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IDENTIFICATION AND CONTROL OF BLACK COLOUR SPECK FUNGAL FORMATION IN VIRGIN COCONUT OIL

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ABSTRACT

A critical issue in virgin coconut oil (VCO) industry is fungal contaminations which leads to black colour speck formation. This study was designed to distinguish the type of fungal growth and to determine the remedial actions. VCO was extracted by cold press method and subjected to eight treatments. Efficacy of the treatments were evaluated in terms of changes in microbial properties [yeast and mould count (YMC) and aerobic plate count(APC)], physicochemical properties [moisture and volatile matter% at 105˚C (MV), specific gravity at 30˚C (SG), saponification value(SV), iodine value(IV), peroxide value(PV), acid value (AV), relative fatty acid profile (RFAP) by gas chromatography and free radical scavenging activity (DPPH assay)] along with a non-treated sample. The results revealed presence of *Aspergillus* sp. as the black colour speck in VCO and among those treatments the combination (X2) where VCO was subjected to 65˚C, 253.7nm UV radiation for 60 seconds was identified as the best because it gave a null YMC, 15CFU/mL in APC, 0.12±0.01 in MV %, 0.9194±0.00 in SG and it was within the APCC standards. Further, IV, SV, PV and AV were obtained as 5.52±0.37mg/g, 262.55±0.16mg KOH/g, 2.96±0.02 Meq/kg and 0.14±0.04mg/g respectively. The X2 sample showed a higher lauric acid percentage (50.489±0.011) compared to the non-treated (NT) sample (49.646±0.001). A lower EC_{50} value was noted in X2 (3.27±0.01mg/L) compared to NT (3.27±0.01 mg/L) sample. Evidently, the present results suggest that combination of heat, UV radiation with time has a significant influence on retarding the black speck formation in VCO.

1. Introduction

VCO is a popular edible oil consumed around the world. It is the purest form of oil extracted from coconut (*Cocos nucifera* Linn.) kernel with unique colourless (water clear) to pale yellowish brown and viscous nature (Dayrit *et al.*, 2007; APCC, 2009). Coconut oil is comprised of a significant level of saturated fatty acids which are in low molecular weight including lauric acid. The chemical properties of VCO increases its potential for use in both edible and non-edible applications (Marina *et al.*, 2009). The unique characteristics of this oil include bland flavor, pleasant odor (fresh natural coconut scent), taste free of rancidity, having a narrow range of melting temperature, improved digestibility with absorbability, and higher potential for foam retention (Che and Mariana, 2006). It is made from the fresh coconut meat (matured kernel 12 months old from pollination) subjected to mechanical press "with or without heat application, which does not lead to alteration of the nature of the oil". (Bawalan and Chapman, 2006; APCC, 2009).

The APCC (Asian and Pacific Coconut Community) and Codex Alimentarius are the major organizations that establish international standards for commercial coconut oil. Both APCC and Codex standards defines "virgin oil" should be edible for humans in its liquid nature and maybe purely obtained by washing with water, settling, filtering, and centrifuging. Even though Codex standards do not have specific standards for VCO, APCC has initialed standards for VCO (Alimentarius, 1999; APCC, 2009; Dayrit *et al*., 2007).

VCO is extracted by wet process directly from coconut milk under controlled temperature conditions and has more beneficial effects over coconut oil because it preserves beneficial constituents (Nevin and Rajamohan, 2004; Marina *et al*., 2009a). It is comprised of natural vitamins resulting in impaired oxidation (hydrolytic and atmospheric). This high antioxidant potential results in lower acid value and peroxide value of coconut oil. VCO has a characteristic fresh coconut aroma (Mansor *el al*., 2012) with a number of health benefits. One of the main issues encountered in VCO industry in Sri Lanka is the "black colour" sediment observed in the bottom of the containers stored under low temperature. It leads to poor consumer perception, retailer un-satisfaction and market returns from the export market thereby declining the demand. Therefore, it is critical and essential for the local VCO manufacturers as well as the exporters to overcome the issue of black speck formation. This study was carried out to distinguish the type of microorganism and to retard the growth by designing treatments changing the heat at cold pressing process (temperature is maintained less than 60˚C) while exposing to UV radiation and changing the time combinations (Table 1). Consequently, physicochemical properties, fatty acid profile, and antioxidant properties of VCO were determined in the given treatments and the best treatment was identified. Furthermore, there have been no previously reported studies conducted in this area locally or internationally. According to authors' knowledge, this is the first time, a research study of this manner has been conducted. In addition, these findings will be beneficial for both commercial scale VCO manufacturers as well as persons who will carry out future studies related to the field of coconut oil and especially, virgin coconut oil.

2. Materials and methods

2.1. Sample Collection

VCO samples (prepared under cold pressed methods) were collected from a local VCO processor in Kurunegala district, North Western province, Sri Lanka.

2.2. Microbial Tests

Initially the microbial species were identified through slide culture technique (Woo *et al*., 2011). Dilution plating method was implemented to evaluate microbial count in VCO samples (Table 1). Both treated and non–treated VCO samples were analysed through total plate count, yeast and mould count using standard plate count agar following AOAC methods (AOAC, 1995).

2.3. Physicochemical Tests

Moisture and volatile matter percentage was determined according to AOAC method (AOAC, 1997), specific gravity was obtained by American Oil Chemists' Society (AOCS) official method (Cc 10a-25, 1999) with slight modifications, saponification value was obtained via AOCS official method (Cd 3-25, 1999), iodine value (Cd 1-25, 1999), peroxide value (Cd 8b, 1999) and acid value (3a-63, 1999) were obtained by AOCS official methods (AOCS,1990).

Relative fatty acid profile was obtained through gas chromatography model-7890 A (GC), equipped with mass spectrometer model-5975 C (MS) inert XL EI/CI MSD with triple-axis detector by preparation of fatty acid methyl esters in oil sample following the programme described in Munasinghe and Wansapala (2015)

2.4. Antioxidant Activity

The free radical scavenging activity of the stable DPPH (1,1-diphenyl-2- picrylhydrazyl) was reported according to the procedure

followed by Marina and others (2009b). This assay was conducted for all VCO samples separately (treated samples and non-treated sample (Table 1). The inhibition activity percentage was calculated as $[(A_0 - A_1) / A_0] \times 100$, where the absorbance of the control (containing no sample extract) is denoted by A_0 and absorbance of the extract is given by A_1. The effective concentration (EC_{50} (mg/mL)) value was obtained depending on the quantity of VCO extracts required to reduce the initial DPPH radical concentration by 50%.All the physicochemical and microbiological tests were carried out to non-treated and treated samples and compared with the values of VCO standards. For the antioxidant study, results were evaluated by one-way analysis of variance (ANOVA) followed by Tukey pairwise comparison test. All the tested samples were triplicated to obtain results and statistical analysis were done by Minitab 17 software.

3.Results and discussions

3.1. Microbial Identification

The microorganism was identified as *Aspergillus* sp. according to its colony morphology (Figure 3) results obtained through slide culture technique (Figure 2). Initially a white colour colony appeared. Later it became black in colour showing a "salt and pepper appearance" (Figure 1). This was resulted from "darkly pigmented conidia born in large numbers on conidiophores and reverse turning pale yellow" (Debets *et al*., 1990). VCO samples obtained from single UV or heat treatment (T1, T2, UV1, and UV2) and non-treated (NT) sample gave positive results for black specks. Negative results were produced for both heat and UV combinations applied samples (X1, X2, X3 and X4).

Non-treated sample showed highest number of CFU/mL for both TPC (155) and YMC (45) which exceeds the APCC (Asian and Pacific Coconut Community) standard limits (<100 CFU/mL for TPC and <10 CFU/mL for YMC). Inability to meet this standard shows the lack of quality and unsafe product which has higher potential to cause health hazards (Uthpala and Navaratne, 2019; Uthpala *et al*., 2021; BFAD, 2004). There was a gradual decrement of microbial load with heat application (Table 2). The implementation of UV technology in the food industry is an alternative to chemical sterilization and it minimizes microbial population (González *et al.*, 2007). Heat and UV combined treatments were absent with fungal growth in PDA media while NT sample noted to have higher amount. Green and others (2007) had reported the impact of UV irradiation over *Aspergillus flavus* and *Aspergillus fumigatus*. In their study, the exposure time of UV radiation had significant impact on disinfectant potential of the above-mentioned fungus (Green *et al.*, 2004). Moreover, Rotem and Aust (1991) had shown that *Aspergillus niger* is highly sensitive to UV and sunlight, even though *Aspergillus* sp. is resistant to high temperatures in dark conditions (Rotem & Aust, 1991).

The results showed the YMC could be reduced to some extent by application of heat and UV combined treatments.

Table 1. Description of treatments and symbols

Treatment No	Symbol	Description
1	NT	Non-treated oil sample
2	T1	Oil sample is heated up to 65˚C for 60 seconds
3	T2	Oil sample is heated up to 85˚C for 60 seconds
4	UV1	Oil sample is exposed to 253.7nm UV radiation for 30 seconds
5	UV2	Oil sample is exposed to 253.7nm UV radiation for 60 seconds
6	X1	Oil sample is heated up to 65˚C for 60 seconds and exposed to 253.7nm UV radiation for 30 seconds
7	X2	Oil sample is heated up to 65˚C for 60 seconds and exposed to 253.7nm UV radiation for 60 seconds
8	X3	Oil sample is heated up to 85˚C for 60 seconds and exposed to 253.7 nm UV radiation for 30 seconds
9	X4	Oil sample is heated up to 85˚C for 60 seconds and exposed to 253.7 nm UV radiation for 60 seconds

T1, T2, UV1, and UV2 are single treatments. X1, X2, X3 and X4 are combined treatments.

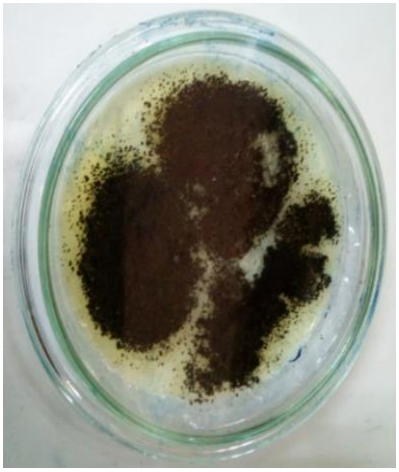

Figure 1.
Fungal growth after a week from inoculation

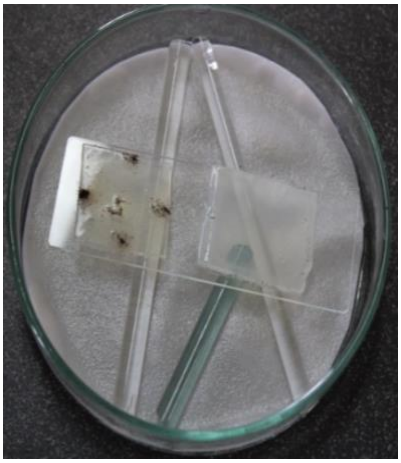

Figure 2.
Slide culture observations after 48 hours.

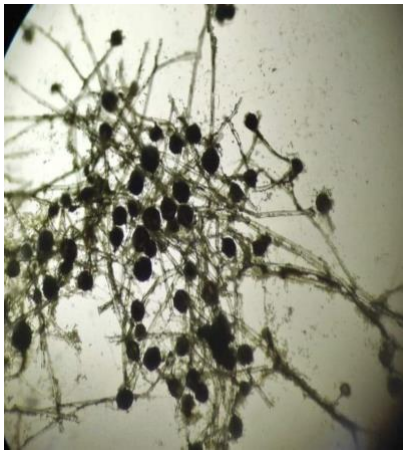

Figure 3.
Microscopic view of the fungus in slide culture under the power of 10x40.

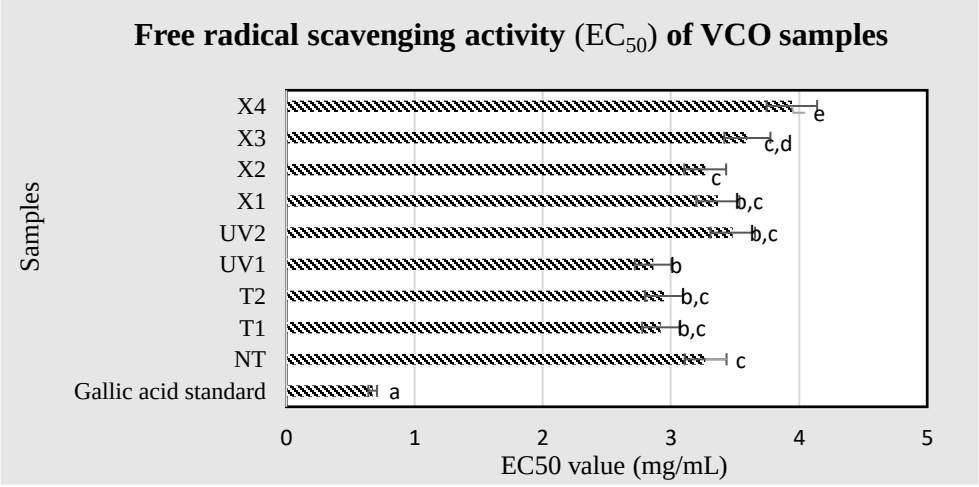

Figure 4.
Antioxidant activity (EC_{50} values) of VCO standard, non-treated sample and treated samples. Each value represents the mean and standard deviations of triplicate determinations.
a,b,c,d,c,d Values with different superscripts are significantly different at 0.05 level.

Table 2. Changes of microbiological count, physicochemical properties of treated VCO samples compared with the non-treated sample and APCC standard

Sample **Parameter**	**Non-Treated (NT)**	**Treatment 1 (T1)**	**Treatment 2 (T2)**	**Treatment 3 (UV1)**	**Treatment 4 (UV2)**	**Combined Treatment 1 (X1)**	**Combined Treatment 2 (X2)**	**Combined Treatment 3 (X3)**	**Combined Treatment 4 (X4)**	**APCC standards**
Microbial Properties										
Total Plate Count (CFU/mL)	155	95	85	65	30	20	15	15	15	<100
Yeast & Mould Count (CFU/mL)	45	10	2.5	7.5	5	Nil	Nil	Nil	Nil	<10
Physicochemical properties										
Moisture & volatile %	0.08±0.01	0.07±0.01	0.07±0.01	0.09±0.01	0.11±0.01	0.07±0.01	0.12±0.01	0.09±0.00	0.08±0.01	0.2
Specific gravity	0.9188± 0.00	0.9200± 0.00	0.9197± 0.00	0.9197± 0.00	0.9198± 0.00	0.9197± 0.00	0.9194± 0.00	0.9196± 0.00	0.9194± 0.00	0.915 – 0.920
Saponification value (mg KOH/g)	262.13± 0.85	262.56± 2.47	262.80± 2.00	262.31± 0.11	262.62± 1.00	262.88± 0.58	262.55± 0.16	262.51± 0.95	262.67± 0.11	250-260 (min)
Iodine value (g I_2/100g sample)	6.94±0.42	6.42±0.52	5.75±0.39	6.27±0.38	6.82±0.53	6.23±0.17	5.52±0.37	5.35±0.28	5.16±0.49	4.1-11.0
Peroxide value (Meq/kg)	0.00±0.00	1.97±0.00	2.96±0.00	2.00±0.00	2.96±0.02	1.98±0.01	2.96±0.02	3.96±0.02	4.94±0.03	3.0 (max)
Acid value (mg KOH/g sample)	0.00±0.00	0.00±0.00	0.17±0.00	0.06±0.00	0.11±0.00	0.06±0.00	0.14±0.04	0.22±0.00	0.28±0.00	0.40 (max)

Relative Fatty Acid Profile (%)											
Carbon number	**Name**	NT	T1	T2	UV1	UV2	X1	X2	X3	X4	APCC standards
C6:0	hexanoic acid /caproic	0.296	0.300	0.479	0.469	0.432	0.182	0.220	0.070	0.222	0.10 -0.95
C8:0	octanoic acid /caprylic	6.023	5.992	7.497	7.417	7.055	4.237	4.574	1.036	3.964	4 -10
C10:0	decanoic acid /capric	4.779	4.917	5.497	5.457	5.154	3.785	3.973	1.022	2.926	4 - 8
C12:0	dodecanoic acid /**lauric**	49.646	48.817	48.393	48.945	48.213	41.591	50.489	30.571	41.925	45 -56
C14:0	methyl tetradecanoic /myristic	18.950	18.658	20.791	21.682	20.947	21.440	22.084	27.665	22.723	16 - 21
C16:0	hexadecanoic /palmitic	8.168	8.355	8.163	7.896	8.397	11.218	8.841	17.142	12.502	7.5 -10.2
C19:0	10,13-octadecadienoic acid	NA	1.066	NA	NA	NA	NA	NA	NA	NA	NA
C18:2	9,12-octadecadienoic acid /linoleic	0.908	NA	NA	0.638	0.752	1.397	0.744	1.706	1.201	0.7 - 2.5
C18:2	9,15 -octadecadienoic acid	NA	NA	0.747	NA	NA	NA	NA	NA	NA	NA
C18:1	Cis-13-octadecadienoic acid	NA	NA	NA	NA	5.417	NA	NA	NA	NA	NA
C18:1	9-octadecenoic acid /oleic	6.637	7.165	5.034	4.508	NA	9.414	5.373	NA	NA	4.5 - 10
C18:1	11-octadecenoic acid /vaccenic	NA	NA	NA	NA	NA	NA	NA	12.477	8.770	NA
C18:0	octadecanoic acid /stearic	4.593	4.731	3.398	2.988	3.634	6.499	3.701	8.312	5.768	2 - 10
	TSFA[a]	92.455	92.836	94.218	94.854	93.832	88.952	93.882	85.818	90.029	NA
	TUFA[b]	7.545	7.165	5.781	5.146	6.169	10.808	6.117	14.183	9.970	NA
	S/U[c]	12.254	12.957	16.298	18.433	15.210	8.230	15.348	6.051	9.030	NA

Each value represents the mean of triplicated samples, NA-not available

Since VCO is stored in airtight containers it maintains anaerobic conditions. Hence there is a high possibility for the growth of anaerobic microorganisms.

3.2. Physicochemical Analysis

Water activity of VCO is very low. Therefore, the freely available moisture content is not enough for the growth and survival of most of the microorganisms. Due to several facts, the moisture content can be increased during the manufacturing process. The MV percentage values obtained from all the treatments are significantly less than or equal to 0.13% (Table 2). Non-treated sample gave 0.08±0.10% moisture and volatile matter value. The high moisture content would initiate hydrolysis reactions (Osawa *et al*., 2007; Lawson, 1985). Therefore, moisture content within the standards ensures safeness of the product.

Specific gravity is a measure of fatty acids available in a unit volume. It is an important parameter for chemical engineering unit operations in the fatty acid industry. All the treated samples showed higher SG values, compared with the non-treated sample. But all samples were within APCC (2009) standards at 30^0C (0.915–0.920) (APCC, 2009).

Saponification is oil hydrolysis under basic conditions to produce glycerol and the salt of the relative FA (Tan *et al*., 2013). SVs of the samples ranged from 262.20 to 263.46 mg KOH/g of fat which was comparable with the VCO research studies done in Malaysia (Mansor *et al.*, 2012). In addition, SVs of all the samples were aligned with the APCC (Table 2) standards (APCC, 2009). SV implies an idea about characteristics of fatty acids available in oil. Lower amount of acid is liberated per gram of fat hydrolysed of fat containing longer carbon chains. It is a measure to get a rough estimation on the molecular weight or chain length of fatty acids available in oil. The longer the chain of fatty acids the lower the SV. They have relatively fewer number of carboxylic functional groups per unit mass of the fat leading to high molecular weight.

Iodine value (IV) is used to evaluate the degree of unsaturation of an oil. Saturated oils take up no iodine leading to zero IV. The IVs obtained for the VCO samples in this study had an average value range of 5.16 to 6.94 g I_2/100 g which is compatible with the APCC (Table 2) standards (APCC, 2009). With both heat and UV treatments the IVs have been reduced. Low IVs reduce the chance of VCO to enhance rancidity (Onyeike and Acheru, 2002; Mansor *et al*., 2012). Since IV is an estimate of overall double bonds in an oil sample, the IVs should be comparable with the relative fatty acid profile results in the absence of other olefinic (double bond containing) compounds (Dayrit *et al.*, 2007).

Double bonds in unsaturated fatty acids can be oxidized and form hydroperoxides which leads to rancidity with time or due to temperature increments (Gunstone, 1996; Dayrit *et al.,* 2007). Codex standards give a peroxide value limit of 15 Meq/kg while APCC specifies 3 Meq/kg for VCO (Alimentarius, 1999; APCC, 2009; Dayrit *et al*., 2007). Even though X3 (3.96±0.02 Meq/kg) and X4 (4.94±0.03 Meq/kg)) treatments exceed the limit of PV compared above APCC specifications, it is compatible with Codex (max 15 Meq/kg) limitations.

Acid value is a quantitative parameter of the free fatty acids (FFA) available in the oil. FFA increment in oil sample indicates hydrolysis of triglycerides which produces glycerol. FFA is a reason for flavors and aroma (Osawa *et al*., 2007). In addition, short chain FFA are volatile and soluble in water with a unique odor. If the oil contains both long chain saturated and unsaturated fatty acids they tend to oxidize and form products (aldehydes, ketones, alcohols, and organic acids) that provide characteristic flavours and aroma (Marina *et al.*, 2009a). All the results obtained in each treatment showed low AVs compared to APCC standard value (0.4 max) (APCC, 2009). Acid values in combined treatment samples are slightly increased compared to the NT sample (Table 2). Degradation of fatty acids due to heat and UV exposure might be the reason because, extrinsic

(light, temperatures and oxygen) and intrinsic (antioxidants, pro-oxidants and water) factors can stimulate lipid oxidation (Kamal, 2006, Barrera *et al*, 2002). Due to chemical or enzymatic mechanisms acid hydrolysis can be initiated. Plant enzymes (lipases) or microbial contaminants are the factors of enzymatic hydrolysis. High levels of FFA are undesirable due to unpleasant mouthfeel and aroma.

The relative fatty acid profile cannot be used to indicate the exact acid value. The ratio of saturation/unsaturation (S/U ratio) had changed significantly with heat and UV applications (Table 2), even main FFA percentages, in most treatments remained within the APCC standard values (APCC, 2009). The treatment X2 was observed with the highest lauric acid percentage (50.49%) while X3 showed the lowest (30%). NT sample had shown 49% lauric acid and single treatments (only heat or UV applied) indicated approximately 48% of lauric acid percentage. Under T1 treatment there is a formation of 10,13-octadecadienoic acid (C19:0), but the amount does not affect the S/U ratio (Table 2).

VCO is predominantly made up of lauric acid (C12:0). Researches have revealed that monolaurin (a monoglyceride form of lauric acid) has antimicrobial properties (Wang *et al*., 1993; Kabara, 1984; Enig, 1998; Mansor *el al*., 2012). The total lauric acid contents obtained for X1, X2 and NT samples are according to the ranges of APCC and Codex (45.10–53.20%) standards (Marina *et a*l, 2009b; Dayrit *et al*., 2007). But lauric acid values for X3 and X4 sample are slightly lower than the above standards. This could be due to application of higher temperature.

Lauric acid (C12) has distinctive biochemical and nutritional properties that are not similar to long-chain saturated fatty acids (SFA). Medium-chain SFA (C6-C12) show different metabolic and physiological properties from long-chain SFA (C14-C18). Lauric acid accounts approximately half of the FA in coconut oil. Further, medium-chain triglycerides account for approximately half of all triacyl glycerides in coconut oil. Hence coconut oil is classified under vegetable oil which has medium-chain FAs (Dayrit *et al.,* 2007)

3.3. Antioxidant potential

As EC_{50} value increases the free radical scavenging ability or the antioxidant potential reduces. According to the EC_{50} value, the effectiveness of free radical scavenging potential in descending order was Gallic acid standard > UV1> T1> T2> X2> NT> X1> UV2> X3> X4 (Figure 4). There was a significant difference ($P<0.05$) in the EC_{50} values among the treatments. The lower E_{50} values compared to NT can be considered as positive impact having treatments. NT sample showed 3.27 mg/mL EC_{50} value and T1, T2, UV1 and X2 showed better antioxidant potential compared to NT sample (Figure 4). González and others (2007) have revealed that the exposure to UV radiation can increase the antioxidant potential in food (González *et al*., 2007). Natural antioxidants can control free radicals which can cause pathological effects such as cancer and vascular diseases. Oxidation is a natural reaction in metabolism and often resulted by reactive compounds such as highly reactive hydroxyl radicals, •OH. Antioxidants quench these free radicals. Natural antioxidants help food manufactures to produce stable products with natural ingredients (Ramadan *et al*., 2006).

Cold press method was implemented to obtain VCO in this manufacturing company from where the samples were obtained for the research study. As from this study it was revealed that black colour speck formation is due to fungal contamination of *Aspergillus* sp. The moisture content, oxygen, temperature and oil as a nutritional source are positive factors for this fungal growth.

According to the study X2 (VCO samples heated up to 65˚C, 60 seconds and exposed to 253.7nm UV, 60 seconds) treatment was evaluated to have better performance having treatment in terms of moisture, TPC, YMC, SV, IV and FAP along with the improved antioxidant properties free of black speck formation. Hence, this treatment can be implemented in controlling

black speck formation in virgin coconut industry while improving product quality simultaneously maintaining within the standard APCC specifications.

4. Conclusions

Virgin coconut oil samples subjected to heat at 65˚C for 60 seconds and expose to 253.7 nm UV radiations for 60 seconds can be implemented as a remedial action to black speck formation. This treatment can be applied to control black speck formation in the virgin coconut industry while improving product quality whilst maintaining the quality parameters within the standard of APCC.

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