Fingerprinting Food Supplements and Their Botanical Ingredients by Coupled UV/Vis/FTIR Spectrometry

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Abstract. Medicinal plants are used as ingredients for a large variety of herbal supplements. Their quality and safety *versus* efficacy, according to present legal requirements, need to meet the minimum quality criteria to support their use. Specific biomarkers to evaluate and screen their authenticity are phenolic derivatives, phytosterols, lipids or alkaloids. We report here the data obtained for two herbal food supplements (A and B) obtained from different mixtures of plants: *Taraxacum officinalis*, *Cynara scolymus* *Silybum marianum* as ingredients for product A) and *Hypericum perforatum*, *Chelidonium majus* and *Lycopodium clavatum* as ingredients for product B).

The combination of UV-Vis and FTIR spectrometry allowed a specific fingerprint of biomarkers in individual plants and derived supplements (A and B), by discriminating the specific areas and peaks of individual plants and mixtures, the significant differences between the methanolic and water extracts.

The data were compared using chemometry (PCA and Cluster analysis).

Using Vis spectrometry combined with FTIR peak intensities at 1732 cm⁻¹ and calibration with gallic acid, the total phenolics concentrations ranged from 5.31 to 9.58 mg gallic acid eq/ml methanol, with a positive and significant correlation between the two methods ($R^2 = 0.979$). The phenolics' concentrations were 2.5 to 4 times lower in water extracts comparing with methanol extracts of products A and B. Finally, we assume that herbal supplements can be adequately characterized for their quality and safety by combined UV-Vis spectrometry/FTIR spectrometry, with good, fast and cheap informations about the main biomarkers of authenticity.

Keywords: herbal supplements, fingerprint, UV-Vis, FTIR, chemometry, authenticity

INTRODUCTION

Medicinal plants are used since centuries, as such or as ingredients for a large variety of herbal mixtures as food supplements. Their quality and safety *versus* efficacy, according to present legal requirements, need to meet the minimum quality criteria to support their use worldwide (Ramawat *et al.*, 2009; Verpoorte, 2009; Gong *et al.*, 2009). Meanwhile, the natural heterogeneity of the plants from herbal medicines determine fluctuations of the type and concentration of different phytochemicals found in medicinal plants, dependent on ontogenic or genotypic factors, influenced by environment, age, time of harvesting, drying and storage (Wichtl, 2004). These fluctuations determine also difficulties in identifying and quantifying the active molecules responsible for the therapeutic effect.

The main phytochemicals, generally used as markers of plant taxonomy and authenticity are secondary metabolites such as carotenoids/chlorophylls/phenolic acids/flavonoids/anthocyanins or monoterpenoids/unsaturated fatty acids/ phytosterols, but also, in some specific cases, saponins, lignans and indoles, thiols and glycosinolates, vitamins

A, C, E. All these classes of molecules have specific roles in plants either for attraction/defence mechanisms or for their antioxidant/antibiotic action. Their functionality and beneficial action for human health, proved to be largely dependent on their supramolecular architecture and environment, solubility and redox potential, stability, and bioavailability (Socaciu *et al.*, 2009). The appropriate identification of the herbs' quality and authenticity is successfully determined either by chromatographic (HPLC or GC) or by simpler methods such as Infrared spectroscopy (FTIR) combined with biostatistical tools (cluster analysis and linear correlation coefficient analysis). The last years, the integrative concept of plant metabolomics involve the use of advanced methodologies for identification and quantification of individual molecules (by MS or NMR) and facilitates an improved understanding of dynamic biochemical composition within the living systems, monitoring plant quality characteristics, identifying potential biochemical markers in individual plants and in mixtures, detecting product authenticity and possible adulterations. A four steps procedure to evaluate plant or food metabolic profile using complementary methods was reported (Socaciu *et al.*, 2009).

These fingerprinting approaches are often combined with multivariate analyses to get most out of the data and need validation. According to plant metabolomics methodology, the hyphenated complementary analytical techniques of liquid or gas chromatography coupled with MS or NMR detectors (GC/MS, LC/MS, LC/MS/MS, LC/NMR, LC/PDA/MS/FTIR in specific platform are yet functional in specialized EU centers, providing spectral libraries and metabolomic databases (www.phenol-explorer.eu; www.liberherbarum.com; www.metabolomics-lab.com; www.kegg.jp)

Beside these sophisticated methods applied to identify or discover new biomarkers of plant recognition, (Socaciu *et al.*, 2009; Mattoli, 2011; Baranska and Schultz, 2006; Fan *et al.*, 2006; Gong *et al.*, 2006; Duron *et al.*, 2009; Giri *et al.*, 2010) many laboratories use rapid and cheaper, available methods to fingerprint and authenticate the composition of such products, e.g. Fourier transform infrared spectroscopy (FTIR), a convenient method to fingerprint herbal medicines, especially when is validated with chromatographic (HPLC-UV or -PDA) analysis (Baranska and Schultz, 2006; Duron *et al.*, 2009).

Among the most important phytochemicals found in medicinal plants are the phenolic derivatives, a large family of secondary metabolites with various roles in plant defense, with antioxidant activity and beneficial health effects (Scalbert *et al.*, 2005). Until now, there were elaborated many analytical protocols to separate, identify and quantify phenolic acids, flavonoids and their glucosides, catechins, tannins (Santos-Buelga and Williamson, 2003; Singleton, 1999). The comparative FTIR/HPLC-PDA profile of European plants (*Echinacea sp. Melissa off.*) combined with chemometric analysis, or other plants from Romanian flora in relation with their phenolic content and antioxidant capacity was recently reported (Socaciu *et al.*, 2009; Spiridon *et al.*, 2011).

In this study we investigated two different herbal medicines with hepatoprotective potential, based on the traditional, longtime use of their ingredients (medicinal plants). For example, dandelion (*Taraxacum officinalis*) is an old remedy in the therapy of liver diseases due to „taraxacin” a mixture of eudesmanolide and germacranolide derivatives and phenolics (apigenol and luteolin) (Schutz *et al.*, 2005). Artichoke (*Cynara scolimus*) is known as colagogue and liver regenerative due to cynarin (Wang *et al.*, 2003). Milk thistle (*Silybum marianum*) acts as hepatocyte activator by „silymarin” flavolignan complex and taxifolin, a flavonoid (Ding *et al.*, 2001; Minakhmetov *et al.*, 2001). St John's wort (*Hypericum perforatum*) is known to contain hypericin, hyperforin and phenolics, mainly flavonoids (rutoside) (Mulinacci *et al.*, 1999; Barnes *et al.*, 2001). Celandine (*Chelidonium majus*) is rich in chelidonin with colecystokinetic and antispastic effects, antihepatotoxic and

anticancerogenic. Wolf's claw (*Lycopodium clavatum*) contains alkaloids and lignans, triterpens and flavonoids with good effects on liver tumor inhibition, anti-inflammatory and antimicrobial action (Pedersen and Ollgaard, 1982).

We report here experimental data obtained by comparative fingerprints of methanol and aqueous extracts of two herbal medicines (A and B) comparative to their individual plant ingredients. The first herbal product (A) was made from a mixture of dandelion, artichoke and milk thistle and the second one (B) contained St John wort, celandine and Wolf's claw.

We used in parallel UV-Vis spectrometry to fingerprint extracts' composition and to quantify total phenolics and Fourier Transform Infrared spectrometry for middle region (FTMIR = 650-4000 cm^{-1}). To validate the quantitative evaluation of phenolics by FTIR as a reliable quantification method for phenolics, calibrations with gallic acid were made and compared with Vis spectrometric data.

MATERIALS AND METHODS

Plant materials and sample preparation

Six medicinal plants were provided from a wild environment from Transylvania, Romania. These were numbered as follows: 1 - *Taraxacum officinalis* (Dandelion), 2- *Cynara scolimus* (artichoke), 3-*Silybum marianum* (milk thistle), 4- *Hypericum perforatum* (St John's wort), 5- *Chelidonium majus* (Celandine), 6- *Lycopodium clavatum* (Wolf's claw). Excepting milk thistle seeds, the other plants were used as a whole plant, without roots.

The dried plant samples were grounded, powdered and submitted to extraction, using duplicated samples from each plant. Two types of herbal supplements were obtained: A - by mixing powders of dandelion, artichoke and milk thistle, 1:1:1 (weight ratios), and B - from St John's wort, Celandine and Wolf's claw , 1:1:1 (weight ratios).

Aliquots of 15 g individual plant powder or herbal mix (A or B) were mixed with 85 ml solvent (methanol 90% in water, containing 1% HCl) and sonicated for 30 min in a bath and centrifuged 10 min at 2000 rot/min. The supernatant was collected and filtered through a 0.45 μm filter, representing the methanolic extract (ME). In parallel, for A and B products we made a water extract (WE), using the same concentration of 15% plant by the same extraction steps.

UV-Vis spectral analysis

The UV-Vis spectra of all extracts were recorded (700-200 nm) using a Jasco V 530 spectrophotometer. From each spectra, the specific fingerprint was recorded, as well the maxima wavelengths, specific to phenolic derivatives (280 and 330 nm). In these conditions, we compared the fingerprint of ME from individual plant extracts with the fingerprint of supplements A and B in both ME and WE.

Determination of Total phenolics by Vis spectrometry

Aliquots of 1 ml ME extracts from each plant and A /B supplements were mixed with water, 5 ml Folin reagent, homogenized with 15 ml Natrium carbonate 7,5 %, according to the method of Singleton (1999). After 2 hrs, the absorbance at $\lambda = 750 \text{ nm}$ was recorded against a blank. In parallel, a calibration curve was built with pure gallic acid as a standard, in the range of 0.0625 to 1 mg/ml methanol. Considering the curve equation $y=0.515x+0.0504$, with $R^2=0.9993$, the total phenolic concentrations of all samples were calculated.

FTIR analysis

The FTMIR spectra of fingerprint regions, specific for ME extracts of the individual plants 1-6 and herbal supplements A and B were determined using a FTIR spectrometer (Shimadzu Prestige 2, Apodization: Happ-Genzel spectrometer). Each spectrum was recorded in the MIR region (4000 to 650 cm^{-1}) with 64 scans were accumulated for each spectrum using the Horizontal Attenuated Total Reflection (HATR) device. The spectra were processed by IR solution Software Overview (Shimadzu) and OriginR 7SR1 Software (OriginLab Corporation, Northampton, USA).

To evaluate quantitatively the phenolic concentration of each sample, the FTIR spectra were calibrated with gallic acid (0.0625 to 1 mg/ml methanol). Two different calibration curves were made previously, considering either the total peak areas of fingerprint region (1610 - 1743 cm^{-1}), or the absorption intensity at 1610 cm^{-1} or at 1732 cm^{-1} . The best correlation IR absorption intensity = $f(\text{gallic acid})$ was found considering the intensity at 1732 cm^{-1} , with a curve equation $y=0.0819x+0.0184$ and $R^2=0.9994$ (data not shown).

RESULTS AND DISCUSSIONS

UV-Vis spectroscopy data

Fig. 1 shows the comparative UV spectral fingerprints (200 - 500 nm) of the herbal supplements A and B in ME.

In these conditions, each ingredient of the product A showed its specific spectral shape. Previously we made the spectra of all ingredients (Zavoi et al., 2011a), the dandelion extract showed three main peaks, at 280 , 330 and 390 nm , while artichoke had also three absorption regions, more intense in the range 240 - 280 nm . Specifically, milk thistle seed extract had a higher peak absorption at 280 nm with a shoulder located at 320 nm and no absorption at 390 nm . In the spectra of product A one can identify the shape of a dominant peak originating from milk thistle (270 - 290 nm), a large peak from 300 to 400 nm and a peak at 420 nm attributed to oxidized forms of phenolics (chinones).

The plants used as ingredients for product B have had different fingerprints: St John wort had a dominant peak in the region 270 - 290 nm and at 520 nm , as indicator of Hypericin. Celandine had three absorption regions, at 270 - 290 nm , 330 - 350 nm and 390 nm , while wolf's claw extract showed no absorptions at 280 nm , but large peaks between 320 and 350 nm and 400 - 420 nm . Looking to the spectra shape of product B extract, one can identify a combination of peaks in the region 270 - 290 nm (from St John wort and celandine) combined with the absorption regions specific to wolf's claw, at 320 - 350 nm and 400 - 420 nm (Zavoi et al., 2011).

Fig. 1. Comparative UV-VIS spectral fingerprints (200 - 500 nm) for methanol extracts (ME) products A (left) and B (right)

When the ME extract was compared with WE, lower extraction yields were obtained in water, e.g. 2.5 times lower for A and 4 times lower for product B, suggesting that B has a

less polar composition than A. We can assume that UV spectra fingerprints give general information about the polar phenolics' solubility and ratios between their subclasses.

According to Tab. 1, dandelion, artichoke and celandine extracts, followed by St John wart extract, were richer in phenolics (from 9.30 to 8.38 mg gallic acid eq. /ml ME) while wolfs' claw and milk thistle were less (7.69 and 6.66 mg gallic acid eq. /ml ME, respectively). The product A had higher concentration (9.37 mg gallic acid eq. /ml ME) than B (9.08 mg gallic acid eq. /ml ME), but not significantly different. In WE *versus* ME, the TP concentrations of A and B were 2.4 and 3.8 times lower, respectively, similarly to the differences observed in UV spectral data.

Tab. 1

Comparative evaluation of total phenolics (expressed in gallic acid/ ml ME) in plant extracts (1-6) and herbal supplements A and B, using Vis spectrometry (VIS) and FTIR, calculated from the peak intensity at 1743 cm^{-1} (I_{1732}). The values represent the average of duplicate measurements. Details are described in *Materials and methods*

Sample	Total Phenolics (mg Gallic acid /ml ME) VIS	I_{1732}	Total Phenolics (mg GAE /ml ME) (FTIR)
Dandelion	9.30	0.930	9.39
Artichoke	9.06	0.906	9.15
Mary Thistle	6.66	0.516	5.31
St. John's Wort	8.38	0.834	8.42
Celandine	9.17	0.918	9.27
Wolf's claw	7.69	0.764	7.71
Product A	9.37	0.950	9.58
Product B	9.08	0.890	8.98

FTIR fingerprints and quantification

Fig. 2 shows the FTIR overlapped spectra ($650\text{-}4000\text{ cm}^{-1}$) of methanol extracts corresponding to the six individual plants : the fingerprints of Dandelion Artichokes and Milk Thistle (Fig.2A), used to obtain A mix and the fingerprints of St. John's Wort, Celandine and Wolf's claw (Fig.2B), used to obtain the B mix. Fig. 2 shows also a comparative FTIR fingerprint of A and B in ME (Fig.2C) and WE (Fig.2D).

Fig. 2. FTMIR overlapped spectra ($650\text{-}4000\text{ cm}^{-1}$) of ME of the six individual plants : upper left – Dandelion, Artichoke and Milk Thistle , upper right- St. John's Wort, Celandine and Wolf's claw, comparatively with ME spectra of herbal supplements in ME (down, left) and WE (down, right) for A (solid line) and B (dots)

One can identify in the methanolic extracts, eight specific regions (marked 1-8 in the figure) corresponding to functional groups which can be found in these extracts, and attributed to stretching and bending IR vibrations.

Tab. 2

Mean values of the peak absorption intensities corresponding to the 8 wavenumber areas of FTIR spectra (4000- 650 cm^{-1}), for herbal supplements A and B, in water (WE) and methanolic (ME) extracts

Wavenumber area (cm^{-1}) / Samples		WE		ME	
		A	B	A	B
3300-3350 (8)		0.95	0.58	0.55	0.74
2800-3000 (7)		0.32	0.29	1.15	1.01
1600-1750 (6)	1732 cm^{-1}	0.82	0.56	0.95	0.89
	1620 cm^{-1}	0	0	0.66	0.67
1500-1520 (5)		0.31	0.25	0.35	0.38
1300-1440 (4)		0.55	0.48	0.75	0.85
1170-1230 (3)		0.28	0.46	0.91	1.15
1000-1100 (2)		0.59	0.65	1.05	1.55
<1000 (1)		0.65	0.62	0.78	0.85

Tab. 2 presents the mean values of the peak absorption intensities corresponding to the 8 wave number areas of FTIR spectra (4000- 650 cm^{-1}), for herbal supplements A and B, in water (WE) and methanol (ME) extracts.

The fingerprint region was localized between 900 and 1750 cm^{-1} (areas 1-6). Area 1 (< 1000 cm^{-1}) corresponds to C-H bending vibrations found in isoprenoids, area 2 (997-1130 cm^{-1}) to stretching vibrations C-O of glucoside bonds, while area 3 (1150-1270 cm^{-1}) corresponds to stretching vibrations of carbonyl C-O or O-H bounds. Area 4 (1300-1450 cm^{-1}) is attributed to stretching vibrations C-O (amide) and C-C bonds from phenyl groups, while area 5 (1500-1520 cm^{-1}) to aromatic domain and N-H bending vibrations. Area 6 (1600-1750 cm^{-1}), is a complex one corresponding to bending vibrations N-H (amino acids), C=O stretching (from aldehydes and ketones, esters) as well to carboxyl groups from free acids (1710 cm^{-1}) or esters (1740 cm^{-1}). The fingerprint of each individual plant, as well for A and B extracts in ME was specific and different in these 1-6 areas (Fig.1). For A and B in WE, many of the specific peaks were lost, the fingerprint was less complex and significantly different comparing with ME.

The area 7 (2800-3000 cm^{-1}) corresponding to C-H stretching vibrations specific to CH_3 and CH_2 from lipids, metoxy derivatives, was identified only in ME and not in WE, being a good indicator of the presence of lipophilic compounds in the extract. Area 8 (3000-3600 cm^{-1}) corresponds to stretching vibrations of OH groups (from water, alcohols, phenols, carbohydrates, peroxides) as well from amides (3650 cm^{-1}), and was not considered for the fingerprint analysis.

Comparing the fingerprints of the six plants, we noticed differences between the peaks 2 3, 4 and 6. Dandelion, artichoke and St John had high intensities of area 2, which can be correlated with their higher concentration in soluble phenolics (glucosides). The ME of white milk thistle and wolfs'claw had 2 peaks in the area 6 (at around 1610 and 1732 cm^{-1}) while the other plants had only one peak, at around 1732 cm^{-1} . The difference can be correlated with the higher concentration in lipid components of milk thistle and wolfs'claw. The fingerprints of A and B supplements differ in the regions 2-4, where B shows higher intensities, correlated with stretching vibrations of C-O bonds from glucosides and esters.

In order to validate these measurements, the FTIR spectra of pure gallic acid, alone or mixed with dandelion extract, identifying the most specific peaks corresponding to

phenolics, which were located at 1610 and 1732 cm^{-1} . The peak intensities at 1610 and 1732 cm^{-1} were better correlated and indicated lower phenolic concentrations for plants no. 3 (milk thistle) and 6 (wolf's claw), in agreement with the spectrometry data. According to the calibration curve made with gallic acid, the best correlation IR absorption intensity = f(gallic acid concentration) was found at 1732 cm^{-1} with a R^2 value of 0.994, then we used the curve equation $y=0.0819x+0.0184$ to calculate the total phenolics based on the peak intensity at 1732 cm^{-1} (Zavoi, 2011b).

As shown in Tab.1, the concentrations of total phenolics (expressed in mg Gallic acid/ ml ME) in plant extracts (1-6) and herbal supplements A and B were compared using Vis spectrometry (VIS) and FTIR, considering the peak intensity at 1732 cm^{-1} . A significant ($p<0.001$) correlation factor ($R^2=0.979$) was obtained, these findings showing that FTMIR absorption intensity at 1732 cm^{-1} is a reliable parameter and FTMIR can offer an adequate evaluation of the phenolics concentration in medicinal plants or herb supplements.

To summarize the data presented in this study showed that UV-Vis spectrometry achieves a general fingerprint and quantification of phenolics as biomarkers of herbal formulas (A and B). The FTMIR fingerprint was very useful in discriminating the specific peaks of functional groups of molecules from individual plants and mixtures, reveals differences between methanolic and water extract, which had a different pattern and composition, in good agreement with the UV-Vis data.

Using Vis spectrometry coupled with FTIR based on peak intensities at 1732 cm^{-1} and calibrated with gallic acid, the total phenolics ranged from 5.31 to 9.58 mg gallic acid eq/ml ME, with a positive and significant correlation between the two methods ($R^2= 0.979$). The TP concentrations were 2.5 to 4 times lower in WE comparative to ME of products A and B.

This study demonstrated that herbal supplements, based on medicinal plant ingredients can be adequately characterized for their quality and safety by combined UV-Vis spectrometry and FTIR spectrometry. These easy-to-handle, cheaper and fast methods gives valuable information about the main biomarkers of authenticity, their concentration, the minor components, the key-molecules of authenticity and to identify the adulteration of herbal supplements.

Our studies can contribute to data cumulated in the European databank of Natural resources, including medicinal plant composition and many derived formulas, many of them known as "traditional medicines". These techniques can offer a good and efficient tool to demonstrate the quality and authenticity of herbal supplements and predict their potential for biomedical applications.

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