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The fingerprinting of *Sedivitax*, a commercial botanical dietary supplement: The classical LC-MS approach vs direct metabolite mapping

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ABSTRACT

The use of phytochemical preparations has shown a massive growth and consequently the related quality control is an important topic. Considering the possible interactions of the active molecules, arising to synergic phenomena, the use of the classical approach, based on the evaluation of the level of one or more active compounds can be limitative. Recently a method, based on the direct infusion of commercial botanical dietary supplements in an ESI source operating in positive and negative ion modes has been proposed. The results so obtained were highly promising, allowing the presence of specific plant extracts in commercial products to be determined. In order to evaluate its validity, the data so obtained have been compared with those achievable by a more consolidated technique, as LC-MS is. For this aim *Sedivitax gocce* (a commercial product composed by extracts of *Passiflora incarnata, Eschscholtzia californica, Melissa officinalis and Valeriana officinalis*) has been considered. Either the plant extracts or *Sedivitax* samples produced in different years have been analyzed by ESI (±) with direct infusion and LC-MS. The data obtained were elaborated with different statistical methods. The results suggest that mass spectrometry linked to statistical methods can be a quick method to assess the overall stability of a botanical dietary supplement, and can be proposed as a promising perspective in quality control.

KEYWORDS

Metabolic fingerprinting;
Botanical dietary supplement;
Electrospray ionization;
LC-MS;
Statistical methods;
Discrepancy factor.

INTRODUCTION

In the past decade the use of phytochemical preparations has shown a massive growth and consequently the related quality control is an important topic. But this is surely not an easy task. In fact, while in a synthetic drug it is enough to verify the level of the described impurities and of ageing-derived products of the active compound, for a natural extract this approach cannot be followed, due to the fact that it is usually composed of thousands of different compounds, with a dynamic range in the order of 10[5]. The classical approach for a phytochemical preparation is based on the evaluation of the level of one or more compounds,

Full Paper

retained responsible for the biological activity of the extract. This approach can be sometimes limitative and different analytical approaches are required, able to describe the phyto-metabolome, i.e. integrated relevant biomarkers in the phytochemical system.

Some years ago we proposed a method based on the direct infusion of plant extracts[1] in an ESI source operating in positive and negative ion modes. By this approach and by multivariate statistical analyses of the mass spectra so obtained, it was shown that characteristic fingerprinting of the different species can be obtained. The same approach was employed by different research groups[2-5], confirming its validity. In a more recent paper, we applied it for the characterization not of plant extracts, but of commercial botanical dietary supplements, constituted by mixtures of different plant extracts, and also in that case specific metabolic profiles were obtained[6].

However, it must be emphasized that by direct infusion of complex mixtures some disadvantages can be present. In fact, due to the ESI conditions employed for the analysis, only partial views of the different components can be put in evidence. In the case of positive ion analysis, the production of protonated molecules of the most basic compounds is privileged, together with the desolvation of the species already present in cationic form in the solution. In the case of negative ion analysis, the production of deprotonated anions of the most acidic compounds is favoured. In both cases suppression effects can take place.

To evaluate its validity, in the present investigation we compare the data obtained with this method with those achieved by a more consolidated technique, as LC-MS is, for a commercial standardized product *Sedivitax gocce* [in which four different plant extracts (*Passiflora incarnata, Eschscholtzia californica, Melissa officinalis, Valeriana officinalis*) are present in a wide concentration range]. Furthermore the data obtained were used to evaluate the validity of the direct infusion approach to investigate by statistical methods on possible inter-batches differences and changes on metabolic profiles due to ageing.

Aside cluster analysis and PCA analysis[7], a different approach has been tested; it is based on the calculation of the discrepancy factor[8] between the ESI spectra of different samples under investigation, in order to have an index, i.e. a numerical value, related to the similarity of the ESI full scan mass spectra.

MATERIALS AND METHODS

Samples

The freeze-dried extract of *Passiflora incarnata* (P), *Eschscholtzia californica* (E), *Melissa officinalis* (M), and *Valeriana officinalis* (V) were provided by Aboca S.p.A. (Sansepolcro, Italy) and were produced according to the extraction procedure described as follows. The dried and ground plant materials of each species were extracted at the same temperature (50°C) by one or two steps. Depending on the characteristics of the natural samples, the extraction was performed with different percentages of ethanol/water and sample/solvent ratios. After 6–12 h, the ethanol/water sample mixtures were dropped for one hour and filtered to remove the exhausted material and the corresponding extracts were concentrated under vacuum to evaporate ethanol. The obtained water extracts underwent to freeze-drying for 72 hours and the resulting freeze-dried extracts were stored at 4 °C until analysis (see TABLE 1).

TABLE 1 : Extraction procedures.

Plant scientific name	Extraction solvent	Sample/solvent ratio	Temperature	Time of extraction
Passiflora incarnata	1°step:EtOH 40° 2°step:EtOH 70°	1/10	50°C	1°step: 4 h 2°step: 6-8 h
Eschscholtzia californica	1°step:EtOH 70°	1/10	50°C	6/8 h
Melissa officinalis	1°step:EtOH 70°	1/13	50°C	6/8 h
Valeriana officinalis	1°step:EtOH 70°	1/10	50°C	12 h

0.5 g of the freeze-dried samples of P, E, M, and V were suspended in 12.5 mL of water:ethanol (30:70). The suspension was left in ultrasonic bath for 22 minutes and centrifuged for 5 minutes at 13000 rpm. The supernatant was collected and the precipitate resuspended in 12.5 mL of water:ethanol (30:70), sonicated

and centrifuged. The supernatant so obtained was added to that previously obtained. For *Passiflora incarnata* the same procedure was applied but in the first step water:ethanol (60:40) was employed, while in the second step water:ethanol (30:70) was used. The solutions so obtained for the four drugs (P, E, M, V) were employed for the determination of their metabolic profiles either by LC-ESI(±)-MS or by direct infusion ESI (±)-MS. Furthermore they were used to prepare an extemporaneous sample (S) of *Sedivitax*, with the same relative concentrations of drug extracts present in the commercially available samples (see TABLE 2).

TABLE 2 : Percentage in weight of plant extracts present in *Sedivitax*

Abbreviation used	Sample name	% in Sedivitax
P	*Passiflora incarnata*	93.93 % (86.73% of P alcoholic extracts and 7.2% of P freeze dried extract)
E	*Eschscholtzia californica*	0.92 %
M	*Melissa officinalis*	1.6%
V	*Valeriana officinalis*	3.3%

All *Sedivitax* samples under investigation were produced by Aboca S.p.A. (Sansepolcro, Italy). Five samples from different batches of the same year of production (2008) (samples 1-5) and further 15 samples from different batches of different years of production (2010: 6,7; 2009: 8-10; 2007: 11-15; 2006: 16-20) were considered.

LC-ESI-MS

The LC-ESI-MS measurements of samples P, E, M, V, S and 6 were carried out by an Agilent 1100 Series LC/MSD ion trap mass spectrometer (Agilent Technologies INC., Santa Clara, CA). The column used was a RP Prodigy 250 x 4.6 mm 5 μm (Phenomenex, Castel Maggiore, Bologna, Italy). The elution was performed by H_2O with 0.1% HCOOH (solvent A) and MeOH (solvent B). The gradient program used was: 0 min 90% A, 35 min 50% A and 75 min 5% A. The flow rate was 0.5 mL min^{-1}.

ESI source was operating in both positive and negative ion mode with a nebulizer gas pressure of 30 psi, a dry gas flow of 8 L min^{-1} and a dry gas temperature of 350°C; in the positive ion mode the capillary voltage was -4500 V, while in the negative ion mode it was 3000 V.

All the samples were filtered and diluted with H_2O with 0.1% HCOOH/MeOH (50:50, v:v) before the analysis. P was diluted 1:100 for positive ion detection and as it was for negative ion detection. M and V were diluted 1:10 for both positive and negative ion detection. E was diluted 1:10 for positive ion detection and as it was for negative ion detection. S and 6 were diluted 1:100 for positive ion detection and 1:10 for negative ion detection. 5 μL of the diluted samples were injected.

Direct infusion ESI (±)-MS

The solutions of the samples P, E, M, V, S and 1-20 were directly infused in the ESI source of an Agilent 1100 Series LC/MSD ion trap at 11 μL min^{-1} with a nebulizer gas pressure of 15 psi for the negative ion mode and 13 psi for the positive ion mode. The dry gas flow and the entrance capillary temperature were 5 L min^{-1} and 325°C respectively for both ion modes. In the positive ion mode the capillary voltage was -4500 V, while in the negative ion mode it was 3000 V.

P was diluted 1:10000 for positive ion detection and as 1:100 for negative ion detection with H_2O with 0.1% HCOOH/MeOH (50:50, v:v). M and V were diluted 1:1000 for both positive and negative ion detection with H_2O with 0.1% HCOOH/MeOH (50:50, v:v). E was diluted 1: 1000 for positive ion detection and 1:100 for negative ion detection with H_2O with 0.1% HCOOH/MeOH (50:50, v:v). The samples 1-20 and S were simply diluted 1:10000 with H_2O with 0.1% HCOOH/MeOH (50:50, v:v).

In order to obtain accurate mass measurements on the ions detected with the LC/MSD instrument, the same solutions of the samples P, E, M, V, S and 1-20 were directly infused in the ESI source of an LTQ Orbitrap (ThermoFisher Scientific, Bremen Germany) mass spectrometer, using a resolution power of 60000 at *m/z* 400 (FWHM). The entrance capillary temperature and voltage were set at 280°C and ±4 kV, respectively. Sheath gas and auxiliary gas flow rates were set at 28 and 3 a.u. respectively

Statistical methods

Before any statistical analysis, the ESI(±)/MS data

were inserted into a matrix of *m/z* value (expressed as percent relative abundance), automatically aligned and converted to ASCII files using SpecAlign software, developed by Dr Jason W.H. Wong (Prince of Wales Clinical School & Lowy cancer Research Centre, Faculty of Medicine, University of New South Wales, Sydney, NSW, Australia). The software characteristics have been described elsewhere[9]; for the present study, the version 2.4.1 was used. The *m/z* values of the various samples were merged together in order to align the *x*-axis of all the samples. Thereafter, by means of SpecAlign, the spectra were binned to a size of 1 unit of *m/z*, in order to reduce the complexity of the spectra. The ASCII files were imported into an Excel spreadsheet, and when necessary, to reduce dimensionality of matrix, data were filtered to a relative abundance greater than 5%.

Multivariate analysis allows to overcome the limits of a simple visual examination of spectra, since it considers the entire set of variables (i.e. the relative abundances of the ionic species) permitting a complete exploratory analysis of the metabolite fingerprinting of the samples[7].

Cluster analysis

Cluster analysis is a multivariate procedure of exploratory data analysis for detecting natural groupings in data. Data classification consists in placing samples into more or less homogeneous groups, in order to reveal any relationship among groups. Standard hierarchical agglomerative clustering techniques were applied using the Ward method. The distance measure was determined using the Euclidean distance. Data were presented as *dendrograms*; also *heat maps* were used in some cases. A heat map is a graphical representation of abundance for each ionic species of the various samples, where the abundances are rendered as colors in a two-dimensional map.

Principal component analysis (PCA)

PCA is a mathematical procedure that transforms a number of possibly correlated variables into a smaller number of uncorrelated variables defined as *principal components*, which are linear combinations of the original variables. The first principal component explains as much of the variability in the data as possible, and each following component accounts for the remaining variability. PCA can be a method of data visualization useful to reduce the dimensionality of the data set; it can help to identify new meaningful underlying variables and also can suggest the presence of clusters within multivariate data. The data are represented in a dimensional space of *n* variables, which are reduced into a few principal components; these principal components are descriptive dimensions indicating the maximum variation within the data. After obtaining the principal components, they can be graphically plotted to observe any groupings in the data set. Covariance matrix was selected for PCA computation and standardized principal component scores were used to characterize the samples; the first 3 components, which accounted for the main variance among data (more than 70%), were considered for the classification of data and presented as 3D-scatter plot.

Discrepancy factor

Alternatively to multivariate methods, a numerical evaluation of differences among sample spectra was obtained by the use of the so-called *Discrepancy Factor*, originally proposed by Crawford & Morrison[8].

RESULTS AND DISCUSSION

LC-ESI (±)-MS

The commercial product employed for this investigation, *Sedivitax gocce*, is constituted by four different plant extracts (see TABLE 2) with concentrations varying from 0.92% for *Eschscholtzia californica* to 93.93% of *Passiflora incarnata*. The components of *Passiflora incarnata* would be expected to lead to the most abundant peaks in the LC chromatogram. In order to detect the presence of low concentration components in the commercial products, LC-MS and LC-MS/MS were firstly performed on the extracts of the different components.

Samples of P, E, M, V natural extracts (see TABLE 2) were analyzed in both ESI positive and negative ion modes, taking into account that the two operative conditions would lead to the detection of chemically different classes of compounds. In fact it is reasonable to assume that in positive ion mode the most basic components are those leading to the most abundant ions (through protonation reactions and/or Na^+ and K^+

cationization), while in negative ion mode the ionization of the most acidic components (through proton extraction) is privileged. Then by the two approaches different, but complementary, views of the phytochemical products present in the extracts can be obtained.

The total ion chromatograms of samples P, E, M, V obtained in LC-ESI(+)-MS are reported in Figure 1. The chromatogram of the sample obtained by the extemporaneous mixture of P, E, M, V with concentrations identical to those present in *Sedivitax* commercial product is reported in the same figure.

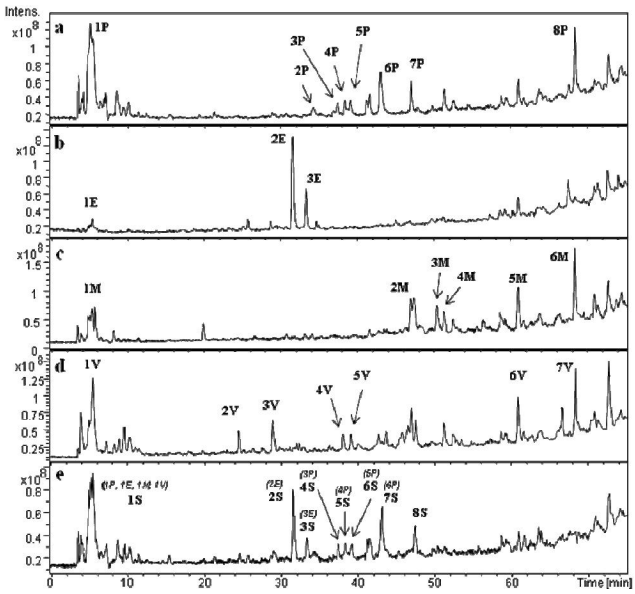

Figure 1 : LC- ESI(+) -MS Chromatogram of: a) *Passiflora incarnata* extracts (P); b) *Eschscholtzia californica* extracts (E); c) *Melissa officinalis* extracts (M); d) *Valeriana officinalis* extracts (V); e) extemporaneous *Sedivitax* (S).

We focused our attention on the components responsible for the most abundant peaks and for them MS/MS experiments were performed. The data so obtained were employed for structural assignments on the basis of what is described in literature for the different plant extracts.

The retention times, the *m/z* values of the ESI generated positive ions and the MS/MS data together with the related structural assignments are reported in TABLE 3. It is at first sight evident the high complexity of the analyzed plant extracts, also considering the fact that some chromatographic peaks are due to different coeluting molecular species (see TABLE 3).

The main component of P extract (Figure 1a) is present at r.t 5.6 min; the related MS/MS spectrum shows losses of neutral moieties of 162 Da and 180 Da, suggesting for it the structure of a Na^+ adduct of a disaccharide. This hypothesis has been confirmed by accurate mass measurement; the experimental accurate mass of this ion was 365.10557 amu, corresponding to the elemental formula $C_{12}H_{22}O_{11}Na$ with an error of 0.376 ppm. As it can be easily seen in Figure 1, this species is not specific for P, but it is present also for V and, in minor extent, for E and M. Other important components of P extract are present at r.t. 34.2, 37.3, 39.2 and 43.4 min. The related ESI spectra show molecular species at *m/z* 595, 565, 449 and 433 respectively. The MS/MS data indicate that they correspond to Vicenin-2, Schaftoside, Orientin and Vitexin. These four compounds have been described in literature[10-12] as components of *Passiflora incarnata* extracts. Furthermore a component of molecular weight 564 Da, the same of Schaftoside, is also present at r.t. 38.4 min. The MS/MS spectrum of its $[M+H]^+$ (*m/z* 565) (see TABLE 3) is partially superimposable with that of Schaftoside, but the lack of fragment ions at *m/z* 427, 409 and 391 indicates that it can be considered a Schaftoside isomer. *Passiflora incarnata* has a long history of use as potent anxiolytic and sedative, besides being a popular traditional medicine for the treatment of insomnia, epilepsy, tetanus and muscular spasms[13].

Eschscholtzia californica leads to the chromatogram shown in Figure 1b. The two species characteristic of this plant extract are present at r.t. 31.5 and 33.2 min and the related MS (indicating molecular species at *m/z* 324 and 338) and MS/MS data (see TABLE 3) lead to their identification as Californidine and Escscholtzine respectively[14].

In the chromatogram of *Melissa officinalis* (Figure 1c) the peak at r.t. 5.5 min, due to the Na^+ adduct of the disaccharide, is present like in all the other plant extracts. At r.t. 50.2 min a peak due to the ion at *m/z* 341 is present; the related MS/MS spectrum evidences only the loss of a H_2O molecule and consequently any structural assignment was impossible. The chromatogram appears poor of components characteristic for M extract; this is probably due to the fact that the major components of *Melissa officinalis* are acidic components, which are better ionized in ESI(-) conditions.

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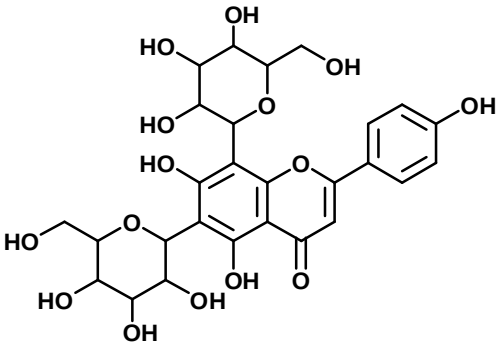
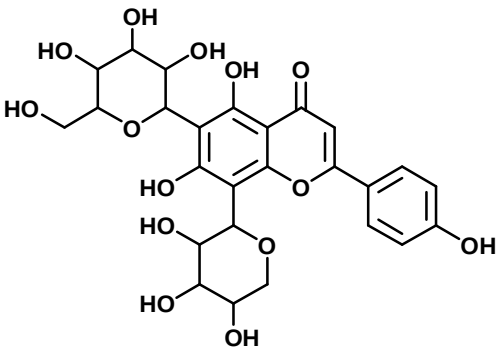
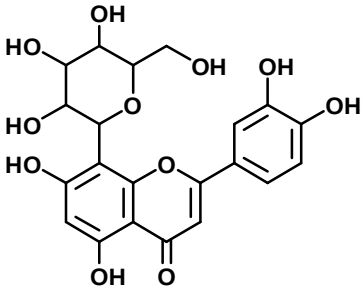
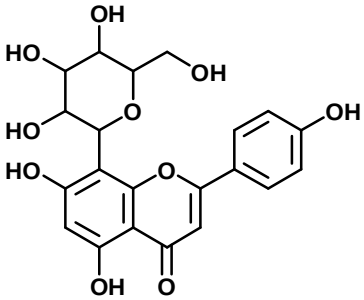
TABLE 3 : Peaks present in the LC chromatograms of Figure 1, their retention times, the related molecular species and product ion spectra, assigned identity and related structure. The presence of the same molecular species in the direct infusion ESI(+) spectra is reported in the last column.

No.	t_R (min.)	Molecular species *m/z*	MS/MS fragments (Rel.Ab. %)	Assigned Identity	Structure	Ion detected in direct infusion spectra
1P	5.6	$[M+Na]^+$ 365	203 (100), 185 (60)	Na^+ adduct of disaccharide	$C_{12}H_{22}O_{11}$	X
2P	34.2	$[M+H]^+$ 595	577 (100), 559 (25), 541 (16), 529 (39), 511 (32), 499 (15), 481 (15), 475 (15), 457 (77), 427 (24)	Vicenin-2		X
3P	37.3	$[M+H]^+$ 565	547 (100), 529 (53), 511 (34), 499 (13), 481 (19), 427 (53), 409 (9), 391 (3)	Schaftoside		X
4P	38.4	$[M+H]^+$ 565	547 (100), 529 (19), 511 (7), 499 (30), 481 (9), 457 (16), 445 (8)	Schaftoside isomer	$C_{26}H_{28}O_{14}$	
5P	39.2	$[M+H]^+$ 449	431 (100), 413 (19), 395 (33), 383 (64), 353 (38), 329 (29), 299 (14)	Orientin		X
6P	43.4	$[M+H]^+$ 433	415 (100), 397 (25), 379 (16), 367 (88), 337 (21), 313 (17), 283 (10)	Vitexin		X

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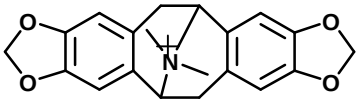
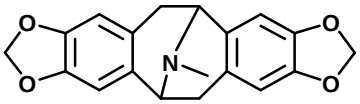
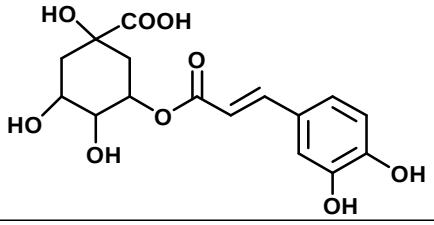
No.	t_R (min.)	Molecular species *m/z*	MS/MS fragments (Rel.Ab. %)	Assigned Identity	Structure	Ion detected in direct infusion spectra
7P	46.9	$[M+H]^+$ 233	215 (100), 207 (26), 173 (18), 150 (70)	unknown		X
		$[M+H]^+$ 535	279 (100)	unknown		X
8P	68.1	$[M+H]^+$ 279	249 (100)	unknown		X
		$[M+H]^+$ 225	197 (100)	unknown		X
1E	5.5	$[M+H]^+$ 365	203 (100), 185 (65)	Na^+ adduct of disaccharide	$C_{12}H_{22}O_{11}$	X
2E	31.5	M^+ 338	293 (100), 263 (28), 235 (19)	Californidine		X
3E	33.2	$[M+H]^+$ 324	293 (100), 235 (14), 188 (65)	Eschscholtzine		X
1M	5.5	$[M+H]^+$ 365	203 (100), 185 (65)	Na^+ adduct of disaccharide	$C_{12}H_{22}O_{11}$	X
2M	46.8	$[M+H]^+$ 433	233 (100)	unknown		X
		$[M+H]^+$ 233	145 (100)	unknown		X
3M	50.2	$[M+H]^+$ 341	323 (100), 297 (31), 195 (22), 181 (6), 163 (17), 147 (42)	unknown		X
4M	46.8	$[M+H]^+$ 433	233 (100)	unknown		
		$[M+H]^+$ 233	145 (100)	unknown		
5M	60.7	$[M+H]^+$ 467	415 (10), 245 (100)	unknown		X
		$[M+H]^+$ 245	171 (100)	unknown		
		$[M+H]^+$ 177	149 (100)	unknown		
6M	68.1	$[M+H]^+$ 535	279 (100)	unknown		X
		$[M+H]^+$ 279	249 (100)	unknown		
		$[M+H]^+$ 225	197 (100)	unknown		
1V	5.5	$[M+H]^+$ 365	203 (100), 185 (70)	Na^+ adduct of Disaccharide	$C_{12}H_{22}O_{11}$	X
2V	24.4	$[M+H]^+$ 268	252 (27), 136 (100)	unknown		X
3V	28.8	$[M+H]^+$ 355	163 (100)	Chlorogenic acid		X

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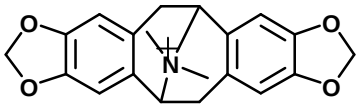
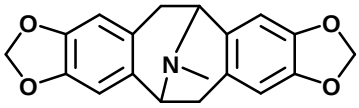
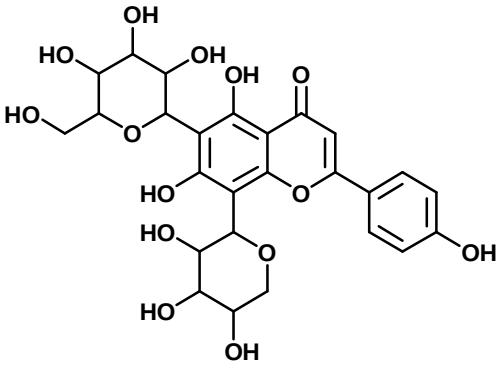
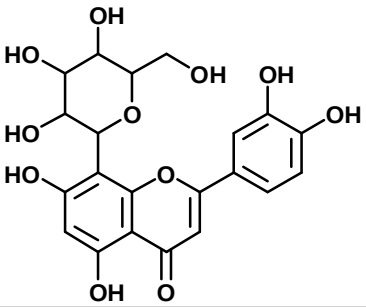
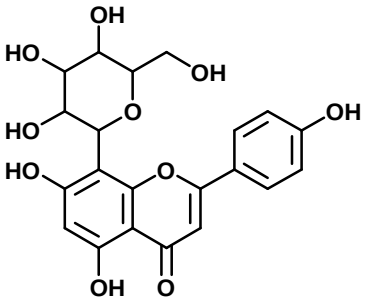
No.	t_R (min.)	Molecular species *m/z*	MS/MS fragments (Rel.Ab. %)	Assigned Identity	Structure	Ion detected in direct infusion spectra
4V	38	$[M+H]^+$ 656	537 (100), 519 (43), 483 (42), 357 (82), 355 (32), 325 (60), 282 (63), 195 (37)	unknown		
5V	39.1	$[M+H]^+$ 647	545 (100), 363 (17)	unknown		X
6V	60.7	$[M+H]^+$ 467	348 (3), 245 (100)	unknown		
		$[M+H]^+$ 245	171 (100)	unknown		
		$[M+H]^+$ 177	149 (100)	unknown		
7V	68.1	535	279 (100)	unknown		
		279	249 (100)	unknown		
		225	197 (100)	unknown		
1S	5.7	$[M+H]^+$ 365	203 (100), 185 (60)	Na^+ adduct of disaccharide	$C_{12}H_{22}O_{11}$	X
2S	31.5	$[M+H]^+$ 338	293 (100), 263 (27), 235 (20)	Californidine		X
3S	33.2	$[M+H]^+$ 324	293 (100), 235 (15), 188 (66)	Eschscholtzine		X
4S	37.3	$[M+H]^+$ 565	547 (100), 529 (50), 511 (32), 499 (13), 481 (19), 427 (53), 409 (10), 391 (3)	Schaftoside		X
5S	38.4	565	547 (100), 529 (20), 511 (7), 499 (30), 481 (9), 457 (18), 445 (7)	Schaftoside isomer	$C_{26}H_{28}O_{14}$	
6S	39.2	$[M+H]^+$ 449	431 (100), 413 (30), 395 (31), 383 (65), 353 (38), 329 (29), 299 (15)	Orientin		X

No.	t_R (min.)	Molecular species *m/z*	MS/MS fragments (Rel.Ab. %)	Assigned Identity	Structure	Ion detected in direct infusion spectra
7S	43.5	$[M+H]^+$ 433	415 (100), 397 (26), 379 (16), 367 (85), 337 (20), 313 (18), 283 (10)	Vitexin		X
8S	47.3	$[M+H]^+$ 383	221 (100), 185 (67)	unknown		
		$[M+H]^+$ 163	144 (100), 135 (22), 121 (21), 117 (29)	unknown		

The chromatogram obtained for the *Valeriana officinalis* extract is reported in Figure 1d. The peak due to the Na^+ adduct of a disaccharide, already described, is present at r.t. 5.5 min. The peak at r.t 28.8 min can be assigned to $[M+H]^+$ ion of Chlorogenic acid[15,16] on the basis of the related MS/MS spectrum, showing the loss of a neutral species of 192 Da, originating from the cleavage of Chlorogenic acid skeleton.

P, E, M, V solutions were mixed in the abundance ratio of 93.93 % (86.73% of P alcoholic extracts and 7.2% of P freeze dried extract), 0.92 %, 1.6% and 3.3 % respectively, i.e. the same ratio employed for the production of *Sedivitax* (see TABLE 2). The sample S so obtained led to the chromatogram reported in Figure 1e. Considering the natural extracts abundance ratio, one could expect that the chromatogram is dominated by the peaks due to P components. In this respect the peak at r.t 5.6 min and the peaks at r.t from 34.2 min to 43 min present in P chromatogram (Figure 1a) are particularly abundant in S (compare Figures 1a and 1e; the peaks 1S, 4S, 5S, 6S, 7S and 8S in the latter just correspond to the peaks 1P, 3P, 4P, 5P, 6P and 7P in the former). However in the S chromatogram (Figure 1e) abundant peaks of E are also detectable at r.t. 31.5 and 33.2 min (2S and 3S; compare Figures 1e and 1b). It must be considered that the E and P percentages are 0.92 and 93.93 respectively; then the abundance of E components in S must be ascribed to their higher ionization yield, due to ionic nature of one metabolite (Californidine) and to high proton affinity of the other (Escscholtzine). In other words the E metabolites seem to exhibit a basicity higher than that of P metabolites. In the S total ion chromatogram the peaks due to components of V and M are detectable in low abundance.

These data show that the chromatogram obtained by ESI(+) of S can be considered valid from the qualitative point of view, allowing to identify the presence of the four plant extracts constituting it, but from the quantitative determination of the different components it cannot be employed as it is, being the specific ion current of the different components related mainly to their basicity, and not to their percentages.

The chromatogram of S and the commercial product 6 are practically superimposable, proving the maintenance of the metabolite profile also in the commercial product.

The LC-ESI(-) ion chromatograms of P, E, M, V and S are reported in Figure 2; the m/z values of the related deprotonated molecules and the corresponding structures are reported in TABLE 4.

Passiflora incarnate[14-16] leads to the chromatogram shown in Figure 2a. In these conditions the peak at r.t. 5.3 min is the most abundant one and it can be assigned, on the basis of MS/MS data and accurate mass measurements, to the presence of a disaccharide, also detected in LC-ESI(+)/MS analysis. The peak at r.t. 34.2 min is due to the anion at *m/z* 593; the related MS/MS data are in agreement with the

Full Paper

structure of Vicenin-2. The peaks at r.t 37.2, 39.1 and 43.4 min correspond to the anions at *m/z* 563, 447 and 431 respectively and, the MS/MS data suggest for them the structures of Schaftoside, Orientin and Vitexin. Compounds of molecular weight 594 and 564 Da are responsible for the peaks at r.t 38.2 min and 41.6 min. The related MS/MS spectra are different from those of Vicenin-2 and Shaftoside, proving that they are isomers of these compounds.

Also for *Eschscholtzia californica* extract (Figure 2b) the main component (r.t. 5.4 min) is due to a disaccharide (*m/z* 341). Others two abundant peaks are at r.t. 45.6 and 49.9 min and MS/MS data indicate that they can be assigned to the structures of Rutin[17] and Isorhamnetin-3-O-rutinoside[18]. The peak due to Rutin glucoside (*m/z* 771) is present at 30.8 min: the related MS/MS spectrum shows the loss of 162 Da and the formation of the ion at *m/z* 609, in agreement with the proposed structure.

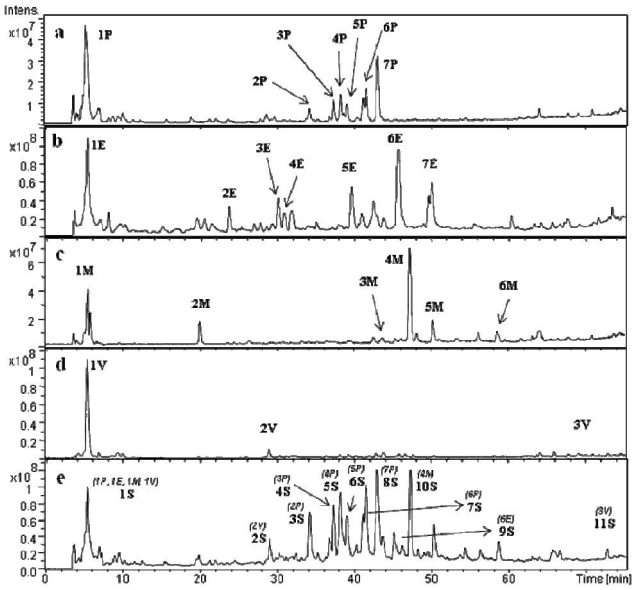
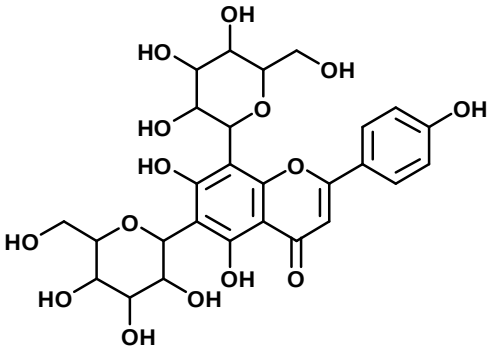
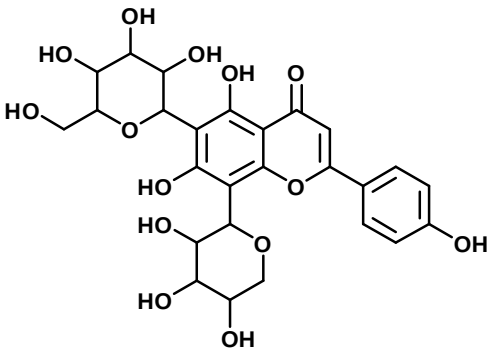
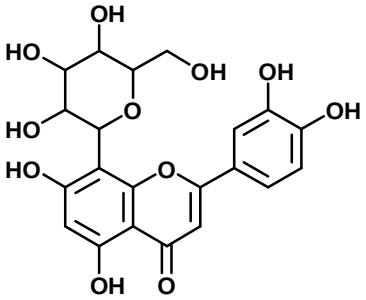
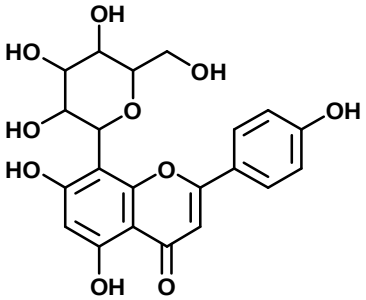
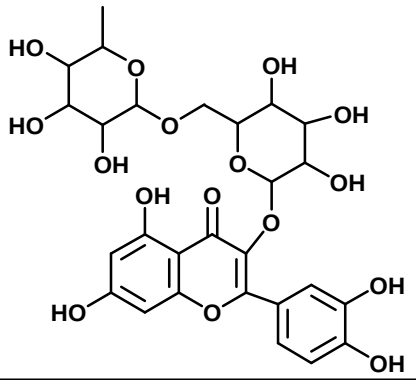


Figure 2 : LC-ESI(-)-MS Chromatogram of: a) *Passiflora incarnata* extracts (P); b) *Eschscholtzia californica* extracts (E); c) *Melissa officinalis* extracts (M); d) *Valeriana officinalis* extracts (V); e) extemporaneous *Sedivitax* (S).

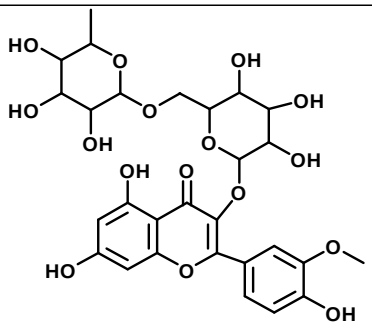
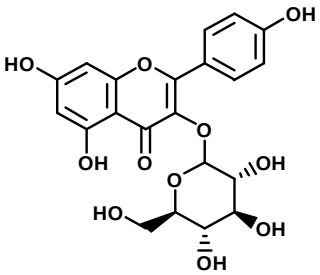
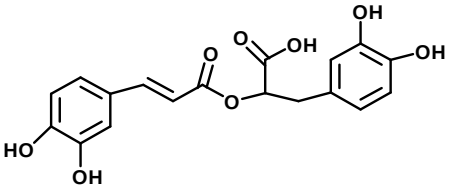
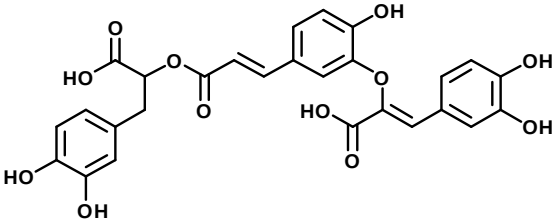
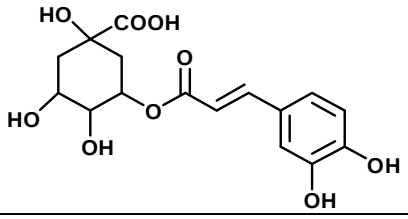
TABLE 4 : Peaks present in the LC chromatograms of Figure 2, their retention times, the related *m/z* value of $[M-H]^-$ species and product ion spectra, assigned identity and related structure. The presence of the same deprotonated molecules in the direct infusion ESI(-) spectra is reported in the last column.

No.	t_R (min.)	$[M-H]^-$ *m/z*	MS/MS Fragments (Rel. Ab.%)	Assigned Identity	Structure	Ion detected in direct infusion spectra
1P	5.3	341	179 (100), 161 (15), 143 (13), 113 (10), 101 (4)	Disaccharide	$C_{12}H_{22}O_{11}$	X
2P	34.2	593	575 (5), 503 (30), 473 (100), 383 (40), 353 (75), 325 (3), 297 (5)	Vicenin-2		X
3P	37.2	563	545 (13), 503 (50), 473 (64), 443 (51), 425 (20), 383 (77), 353 (100), 325 (4), 297 (3)	Schaftoside		X

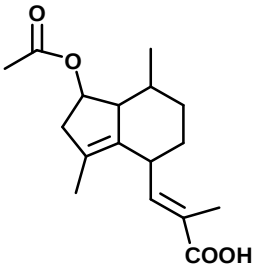
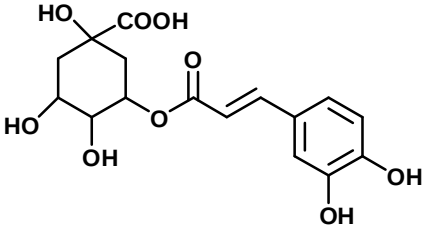
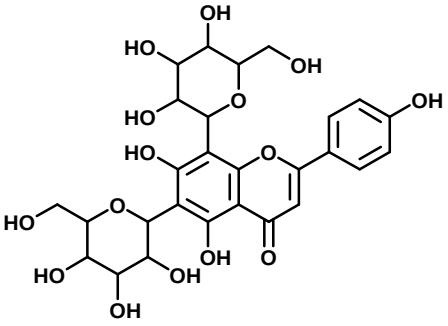
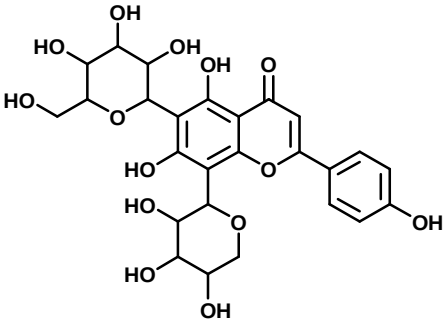
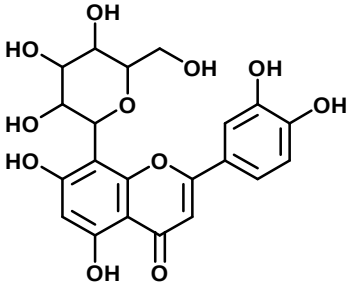
Full Paper

No.	t_R (min.)	$[M-H]^-$ *m/z*	MS/MS Fragments (Rel. Ab.%)	Assigned Identity	Structure	Ion detected in direct infusion spectra
4P	38.2	563	545 (10), 473 (59), 443 (100), 383 (48), 353 (75), 325 (4), 297 (3)	Schaftoside isomer	$C_{26}H_{28}O_{14}$	
5P	39.1	447	429 (12), 411 (2), 357 (60), 327 (100), 285 (2)	Orientin		X
6P	41.6	593	473 (62), 413 (100), 293 (10)	Vicenin -2 isomer		
7P	43.4	431	413 (7), 395 (5), 341 (39), 311 (100), 283 (5)	Vitexin		X
1E	5.4	341	179 (100), 161 (12), 143 (8), 113 (17), 101 (4)	disaccharide	$C_{12}H_{22}O_{11}$	X
2E	23.8	341	203 (5), 179 (100), 161 (29), 135 (9)	Caffeic Acid Glucoside	$C_{15}H_{18}O_9$	
		355	337 (2), 295 (1), 265 (7), 235 (5), 217 (46), 193 (100), 175 (30)	unknown		X
3E	30.1	385	325 (4), 295 (2), 277 (2), 265 (8), 247 (47), 223 (100), 205 (57), 190 (4)	unknown		X
4E	30.8	771	609 (100)	Rutin glucoside	$C_{33}H_{40}O_{21}$	X
5E	39.3	755	609 (6), 591 (14), 489 (15), 343 (8), 300 (100), 271 (26), 255 (12)	unknown		X
6E	45.6	609	563 (12), 464 (15), 301 (100), 284 (26), 179 (5)	Rutin		X

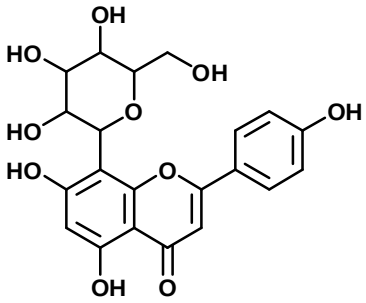
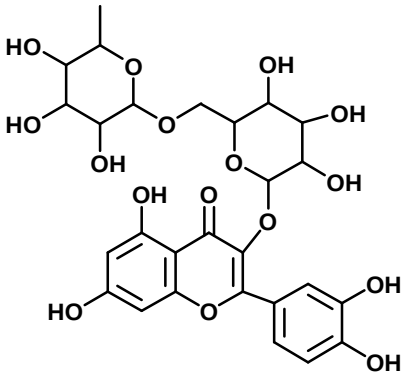
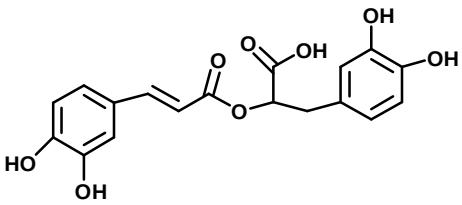
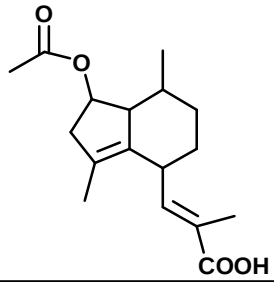
Full Paper

No.	t_R (min.)	$[M-H]^-$ m/z	MS/MS Fragments (Rel. Ab.%)	Assigned Identity	Structure	Ion detected in direct infusion spectra
7E	49.9	623	315 (100), 300 (22), 271 (14), 255 (5)	Isorhamnetin-3-O-rutinoside		X
1M	5.4	341	179 (100), 161 (10), 143 (7), 113 (17), 101 (3)	disaccharide	$C_{12}H_{22}O_{11}$	X
2M	19.5	197	179 (100), 153 (3), 135 (5), 123 (3), 73 (5)	unknown		X
3M	43.4	447	285 (100)	Kaempferol Glucoside		X
4M	46.9	359	223 (13), 197 (25), 179 (25), 161 (100), 133 (4)	Rosmarinic acid		X
5M	49.4	537	493 (100), 359 (18), 313 (2)	3'-O-(8"-Z-caffeoyl) rosmarinic acid		X
6M	55.2	439	391 (2), 359 (21), 259 (71), 231 (35), 215 (100), 179 (2), 161 (2), 151 (3), 135 (15)	unknown		X
1V	5.4	341	179 (100), 161 (10), 143 (12), 113 (10), 101 (4)	disaccharide	$C_{12}H_{22}O_{11}$	X
2V	28.8	353	191 (100)	Chlorogenic acid		X

Full Paper

No.	t_R (min.)	[M-H]$^-$ *m/z*	MS/MS Fragments (Rel. Ab.%)	Assigned Identity	Structure	Ion detected in direct infusion spectra
3V	72.6	291	249 (100), 231 (50)	Acetoxyvalerenic acid		X
1S	5.4	341	179 (100), 161 (10), 143 (11), 113 (10), 101 (4)	disaccharide	$C_{12}H_{22}O_{11}$	X
2S	28.8	353	191 (100)	Chlorogenic acid		X
3S	34.2	593	575 (9), 503 (20), 473 (100), 383 (48), 353 (80), 325 (3), 297 (6)	Vicenin-2		X
4S	37.2	563	545 (26), 503 (49), 473 (82), 443 (50),425 (20), 383 (85), 353 (100), 325 (4), 297 (3)	Schaftoside		X
5S	38.2	563	545 (9), 473 (67), 443 (100), 383 (44), 353 (82), 325 (4), 297 (3)	Schaftoside	$C_{26}H_{28}O_{14}$	
6S	39.1	447	429 (10), 411 (2), 357 (71), 327 (100), 285 (2)	Orientin		X

Full Paper

No.	t_R (min.)	$[M-H]^-$ *m/z*	MS/MS Fragments (Rel. Ab.%)	Assigned Identity	Structure	Ion detected in direct infusion spectra
7S	41.4	593	473 (70), 413 (100), 293 (12)	$C_{27}H_{30}O$		
8S	43.4	431	413 (5), 395 (2), 341 (32), 311 (100), 283 (3)	Vitexin		X
9S	45.6	609	563 (10), 464 (16), 301 (100), 284 (25), 179 (4)	Rutin		X
10S	46.9	359	223 (10), 197 (20), 179 (25), 161 (100), 133 (6)	Rosmarinic acid		X
11S	74.4	291	249 (100), 231 (55)	Acetoxyvalerenic acid		X

The chromatogram of *Melissa officinalis* is reported in Figure 2c. The most abundant peak is at r.t. 46.9 min, corresponding to the structure of Rosmarinic acid[19] (*m/z* 359), as confirmed by MS/MS data. At r.t. 5.4 min the peak due to the anion of a disaccharide (*m/z* 341) is present, as observed for P and E. The peak due to an anion at *m/z* 447 is present at 43.4 min; the MS/MS data indicate for it the structure of Kaempferol glucoside. The anion at *m/z* 537 is responsible for the peak at r.t. 49.4 min and for it the structure of the 3'-O-(8''-Z-caffeoyl) rosmarinic acid can be assigned on the basis of MS/MS spectrum.

The chromatogram of *Valeriana officinalis* is reported in Figure 2d. The most abundant peak is at r.t. 5.4 min and corresponds to the anion at *m/z* 341 ($[M-H]^-$ of a disaccharide)[20]. The peak at r.t 28.8 min corresponds to the anion at *m/z* 353; it can be assigned the structure of Chlorogenic acid[15,16]. The peak due to the presence of the Acetoxyvalerenic acid (*m/z* 291) is present[21] at r.t. 72.6 min.

In the chromatogram of sample S, containing the four plant extracts with relative percentage identical to that of commercial product *Sedivitax*, at least one peak of the four plant extracts is present; it is dominated by the peaks of P [unlike in the ESI(+) chromatogram] which is the most abundant plant extract in *Sedivitax*; in fact the peaks due to the ions at *m/z* 593, 563, 431 and 447 described for *Passiflora incarnata* extract are present in the chromatogram. An exception is observed for Rosmarinic acid; the peak due to the ion at *m/z* 359 is the most abundant, also if *Melissa officinalis* extract (M) is present in a low abundance in S.

Also in this case the chromatogram of S and the commercial product 6 are practically superimposable, proving the maintenance of the metabolite profile also in the commercial product.

The total ion chromatogram of S obtained in both ESI(+) and ESI(-) conditions are, as expected, highly complex and the possible overlapping of different component from different plant extracts can occur. However this negative aspect can be easily overcome by extracted ion chromatograms. As examples the extracted ion chromatograms of some ions characteristic of the different plant extracts detected in ESI(+) and ESI(-) chromatograms of S are reported in Figures 3 and 4. These data indicate that, as usual, extracted ion chromatograms are an useful tool to perform quantitative measurements and to verify the composition of sample S.

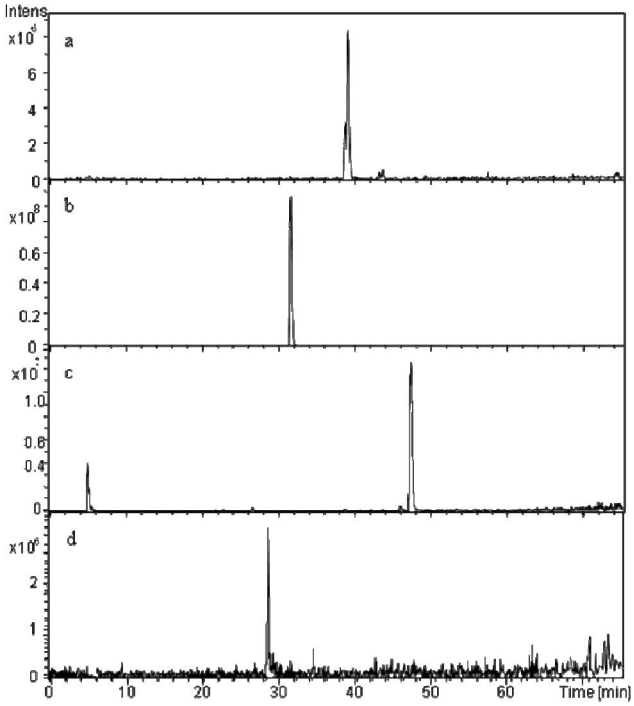


Figure 3 : ESI(+) Reconstructed ion chromatogram of ions at: m/z 449 [characteristic for *Passiflora incarnata* extracts (a)], m/z 338 [characteristic for *Eschscholtzia californica* extracts (b)], m/z 383 [characteristic for *Melissa officinalis* extracts (c)] and m/z 355 [characteristic for *Valeriana officinalis* extracts (d)] detected in sample S.

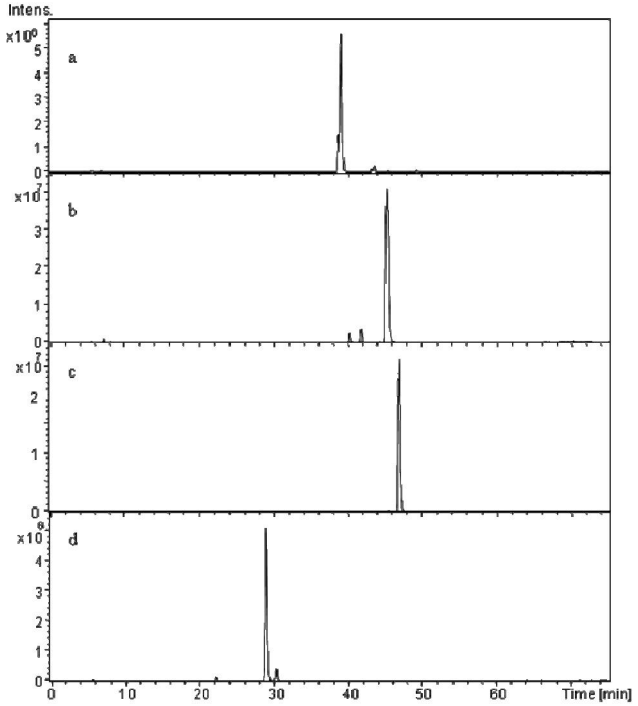


Figure 4 : ESI(-) Reconstructed ion chromatogram of ions at: m/z 447 [characteristic for *Passiflora incarnata* extracts (a)], m/z 609 [characteristic for *Eschscholtzia californica* extracts (b)], m/z 359 [characteristic for *Melissa officinalis* extracts (c)] and m/z 353 [characteristic for *Valeriana officinalis* extracts (d)] detected in sample S.

Direct infusion-ESI(±)-MS

Alternatively to the LC-ESI-MS approach, it was considered of interest to undertake the analysis on the same samples (P, E, M,V and S) by direct infusion of the related solutions in the ESI source operating in both positive and negative ion mode. This approach has been already successfully employed to characterize extracts for different botanic species[1-5]. The ESI data were evaluated by statistical methods and the results showed a clear differentiation among different species. More recently the same approach was also used to obtain a fingerprinting of phytochemical preparations produced

Full Paper

by the use of different plant extracts[6]. Even if the plant metabolic profiles are in these cases highly complex, specific fingerprintings have been obtained.

The data related to samples P, E, M, V, S and 6 fully confirm these findings. The ESI (+) spectra of the samples P, E, M, V, S are reported in Figure 5.

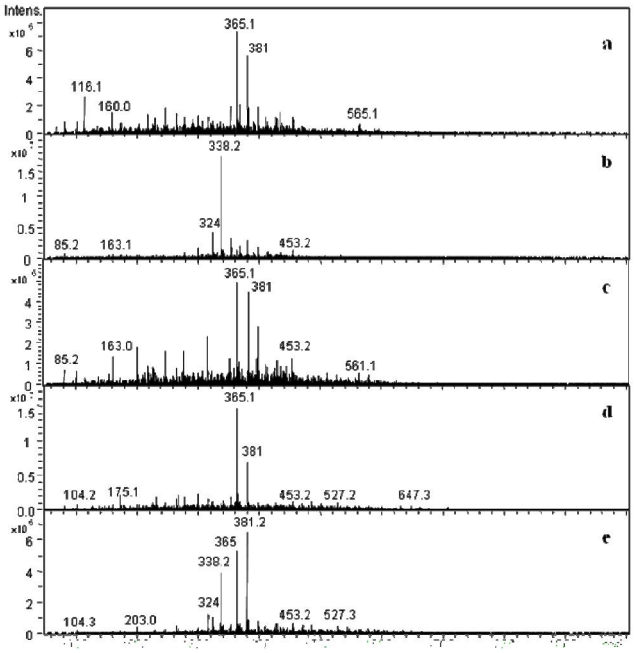


Figure 5 : ESI(+) full scan mass spectra obtained by direct infusion of: a) *Passiflora incarnata* extracts (P); b) *Eschscholtzia californica* extracts (E); c) *Melissa officinalis* extracts (M); d) *Valeriana officinalis* extracts (V); e) extemporaneous *Sedivitax* (S).

In all the spectra peaks up to *m/z* 600 are present. It is to emphasize that not necessarily to one peak corresponds only one molecular species. In fact, due to the low mass resolution of the instrument employed for the investigation (ion trap), isobaric ions with different structures, different elemental compositions and different exact mass values can contribute to the same peak. However, for the aim of the present work, i.e. the evaluation of fingerprinting specificity, this aspect is not essential.

In the case of P, M, V (Figures 5a, 5c and 5d respectively) strongly analogous spectra are obtained in ESI(+) conditions: the most intense peaks at *m/z* 365 and 381 are due to Na^+ and K^+ adducts of the disaccharide already detected in LC-ESI-MS conditions, as proved by MS/MS and accurate mass measurements. On the contrary E (Figure 5b) leads to a specific spectrum, due to the presence of ions at *m/z* 338 and 324 due to Californidine and Escscholtzine[14]. The spectrum of S (Figure 5e) is characterized by the ions above described, and it is exactly superimposable with that of the commercial products 6. It is worth of noting that the most of ionic species detected in the spectrum obtained by direct infusion were those leading the most intense chromatographic peaks (see TABLE 3, last column on the right). Then direct infusion can give, very rapidly (few minutes vs 75 minutes for the LC run) a high number of information on the natural extracts. The strict analogies among P, M, and V were also present in the total ion (+) chromatograms. This ineffectiveness of ESI(+) in the differentiation of P, M, V is due to the presence of the disaccharide species which are ionized in high yield, leading in LC/MS conditions to abundant protonated molecules while, in direct infusion conditions, depress the formation of charged molecular species from the other components of plant extracts.

The situation shows a clear change by direct infusion of P, E, M and V in ESI(-) conditions. In this case (see Figure 6) four strongly different spectra are obtained, due to the privileged production of anions from the different, more acidic species present in the extracts.

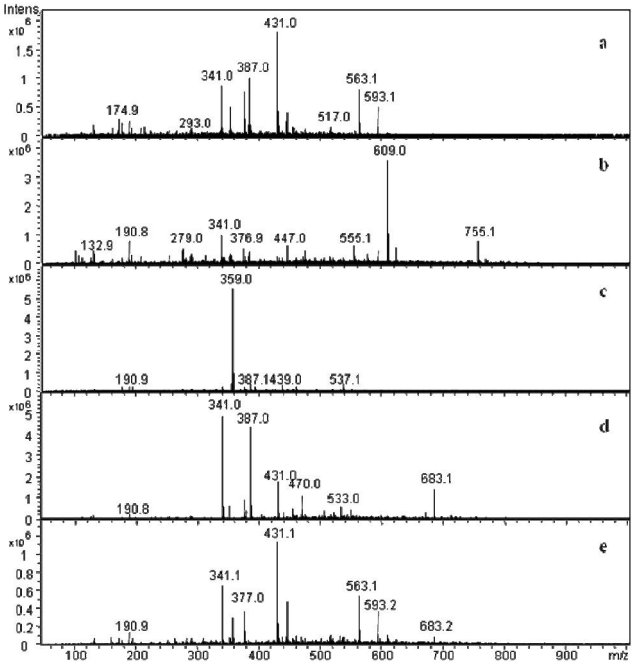


Figure 6 : ESI(-) full scan mass spectra obtained by direct infusion of: a) *Passiflora Incarnata* extracts (P); b) *Eschscholtzia californica* extracts (E); c) *Melissa officinalis* extracts (M); d) *Valeriana officinalis* extracts (V); e) extemporaneous *Sedivitax* (S).

For the most abundant ion MS/MS spectra were obtained and they resulted perfectly the same obtained by the LC-MS/MS experiments.

The ESI(-) spectrum of *Passiflora incarnate*[10-12] is reported in Figure 6a. The most abundant ion is at *m/z* 431, due to Vitexin. The ions at *m/z* 341, 563 and 593 correspond to the $[M-H]^-$ anion of a disaccharide, Schaftoside and Vicenin-2 respectively. Both the ions at *m/z* 387 and 377 may be related to the disaccharide; the related MS/MS spectra showing the formation of the ion at *m/z* 341, i.e. the molecular species of disaccharide. Furthermore the ion at *m/z* 447 was detected in the direct infusion analysis of P; as confirmed by MS/MS, it can be assigned to the deprotonated molecule of Orientin.

The spectrum of *Eschscholtzia californica* is reported in Figure 6b. The most abundant ion is at *m/z* 609 and it can be assigned the structure of Rutin[17], as proved by the related MS/MS data. The ions at *m/z* 191, present also in the spectrum of M and V, and those at m/z 341 correspond to the deprotonated molecules of Quinic acid[18] and of the disaccharide. It is to underline that the ion at m/z 191 seems to be undetectable in LC-ESI(-)-MS analysis of E, M and V, but by extracted ion chromatogram, its presence is observed at r.t. 64.1 min.

The spectrum of *Melissa officinalis* (see Figure 6c) is characterized by the ion at *m/z* 359, which corresponds to the structure of Rosmarinic acid[19]. The ions at *m/z* 341 are present at lower intensity, corresponding to the deprotonated molecule ($[M-H]^-$) of the disaccharide.

The ions present in the spectrum of *Valeriana officinalis*[20] (Figure 6d) at *m/z* 387, 377, 431, 470 and 683 are related with the most abundant ion, which is at m/z 341 and corresponds to the disaccharide structure. In fact the related MS/MS data show for all them the formation of the ion at m/z 341. Furthermore the ion at m/z 291 (Acetoxyvalerenic acid)[21] is present at low intensity; this aspect may be due to signal suppression phenomena often observed in ESI condition.

Finally the spectrum of the extemporaneous *Sedivitax* (sample S) is reported in Figure 6e; the ions present in the spectrum are the same ion just described for the four plant extracts, but with different intensity.

The data above reported show that by direct infusion a high number of information can be gained, with an analysis time one order of magnitude lower than that needed for LC-MS analysis. Of course we are conscious that by direct infusion the quali- and quantitative description of the complex mixture are at a level lower than that achievable by LC-MS. However, the former approach could be of interest to establish, in preliminary form, inter-batches variations and the possible changes in metabolite composition related to ageing. In other words the direct infusion approach can be considered, in principle, a valid analytical method for quality control of phytochemical products.

To be confident on this aspect some investigation are needed, so to evaluate: i) day-by-day instrumental changes; ii) inter-batches variation; iii) variations observed for phytochemical products produced in different years. It must be considered that point iii) covers both i) and ii) and, in this turn, point ii) covers i).

For this aim a first investigation was performed to evaluate the day-by-day variation of the phytochemical fingerprinting due to instrumental factors. The *Sedivitax* sample #16 (produced in 2006; this batch was used as exemplificative of maximal degradation phenomena occurring with ageing) was analyzed for 3 times in 5 different days. Both ESI(-) and ESI(+) spectra were obtained. To evaluate the differences among different spectra, statistical methods are required. In particular, since the data are obtained from complex matrices, multivariate procedures represent an appropriate approach.

Figures 7 and 8 show the results of cluster analysis and of PCA performed on relative abundances in the 15 ESI (-) and 15 ESI (+) spectra, respectively, of sample #16.

Although cluster analysis shows for both negative and positive ion spectra an apparent diversity, conversely PCA suggests that the true differences are quite limited, as indicated by the strict crowding of samples in the three-dimensional plot of the first three principal components. The changes observed in different days, amplified by cluster analysis procedure, are actually due to small variations of the spectrum; the fact that differences are not progressively linked to consecutive days, but look randomly dispersed, indicates that the changes are not due to degradation of the extracts, but are consequences of undetermined instrumental bias, suggesting careful evaluation of instrumental conditions, and eventually need of repeated measurements.

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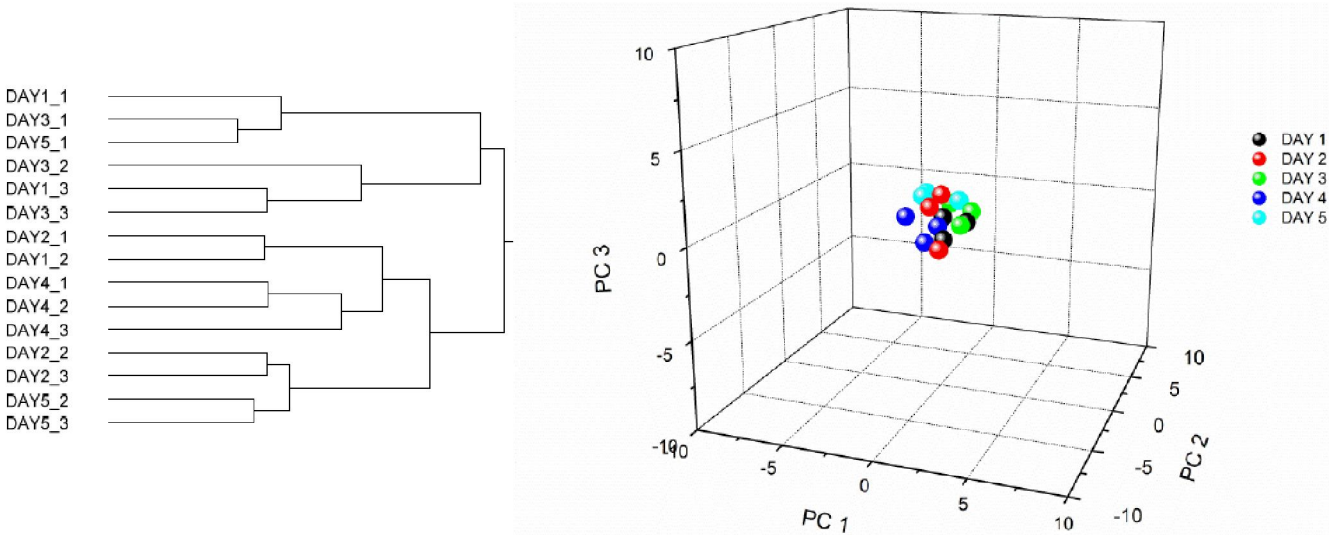

Figure 7 : Cluster analysis (graph on left reports the dendrogram) and PCA (graph on right shows 3D-plot of the scores of the first 3 principal components) obtained with ESI(-) full scan mass spectra of sample no. 16. Each sample is named with a first number representing the day of assay, followed by another number, indicating the replicate.

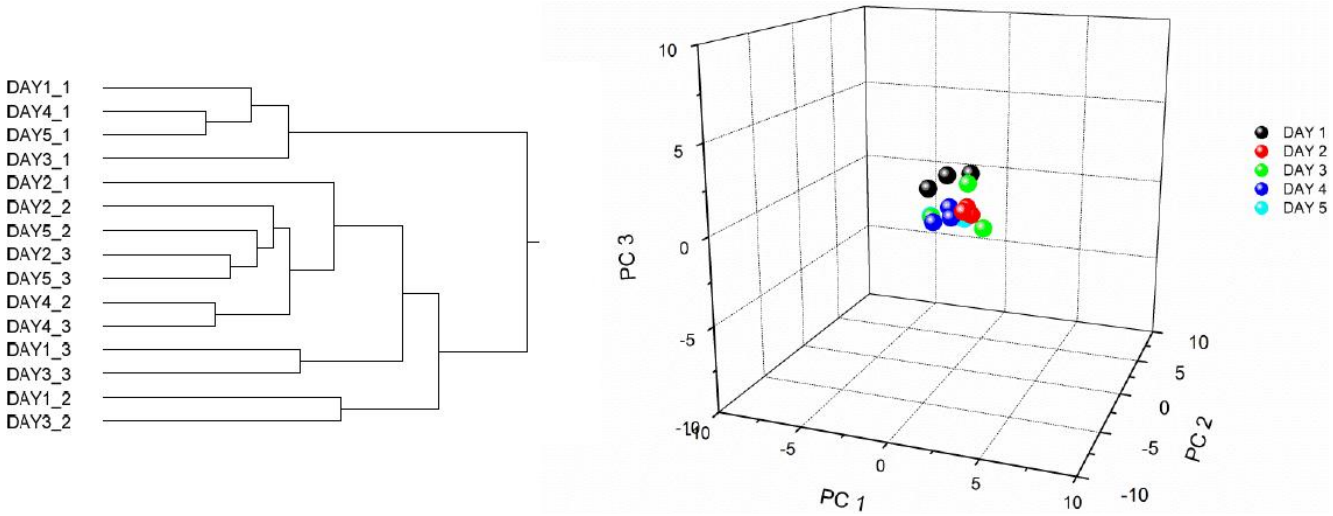

Figure 8 : Cluster analysis (graph on left reports the dendrogram) and PCA (graph on right shows 3D-plot of the scores of the first 3 principal components) obtained with ESI(+) full scan mass spectra of sample no. 16. Each sample is named with a first number representing the day of assay, followed by another number, indicating the replicate.

An alternative numerical evaluation of these differences can be performed and can be obtained by the evaluation of the Manhattan distance[22] or by the so-called *Discrepancy Factor (D)*, originally proposed by Crawford & Morrison[8], that can be applied to normalized spectral data obtained by considering the sum of the abundances of all the *k* peaks present in the spectrum, *Pn*, equal to unity:

$$\sum_{n=1}^{k} Pn = 1 \qquad (1)$$

The comparison of the normalized mass spectra of two samples *w* and *y* can be obtained by the *Discrepancy Factor*, defined as[12]:

$$D = \sum_{n=1}^{k} | Pn_w - Pn_y | \qquad (2)$$

where *n* is now taken over all observed *k* peaks in both spectra, the *w* and *y* ones, normalized by Equation 1. For complete similarity of the two spectra, *D* will be equal 0.0; for the complete dissimilarity – i.e., no peaks at a common *m/z* – D will equal 2.0.

Two different approaches can be employed to evaluate *D* values for a *m* number of spectra. The first, *(i)*, is based on the calculation of the "mean spectrum" and the comparison of all the *m* spectra with it (obtaining

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n_1=m values of D according to Equation 2, with sum here assumed to be D_{sum1}). With this method, the average D value is calculated as:

$$\mathbf{D_{aver1} = D_{sum1} / n_1} \quad (3)$$

which can be used as an estimate of the difference *vs* mean reference spectrum.

Alternatively, *(ii)*, the D factor can be obtained through a more integrated procedure, taking into account the combined comparisons of each spectrum with all the others; in this case, the total number of comparisons n_2 from m spectra will be:

$$\mathbf{n_2 = (m^2 - m)/2} \quad (4)$$

A cumulative D_{sum2} value will be obtained, as combinatory differences from each sample and for each ionic species:

$$\mathbf{D_{sum2} = \sum_{n=1}^{k} \sum_{i=1}^{m} \sum_{j=1}^{m} | Pn_{ijk} - Pn_{jik} |} \quad (5)$$

where Pn is the abundance normalized to 1 for each ionic species and for each sample under comparison, k is the total number of ionic species observed in the spectra, and m is the total number of samples, considering for each ionic species a symmetric matrix composed of i rows and j columns. Since the matrix is symmetrical, to avoid unnecessary duplicates, only half of the contrasts will be considered.

The final D_{aver2} value will be the value of cumulative D_{sum2}, averaged to the total number of comparisons n_2:

$$\mathbf{D_{aver2} = D_{sum2} / n_2} \quad (6)$$

Some aspects need to be discussed. First of all D was proposed to compare the spectra obtained in electron ionization (EI) conditions, i.e. to the set of molecular and fragment ions originating by the gas-phase interaction of an electron beam with the neutral molecules of the analyte. In these conditions the same compound leads to practically superimposable mass spectra and the D factor is close to zero; on the contrary two structurally different compounds lead to high D factor values. In the present case the ESI spectra obtained for the different samples represent the set of molecular species ($[M+H]^+$ for positive ions, $[M-H]^-$ for negative ions) produced by direct infusion of the sample inside the ESI source.

The possible change in D value of EI spectra of the same compound can be related only to instrumental factors (electron energy, ion source temperature, ion source defocusing), while in the case of ESI of complex mixtures it can be related also to instrumental factors (and this point must be necessarily evaluated), but mainly to changes of concentration of the different component present in the complex mixture.

According to approach *(i)*, the discrepancy factor (Equation 3) for the 15 replicates of sample #16, in comparison to their mean spectrum, considered as reference, for negative and positive ions was found to be 0.17±0.04 and 0.26±0.06, respectively (see figures 9a

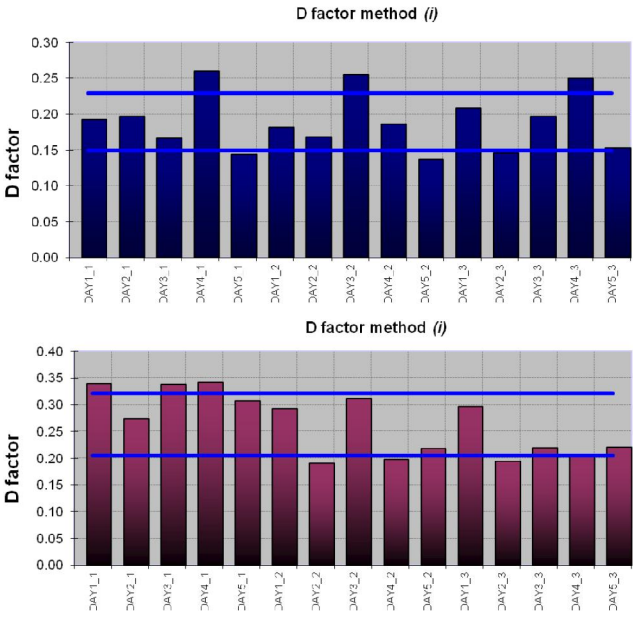

Figure 9 : a: D factor calculated for each sample, using negative ions. Horizontal lines indicate the interval mean ±SD. Data legend refers to the day of analysis followed by the replicate number; b: D factor calculated for each sample, using positive ions. Horizontal lines indicate the interval mean ±SD. Data legend refers to the day of analysis followed by the replicate number.

TABLE 5 : *Discrepancy factor* of sample #16 determined according to method *(i)* for both ESI negative and positive ion mode. Relative abundances were normalized to 1. As reference spectrum the mean spectrum of all the m=15 replicates was considered. D_{aver1} was calculated dividing D_{sum1} by n_1 comparisons.

	No. of sample spectra (m)	No. of ionic species in spectra (k)	No. of comparisons (n_1)	D_{sum1}	D_{aver1}±SD
ESI (-) ionic species	15	288	15	2.84	0.17±0.04
ESI (+) ionic species	15	270	15	3.94	0.26±0.06

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and 9b and TABLE 5 for details of calculation and values obtained with each sample).

Using the approach *(ii)*, the discrepancy factor (Equation 6) from all the possible combinations of samples was 0.21±0.13 and 0.31±0.15 for negative and positive ions, respectively (TABLE 6).

TABLE 6 : *Discrepancy factor* of sample #16 determined according to method *(ii)* for both ESI negative and positive ion mode. Relative abundances were normalized to 1. D_{aver2} was calculated dividing D_{sum2} by n_2 comparisons. The total number of binary contrasts operated is ($k \cdot n_2$), that is 29400 for negative ions and 28350 for positive ions.

	No. of sample spectra (*m*)	No. of ionic species in spectra (*k*)	No. of comparisons (n_2)	D_{sum2}	D_{aver2}±SD
ESI (-) ionic species	15	288	105	25.62	0.21±0.13
ESI (+) ionic species	15	270	105	37.43	0.31±0.15

Both methods of *D* factor calculation appeared to give comparable results, and suggest that positive ion spectra have an overall larger difference among replicates than negative ion ones. The D values so obtained express the variation due to instrumental factors; it has not a direct meaning but it represents the starting point to evaluate, in *toto*, the variation of *Sedivitax* composition present in different batches and due to ageing of the products.

Different batches, although belonging to the same year, could have been produced by different plant extracts (different harvest and/or different plant production areas). To verify the validity of our approach, five different *Sedivitax* batches (1-5) produced in 2008 were analyzed 5 times in 5 different days. Both ESI (-) and ESI (+) full scan mass spectra were obtained and the related data were employed for statistical treatment and *D* value calculation.

Cluster analysis and PCA conducted on ESI (-) and ESI (+) full scan mass spectra of batches (1-5) are reported in Figures 10 and 11.

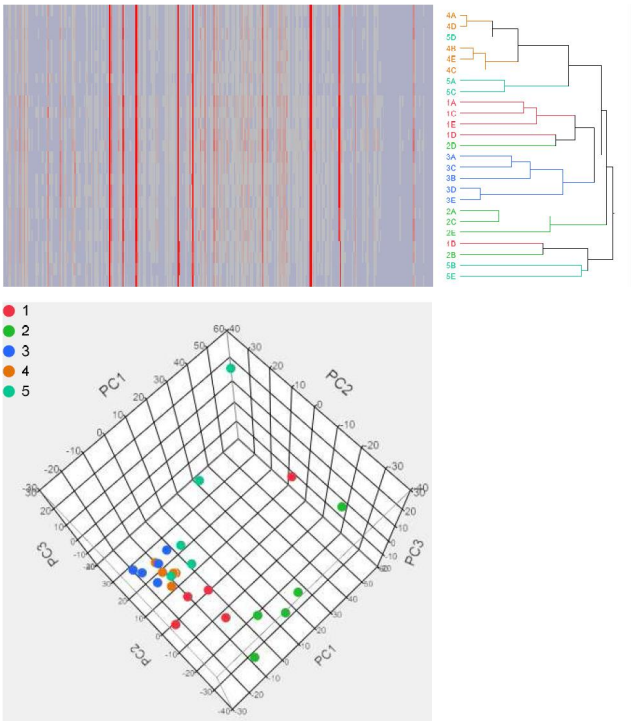

Figure 10 : Cluster analysis (graph above: dendrogram with heat map) and PCA (graph below: 3D-plot of the scores of the first 3 principal components) obtained with ESI(-) full scan mass spectra of batches no. 1-5. Data for each sample in the heat map are on a graduated color scale, with values increasing from blue to gray to red. Each sample is named with a number representing the batch, followed by a letter, indicating the replicate.

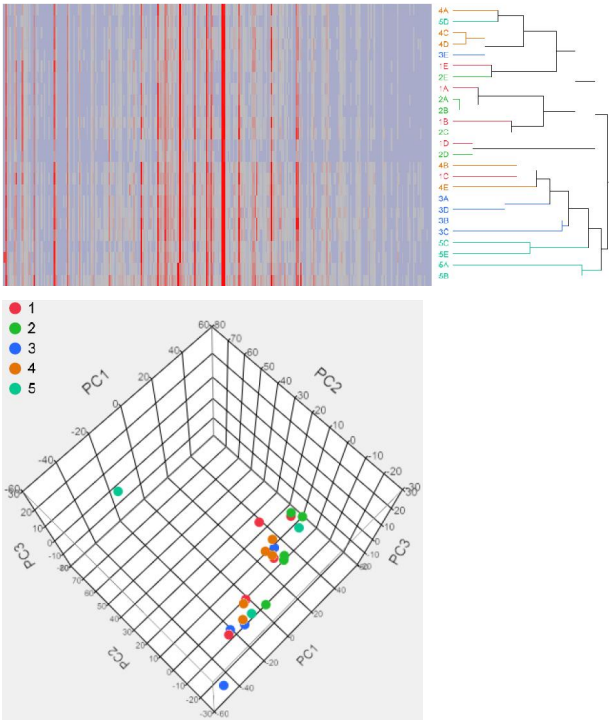

Figure 11 : Cluster analysis (graph above: dendrogram with heat map) and PCA (graph below: 3D-plot of the scores of the first 3 principal components) obtained with ESI(+) full scan mass spectra of batches no. 1-5. Data for each sample in the heat map are on a graduated color scale, with values increasing from blue to gray to red. Each sample is named with a number representing the batch, followed by a letter, indicating the replicate.

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The heat maps, indicating at a glance the abundance of each ionic species in each sample, show an overall similarity among samples, both for negative and positive ions; however, considering all the *m/z* abundances (expressed as relative abundances), some differences were detected and highlighted by the cluster analysis, both with ESI(-) and ESI(+) mode (Figures 10 and 11), and confirmed with PCA. The average D factor values, calculated according the two different approaches above described, are presented in TABLES 7 and 8, respectively.

Both ESI (-) and ESI (+) spectra were considered. The values of D factors, although slightly higher than those previously seen for instrumental variability, appear still moderate, and confirm a greater discrepancy factor for positive ions. Moreover, also with this analysis, both methods of D factor determination appear consistent with each other.

TABLE 7 : *Discrepancy factor* of batches #1-5 determined according to method *(i)* for both ESI negative and positive ion mode. Relative abundances were normalized to 1. As reference spectrum the mean spectrum of all the *m*=25 replicates was considered. D_{aver1} was calculated dividing D_{sum1} by n_1 comparisons.

	No. of sample spectra (*m*)	No. of ionic species in spectra (*k*)	No. of comparisons (n_1)	D_{sum1}	D_{aver1}±SD
ESI (-) ionic species	25	288	25	2.38	0.26±0.06
ESI (+) ionic species	25	270	25	7.76	0.31±0.08

TABLE 8 : *Discrepancy factor* of batches #1-5 determined according to method *(ii)* for both ESI negative and positive ion mode. Relative abundances were normalized to 1. D_{aver2} was calculated dividing D_{sum2} by n_2 comparisons.

	No. of sample spectra (*m*)	No. of ionic species in spectra (*k*)	No. of comparisons (n_2)	D_{sum2}	D_{aver2}±SD
ESI (-) ionic species	25	288	300	99.21	0.33±0.60
ESI (+) ionic species	25	291	300	122.17	0.41±0.59

Finally the effect of aging in *Sedivitax* metabolic profile was considered by analyzing 20 samples produced in different years (2010: 6,7; 2009: 8-10; 2008: 1-5; 2007: 11-15; 2006: 17-20). For all the samples ESI (+) and ESI (-) spectra were recorded and the data were analyzed by both multivariate statistics and *D* value calculation. The latter parameter was calculated with respect to the mean spectrum of sample 16, obtained for the instrumental variation evaluation. Again, this mean spectrum was considered as reference since obtained with a pool of repeated measures on a batch that, being older than the others, can represent an example of most pronounced ageing processes. For negative ions, D_{aver1} = 0.55±0.13 (n=67) and for positive ions, D_{aver1} = 0.63±0.16 (n=67).

Cluster analysis (Figure 12) shows the differences among different samples without suggesting a peculiar pattern linked to ageing of samples. PCA was able to show a pattern for batches belonging to different years; specifically, with negative ions, the oldest samples (16-20) appeared as the most different ones (Figure 12). Positive ions failed to show any specific behaviour due to age progression (Figure 13). Considering positive ions, the spectra look more differentiated and clustering shows random grouping of samples, not assignable to homogeneous batches according to ageing (Figure 13). The datum is in agreement with the D factor, that being greater for positive ion spectra, suggests a larger distribution of values.

From our experience, therefore, the D values can be considered a valid tool to quality control, even more flexible than cluster analysis and PCA. The data obtained in the present investigation indicate that for D_{aver1} = 0.3 any effect of ageing is not present in the commercial product, but the observed differences can originate by inter-batches variability. Eventually, the data analysis can be completed further by the use of cluster analysis/PCA to identify also outliers.

In conclusion, the present investigation suggests that mass spectrometry can be a quick method to assess the overall stability of a phytochemical preparations, and can be proposed as a promising perspective in production quality control.

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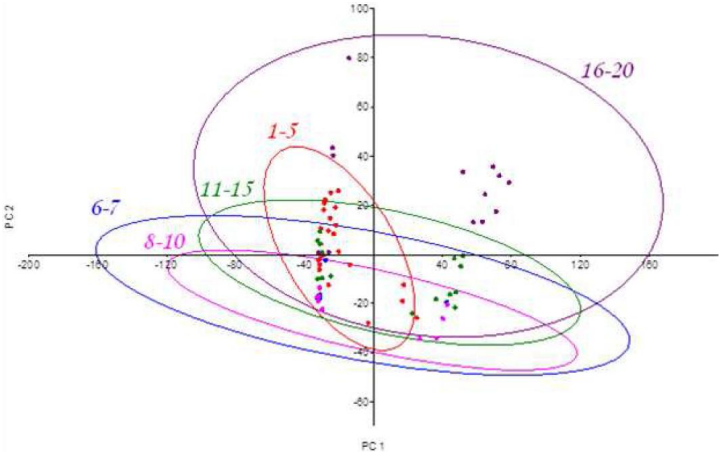
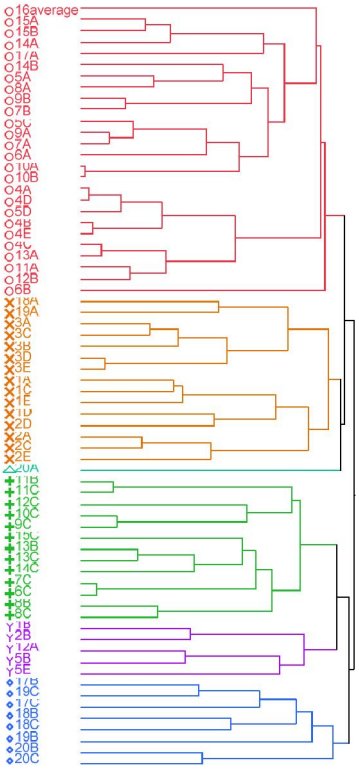
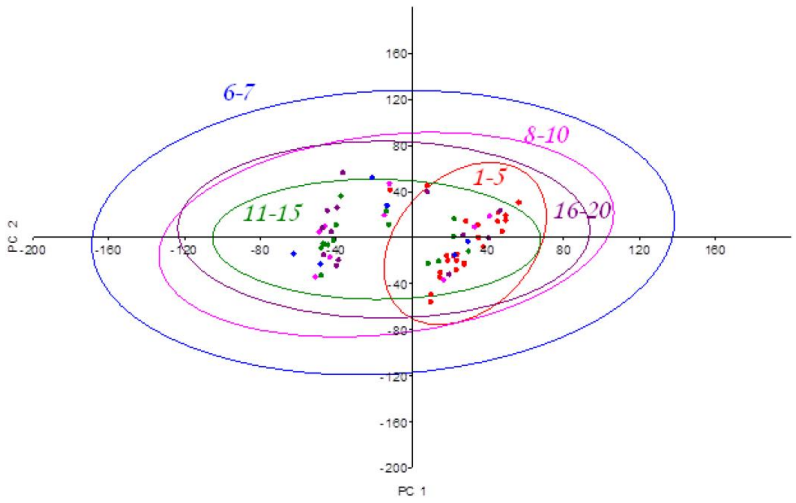
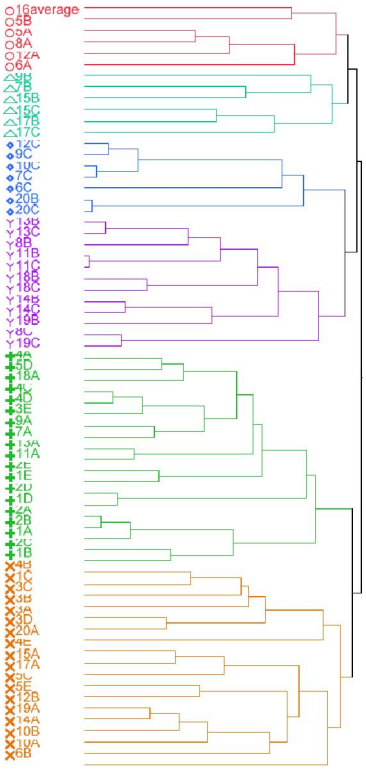




Samples 1-5: year 2008; samples 6-7: year 2010; samples 8-10: year 2009; samples 11-15: year 2007; samples 16-20: year 2006.

Figure 12 : Principal component score plot of the negative ion abundances of all the samples divided according to batch from different years of production. Each ellipse includes the 95% of the data divided according to age groups. The first two PCs accounted for 48% and 15% of total variation among the data.





Samples 1-5: year 2008; samples 6-7: year 2010; samples 8-10: year 2009; samples 11-15: year 2007; samples 16-20: year 2006.

Figure 13 : Principal component score plot of the positive ion abundances of all the samples divided according to batch from different years of production. Each ellipse includes the 95% of the data divided according to age groups. The first two PCs accounted for 37% and 18% of total variation among the data.

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