Complementary Advanced Techniques Applied for Plant and Food Authentication

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Abstract: Our studies focus on the characterisation of specific metabolic profiles of some representative plants from Romania (St. John wort, soybean and seabuckthorn berries) and their derived products (oils, hydrophilic and lipophilic extracts) with biomedical and food applications. Four different, complementary methods were applied successively: UV-Vis and FTIR spectroscopy versus chromatography (HPLC-PDA, GC-FID). To investigate accurately the main biomarkers specific to each plant tissue (secondary metabolites) or to final products were determined as useful quality and authenticity indicators: carotenoid pigments, phenolic derivatives and flavonoids, fatty acids and sterols or other specific metabolites. We found that spectroscopy can give direct and ready-to-use preliminary information on the fingerprint of functional groups in the plant tissue or extracts, while chromatography gives qualitative and quantitative information related to individual molecules which characterise the plant or specific extract. Such complementary methods have a good performance/price ratio and may be used either in agrifood metabolomics research or in laboratories for food and phytopharmaceuticals' control, in order to evaluate their quality, authenticity/adulteration and traceability in the production and marketing chain (from field to consumer).

Keywords: St. John wort; soybean; seabuckthorn berries; Romania; UV-Vis and FTIR spectroscopy; HPLC-PDA; GC-FID; fingerprint; food authenticity

INTRODUCTION

The determination of food quality, authenticity or traceability of some markers in the production chain has improved dramatically the last 15 years due to the large extension of advanced analytical techniques which offer information about main characteristics of nutrients and minor components originating from raw materials (plants, animal tissue). For plant-derived food, the secondary metabolites are used as markers of biological or geographical authenticity, as well for adulteration and traceability studies, representing the metabolic profile or fingerprint (Hall *et al*. 2002; Lees 2003).

There are four important issues which are addressed for metabolite analysis: an efficient and unbiased extraction of metabolites from specific plant tissue of food matrix (1), the identification of specific groups (fingerprint) of compounds without separation, directly based on their absorption characteristics (using UV-Vis, FTIR, NIR or Raman spectroscopy) (2) (Baeten & Aparicio 2000), the identification and quantification of specific unique metabolites by succesive enzymatic reactions (3) (Baranska & Schultz 2006) or a combination of accurate chromatographic or electrophoresis separations coupled generally with photodiode array (PDA), mass-spectrometry (MS), fluorescence or IR spectrometry detection and quantification (Barnes 2001) (4).

The impressive development of chromatographic methods (GC-FID, GC-MS, HPLC-PDA, LC-MS) widely used now allow high resolution separation of plant and food biomarkers. More than ten types of edible oils of different origin, quality and sales volume are sold in Europe. Their functionality is often related to the attractiveness to fraud, olive oils being a good example. The standards of the Codex Alimentarius Commission (CAC) for fats

and oils helps to verify mainly their quality parameters (moisture, impurities, free fatty acids and peroxide value) but not their functionality markers related to their origin and nutritional quality (crude, extra-virgin, or refined) (Aparicio & Aparicio-Ruiz 2000). A considerable number of chromatographic protocols have been developed for the determination of the composition of various vegetable oils by accurate fingerprint of fatty acids, sterols or carotenoids, chlorophylls, phenolics and tocopherols (Cserháti *et al*. 2005).

Another area of high interest for human health is represented by botanical supplements, obtained from medicinal plants or cereals, used as ingredients for pharmaceuticals, nutraceuticals and functional food. Advanced methods, similar to ones mentioned in oils, are needed nowadays to characterise the most relevant biomarkers (especially polyphenolic derivatives, pigments and vitamins) useful to standardise, authenticate and to identify adulteration of such products.

Our previous experience and results (Socaciu 2003; Socaciu *et al*. 2005) are related to phytochemicals fingerprint in functional oils or plant extracts, at the best performance/price/time methods.

St. John wort is used for centuries, orally and topically, as an anti-inflammatory, sedative, antiviral, enhancer of wound healing, but especially as anti depression plant (Barnes *et al*. 2001). The effects are due mainly to its high content of naphthodianthrones (hypericin**,** pseudohypericin**,** isohypericin, protohypericin), and many polyphenol derivatives (Kurth & Spreemann 1998).

Soybeans are known to be rich in functional lipids (unsaturated fatty acids, carotenoids and sterols) as well in antioxidant phenolics (especially isoflavones) with estrogen-like effects (Patel *et al*. 2004; Kanamaru *et al*. 2006). Sea Buckthorn fruits contain many hydrophilic and lipophilic bioactive components, the berry oil or powder or juice being considered functional foods due to their rich content in vitamin C and flavonoids, unsaturated fatty acids, carotenoids, sterols,tocopherols and plant sterols (Socaciu 2003, 2008; Zadernowski *et al*. 2005; Chen *et al*. 2007; Ranjith *et al*. 2008).

We present here results obtained from the application of 3 complementary methods to characterise a botanical supplement made of St. John wort (*Hypericum perforatum*) extract (HE) and 3 functional extracts and oils obtained from soybean (*Glycine max*) (S) and seabuckthorn (*Hippophae rhamnoides*) (SB), interesting for their health-protective actions. We used the most advanced chromatographic methods to fingerprint and evaluate quantitatively the authenticity biomarkers, complemented by the cheaper, non-destructive FTIR-ATR spectrometry.

MATERIALS AND METHODS

Soybean (provided from a Romanian farm) was analysed as powder (S), as methanol extract (methanol 95% + 1% HCl conc.) (Sm) or as ethyl ether extract (So) with 15% plant tissue per 100 ml. The soybean crude oil (SO) (provided from Biolon-Gasomil Blaj, Romania) was analysed as such or saponified.

St. John wort was analysed as a methanolic extract (methanol 95% + 1% HCl conc.) (SJm) or acethyl ether extract (SJo), 15% plant tissue per 100 ml. The oil (SJO) was obtained by long-time (6 months) maceration under sun light, in sunflower oil.

Seabuckthorn fruits extracted in the same acid-methanol solution (SBm) and in ethyl ether extract (SBo) were analysed using 20 mg fruit per 100 ml solvent. The crude berry oil (SBO) (provided from SC Chimica Bistrita, Romania) was obtained by solvent extraction and vacuum evaporation.

Instrumentation and protocols

To fingerprint the UV-Vis spectra, a Jasco V 530 spectrophotometer was used and the spectra were recorded from 200 to 600 nm.

Carotenoids were analysed using a HPLC-PDA Shimadzu chromatograph and a Restek Ultra Aqueous C18 column while phenolics were determined using HPLC Agilent 1200 coupled with UV detection and a 5 μm Supelcosil LC18 column, according to our own protocol (Socaciu *et al*. 2005). For total fatty acids analysis, fatty acid methyl esters (FAMEs) were prepared by transesterification of the oil samples by sodium methoxide catalysis (Christie 1982). The GC-FID analysis was made by Shimadzu GC-17-A and 30 m Alltech AT-WAX capillary column.

The sterols (esterified and free) were determined as TMS derivatives by GC-FID separations using RTX-5^{TM} fused silica capillary columns. Identification of sterols was based on their relative retention times (R_t to β-sitosterol) comparing with literature data (Laakso 2005; Phillips *et al*. 2005). The ste-

rol concentrations were calculated using the area of the internal standard peak. All determinations were performed in triplicate. The mean values and standard deviations were calculated.

The FT(ATR)MIR spectra were obtained with a Shimatsu-Prestige spectrometer, equipped with horizontal Attenuated Total Reflectance (HATR) accessory and registered from 3200 to 650 cm^{-1}. The spectra interpretation was made in accordance with literature data (Baeten & Aparicio 2000; Ellis *et al*. 2003; Baranska & Schultz 2006; Schultz & Baranska 2007).

RESULTS

To characterise comparatively the phytochemicals found in these plants, we used complementary measurements by UV-Vis and FTIR spectrometry (fingerprints of functional groups) and in parallel HPLC-PDA or GC-FID analysis to identify and quantify individual molecules.

1. UV-Vis spectra of metanolic extracts (SJm, SBm, Sm) (dil. 1:100) are shown in Figure 1. These spectra are useful to identify the specific bioactive classes of molecules found in different concentrations: carotenoids ((λ_{max}: 420–480 nm) (Figure 1A) and polyphenols (λ_{max}: 260–280 nm for phenolic acids and and 350–360 nm for flavonoids) (Figure 1B). The richest extract was SBm with high absorptions in the carotenoid and flavonoid regions, while Sm was rich in carotenoids and phenolic acids, but poor in flavonoids.

2. The GC-FID fingerprint of fatty acid composition of oils (SO, SBO) was presented comparatively with sunflower oil (SFO) (Figure 2).

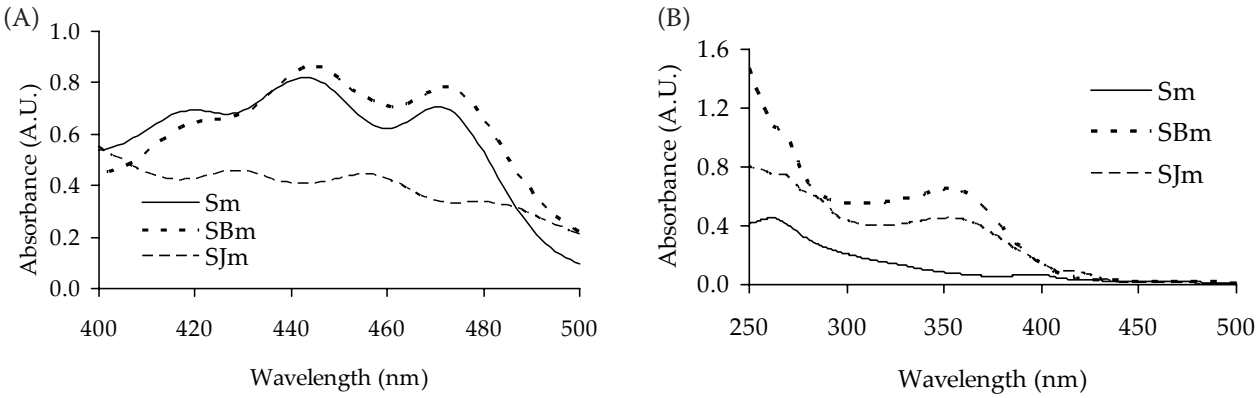

Figure 1. UV-Vis spectra of oil extracts (dilution 1:100), useful to identify their zspecific fingerprint for 2 classes of bioactive molecules: (A) carotenoids ((λ_{max}: 420–480 nm) and (B) polyphenols (λ_{max}: 260–280 and 350–360 nm)

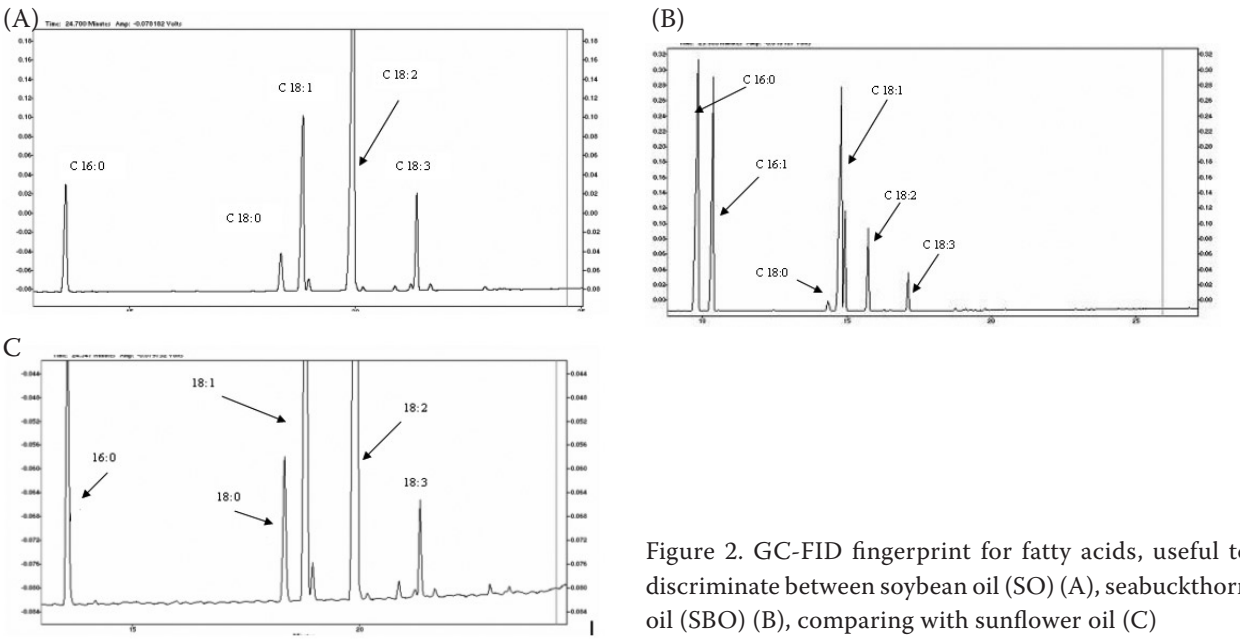

Figure 2. GC-FID fingerprint for fatty acids, useful to discriminate between soybean oil (SO) (A), seabuckthorn oil (SBO) (B), comparing with sunflower oil (C)

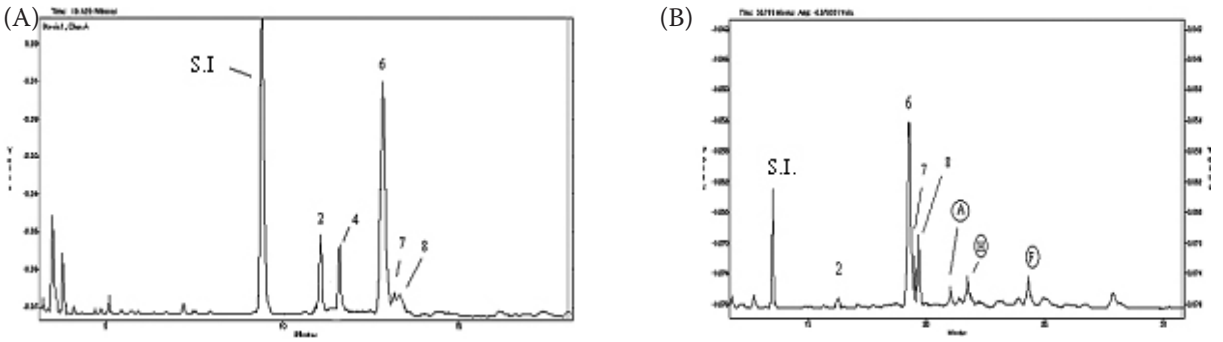

Figure 3. GC-FID fingerprint of esterified sterols useful to discriminate between soybean oil (SO) (A), seabuckthorn oil (SBO) (B). Peak identification: S.I.: 5α-colestan-3β-ol; 2: campesterol; 4: Stigmasterol; 6: β-sitosterol; 7: sitostanol; 8: Δ5-avenasterol; A and X – unidentified sterols

One can recognise easyly their specific qualitative composition but also the relative ratios between major markers. For example, SO is characterised by high C18:2 (45.2%) and C18:1 (27.73%) out of total fatty acids, while SBO was rich in C16:0 (32.27%), C16:1 (25.46%) and C18:1 (23.31%). Such compositions differ significantly from SFO.

3. The GC-FID fingerprint of esterified sterols (Figure 3) allows also a good discrimination between SO (A), and SBO (B). Both oils are rich in β-sitosterol (6) but SO contains campesterol (2) and stigmasterol (4) in high concentrations aprox. ratio 1:1.Meanwhile SBO contains other, non-identified sterols (A and X).

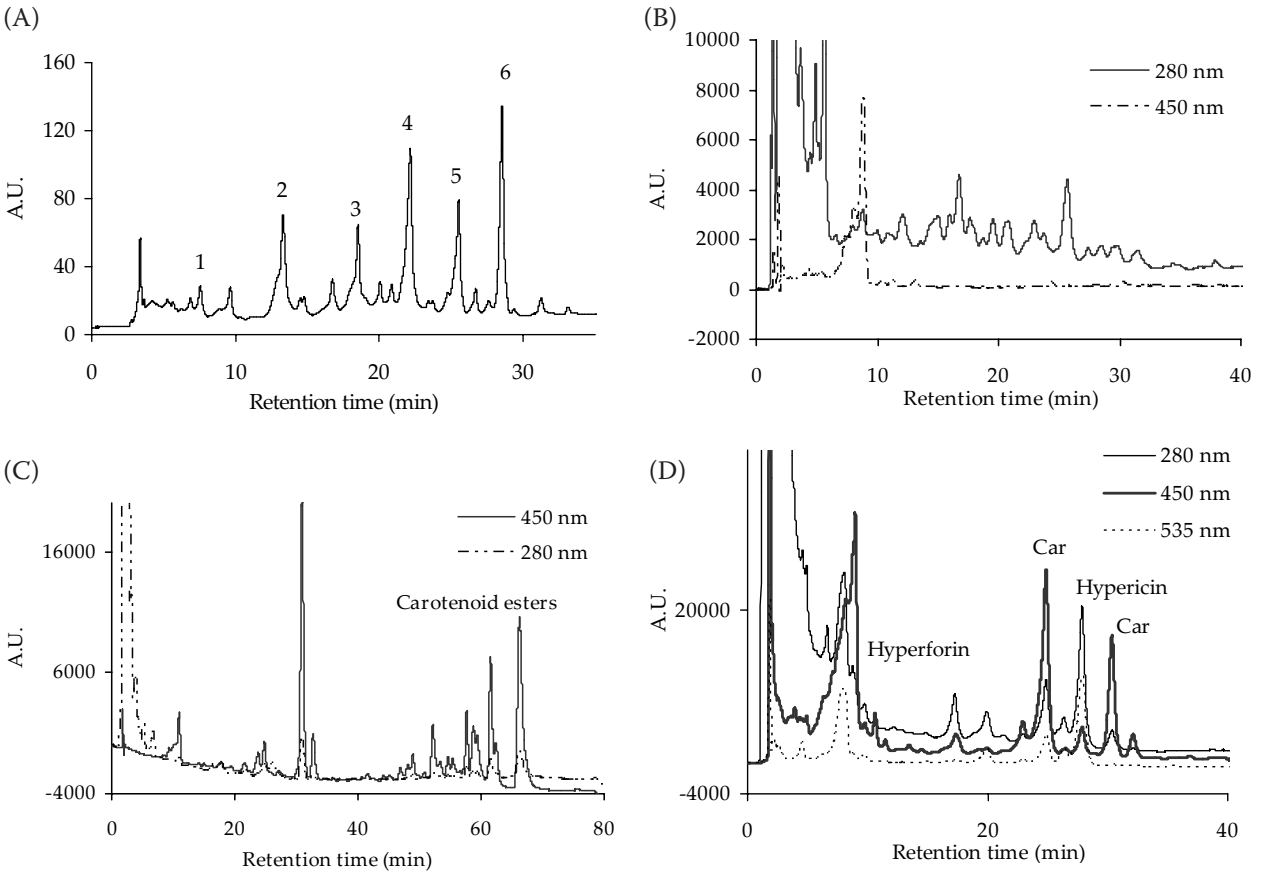

Figure 4. A–D. HPLC-DAD applied to fingerprint concomitantly phenolics and carotenoid profile of Sm and So (A and B), SBo (C), and SJo (D) extracts, recorded at 280 and 450 nm. By parallel screening at 535 nm in SJo we identified its typical biomarkers. Similar HPLC-DAD for SJo, reveres concomitantly carotenoid fingerprint and specific markers hypericine and hyperforine (D). For peak identification see Results

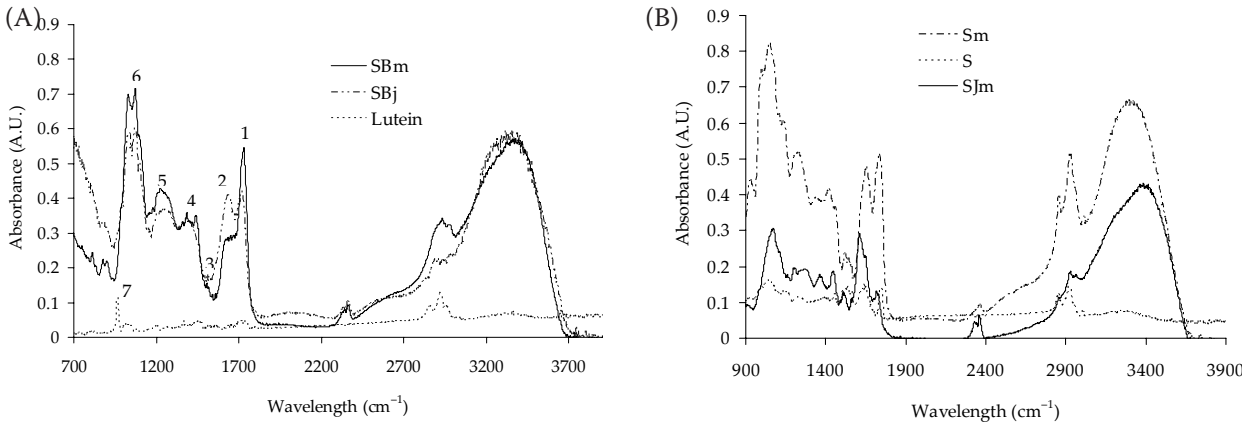

Figure 5. Comparative FT(ATR)MIR fingerprint regions of SB extract (SBm), juice (SBj) and pure Lutein (A) and soybean powder (S0 or extract (Sm) comparatively with SJm (B). The distinct peaks correspond to 1720–1734 cm^{-1} (1), 1620–1695 cm^{-1} (2), 1516–1550 cm^{-1} (3), 1238–1396 cm^{-1} (4), 1132–1134 cm^{-1} (5), 1024–1029 cm^{-1} (6), 961–964 cm^{-1}(7). For assignments see Results section

4. As it is shown in Figure 4 (A–C) by HPLC-PDA we realised a fingerprint of phenolics and carotenoids or other markers either in Sm and So (A and B) and SBo (C) extracts, registerd at 280 and 450 nm. For soy extracts we had different fingerprints in methanol (A) and ethyl ether (B), identifying caffeic acid (2), *p*-coumaric (3), syringic (4) acids as well isoflavones like daidzein (5) and genistein (6). The non-polar solvent extracted differently the phenolic molecules, more difficult to identify (B-solid line), but easier for lutein and zeaxanthin, as major carotenoids (B, dotted line). For SBo, a clear image of carotenoid fingerprint (β-carotene is the major peak at 450 nm) (C-solid line). For SJo (D) we had a concomitant fingerprint at 280, 450 and 535 nm, identifying *p*-coumaric acid (t_R = 18.5 min), lutein (t_R = 18.5 min), zeaxanthin and β-carotene and hyperforine and hypericine, respectively.

5. The FT-ATR-MIR spectroscopy measurements (Figures 5 A,B) reveal the fingerprint regions (A) specific to carotenoids (961–970 cm^{-1} and 2800 to 3000 cm^{-1}, as confirmed by pure lutein (7), oil-specific lipids (peaks 1, 2 and 3), glucosides of phenolics, organic acids and alcohols (including the solvent used) (peaks 4, 5 and 6). It is possible to discriminate between peak 6 (glucosides and carbohydrates) and peaks 1 and 2 (fatty components) when S was compared with Sm. The SJm extract was poor in bioactive molecules comparing with Sm, while SBm and SBj are similar in their IR fingerprints.

CONCLUSION

We were able to use complementary methods (chromatographic and spectroscopic) to identify specific markers of soybean, seabuckthorn or St. John wort. Each method brought specific data, and by complementary information they are relevant for the plant authentication, as well for the establishment of the quality of derived food or food ingredients obtained by selective extraction in hydrophilic or lipophilic solvents. The case-studies we used here may demonstrate that complementary methods such as UV-Vis and FTIR spectroscopy coupled with GC and HPLC-accurate chromatography can offer important, valuable information on agrifood quality and authenticity.

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