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^{1}H Nuclear Magnetic Resonance, Infrared, and Chemometrics in Lipid Analysis of Brazilian Edible Oil-Based Nutraceutical

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Article

¹H Nuclear Magnetic Resonance, Infrared, and Chemometrics in Lipid Analysis of Brazilian Edible Oil-Based Nutraceutical

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Abstract: Edible oils have commercial and nutritional value due to the presence of essential fatty acids. They can be consumed fresh in the form of capsules known as nutraceuticals. The quality of such products is of interest to the consumer. In this context, this study describes a method based on high resolution nuclear magnetic resonance (NMR) and Fourier transform mid-infrared spectroscopic analysis (FTIR), combined with statistical analyzes to differentiate different edible oils used as nutraceuticals in Brazil by fatty acid content. Through the analysis of ¹H NMR spectra, the levels of saturated and unsaturated fatty acids in edible oils were characterized and quantified. Statistical analysis of the data confirmed the real distinction between nutraceutical raw materials, with emphasis on ω-9, ω-6 and ω-3 fatty acids. The analytical approach presented also demonstrated potential to identify the origin (animal or vegetable) of edible oils used as nutraceuticals.

Keywords: lipid composition; ¹H-NMR; chemometrics; edible oils quality control

1. Introduction

Vegetable and animal edible oils have great economic relevance since they are lipid sources widely used as raw material in the processing industry (pharmaceutical, food, and chemical) and for the human diet. They can be present in diets in different forms, including the *in natura* encapsulated formula known as nutraceuticals [1,2]. This term was introduced in 1989 and refers to foods of plant or animal origin with pharmaceutical and nutritional activities [3,4]. Therefore, nutraceuticals have a functional food appeal.

The high commercial and nutritional value of these oils is closely related to the specific properties linked to their lipid composition, marked by triacylglycerides (TG) constituted by saturated (SFA), monounsaturated (MUFA), and polyunsaturated (PUFAs) fatty acids. Free fatty acids, tocopherols, tocotrienols, and vitamins A and E may also be present in smaller amounts [5]. A wide variety of edible oil-based nutraceuticals can be found in Brazil. The most common are the oils from soybean (*Glycine max*), sunflower (*Helianthus annuus*), garlic (*Allium sativum*), corn (*Zea mays*), almonds (*Prunus dulcis*), andiroba (*Carapa guianensis*), safflower (*Carthamus tinctorius*), Brazil nut (*Bertholletia excelsa*), coconut (*Cocos nucifera*), linseed (*Linum usitatissimum*), primrose (*Oenothera biennis*), borage (*Borago officinalis*), chia (*Salvia hispanica*), and palm oil (Elaeis guineensis) [5]. Nutraceuticals formulated based on fish oils, known to be rich in PUFAs, are also common [5-7].

The number of available nutraceuticals has continuously grown, reflecting market developments, research, and consumer interest. However, they are often met with skepticism due to efficacy and safety concerns, in part due to the well-documented lack of regulation and oversight in the sector [4]. Therefore, regulating quality, efficacy, mechanism of action, and safety requirements can benefit potential consumers and the industry. In this context, studies referring to the composition

of different raw materials used in the production of nutraceuticals converge to determine the quality of the product in question.

Classically, the quality of edible oils used in nutraceutical manufacturing is evaluated by moisture, refraction index, viscosity, acidity, peroxide, iodine index, and saponification [2,8,9]. Although they are analytically robust and easy to perform, these conventional methods are time-consuming and only allow the simultaneous analysis of a few classes of compounds. On the other hand, modern analytical instrumentation, especially chromatographic and spectroscopic techniques, is more accurate and informative in evaluating the complex chemical profiles of these oils [1,10-12]. Among the spectroscopic techniques, nuclear magnetic resonance (NMR) and medium infrared with Fourier transform (FTIR) stand out in this analytical context. These methods allow the simultaneous collection of qualitative and quantitative chemical information without quantification standards, common in chromatographic analysis routines [13-15].

The non-selective and highly reproductive nature of the NMR allows the collection of molecular fingerprints without the need for elaborate separation methods. The NMR, as a result of the direct relationship between the signal area and the number of nuclei responsible for that signal, allows the quantification of many compounds in complex matrices concomitantly with qualitative analysis, which leads to a reduction in sample handling and exposure time [13,15-18]. Consequently, NMR can be applied in assessing the quality of edible oils since such investigations are based on the lipid composition and its relationship with the physicochemical and organoleptic characteristics of the matrix.

A suitable quantitative approach by NMR allows the simultaneous collection of information about the chemical profile as well as the contents of such components. This fact for an analytical context of edible oils represents a significant advantage since the time of exposure and manipulation of the sample can determine the onset of unwanted reactions such as lipid oxidation [15]. Quantification by NMR (qNMR) is possible due to the direct proportion between the intensity of a signal and the number of nuclei responsible for that signal. This information is translated by integrating the analyte NMR signal. Thus, the higher the concentration of a given substance, the greater the number of nuclei responsible for that signal and, therefore, the greater the area of the signal [18,19]. Commonly, quantification via NMR requires the use of internal standards that are inserted directly into the solution containing the analyte [18]. However, the choice of a quantification standard must meet requirements such as purity, chemical inertness towards the sample components, low volatility and solubility similar to the analyte, and, in the particular case of NMR, present relaxation times (which must be adequate to the time available for analyses) as well as its chemical structure, to avoid overlapping of standard x analyte signals [16,18].   Alternatively, Akoka *et al*. developed a computational protocol called Electronic Reference to access In-vivo Concentration (ERETIC), which consists of electronically synthesized reference signals from calibration experiments with known concentration solutions [19]. Such "synthetic" signals can be inserted in any region of the sample spectrum, thus eliminating possible effects of analyte versus internal standard interaction and signal overlap [18-21].

The use of NMR in studies of complex matrices (e.g., edible oils) generates a range of information that may not be readily interpretable. The simple comparative method may not recognize the grouping or separation tendencies between samples caused by possible processing errors or tampering [13,14,22]. In this context, chemometric treatment of the NMR and FTIR data can help identify and quantify potential authenticity biomarkers. The main chemometric protocols used in the aforementioned analytical approaches refer to exploratory analysis *via* Principal Component Analysis (PCA) and Hierarchical Class Analysis (HCA) [23]. In PCA, the multivariate dataset is projected into a new space with a reduced statistical dimension, with minimal damage to sample relationships. The new dimension, expressed by new variables called principal components (PCs), aims to highlight clustering or separation trends not previously identified in the spectra [17,24]. HCA, on the other hand, is a hierarchical process in which the reduction of the data matrix results in a two-dimensional dendrogram that emphasizes the clusters of samples by similarity. Thus, it is understood that neighboring samples are statistically and chemically similar. In contrast, distant samples, even

from the same matrix, may indicate possible tampering processes since they were classified as statistically and chemically distinct [18,25-27].

In the context presented, our study describes an analytical protocol based on the synergy between ^{1}H NMR and FTIR applied in the analysis of Brazilian edible oil-based nutraceuticals. The data, derived from the investigation of different matrices of edible oils, were submitted to unsupervised chemometric analyzes that allowed the distinction between the types of oils. The discrimination between them indicated the existence of different contents of saturated and unsaturated fatty acids, and this observation motivated the relative quantification of the cited fatty acids.

2. Materials and Methods

2.1. Sample Collection

The sampling comprised pure edible oils provided by Brazilian industries in Goiânia and Anápolis and commercial oils purchased in the same cities. Samples of garlic (*Allium sativum*), almonds (*Prunus dulcis*), andiroba (*Carapa guianensis*), sunflower (*Helianthus annuus*), safflower (*Carthamus tinctorius*), Brazil nut (*Bertholletia excelsa*), palm (Elaeis guineensis), coconut (*Cocos nucifera*), linseed (*Linum usitatissimum*), primrose (Oenothera biennis), chia (Salvia hispanica), and soybean (*Glycine max*) and fish oils pure and in mixtures, were used in this study.

2.2.1. H NMR Experiments

For each ^{1}H NMR analysis, 100 µL of the oil sample was solubilized in 400 µL of deuterated chloroform ($CDCl_3$). The NMR spectra were acquired on a Bruker Avance III 500 spectrometer (Bruker, Germany) operating at 11.75 Tesla, fitted with a three-channel broadband inverse (TBI) probe. The ^{1}H NMR experiments were performed in triplicates, acquired with an accumulation of 256 spectra, 65,536 scanning points during acquisition and processing, the total acquisition time of 7.1 seconds, the spectral width was 16 ppm, receiver gain fixed at 11.3 and constant temperature of 25 °C during acquisition. The zg30 pulse sequence (Bruker) was used, with automatic pulse calibration and magnetic field homogeneity (shimming). An exponential correction factor was applied in processing to broaden the spectral line of 0.3 Hz. Baselines and phases were automatically corrected in the TopSpin working software (v 3.5, Bruker, Germany). All NMR spectra were given in ppm related to the TMS signal used as an internal reference at δ 0.00.

2.3. ERETIC Signal Calibration

The lipid composition of the oils was determined by relativizing the analyte signals versus the ERETIC signal. Therefore, it was necessary to calibrate the ERETIC signal by acquiring a ^{1}H NMR spectrum under quantitative conditions using a standard solution of known concentration. A 2.0 mmol.L^{-1} caffeine solution in $CDCl_3$ was used as a calibration standard. The spectral acquisition and processing parameters strictly followed the experimental protocol of the ^{1}H NMR experiments.

2.4. Fourier-Transform Infrared Spectroscopy (FTIR)

FTIR spectra of edible oils were acquired on a Spectrum 400 FTIR spectrometer (PerkinElmer, USA). Samples were mixed with potassium bromide (KBr), and the pellets were analyzed in the range of 4000–400 cm^{-1} (resolution of 2 cm^{-1}), with 12 scans recorded. All samples were analyzed in triplicate and measured at room temperature (25 °C) under identical conditions.

2.5. Exploratory Analysis by PCA and HCA

Data analysis by PCA was performed using the AMIX 3.9.15 software (Bruker BioSpin, Germany). The data matrix was obtained by the spectral bucketing procedure of the ^{1}H NMR data, using the rectangular shape and optimized width of 0.05. The spectral regions referring to the residual signals to non-deuterated solvent at δ 7.0-7.5 ($CHCl_3$) and the TMS at δ - 0.3-0.3 were

excluded from the data matrix. The PCA was performed with a data matrix mean-centered and a 95.0% confidence interval and validated with the full-cross validation method. The HCA was performed from the data matrix constructed with each oil sample's average FTIR spectral data. The nearest neighbor method to create the Clusters and their correlations as the type of distance was used to construct the dendrogram.

3. Results and Discussion

3.1. Characterization of Lipid Profile by ^{1}H NMR

Figure 1 shows ^{1}H NMR spectrum of a linseed oil sample. The spectrum showed regions with broad signals, reflecting the cause-and-effect relationship between the low mobility of lipids in solution and the consequent short spin-spin relaxation times [28]. The spectral characterization was performed based on the literature [5,13-15,27-30]. Table 1 shows the assignment of the main signals of this spectrum, their multiplicities and coupling constants.
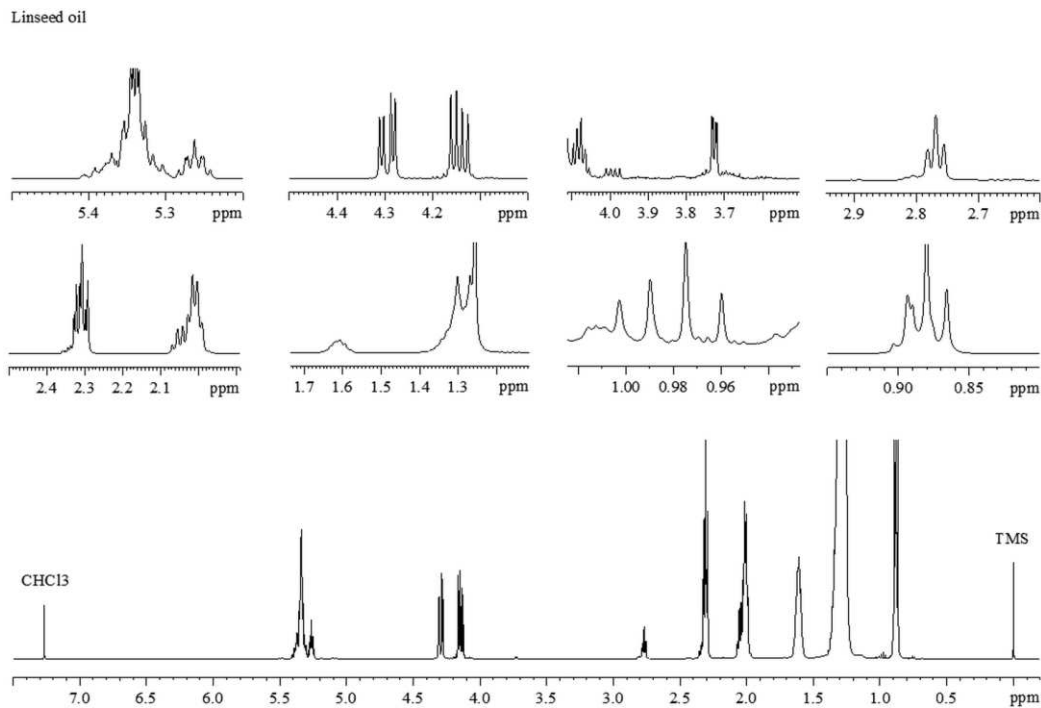

Figure 1. ^{1}H NMR spectrum of a linseed oil sample (CDCl$_3$, 500 MHz). Highlighted, expansions of spectral regions useful in the characterization of lipid profiles were presented.

Table 1. ^{1}H NMR spectral data assignment for the relevant signals observed in edible oils samples analyzed (CDCl$_3$, 500 MHz).

δ ^{1}H (ppm)	Multiplicities (J Hz)	Group	Assignment
0.87	t (6.7)	-CH_3	SFAs and MUFA (omega-9) acyl group
0.89	t (6.7)	-CH_3	linoleic acid (omega-6) acyl groups
0.97	t (7.5)	-CH_3	omega-3 acyl groups
1.20-1.40	m	-(CH_2)$_n$	acyl groups of all fatty acids
1.55-1.65	m	–CH_2–CH$_2$-COO-	PUFAs, except DHA
1.95-2.10	m	-CH_2-CH=CH-	PUFAs, except for DHA
2.30	t (7.5)	–CH_2-COO-	acyl group, except DHA
2.33	t (7.5)	–CH_2-COO-	Free fatty acids

2.43	m	–CH_2-COO-	DHA acyl group
2.77	t (6.5)	=HC-CH_2-CH=-	bis-allylic hydrogen of omega-6 PUFAs
2.80	t (6.3)	=HC-CH_2-CH=-	bis-allylic hydrogen of omega-3 PUFAs
3.73	dd (1.4, 4.9)	-CH_2-OH	1,2-diglycerides
4.07	m	-CH-OH	1,3-diglycerides
4.14	dd (6.0, 11.8)	-CH_2-OCOR-	glyceryl group of TG
4.30	dd (4.4,11.8)	-CH_2-OCOR-	glyceryl group of TG
5.26	m	-CHOCOR	glyceryl group of TG
5.34	m	-CH=CH-	PUFAs acyl groups

Multiplicities: d – doublet, dd – doublet of doublet, m – multiplet, t – triplet.

3.2. Statistical Analysis

Due to the extreme visual similarity between the ^{1}H NMR spectra, previous exploratory analysis by PCA was performed to verify if the samples of edible oil-based nutraceuticals could be statistically differentiated. The PC1 x PC2 score chart indicated clear discrimination tendencies between the samples (Figure 2A), with the positive sense of PC1, the statistical region responsible for discriminating the most significant number of samples. In contrast, the negative PC1 discriminated the soy, linseed, and chia samples. The accumulated variance explained by the first two components used in the construction of the graph was 86.9%.

Figure 2. Score plot (a) and loadings (b) of PC1 *versus* PC2 obtained from the ^{1}H NMR data of Brazilian edible oil-based nutraceuticals.

Analyzing the PCA loadings (Figure 2B), the principal chemical descriptors responsible for discriminating the samples presented in the score plot were identified. The positive scores of PC1, the statistical region where most of the sampling were discriminated, was strongly influenced by the signals of hydrogens with chemical shifts at δ 0.88, assigned to the hydrogens of terminal methyl

groups of oleic (ω-9) and SFAs. On the other hand, the trend observed along with the negative PC1 scores, the statistical region populated by soybean, linseed, and chia samples, was strongly influenced by the signals at δ 5.38, 2.83, 2.08, and 0.98, assigned to vinylic, allylic, bis-allylic, and terminal methyl hydrogens of α-linolenic acid (ω-3), respectively.

Extrapolating the results from PCA, we can infer that the distinction between the oil-based nutraceuticals samples was strongly influenced by the different contents of MUFAs (ω-9) and PUFAs (ω-6 and ω-3) in addition to signals attributed to SFAs. Therefore, an adaptation between the equations described by Miyake et al. (1998) and the ERETIC method to calculate the relative percentages of the lipid descriptors (SFA, ω-9, ω-6, and ω-3) indicated as PCA discriminators for each nutraceutical sample evaluated in the present study [31]. It is worth noting that the application of NMR in the quantification of fatty acids relies on other protocols, such as those described by Martinez-Yusta et al. (2014) and Santos et al. (2018) [5,32]. The equations used in the aforementioned calculations, as well as the integrations of the signal areas, are shown in Figure 3.
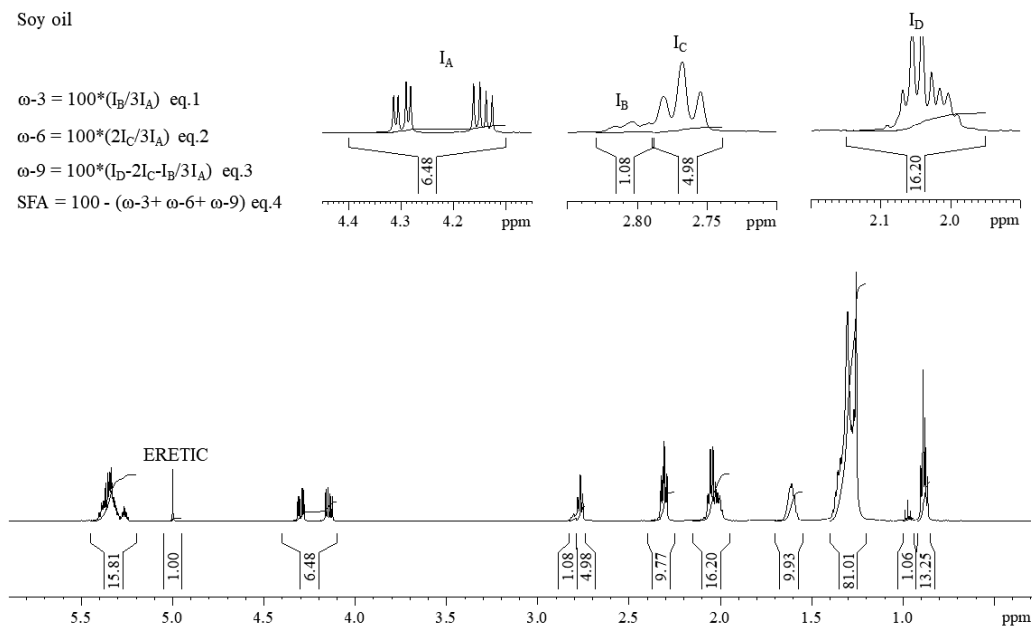

Figure 3. ^{1}H NMR spectrum of soy oil-based nutraceutical. The integrations of the signals of the chemical groups were presented concerning the ERETIC signal, positioned at δ 5.00 ± 0.15 of precision related to the signal's center. Also highlighted were the expansions of the regions of interest for calculating the percentages of SFA, ω-9, ω-6, and ω-3, calculated from equations 1, 2, 3, and 4, positioned in the upper left portion.

Table 2 presents the results of the quantifications for each type of sample. Analyzing the percentages of ω-9 fatty acids and SFAs of the different matrices, it was found that nutraceuticals based on coconut, palm, andiroba, Brazil nut, and safflower oils, previously divided into positive PC1 scores (Figure 2A) had low percentages of ω-3 fatty acids. On the other hand, these samples showed the highest levels of SFAs and ω-9 fatty acids. This information is consistent with the analysis of PCA loadings (Figure 2B), which indicated as the main descriptor for the region, the signal in δ 0.88, previously assigned to terminal methyl groups of SFAs and ω-9 fatty acids.

Table 2. Percentages of saturated, ω-3, ω-6, and ω-9 fatty acids by ^{1}H NMR data of edible oil-based nutraceutical samples used as nutraceuticals in Brazil.

Vegetable source				
Sample	**SFA**	**PUFA (ω-3, %)**	**PUFA (ω-6, %)**	**MUFA (ω-9, %)**
Almond	45.3±1.0	4.8±1.5	10.7±0.7	39.1±0.9
Andiroba	57.5±0.6	3.4±0.3	12.3±1.1	26.8±0.7

Brazil nut	56.0±0.9	6.5±0.3	12.9±1.2	24.6±1.4
Chia	21.4±1.1	48.5±1.1	19.0±0.9	11.1±1.2
Coconut	68.8±0.4	3.7±0.5	2.0±0.2	30.5±0.5
Copaiba	39.8±0.8	6.4±0.5	29.6±1.0	24.2±1.1
Garlic	35.4±1.1	5.5±1.0	15.9±0.8	43.2±1.5
linseed	19.1±1.0	46.3±1.1	17.9±0.8	16.7±1.3
Palm	63.9±1.3	5.1±0.8	5.8±0.6	25.2±0.7
Primrose	21.6±0.5	50.9±0.5	4.2±0.4	12.8±0.8
Table 2. Continued.				
Safflower	55.0±0.6	4.2±0.4	7.2±0.9	33.6±0.7
Soy	16.9±1.1	9.6±0.3	41.0±1.3	32.5±1.8
Sunflower	28.9±0.6	14.3±0.3	14.4±0.7	42.4±0.8
Animal source				
Fish (blends)	43.7±0.4	10.1±0.6	14.9±0.7	31.3±0.5
Fish	51.4±0.5	22.0±0.5	13.8±0.8	12.8±0.3

The matrices with the highest percentages of ω-3 acids were the samples of chia and linseed. These samples were broken down into negative PC1 scores, as shown in Figure 2A. Again, the descriptors (δ 5.38, 2.83, 2.08, and 0.98) indicated in the loading plot (Figure 2B), previously assigned to hydrogens of the *α*-linolenic acid (ω-3) and others unsaturated chemical groups, support the observation. The large group in the center of the graph in Figure 2A, composed of oil-based nutraceuticals sunflower, fish, soy, primrose, garlic, copaiba, and almond samples, showed intermediary levels of saturated and unsaturated fatty acids when compared with the other samples. No apparent correlation between the percentages of saturated and unsaturated fatty acids and the descriptors indicated in the loading graph was observed for the large group of samples centered on the PCA score graph (Figure 2A). For comparative analytical purposes of the results from the NMR-PCA model presented, the profiles of oil-based nutraceuticals were also evaluated via the FTIR technique, which is recognized as a more accessible for the industry. Figure 4 shows the score plot for PC1 x PC2, with accumulated variance explained by the first two components equal to 72.5%.
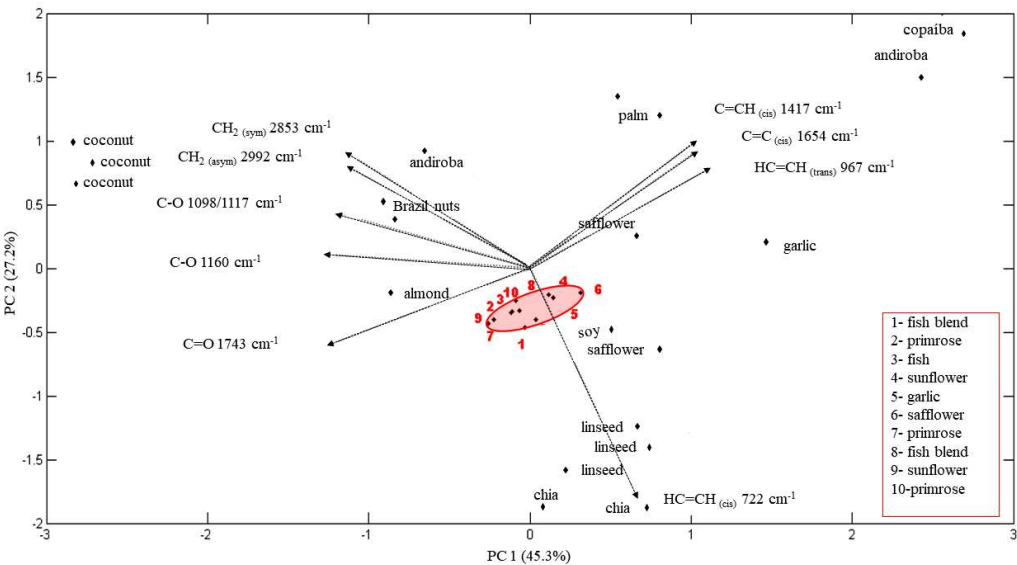

Figure 4. Score plot of PC1 *versus* PC2 obtained from the FTIR data of Brazilian edible oil-based nutraceuticals. The main numbers of waves and chemical groups responsible for the observed discrimination were highlighted.

The data dispersion was similar to that obtained by the ^{1}H NMR data. Along the positive PC1 scores, safflower, palm, garlic, andiroba, and copaiba samples were discriminated, and the statistical region was strongly influenced by signals with wave numbers equal to 1417 cm^{-1} (C=CH), 1654 cm^{-1} (C=C), and 967 cm^{-1} (HC=CH). These signals are characteristic of absorptions (folding and stretching) of PUFAs. The coconut samples were discriminated in the negative scores of PC1, influenced mainly by signals referring to the stretching of C-O and C=O bonds, typical of ω-6 and ω-9 fatty acids, highlighting again the contents of these acids, previously determined by ^{1}H NMR. As in the NMR analyses, chia and linseed samples were discriminated similarly. However, now it was along the negative PC2 scores, with the C=C link (ν 722 cm^{-1}) as the main descriptor.

Regarding the similarity between the edible oils, the multivariate analysis of the FTIR data via HCA highlighted the remarkable similarity between the chia and linseed samples (Figure 5), previously identified in the PCA treatment of the NMR data. The resulting dendrogram also indicated the existence of significant similarity between the samples of soybean, sunflower, and fish (pure and declared mixture). This similarity between the samples is the principal basis of the adulteration of fish oils, commonly practiced with these vegetable oils (soybean and sunflower) of lower market value. However, despite HCA presenting data in agreement with the PCA-NMR and PCA-FTIR models, the resulting dendrogram indicated a more remarkable similarity between copaiba and coconut oils, information not evidenced in the exploratory analyzes by PCA of NMR and FTIR data.
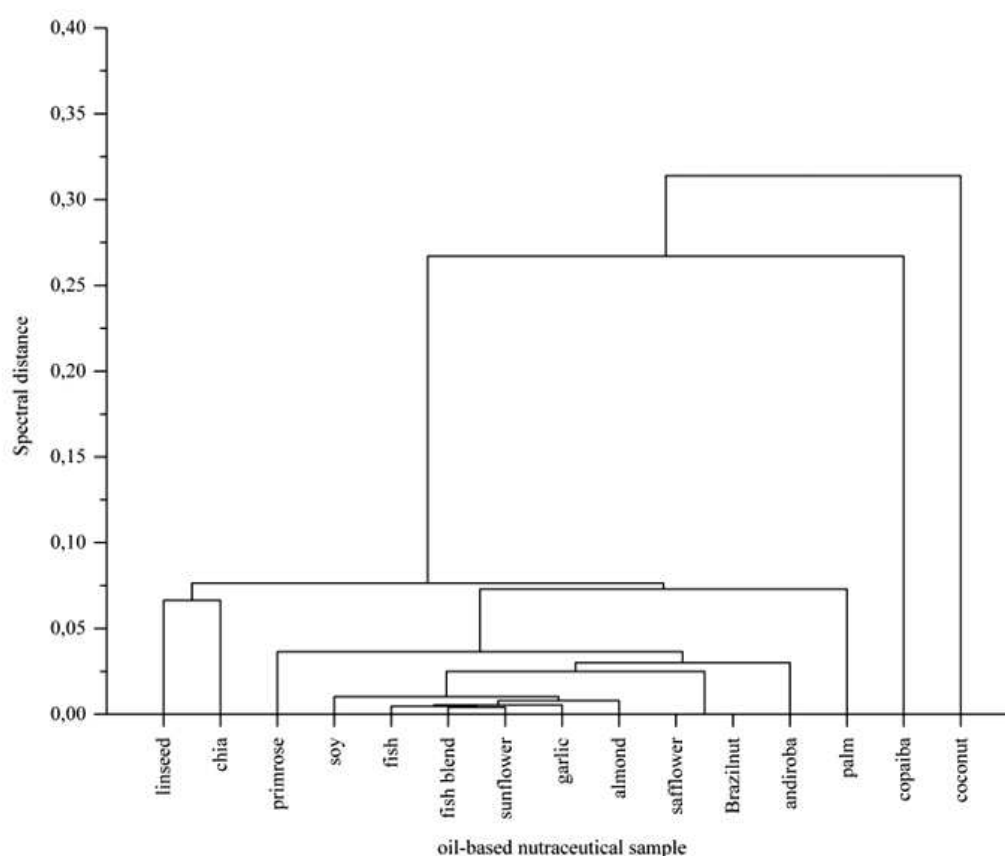

Figure 5. Resulting dendrogram from the hierarchical classes analysis of FTIR analysis of oil-based nutraceuticals.

Although NMR allows the simultaneous obtaining of a qualitative (determination of constituents) and quantitative (relative lipid percentages) lipid screening, the FTIR technique showed concordant and rapid results, confirming analytical complementarity since similarities were observed in the discrimination of nutraceuticals by composition. The interpretation of the results can still be extrapolated in terms of clear distinctions between nutraceuticals regarding the origin of the

oilseed matrices used, as well as peculiar results from the FTIR-HCA model that indicated the possibility of verifying the authenticity of the edible oils used as raw materials.

5. Conclusions

this study demonstrated the ^{1}H NMR data allowed the qualitative (signal assignment) and quantitative (via relative signal integration) determination of the lipid profiles of different Brazilian edible oils commonly used to manufacture nutraceuticals. Additionally, the chemometric treatment (PCA) of the NMR data allowed the identification of discriminatory statistical trends between the analyzed lipid profiles. Such information was corroborated in the treatment of the data obtained by analyzing the oils by FTIR. The evaluation of PCA loadings indicated that the signals of SFAs, MUFAs (ω-9), and PUFAs (ω-6 and ω-3) showed great relevance for data dispersion. As the signal areas influence the intensities of the ^{1}H NMR signals, the descriptors identified in the loadings were quantified, making it possible to build a database that can be used for future studies to authenticate the edible oils studied. The HCA-FTIR model evaluated the oil similarities, and it allowed the confirmation of the significant nearness of the principal adulterants (soybean and sunflower oils) of fish oils. Notably, the analytical protocol presented here can be a promising analytical alternative for regulatory and certifying bodies since the quality authentication of edible oils used as raw materials in the industry is a relevant issue for the economy and health of the final customer.

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