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Untargeted Metabolomics for Sea Buckthorn (*Hippophae rhamnoides* ssp. *carpatica*) Berries and Leaves: Fourier Transform Infrared Spectroscopy as a Rapid Approach for Evaluation and Discrimination

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Abstract

Untargeted metabolomics coupled with chemometric analysis was applied to evaluate and discriminate six Romanian sea buckthorn (*Hippophae rhamnoides* L.) berries and leaves. Total carotenoids and total phenolics were determined quantitatively by UV-Vis spectrometry. The qualitative evaluation and discrimination was obtained using the FTIR fingerprints (by using Fourier Transform Infrared spectroscopy) of raw carotenoid and phenolic extracts. The average concentration of total carotenoids was 54 and 3.9 mg carotenoids/ 100g DW in berries and leaves, respectively. The average concentration of total phenolics was 746 mg GAE/100g DW in berries, approximately 1.8 times lower than total phenolics found in leaves. By PCA (Principal Component Analysis) of fingerprints (900-1800 cm^{-1}), the responsible bands for samples discrimination were identified. In case of total carotenoids extract the biomarker bands were: 1745, 1743, 1500 cm^{-1} for berries and 1458 cm^{-1} and 1735 cm^{-1} for leaves, while for total phenolic extract the key bands were 1731, 1033, 1622 cm^{-1} for berries and 1047 cm^{-1}, 1616, 1512 and 1454 cm^{-1} for leaves. FTIR spectroscopy proved to be a simple and sensitive analytical technique that can be successfully used in sample discrimination and classification.

Keywords: authentication biomarkers, principal component analysis, untargeted analysis, carotenoids, phenolic compounds, FTIR

Introduction

In the past few years, the emerging field of metabolomics has become an important technology in many research areas such as toxicology and clinical chemistry including disease diagnosis and also therapeutic drug monitoring (Madsen *et al*., 2009; Roux *et al*., 2011) as well for food quality related to nutrition (Hall *et al*., 2008), ecology, plant biochemistry or chemotaxonomy (Fischedick *et al*., 2010). Metabolomics is a novel experimental methodology, representing an 'omics' approach along with genomics, transcriptomics and proteomics (Oksman-Caldentey and Saito, 2005), often used in combination with the other omics approaches for deeper understanding of complex biological processes, especially metabolism (Sawada *et al*., 2009). Metabolomics offers a comprehensive chemical analysis of all metabolites in biological systems (Madsen e*t al*., 2009), followed by the computation and chemometric analysis of large data sets.

Considering the chemometric approach, both untargeted analysis (rapid and global analysis of sample's fingerprint) and targeted analysis, (qualitative and quantitative analysis of one or a few metabolites related to a specific metabolic pathway) can provide relevant features that can distinguish between sample classes. Such multivariate projection methods for data exploration (e.g., Principal Component Analysis, PCA) and Cluster Analysis have been shown to be very useful to point out the systematic changes between many samples using their complex map of metabolites (Frisvad *et al*., 2008).

Advanced analyses by complementary methods are applied for different categories of metabolites, such as mass spectrometry (MS), nuclear magnetic resonance (NMR), infrared (IR) and Raman spectroscopy in order to achieve a complex visualization of the metabolome (Hall, 2006).

In case of untargeted analysis, Fourier transform infrared (FTIR) spectroscopy is one of the most available methods used for this approach (Johnson *et al*., 2004), being simple, fast and non-destructive, suitable for investigations which

assure high reproducibility, specificity and easy preparation of samples.

So far it has been mostly used to authenticate, identify or classify fats and oils (Abdul *et al*., 2010), in chemotaxonomy to discriminate between samples of various origin (Lu et al., 2008) and in food quality and control (Sinelli *et al*., 2008; Socaciu *et al*., 2009). To our knowledge, the FTIR metabolic fingerprinting has never been used so far, to discriminate the Romanian sea buckthorn varieties.

In this article, the applications of untargeted metabolic fingerprintig along with chemometrics was applied for sea buckthorn (*Hippophaë rhamnoides* L.) classification and evaluation.

Sea buckthorn is well known for its large scale utilization in industry, pharmacology, forest and land rehabilitation provided by its unique rich biochemical composition, variety of species, fast fruiting, high productivity and ecological adaptation (Bal *et al*., 2011; Pop, 2012). Moreover, the high diversity of bioactive compounds like vitamins, phenolics, lipids, tocopherols, carotenoids and phytosterols contribute to the high, increasing interest for sea buckthorn utilization in human health and nutrition. Among bioactive compounds one of the most important are phenolics which play an important role as strong antioxidants, with nutritional and health promoting abilities (Gupta *et al*., 2006) and carotenoids known as anti-oxidant, anti-mutagenic and anti-tumour (Johnsons, 2002).

For this purpose, the metabolomic analysis (untargeted fingerprinting) focused on phenolic metabolites, and carotenoids will be used in sea buckthorn classification using a chemometric approach (PCA Analysis).

Materials and methods

Sea Buckthorn samples

Fresh sea buckthorn berries and leaves (*Hippophaë rhamnoides* L. ssp. *carpatica*) were collected from an experimental field at the Fruit Research Station from Bacău (Romania), in the first week of October 2011. The established cultivars taken for evaluation were: 'Victoria', 'Tiberiu', 'Sf. Gheorghe', 'Serpenta', '□erbănești 4' and 'Ovidiu' which were previously described for their carotenoid and flavonols composition (Pop *et. al*, 2013, 2014). The berries and leaves were lyophilized and then stored at -20 °C.

Reagents and standards

Organic solvents used for extraction were of analytical grade. Hexane and acetone were purchased from Sigma-Aldrich (Steinheim, Germany). All other chemicals were purchased from Merck (Darmstadt, Germany).

Carotenoids extraction

Total carotenoids were extracted from lyophilized berries and leaves (1g) using methanol: ethyl acetate: petroleum ether (1:1:1, *v/v/v*) accordingly to Pop *et al*. (2014). The obtained extracts were analyzed for their total carotenoid content and used as such for the FTIR analysis. All extractions were done in triplicate.

Phenolic compounds extraction

For extraction, the lyophilized berry or leaf samples (1 g each) were extracted in 70% (v/v) aqueous acetone as described by Pop *et al*. (2013). The extracts were analyzed for their total phenolic content using Folin-Ciocalteau reagent. The lyophilized samples were solubilised in 50% (v/v) aqueous MeOH and centrifuged, and the supernatant was used for FTIR analysis. All extractions were done in triplicate.

FTIR-HATR analysis

The FTIR spectra were obtained with a Shimatzu IR Prestige- 21 spectrometer using attenuated total reflectance (ATR) and an internal reflection accessory made of Zinc Selenide (ZnSe) Composite. The samples were recorded by co-adding 64 scans. Each spectrum was registered from 4000-650 cm^{-1}. As reference, the background spectrum of air was collected. The carotenoids and total phenolic extracts samples were measured without any preparation, directly on the ZnSe ATR crystal. Between measurements, the ATR crystal was carefully cleaned using acetone.

Statistical analysis

Advanced chemometrics was applied to discriminate between berries and leaves varieties using their FTIR metabolic fingerprints. Therefore, the discrimination was done using total carotenoids and phenolics extracts specific IR absorption spectrum zones (900-1800 cm^{-1}) by principal component analysis (PCA) with UnscramblerX 10.1 Software, version 10.1 (CAMO Software AS, Norway). Spectra were transformed by normalization of the absorbance spectra to the most intense band. PCA was carried out using 365 point for carotenoids and 494 points (normalized absorbance) for phenolic compounds.

Results and discussions

Sea buckthorn berry and leaf total carotenoids and total phenolics content

Table 1 represents the total carotenoid and phenolics' content compared for berries and leaves. In berries, total carotenoids concentration ranged between 22 and 98 mg carotenoids/ 100g DW with the lowest content in 'Sf Gheorghe' variety and the highest in 'Tiberiu' variety. Compared with berries, leaves had the average concentration 32 times lower, being 3.8 mg carotenoids/ 100g DW among varieties. Total phenolics concentration found in berries ranged between 204 and 1060 mg GAE/ 100g DW. These results were in accordance with other investigations reported in the literature (Bal *et al*., 2011; Pop *et al*., 2013, 2014).

Comparative FTIR metabolic fingerprinting of sea buckthorn berry and leaf carotenoid and phenolic extracts

Figures 1 and 2 represents the comparative FTIR spectra (3500-600 cm^{-1}) and the comparative FTIR fingerprint zone spectra (1850-600 cm^{-1}) of total carotenoid and total phenolic extracts in berries *vs* leaves, for 'Victoria' variety.

The major peak at 1743 cm^{-1} in the berries carotenoids fingerprint zone (Fig. 1A) appears due to the stretching vibrations of carboxylic groups (C=O) in the volatile oils, triglycerides, aliphatic esters or other compounds in the extracts (Socaciu *et al*., 2009). Also, the peak at 1743 cm^{-1} suggests the presence of high carotenoid esters, β carotene or lycopene in the carotenoid extract. The same band was attributed for

lycopene and β carotene pigments in marigold extract (Bunghez and Ion, 2011). The next representative band at 1463 cm^{-1} due to the bending vibration of methylene $–CH_2$ (scissoring), may indicate significantly higher concentrations of total lipids in the extracts (Socaciu *et al*., 2009). This band can also be assigned to lycopene pigments (1463 cm^{-1}), while 1377 cm^{-1} band to the *b*-ionone ring of β -carotene or due to the C-H ($–CH_3$) symmetrical bending (Abdul *et al*., 2010). The small peak at 1418 cm^{-1} could be assigned to carboxyl groups (COO) of esters. The peaks between 1170-930 cm^{-1} are specific for carbohydrates with C-O and C-OH stretching vibrations. Since the carbohydrates content is reduced due to the extraction procedure, the three bands at 1161, 1116 and 1093 cm^{-1} are assumed to be produced by the C-O stretching of the ester group (Stuart, 2004). The variety of conjugated *cis* and *trans* vinylenes (–CH=CH–) present in carotenoids determine the presence of 951 cm^{-1} band (Rubio-Diaz *et al*., 2010). The peak at 721 cm^{-1} is probably due to the methylene CH_2 rocking band from long CH_2 chains (Stuart, 2004).

In case of leaves carotenoids fingerprint zone (Fig. 1B) the first intense peak at 1735 cm^{-1} is due to the presence of phospholipids or due to ester carbonyl absorption. Next, the peaks at 1652, 1608, 1550 and 1515 cm^{-1}, which are totally absent in berries, are caused by the chlorophylls and/or protein content. Therefore, the high diversity bands are produced by the carbonyl groups from chlorophyll molecules (e.g. esters or keto C=O from chlorophyll *a*), or from proteins (peptides and side chain C=O groups) (Pop, 2012; Stuart, 2004).

Regarding berries phenolics fingerprint zone (Fig. 2A), the region from 1850-1500 cm^{-1} is characteristic to amide group from proteins (Barth, 2007; Zavoi *et al*., 2011). Comparing the intensity of the protein band at 1622 cm^{-1} with the previous

Tab. 1. The mean values (x± s) of total carotenoid and phenolics' concentration (mg/100 g dry weight) of different sea buckthorn berries and leaves varieties

Cultivar	Total carotenoids		Total phenolics	
	berries	leaves	berries	leaves
'Victoria'	44.72 ± 3.94	3.83 ± 0.22	835.37 ± 53.69	1615.26 ± 114.54
'Tiberiu'	97.80 ± 5.23	3.48 ± 0.05	1059.84 ± 68.86	1420.03 ± 21.3
'SfGheorghe'	21.98 ± 5.65	3.92 ± 0.19	959.77 ± 53	1335 ± 47.1
'Serpenta'	65.16 ± 0.43	4.22 ± 0.27	204.24 ± 5.87	1347.2 ± 180.62
'□erbanesti'	49.88 ± 7.78	4.19 ± 0.2	956.56 ± 54.34	1274.6 ± 33.63
'Ovidiu'	42.87 ± 0.55	4.22 ± 0.06	457.82 ± 37.49	1307.6 ± 84.24

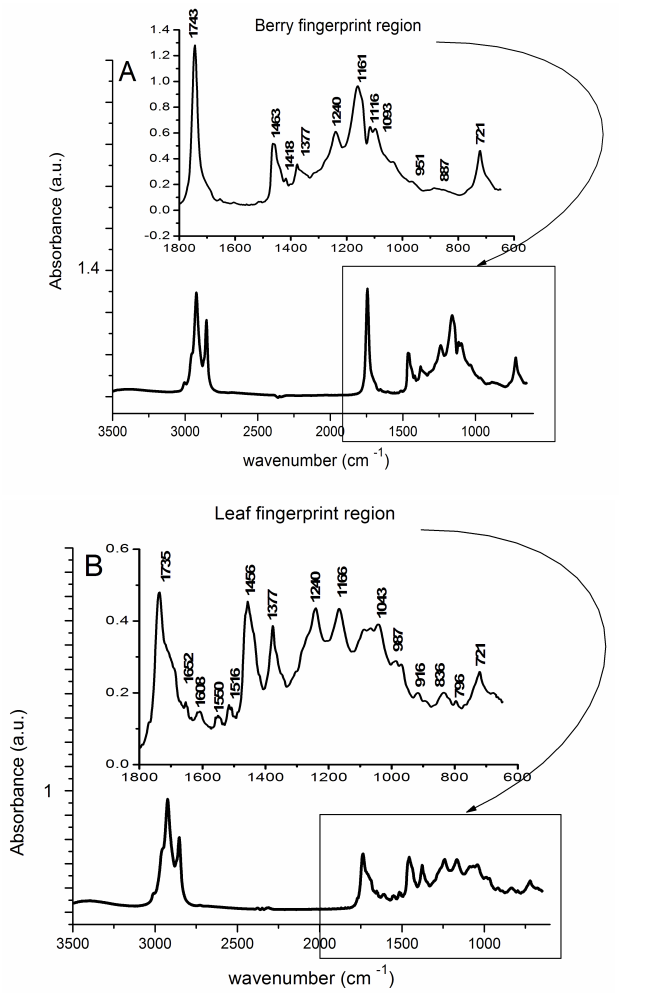

Fig. 1. General FTIR spectra (600-3500 cm^{-1}) and FTIR fingerprint region (600-1800 cm^{-1}) of sea buckthorn berry (A) and leaf (B) carotenoid extracts from 'Victoria' variety

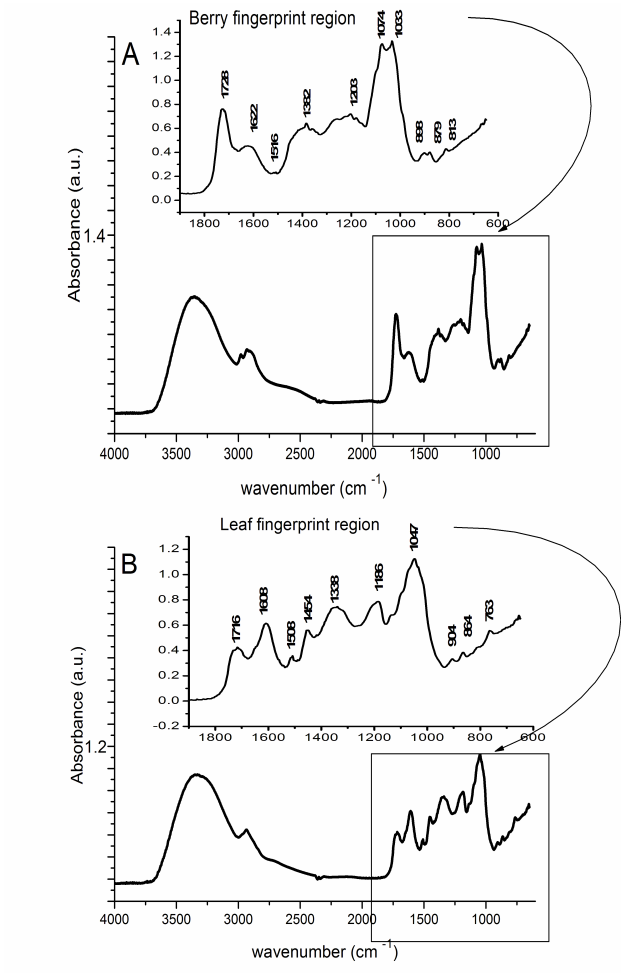

Fig. 2. General FTIR spectra (600-3500 cm^{-1}) and FTIR fingerprint region (600-1800 cm^{-1}) of sea buckthorn berry (A) and leaf (B) phenolics extract from 'Victoria' variety

band at 1728 cm^{-1}, which appears due to the C=O stretching from lipids, we can conclude that lipids are found in higher concentrations than proteins. The presence of the absorption band at 1622 cm^{-1} can be assigned to the conjugated carbonyl bonds from flavonoids, which commonly appear within this area (Adiana and Mazura, 2011).

The bands between 1500 – 1170 cm^{-1} might be assigned to proteins, lipids or phosphate compounds CH_2, CH_3, P=O. The band at 1203 cm^{-1} can be attributed to the phenolic -OH (Ficarra *et al*., 2002). Next, the bands between 1170-930 cm^{-1} can be predominantly assigned to polysaccharides or flavonoids (Lu *et al*., 2011). The high intensity bands in this region can be explained by the presence of flavonol glycosides in high amounts, compounds which have alcohol as primary functional group. The last bands between 930-700 cm^{-1} can be assigned to the out-of-plane C–H bending from the aromatic compounds (Stuart, 2004).

The differences between leaf (Fig. 2B) and berry (Fig. 2A) FTIR fingerprints mainly consist in the different band intensity. For example, the protein band at 1608 cm^{-1} has higher signal in leaves and smaller in berries, while the lipids band at 1716 cm^{-1} is higher in berries and smaller in leaves. As expected, previous characteristic bands are due to the higher content of oils versus proteins in berries and leaves, respectively.

The phenolic compounds are well represented in leaves as well (Fig. 2B). The peak at 1454 cm^{-1} is produced by the bending C-H vibration or stretching aromatic ring vibrations from flavonoids (Sinelli *et al*., 2008). The peak at 1338 cm^{-1} is due to the in-plane C-O stretching vibration combined with the ring stretch of phenyl (Lu *et al*., 2011). Finally, the most intense peak at 1047 cm^{-1} suggests the presence of flavonoids or compounds containing alcohol groups trough the stretching vibrations of =C-O-C, C-C or bending vibration of C-OH bounds (Sinelli *et al*., 2008).

Chemometric approach of sea buckthorn berry and leaf carotenoids and phenolics by untargeted PCA analysis

The derivatized FTIR spectra enabled PCA to generate in the score plot six groups based on their predominant carotenoid profile (Fig. 3). The first two principal components (PCs) explained 92% and 89% of the spectra total variance level in case of berries and leaves, respectively. The score plot, generated from comparisons of the first two PCs, was as follows: PC1 explained 86% and 78% of the total variance in the data set and PC2 explained 6% and 11% for berries and leaves, respectively (Figs. 3A, B.).

Therefore, in the area defined by the first two principal components, a satisfactory sample distribution was found according to their FTIR fingerprint. Overall, each sample was able to form distinct clusters in the two dimensional plot.

Comparing group's formation in the PCA score plot of berry (Fig. 3A) and leaf (Fig. 3B), we can observe their opposite position along PC2. Excepting Victoria and Serbanesti groups, which were in both cases positioned in the centre, the groups which were situated in the top of one plot were found in the bottom of the other plot. According with the loadings plot

Tab. 2. Principal biomarker IR bands which characterize the specific absorptions of functional groups from carotenoids and phenolics, from the score loadings, that discriminates the sea buckthorn berries and leaves groups

PC loadings	Carotenoids key bands in sample clustering (cm^{-1})		Phenolic compounds key bands in sample clustering (cm^{-1})	
Berries	1745	Stretching vibrations of carboxylic groups (C=O) from carotenoid esters	1731	Stretching vibrations of C=O carbonyl group
	1743	The stretching vibrations of carboxylic groups (C=O) in the volatile oils, triglycerides, aliphatic esters	1622	Conjugated C=O bonds included in flavonoids
	1500	C–H, ($-CH_3$) symmetrical bending or the carboxyl groups (COO) of carotenoid esters	1033	Stretching C-OH vibrations of carbohydrates and glucosyl moieties
Leaves	1735	Stretching vibrations of -C=O ester groups from leaf lipid fraction	1616151 2 1454	Stretching vibrations (C=C) of the phenyl rings of flavonoids
	1458	Bending vibrations of CH_2) groups from carotenoids	1047	Stretching vibrations =C-O-C, C-C, and bending vibration of C-OH bounds or (C-H) in plane vibrations from flavonoids or hydroxyl groups

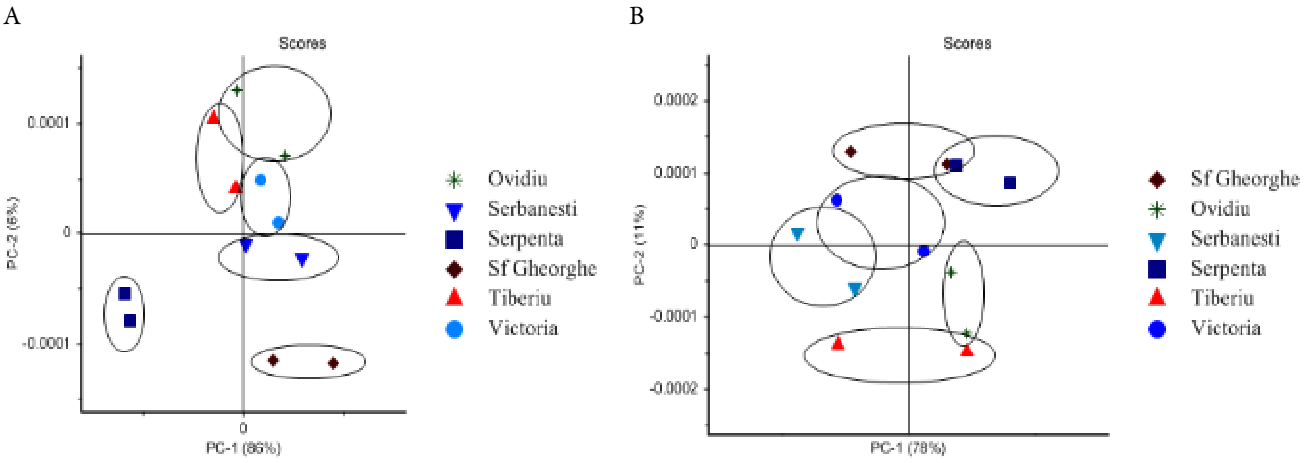

Fig. 3. Sea buckthorn berries (A) and leaves (B) score plots of the first two principal components, PC1 and PC2 for pre-treated based on the peak areas (normalized and second derivative), of IR fingerprint region (900-1800 cm^{-1}) specific to carotenoid extracts, from 'Victoria' variety

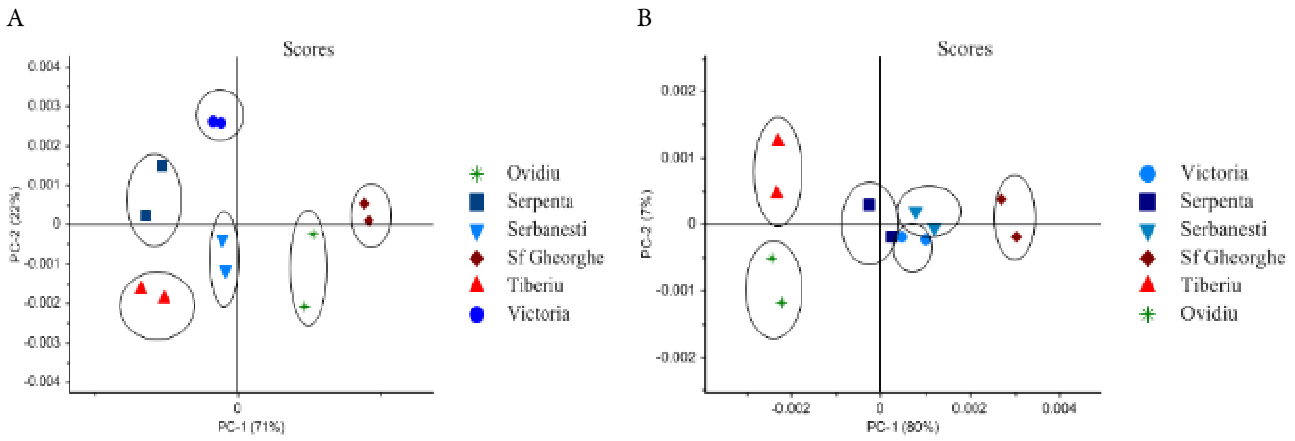

Fig. 4. Sea buckthorn berries (A) and leaves (B) score plots of the first two principal components, PC1 and PC2 for pre-treated based on the peak areas (normalized and second derivative), of IR fingerprint region (900-1800 cm^{-1}) specific to phenolic extracts from 'Victoria' variety

(data not shown), we can conclude that the carboxylic groups (C=O) had a big influence in the opposite sample clustering. Therefore, we can assume that berries are richer in carotenoid esters with fatty acids (1742 cm^{-1}), while leaves are rich in ethyl esters of fatty acids (1735 cm^{-1}). The corresponding biomarkers indicated by the loadings plot that influenced sample clustering are synthesized in Tab. 2.

When total phenolics metabolic FTIR fingerprints were used (Fig. 4), the first two principle components (PC1 and PC2) made up 93% and 87% of the total variance in case of berries and leaves, respectively. PC1 explained 71% and 80% of the total variance in the data set and PC2 explained 22% and 7% for berries and leaves, respectively. Six groups, corresponding to the number of investigated berries and leaves varieties, were clearly separated from negative to positive values of the two PC1 and PC2 scores (Figs. 4 A, B.).

In this case, the distribution of groups was made exclusively along the PC1 axis. 'Tiberiu', 'Ovidiu' and 'Serpenta' varieties were situated in the negative part, while Victoria, Serbanesti and 'Sf Gheorghe' varieties lied towards the positive part. Among the groups 'Sf Gheorghe' was negatively correlated with 'Tiberiu' and 'Ovidiu' because of their opposite position along the PC1 axis. The other groups, represented by 'Serpenta', Victoria and Serbanesti had a strong positive correlation, being closed to each other.

Conclusions

According to our experimental data, FTIR spectroscopy proved to be a simple and sensitive analytical technique successfully used as a rapid, alternative technique to the expensive chromatographic techniques, providing significant data for sample classification. Both carotenoids and phenolics fingerprints of sea buckthorn berries and leaves were different, dependent on variety. The complementarities were explained by the different ratios between lipophylic and hydrophilic compounds in berries and leaves, as well their specific inclusion in pigment-protein complexes, more relevant for leaves than for berries.

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