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ANALYSIS OF THE TOTAL LIPID CONTENT IN THE KERNELS OF SEVERAL TEMPERATE NUT CROPS ACCESSIONS FROM THE GERMPLASM COLLECTIONS OF UCv-SCDP VÂLCEA, ROMANIA

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Abstract

Lipids are an important group of compounds that provide several biological functions such as: energy storage, cell membrane structure, and signaling. This study has the aims to investigate the total lipid content of fruits belonging to temperate nut crops like walnut (Juglans regia L.*), pecan (Carya illinoinensis), and hazelnut (Corylus avellana* L.*) using the extraction method based on different solvents. The analyzed kernels were collected from six walnut accessions ('Valcor', 'Jupâneşti', 'VL 51 B', 'Payne', 'Lara', and 'Franquette'), one pecan hybrid selection (H 21-13 - 2008) and six hazelnut cultivars ('Valcea 22', 'Romavel', 'Ennis', 'Daviana', 'Du Chilly', and 'Purple Filbert'), all from the germplasm collections of Fruit Growing Research and Extension Station (SCDP) Valcea. The FTIR spectroscopy using attenuated total reflectance and suitable variables (absorbance values at certain wavenumbers) of nut oil samples was utilized at frequency regions of 4000-400 cm^{-1}. The colorimetric sulfo-phospho-vanillin method developed by Van Handel (1985) was used in order to determinate the total lipid content. The results obtained after analyzing the nut kernels emphasized inter- and intraspecific variation depending on the genotype and the solvent used.*

Key words*: walnut (Juglans regia* L.*), pecan (Carya illinoinensis), hazelnut (Corylus avellana* L.*), total lipid content, lycopene, β-carotene, ATR-IR spectroscopy*

INTRODUCTION

The edible seeds of tree nut crops are used as a diet complement and are highly consumed worldwide. The production and consumption of these foods have been increasing, and an enormous global market value is forecasted for the next years. In addition to their high lipid content and nutritional value, they provide health benefits to fat metabolism, heart, skin, and brain (Bizera et al., 2019; Vîjan et al., 2023).

Due to their high fat content, there was a belief that foods high in lipids, such as walnuts, hazelnuts, almonds, and olives, shouldn't be consumed frequently. Nonetheless, an abundance of epidemiological and clinical research has demonstrated that fatty fruits and tree nuts are foods that promote health and are now regarded as important food groups and necessary elements of a balanced diet (Widmer et al., 2015). Additionally, research shows an inverse relationship between these foods' intake and the prevalence of coronary heart and cardiovascular diseases, as well as a positive correlation with blood pressure, visceral adiposity, oxidative stress, inflammation, insulin sensitivity, and cancer, among other factors (Banel and Hu, 2009; Guasch-Ferré et al., 2014; Mericli et al., 2017; Massaro et al., 2020).

Having a low glycemic index, the fruits belonging to temperate nut crops have protective properties in accordance with their chemical composition. Beside to water (2-5%), these conventional functional foods contain proteins (10-25%), biogenic amines (melatonin and serotonin), polyunsaturated fatty acids (omega-3 and 6), vitamins (retinol, niacin, thiamine, biotin, and pyridoxine), minerals (potassium, calcium, magnesium, iron, copper, zinc, and manganese), and fiber (Bizera et al.,

2019; Giura et al., 2019; USDA Food Data Central, 2024).

Lipids are an important group of compounds that provide several biological functions such as energy storage, cell membrane structure and signaling (Green and Tzagoloff, 1966). Lipids are esters of fatty acids with different alcohols, the products of metabolism, which in plants are found in almost all organs in variable quantities. Some fatty acids have shown an important role in the development of obesity, diabetes, cardiovascular disease, cancer, and mental illness (Alabdulkarim et al., 2012; Julvez et al., 2021). Due to their excellent biocompatibility, lipids are also used as efficient carriers for drug delivery (Yang and Merlin, 2020).

Lipids include various categories of organic substances such as: oils, fats, waxes and phosphatides, which are included in two classes: 1) saponifiable, which are either esters of glycerin with saturated and unsaturated fatty acids (glycerides) and phosphoric acid (phosphoglycerides), or esters of sphingosine with a fatty acid and a phosphoric acid to which a compound is linked with nitrogen (sphingolipids) or esters of primary and secondary alcohols with fatty acids (cerides), 2) unsaponifiable, which are compounds with an isoprenoid structure. The last category includes phytosterols, steroid hormones, carotenoids and fat-soluble vitamins (Popescu et al., 1980).

Due to their chemical heterogeneity, the classification of lipids is more or less arbitrary. However, lipid constituents, regardless of chemical structures, are similar in terms of common solubility characteristics: they are hardly soluble in water, but they dissolve easily in non-polar solvents.

Carotenoids are a group of natural pigments produced by all photosynthetic organisms. Some of these compounds serve as vitamin A precursors, and other are crucial for visual health (lutein) while most carotenoids act as antioxidant molecules in lipid-rich environments (Popescu et al., 1980).

In plant materials, lipid compounds can be found freely, easily separated by extraction with a suitable organic solvent (petroleum ether, hexane, chloroform, dichloromethane, diethyl ether, methanol, etc.), or bound to other compounds, from which they can be separated only after cleavage in the presence of hydrochloric acid. In the absence of a UV-Vis absorption spectrophotometer and a suitable lipid assay standard, the gravimetric method is used to determine the total lipid content, despite the fact that this method is time- and reagent-consuming. The sample is brought to a fine granulation with a grinder, it is refluxed with a solution of hydrochloric acid at low heat (for one hour), the filtered residue is extracted with a suitable solvent in a Soxhlet extractor (for 3-4 hours), the traces of solvent are removed by drying and, finally, by weighing, the mass of extracted fat is determined.

Over the last years, several extraction methods have been developed (Bligh and Dyer, 1959; Gardner et al., 1985; de Boer, 1988; Booij and van den Berg, 1994; Smedes, 1999). The method called liquid - liquid extraction has been used most frequently. Because of the difficulties in the phase separation and the high number of manual operations, the hot Soxhlet extraction is preferred over liquid - liquid extraction (Manirakiza et al., 2001). However, without a complete extraction of the lipid compounds from samples, the results should be interpreted with care.

Due to the great diversity of lipid compounds and its possibility to bind to other molecules, many researchers (Carlson, 1985; Smedes, 1999; Manirakiza et al., 2001; Cequier-Sánchez et al., 2008; Ramírez, 2022) have tested the ability of different solvents and solvent mixtures to totally solubilize the lipids. These studies have shown that solvents used for lipids extraction should have a high solubility for all lipid compounds and be sufficiently polar to remove them from their binding sites with cell membranes, lipoproteins and glycolipids.

This study intends to investigate the carotenoids (lycopene and β-carotene) and the total lipid content of fruits belonging to several nut crops like walnut (*Juglans regia* L.), pecan (*Carya illinoinensis* (Wangenh.) K. Koch) and hazelnut (*Corylus avellana* L.) by using a reliable and economical colorimetric method. Taking into account that neutral lipids (triglycerides, diglycerides, monoglycerides, and sterols) dissolve well in non-polar organic solvents while more polar lipids (free fatty acids, phospholipids, and sphingolipids) dissolve only in relatively polar solvents, the

lipid extraction from the fruits belonging to temperate nut crops was performed in different solvents. Moreover, ATR-FTIR spectroscopy provide fingerprinting information about the molecules in the composition of investigated compounds.

MATERIALS AND METHODS

Plant Materials and Sampling

The analyzed kernels were collected from six walnut cultivars ('Lara'-N1, 'Valcor'-N2, 'Jupânești'-N3, 'Payne'-N4, 'VL 51 B'-N5, and 'Franquette'-N6), one pecan hybrid selection (H 21-13-2008, P) and six hazelnut cultivars ('Romavel'-H1, 'Ennis'-H2, 'Vâlcea 22'-H3, 'Daviana'-H4, 'Du Chilly'-H5, and 'Purple Filbert'-H6), all from the germplasm collections of Fruit Growing Research and Extension Station (UCv-SCDP) Vâlcea. 'Valcor', 'Romavel' and 'Vâlcea 22' are cultivars and 'VL 51 B' and H 21-13-2008 are selections issued from UCv-SCDP Vâlcea breeding programs.

The nuts were kept in their shells between the harvest moment and mid-December 2023 in boxes in a ventilated room. Then, the nuts were cracked and the nut kernels (about 100 g) were transformed into a homogeneous mixture with a grinder and were stored at 3-4°C to perform all analyses.

Chemicals and Reagents

The following chemicals and reagents were used: n-hexane, ethanol, acetone, distilled water, petroleum ether, chloroform, vanillin, phosphoric acid, sulfuric acid, and the standards: triolein, lycopene and β-carotene. All chemicals and reagents were purchased from Merck, Darmstadt, Germany.

Extraction Procedures, Biochemical Determinations and Equipment

For the extraction of lipids, samples from one gram of nut kernels powder in 10 mL solvent (chloroform, n-hexane, and petroleum ether) were used. The mixtures were subjected to the following procedures: vortexing for 2 minutes at 3000 rpm, ultrasonication for 30 minutes at 40 kHz, and centrifugation at 6500 rpm for 30 minutes. Next, the extracts were filtered and the lipid determination was made immediately after extraction. Total lipid content was determined using the method proposed by Van Handel, 1985. The results were expressed in mg triolein per 100 g nut kernel powder.

For the quantitative determination of carotenoids, the supernatant obtained by magnetic stirring for 30 minutes at 1500 rpm of 2 g nut kernels powder in 25 ml volumetric mixture of hexane: ethanol: acetone in a 2:1:1 ratio was used, followed by magnetic stirring for another 10 minutes at 1500 rpm of the mixture after adding 10 ml distilled water. After the phase separation, in 10-15 minutes of rest, the volume of the supernatant was measured. Then, the absorbance spectrum of the supernatant was recorded in the 350-550 nm range, using n-hexane as a control. The lycopene and β-carotene contents were calculated, taking into account the amount of nut kernel powder taken in the analysis and the volume of the supernatant obtained after the separation of the phases in accord with Tudor-Radu et al., 2016. The results were expressed in μg lycopene or β-carotene per 100 g nut kernel powder.

The spectral measurements were made with a UV-Vis Perkin-Elmer Lambda25 and an FTIR Jasco 6300 spectrometer. An ATR accessory equipped with a diamond crystal (Pike Technologies) allows the collection of FTIR spectra directly on a sample without any special preparation. The FTIR spectra were recorded in the region of 4000-400 cm^{-1}, with a detector TGS and apodization Cosine. The spectral data were processed with the JASCO Spectra Manager II software. Samples were scanned at 4 cm^{-1} resolution, accumulation: 100 scans. Background reference spectra were recorded using air after every sample to minimize the interference due to carbon dioxide and water vapor in the atmosphere. Between measurements, the ATR crystal was carefully cleaned using pure acetone (Sigma-Aldrich Co.) and then dried with soft tissue (Topală and Tătaru, 2019; Topală et al., 2020).

Data Analysis

All measurements were taken at room temperature (23°C). All analyses were performed in three replicates. Practically, the extracts necessary for the determinations were analyzed in triplicate.

Statistical analysis was performed with the IBM SPSS Statistics 29.0 software package.

Data were reported as mean ± standard deviation.

RESULTS AND DISCUSSIONS

Table 1 show the values for contents of carotenoids (lycopene and β-carotene) and total lipids extracted in different solvents (chloroform, petroleum ether and n-hexane) with indication of the values of mean and standard deviation for the six walnut accessions ('Valcor', 'Jupâneşti', 'VL 51 B', 'Payne', 'Lara', and 'Franquette'), one pecan hybrid selection (H 21-13 - 2008) and six hazelnut cultivars ('Valcea 22', 'Romavel', 'Ennis', 'Daviana', 'Du Chilly', and 'Purple Filbert').

Table 1. The content of lycopene, β-carotene and total lipids in nut kernel powder belonging to several nut crop accessions

Genotype	Lycopene (μg/ 100 g)	β-carotene (μg/ 100 g)	Total lipids (g triolein/100 g)		
			Petroleum ether	n-hexane	Chloroform
Valcor	354.67±4.51^{a}	26.67±2.52^{c}	55.33±0.45^{a}	59.50±0.05^{b}	62.91±0.08^{d}
Payne	306.99±3.01^{d}	45.33±4.04^{b}	51.56±0.12^{d}	55.15±0.27^{d}	60.51±0.01^{e}
Jupâneşti	294.01±3.98^{e}	28.02±3.01^{c}	55.85±0.09^{a}	60.86±0.02^{a}	66.95±0.02^{a}
VL 51 B	316.33±3.51^{c}	28.67±3.06^{c}	54.27±0.43^{b}	58.64±0.03^{c}	64.52±0.03^{b}
Lara	249.33±2.52^{f}	63.33±2.08^{a}	54.28±0.34^{b}	60.41±0.44^{a}	63.39±0.21^{c}
Franquette	344.67±5.51^{b}	12.03±1.98^{d}	52.71±0.36^{c}	54.45±0.06^{e}	59.23±0.50^{f}
H 21-13 - 2008	425.98±1.73	43.67±3.06	54.14±0.03	63.31±0.04	72.54±0.03
Romavel	304.67±5.03^{d}	13.67±2.08^{ab}	36.38±0.48^{e}	41.18±0.15^{e}	45.28±0.17^{d}
Ennis	307.67±6.03^{d}	14.67±2.31^{ab}	43.17±0.49^{b}	44.96±0.02^{c}	46.21±0.03^{c}
Daviana	463.04±6.11^{b}	12.33±1.53^{b}	41.54±0.17^{c}	42.51±0.03^{d}	45.56±0.12^{cd}
Valcea 22	458.33±4.04^{b}	14.33±2.52^{ab}	38.98±0.02^{d}	40.63±0.17^{f}	46.07±0.83^{c}
Du Chilly	482.33±9.05^{a}	15.01±2.01^{ab}	41.74±0.04^{c}	49.26±0.14^{b}	55.02±0.02^{b}
Purple Filbert	304.67±5.03^{c}	17.33±1.53^{a}	52.18±0.02^{a}	55.59±0.02^{a}	58.17±0.01^{a}

Data are presented as mean ± SD (standard deviation).
Different letters between cultivars denote significant differences (Duncan test, $p < 0.05$)

Regarding the content of carotenoids, the lowest level of β-carotene was 12.03 μg/100 g in 'Franquette' (a walnut cultivar) and 12.33 μg/100 g in 'Daviana' (a hazelnut cultivar), while the highest level was 43.67 μg/100 g in H 21-13-2008 (a pecan hybrid selection), respectively 45.33 μg/100 g for 'Payne' and 63.39 μg/100 g for 'Lara' (two walnut cultivars). The lycopene content ranged from 249.33 μg/100 for 'Lara' walnut cultivar to 482.33 μg/100 g for 'Du Chilly' hazelnut cultivar. Similar results were reported by Bolling et al., 2011; Alasalvar and Bolling, 2015; Bizera et al., 2019; Vîjan et al., 2023.

Regarding the total lipids, the best extraction was in chloroform, followed by n-hexane and, finally, petroleum ether. In agreement with the observations of Carlson, 1985; Smedes, 1999; Cequier-Sánchez et al., 2008; Miraliakbari and Shahidi, 2008; Ramírez, 2022, it appears that an efficient extraction of lipids from biological tissues requires a solvent sufficiently polar to remove the lipids from their association with cell membranes and lipoproteins, but sufficiently non-polar to dissolve neutral lipids. Our results indicate chloroform as the suitable solvent for an efficient extraction of lipids from walnut, pecan and hazelnut kernels. Similar results were reported by Carlson, 1985; Smedes, 1999; Cequier-Sánchez et al., 2008; Miraliakbari and Shahidi, 2008; and Ramírez, 2022.

In chloroform, the six walnut accessions they presented values between 59.23 g triolein/100 g ('Franquette') and 66.95 g triolein/100 g ('Jupâneşti') whereas the six hazelnut cultivars ranged between 45.28 g triolein/100 g ('Romavel') and 58.17 g triolein/100 g ('Purple Filbert'). Iordănescu et al., 2021 highlighted a lipids content between 56.09% and 66.56% for twenty samples of walnuts from three different locations from the west and north-west of Romania. Köksal et al., 2006 reported the values between 56.07% and 68.52% for the total lipid content in seventeen Turkish hazelnut varieties, using n-hexane for six hours in a Soxhlet extractor. The pecan hybrid selection (H 21-13-2008) showed the highest value for total lipid content (72.54 g triolein/100 g) among the analyzed nuts and recommends the pecan kernels in the human diet. This result is consistent to those published by Rudolph et al., 1992; Wakeling et al., 2001; Miraliakbari and Shahidi, 2008, but higher than those reported by Amaral et al., 2005 and Ribeiro et al., 2020.

Differences of the total lipid content in the fruits belonging to temperate nut crops could be attributed to many factors, such as cultivar, crop location, climate, year of production, soil composition, harvest time and extraction methods (Rudolph et al., 1992; Parcerisa, 1993; Ribeiro et al., 2020; Goodarzi et al., 2023).

The main characteristic vibrations in the ATR FTIR spectra are presented in Figures 1-3 for samples N1 ('Valcor'), P (pecan selection) and H1 ('Romavel'). Figures 4 and 5 show the overlapped spectra of walnut and hazelnut samples.

The 400-4000 cm^{-1} region of FTIR spectra shows several characteristic peaks (bond oscillations) at certain wavenumbers that are assigned to specific functional groups of their respective components (Tables 2 and 3).

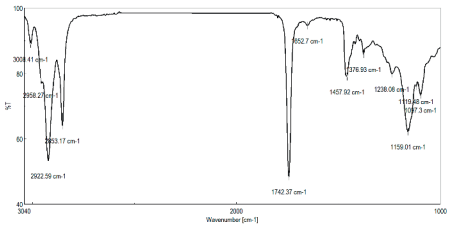

Figure 1. ATR-FTIR Spectrum for 'Valcor' (N1) walnut cultivar sample - chloroform extract

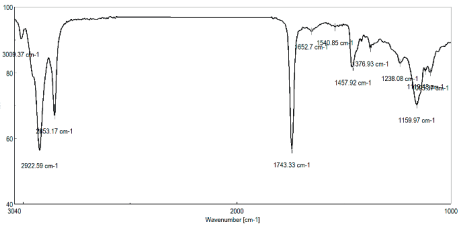

Figure 2. ATR-FTIR Spectrum for pecan hybrid selection (P) sample - chloroform extract

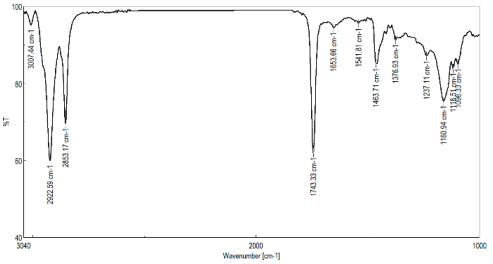

Figure 3. ATR-FTIR Spectrum for 'Romavel' (H1) hazelnut cultivar sample – chloroform extract

The unsaturated C atom of olefins in nuts exhibits C–H stretching vibrations, which peak at 3008–3010 cm^{-1}.

The peaks at 2922 and 2853 cm^{-1} are assigned to asymmetric stretching vibrations of methylene (–CH_2), while peak at 2953–2956 cm^{-1} is attributed to symmetric stretching vibration of methyl (–CH_3) (Topală et al., 2020).

In the region of 1800-1000 cm^{-1} are observed protein amide C=O stretching and amide N–H bending vibrations (60%) coupled to C–N stretching vibration (40%) mode of the polypeptide and protein backbone, respectively. In the same region, the absorption band at 1746 cm^{-1} is due to C=O stretching vibration of ester groups in triacylglycerols and two bands at 1462 cm^{-1} and 1378 cm^{-1} corresponding to the CH absorption bending vibration of CH_2 and CH_3 groups, respectively (Che Man and Setiowaty, 1999; Guillén and Cabo, 1999), are also lipid-related bands.

The bands at 1462 cm^{-1} are correlated to the content of fatty acid chains (Melin et al., 2000).

Table 2. The location of the maxima of the absorption bands FTIR in the tested nuts accessions samples

Attribution	Samples						
	N1	N2	N3	N4	N5	N6	P
C-H ring vas (=C-H), lipids, fatty acids	3008	3008	3008	3008	3009	3010	3009
asymmetric stretching vibration of CH_3	2956	2953	2956	2953	2953	2956	2953
asymmetric stretching vibration of CH_2	2922	2922	2921	2922	2922	2921	2922
symmetric stretching vibration of CH_2	2853	2853	2853	2853	2853	2854	2853
C=O stretching vibration of ester groups	1742	1742	1742	1742	1742	1746	1743
Amide I absorption (predominantly the C=O stretching)	1652	1652	1652	1652	1652	1646	1652
Protein amide II absorption (δN-H, νC-N)	1540	1540	1540	1540	1540	1549	1540
CH_3 bending vibration (lipids and proteins)	1457	1456	1457	1456	1456	1456	1457
vibration of CH_2 and CH_3 groups	1376	1376	1376	1376	1375	1376	1376
Amide III	1238	1237	1237	1238	1238	1238	1238
νC-O of proteins and carbohydrates	1159	1159	1159	1159	1158	1159	1159
Symmetric stretching P-O-C; C-O stretching vibration of C-OH	1119						1119
Stretching PO_2^{2-} symmetric	1097	1098	1098	1098	1098	1098	1095

Codifications: N1–N6 for six walnut cultivars ('Lara'-N1, 'Valcor'-N2, 'Jupânești'-N3, 'Payne'-N4, 'VL 51 B'-N5, and 'Franquette'-N6), and P for one pecan hybrid selection (H 21-13-2008)

Table 2. The location of the maxima of the absorption bands FTIR of the tested hazelnuts samples

Attribution	Samples					
	H1	H2	H3	H4	H5	H6
C-H ring vas (=C-H), lipids, fatty acids	3007	3007	3004	3004	3006	3006
asymmetric stretching vibration of CH_3	2953	2953	2956	2953	2952	2956
asymmetric stretching vibration of CH_2	2922	2922	2921	2922	2922	2922
symmetric stretching vibration of CH_2	2853	2853	2852	2852	2852	2853
C=O stretching vibration of ester groups	1743	1743	1743	1743	1743	1743
Amide I absorption (predominantly the C=O stretching)	1653	1652	1652	1652	1648	1651
Protein amide II absorption (δN-H, νC-N)	1541	1540	1541	1540	1540	1540
CH_3 bending vibration (lipids and proteins)	1463	1463	1456	1456	1456	1456
vibration of CH_2 and CH_3 groups	1376	1375	1376	1376	1376	1375
Amide III	1237	1238	1237	1236	1236	1237
νC-O of proteins and carbohydrates	1160	1160	1160	1159	1159	1160
Symmetric stretching P-O-C; C-O stretching vibration of C-OH	1118	1118	1117	1118	1118	1118
Stretching PO_2^{2-} symmetric	1096	1096	1095	1095	1095	1095

Codifications: H1–H6 for six hazelnut cultivars: 'Romavel'-H1, 'Ennis'-H2, 'Vâlcea 22'-H3, 'Daviana'-H4, 'Du Chilly'-H5, and 'Purple Filbert'-H6

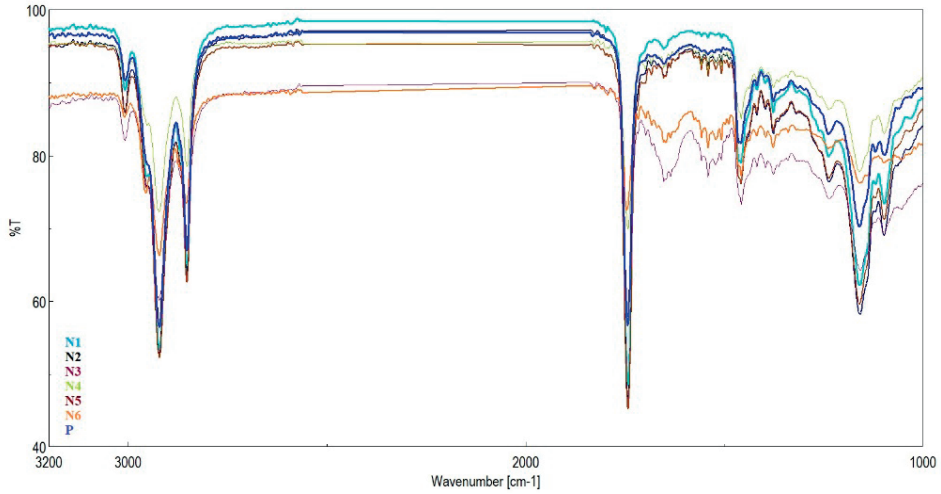

Figure 4. ATR-FTIR spectra of overlapped walnut samples (chloroform extracts)

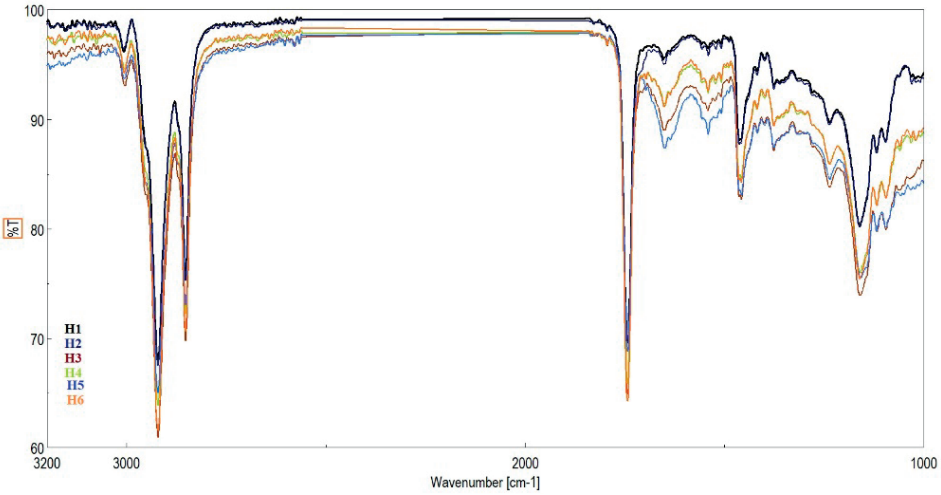

Figure 5. ATR-FTIR spectra of overlapped hazelnut samples (chloroform extracts)

CONCLUSIONS

In the present research, a reliable and economical colorimetric method has been developed for quantitative analysis of total lipid. There are multiple advantages of the modified Van Handel method, such as: 1) the reagents used in analysis are inexpensive and easy to handle, 2) the amount of sample required for the determinations is small and it can be adjusted to fit in the standard range of concentrations of the spectrophotometric analysis, 3) a large number of samples can be analyzed in a short period of time (about two hours), with less labor for the samples preparation and analysis, and 4) the color development of the mixture of lipids with the sulfo-phospho-vanillin reagent is rapidly and consistent.

Regarding the total lipids, the best extraction was in chloroform, followed by n-hexane and, finally, petroleum ether. The pecan hybrid selection (H 21-13 - 2008) presented the highest lipid content (72.54 g triolein/100 g in chloroform), followed by the walnut samples (59.23-66.95 g triolein/100 g in chloroform) and finally the hazelnut samples, with values between 45.28 and 58.17 g triolein/100 g in chloroform. Moreover, the lipid fingerprint was highlighted by FTIR spectroscopy.

As regards to the carotenoid content of the analyzed samples, there is no differentiation between the three plant materials in terms of lycopene content. However, with the exception of the walnut sample from 'Franquette', one can notice that the walnut samples had 1.5 to 3.7 times higher values for the β-carotene content compared to the hazelnut samples.

Nowadays, in order to guarantee the quality and safety of food and agricultural goods, it is crucial to obtain important information on structural elements.

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