Modulation of essential fatty acid levels in coconut oil with flaxseed oil

OA.A. Joshi^a, OM.V. Hegde^a and OA.A. Zanwar^{a,⊠}

^aCentre for Innovation in Nutrition Health Disease, Interactive Research School for Health Affairs, Bharati Vidyapeeth (Deemed to be University), Pune-411 043, India ^{III}Corresponding author: anand.zanwar@bharatividyapeeth.edu

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SUMMARY: Coconut oil (CO) is a popular cooking medium but its lack of essential fatty acids (FA) is a health concern. Therefore, the aim of this work was to improve the FA profile of CO by blending with flaxseed oil (FO). Blends with various percentages of FO were prepared and studied for physicochemical characterization, thermal and long-term storage stability. The results indicated that the blends made favorable alterations in FA composition without adverse effects to the oxidative stability of the fatty acids and they resisted secondary thermal deterioration up to two hours at 180 °C. The blend with the highest percentage of FO was stable for nine months. THP-1 cell line studies showed that ω -3 FA from the blend was incorporated into the cells with no adverse effect on cell viability but the inflammatory markers studied remained unaltered. Thus, CO and FO blends could be stored for at least nine months and could be used as cooking medium when prolonged heating is not involved.

KEYWORDS: Blending; Coconut oil; Essential fatty acids; Flaxseed oil; ω -3 fatty acids; ω -6: ω -3 ratio

RESUMEN: *Modulación de los niveles de ácidos grasos esenciales en aceite de coco con aceite de linaza.* El aceite de coco (CO) es un medio popular para cocinar, pero su falta de ácidos grasos esenciales (AG) supone un problema para la salud. Por tanto, el objetivo de este trabajo fue mejorar el perfil de AG de CO mediante la mezcla con aceite de linaza (AL). Se prepararon y estudiaron mezclas con diversos porcentajes de AL para su caracterización fisicoquímica, estabilidad térmica y almacenamiento a largo plazo. Los resultados indicaron que las mezclas tuvieron una alteración favorable en la composición de AG, sin efectos adversos en su estabilidad oxidativa y resistieron el deterioro térmico secundario hasta dos horas a 180 °C. La mezcla con el mayor porcentaje de AL se mantuvo estable durante nueve meses. Los estudios de la línea celular THP-1 mostraron que los ácidos grasos ω -3 de la mezcla, se incorporaron en las células sin efectos adversos sobre la viabilidad celular, pero los marcadores inflamatorios estudiados permanecieron inalterados. Por lo tanto, las mezclas de CO y AL podrían almacenarse al menos durante nueve meses y podrían usarse como medio de cocción donde no se requiere un calentamiento prolongado.

PALABRAS CLAVE: Aceite de coco; Aceite de linaza; Ácido graso ω-3; Ácidos grasos esenciales; Mezclas; Relación ω-6:ω-3

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1. INTRODUCTION

In the tropical countries coconut oil (CO) has been used as cooking oil since long ago. In recent years, CO has become popular globally. CO is available as virgin coconut oil (VCO) and copra oil. The FA compositions of these two varieties are almost the same but the former contains higher levels of biologically active compounds (Wallace, 2019). CO is dominant in medium-chain saturated fatty acids (MC-SFA). CO belongs to the lauric oil group in which oils rich in lauric acid are included. CO has a long shelf-life and is very stable against atmospheric oxidation (Bhatnagar et al., 2009). However, regulatory authorities in many countries are concerned about the adverse health effects of CO mainly because of its high SFA content and lack of essential FA (omega-3 (ω -3) and omega-6 $(\omega$ -6)) (Lima and Block, 2019). Dietary intake of Linoleic acid (ω -6 FA, LA) and Alpha linolenic acid (ω -3 FA, ALA) is required as the human body cannot synthesize these FA. Unfortunately, today, the human diet is low in ω -3 FA (anti-inflammatory nature) and loaded with ω -6 FA (inflammatory nature) (Kaur et al., 2014). Therefore, improving the consumption of ω -3 FA and simultaneously reducing the consumption of ω -6 FA is important. Additionally, Bhatanagar et al. (2009) have suggested that prolonged use of CO may result in diets deficient in MUFA and PUFA.

Edible oils serve as an excellent source of FA including essential FA. They provide FA in the form of triacylglycerols. (Kaur *et al.*, 2014). It is possible to alter CO's nutritional value (i.e. FA composition) by blending it with an oil rich in MUFA/PUFA (Chandrashekar *et al.*, 2010). ALA is known to possess many health benefits and flaxseed oil (FO) is a major plant source of ALA. It also contains a good amount of ω -6 FA; LA (Kaur *et al.*, 2014). Because of its high PUFA content, FO is oxidatively and thermally very unstable (Symoniuk, 2016).

Therefore, the development of oxidatively and thermally stable CO blends to provide essential ω -3 and ω -6 FA was the objective of this study. CO blends containing various percentages of FO were prepared to achieve different ω -6 and ω -3 FA percentages. Here, we report changes in the FA composition (modulation of nutritive parameter), oxidative and thermal stability of the developed CO blends. The storage stability of ω -3 FA contained in the blend (with FO 20%) was studied for up to nine months. THP-1 cell line was used to study the effects of the CO blends at the cellular level.

2. MATERIALS AND METHODS

2.1. Materials used

The Real World Nutrition Laboratory Foundation, Pune (India) had supplied cold-pressed FO and cold-pressed CO was bought from local market. Analytical grade reagents were procured from SRL Laboratories. Fine chemicals (like MTT and LPS) were purchased from Sigma-Aldrich (Saint Louis, MO, USA). ELISA kits for Human TNF α and IL-6 were bought from eBiosciences (Vienna, Austria). Cell culture grade reagents like RPMI 1640 medium (Cell culture medium), fetal bovine serum (FBS), DMSO and other reagents required for THP-1 cell line studies were obtained from Hi-Media (India).

2.2. Preparation of CO blends

Three blends containing either 20, 10 or 5% FO (v/v) were prepared using CO as the base oil. For proper blending of the two oils, a mechanical homogenizer (Remi Elektrotechnik Ltd., India) was used (15-20 minutes, three cycles of five minutes, mixing with two-minute breaks). Blending was done at room temperature and no rise in temperature was detected in any of the cases.

2.3. Characterization of CO blends: Study of initial physicochemical parameters

2.3.1. Determination of FA composition of CO blends

The Ichihara and Fukubayashi (2010) method with some modifications was used to determine the FA compositions of the oils and blends. To summarize, 50 mg oil/blend were used for the preparation of fatty acid methyl esters (FAME) by adding methanolic HCl containing BHT (antioxidant, 50 mg/mL) and subjecting the mixture to 80 °C (in a water bath) for 2 h. n-hexane was used to extract the formed FAME. After drying the FAME (using nitrogen gas), they were reconstituted in n-hexane and subjected to FA analysis by gas chromatography (GC) (7820A, Agilent, Santa Clara, CA, USA), which was coupled with a Flame Ionization Detector (FID). The stationary phase was HP88 capillary column; 30 m long with i.d. 0.25 mm and the thickness was 0.2 μ m. The injector and FID temperature was set at 250 °C. Oven temperature was set at 140 °C for an initial 5 min and with 4 °C/min rise, until reaching 230 °C and held at 230 °C for 12.5 min. 1:25 was the split ratio and nitrogen was the carrier gas at a flow rate of 1.1 mL/min. The FA composition is expressed as % FA.

2.3.2. Physico-chemical parameter determination

The acid value (AV, expressed as mg KOH/ g oil), % free fatty acid (% FFA, expressed as % FFA (as lauric acid)) and peroxide value (PV, expressed as milliequivalent O_2 / kg oil) were determined using AOAC official Method 940.28, Ca 5a-40 and Cd 8b-90, respectively (Jagtap *et al.*, 2021, Joshi *et al.*, 2022 and De Boer *et al.*, 2018). Smoke point (SP) was determined according to a method reported by Das *et al.* (2013).

2.4. Effect of heat on various chemical parameters of CO blends

2.4.1. Evaluation of K232 and K268 of oils/blends

The CO blends were subjected to heat at 180 °C for 240 minute (four hours). Samples were collected at 0, 60, 120 and 240 minutes of heating. Using UV/VIS spectrophotometer (UV 3000⁺LABINDIA ANA-LYTICAL, India), the absorbance of the oils/blends (dissolved in iso-octane) was measured at 232 nm and 268 nm. Values for K_{232} and K_{268} were estimated using the formula cited by Malvis *et al.* (2019).

2.4.2. Evaluation of oxidation status of oils/blends

The oxidation status of CO blends after four hours of heating was analyzed by estimating the PV, the para-anisidine Value (*p*-AV) and total oxidation value (TOTOX Value) using AOCS official methods.

2.5. Determination of storage stability of CO blends at room temperature

A storage stability study was conducted for the blend containing the highest level of oxidatively susceptible FO (20 %). During this study, the oils and blends were stored at room temperature. The study was continued up to nine months by evaluating the PV, AV and FA composition of the blends at specified time points.

2.6. Assessment of effects of CO blends in THP-1 cell line

The human monocytic leukemia cell line, THP-1 was cultured in 10% FBS containing a RPMI 1640 medium under standard incubation conditions. The oils or blends were dissolved in DMSO (50 mg/mL). DMSO stocks were diluted in FBS under sterile conditions and pre-incubated (before addition to cells) at 37 °C for one hour. When the FBS stocks were added to the cells, the final FBS concentration was maintained at 10 % and the final DMSO concentration was 0.5% in all the studies. Only DMSO pre-incubated with FBS served as the control (Joshi *et al.*, 2022).

2.6.1. Effect of CO blends on THP-1 cell viability

THP-1 cells were seeded at the density of 1×10^5 cells/well on the transparent 96W plate. The concentrations of oils or CO blends used to treat the cells were 125, 62.5 and 31.25 µg/mL. A MTT assay was performed to assess cell viability at the end of 24. 48 and 72 h treatment. In brief, 1 mg/mL MTT stock was added to the cells (200 µL/well) after careful removal of the culture medium. The plates were incubated in the CO₂ incubator for three hours followed by careful removal of the MTT solution from all the wells. DMSO was added to all the cells (100 μ L/ well) and the plates were kept at room temperature for 20 minute to allow dissolution of formazan crystals. Absorbance was measured at 570 nm. Viability percentage was calculated, assuming the viability of control cells (cells treated with DMSO) to be 100%.

2.6.2. Effect of CO blends on fatty acid composition of THP-1 cells

At the end of 48 h treatment with the CO blends (125 μ g/mL), THP-1 cells were harvested followed by two, 1X PBS washes. As per Folch *et al.* (1957), the total lipid extraction was done and the Ichihara and Fukubayashi (2010) method was used for the esterification of the lipids extracted from the cells. The THP-1 FA composition was determined by performing GC on the esterified lipids. The GC conditions as mentioned in section '2.3.1' were used. The data is presented as % FA of the total lipids extracted from the cells.

2.6.3. Effect of the CO blends on TNFa and IL-6

THP-1 cells (1×10^5 /well in 24 W plate) were treated with individual oils or CO blends ($125 \mu g$ /

mL) for 48 h. For TNF α and IL-6 estimation, oil or blend treated cells were LPS (25 ng/mL) stimulated. The cell supernatant was collected (after LPS stimulation) at 6 h for TNF α and at 24 h for IL-6. ELISAs were performed as per manufacturer's instructions to determine cytokine levels. The data is graphically represented as fold change compared to +LPS (only LPS stimulation).

2.7. Statistical analysis

Each measurement was taken in triplicate. Data is represented as Mean \pm SD (n=3). GraphPad Prism (version 5.02) software was used for statistical analysis. Details of the statistical analyses and tests used are reported in the footnote and caption for each data.

3. RESULTS AND DISCUSSION

3.1. Preparation of CO blends

The aim of this study was to improve ω -3 and ω -6 levels in CO through blending with FO. Blends of CO with soybean, safflower, rice bran oil, tiger nut oil, and groundnut oil have been prepared and studied in various animal models (Chandrashekar *et al.*, 2010). As the richest plant source of ALA, FO (5-

52 %) was used in the blends (Hintze *et al.*, 2016). Here, 20, 10 and 5 FO (v/v) were used to prepare blends with CO to achieve various percentages of essential FA. Depending on the FO percentage in the blend, they were labeled C20, C10 and C5.

3.2. Characterization of CO blends: Study of initial physicochemical parameters

3.2.1. Fatty acid composition of CO blends

GC-FID analysis was used to confirm altered essential FA levels in the blends. The FA composition of the individual oils and CO blends is presented in Table 1. The FA compositions of CO and blends were statistically different than FO which was also reflected in the total SFA, MUFA and PUFA contents (#; p < 0.001 versus FO, \$; p < 0.05 versus FO). FO had the highest PUFA content while CO had the highest saturated FA content with lauric acid as the major FA. The reported percentages in lauric acid are in the range of 45.9-52.6 (Kumar et al., 2015; Bhatnagar et al., 2009). CO had meager amounts of both MUFA and PUFA, especially essential FA and LA. CO had no ALA. A similar FA composition for CO has been reported in the literature (Kumar et al., 2015; Bhatnagar et al., 2009; Guillaume et al., 2018,

Table 1. Fat	ty acid	l composition	of CO	blends
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Fatty acid	FO	СО	C5	C10	C20
Caprillic acid	0.00 ± 0.00	$6.69 \pm 0.03^{\#}$	$5.78\pm0.04^{\text{H},\omega}$	$4.89\pm0.01^{\text{\#, }\omega,\mu}$	$4.28\pm0.04^{\text{H},\omega,\mu}$
Capric acid	0.00 ± 0.00	$5.5\pm0.04^{\scriptscriptstyle\#}$	$4.99\pm0.06^{\text{H},\text{A}}$	$4.4\pm0.01^{\text{\#, }\omega,\mu}$	$3.98\pm 0.02^{\#,\omega,\ \mu,*}$
Lauric acid	0.00 ± 0.00	$47.94 \pm 0.07^{\#}$	$45.2\pm0.07^{\text{\#,}\omega}$	$42.26\pm0.2^{\text{\#, }\omega,\mu}$	$36.36\pm 0.02^{\#,\omega,\ \mu,\ *}$
Myristic acid	0.00 ± 0.00	$20.64 \pm 0.04^{\#}$	$19.7\pm0.04^{\text{\#,}\omega}$	$19.21 \pm 0.03^{\text{\#, }\omega,\text{`'}}$	$16.05\pm 0.08^{\text{H},\omega,\mu,*}$
Palmitic acid	6.61 ± 0.08	$8.7\pm0.23^{\scriptscriptstyle\#}$	$8.72 \pm 0.06^{\#}$	$8.98 \pm 0.04^{\text{#,"}}$	$8.7\pm0.01^{\scriptscriptstyle\#}$
Stearic acid	6.59 ± 0.07	$3.76\pm0.02^{\scriptscriptstyle\#}$	$3.91\pm0.03^{\scriptscriptstyle\#}$	$4.22\pm0.01^{\text{H,A},\text{``}}$	$4.68\pm0.04^{\text{\#,}\omega,\mu,\alpha}$
Oleic acid	21.61 ± 0.01	$5.54\pm0.04^{\scriptscriptstyle\#}$	$6.61\pm0.02^{\text{\#,}\omega}$	$7.39\pm0.22^{\text{\#, }\omega,\mu}$	$9.09\pm 0.11^{\#,\omega,\mu,*}$
Linoleic acid (LA)	14.47 ± 0.1	$0.87\pm0.03^{\scriptscriptstyle\#}$	$1.65\pm0.08^{\text{H},\omega}$	$2.37\pm0.02^{\text{\#, }\omega,\mu}$	$3.97\pm 0.02^{\text{\#,}\omega,\mu,*}$
Alpha Linolenic acid (ALA)	50.72 ± 0.23	$0.00\pm0.00^{\#}$	$3.14\pm0.01^{\text{\#,}\omega}$	$5.9\pm0.01^{\text{\#, }\omega,\mu}$	$12.1\pm 0.02^{\text{\#,}\omega,\mu,*}$
ΣS	13.2 ± 0.16	$93.22\pm0.42^{\#}$	$88.29\pm0.3^{\text{\#,}\omega}$	$83.95 \pm 0.25^{\#,\omega,\mu}$	$74.03\pm0.09^{\#,\omega,\ \mu,\ *}$
ΣΜ	21.61 ± 0.01	$5.54\pm0.04^{\scriptscriptstyle\#}$	$6.61\pm0.02^{\text{\#,}\omega}$	$7.39\pm0.22^{\text{\#, }\omega,\mu}$	$9.09\pm 0.11^{\text{\#,}\omega,\mu,*}$
ΣΡ	65.19 ± 0.13	$0.87\pm0.03^{\scriptscriptstyle\#}$	$4.24\pm0.83^{\text{\#,}\omega}$	$8.26\pm0.03^{\text{\#, }\omega,\mu}$	$16.06\pm 0.04^{\text{\#,}\omega,\mu,*}$
LA: ALA	0.29 ± 0.00	ND	$0.52 \pm 0.03^{\#}$	$0.40\pm0.00^{\text{\#},\beta}$	$0.33 \pm 0.00^{\text{s},\mu,\&}$

The FA compositions of the individual oils and CO blends were estimated by GC-FID. Data are presented as Mean \pm SD (n=3). FO: Flaxseed oil alone, CO: Coconut oil alone, C5: Coconut oil blend containing 5% flaxseed oil, C10: Coconut oil blend containing 10% flaxseed oil, C20: Coconut oil blend containing 20% flaxseed oil, Σ S: total saturated fatty acids, Σ M: total monounsaturated fatty acids and Σ P: total unsaturated fatty acids. Statistically significant differences were determined by Two Way ANOVA and Bonferroni posttests. #; p < 0.001 versus FO, \$; p < 0.05 versus FO, ω ; p < 0.001 versus CO, Δ ; p < 0.05 versus CO, μ ; p < 0.001 versus C5, "; p < 0.05 versus C5, β ; p < 0.001 versus C10, α ; p < 0.05 versus C10, ω ; p < 0.01 versus C10

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Maurikaa *et al.*, 2020). FO showed ALA as the dominant FA and OA and LA were the next abundant FA. When compared to CO, the blends had significantly modified FA composition except for PA content. As the FO percentage in the CO blends increased, ALA content also increased, resulting in significantly lower LA:ALA. Therefore, from Table 1, it is clear that by blending FO and CO, the FA profile, especially ALA and LA contents and LA:ALA ratio were significantly altered. In addition, total SFA was significantly lower with a significant increase in total MUFA and PUFA contents just by adding 5% FO into the blend.

3.2.2. Physico-chemical characterization of CO blends

The physico-chemical parameters of the prepared blends were estimated immediately after blend preparation. These parameters (AV, % FFA, PV and SP) are represented in Table 2. The quality of any oil is indicated by its AV and % FFA (Mahesar et al., 2014). The AV for FO was significantly higher than CO and the blends. Maurikaa et al. (2020) reported the AV of CO at 0.9 mg KOH/g oil. For FO, the reported AV range from 0.21 (Symoniuk et al., 2016) to 0.53 (Bhardwaj et al., 2015). There were no significant differences between and among the AV of the CO and the blends. % FFA values were similar for FO, CO and the blends except for C20, which had a significantly higher value than FO. In the case of CO, reported % FFA values were in the range of 0.53 - 0.65 (Kumar *et al.*, 2015) and Perera et al., 2020) while 0.32 was the % FFA reported for FO by Bhardwaj et al. (2015). PV represents oxidative deterioration of the oil (Perera et al., 2020). No peroxides were detected in CO but blending with FO resulted in significant increase in PV for the blends compared to CO. Among the blends, there were statistically significant differences. The PV for CO was reported in the range of 0.00 -3.9 (Kumar et al., 2009; Moigradean et al., 2012; Kumar et al., 2015; Perera et al., 2020), while the reported values for the PV of FO were 0.98 (Bhardwaj et al., 2015) and 2.34 (Symoniuk et al., 2016). A temperature at which various components of oil undergo breakdown and are noticed in the form of fumes is known as the SP (Guillaume et al., 2018). The SP of FO was significantly lower than CO and its blends. Compared to CO, the blends had significantly lower SP. But compared to the SP of FO, the SP for the blends were significantly higher (p < 0.01 versus FO). The literature reports the SP of CO at 191±3.6 °C (Guillaume et al., 2018) and 193±3.0 °C by Perera et al., (2020). The observed differences in the values of the studied parameters may be due to differences in the raw material, handling, processing and storage conditions and duration (Mahesar et al., 2014).

For the blends, AV, % FFA and PV increased as the contribution (percentage) of FO in the blend increased, although the SP of the blends decreased. The observed increase in the studied parameters may be because of the presence of pro-oxidants in the oil (Nering 2016). It is important to note that, though the SP for the blends were lower than CO, they were still higher than the routinely used cooking temperatures. Thus, the data from Table 2, indicates that the CO blends containing FO at up to 20% can be used as cooking oils without disturbing their physicochemical characteristics adversely.

TABLE 2. Physico-chemical parameters of FO, CO and their blends determined immediately after the blend preparation.

Oil/Blend	AV (mg KOH/ g oil)	% FFA (as Lauric acid)	PV (meq O ₂ /kg oil)	SP (°C)	-
FO	2.52 ± 0.48	0.49 ± 0.01	0.80 ± 0.00	104.0 ± 1.41	
СО	$0.84 \pm 0.08^{**}$	0.59 ± 0.09	$0.00\pm 0.00^{***}$	$205.0\pm1.41^{\mu}$	
C5	$0.90 \pm 0.00^{**}$	0.67 ± 0.06	$0.65\pm0.07^{\scriptscriptstyle\#}$	$198.5 \pm 0.71^{\mu,\pi}$	
C10	$0.90 \pm 0.00^{**}$	0.73 ± 0.11	$0.60 \pm 0.00^{*,\#}$	$198.5 \pm 0.71^{\mu,\pi}$	
C20	$1.09 \pm 0.04^{**}$	$0.85 \pm 0.00^{*}$	$0.85\pm 0.07^{\text{\#,@,\%}}$	$196.5 \pm 0.71^{\mu,\pi}$	

Data are Mean \pm SD (n=3). FO: Flaxseed oil alone, CO: Coconut oil alone, C5: Coconut oil blend containing 5% flaxseed oil, C10: Coconut oil blend containing 10% flaxseed oil and C20: Coconut oil blend containing 20% flaxseed oil. Statistically significant differences were determined by One-way ANOVA and Tukey's Multiple Comparison Test. *and **and ***; p < 0.05 versus FO, #; p < 0.05 versus CO, @; p < 0.05 versus C5, %; p < 0.05 versus C10, μ ; p < 0.01 versus FO and π ; p < 0.01 versus CO

3.3. Effect of heat on various chemical parameters of CO blends

Heating oil results in the formation and accumulation of various primary and secondary oxidation products. This results in declined oil quality. K_{232} and K_{268} are used to determine the levels of conjugated dienes and conjugated trienes formed during the heating of oil (Malvis *et al.*, 2019). PV indicates initial oxidation status of the oil while *para*-anisidine (*p*-AV) represents secondary oxidation of the oil. Additionally, TOTOX value gives overall oxidation status of the oil.

3.3.1. Effect on K₂₃₂ and K₂₆₈ of oils/blends

Figure 1 represents the changes in the K_{232} and K_{268} values for the oils and blends heated up to 240 min (four hour) at 180 °C. As shown in Figure 1a, before heating (0 min) as well as after heating, FO had a significantly higher K_{232} value when compared to CO and all the blends (*; p < 0.001) at respective time points. No statistically significant differences

were observed between or among CO and all the blends at 0 min. FO, CO and all the blends started to show a significant rise in $K_{_{232}}$ right from 60 min (#; p < 0.001 versus 0 min). Though the fold rises (0 and 240 min) for CO and the blends were higher than FO, FO had higher values than the blends. It is significant to note that when compared to CO, C10 and C20 showed significant deterioration after 120 minutes; whereas in the case of C5, it was at 180 minutes (μ ; p < 0.001 and β ; p < 0.05 versus CO at respective time points).

Figure 1b represents the effect of heating on K_{268} . Before heating (0 min), no significant differences for K_{268} were detected among the individual oils or the CO blends. FO started showing a significant rise in K_{268} right from 60 min (&; p < 0.001 versus 0 min and previous time point). CO did not show any rise in K_{268} at any of the time points of heating. Although the blends did show resistance for significant rise (at 60 min), the significance level rise at later time points (#; p < 0.001 versus 0 min). FO K_{268} values were significantly high at all the time points of heat-



Figure 1. Effect of heat (180 °C for four hours) on K_{232} and k_{268} of the oils and blends. K_{232} (a) and K_{268} (b) were estimated for individual oils or CO blends when heated at 180 °C for up to 240 mins for above specified time points (n=3). FO: Flaxseed oil alone, CO: Coconut oil alone, C5: Coconut oil blend containing 5% flaxseed oil, C10: Coconut oil blend containing 10% flaxseed oil and C20: Coconut oil blend containing 20% flaxseed oil. Statistically significant differences were determined by Two-Way ANOVA and Bonferroni posttests. *; p < 0.001 versus CO and all blends 0 min and respective time points, #; p < 0.001 versus 0 min, μ ; p < 0.001 versus CO at respective time points, &; p < 0.001 versus 0 min and previous time point, &; p < 0.001 versus CO and blends at respective time points, &; p < 0.001 versus 0 min and previous time point, &; p < 0.001 versus CO and blends at respective time points.

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Oil / Blend	PV (meq O ₂ /kg oil)		p-AV		TOTOX value	
	Before heating	After heating	Before heating	After heating	Before heating	After heating
FO	0.80 ± 0.00	9.13 0.81#	0.338 ± 0.04	$9.586 \pm 0.05^{\#}$	1.94 ± 0.04	$27.85 \pm 1.63^{\#}$
CO	0.00 ± 0.00	$6.33\pm0.12^{\scriptscriptstyle\#}$	0.074 ± 0.01	1.65 ± 0.00	$0.07\pm0.01^{@}$	$14.31 \pm 0.23^{\#}$
C5	0.65 ± 0.07	9.07 0.61#	0.082 ± 0.01	$5.31\pm0.39^{\scriptscriptstyle\#}$	1.38 ± 0.1	$21.67\pm3.2^{\scriptscriptstyle\#}$
C10	0.60 ± 0.00	$18.6\pm0.2^{\scriptscriptstyle\#}$	0.064 ± 0.02	$6.16\pm0.19^{\scriptscriptstyle\#}$	1.26 ± 0.02	$43.36 \pm 0.48^{\#}$
C20	0.85 ± 0.07	18.63 0.95#	0.096 ± 0.02	$6.29\pm0.02^{\scriptscriptstyle\#}$	1.8 ± 0.09	$43.55 \pm 1.91^{\#}$

TABLE 3. Effect of heat (180 °C for four hour) on oxidative status of CO blends

Peroxide value (PV), *para*-anisidine value (*p*-AV) and total oxidation value (TOTOX value) for oils/blends were determined before and after heating at 180 °C for four hours (240 min). Data is presented as Mean \pm SD (n=3).

FO: Flaxseed oil alone, CO: Coconut oil alone, C5: Coconut oil blend containing 5% flaxseed oil, C10: Coconut oil blend containing 10% flaxseed oil and C20: Coconut oil blend containing 20% flaxseed oil

Statistically significant differences were confirmed by Two Way ANOVA and Bonferroni posttests.

#; p < 0.001 versus 0 h, @; p < 0.05 versus FO and C20 before heating

ing (except 0 min) when compared to CO and all the blends for respective time points (%; p < 0.001). It is also important to note that, when compared to CO, all the blends showed significant deterioration from 120 min (μ ; p < 0.001 versus CO at respective time points). It is clear that though there was no statistical difference at 0 min for K₂₆₈ values, the rise at 240 min was in the order FO > C20 > C10 > C5 > CO, indicating that as the FO levels increased in the blend, K₂₆₈ also increased upon heating. Thus, from Figure 1, it is clear that though CO and the blends displayed significant rises in both K₂₃₂ and K₂₆₈, their values were always lower than FO. In addition, the blends resisted these rises up to a certain time period compared to CO.

3.3.2. Effect on oxidation status of oils/blends

Table 3 represents the PV, *p*-AV and TOTOX values for the individual oils and CO blends initially and at the end of four hours heating at 180 °C. It is clear that all three parameters after four hours heating were very significantly high for all the oils and blends except the *p*-AV for CO when compared to the before-heating values. Although the fold rise in TOTOX value was the highest for CO, it had the lowest actual value. It is important to note that fold rise for all these three parameters in the blends were higher than FO. This is probably due to the fact that thermo-oxidative deterioration products in the blends are further attacking oxidatively and thermally susceptible components present in the blends. In

another study, when CO was heated to 180 °C, the oxidative stability significantly dropped within the first hour of heating, indicating thermo-oxidative deterioration (Guillaume et al., 2018). The same authors evaluated the performance of coconut oil under two temperature conditions. In the first one, they heated the oil to 180 °C for six hours and in the second, oil was heated up to 240 °C. Samples were collected at specific time points or temperatures. In both the cases, CO showed a rise in K_{232} at later time points or temperatures after initial resistance to rise but there was no rise in K_{268} in both cases. Thus, our data is in line with Guillaume et al. (2018), where we also see a rise in K_{232} but no change in K_{268} . A significant rise in the primary oxidation but no significant deterioration measured as secondary oxidation products was also well supported by the before and after heating PV and the *p*-AV values in the case of CO.

From Table 3 and Figure 1 it is clear that though CO alone resisted secondary oxidation, it showed significant deterioration upon heating (PV and K_{232}). The thermo-oxidative deterioration reflected by the TOTOX value was highest for the CO blends. In one study, Subramanian (2019) found that CO was the least thermally and thermo-oxidatively stable oil when compared to sunflower oil, groundnut oil and gingelly oil. The thermal and oxidative susceptibility were attributed to factors other than the unsaturation level of the oil. Bhatnagar *et al.* (2009) experimentally showed that CO had low levels of natural anti-oxidants, especially total tocopherols. Researchers could successfully lower the oxidative

deterioration of CO during heating by adding essential oils which were rich in antioxidants. (Perera *et al.*, 2020).

Thus, various chemical parameters were used to determine and confirm the thermal stability of the CO blends. As the FO contribution (percentage) in the CO blends increased, thermal degradation also increased. K_{232} and K_{268} indicated that the blends tolerated different heating durations before demonstrating significant deterioration. The extent of deterioration was indicated either as the level of significance and or the time required to reach that level of significance. Eyres *et al.* (2016) suggested the use of CO as a cooking oil where intense heat for long duration (like continuous deep frying) was not used but mild or medium heat for short duration (like single shallow fry) was applied.

3.4. Determination of storage stability of the CO blend at room temperature

At room temperature, CO is known to have a long shelf-life, while FO is known to suffer significant oxidative deterioration. Therefore, the effect of blending FO with CO on long-term storage stability was studied for the blend containing the highest percentage of FO (i.e. C20). Table 4 presents the effects of storage on PV and AV for up to nine months. As the data indicates, there was a significant rise in both the parameters right from third month to the ninth month for CO when compared to the 0-month values. A similar trend was seen in the case of C20 for PV but for AV, a significant change was noticed from

the sixth month. At the end of nine months, there were 3-fold and 4.4-fold rises in PV for CO and C20, respectively, while 2.4 and 1.1-fold rises in AV were observed for CO and C20, respectively. Moigradean et al. (2012) conducted a similar study on CO and noticed an approximate two-fold rise in the PV at the end of nine months when compared to the initial value. We also observed similar trend. Pazzoti *et al.* (2018) studied the performance of CO, FO and their blend (1: 1 v/v) under accelerated storage conditions (60 °C for 20 days). They found that FO showed the least oxidative and thermal stability followed by the blend. Here, we observed that though there were significant rises in PV and AV, these values were significantly lower than CODEX (1999). According to Codex Stan 19-1981 for refined oils, PV and AV should be lower than 10 milliequivalents of active oxygen/ kg oil and 0.6 mg KOH/g fat or oil, respectively. The values for these parameters in cold-pressed oils are 15 milliequivalents of active oxygen/kg oil and 4 mg KOH/g fat or oil respectively.

Thus, from Table 4, it is clear that both CO and C20 are stable at room temperature for at least up to nine months, as indicated by their PV and AV.

A FA analysis was done for CO and C20 at specified time points. We did not see any significant changes in FA composition at these time points compared to the initial (0 month) data (data not shown). Srivastava *et al.* (2017) also reported no significant change in the FA profile of VCO and refined soybean oil blend or VCO and safflower oil blend which were stored at room temperature for one year. Thus,

TABLE 4. Effect of storage at room temperature on peroxide value and acid value for CO and C20

Oil/Blend	0 Month	3 rd Month	6 th Month	9 th Month			
Peroxide value (PV, meq O ₂ / kg oil)							
СО	0.3±0.0	$0.46{\pm}0.06^{\mu}$	$0.74\pm0.06^{\mu}$	$0.9\pm0.0^{\mu}$			
C20	$0.5 {\pm} 0.0^{\beta}$	0.82±0.06 ^{µ β}	$0.98\pm0.01{}^{\mu\beta}$	2.2 ± 0.1 ^{μ β}			
Acid value (AV, mg KOH/g oil)							
СО	0.24±0.00	$0.37 \pm 0.01^{\mu}$	$0.46\pm0.06^{\mu}$	$0.58 \pm 0.01 ^{\mu}$			
C20	$0.48{\pm}0.00^{\beta}$	$0.49\pm0.01^{~\beta}$	$0.46 \pm 0.06^{*}$	$0.55\pm0.00^{\mu\beta}$			

Peroxide value and acid value were evaluated for CO and C20 when stored at room temperature for up to nine months at specified time intervals. Data are presented as Mean \pm SD (n=3).

CO: Coconut oil alone and C20: Coconut oil blend containing 20% flaxseed oil

Statistical significance was confirmed by Two-way ANOVA and Bonferroni posttests.

 μ ; p < 0.001 versus 0 Month, *; p < 0.05 versus 0 Month, β ; p < 0.001 versus CO respective time points

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Table 4 indicates that CO and C20 were oxidatively stable at least for nine months when stored at room temperature without significant alteration in their FA composition.

3.5. Assessment of the effects of CO blends on the THP-1 cell line

As CO and blends showed good physicochemical characterization and storage stability, we assessed the biological effects of the CO blends in the THP-1 cell line. The THP-1 cell line is widely used for lipo-toxicity studies and the induction of inflammation studies. Few studies have evaluated effect of VCO, lauric acid and ALA on the viability and inflammation of THP-1 cells (Varma *et al.*, 2019; Tham *et al.*, 2020), but to our knowledge there are no reports on CO and FO blends with the percentages of FO we used.

3.5.1. Effect of CO blends on THP-1 cell viability

Varma *et al.* (2019) used VCO to demonstrate anti-inflammatory and skin protective effects. They found that 200 μ g/mL and lower concentrations of VCO were non-toxic in THP-1 cells (24 h treatment). Here, we used commercially available CO. We used three doses of oils and the blends (i.e. 125, 62.5 and 32.25 μ g/mL) for three different treatment periods (24 h, 48 h and 72 h). Here, Figure 2 represents data for 125 μ g/mL. For the other two concentrations, there were no statistically significant differences wrt Control (data not shown). From Figure 2, it is clear that at 24 h, CO and blends showed higher % viability, which was significant for C10 and C20. But for CO, blends and FO, at later time points, % viability was not significantly different than their respective Controls. Thus, at early time points we observed increased viability, which came down to the level of control cells at later time points. Similar rapid response (after 24 h treatment with CO) for elevated mitochondrial functioning was observed by Gil-Villarino et al. (1999) in hepatic tissue. The authors showed that in chicks fed with CO oil, up-regulation in the mitochondrial function was seen after 24 h but the up-regulated mitochondrial functioning returned back to the level of controls at longer treatment time points of CO feeding. Indeed, lauric acid from CO has been shown to improve mitochondrial functioning and biogenesis in insulin resistant THP-1 derived macrophages (Tham et al., 2020). From Figure 2, it is clear that oils and their blends do not adversely affect cell viability.

3.5.2. Effect of CO blends on FA composition of THP-1 cells

The enrichment of FA within the cells is possible by the external supply of that particular FA to the cells. Here, the FA analysis of THP-1 cells was done after treating the cells with individual oils or CO



Figure 2. Effect of CO blends on THP-1 cell viability determined by MTT assay after treating the cells with oils/blends (125 μ g/mL) for specified time points. Data are presented as % viability wrt Control (n=3). FO: Flaxseed oil alone, CO: Coconut oil alone, C5: Coconut oil blend containing 5% flaxseed oil, C10: Coconut oil blend containing 10% flaxseed oil and C20: Coconut oil blend containing 20% flaxseed oil. Statistical analysis was done by Two-way ANOVA and Bonferroni posttests. *; *p* < 0.05 versus Control, **; *p* < 0.01 versus Control

	<u> </u>	20		~ ~		CA
FA	Control	FO	CO	<u>C5</u>	C10	C20
Lauric acid	8.02 ± 1.43	7.47 ± 1.65	8.94 ± 1.51	8.8 0.95	8.43 ± 2.08	8.50 ± 1.01
Myristic acid	0.96 ± 0.46	0.91 ± 0.36	1.11 ± 0.63	2.39 ± 0.19	2.40 ± 1.11	1.97 ± 0.24
Palmitic acid	23.38 ± 3.17	20.71 ± 1.51	$24.33\pm0.21^{\ast}$	$24.93\pm1.06^{@}$	$25.29\pm1.34^{\textcircled{@}}$	23.87 ± 0.23
Stearic acid	15.86 ± 1.53	14.21 ± 1.74	16.91 ± 4.83	15.58 ± 1.53	15.67 ± 2.93	15.04 ± 3.37
Oleic acid	4.52 ± 0.04	7.15 ± 2.36	4.03 ± 0.20	4.62 ± 1.81	4.8 ± 1.09	6.7 0.64
Linoleic acid (LA)	0.23 ± 0.05	2.00 ± 0.92	0.21 ± 0.06	0.4 ± 0.17	0.34 ± 0.04	0.45 ± 0.1
Alpha linolenic acid (ALA)	0.00 ± 0.00	$4.48\pm1.49^{\#}$	0.00 ± 0.00	$0.11\pm0.08^{@}$	$0.125 \pm 0.05^{@}$	$0.47\pm0.1^{\ast}$
Arachidonic acid	0.9 ± 0.33	1.18 ± 0.62	0.36 ± 0.16	0.90 ± 0.56	0.87 ± 0.17	0.77 ± 0.17
Eicosapentaenoic acid	0.00 ± 0.00	0.62 ± 0.16	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.48 ± 0.09
Docosahexanoic acid	0.86 ± 0.3	0.54 ± 0.19	0.21 ± 0.08	0.53 ± 0.06	0.54 ± 0.14	0.71 ± 0.33
LA: ALA	ND	0.46 ± 0.05	ND	4.33 ± 1.80	3.02 ± 1.54	1.19 ± 0.65
Σω-6	1.13 ± 0.28	3.18 ± 1.53	0.56 ± 0.23	1.30 ± 0.73	1.21 ± 0.21	1.22 ± 0.27
Σ ω-3	0.86 ± 0.30	$5.63\pm2.84^{\#}$	$0.21\pm0.29^{\&}$	$0.64\pm0.15^{\&}$	$0.67\pm0.09^{\&}$	$1.66 \pm 0.57^{*}$
ω-6:ω-3	1.34 ± 0.16	0.57 ± 0.01	2.73 ± 0.78	1.96 ± 0.69	1.81 ± 0.07	0.75 ± 0.1

TABLE 5. Effect of CO blends on fatty acid composition of THP-1 cells

Total lipids extracted from THP-1 cells were subjected to FAME preparation after 48 h treatment of cells with the individual oils and CO blends. FA analysis of the FAME was done by GC-FID. Data are presented as Mean \pm SD (n=3). FO: Flaxseed oil alone, CO: Coconut oil alone, C5: Coconut oil blend containing 5% flaxseed oil, C10: Coconut oil blend containing 10% flaxseed oil, C20: Coconut oil blend containing 20% flaxseed oil, $\Sigma \omega$ -6: total omega-6 fatty acids, $\Sigma \omega$ -3: total omega-3 fatty acids. Statistical significance was confirmed by Two-way ANOVA and Bonferroni. #; p < 0.01 versus Control, *; p < 0.05 versus FO, @; p < 0.01 versus FO, &; p < 0.001 versus FO

blends. Total lipids were extracted from the treated cells, esterified and subjected to GC-FID. Table 5 presents FA levels represented as a % FA of the total extracted lipids. Here, the major FA available in the individual oils and ALA and LA derived major FA were identified and presented. The FA analysis of the Control and CO-treated cells indicated that ALA was absent in these cells. Compared to these cells, FO-treated cells had significant incorporation of ALA within the cells. It was also reflected in the total ω -3 FA content. CO was the major contributor in the blends with lauric acid as major FA. In CO or blend-treated cells, no alteration in the FA related to CO was observed. In addition, as the FO percentage in the blend increased, there was a rise in the ALA (0.11, 0.13 and 0.47% for C5, C10 and C20, respectively) and total ω -3 FA content (0.64, 0.67) and 1.66% for C5, C10 and C20, respectively). An increase in ALA and total ω -3 FA content was also reflected in the LA: ALA ratio (from 4.3 for C5 to 1.2 for C20), although these changes were not statistically significant. This might be because of the preferential uptake and utilization of MC-SFA present in the CO for energy generation as seen in the liver. It is important to note that percentages of ω -6 FA remained relatively constant in all treatments.

Therefore, it is clear that the external addition of ALA in the form of CO and FO blend can result in a dose-dependent incorporation and rise in ω -3 FA content with a simultaneous lowering of ω -6: ω -3 ratio in THP-1 cells.

3.5.3. Effect of the CO blends on TNFa and IL-6

ALA (present in FO) is a precursor for Eicosapentaenoic acid and Docosahexaenoic acid. These ω -3 FA are known for their anti-inflammatory potential (Zhao *et al.*, 2007). From the data presented in Table 5, it is clear that externally added ALA as (alone FO or as a CO blend) could alter LA: ALA as well as ω -6: ω -3 ratio in THP-1 cells. Therefore, it was interesting to investigate the effect of CO blends on the release of TNF α and IL-6 inflammatory markers.

As shown in Figures 3a and 3b, when THP-1 cells were stimulated by LPS, there were significant rises in the release of TNF α and IL-6 in the supernatant (denoted as +LPS). For FO pre-treated (before LPS addition) THP-1 cells, there was a significant decrease in the TNF α level in the supernatant but there was no significant alteration in IL-6 level. The blends did not affect either inflammatory marker. This might be because



Figure 3. Effect of CO blends on inflammatory markers: TNF α and IL-6 in THP-1 cells. THP-1 cells pre-treated with the individual oils or CO blends (125 µg/mL) for 48 h were stimulated by co-incubation with LPS (25 ng/mL). Cell supernatants were collected at 6 h for TNF α and at 24 h for IL-6 estimation. ELISA was performed for quantitative analysis of these inflammatory markers. Data are presented as fold change wrt +LPS (n=3). FO: Flaxseed oil alone, CO: Coconut oil alone, C5: Coconut oil blend containing 5% flaxseed oil, C10: Coconut oil blend containing 10% flaxseed oil, C20: Coconut oil blend containing 20% flaxseed oil, -LPS: without LPS stimulation and +LPS: with LPS stimulation only. Statistically significant differences were determined by One-way ANOVA and Dunnett's Multiple Comparison Test.*,**; p < 0.01 versus+LPS

the ω -3 FA levels achieved in the cells are not sufficient to down-regulate LPS-induced inflammation. Indeed, Hintze et al. (2016) have shown that not only ω -6: ω -3 ratio but concentrations of individual FA also regulate cytokine production. In addition, when VCO-treated THP-1 cells were LPS-stimulated, VCO displayed anti-inflammatory potential at the transcriptional and translational levels (Varma et al., 2019). It is known that CO has lower levels of biologically active compounds compared to VCO (Lima and Block, 2019). Thus, the observed differences in the results might be due to the availability of anti-inflammatory bioactive compounds in the commercially-available CO compared to VCO. Here, we observed that although the ω -6: ω -3 ratios were favorably altered in the case of the blends, the concentrations of individual FA were probably not high enough to show anti-inflammatory effects.

4. CONCLUSIONS

The aim of this study was to improve the ω -3 and ω -6 levels in CO using the blending technique. We selected FO as a rich source of essential FA for blending. The physico-chemical characteristics of edible oil govern the observed health effects of cooking oil. The blending of FO and CO resulted in significantly improved ALA and LA levels, which is also reflected in LA: ALA ratios. SFA, MUFA and PUFA levels were also favorably altered. The storage stability study of C20 (blend with highest FO percentage) showed good oxidative stability. The thermal stability study indicated that the blends could resist thermal deterioration up to a certain time point only, probably indicating that the blends can be used for cooking which does not involve intense heating for a long time. The THP-1 cell line study indicated that it was possible to improve the LA: ALA ratio without adverse effects on cell viability. Still, future studies warrant that not only the FA profile but also the bioactive compound profile of the blends need to be studied and improved to ensure thermo-oxidative stability. In additionally, the uptake of essential FA from the blends by the cells needs to be increased in order to observe biological effects.

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