

Article

Vibrational Spectroscopic Evaluation of the Composition of Flax (*Linum usitatissimum* L.) Seeds: Differences for Cultivars

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Featured Application

This study contributes to the use of vibrational spectroscopic methods to characterize oilseeds and other crops, enabling the screening of seeds with desired compositions and properties.

Abstract

Flax (*Linum usitatissimum* L.) is a crop widely cultivated for fiber and oil production. The screening method for flax breeding must effectively address the biochemical characteristics of flaxseeds. In this study, to characterize flax cultivars, we extracted oil, defatted kernel, hull, and mucilage from whole seeds for the ATR-FT-MIR and FT-Raman spectroscopic measurements. In addition, for ATR-FT-MIR analysis, oil samples were obtained by pressing the flaxseed directly onto the crystal surface. After removing any seed residues, a grease stain was used for the measurement, allowing for the acquisition of the oil spectrum from a single seed. This method also enabled the detection of free fatty acids, serving as evidence of seed damage. Both methods effectively estimated the degree of unsaturation as a cultivar marker. The vibrational spectra of defatted kernels showed strong protein features; polysaccharide bands dominated in hull and mucilage spectra. Discrimination of flax cultivars using principal component analysis of vibrational spectra in specific regions was the most promising for flaxseed oil and mucilage. Multivariate analysis of a set of selected variables sensitive to the flaxseed oil composition successfully distinguished all flax cultivars of this study. The strong correlation observed between ATR-FT-MIR and FT-Raman results confirmed that these methods are comparable for characterizing different grades of flaxseed oil.

Keywords: flaxseed oil; cultivars; FTIR; Raman; multivariate analysis



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1. Introduction

Flax (genus *Linum*, family Linaceae) is a well-known agricultural plant that originated in the Mediterranean and Southwest Asia [1]. There are about 22 different species of flax, but the most common is *Linum usitatissimum*. The main nutritional components of flaxseed are oil, proteins, and dietary fibers. Flaxseed also contains phenolic acids, lignans, vitamins,

and minerals [2,3]. Due to biologically active compounds, flax seeds are considered a functional food [4].

The chemical composition of flaxseed varies among different flax cultivars and depends on climatic conditions and harvesting regions [5]. The main nutritional components include fat (38–45%), protein (10–23%), total dietary fibers (28%), moisture (7.7%), and ash (3.4%) [3,6–8]. Carbohydrates are present as polysaccharides and in glycosides of phenolic acids, cyanogenic glycosides [9], and lignans [10].

- **Fat (oil)**, primarily composed of triacylglycerols (TAGs), has oleic, linoleic, and α -linolenic acids as the main unsaturated fatty acids (UFAs), and palmitic and stearic acids as saturated fatty acids (SFAs) [2–4,6,11]. The FA profile of flaxseed oil, particularly the linoleic acid to α -linolenic acid ratio, can be altered through breeding [12]. These two UFAs are considered the main polyunsaturated fatty acids (PUFAs) in flaxseeds.
- **Proteins** account for 35–45% of the dry, oil-free material, mainly located in the aleurone grains of the cotyledons and to a lesser extent in the endosperm [13]. Flaxseed protein isolate obtained from defatted flaxseed meal contains nearly all amino acids required for human nutrition; the limiting amino acids are leucine and threonine [14].
- **Dietary fibers** include water-soluble (arabinoxylan, pectins) and water-insoluble (cellulose, hemicelluloses) polysaccharides, both of which are in cell walls. Partially, flaxseed mucilage originates from the secondary cell wall material in the outermost layer and consists of highly branched arabinoxylan (AX) and rhamnogalacturonan type I (RG-I) [15]. The variation in monosaccharide profile of mucilage depends on the flax cultivar, harvesting year, raw material, and extraction conditions [16–18].

Flaxseed processing produces several value-added products (Figure 1). Typically, the first step involves powder production by grinding whole seeds [5]. However, the dehulling step may precede milling, removing hulls from the seeds. As a result, we obtain fully or partially dehulled kernels and hulls. The latter is suitable for feed formulations or further extraction of phytochemicals [19,20]. The flaxseed kernel is rich in oil [21] and proteins [22] and also contains some polysaccharides [23]. High protein and oil content, together with low carbohydrate level, make dehulled flaxseed (kernel) an ingredient desirable for improved food and feed production [20]. Chang et al. [24] reviewed advanced flaxseed dehulling technologies, including chemical, enzymatic, mechanical, and ultrasonic methods. The hulls, which include the seed coat and endosperm, contain ~25% proteins, 15–30% oil, polysaccharides—mainly cellulose and hemicelluloses—and phenolic acids [25].

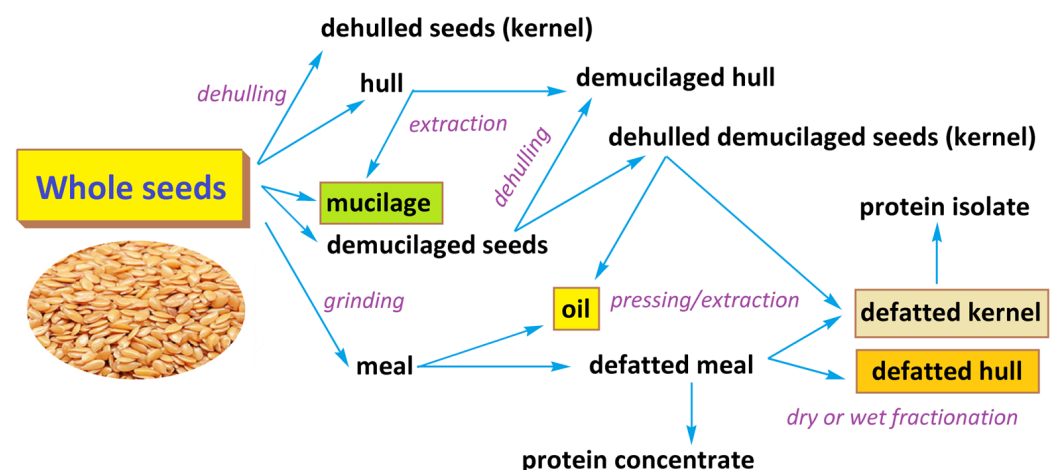


Figure 1. Flaxseed processing and flaxseed-derived products.

The next step is pressing the whole or dehulled flaxseed to produce oil and defatted meal (seeds or kernels) [26,27]. Flaxseed oil is extracted from the seeds using various methods, including solvent extraction, supercritical carbon dioxide extraction, and both hot and cold pressing [28]. Liang et al. [29] described a simple wet-screening approach for flaxseed dehulling and fractionation using ethanol washing and wet sieving. This method successfully separates whole flaxseeds into their components: oil, hull, and kernel. The oil was highly pure, exhibited good rheological properties, and contained essential FAs, vitamin E, phytosterols, and cyclopeptides [29]. Defatted flaxseed meal, a by-product of oil processing, contains gluten-free proteins, fiber, and minerals and can therefore impart functional properties to foods [27]. It is also the source of HG-rich pectin [30]. Carbohydrate-rich defatted hull was considered a source of dietary fiber and mucilage for human nutrition, and the kernel, freed from oil and hull, consisted mainly of proteins [29]. Protein concentrate can be obtained from defatted flaxseed meals by ethanol fluidization; protein isolate can be derived from dehulled or demucilaged seeds (kernel) via isoelectric precipitation of the aqueous extract. Lastly, mucilage is obtained by aqueous extraction from whole seeds, defatted seed meal, or hulls [14,31,32]. Mucilage obtained from seed meals is of low quality due to the presence of proteins and tannins [32]. Extraction of mucilage from whole seeds is more effective at higher temperatures. However, heating leads to the co-extraction of hull proteins and pectin, primarily homogalacturonan (HG), increasing the uronic acid content in the extract. A continuous wet process was proposed as an alternative for extracting the mucilage and hulls from flaxseed [33]. In this process, mucilage is obtained from whole seeds by aqueous extraction, and demucilaged seeds were ground and fractionated using an aqueous method, resulting in the hull and kernel parts. Flaxseed mucilage is a unique hydrocolloid complex with distinct composition and properties [4,32,34].

Vibrational spectroscopic methods, especially Fourier-transform infrared spectroscopy (FTIR) in near-infrared (NIR, 10,000–4000 cm^{-1}) and mid-infrared (MIR, 4000–400 cm^{-1}) regions—referred to as FT-NIR and FT-MIR—are widely used to characterize edible seed oils [35–41] and oilseeds [42,43]. Petraru et al. [44] reported ATR-FT-MIR spectra of flaxseed cake, a by-product of the cold pressing of flaxseed oil, observing characteristic bands of lipids at 2923, 2853, and 1743 cm^{-1} , proteins at 1636 and 1540 cm^{-1} , and polysaccharides at 1038 cm^{-1} . Minevich et al. [45] studied the protein–polysaccharide extracts from flaxseeds of two cultivars using ATR-FT-MIR spectroscopy.

Multivariate analysis of FT-NIR and FT-MIR spectra has been employed to characterize linoleic and α -linolenic acids in sunflower seeds [42], as well as in yellow and brown flax seeds and flaxseed flours [46]. Elzey et al. [47] evaluated the composition of adulterated neem and flaxseed oils using ATR-FT-MIR spectroscopy coupled with partial-least-squares (PLS) multivariate regression analysis. Nadimi et al. [48] assessed mechanical damage and germination of flax seeds using visible and NIR hyperspectral imaging combined with multivariate analysis. Using key wavelengths, they developed discrimination models to differentiate seeds based on the degree of mechanical damage. A similar approach was used to assess the biochemical composition (oil, protein, linoleic acid, and lignan content) and some quality parameters of flax seeds, which depend on the cultivar [49].

Additionally, Raman spectroscopy, often combined with FT-MIR, has been used to assess seed oil authenticity and detect adulteration [50–54]. Hua et al. [55] evaluated the effect of antioxidants on the quality of fried flaxseed oil using Raman spectroscopy. When added, antioxidants effectively reduce the formation of *trans*-C=C bonds associated with the 970 cm^{-1} band and protect the ester group (1745 cm^{-1}), *cis*-C=C bonds (1265 cm^{-1}), and CH_2 groups (1300 and 1440 cm^{-1}) in flaxseed oil during frying. Punzalan et al. [56] investigated flaxseed proteins obtained by pulsed electric field (PEF)-assisted extraction at

various conditions. Several alterations in secondary and tertiary structures of proteins were observed using Raman band markers and multivariate analysis. The α -helical (1659 cm^{-1}) and β -sheet (1671 cm^{-1}) structures in PEF-treated flaxseed proteins, which predominated at neutral and alkaline conditions, respectively, were well distinguished.

In earlier studies [57,58], we showed how diffuse reflectance (DR) spectroscopy, in the visible (Vis, 380–770 nm) and NIR regions, combined with multivariate analysis, can effectively differentiate flax cultivars based on seed color and chemical composition, respectively. In both cases, whole seeds from yellow- and brown-seeded cultivars were analyzed, and, for comparison, four fractions (oil, oleaginous kernel, hull, and defatted flour) were extracted from flaxseeds for FT-NIR spectroscopic analysis. These cultivars varied not only in seed color parameters [57] but also in flaxseed oil fatty acid (FA) composition [58] and the composition and rheological properties of flaxseed mucilage [59]. The spectroscopic techniques used for flaxseed characterization proved effective in distinguishing between different flax cultivars through hierarchical cluster analysis (HCA) and principal component analysis (PCA). The use of vibrational spectroscopy in the mid-infrared range enables more effective structural analysis of flaxseed fractions by interpreting prominent bands characteristic of key biochemical components, including fats, proteins, polysaccharides, and polyphenols. Flax cultivars can be differentiated using multivariate analysis, focusing on specific IR/Raman bands and spectral regions associated with the respective biochemical components.

This study aimed to evaluate the potential of vibrational spectroscopy methods for cultivar-specific analysis of flaxseed fractions using multivariate statistics. The flaxseeds of four yellow- and brown-seeded cultivars and different harvesting years (2015–2018) were characterized using ATR-FT-MIR and FT-Raman spectra of the seed fractions (oil, hulls, defatted kernel, and mucilage). We improved the flaxseed fractionation method earlier used [58], yielding oil, defatted kernel, and hull. Also, mucilage was isolated from whole seeds as described above [59–61]. These flaxseed fractions are characteristic of cultivars due to their specific composition and possible use in food, medicine, or cosmetics. Discrimination of flax cultivars was performed using multivariate analyses of the vibrational spectra (HCA, PCA) in specific wavenumber regions.

2. Materials and Methods

2.1. Flaxseed Cultivars

Ten samples of flaxseed cultivars, harvested from 2015 to 2018 and characterized by yellow and brown seeds, were provided by the local producer Agritec, s.r.o. (Šumperk, Czech Republic). These cultivars were grown at Rapotín locality in the Czech Republic. The details of these samples are summarized in Table 1. The oils extracted from these cultivars vary in the ratio of two major polyunsaturated fatty acids, specifically linoleic and α -linolenic acids [57,58]. A similar set of flaxseed samples was previously analyzed using diffuse reflectance Vis and FT-NIR spectroscopy [57,58]. Earlier, samples, including mucilage, were isolated from the whole seeds of these cultivars, and their chemical composition and rheological properties were described [58,59].

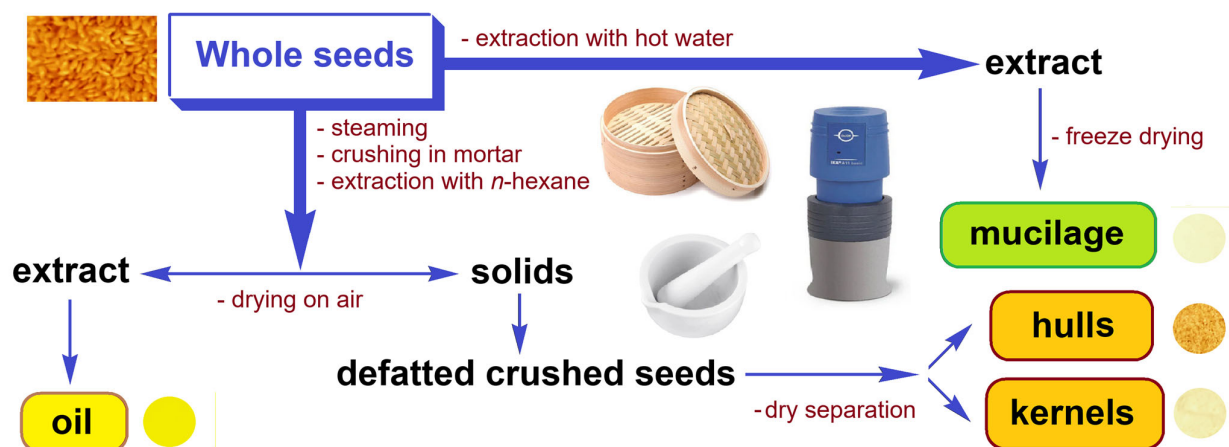
Table 1. Specification of flaxseed samples [58,59].

Sample	Cultivar	Breeder	Harvesting Year	Seed Color	Composition *	
					Oil	Mucilage
a15	Amon	Agritec (Šumperk, Czech Republic)	2015	Yellow	High linoleic, low α -linolenic	Medium AX/ medium-to-high RG-I/ low proteins
a16			2016			
a17			2017			
r15	Raciol/ Raciol Bio		2015		Medium linoleic, medium α -linolenic	High AX/low RG-I and proteins
r16			2016			
r17			2017			
rb15			2015			
lb17	Libra Bio	Limagrain Advanta Nederland, B.V.	2017	Brown	Low linoleic, high α -linolenic	Low AX/high RG-I/ medium proteins
lb18		(Rilland, Holland)	2018			
rt15	Recital	Laboulet Semences (Airaines, France)	2015			Medium AX/RG-I/proteins

* AX, arabinoxylan; RG-I, rhamnogalacturonan type I.

2.2. Preparation of Flaxseed-Derived Samples

Different seed compartments, i.e., oil, hulls, kernels, and mucilage, were isolated from whole seeds of flax cultivars specified in Table 1. The isolation procedures are represented in Scheme 1. Whole seeds were steamed for 15 min in a traditional bamboo steamer set over a pot of boiling water, using a steam temperature of 100 °C. Then, they were crushed in a mortar, and oil was extracted by vigorously stirring with hexane for 15 min (6:1 *v/w*). Then, the hexane extract was filtered through a paper filter. The air-dried filtrates (oil) were placed in Eppendorf tubes and stored in the dark at 4 °C, and the solids (defatted meal) were air-dried and stored at room temperature. The hulls were manually separated from the kernels of larger fragments of defatted, crushed seeds using tweezers, a scalpel, and a magnifying glass, as previously described [58]. As an alternative method, defatted, crushed seeds were ground in an IKA® A11 basic analytical mill (IKA-Werke GmbH & Co., Staufen, Germany), washed several times with *n*-hexane to remove residual oil, and then air-dried. The kernel and shell particles were separated by sifting the meal on a sheet of paper using vibratory motions. Lighter hull fragments were easily separated from the dense kernel particles. Completeness of purification was monitored visually.

**Scheme 1.** Fractionation of flaxseeds used in the current work.

Flaxseed mucilage was extracted from whole seeds using the previously described procedure [60,61]. The flaxseeds were suspended in the distilled water at a solid/water ratio

of 820 g/4000 g and a temperature of 85 °C for 30 min under continuous manual stirring using the machine Stephan (Stephan CombiCut TC300/SK400, Hameln, Germany), then the viscous mixture was filtered through a sieve to remove the seeds and some impurities. Fresh flaxseed mucilage extract was freeze-dried in a Freeze Dryer ALPHA 1-4 LSC (Martin Christ Gefriertrocknungsanlagen, Osterode am Harz, Germany) for 48 h and stored at room temperature.

2.3. Vibrational Spectroscopy

ATR-FT-MIR spectra (4000–650 cm^{-1} , 64 scans, resolution 2 cm^{-1}) of the flaxseed fractions were recorded on a Nicolet 6700 spectrophotometer (Thermo Fischer Scientific, Waltham, MA, USA) using a KBr beam splitter and a “smart” HATR holder with ZnSe crystal (Thermo Fischer Scientific, Waltham, MA, USA). The spectra were smoothed using the Savitzky–Golay algorithm, ATR-corrected, and multi-point linear baseline-corrected using Omnic 8.0 software (Thermo Fisher Scientific, Waltham, MA, USA).

FT-Raman spectra (100–4000 cm^{-1} , 1000 scans, resolution 4 cm^{-1}) of the flaxseed fractions were recorded on a Nicolet iS50 FT-IR spectrometer with FT-Raman module (Thermo Fisher Scientific, Waltham, MA, USA), Nd:YAG laser ($\lambda_{\text{ex}} = 1064 \text{ nm}$, power 500 mW), CaF_2 beam splitter, and InGaAs detector. The spectra were recorded and processed similarly to ATR-FT-MIR spectra using Omnic 9.0 software (Thermo Fisher Scientific, Waltham, MA, USA).

The ATR-FT-MIR and FT-Raman spectra of oil, kernel, hull, and mucilage samples were recorded in triplicate per cultivar. For the ATR-FT-MIR measurement of the oil, between 4 and 15 seeds were randomly selected from each cultivar. For additional processing, such as scale normalization at the reference band, statistical evaluation, and preparation of graphical outputs, the spectra were converted to ASCII format and exported to Origin 6.0 software (OriginLab, Northampton, MA, USA). For scale normalizing, the following bands were used for the seed fractions:

- Oil: 1745 cm^{-1} (IR, C=O stretch); 2902 cm^{-1} (Raman, C–H stretch)
- Hull: 1596 cm^{-1} (IR, COO^- antisymmetric stretch); 2933 cm^{-1} (Raman, C–H stretch)
- Kernel: 1647 cm^{-1} (IR, amide I); 2933 cm^{-1} (Raman, C–H stretch)
- Mucilage: 1039 cm^{-1} (IR, C–O and C–C stretch); 2933 cm^{-1} (Raman, C–H stretch).

The selected IR bands characterize the main biochemical components of the fraction, i.e., fats for the oil, proteins for the kernel, pectin (HG) for the hull, and AX for the mucilage. In the case of FT-Raman, we used the most intense band in the spectrum, relating to the aliphatic groups. This choice is explained by the fact that in the corresponding spectra, the IR bands of functional groups and Raman bands of the hydrocarbon base of organic molecules are most clearly expressed.

2.4. Multivariate Statistics

Flax cultivars were differentiated using PCA on normalized ATR-FT-MIR and FT-Raman spectra of flaxseed fractions (oil, hull, kernel, and mucilage) analyzed with Statistica 14.1.0 software (TIBCO Software Inc., San Ramon, CA, USA). The spectral regions from IR/Raman spectra that display multiple bands with cultivar sensitivity, which generate strong positive or negative peaks in the loading plots, were chosen for PCA. Oppositely, spectral regions containing bands whose contribution did not correlate with cultivar, such as polysaccharide bands for the kernel or phenolic acid bands for the hull or mucilage, were excluded. The results were presented through graphs of component loading and component scores. Comparing the vibrational spectra and corresponding PC1 loading curves of the flaxseed oil samples, a set of 24 variables (15 from ATR-FT-MIR and 8 from FT-Raman) was selected, normalized, and utilized for multivariate statistical discrimination

of the flax cultivars based on their fatty acid composition. The normalized spectroscopic data were processed using hierarchical cluster analysis (HCA) (Ward method of clustering, square Euclidean distances) and principal component analysis (PCA) (covariation matrix) with the help of Statistica 12.0 software (Statsoft, Tulsa, OK, USA). The graphical outputs, including dendrograms of similarity and component loading and score plots, were created using Origin 6.0 software (OriginLab, Northampton, MA, USA).

3. Results

The ATP technique is not appropriate for measuring FT-MIR spectra of whole flax seeds. The pressure required to ensure contact with the crystal surface causes the seeds to break apart and release oil from the kernel. Seed fractionation was applied to avoid this complication, as in the case of diffuse reflectance FT-NIR used in the previous article [59]. The whole seeds and derived fractions of cultivar Amon a16 are represented in Figure S1.

3.1. Flaxseed Oil

The average normalized ATR FT-MIR and FT-Raman spectra of the oil fractions isolated from flaxseeds of various cultivars are shown in Figure 2, panels (a) and (b); the band assignments are summarized in Table 2. In comparing the spectra of three groups (Amon, Raciol/Raciol bio, and Libra bio/Recital) demonstrated in Figure S2, panels (a) and (b), several IR and Raman bands that are sensitive to the PUFA composition of flaxseed oils were identified and assigned to specific vibrations.

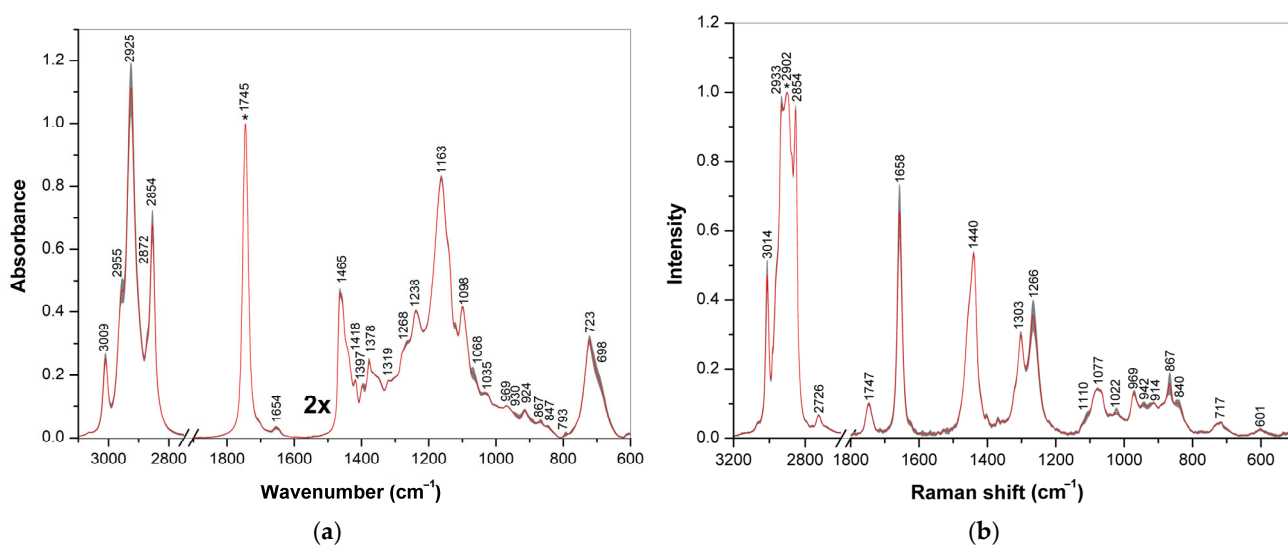


Figure 2. The average ATR-FT-MIR (a) and FT-Raman (b) spectra of flaxseed oils with the SD intervals (gray bars); asterisks * show the normalized band of C–H/C=O stretching vibrations.

Table 2. IR and Raman band assignments for flaxseed oils [41,50,55,62–68].

Wavenumber (cm ^{−1}) *		Assignment †	Origin ‡
IR	Raman		
3290br		$\nu_{as,s}(H_2O)$	water
3009 → 3011 ↑	3010 → 3014 ↑	$\nu_s(=CH)_{cis}$	UFA
2954 → 2958	2964 → 2967sh	$\nu_{as}(CH_3)$	acyls
2925; 2932sh ↑	2933 ↑	$\nu_{as}(CH_2)$	UFA
	2902	$\nu_{as}(CH_2), \nu_s(CH_3)$	acyls
2872sh	2875sh	$\nu_s(CH_3)$	acyls
2854	2854 ↓	$\nu_s(CH_2)$	acyls
	2726	$\delta(CH_2) + \tau(CH_2)$	acyls

Table 2. Cont.

Wavenumber (cm ⁻¹) *		Assignment †	Origin ‡
IR	Raman		
1745	1747	$\nu(\text{C}=\text{O})$	esters
1653	1658 ↑	$\nu(\text{C}=\text{C})_{\text{ip}}$	UFA
1465		$\delta(\text{CH}_2)$	acyls
1456 ↓	1457sh	$\delta_{\text{as}}(\text{CH}_3)$	acyls
	1440	$\delta(\text{CH}_2), \delta_{\text{as}}(\text{CH}_3)$	acyls
1418		$\rho(\text{=CH})_{\text{cis}}$	UFA
1397, 1393 ↑		$\rho(\text{=CH})_{\text{cis}}$	UFA
1378 ↓		$\delta_{\text{s}}(\text{CH}_3)$	acyls
1319		$\omega(\text{CH}_2)$	acyls
1301sh → 1307sh	~1302	$\tau(\text{CH}_2)_{\text{ip}}$	acyls
1265sh → 1267sh	1266 ↑	$\delta(\text{=CH})_{\text{cis}}$	UFA
1238		$\nu(\text{C}-\text{O})$	esters
1162		$\nu(\text{C}-\text{C})$	acyls
1139sh → 1137sh ↑		$\nu(\text{C}-\text{C})$	acyls
1120 ↓		$\nu(\text{C}-\text{C})$	acyls
1100	1100sh ↓	$\nu(\text{C}-\text{O}), \nu(\text{C}-\text{C})_{\text{ip}}$	esters; acyls, all-trans
1062sh, 1068 ↑	1067–1079 ↑ ↓	$\nu(\text{C}-\text{C})_{\text{g,op}}$	acyls, all-trans
1034		$\nu(\text{C}-\text{C})$	acyls
	1022 ↑	$\nu(\text{C}=\text{C})$	UFA
984, 970	973 → 969 ↑	$\gamma(\text{=CH})_{\text{cis}}$	UFA
942 ↑	~942 ↑, ~925 ↑	$\gamma(\text{=CH})_{\text{cis}}$	UFA
914	914	$\nu(\text{C}-\text{C}), \delta(\text{C}-\text{C}-\text{C})$	acyls
874	873	$\rho(\text{CH}_3)$	acyls
866 ↑	867 ↑	$\gamma(\text{=CH})_{\text{cis}}$	UFA
845 ↓	846 ↓	$\nu(\text{C}-\text{C})$	acyls
793 ↑		$\gamma(\text{=CH})_{\text{cis}}$	UFA
772 ↓		$\nu(\text{C}-\text{C})$	acyls
723	715	$\rho(\text{CH}_2)$	acyls, all-trans
695br, sh ↑		$\omega(\text{=CH})_{\text{cis}}$	UFA
	601	$\omega(\text{=CH})_{\text{cis}}$	UFA

* arrows →, ↑, and ↓ show wavenumber/intensity changes in the order Amon—Raciol/Raciol bio—Libra bio/Recital cultivars; br, broad; sh, shoulder. † ν , stretching; δ , bending; ρ , rocking; τ , twisting; ω , wagging; γ , out-of-plane deformation; as, antisymmetric; s, symmetric; ip, in-plane, op, out-of-plane, g, gauche. ‡ UFA, unsaturated fatty acids.

The IR band at 3009–3011 cm⁻¹ originated from the =C–H stretching vibration of the *cis*-unsaturated fatty acids. In the order Amon—Raciol/Raciol bio—Libra bio/Recital, the latter band increased in intensity and slightly shifted to higher wavenumbers. This shift indicated a higher contribution of polyunsaturated acyl groups [62–64]. The linolenic acid-rich flaxseed oils of Libra bio and Recital cultivars show this IR band at a higher frequency (~3011 cm⁻¹) than olive oil (~3005 cm⁻¹), rapeseed oil (~3007.5 cm⁻¹), and even corn oil (~3009 cm⁻¹) [41]. Two intense bands at 2925 and 2854 cm⁻¹ corresponded to the antisymmetric and symmetric stretching vibrations of CH₂ groups, and the band at 2954–2958 cm⁻¹ and the shoulder near 2872 cm⁻¹ arose from the corresponding vibrations of terminal CH₃ groups in acyls. The shift of the CH₂ antisymmetric band to higher wavenumbers and the appearance of a shoulder near 2932 cm⁻¹ in the order Amon—Raciol/Raciol bio—Libra bio/Recital cultivars are associated with a higher contribution of *cis*-unsaturated groups.

The strong IR band at 1745 cm⁻¹ was assigned to the C=O stretching vibration of esters, the weak band near 1653 cm⁻¹ to the C=C stretching vibration in *cis*-unsaturated fatty acid residues [41]. The bands at 1465 and 1378 cm⁻¹, along with a shoulder near 1456 cm⁻¹, correspond to CH₂ scissoring, CH₃ symmetric, and CH₃ antisymmetric bending vibrations, respectively. Two CH₃ bending bands decreased slightly and shifted to lower wavenumbers by 2 cm⁻¹ in the order: Amon—Raciol/Raciol bio—Libra bio/Recital. The

band at 1120 cm^{-1} , assigned to C–C stretching vibration in saturated acyls, is sensitive to the degree of unsaturation. It decreased significantly for Raciol and disappeared for Libra bio and Recital cultivars. The absence of this band indicates that the oil is highly unsaturated [41].

The IR band near 1100 cm^{-1} , associated with the C–O stretching vibration in esters, was sensitive to the proportion of mono- and polyunsaturated acyl groups [44,45]. However, the position of this band did not show significant changes among the flaxseed cultivars. The IR band observed near 914 cm^{-1} is also sensitive to the degree of unsaturation. This band broadened for cultivars with higher levels of α -linolenic acid. For comparison, FT-MIR spectra of pure tristearin, tripalmitin, trilinolenin, trilinolein, and triolein showed bands at 921, 917, 916, and 914 cm^{-1} (shoulder), respectively [41]. Thus, the band near 914 cm^{-1} obtained for all flaxseed oils could be attributed to a significant amount of polyunsaturated acyl groups [65]. The band perturbations at $845\text{--}866\text{ cm}^{-1}$ and $772\text{--}793\text{ cm}^{-1}$ (out-of-plane =C–H deformations) also indicate the relationship between the unsaturated fatty acids in the flaxseed oils. The band at 723 cm^{-1} and a broad shoulder near 695 cm^{-1} corresponded to the overlapping in-phase CH_2 rocking and the out-of-plane =C–H wagging vibrations of *cis*-unsaturated acyls, respectively. The shoulder was more pronounced for oils from Raciol and, especially, Libra and Recital cultivars, having more *cis*-unsaturated groups.

As mentioned above for the ATR-FT-MIR spectra of flaxseed oils, the FT-Raman spectra also showed an evident dependence on FA composition and cultivar (Figure 2b). The Raman bands at $3010\text{--}3014$, 1658 , 1266 , and 867 cm^{-1} , originating from vibrations of *cis*-CH=CH groups in UFA [55,66,67], increased in relative intensity in the order: Amon—Raciol/Raciol bio—Libra bio/Recital. The same trend was observed for Raman bands at 2933 , 1022 , 969 , and 942 cm^{-1} , which are assigned to the stretching vibrations of CH_2 and C–C near the non-conjugated double bonds =CH– CH_2 –CH=. Conversely, the bands and shoulders at 2854 , 2726 , 1440 , ~ 1302 , 1100 , 914 , and 615 cm^{-1} —mainly associated with vibrations of saturated parts in acyl groups—showed no significant changes or gradually declined in the same order.

3.2. Flaxseed Hulls

Average normalized ATR-FT-MIR and FT-Raman spectra of hulls isolated from flaxseeds of various cultivars are shown in Figure 3, panels (a) and (b); band assignment is summarized in Table 3. Vibration bands of pectic compounds predominated in these spectra observed for the hulls of various cultivars, as demonstrated in Figure S3, panels (a) and (b). The broad IR band centered at $3440\text{--}3390\text{ cm}^{-1}$ arose from O–H stretch vibrations of hydroxylic groups and bound water, and the C–H stretch bands at $3000\text{--}2800\text{ cm}^{-1}$. The bands at $1750\text{--}1710\text{ cm}^{-1}$ were assigned to C=O stretching vibrations of carboxylic and ester groups. Two intense bands of COO^- antisymmetric and symmetric stretching vibrations were observed at $1598\text{--}1592$ and 1415 cm^{-1} , respectively; a band near 860 cm^{-1} was assigned to carboxylate in-plane bending vibration [69]. The intense carboxylate bands and the absence of characteristic methyl ester bands confirmed that flaxseed hull pectins are either slightly or not esterified. Bands at 1330 and 1238 cm^{-1} corresponded to in-plane C–H and O–H bending vibrations in pyranoid rings. The band near 1142 cm^{-1} indicates C–O–C stretching vibrations in glycosidic bonds of pectins [70], and the bands at $1100\text{--}980\text{ cm}^{-1}$ arose from C–O and C–C stretching and C–O–H bending vibrations in the pyranoid ring. Two bands near 1100 and 1013 cm^{-1} are characteristic of the homogalacturonan (HG) part of pectin, while two bands at 1074 and 1045 cm^{-1} characterize galactopyranosyl and arabinofuranosyl units in arabinogalactans' side chains of RG-I [70]. Bands at 916 , 894 , 835 , and 822 cm^{-1} originated from C1–H bending, out-of-plane C–O–H bending, and ring vibrations of pectic

polysaccharides. Therefore, pectins and, to a lesser extent, hemicelluloses (xylans) are the main components of flaxseed hull.

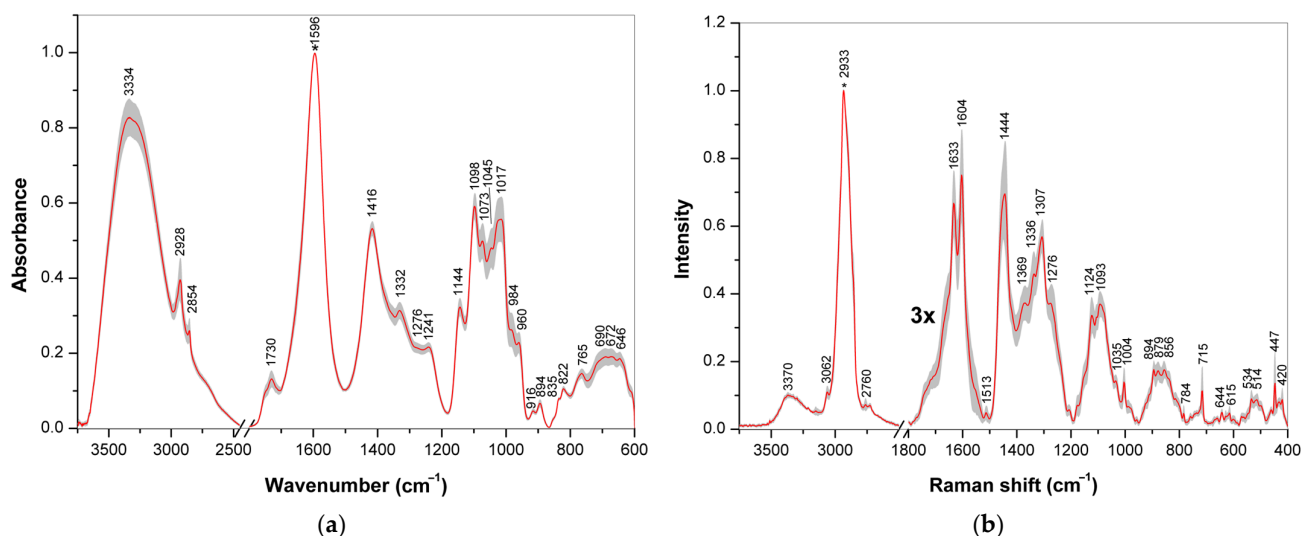


Figure 3. The average ATR-FT-MIR (a) and FT-Raman (b) spectra of flaxseed hulls with the SD intervals (gray bars); asterisks show the normalized band of COO[−]/C–H stretching vibrations.

Table 3. IR and Raman band assignment for flaxseed hulls [30,69–78].

Wavenumber (cm ^{−1}) *		Assignment †	Origin ‡
IR	Raman		
3334br	3370br	$\nu(\text{OH})$, $\nu_{\text{as,s}}(\text{H}_2\text{O})$	ROH, water
	3062	$\nu(=\text{CH})$	tannins
2928	2933	$\nu_{\text{as}}(\text{CH}_2)$, $\nu(\text{CH})$	CH ₂ OH, AX
2893sh	2892sh	$\nu(\text{CH})$, $\nu_{\text{s}}(\text{CH}_3)$	proteins
2873sh		$\nu_{\text{s}}(\text{CH}_3)$	proteins
2854	2854sh	$\nu_{\text{s}}(\text{CH}_2)$	CH ₂ OH, AX
	2760	$\delta(\text{CH}_2) + \tau(\text{CH}_2)$	CH ₂ OH, AX
1730	1745–1708sh	$\nu(\text{C}=\text{O})$	pectins, tannins
	1673–1656sh	amide I, $\nu(\text{C}=\text{C})$	proteins, tannins, lignin
	1633	$\nu(\text{C}_\alpha=\text{C}_\beta)$	phenolic acids
	1604	$\nu(\text{C}=\text{C})$ ring	phenolic acids, lignin
1596		$\nu_{\text{as}}(\text{COO}^-)$	pectin
	1513	$\nu(\text{C}=\text{C})$	phenolic acids
1463sh	1461sh	$\delta(\text{CH}_2)$, $\nu(\text{C}=\text{C})$	CH ₂ OH, tannins
1442sh	1444	$\delta_{\text{as}}(\text{CH}_3)$, $\nu(\text{C}=\text{C})$	proteins, tannins
1416		$\nu_{\text{s}}(\text{COO}^-)$	pectin
1371sh	1369	$\delta_{\text{s}}(\text{CH}_3)$, $\delta(\text{COH})$	proteins, xylan, tannins
1353sh		$\delta(\text{C}-\text{H})$	pyranoid ring (xylan)
1332	1336	$\delta(\text{C}-\text{H})$	pectin, lignin
1304sh	1307	$\tau(\text{CH}_2)$	CH ₂ OH
1276sh	1276	amide III, $\nu, \delta(\text{C}-\text{OH})$	proteins
			phenolic acids, lignin
1241	1237sh	$\delta(\text{C}-\text{H})$	pyranoid ring (pectin)
	1205sh	$\delta(\text{C}-\text{H})(\text{O}-\text{H})$, $\nu(\text{C}-\text{O})$	tannins
1144	1157sh	$\nu(\text{C}-\text{O}-\text{C})$	pectin
	1124	$\nu(\text{C}-\text{O}-\text{C})$	cellulose, xylan
1098	1093	$\nu(\text{C}-\text{O})(\text{C}-\text{C})$	cellulose, xylan
1073	1076sh	$\nu(\text{C}-\text{O})(\text{C}-\text{C})$	pyranoid ring (HG)
1045	1050sh	$\nu(\text{C}-\text{O})(\text{C}-\text{C})$	pyranoid ring

Table 3. Cont.

Wavenumber (cm ⁻¹) *		Assignment [†]	Origin [‡]
IR	Raman		
1027sh	1035	$\nu(\text{C-O})(\text{C-C})$	pyranoid ring (HG), lignin
1013		$\nu(\text{C-C}), \delta(\text{C-O-H})$	pyranoid ring (HG)
	1004	Phe ring	proteins
984	~990	$\delta(\text{COO}^-)$	pectin
960	958	$\nu(\text{C-O}), \delta(\text{C-O-H})$	pyranoid ring (xylan)
916			
894	894	$\delta(\text{C1}\beta\text{-H}), \text{ring}$	cellulose, xylan
	879	$\delta(\text{C1}\beta\text{-H})$	galactan
	856	$\delta(\text{C1}\alpha\text{-H}), \text{ring}$	pectin
835	836sh	$\gamma(\text{C-O-H})$	pectin
822		$\gamma(\text{C-O-H})$	
	784	$\gamma(\text{C-H})(\text{O-H})$	tannins
765	~760	skeletal	pyranoid ring (pectin)
	715	$\gamma(\text{C-O-H})$	pectin
690			
672		ring deformation	pyranoid ring
646	644		
	615		
	574	$\delta(\text{C-O-C})$	AX (Xyl)
	534	$\delta(\text{C-O-C})$	AX (Xyl)
	514	$\delta(\text{C-C-C})$	AX (Xyl)
	447	$\delta(\text{C-O-C})$	pectin (HG)
	420	$\delta(\text{C-C-C}), \delta(\text{C-C-O})$	cellulose, xylan

* br, broad; sh, shoulder. [†] ν , stretching; δ , bending; τ , twisting; ω , wagging; γ , out-of-plane deformation; as , antisymmetric; s , symmetric. [‡] AX, arabinoxylan; HG, homogalacturonan; Xyl, xylose.

Currently, there is no information regarding the Raman patterns of flaxseed hull. As previously found out for flax stem [71–73], Raman bands from 3000 to 2800 cm⁻¹ arose from C–H stretching vibrations in cellulose and other cell-wall polysaccharides, bands at 1444, 1369, and 1307 cm⁻¹ were assigned to O–H/C–H, CH₂ and CH₃ bending vibrations, bands at 1200–950 cm⁻¹ to C–O/C–C stretching vibration, bands at 950–700 cm⁻¹ to C–OH, C–CH, and O–CH bending. The band at 894 cm⁻¹ to C1 β –H bending in cellulose and xylan [70,74]. Two Raman bands at 1124 and 1093 cm⁻¹ were assigned to skeletal symmetric and asymmetric C–O–C vibrations of the (1→4)- β -glycosidic linkages in cellulose. However, non-cellulosic polysaccharides and lignin also have a band near 1120 cm⁻¹. Sharp Raman bands at 1004 and 1035 cm⁻¹ (Phe ring vibrations) are characteristic of proteins [75,76], and two intense bands at 1633 and 1604 cm⁻¹ arose from C=O/C=C stretching vibrations of phenolic acids [70,71,77].

3.3. Defatted Flaxseed Kernels

Average normalized ATR-FT-MIR spectra of defatted flaxseed kernel samples are shown in Figure 4, panels (a) and (b); band assignments are summarized in Table 4. The corresponding spectra observed for the kernels of various cultivars are demonstrated in Figure S4, panels (a) and (b). The protein bands predominated in these spectra, while the polysaccharide bands were less pronounced. The IR bands at 3280–3290, 3080, 1627–1651, 1538–1555, and 1240–1316 cm⁻¹ were assigned to the principal vibrations of the polypeptide backbone [79,80]. The spectral differences observed in the amide I, II, and III and C α –C stretching regions are attributable to the contributions of α -helix, β -sheet, and random coil in proteins' secondary structure [76,81]. Also, several amino acid side-chain bands were observed for flaxseed kernel [82]. In the FT-Raman spectra, the bands observed at 1666, 1265, 1033, and 1004 cm⁻¹ are characteristic of proteins [66]. For flaxseed proteins [83],

two Raman bands of amide vibrations have been observed at 1245 cm^{-1} (amide III) and 1665 cm^{-1} (amide I). The C–H stretching ($3000\text{--}2800\text{ cm}^{-1}$) and C–H bending ($1467\text{--}1340\text{ cm}^{-1}$) regions have several bands originating from proteins and polysaccharides. The shoulder at $1550\text{--}1600\text{ cm}^{-1}$ and the band at $1389\text{--}1415\text{ cm}^{-1}$ were assigned to antisymmetric and symmetric stretching vibrations of COO^- , respectively. Carboxylate anions originate from proteins (Asp, Glu) and polysaccharides (pectins), and their contributions can be distinguished by wavenumbers [79,80,82,84]. Highly overlapped bands at $996\text{--}1161\text{ cm}^{-1}$ originate mainly from C–O and C–C stretching vibrations of cell wall polysaccharides (cellulose, hemicelluloses, and pectins) [52,53,56,66], and these bands differ from those observed in the spectra of flaxseed hull.

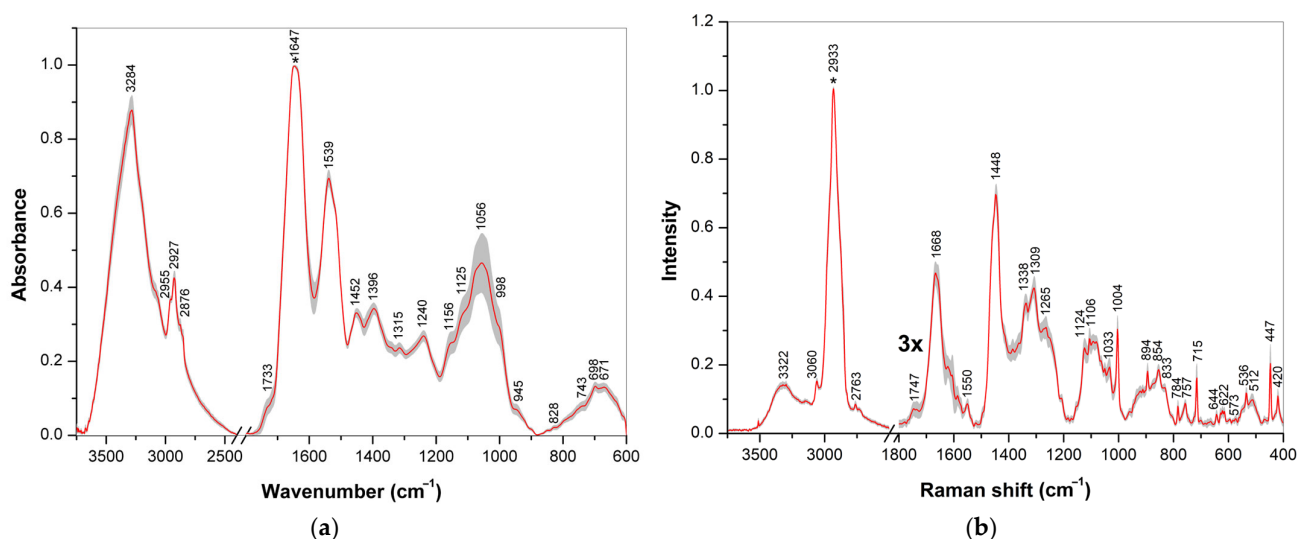


Figure 4. The average ATR-FT-MIR (a) and FT-Raman (b) spectra of defatted flaxseed kernels with the SD intervals (gray bars); asterisks show the normalized band of C–H/C=O stretching vibrations.

Table 4. IR and Raman band assignment for flaxseed kernels [30,56,70,71,74–76,79,80].

Wavenumber (cm^{-1}) *		Assignment ^{†, ‡}	Origin [‡]
IR	Raman		
3284br	3322br	amide A	proteins
3080sh		amide B	proteins
	3060	$\nu(\text{=CH})$	proteins (Tyr, Trp, Phe)
2955		$\nu_{\text{as}}(\text{CH}_3)$	proteins
2927	2933	$\nu_{\text{as}}(\text{CH}_2)$	CH_2OH , fat reminder
2893sh		$\nu(\text{CH})$	proteins, CHOH
2876	2873sh	$\nu_{\text{s}}(\text{CH}_3)$	proteins
2853sh	2853sh	$\nu_{\text{s}}(\text{CH}_2)$	CH_2OH , proteins
	2763	$\delta(\text{CH}_2) + \tau(\text{CH}_2)$	proteins
1733sh	1747	$\nu(\text{C=O})$	fat reminder (COOR)
~1670sh	1668	amide I	proteins (β -turn)
1647	1654sh	amide I	proteins (α -helix)
1633–1627sh	1634sh	amide I	proteins (β -sheet)
	1606–1621	$\nu(\text{C=C})$	proteins (Tyr, Trp, Phe)
1567–1597sh	1587	$\nu_{\text{as}}(\text{COO}^-)$	proteins (Asp, Glu)
1555sh		amide II	proteins (α -helix)
	1550	indole ring	proteins (Trp)
1539		amide II	proteins (β -sheet)
1516sh		$\nu(\text{C=C})$	Tyr, Trp (proteins)

Table 4. Cont.

Wavenumber (cm ⁻¹) *		Assignment ^{†,‡}	Origin [‡]
IR	Raman		
~1470sh	~1460sh	δ(CH ₂)	proteins
1452	1448	δ _{as} (CH ₃)	CH ₂ OH, proteins
1417sh	1413sh	ν _s (COO ⁻)	proteins (Asp, Glu)
1396	1386	ν _s (COO ⁻), δ _s (CH ₃)	proteins
1367sh	1365sh	δ _s (CH ₃)	proteins
1339sh	1338	δ(Cα-H)	proteins (Trp)
1315	1309	δ(C-H)	proteins
1276	1276sh	amide III	proteins (α-helix)
	1265	amide III	proteins (β-sheet, random)
1240	1247sh	amide III	proteins (β-sheet, random)
1161sh	1154	ν(C-O-C)	cellulose, AX
1120sh	1124	ν(C-N)	proteins
	1106		
1078sh	1081	ν(C-O)(C-C)	hemicelluloses
1056sh	1050	ν(C-O)(C-C)	cellulose, AX
1038sh		ν(C-O)(C-C)	cellulose
	1033	Phe ring	proteins
	1004	Phe ring	proteins
996		ν(C-O)(C-C)	AX
945sh	910–946sh	ν(Cα-C)	proteins
896sh	894	δ(C1β-H)	xylan, cellulose
852sh	854	δ(C1α-H), Tyr ring	pectin, proteins
828	833	Tyr ring	proteins
798sh	784		
757sh	757	Trp ring	proteins
743sh			
	715		
698		skeletal	proteins, carbohydrates
671		skeletal	proteins, carbohydrates
	644	Tyr ring	proteins
	622	Phe ring	proteins
	536	ν(S-S)	proteins
	512	ν(S-S)	proteins
	447		
	420		

* br, broad; sh, shoulder. [†] ν, stretching; δ, bending; _{as}, antisymmetric; _s, symmetric. [‡] AX, arabinoxylan; HG, homogalacturonan; Xyl, xylose; Asp, aspartic acid; Glu, glutamic acid; Phe, phenylalanine; Tyr, tyrosine; Trp, tryptophan.

3.4. Flaxseed Mucilage

Average normalized ATR-FT-MIR and FT-Raman spectra of flaxseed mucilage from various cultivars are shown in Figure 5, panels a and b; band assignments are summarized in Table 5. The corresponding spectra observed for the mucilage of individual cultivars are demonstrated in Figure S5, panels (a) and (b). There are intense IR bands or groups of bands corresponding to water and hydroxylic groups (3300 cm⁻¹, O-H stretching), polysaccharides (1200–950 cm⁻¹, C-O and C-C stretching), uronic carboxylate groups (1610 and 1410 cm⁻¹, COO⁻ antisymmetric and symmetric stretching), and proteins (shoulders at 1660 and 1540 cm⁻¹, amide I, II vibrations) [85–88]. The bands of C-H and C=O stretching vibrations were observed at 2929 and 1733 cm⁻¹, respectively [86]. The band near 1147 cm⁻¹ was assigned to the C-O-C vibration of glycosidic bonds in AX [89]. The intense, sharp band at 1039 cm⁻¹ may contribute to C-OH bending in AX, typically observed near 1035–1047 cm⁻¹ [90–92]. The shift of this band towards lower wavenumbers may be associated with the structural features of the flaxseed AX, a part of flaxseed mucilage.

Two weak bands or shoulders at 978 and 951 cm^{-1} are also associated with AX vibrations. The corresponding branching-sensitive bands were previously observed for cereal AX at 981 and 958 cm^{-1} [92]. The band at 899 cm^{-1} was attributed to the β -xylopyranosyl residues in AX [92,93]. Ren et al. [94] reported FTIR spectra of flaxseed mucilage of six cultivars. They observed several polysaccharide bands at 3275 cm^{-1} (O–H stretch), 1605 and 1418 cm^{-1} (antisymmetric and symmetric COO^- stretch), 1145 cm^{-1} (C–O–C stretch in glycosidic bonds), 1024 cm^{-1} (C–O and C–C stretch and C–OH bend in pyranoid rings), and 527 cm^{-1} (skeletal). For some cultivars, the protein bands at 1635 cm^{-1} (amide I) and around 1540 cm^{-1} (amide II) bands of proteins were pronounced. The authors explained the spectral differences between the mucilage samples by the specific composition of monosaccharides, depending on the cultivar, while the contribution of proteins was also evident.

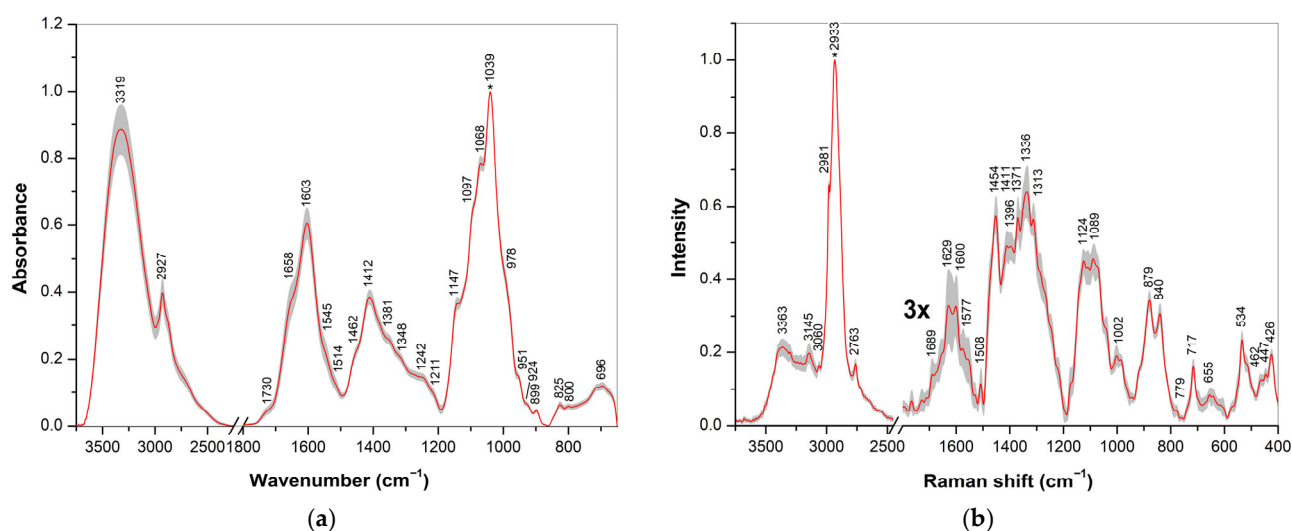


Figure 5. The average ATR-FT-MIR (a) and FT-Raman (b) spectra of flaxseed mucilage with the SD intervals (gray bars); asterisks show the normalized band of C–O/C–H stretching vibrations.

Table 5. IR and Raman band assignments for flaxseed mucilage [45,69,70,84,85,95–98].

Wavenumber (cm^{-1}) *		Assignment †	Origin ‡
IR	Raman		
3319br	3363br	$\nu(\text{OH})$, $\nu_{\text{as,s}}(\text{H}_2\text{O})$	R–OH, water
	3060	$\nu(=\text{CH})$	phenolic acids
2979sh	2981	$\nu_{\text{as}}(\text{CH}_2)$	methyl esters, Rha
2927	2933	$\nu_{\text{as}}(\text{CH}_2)$	CH_2OH , Xyl
2877sh	2886sh	$\nu_{\text{s}}(\text{CH}_3)$	methyl esters, Rha
2852sh	2855sh	$\nu_{\text{s}}(\text{CH}_2)$	CH_2OH , Xyl
1730sh		$\nu(\text{C}=\text{O})$	COOH , methyl esters
	1689	amide I	proteins
1658sh	1656sh	amide I	proteins
	1629	$\nu(\text{C}_\alpha=\text{C}_\beta)$	phenolic acids
1603		$\nu_{\text{as}}(\text{COO}^-)$	HG, RG-I
	1600	$\nu(\text{C}=\text{C})$ ring	phenolic acids
1545sh		amide II	proteins
1514sh	1508	$\nu(\text{C}=\text{C})$	phenolic acids
	1481sh	$\delta(\text{CH}_2)$	CH_2OH , Xyl
1462sh	1454	$\delta_{\text{as}}(\text{CH}_3)$	methyl esters, Rha

Table 5. Cont.

Wavenumber (cm ⁻¹) *		Assignment [†]	Origin [‡]
IR	Raman		
1412	1411	$\nu_s(\text{COO}^-)$	HG, RG-I
	1396	$\nu_s(\text{COO}^-), \delta_s(\text{CH}_3)$	proteins
1381sh		$\delta_s(\text{CH}_3)$	methyl esters, Rha
	1371	$\delta(\text{CH}), \delta(\text{C3OH})$	AX
1348sh	1336	$\omega(\text{CH}_2)$	CH ₂ OH, Xyl
1313sh	1313	$\delta(\text{C-H}), \delta(\text{C3-O-H})$	AX
1242sh	1243sh	amide III	proteins
1211sh	1214sh	$\nu(\text{C-O-C})$	esters
	1195sh	$\nu(\text{C-O-C})$	glycosidic bonds
1147	1143sh	$\nu(\text{C-O-C})$	glycosidic bonds
1122sh	1124	$\nu(\text{C-O-C})$	glycosidic bonds (AX)
	1108	$\nu(\text{C-O-C})$	glycosidic bonds
1097sh	1089	$\nu(\text{C-O})(\text{C-C})$	AX
1068	1068sh	$\nu(\text{C-O})(\text{C-C})$	HG, RG-I
1039	1037sh	$\nu(\text{C-O})(\text{C-C}), \text{Phe ring}$	AX, proteins
	1002	Phe ring	proteins
978sh	987	$\nu(\text{C3-O})$	AX
951sh	944–962sh	$\delta(\text{COO}^-)$	HG, RG-I
924sh	921sh	$\rho(\text{CH}_3)$	methyl esters, Rha
899	879	$\delta(\text{C1}\beta\text{-H})$	galactan
825	840	$\gamma(\text{C-O-H})$	HG, RG-I
779sh	779		
717sh	717	$\gamma(\text{C-O-H})$	HG, RG-I
658	655		
	642		
	617		
	574sh	$\gamma(\text{C-C-O})$	AX
	534	$\gamma(\text{C-C-O})$	AX
	514sh	$\gamma(\text{C-C-O})$	AX
	447	$\tau(\text{C-O-C})$	pectin (HG)
	426	$\tau(\text{C-O-C})$	

* br, broad; sh, shoulder. [†] Phe, phenylalanine; ν , stretching; δ , bending; ρ , rocking; τ , twisting; ω , wagging; γ , out-of-plane deformation; *as*, antisymmetric; *s*, symmetric. [‡] AX, arabinoxylan; HG, homogalacturonan; RG-I, rhamnogalacturonan type I; Rha, rhamnose; Xyl, xylose.

The most intense Raman bands at 2933 cm⁻¹ corresponded to C–H stretching vibrations in aliphatic groups. The bands at approximately 1630 and 1600 cm⁻¹ (C=C stretching) originated from phenolic acids [70,77]. Two Raman bands at ~1480 cm⁻¹ (notably for Libra bio and all Amon cultivars) and 1454 cm⁻¹ were attributed to CH₃ bending and CH₂ scissoring vibrations, respectively [71]. The bands at 1200–940 cm⁻¹ (C–C, C–O, and C–N stretching) mainly arose from polysaccharides and, to a lesser extent, proteins. Weak bands near 980 and 534 cm⁻¹ were assigned to skeletal vibrations of AX. The notable differences observed in these bands among the cultivars highlight a source-dependent variation in the structure and composition of mucilage [59]. The weak-to-medium bands at 1660 cm⁻¹ (amide I) and 1630–1600 cm⁻¹ (C=C stretching) originated from proteins and phenolic acids, respectively. Two Raman bands at 1480 cm⁻¹ (pronounced for Libra bio and all Amon cultivars) and 1450 cm⁻¹ were assigned to CH₃ bending and CH₂ scissoring vibrations, respectively. A similar band at 1455 cm⁻¹ was observed for tomato fruit RG-I fraction [95]. The bands at 1200–940 cm⁻¹ (C–C, C–O, and C–N stretching) originated mainly from polysaccharides and proteins. Weak bands near 980 and 534 cm⁻¹ were assigned to skeletal vibrations in AX. There are some similarities with the Raman spectrum of wheat AX, particularly in the region of 1000–1200 cm⁻¹ assigned to the C–O, C–O–C, and C–C stretching vibrations in carbohydrates, and two C=O/C=C stretching bands of ferulic acid observed at 1628 and 1598 cm⁻¹ [96,97].

3.5. Evaluation of Seed Integrity

The ATR FT-MIR spectra were also measured by directly squeezing the flaxseed onto the crystal surface, then removing seed residues and applying a grease stain. This simple method enables the extraction and measurement of oil from a single seed, facilitating the assessment of triacylglycerols (TAG).

Improper storage can trigger the germination process of oilseeds, leading to the enzymatic hydrolysis of lipids. In this process, seed lipase (EC 3.1.1.3) successively breaks down TGA into glycerol and FA. Consequently, the oil quality deteriorates, leading to oxidative damage and a shorter shelf life for the finished product [99]. The acid value, based on the amount of free fatty acids (FFA), characterizes the extent of oil deterioration.

Comparison of the ATR-FT-MIR spectra of oil stains expelled from individual seeds revealed the presence of FFA in some seeds of cultivar **Ib17** (Figure 6). We did not detect any mechanical damage or mold in these seeds, so this is most likely the result of spoilage during storage. Warm and humid conditions promote seed germination and activate seed lipase, which hydrolyzes TGA, releasing FFA. The bands of carboxylic vibrations are located at 2684 cm^{-1} (O–H stretching), 1711 cm^{-1} (C=O stretch), 1416 cm^{-1} (C–O–H in-plane bend), $1280, 1243\text{ cm}^{-1}$ (C–O stretch), and 938 cm^{-1} (C–O–H out-of-plane bend) [41,100]. The broad band centered near 3290 cm^{-1} results from O–H stretching vibrations in di- and monoacylglycerols (DAG, MAG) [101].

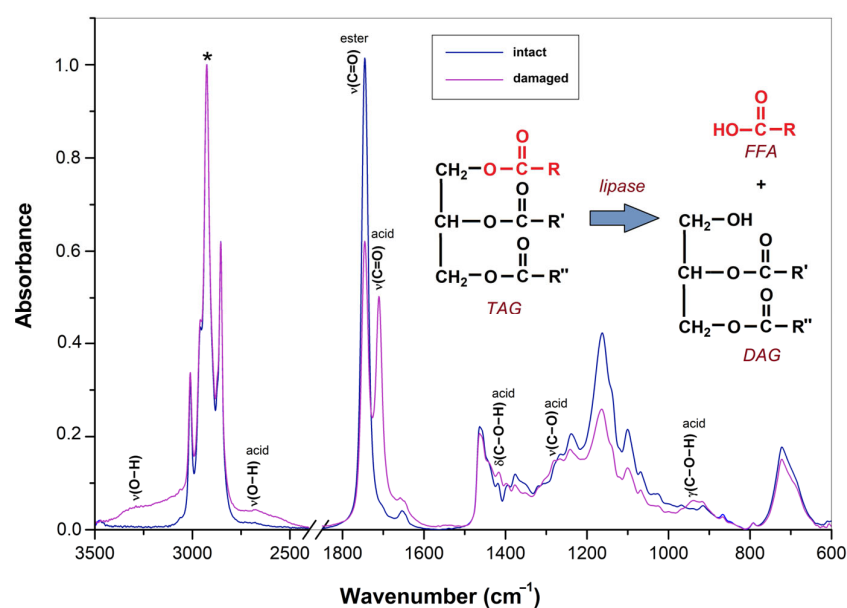


Figure 6. ATR-FT-MIR spectra of flaxseed oil obtained from intact and damaged seeds; the asterisk * shows the normalized band of CH_2 antisymmetric stretching vibration. In the damaged seed, partial fat hydrolysis, catalyzed by seed or mold lipase (EC 3.1.1.3), occurs, converting triacylglycerols (TAG) to diacylglycerols (DAG) and releasing free fatty acids (FFA).

As seen in the C=O/C=C stretching region ($1840\text{--}1600\text{ cm}^{-1}$) of ATR-FT-MIR spectra (Figure S6), among the 10 randomly selected seeds of cultivar **Ib17**, up to 3 showed the intense FFA marker band at $\sim 1710\text{ cm}^{-1}$. The appearance of this band is followed by a decrease in the ester band at 1745 cm^{-1} , which is characteristic of fat molecules, including TAG, DAG, and MAG, and thus was used as the fat reference band. The peak decomposition at $1840\text{--}1600\text{ cm}^{-1}$ allowed us to determine the areas of these peaks, assigned as A_{1710} and A_{1745} (Figure S7). We used the peak area ratio $A_{1710}/(A_{1710} + A_{1745})$ to estimate the FFA release in damaged seeds; the obtained values are summarized in Table S1. The FFA release ranged from 30% to 50%, indicating a significant breakdown of fats in these seeds. Among

the 10 chosen seeds, 3 showed abnormalities. However, since it is a small selection and the oil extracted from **lb17** seeds did not display the FFA marker peak at 1710 cm^{-1} , we can conclude that the presence of damaged seeds in the raw material is not significant.

3.6. The Degree of Flax Oil Unsaturation Depended on the Cultivar

The flax varieties used in this work differ primarily in the composition of the oil, expressed as the degree of unsaturation (DU), which is the proportion of $C=C$ double bonds in fatty acid residues. Using vibrational spectra, this degree can be calculated, for example, as the ratio of the heights or areas of the stretching vibration bands of $C=C$ bonds and ester $C=O$ bonds at 1654 and 1740 cm^{-1} , respectively. However, in the ATR-FT-MIR spectra of flaxseed oils, the first band is weak, while in the corresponding FT-Raman spectra, the latter peak is weak instead. Therefore, in the Raman spectra, the CH_2 scissor vibration band was used instead of the $C=O$ stretching band [102]. As an alternative, to evaluate DU by both methods, we choose two $C-H$ stretching vibration bands at $3010\text{--}3014\text{ cm}^{-1}$ (analytical band) and 2854 cm^{-1} (reference band), indicating $=CH-$ and CH_2 groups, respectively [103]. The heights of these peaks, designated as H_1 and H_0 , were determined as shown in Figure 7a; we used ATR-FT-MIR spectra obtained for the randomly chosen seeds. Obtained values of the H_1/H_0 ratios are summarized in Table S2. The unsaturation degree increased in the order Amon—Raciol/Raciol bio—Libra bio/Recital; the corresponding mean $\pm$ SD values for these cultivars were 0.295 ± 0.002 , 0.362 ± 0.017 , and 0.445 ± 0.014 . The H_1/H_0 values from ATR-FT-MIR and FT-Raman spectra of the oils showed a good correlation, separating the clusters by grade (Figure 7b). These height ratios were used to calculate DU via linear regression of the reference values (Figure S8, Table S3); the obtained and reference values are also presented in Table S2. The DU values of flaxseed oils obtained by the ATR-FT-MIR and FT-Raman methods were very close to the values obtained from contents of five predominant FA: ~ 1.68 for Amon, 1.94 for Raciol and Raciol bio, and 2.25 for Recital and Libra bio, with Recital showing a slightly lower value (2.17) than Libra bio (~ 2.30).

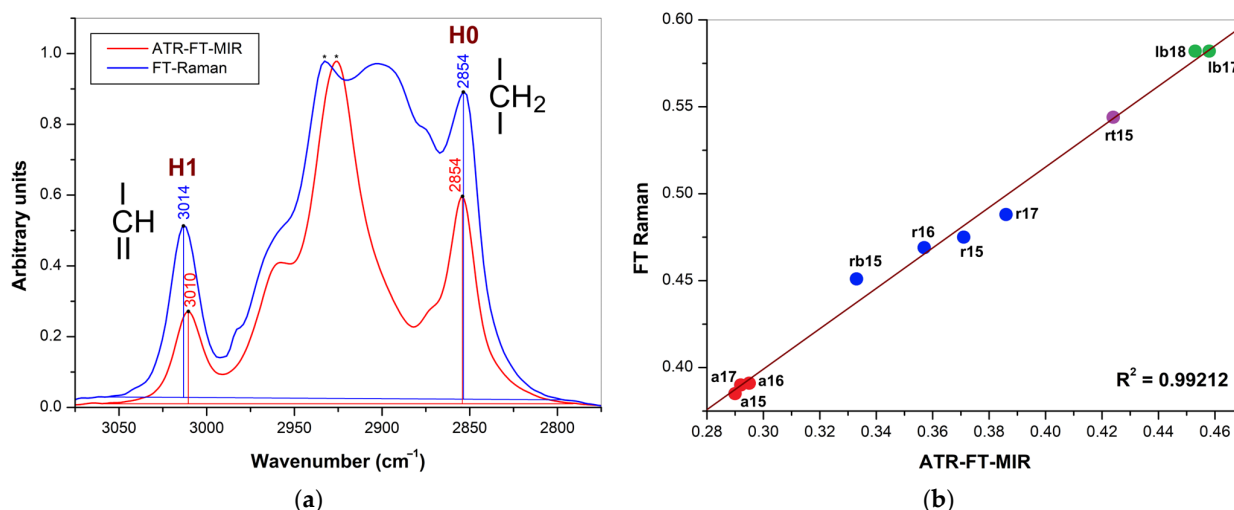


Figure 7. (a) ATR-FT-MIR and FT-Raman spectra ($2775\text{--}3075\text{ cm}^{-1}$) of flaxseed oils: determination of H_1 and H_0 ; asterisks * show the normalized band of $C-H$ stretching vibrations; (b) correlation between the H_1/H_0 values obtained from ATR-FT-MIR and FT-Raman spectra.

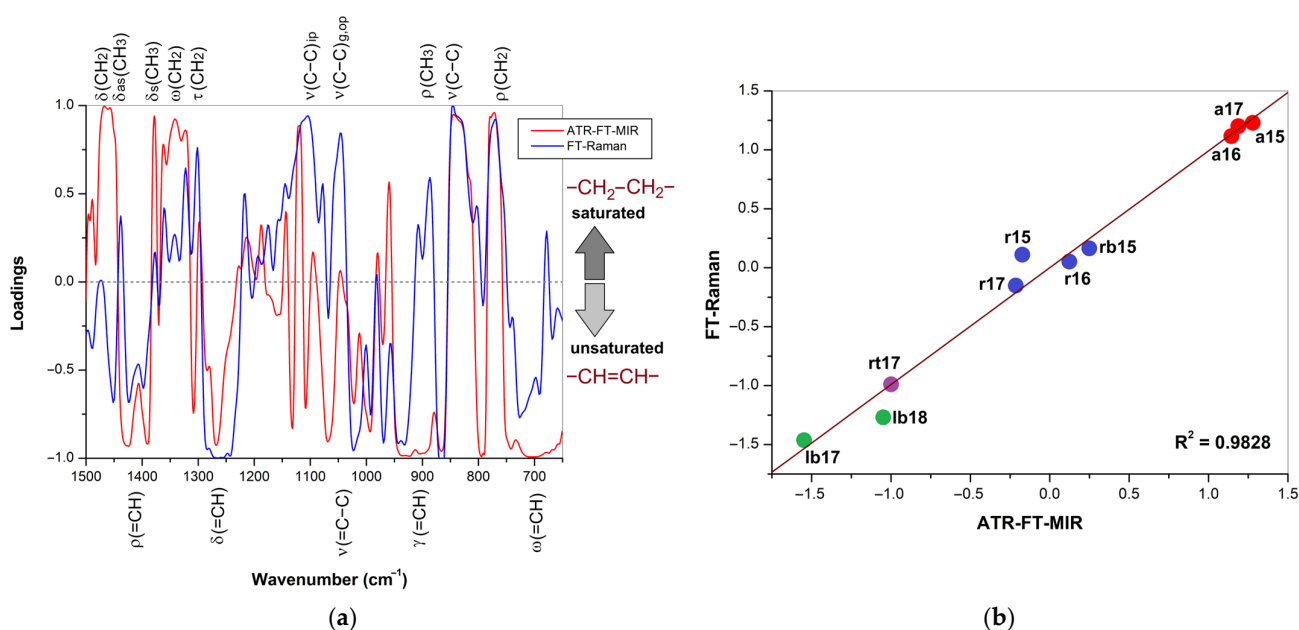
3.7. Discrimination of Flax Cultivars Based on Vibration Spectroscopic Data

Table 6 summarizes the discrimination of flax cultivars using PCA of the vibrational spectra obtained from flaxseed fractions (oil, hull, kernel, and mucilage).

Table 6. Discrimination of flax cultivars using PCA of the vibrational spectra obtained from the flaxseed fractions.

Method	Fraction	Region (cm ⁻¹)	PCs (Variance)	Cumulative Variance
ATR-FT-MIR	oil	650–1500	PC1 (77.05%)	77.05%
	kernel	1200–1500	PC4 (2.71%) + PC5 (0.38%)	3.09%
	hull	930–1500	PC1 (79.90%) + PC2 (11.46%) + PC3 (4.81%)	94.17%
	mucilage	1050–1165	PC1 (64.76%) + PC3 (7.47%)	72.23%
FT-Raman	oil	650–1500	PC1 (83.57%)	83.57%
	kernel	800–1190	PC1 (53.31%) + PC2 (17.53%) + PC5 (4.56%)	75.40%
	hull	1165–1500	PC3 (6.06%) + PC4 (2.59%)	8.65%
	mucilage	950–1500	PC1 (52.81%) + PC2 (19.86%)	72.67%

For the flaxseed oils, PC1 calculated for both methods distinguished the cultivars based on the PUFA composition, forming clusters of Amon (positive PC1), Raciol/Raciol bio (PC1 near zero), and Libra bio/Recital (negative PC1). As evident from the loading graph, the maxima and minima of both curves indicate vibrations of saturated and unsaturated residues, respectively (Figure 8a). The PC1 scores obtained from the analysis of ATR-FT-MIR and FT-Raman spectra of the oils correlated well (Figure 8b).

**Figure 8.** PCA of ATR-FT-MIR and FT-Raman spectra (650–1500 cm⁻¹) of flaxseed oils: (a) PC1 loading graph; (b) correlation of the PC1 score values.

For the flaxseed mucilage, a combination of two PCs, namely PC1 with PC3 (ATR-FT-MIR) (Figure 9a,b) or PC1 with PC2 (FT-Raman) (Figure 9c,d), was enough to differentiate the cultivars based on the contribution of major polysaccharides, specifically AX and RG-I, as well as proteins, as previously described [59]. The effect of HG on this separation was probably minor because of the uncertainty in its content, which was clearly much less pronounced than in the hulls. The distribution of individual cultivars across quadrants (Figure 9a) was influenced by the relationships among these compounds, as shown in the loading graphs (Figure 9b).

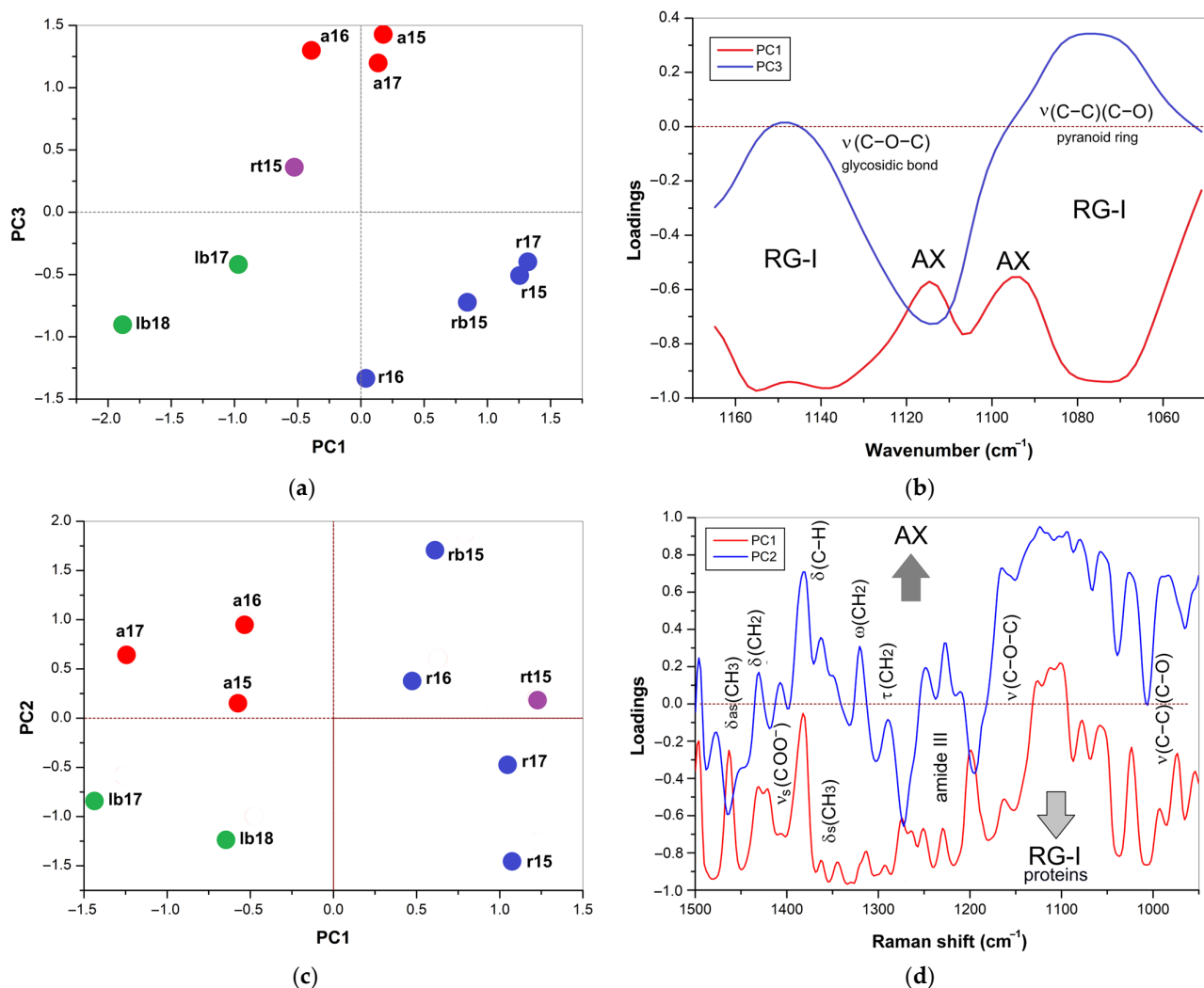


Figure 9. PCA of the vibrational spectra obtained for flaxseed mucilage: component score (a) and loading (b) graphs for PC1 and PC3 obtained for ATR-FT-MIR spectra (1050–1165 cm^{-1}); component score (c) and loading (d) graphs for PC1 and PC2 obtained for FT-Raman spectra (950–1500 cm^{-1}).

For defatted kernels and hulls, a combination of 2 or 3 PCs was used to distinguish all 4 cultivars. For the ATR-FT-MIR spectra of hulls (1160–1500 cm^{-1}), did a 3D score plot of the first three PCs reach the effective separation of the cultivars (Figure 10a), where PC1 separated the brown-seeded cultivars (positive PC1) from the majority of yellow-seeded samples, PC2 separated Recital (negative PC2) from Libra bio (positive PC2), and, finally, a combination of PC1 and PC3 placed the Amon samples in quadrant III (negative values for both PCs). The loading curves of these PCs (Figure 10b) have positive and negative peaks, indicating specificity in contributions of polysaccharides (pectins, cellulose, xylan), polyphenols (tannins, phenolic acids), and proteins. Thus, the main maxima of PC1 at 1482, 1408, 1336, 1222, 1135, and 1024 cm^{-1} indicate the vibrations of pectins [84] and, to a lesser extent, tannins [104], while the minima at 1441, 1388, 1262, 1189, and 1113 cm^{-1} correspond to the vibrations of proteins [79,80], cellulose, and xylan [70,90,91]. By contrast, positive peaks of PC2 at 1458, 1384, 1377, 1269, 1190, and 1114 cm^{-1} indicate protein vibrations, while its negative peaks at 1415, 1330, 1232, 1143, and 1005 cm^{-1} were attributed to pectins and other polysaccharides. For the PC3 curve, the perturbations around 1426–1386, 1240–1158, 1123–1102, and 1038–960 cm^{-1} indicate differences between the COO^- -stretching/ CH_3 -bending, the amide III/ C-O-C stretching, and the C-O , C-C , and C-N stretching vibrations in proteins and polysaccharides, respectively.

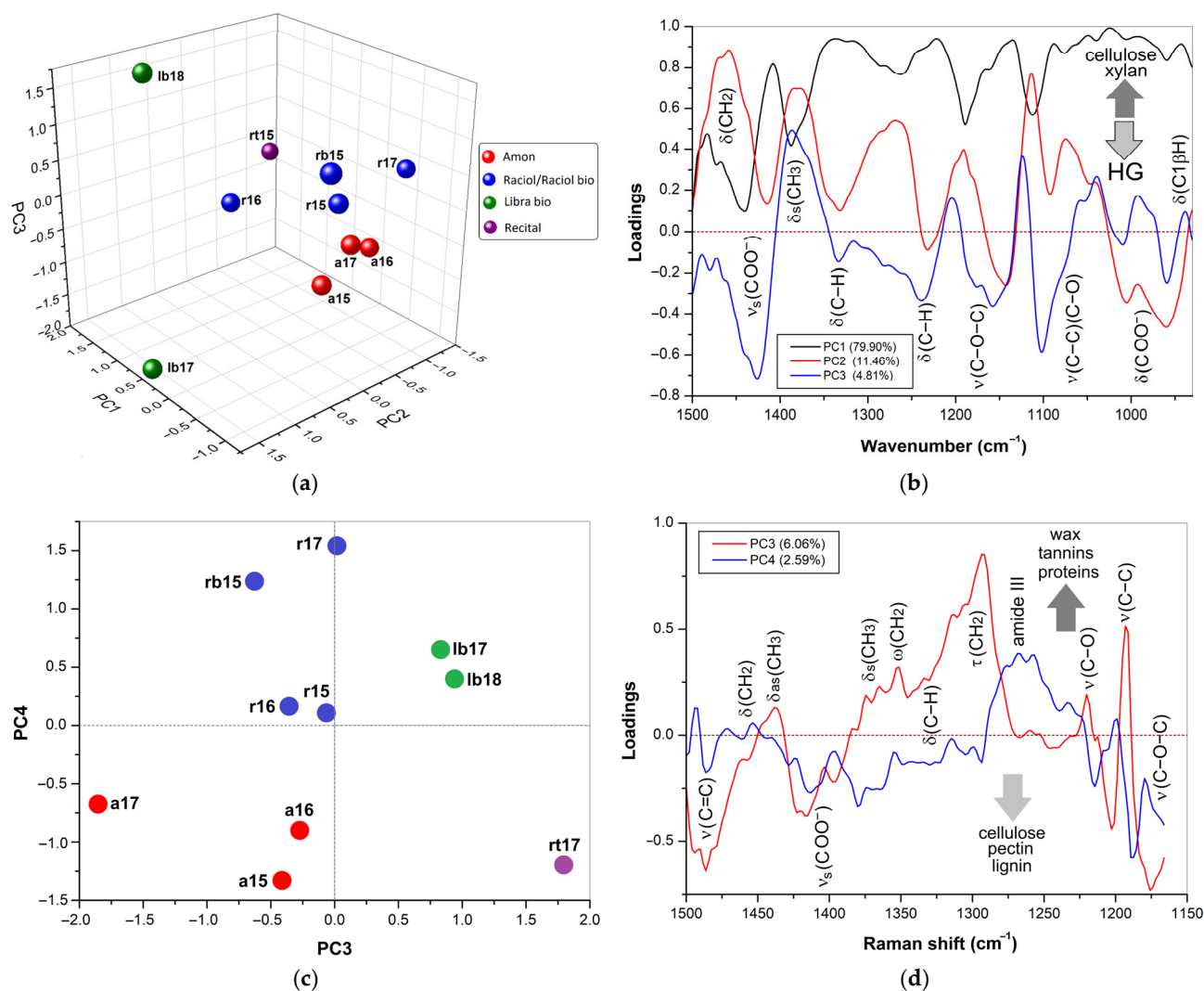


Figure 10. PCA of the vibrational spectra obtained for flaxseed hulls: component score (a) and loading (b) graphs for PC1, PC2, and PC3 obtained for ATR-FT-MIR spectra ($930\text{--}1500\text{ cm}^{-1}$); component score (c) and loading (d) graphs for PC3 and PC4 for FT-Raman spectra ($1165\text{--}1500\text{ cm}^{-1}$).

For the FT-Raman spectra of hulls ($1160\text{--}1500\text{ cm}^{-1}$), did a 2D score plot of PC4 versus PC3 achieve complete discrimination (Figure 10c), where PC3 differentiated the yellow-seeded and brown-seeded cultivars, and PC4 further separated these into individual cultivars, each occupying a distinct quadrant. For the loading curves of both PCs (Figure 10d), the perturbations at the edges of the spectrum region used indicate differences between the stretching vibrations of C=C bonds and deformation vibrations of CH_2 and CH_3 groups ($\sim 1430\text{--}1500\text{ cm}^{-1}$) and the stretching vibrations of C-C and C-O bonds ($\sim 1165\text{--}1230\text{ cm}^{-1}$) for different structural components, primarily polysaccharides, but also proteins and polyphenols. The brown pigmentation of flaxseed husks is due to tannins produced by pigment cells located in the inner layer of the flaxseed hull [105]. In contrast, yellow flaxseeds lack tannins and instead contain yellow carotenoids in the cotyledons, which are visible through the transparent hull [106]. The maxima of the PC3 loading curve at 1193, 1220, and $1351\text{--}1374\text{ cm}^{-1}$ indicated the presence of tannins [107]. Additionally, the positive PC3 peaks at 1293 and 1438 cm^{-1} are likely due to CH_2 twisting and CH_2 antisymmetric bending in waxes, respectively [71,108]. The minima of the PC3 and PC4 loading curves at 1413–1417 and 1398 cm^{-1} corresponded to the symmetric stretching band of COO^- in pectin and, to a lesser extent, proteins [74,84]. Cellulose vibrations contributed to the negative PC4 peaks at 1295 and 1380 cm^{-1} and to the positive PC3/negative PC4

peak at 1486 cm^{-1} [78,109], while lignin vibrations were involved in the PC4 minima at 1187 , 1214 , and 1428 cm^{-1} [78]. Finally, positive PC4 peaks at 1234 , 1259 , 1268 , and 1454 cm^{-1} may indicate the amide III and C–H deformation vibrations in proteins [75].

As mentioned above, for ATR-FT-MIR spectra of kernels ($1200\text{--}1500\text{ cm}^{-1}$), a component score plot of PC5 versus PC4 shows how the cultivars are distinguished (Figure 11a). The latter component separates Amon (positive PC4) from most other cultivars, except sample r18 (negative PC4), while the former component distinguishes Raciol/Raciol bio (positive PC5) from the others (negative PC5). In the spectral region, the loading curves of PC4 and PC5 exhibit several perturbations related to C–H, CH_2 , and CH_3 deformations, COO^- symmetric stretching, and amide III vibrations in proteins, which are the main components of the defatted kernel (Figure 11b). In the amide III region, PC4 showed two positive peaks around 1239 and 1268 cm^{-1} , assigned to the α -helix and β -sheet/random structures of protein, respectively, and PC5 showed one positive peak at 1230 cm^{-1} . Therefore, discrimination between cultivars is based on differences in protein structure and amino acid composition of the kernel.

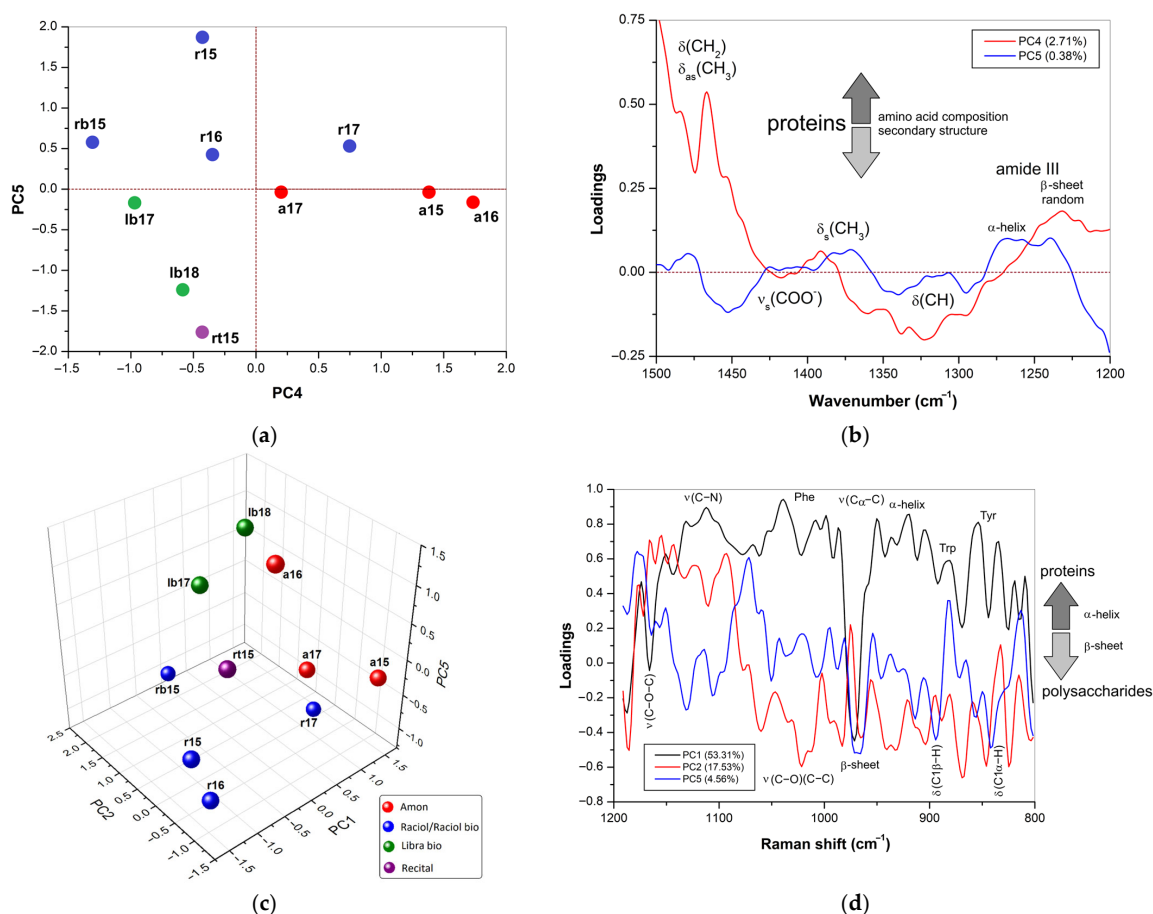


Figure 11. PCA of the vibrational spectra obtained for defatted kernels: component score (a) and loading (b) graphs for PC4 and PC5 obtained for ATR-FT-MIR spectra ($1050\text{--}1165\text{ cm}^{-1}$); component score (c) and loading (d) graphs for PC1, PC2, and PC5 obtained for FT-Raman spectra ($800\text{--}1190\text{ cm}^{-1}$).

For the FT-Raman spectra of kernels ($800\text{--}1190\text{ cm}^{-1}$), three PCs, namely PC1, PC2, and PC5, were used to discriminate the cultivars (Figure 11c). The combination of the first two PCs placed Amon samples in quadrant II (positive PC1, negative PC2), and PC5 separated Libra bio and Recital (positive PC5) from Raciol/Raciol bio (negative PC5). In the loading graphs of these PCs (Figure 11d), the main PC1 maxima at 1112 , 1039 , 919 , 853 , and 834 cm^{-1} indicate C–N stretching, Phe ring, $\text{C}\alpha\text{--C}$ stretching (α -helix), and Tyr ring

vibrations [57,110], while the minima have a contribution of polysaccharide vibrations, i.e., C–O–C, C–O, and C–C stretching ($1187\text{--}1143\text{ cm}^{-1}$) and C1–H bending ($892\text{--}811\text{ cm}^{-1}$). The most intense negative peak at 971 cm^{-1} , observed for all these PCs, was assigned to C α –C stretching vibrations (β -sheet) [110]. The PC2 curve contains a complex combination of positive and negative peaks, corresponding mainly to the stretching of C–C, C–N, and C–O bonds, the bending of C1–H bonds, and the vibrations of the Tyr and Trp rings, indicating differences in the composition of proteins and polysaccharides. Unlike the two previous ones, the PC3 curve shows the features of the polysaccharide composition, since the main maxima at 1178, 1072, 883, and 813 cm^{-1} and minima at 1131, 1106, 966, 914, 894, and 842 cm^{-1} indicate different vibrations of cellulose, xylan, and pectin, and, to a lesser extent, proteins. Thus, in this case, PCA-assisted differentiation of the cultivars is based on differences in structure and amino acid composition of proteins, as well as on the contribution of cell wall polysaccharides.

The ATR-FT-MIR/FT-Raman dataset includes 23 normalized variables related to selected wavenumbers (Table 7). Discrimination among the chosen flax cultivars was based on this dataset, using multivariate statistical methods (HCA and PCA). Figure 12 demonstrates the graphical outputs from HCA and PCA of the dataset. The dendrogram of similarity clearly displayed the clustering of flax cultivars based on the FA composition (Figure 11a). Three main clusters corresponded to cultivars Amon, Raciol, and Libra/Recital. The PCA method effectively distinguished the Amon, Raciol, and Libra/Recital clusters along PC1, while PC2 further separated Recital from Libra (Figure 11b). Additionally, PC2 indicated differences within the cultivar, which were related to the harvest year or other environmental factors.

Table 7. Variables (cm^{-1}) of the ATR-FT-MIR and FT-Raman spectra of flaxseed oils used for the discrimination of flax cultivars.

Method	Variables: Wavenumber (cm^{-1})
ATR-FT-MIR	700, 771, 795, 845, 866, 923, 993, 1068, 1119, 1323, 1341, 1379, 1390, 1425, 1468
FT-Raman	726, 771, 846, 863, 933, 1022, 1104, 1266

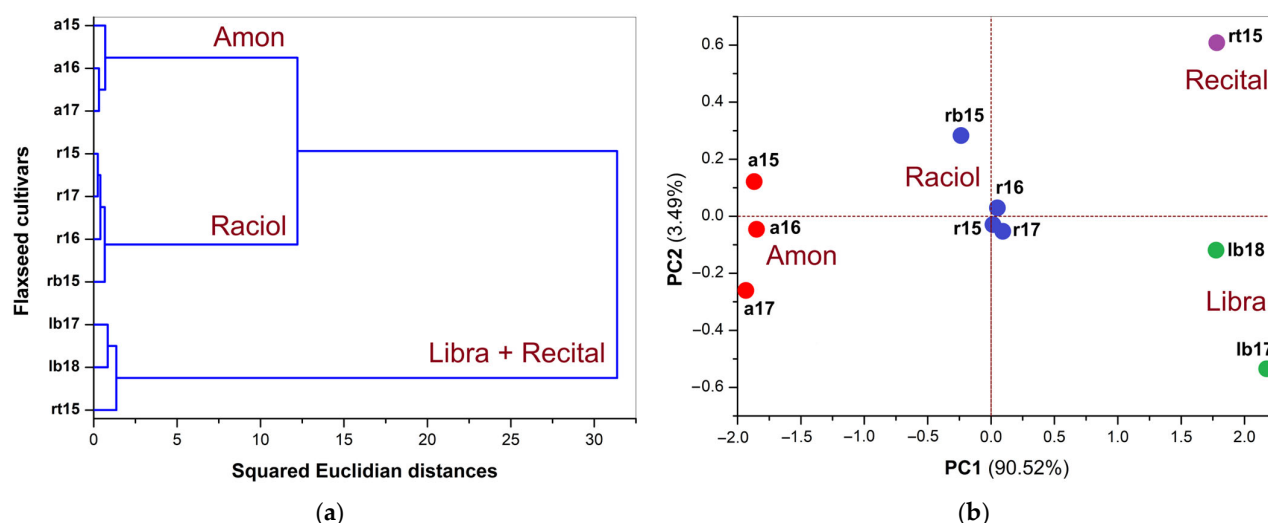


Figure 12. Graphical outputs from HCA and PCA of the ATR-FT-MIR/FT-Raman dataset obtained for flaxseed oils: (a) dendrogram of similarity; (b) component score graph PC2 versus PC1.

4. Discussion

The current study comprises two parts: preparative and analytical, focused on obtaining the individual seed fractions from selected flax cultivars and conducting a comparative analysis of these fractions using vibrational spectroscopic methods and multivariate statistics.

4.1. Fractionation of Flaxseeds

In the current study, fractionation of flaxseeds was applied for analytical purposes as a method of sample preparation. To carry out spectroscopic measurements, we need to obtain samples of individual fractions weighing from tens to hundreds of milligrams. The desired fractionation approach should be fast, simple, and efficient, with minimal use of expensive laboratory devices.

We abandoned the dehulling step [20,24] due to the lack of necessary equipment. Instead, the seeds were steamed in the bamboo steamer, crushed in a mortar, and then fractionated. Steaming softens the seeds, making subsequent grinding and oil extraction easier. We used *n*-hexane for the extraction of flaxseed oil from crushed seeds because it is a simple method that does not require specialized equipment. This non-polar solvent is more effective at extracting fats than ethanol, which is also used for this purpose [29], and it evaporates quickly from the extract. Then, we dried the defatted meal in the air and further crushed it to break up the pieces of kernel attached to the hull.

In the previous work [58], hulls were manually separated from kernels using tweezers, a scalpel, and a magnifying glass. The pieces of kernel or hull obtained in this way can be measured easily as independent samples. However, this approach is not optimal for preparing multiple samples, which are non-uniform. Wet separation of the kernel and hull has also been proposed [29,33], which, although effective, is a labor-intensive process more suitable for preparative purposes. Separating the hull from the kernel of dry defatted flaxseed meal is quicker and easier. The kernel particles differ from the hull fragments in their density, so the lighter hulls were easily removed from the dense crushed kernels by sifting them using a vibrating sheet of paper. A weak stream of air, such as from a hair dryer, could be used to separate the shell particles from the kernel. Unfortunately, the common hair dryer was unsuitable for small quantities of meal (grams or tens of grams) v used in the current work, as its air pressure was too strong, blowing away all the material. It might have been suitable for much larger meal amounts. Like our laboratory method, vibrating sieves or cyclones can be used in industrial production to fractionate defatted flaxseed meal as an alternative to the dehulling of whole seeds [24]. As seen in Figure S1, even in yellow seeds, the kernel particles are significantly lighter than the hull fragments, which were clearly visible during the cleaning process. The resulting purified kernel fraction appears to be quite uniform.

In the current protocol, mucilage was extracted from whole seeds using hot water and lyophilized. Kaushik et al. [111] reported that extraction at higher temperatures resulted in mucilage with higher protein and uronic acid contents, but lower amounts of neutral monosaccharides. Despite heating leading to additional extraction of pectin and proteins from hulls, this method of extraction significantly reduces the time and increases the yield of the product [60,61]. Also, compared to oven drying, evaporation, or ethanol precipitation, lyophilization is a gentler and more optimal method for drying aqueous extracts of flaxseed mucilage, preserving its composition, rheological, and functional properties [112,113].

ATR-FT-MIR and FT-Raman spectra of the flaxseed fractions (Figures S2–S5) clearly detect the main biochemical components, namely, fats in the oil, proteins in the kernel, polysaccharides, and, to a lesser extent, proteins and polyphenols in the hull and mucilage, based on the presence of vibrational bands characteristic of these compounds, which is in agreement with previous reports [29,59,60,114]. Table 8 summarizes the prominent

IR/Raman marker bands of the main biochemical constituents in the flaxseed fractions. According to this table, the ATR-FT-MIR method is more suitable for assessing polysaccharides, whereas the FT-Raman method is more effective at detecting polyphenols. Both methods, however, are equally efficient for evaluating fats and proteins.

Table 8. The prominent IR/Raman marker bands of the main biochemical constituents in the flaxseed fractions.

Compounds *		Wavenumber (cm ^{−1})		Fraction	References
		ATR-FT-MIR	FT-Raman		
Fats	Esters	1745	1747	Oil	[35,50,55,63–68]
	SFA	2925, 2854,1465, 723	2933, 2902, 2854, 1440, 1303		
	UFA	3009, 1654	3014, 1658, 1266		
Proteins		3284, 1647, 1539	1668, 1265, 1004	Kernel	[75,76,79–83]
Polysaccharides	HG	1596, 1416, 1144, 1098, 1017	1035	Hull	[69–74,84,90–93,95,96,98]
	RG-I	1603, 1412	840	Mucilage	
	AX	1147, 1039, 899	1089, 534		
Polyphenols			1633, 1604	Hull	[77,97]
			1629, 1600	Mucilage	

* SFA, saturated fatty acids; UFA, unsaturated fatty acids; AX, arabinoxylan; HG, homogalacturonan; RG-I, rhamnogalacturonan type I.

4.2. Spectroscopic Evaluation of Flaxseed Cultivars

Although flax breeding is primarily aimed at changing the oil composition, other parts of the seed are also subject to change to varying degrees. According to Abtahi and Mirlohi [115], the nutritional properties of flax expressed in the targeted metabolite contents depended primarily on hereditary predisposition, and then on environmental conditions, like temperature, humidity, and sunshine time. It has been demonstrated that the cultivar influences not only the composition of flaxseed oil [12,114,116] but also that of its proteins [117,118], polysaccharides [118,119], and polyphenols [120,121]. Moreover, the composition and rheological properties of flaxseed mucilage varied for cultivars [59,122,123]. Minevich et al. [45] observed that, for the aqueous flaxseed extracts (mucilage) obtained at different extraction times, the band intensity at 1540–1680 cm⁻¹ correlated with the total protein content. Moreover, the secondary extraction gave a product characterized by more intense protein bands than those of the primary extraction. The amide bands of proteins observed for the extracts were also sensitive to the pH of the medium, reaction temperature, and cultivar.

In this study, we proposed a sample preparation method for measuring the ATR-FT-MIR spectra of oil from individual seeds. The single-seed or single-kernel non-destructive measurement method is suitable for NIR equipment, which does not require complex sample preparation [124]. For ATR-FT-MIR, establishing close contact between the sample and the ATP crystal is necessary. Therefore, measuring the entire seed is impossible, but the oil squeezed from crushed seed can be analyzed. This method allows the analysis of a set of randomly selected seeds, thereby characterizing the heterogeneity within the sample. The results of the single-seed method were compared with those obtained for the flaxseed oil extracted with *n*-hexane when determining the value of H_1/H_0 (see Table S2). Despite some differences among individual samples, the average values for the groups with approximately the same FA composition (Amon, Raciol/Raciol bio, and Libra bio/Recital) were quite close. Furthermore, the calculated DU values for the single-seed ATR-FT-MIR method correlated well with the results of FT-Raman. It is interesting to note that, for cultivar **lb17**, the presence of FFA in some seeds ($N = 15$) did not influence the H_1/H_0 value obtained. Comparing these two sample preparation methods for ATR-FT-MIR reveals

limitations to their optimal use. If we want to characterize a desired product (oil) based on its unsaturation and fatty acid composition, the extract analysis is the best solution. However, when analyzing material heterogeneity, such as the presence of seeds from other cultivars, the statistical distribution of oil properties across individual seeds, and assessing potential seed spoilage, a single-seed measurement is the optimal approach. The more seeds analyzed, the more comprehensive the characterization of the entire material's potential as a source for oil extraction will be. Seed material collected from fields often contains admixtures of other cultivars or hybrids with seeds of different colors, which is not obligatory. Such admixtures may be the same color but differ in FA composition, and a single-seed measurement of the oil can help identify admixtures and assess their impact on the overall yield. Similarly, this approach can be used to measure both shell and kernel if they can be obtained from the same seed and successfully defatted.

The spectral differences among flax cultivars were most notable in oils, as PUFA composition was the key factor in flax breeding. For example, Walkowiak et al. [125] identified several promising F1 hybrid crosses for further breeding aimed at modifying the FA composition of flaxseed oil, selecting varieties with ω -6: ω -3 PUFA ratios of 1:1 and 2:1, which are beneficial for human health and help extend the oil's shelf life. This allowed us to use specific vibration bands to assess DU, an effective cultivar marker, and to detect FFA with ATR-FT-MIR as indicators of seed damage during harvesting or storage.

Vibrational spectroscopic evaluation of the cultivar-sensitive characteristics of flaxseed hulls and kernels is of primary importance for their use as sources of food proteins [14,126] and polysaccharides [110,127], respectively. In addition to proteins, flaxseed kernels also contain dietary fibers, including soluble polysaccharides [128]. Flaxseed mucilage, a natural mixture of polysaccharides with some proteins and polyphenols, exhibits compositional, physical, and rheological properties influenced by its cultivar [59,123] and reflected in the vibrational spectra, which are crucial for its applications in the food industry, medicine, and cosmetics [32,34]. However, for these seed fractions, the cultivar-dependent spectral differences were less obvious than for the oil, so we used PCA to extract more information from the spectra. As a result, the flax cultivars were distinguished using PCA of the vibrational spectra obtained for all flaxseed fractions (Table 6). To separate the oil samples by cultivar, only a single PC1 was needed. For both spectroscopic methods, PC1 explains up to 77% of the variance in oil composition, and they strongly correlate due to the complementarity of FTIR and Raman spectroscopy. Consequently, these components, as well as the spectra themselves, can be applied to quantify FA composition. For mucilage, two components, namely PC1 versus PC2 or PC3, were used. Additionally, for kernel and hull samples, two or three PCs, including PC4 and/or PC5 in some cases, were employed. For these fractions, genotypic differences may align with higher PCs, whereas the first three components obviously reflected phenotypic variability related to the harvest year or growing location.

In any case, a comparison of the vibrational spectra observed for seed fractions revealed that flax breeding impacts various biochemical and structural characteristics of the seed. The ATR-FT-MIR and FT-Raman outputs, such as H_1/H_0 or PC1, obtained for oils, showed significant correlations. These findings demonstrated that the two methods of vibrational spectroscopy are interchangeable for characterizing flaxseeds from various cultivars.

5. Conclusions

Fractionating flaxseeds into oil, mucilage, kernel, and hull produced samples better suited for vibrational spectroscopic analysis in the mid-infrared region than the whole seed. The fractionation method used in this work can be used to develop functional ingre-

dients from flaxseed, such as flaxseed protein concentrate/isolate or dietary fibers, and support the isolation of numerous biologically active substances contained in flaxseed. As previously studied, the flaxseed varieties used in this research varied significantly in the composition of unsaturated fatty acids in their oils and the biopolymeric composition of their mucilage. Vibrational spectroscopic analysis confirmed these differences in oil and mucilage originated from different cultivars, in agreement with earlier reports. Moreover, cultivar-sensitive features of the hull and kernel fractions were also observed using vibrational spectroscopy coupled with multivariate analysis. Interestingly, analyzing these fractions also allowed for differentiation of flax varieties based on the vibrational spectra, though not as effectively as with oil or mucilage.

Characteristic vibrational bands of biochemical components were identified in the spectra of the fractions. These features can be used to monitor flax breeding towards more promising forms for cultivation and assess seed quality for culinary and industrial applications. Combining vibrational spectroscopy with chemometrics distinguished flaxseed samples according to their biochemical composition, determined by genotype and variety. The most effective separation was achieved through differences in unsaturated fatty acids in the oil and, to a lesser extent, the polysaccharide and protein contents of the mucilage. These results can serve as an example for screening flax varieties, setting selection criteria, and guiding their use. Clearly, a low α -linolenic acid level in the oil is preferable for culinary purposes, and the composition of flaxseed mucilage is crucial for these hydrocolloids applied in food, medicine, or cosmetics. Additionally, a simple and effective method for analyzing the fat content of individual seeds using ATP-FT-MIR was proposed to assess damage related to free fatty acid release by lipases. Despite difficulties in interpreting spectra and loading curves of principal components, this study demonstrates that combining vibrational spectroscopy with multivariate statistical methods can be a powerful tool for analyzing fatty acid composition and the profiles of proteins, polysaccharides, and polyphenols in flaxseeds. The versatility of vibrational spectroscopy allows these techniques to examine various seed types based on their compositional traits. This method could be a viable alternative to traditional, expensive, and labor-intensive analytical approaches.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/app16031612/s1>, Figure S1: The flaxseed samples of cultivar Amon a16: (a) whole seeds, (b) oil, (c) kernel, (d) hull, (e) mucilage; Figure S2. (a) ATR-FT-MIR and (b) FT Raman spectra of flaxseed oils obtained from various cultivars; asterisks show the normalized bands of C=O/C-H stretching vibration, arrows show changes in the order Amon (red)—Raciol/Raciol bio (blue)—Libra bio/Recital (green) cultivars; Figure S3. (a) ATR-FT-MIR and (b) FT Raman spectra of flaxseed hulls obtained from various cultivars; asterisks show the normalized bands of COO[−]/C-H stretching vibrations; Figure S4. (a) ATR-FT-MIR and (b) FT Raman spectra of defatted flaxseed kernels obtained from various cultivars; asterisks show the normalized bands of amide I/C-H stretching vibrations; Figure S5. (a) ATR-FT-MIR and (b) FT Raman spectra of flaxseed mucilage obtained from various cultivars; asterisks show the normalized bands of amide I/C-H stretching vibrations; Figure S6: The C=O/C=C stretch region (1840–1600 cm^{−1}) of ATR-FT-MIR spectra of flaxseed oil obtained from 10 intact and damaged seeds of Ib17; Figure S7: Peak decomposition for the C=O/C=C stretch region (1840–1600 cm^{−1}) of ATR-FT-MIR spectra of flaxseed oil obtained from 10 intact and damaged seeds of Ib17; Table S1: FFA release (mol%) in damaged seeds of Ib17 calculated from the Voigt component areas; Table S2: ATR-FT-MIR and FT-Raman estimation of the DU values of flaxseed oils using the ratio H₁/H₀; Figure S8: Linear regression curves for the H₁/H₀ values obtained from the ATR-FT-MIR and FT-Raman spectra used for calculation of DU; Table S3: Linear regression parameters used for calculation of DU from the H₁/H₀ values.

Author Contributions: Conceptualization, A.S. (Andriy Synytsya) and Y.T.; methodology, Y.T., R.B., and A.S. (Andriy Synytsya); software, A.S. (Alla Synytsya); validation, M.S., R.B., and A.S. (Alla Synytsya); formal analysis, Y.T., R.B., and N.P.; investigation, A.S. (Andriy Synytsya); resources, J.Š.; data curation, R.B.; writing—original draft preparation, Y.T.; writing—review and editing, Y.T. and A.S. (Andriy Synytsya); supervision, J.Š., A.S. (Andriy Synytsya), R.B., and M.S.; project administration, J.Š.; funding acquisition, J.Š. All authors have read and agreed to the published version of the manuscript.

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Abbreviations

The following abbreviations are used in this manuscript:

as	antisymmetric
ATR	Attenuated Total Reflectance
ATR-FT-MIR	Attenuated Total Reflectance Fourier-transformed Mid-infrared
Asp	aspartic acid
AX	arabinoxylan
br	broad
DAG	diacylglycerol
DR	Diffuse reflectance
DU	Degree of unsaturation
FA	Fatty Acid
FFA	Free Fatty Acids
FT	Fourier-transformed
FT-MIR	Fourier-transformed Mid-infrared
FT-NIR	Fourier-transformed Near-infrared
FT-Raman	Fourier-transformed Raman
Glu	glutamic acid
HCA	Hierarchical Cluster Analysis
HG	homogalacturonan
IR	infrared
MAG	monoacylglycerol
MIR	Mid-infrared
NIR	Near-infrared
PC	Principal Component
PCA	Principal Component Analysis
PEF	Pulsed electric field
Phe	phenylalanine
PLS	Partial-Least-Square

PUFA	polyunsaturated fatty acid
RG-I	rhamnogalacturonan type I
SFA	saturated fatty acids
sh	shoulder
s	symmetric
TAG	triacylglycerol
Trp	tryptophan
Tyr	tyrosine
UFA	unsaturated fatty acid
Vis	visible

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