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Application of ^{13}C NMR and Untargeted Multivariate Analysis for Classification of Virgin Coconut Oil [†]

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Abstract: Virgin coconut oil (VCO) is produced from fresh mature coconut meat without the use of chemicals or high heat. VCO can be made using three processes: fermentation, centrifuge, and expeller. To determine quality, it is important to be able to differentiate Control VCO (fresh) from old VCO, refined bleached and deodorized coconut oil (RBDCO), and VCO which has been adulterated with RBDCO. Differentiating these types of samples has remained a challenge because of their chemical similarity. This study investigated the ability of ^{13}C NMR and multivariate analysis to differentiate these different coconut oil samples. The methodology used the standard ^{13}C NMR pulse sequence with broadband ^1H decoupling with dioxane as internal standard (IS). After pre-processing of the spectra (alignment, bucketing/binning, normalization with respect to dioxane IS peak), untargeted multivariate analyses, both unsupervised and supervised, were done on the bins of the ^{13}C peaks. Principal components analysis (PCA), a linear unsupervised method, was able to differentiate Control VCO ($n = 57$) from RBDCO ($n = 21$), adulterated VCO ($n = 9$), and old VCO ($n = 11$). Partial Least Squares Discriminant Analysis (PLS-DA) was used as supervised linear binary classifiers. Using overall accuracy and AUC-ROC curves (by 100 cross validation and single validation using manual holdout), the supervised dataset with an optimized model gave better than 99%, 95% and 80% performance in differentiating control VCO vs RBDCO, old VCO, and adulterated VCO (one vs one), respectively. Predictive ability ($Q^2 < 0.20$) and overall accuracy (< 0.80) were poor compared to the previous models for binary classifier models (one vs rest) to differentiate among the three VCO processes. This may be due to the variations in production conditions and methods that different VCO producers use. We conclude that ^{13}C NMR combined with linear techniques can be used to accurately differentiate fresh VCO from RBDCO, old VCO, and adulterated VCO.

Keywords: virgin coconut oil; ^{13}C NMR spectroscopy; multivariate analysis; ROC curves

1. Introduction

Virgin coconut oil (VCO) is an oil that is recognized as a functional food which has been gaining in popularity worldwide. VCO is defined as the oil that is obtained directly from fresh mature coconut meat without the use of chemicals and high heat [1] and can be produced using three main processes: fermentation, centrifuge and expeller. In the fermentation and centrifuge processes, coconut milk is prepared from the fresh coconut meat. The fermentation process takes advantage of the presence of natural microorganisms that release lipase and other demulsifiers to destabilize the emulsion and separate the coconut oil layer. The crude coconut oil is filtered, dried, and is sometimes subjected to centrifugation. In the centrifuge process, the coconut milk is centrifuged to directly

separate the oil from the aqueous layer. Many producers use a three-pass centrifuge system. The expeller process passes the coconut meat directly through an expeller press to squeeze out the oil [2]. In contrast, coconut oil that is used for frying is produced from copra and is refined, bleached and deodorized and is referred to here as RBDCO.

Because VCO and RBDCO have similar physico-chemical characteristics, it is difficult to distinguish them using classical techniques, and it is even more difficult to detect adulteration of VCO with RBDCO. The reported methods of detecting adulteration that use a different vegetable oil as adulterant are inadequate for this purpose. Fourier transform infrared (FTIR) spectroscopy and differential scanning calorimetry (DSC) have been used to differentiate VCO from non-coconut oil samples, but these have not been applied to adulteration of VCO with RBDCO [3,4].

NMR is a nondestructive, unbiased method of analysis for organic compounds. Comparing ^1H and ^{13}C NMR, the latter has a wider chemical shift range and gives singlets resulting in simpler spectra with less problems of overlapping peaks with comparable spectra across different magnetic field strengths. ^{13}C NMR is also less susceptible to solvent and temperature effects. As ^{13}C NMR is less sensitive than ^1H NMR, it requires longer acquisition time. Although broadband ^1H -decoupled ^{13}C NMR cannot be integrated, ^{13}C NMR analysis is reproducible and can be used for profiling of organic samples. Quantitative ^{13}C NMR analysis can be done using the inverse-gated decoupling pulse sequence, but this requires long recycle delays. Although the use of relaxation agents may shorten repetition time when using quantitative ^{13}C NMR, the ^{13}C NMR profile is suitable only for chemometric pattern recognition and untargeted multivariate analysis, but not for targeted quantification [5].

The statistical pipeline follows a typical multivariate method used in metabolomics [6,7]. ^{13}C NMR profiles were pre-processed, aligned, bucketed, normalized, and auto scaled. Linear methods were applied to the data. Exploratory unsupervised analysis, such as principal component analysis (PCA), was used to infer patterns and clustering within the dataset. Binary classifiers were developed for better evaluation of model performance compared to multi-class classifiers in supervised analysis. Partial Least Squares–Discriminant Analysis (PLS-DA), a linear method, was then used for supervised analysis. The suitability of using a PLS-DA model (as it tends to overfit) was evaluated by the overall accuracy, R^2 (linear fit of training data) and Q^2 (linear fit of predicted data/prediction performance on new data) parameters. The resulting models were then optimized for number of PLS-DA variables and number of features by being internally cross-validated by random class assignments, manually single validated using manual holdout, and evaluated on ROC-based performance and predictive ability [8].

Receiver operating characteristics (ROC) is a diagnostic tool used to discriminate two samples based on binary classifier system and a discrimination threshold for organizing classifiers and visualizing their performance. A ROC graph is a plot used to visualize the performance of the differentiation. The ROC curve plots the true positive rate (sensitivity) on the y axis against the false-positive rate (1-specificity) on the x axis. The Area under the Curve (AUC) of an ROC curve is used to quantify the performance of a binary classifier, a normal or control versus abnormal or not control characteristic. Both the ROC curve and its corresponding AUC are functions of sensitivity and specificity of a prediction model. A perfect test will have an AUC value of 1.0, whereas a random chance will have a value of 0.5. Interpretation of AUC values we used are the following: 1.0 is a perfect test, 0.9–0.99 is an excellent test, 0.8–0.89 is a good test, 0.7–0.79 is a fair test, 0.51–0.69 is a poor test, and 0.5 is of no value or is an unusable test [9].

The predictive performance of a model can be measured by permutation testing to determine whether it is statistically significant or not. A p -value < 0.05 means that given a randomly permuted outcome variable, there is less than 5% chance that a model of similar performance to the “true” non-permuted model will be produced.

2. Research Objectives

The goal of this research is to develop a ^{13}C NMR method that can be used to differentiate VCO from RBDCO, from VCO adulterated with RBDCO, and old VCO (VCO which is beyond the shelf

life of two years). Additionally, we also investigated whether this method will be able to differentiate VCO according to the type of production process, that is, fermentation, centrifuge or expeller.

3. Materials and Methods

3.1. Oil Samples

Total VCO samples (n = 98) were divided into two groups: Sample Types—Control VCO (n = 57) and Not Control VCO (n = 41). The Control VCO (n = 57) comprised of two VCO Oil Types—the Observed (n = 42), and the Submitted (n = 15) samples. The Observed and the Submitted samples were acquired from the VCO producers of the Philippines who committed to participate in the study. Three to four manufacturers per process (Fermentation, Centrifuge, and Expeller) were enrolled. Each manufacturer provided two Oil Type samples: Observed and Submitted. The Observed samples were gathered by the researchers while observing the full VCO production process. The Submitted were produced unobserved by the researchers. The Observed samples constituted the Training samples, while the Submitted samples were the Validation samples.

The sample type Not Control VCO (n = 41) consisted of the following oil types: Old VCO (n = 11), Adulterated VCO (n = 9), and RBDCO (n = 21). The old/degraded VCO samples included samples which were subjected to accelerated degradation at 40 °C for 6 months and samples which were over 2 years old. The adulterated VCO samples were composed of fermentation, centrifuge, and expeller samples that were adulterated with 25%, 50%, and 75% of an RBDCO sample.

3.2. Chemicals, Reagents and Sample Preparation

Deuterated chloroform (CDCl₃) with 0.05% *v/v* trimethyl silane (TMS) was used as NMR solvent (cat# DLM-7TB-100S, Cambridge Isotope Laboratories, Inc.). 1,4-dioxane (cat# 1.09671, Merck ACS, ISO grade) was used as internal standard (IS).

Approximately 350 µL of oil sample was transferred into a 5 mm Wilmad High Throughput NMR tube (WG-1000-8-50) and added with about 230 µL of CDCl₃ solvent containing 2.9% *w/w* 1,4-dioxane. 1,4-dioxane was added as IS for normalizing the metabolite bucket integrations. The mixture was shaken to homogenize the sample.

3.3. Instrumentation and Experimental Parameters

An Avance Neo 400 Nuclear Magnetic Resonance (NMR) spectrometer (Bruker Biospin) was used operating at 100.097 MHz ¹³C. Standard Bruker ¹³C pulse sequence was used (C13CPD/zgpg30) with 64K acquisition points, 4K scans, 1.38 s acquisition time, spectral width from -18.6 ppm to 219.3 ppm (23,809.523 Hz) with autogain settings and run at VT = 300K.

3.4. Data Processing

Processed NMR spectra used standard automatic Bruker Topspin 4.0.7 ¹³C post-processing Third party packages and statistical frameworks to the R Statistical software were used for batch processing of spectra and in the unsupervised and supervised analyses. NMRPROCFLOW [10] was used for batch processing of peak shifting, spectral alignment and variable bucketing. MetaboAnalyst 4.0 [11] was used for the data normalization and statistical analyses (untargeted, multivariate; unsupervised and supervised).

4. Results

Unsupervised Analysis: Preliminary exploratory unsupervised analyses were done in the context of the research objectives. Using ¹³C NMR untargeted profiling and linear methods: Can we differentiate Control VCO samples from RBDCO samples and other VCO samples not considered Control? Can we differentiate by VCO process?

Binary classifiers were designed so that model performance can be easily evaluated by ROC curves. These binary classifiers were then used in supervised analyses and on the test samples:

Control vs. Not Control VCO (one vs one)-Control VCO vs. RBDCO, Control VCO vs. Old VCO; Control VCO vs. Adulterated VCO. Control VCO samples were used for the VCO Process binary classifiers (one vs. rest): Fermentation VCO vs. Not Fermentation VCO; Centrifuge VCO vs. Not Centrifuge VCO and Expeller VCO vs. Not Expeller VCO.

2D unsupervised exploratory PCA plots for the binary classifiers for Control VCO vs. NOT Control VCO is shown in Figure 1 Control VCO vs. RBDCO is clearly separated. Some overlap is seen for Control VCO vs. Old VCO. There is significant overlap and no clear separation seen for the binary classifiers for the VCO processes.

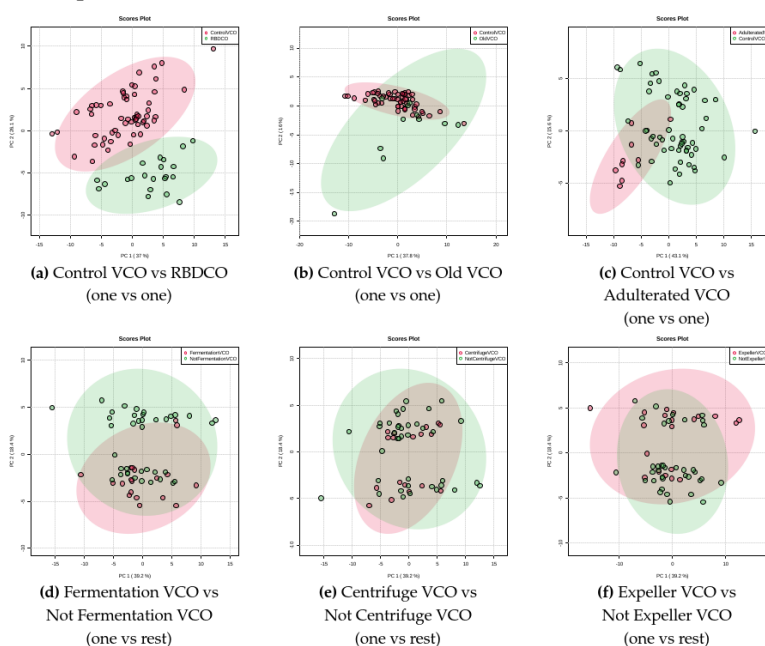


Figure 1. 2D Unsupervised Exploratory PCA plots for the binary classifiers.

Supervised Analysis: PLS-DA performance of Control VCO vs RBDCO indicates it is a perfect model and statistically significant (Figure 2). The optimal Q2 is determined to be 4 PLS-DA components with overall accuracy of about 1. Preliminary permutation tests indicate a p -value < 0.001 (0/1000 permutations). Monte Carlo Cross Validation (MCCV) indicates the model is a perfect classifier with most of the AUC for the ROC curves being 1. We get the same performance for a model built with buckets with AUC > 0.99 for 100 CV and for holdout data. There is good and clear separation between samples of the two classes. Predictive Accuracy of the assembled model using Permutation Test with 1000 permutations is statistically significant, $p < 2.5024e-05$.

The PLS-DA classifiers for the Control VCO processes (Fermentation vs Not Fermentation VCO, Centrifuge VCO vs Not Centrifuge VCO, and Expeller VCO vs Not Expeller VCO) indicated poor model performance with some having comparatively high p -values.

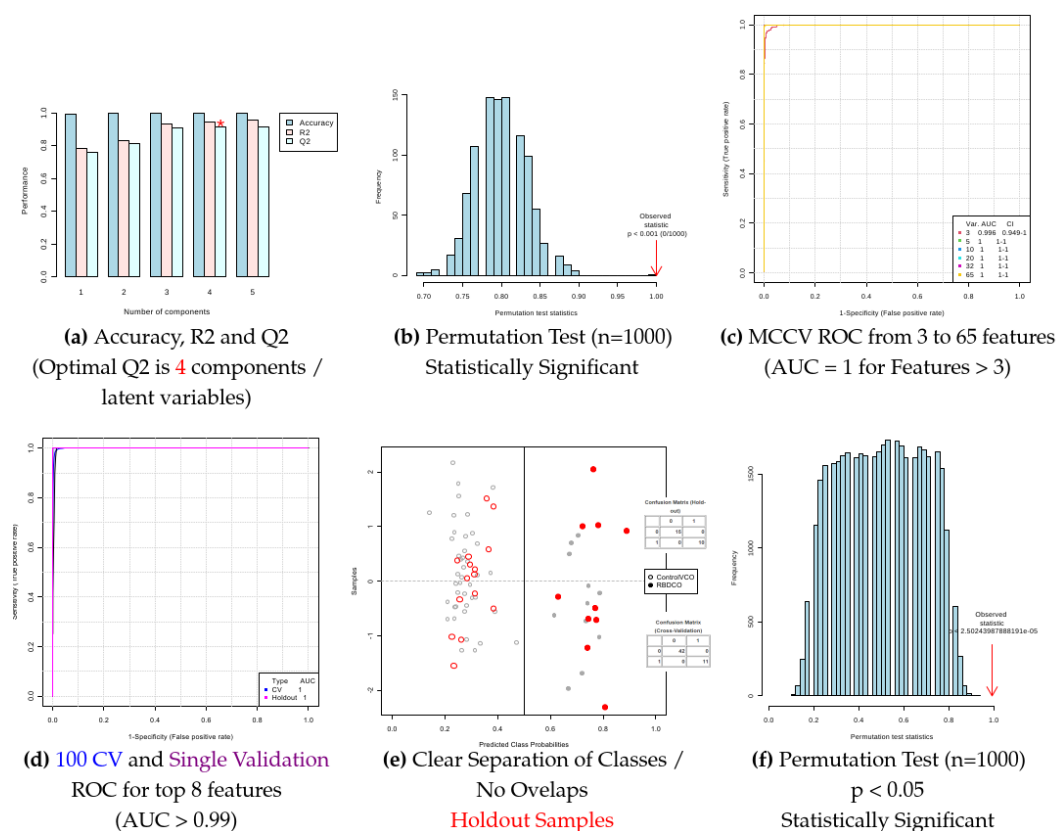


Figure 2. PLS-DA Performance of Control VCO vs RBDCO indicates it is a Perfect Model and Statistically Significant.

5. Discussion

Based on the unsupervised analyses, we expect that differentiating control VCO from RBDCO, from VCO adulterated with RBDCO, and from old VCO samples is feasible, but differentiating by VCO process is not. The Control VCO vs RBDCO classifier is essentially a perfect model and very highly statistically significant. The Control VCO vs Old VCO classifier may be considered also an excellent model, and marginally close to the cutoff of p -value = 0.05. This may be improved with more Old VCO samples.

Control VCO vs Adulterated VCO classifier is a mixed bag. Although the model performance may be considered good from a practical application, it was not statistically significant, meaning that there may be a significant number of models with random labeling assignments that will perform better than the optimized model. We hope to determine in future studies if the statistical significance of the model can be improved with more samples of adulterated VCO.

6. Summary and Conclusions

The use of ¹³C NMR and multivariate linear statistical methods were sufficient to discriminate the following: Control VCO from RBDCO; Control VCO from old VCO, and Control VCO from VCO adulterated with RBDCO. The accuracy to discriminate VCO samples produced by different processes proved to be inadequate. Figure 3 summarizes the results and conclusions.

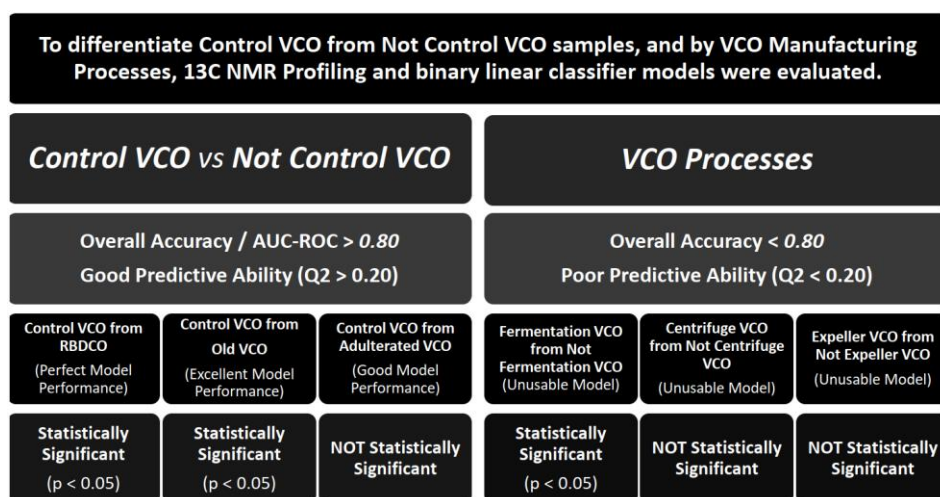


Figure 3. Graphical Summary of the Results and Conclusions.

Author Contributions: Conceptualization: F.M.D.; methodology: L.G.L. and M.J.M.G.; software and formal analysis: M.J.M.G.; writing and editing: M.J.M.G., L.G.L. and F.M.D.; supervision and project administration: F.M.D. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: The authors declare no conflict of interest.

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