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An Integrated Approach to the Evaluation of a Metabolomic Fingerprint for a Phytocomplex. Focus on Artichoke [*Cynara cardunculus* subsp. *scolymus*] Leaf

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The availability of reliable herbal formulations is essential in order to assure the maximal activity and to limit unwanted side-effects. The correct concentration of declared components of herbal products is a matter of health legislation and regulation, but is still a topic under debate in the field of quality control assessment. In the present work specific constituents of artichoke leaf extracts, considered as a test herbal product, were measured by standard spectrophotometric and HPLC methods (for quantitative determination of some components only), and results were correlated with the ESI-MS (showing the full metabolomic fingerprint). Phytocomplex stability over time was also investigated in batches submitted to different storage conditions. The results indicated excellent agreement between the two approaches in the measurement of total caffeoylquinic acids and chlorogenic acid contents, but the metabolomic ESI-MS method approach provides a more complete evaluation and monitoring of the composition of a herbal product, without focusing only on a single/few compound measurements. Therefore, the ESI-MS method can be proposed for the evaluation of the quality of complex matrices, such as those in a phytocomplex. Another aspect lies in the possibility to obtain a broad-spectrum stability control of herbal formulations, requiring minimal sample pre-processing procedures.

Keywords: ESI-MS, Phytocomplex, Artichoke, Herbal product stability, Metabolomics, Multivariate analysis.

Although used since antiquity, herbal remedies have been considered under a scientific, evidence-based methodology only in recent times. Statistical surveys have demonstrated that resorting to natural remedies is a growing aspect of self-medication and also a choice of many physicians who recognize a useful and gradual approach to either the cure of illnesses or to help maintain a healthy status [1-3]. Under this view, herbal medicine remains the most popular among complementary and alternative medicines (CAM) [4]. Moreover, the investigation of locally available herbal medicines opens interesting frontiers in rational medicinal plant use and in economical appreciation of local resources as a modern development of ethnopharmacology [5, 6]. Under this scenario, the availability of reliable herbal formulations is essential, in order to assure the maximal activity, limit unwanted side-effects, and avoid dangerous interactions [7,8]. The presence of a correct concentration of declared components is also a matter of health legislation and regulation [9,10]. However, the field of quality control assessment of herbal products is still in an evolving state which encourages further scientific contributions [11-15].

The aim of this work is the comparison of single markers of a phytocomplex, as conventionally determined in quality control, with the overall metabolomic fingerprint, i.e., a new method of global evaluation of the phytocomplex components. For this purpose, leaves of artichoke, a well characterized herb widely employed for digestive complaints [16], were used. The measurements were carried out on extracts by using spectrophotometric and HPLC methods (for specific constituents' quantitative determination), and results were correlated with the ESI spectrum of all constituents (metabolomic fingerprint). A comparison of the different methods was carried out, also focusing on phytocomplex stability over time. Since the above mentioned conventional quantitative methods are currently used as a standard reference, the comparative role of newly proposed global metabolomic fingerprint determination [12,13] was evaluated.

The quantitative analysis of extracts was evaluated by a spectrophotometric method on samples undergoing different simulated shelf-life conditions, i.e. "conventional storage conditions" (CSC) and "accelerated storage conditions" (ASC) (see Experimental section for details). CSC and ASC samples gave the results reported in Table 1, expressed as total caffeoylquinic acids content. Although the three batches significantly differed (P<0.001, ANOVA), the basal value remained substantially unchanged within each batch, both under CSC and ASC conditions. The subsequent analysis by using HPLC to quantify the amount of chlorogenic acid gave the results reported in Table 2. Also in this case, the three batches significantly differed (P<0.001, ANOVA) but the basal value of chlorogenic acid remained substantially unchanged within each batch in both CSC and ASC conditions (Table 2).

By the ESI-MS method, the extracts were directly introduced into the instrument by Flow Injection Analysis (FIA), without any chromatographic separation, and spectra were obtained in negative mode in the range 50-1500 *m/z*. In previous investigations it has been found that the negative ion spectra of natural products are much more informative than those achieved in positive ion mode [12]. In the latter case, the spectra are highly complex, due to a particularly intense chemical background, which results in difficult interpretation. A typical spectrum obtained in ESI(-) mode is

reported in Figure 1; the analysis permitted the evaluation of more than 1000 ionic species in a single data capture. For this reason, the multivariate statistical approach was used, in order to track any change in the overall profile of the spectrum within batches and after different conditions of storage.

Table 1: Spectrophotometric determination of total caffeoylquinic acids in the three different batches (11G, 11F and 12B) of artichoke extract at basal condition (T0) and after different simulated shelf-life conditions (T7: 7 days; T30: 30 days; T60: 60 days). CSC: "conventional storage conditions", samples stored at 25±2°C and 60±5% relative humidity. ASC: "accelerated storage conditions", samples stored at 40±2°C and 75±5% relative humidity. Data are reported as mean ± SD of three replicates.

Time and storage conditions	Caffeoylquinic acid (%) ± SD		
	11G	11F	12B
T0	1.62 ± 0.11	3.17 ± 0.20	2.32 ± 0.18
T7 CSC	1.65 ± 0.10	3.05 ± 0.22	2.37 ± 0.16
T30 CSC	1.60 ± 0.10	3.09 ± 0.19	2.45 ± 0.13
T60 CSC	1.71 ± 0.13	3.04 ± 0.22	2.54 ± 0.17
T7 ASC	1.63 ± 0.11	3.19 ± 0.18	2.38 ± 0.12
T30 ASC	1.74 ± 0.13	3.20 ± 0.20	2.32 ± 0.16
T60 ASC	1.73 ± 0.12	3.24 ± 0.21	2.42 ± 0.14

Table 2: Chlorogenic acid content measured by HPLC in the three different batches (11G, 11F and 12B) of artichoke extract at basal condition (T0) and after different simulated shelf-life conditions (T7: 7 days; T30: 30 days; T60: 60 days). CSC: "conventional storage conditions", samples stored at 25±2°C and 60±5% relative humidity. ASC: "accelerated storage conditions", samples stored at 40±2°C and 75±5% relative humidity. Data are reported as mean ± SD of three replicates.

Time and storage conditions	Chlorogenic acid (%) ± SD		
	11G	11F	12B
T0	0.63 ±0.03	2.21 ± 0.03	0.93 ± 0.03
T7 CSC	0.65 ± 0.02	2.43 ± 0.05	0.96 ± 0.03
T30 CSC	0.67 ± 0.03	2.19 ± 0.05	0.92 ± 0.05
T60 CSC	0.68 ± 0.03	2.27 ± 0.02	1.01 ± 0.04
T7 ASC	0.60 ± 0.02	2.16 ± 0.05	0.96 ± 0.03
T30 ASC	0.65 ± 0.03	2.35 ± 0.04	0.98 ± 0.04
T60 ASC	0.66 ± 0.02	2.28 ± 0.02	0.92 ± 0.05

In order to reduce the dimensionality of spectra, Principal Components Analysis (PCA) was first applied. PCA (an unsupervised technique) uses linear combinations of the original variables (*m/z* values) to generate new axes, also known as principal components, or PCs, of linearly uncorrelated variables. Figure 2 shows the score plot of the first two PCs obtained with the ESI(-) spectra of all the samples. Overall R2X for negative ions was 0.843; Q2 parameter was 0.694. Hotelling's T2 did not find any outlier, thus confirming the suitability of the model. The different location of samples in PCA plot indicates the difference in the overall metabolomic composition of the three different batches of extracts, as observed with the two previous analytical methods focused on specific constituents. With respect to the various storage conditions of samples, it is noteworthy that the overall metabolomic changes induced on the phytocomplex by the treatments were quite modest, as suggested by the close location of the different values within samples from each batch. The loading plot, showing the relevance of the single variables in conditioning the global performance of data distribution in the PCA analysis, suggests some ionic species as the most important in describing the metabolome (Figure 3). In particular, species at *m/z* 514.8 (identified as dicaffeoylquinic acid), 532.8, 580.3, 515.9, 376.9, 638.2 and 533.9 (all due to dicaffeoylquinic acids) appeared as the most relevant ones, suggesting that the metabolomic approach relies on these species that are measured by the two previous conventional analytical methods [according to 17, 18]. By using another multivariate technique (supervised) of data evaluation, the PLS-DA, applied to spectral data after preventive grouping obtained by means of cluster analysis (thus focusing on most relevant variables within each batch) the ionic species presenting larger variance were identified (Figure 4). The species at *m/z* 352.1 was emerging, and found to correspond to chlorogenic acid, a constituent of the extract that was monitored through the HPLC method, too. From the evaluation of the intensity of all the variables considered by PLS-DA among the different batches (data not shown), the value of the variable corresponding to *m/z* 352.1 (chlorogenic acid) was higher in the batch 11F, as previously found with HPLC analysis (see Table 2), confirming similar findings with the two methods.

In conclusion, the present study suggests that a global chemometric approach to investigate complex mixtures, such as herbal extracts, is adequate and recommended in order to provide a more complete evaluation of the composition of a product, without focusing only on a single/few compound measurement. The new metabolomic fingerprint approach includes the monitoring of the main constituents, giving weighted relevance to the most abundant ones, but also considering minor components, that might be also notable in view of an integrated – often synergistic – effect on the biological system. The metabolomic fingerprint data were confirmed by analysis of specific metabolites of the artichoke leaves as caffeoylquinic acids and chlorogenic acid. This method can therefore be used for the evaluation of the quality of complex matrices. Another aspect to be considered is the possibility to obtain a broad-spectrum stability control of herbal formulations, requiring minimal sample pre-processing procedures.

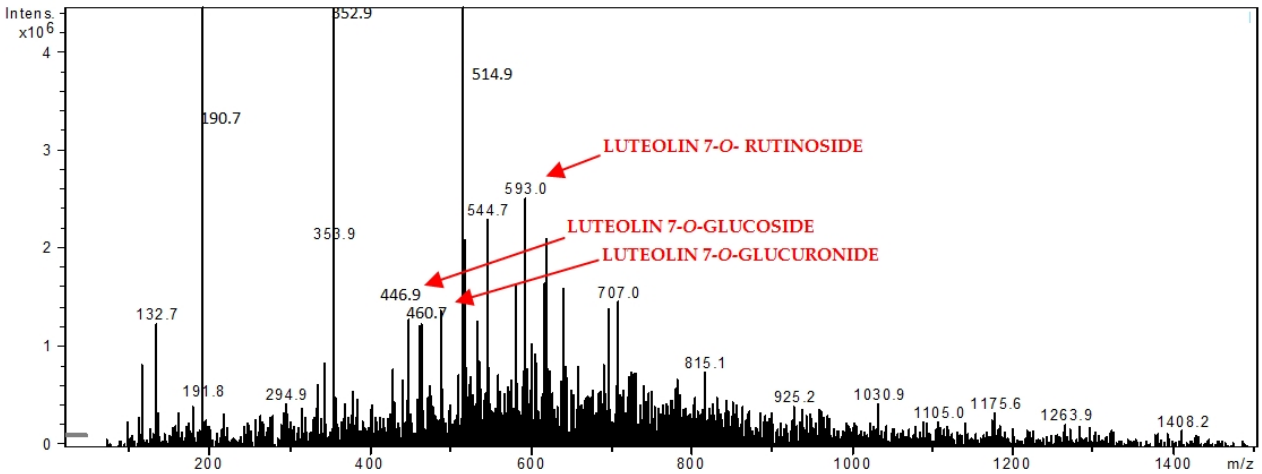

Figure 1: Typical appearance of ESI (-) spectrum of artichoke extract.

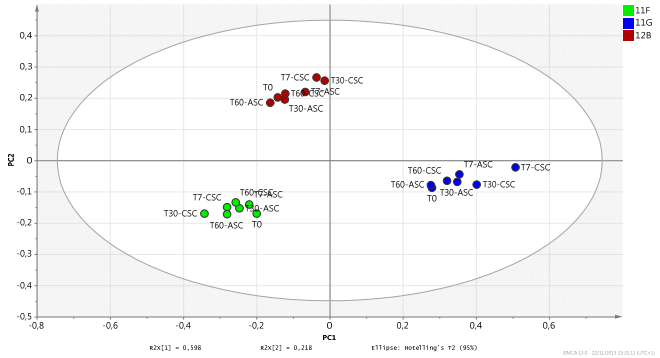

Figure 2: Score plot of PCA obtained with the ESI(-) spectra. Samples are labeled according to batch provenance (11F, 11G, 12B) and condition of storage (see Experimental section).

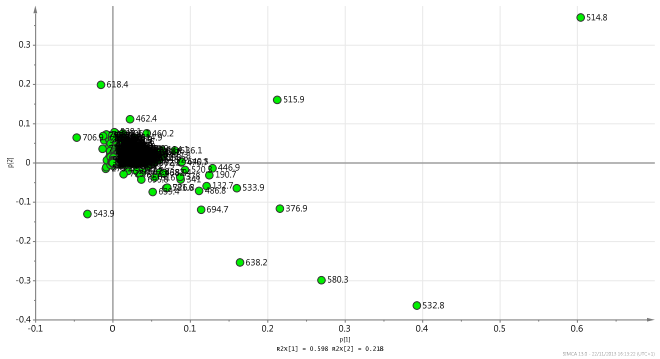

Figure 3: Loading plot of PCA obtained with the ESI(-) spectra.

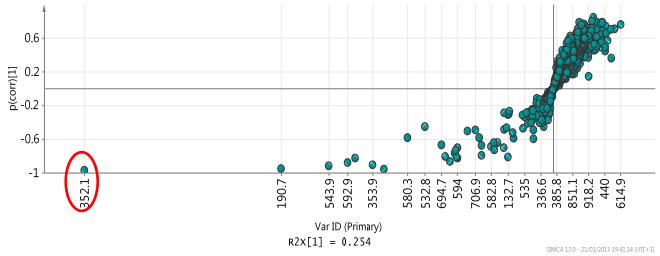

Figure 4: S-plot from PLS-DA analysis performed on samples' ESI(-) spectra. indicating the magnitude (intensity, x-axis) and respective reliability (y-axis) of the ionic species.

Experimental

Plant material: Experiments have been conducted on a series of artichoke [*Cynara cardunculus* L. subsp. *scolymus* (L.) Hegi] leaf extracts, obtained from three different batches (Aboca) 11G0125, 11F0085, 12B1486, in the present paper abbreviated as 11G, 11F and 12B. The material of origin consisted of leaves harvested in April/May 2011 and 2012, dried under air flow at standard temperature and humidity conditions (Aboca). From each batch, samples were collected and submitted to different simulated shelf-life conditions (each in triplicate): permanence at 25±2°C and 60±5% relative humidity for 7, 30 and 60 days (here defined as "conventional storage conditions", CSC), or at 40±2°C and 75±5% relative humidity for 7, 30 and 60 days (here defined as "accelerated storage conditions", ASC). See also Table 1 and 2 headings.

Spectrophotometric measurements: Spectrophotometric quantitative determination was obtained on 2 mL of acetic acid extracts (11.4%, v/v) diluted to 25 mL with methanol through absorbance measurement at 325 nm for total caffeoylquinic acids, expressed as chlorogenic acid, considered as a standard method for quality control of artichoke herbal products [17].

HPLC measurements: HPLC analysis was carried out on 75% methanol/0.1% formic acid extract, using a mobile phase of water/0.1% formic acid (eluent A) and acetonitrile (acidified with formic acid 0.1%) (eluent B) as gradient from 92/8 to 52/48 (15 min). The UV-visible spectrum was measured in the range 190-600 nm in order to detect chlorogenic acid (325 nm) [18]. The measurement coefficient of variation never exceeded 5%.

Metabolomics: The metabolomic fingerprint was obtained with an HPLC/ESI/MS/Iontrap set-up on a 50% methanol extract. Considering results obtained in previous studies [12, 13], a standard non-HR equipment (Agilent 1100 Series LC/MSD ion trap mass spectrometer) offers an acceptable resolution in order to obtain a quality control of samples. ESI(-) analysis was determined using a capillary voltage of +3500 V, end plate offset -500 V, nebulized 40 psi, dry gas 8 L/min, dry temperature 350°C. Spectral data were transformed into numerical matrices and aligned using SpecAlign software [19]. Multivariate analysis was carried out using SimcaP (Umetrix) software on variables centered on their mean value. The goodness of fit and predictivity for each model were assessed by R2X and Q2 parameters. Hotelling's T2 was used to determine the presence of outliers.

The species at *m/z* 514.8 was identified on the basis of its MS-MS collisionally-induced decomposition pathways, showing the formation of ions at *m/z* 352.8, 190 and 334.9. The same fragment ions are present in the MS/MS spectrum of a standard sample of 1,5-dicaffeoylquinic acid (cynarin, Sigma Aldrich). The ionic species at *m/z* 352.9 was identified as chlorogenic acid through MS/MS generation of the ionic species at *m/z* 190.6 and 172.7, which are also detected in the MS/MS spectrum obtained from a pure chlorogenic acid standard (Sigma Aldrich).

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