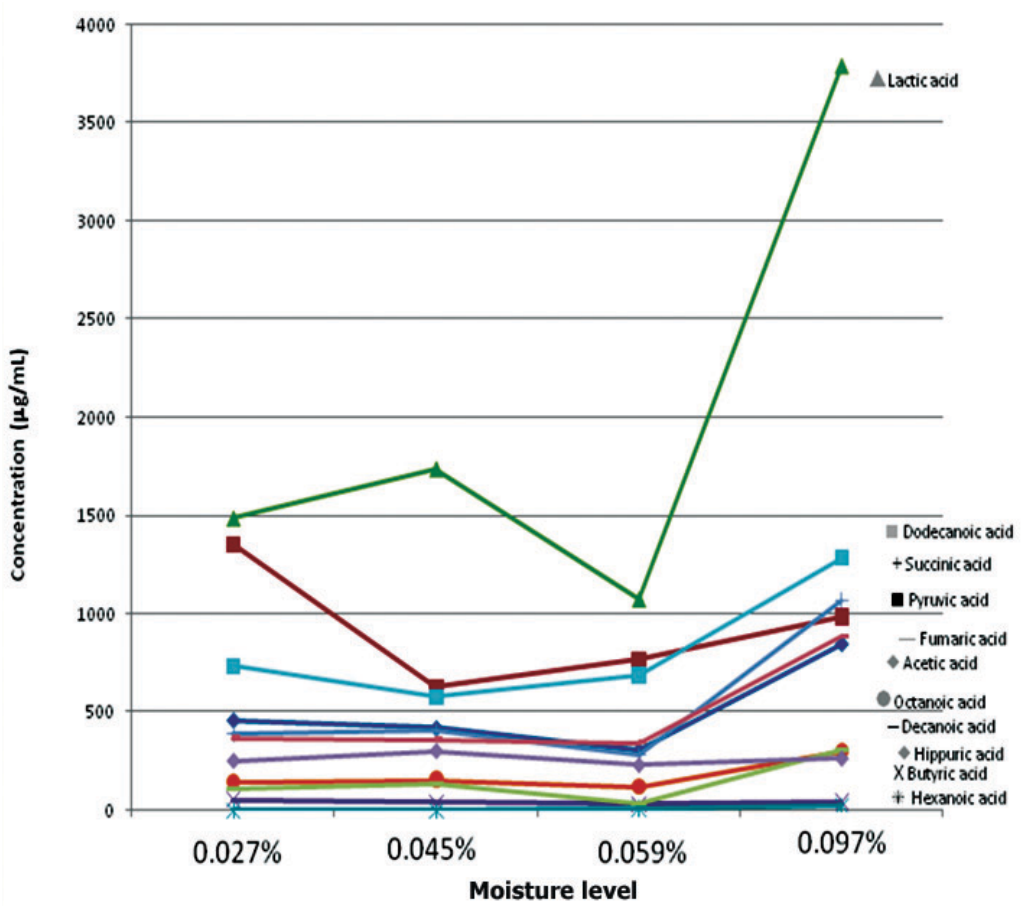

Figure 7. Trends in the concentrations of organic acids in VCO samples which have been inoculated with microorganisms in coconut milk, at different moisture levels after 4 days of incubation at 37°C. The data are plotted from Table 6A.

DISCUSSION

The degradation of virgin coconut oil via physico-chemical oxidation and hydrolysis, and microbial action was studied. Microbial degradation can occur by both simple and complex oxidative and hydrolytic transformations. The key results can be summarized as follows:

1. The physico-chemical oxidation of VCO in the air at room temperature is negligible. Oxidation of VCO was significant only when air, UV radiation, and Fe^{3+} were present; the presence of high levels of FFA further promotes oxidation by Fe^{3+}. Hexanal is the most significant product of oxidation. In general, VCO shows better stability than vegetable oils which have high levels of polyunsaturated fatty acids.

2. VCO contains relatively low levels of phenolic antioxidants (around 6 mg GAE/100 g oil). α-Tocopherol was not detected.

3. The rate of chemical hydrolysis of VCO was measured through the increase in FFA under various conditions. At room temperature and 0.06% moisture, it will take more than 197 days to produce 0.1% FFA. On the other hand, at 80°C and 0.22% moisture (saturated), it will only take three days to form 0.1% FFA.

4. At low moisture level (less than 0.06%), VCO is stable to microbial decomposition. However, at higher moisture levels, microbial action leads to the formation of organic acids, in particular, acetic acid, lactic acid, hexanoic acid, succinic acid, fumaric acid, decanoic acid, and dodecanoic acid. Thus, it is worth noting that while the standard analysis by titrimetry assumes that the titratable acidity is primarily due to FFA, the results of this study indicate that, when microbial action is occurring, over half of the titratable acidity may be due to organic acids from microbial processes.

In summary, the most important conditions that influence the physico-chemical and microbial degradation of VCO are moisture, temperature, and the presence of microorganisms. However, these degradation processes can be minimized if the moisture level is maintained below 0.06%.

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