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MALDI mass spectrometry in the characterization of phytochemical products

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ABSTRACT

Five commercial botanical dietary supplements have been directly analyzed by mass spectrometry by laser irradiation with and without the presence of matrix (in MALDI and LDI conditions). The data so obtained allowed to obtain the characterization of the different samples on the basis of metabolite specific for the different botanic species contained. Furthermore, in the case of positive ion analysis the presence of polysaccharides is put in evidence by using suitable matrices (DHB, super-DHB). If the MALDI experiments are carried out with 1,5-diaminonaphtalene as matrix with basic character, negative ion spectra of good quality are obtained, with the detection of an high number of metabolites, so leading to a clear characterization of the different dietary supplements.

INTRODUCTION

Nowadays, matrix assisted laser desorption ionization (MALDI) mass spectrometry is widely employed in the investigation of protein structure and characterization and, in smaller extent, to nucleic acid and synthetic polymer fields. The reason of this is mainly related to the largest mass range exhibited by MALDI-TOF instrument, which allows to determine the singly charged molecular species to 10^6 Da. The privileged formation of singly charged species (i.e. usually $[M+H]^+$ and $[M-H]^-$ species for positive and negative ions respectively) suggests the use of this technique to obtain the fingerprint of mixture constituted by low molecular weight compounds. The negative aspect of this approach could lie in the small dynamic range of MALDI measurements (10^3) and to possible suppression effect due, in the case of positive ion detection, to the privileged protonation of the components exhibiting the highest proton affinity values. However MALDI mass spectrometry has been described as a valid analytical tool also for low molecular species: the review from Burgers et al[1] report a series of examples of this power.

Botanical dietary supplements products are of wide interest, exhibiting a high therapeutic activity without any side effects, usually observed with synthetic drugs. Considering their wide use and their high complexity (usually they are constituted of mixtures of natural extracts and consequently composed by thousands of different molecules), the development of analytical methods able to give an effective view at molecular level of the phytochemical products is surely of interest. For this aim, the classical approach is based on the quantitative measurements of the compounds responsible of the pharmacological activity but this approach can be somehow reductive. In fact very often the natural extract exhibits an ac-

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tivity higher than that of the single active component. For example Bilia et al[2] have recently investigated on the activity of different extracts of *Artemisia Annua*, all containing artemisinin, responsible for antimalarial activity. The acetone and n-hexane extracts were found to be the most potent ones against different parasitic protozoa and this behavior was rationalized considering, in these extracts, the presence of other bioactive compounds and/or of compounds having a significant synergism with artemisinin.

Then different analytical approaches, able to describe the phyto-metabolome, (i.e. integrating relevant biomarkers in the phytochemical system) are to be tested.

To obtain a more enlarged view of the botanical dietary supplements extracts we proposed a method based on the direct infusion of plant extracts in an Electrospray Ionization (ESI) source operating in positive and negative ion modes (ESI + and ESI -). This approach allowed to obtain spectra well characterizing the different plant extracts, as evaluated by statistical analysis of the data[3]. The same approach was employed by different research groups, confirming its validity[4-7]. In a further investigation the same approach was used for characterization of botanic dietary supplements, constituted by mixtures of plant extracts, and also in this case specific metabolic profiles were obtained[8]. More recently a comparison of the results obtained by LC/ESI/MS and direct infusion/ESI/MS has been done, showing that analogous results can be obtained by the two approaches, being both able to identify the presence of specific plant extracts in commercial products[9].

TABLE 1 : Ingredients of the commercial botanical dietary supplements reported in decreasing quantity order.

Commercial Name	Ingredients
Sedivitax gocce (drops)	*Passiflora Incarnata* (hydroalcholic leaf exctract) *Passiflora Incarnata* (freeze-dried leaf extract, Passiflò 2-LMF) *Valeriana Officinalis* (freeze-dried root extract) *Melissa Officinalis* (freeze-dried leaf extract) *Eschscholtzia Californica* (freeze-dried top flowered plant extract) *Citrus Aurantonum var. dulcis* (essential oil) *Lavandula angustifoglia* (essential oil)
Ruscoven Gocce (drops)	*Hamamelis Virginiana and Vitis Vinifera* (hydroalcholic leaf extract) *Ruscus Aculeatus* (freeze-dried root extract) *Centella Asiatica* (freeze-dried leaf extract) *Ginko Biloba* (freeze-dried leaf extract)
Sollievo Bio Tavolette (tables)	*Cassia Angusti Foglia* (powdered leaf) *Taraxacum Officinalis* (powdered root) *Aloe Ferox* (powdered dried leaf juice) *Cichorium intybus* (powdered root) *Foeniculum vulgare* (powdered fruit) *Carum Carvi* (powdered fruit) *Peumus boldus* (leaf) *Cuminum Cyminum* (powdered fruit) *Foeniculum vulgare* (essential oil)
Finocarbo Plus Opercoli (capsules)	*Foeniculum vulgare* (powdered fruit) *Charchoal Gelatine,* *Chamomilla Recutita* (freeze-dried flower extract) *Carum Carvi* (powdered fruit) *Cuminum cyminum* (powdered fruit) *Foeniculum Vulgare* (essential oil) *Mentha Piperita* (essential oil)
MiniMas Opercoli (capsules)	*Olea Europea* (powdered leaf) *Crataegus monogyna* (powdered leaf and flower) *Olea Europea* (freeze-dried leaf extract) *Gelatine* *Crataegus monogyna* (freeze-dried flowering summit extract)

We were conscious that by that approach only a partial view of plant extracts content was obtained, due to the detection of ionic species exhibiting the highest ionization yields in ESI ± conditions. In order to obtain a more complete view, ionization methods based on different approaches must be used and in the present investigation we evaluate the results achievable for the characterization of different phytochemical commercial products by both laser desorption ionization (LDI) and MALDI methods and compare them with those already obtained in ESI conditions for the same products.

EXPERIMENTAL

Samples

Five commercial botanical dietary supplements produced by Aboca S.p.A., Società Agricola, San Sepolcro Italy, were analyzed: Sedivitax gocce (1); Ruscoven gocce (2); Sollievo Bio Tavolette (3); Finocarbo Plus Opercoli (4); MiniMas Opercoli (5).

The ingredients in decreasing quantity order are reported in TABLE 1.

Analytical sample preparation

Sedivitax Gocce (1) and Ruscoven gocce (2) products, available as water:ethanol (55:45 and 35:65 v/v respectively) solutions, were simply diluted 1:100 (v/v) with H_2O containing 0.1% of Trifluoracetic acid (TFA). Finocarbo Plus Opercoli (4) and Minimas Opercoli (5) are available as opercula. 1 opercula (500 mg) was suspended in 4 mL of water:methanol, (50:50, v/v), sonicated for 20 minutes and let standing overnight. The supernatant was centrifuged (13,000 turns/min) for 15 min and the supernatant so obtained diluted 1:10 (v/v) with H_2O containing 0.1% TFA. Sollievo Bio Tavolette (3) is available as tablets. One tablets (400 mg) was powdered and mixed with 5 mL water:methanol 50:50 (v/v) sonicated for 20 minutes and let standing overnight. The supernatant was centrifuged (13,000 turns/min) for 15 min and the supernatant so obtained diluted 1:10 (v/v) with H_2O containing 0.1% TFA.

One microliter of each diluted solutions of the five commercial botanical dietary supplements considered was deposited on the sample plate for the Laser Desorption Ionization (LDI) analysis.

The same solutions were deposited on the sample plate with three different MALDI matrix (α-cyano-4-hydroxycinnamic acid [HCCA], 2,5-dihydroxybenzoic acid [DHB] and Super-DHB [95% 2,5-dihydroxybenzoic acid and 5% 2-hydroxy-5-methoxybenzoic acid]) by the traditional Dried Droplet deposition method[10]. Five μL of each sample were mixed with 5 μL of matrix solutions. The three matrices employed were HCCA, DHB and super-DHB 10 mg/mL in H_2O/Acetonitrile (50/50; v/v) containing 0.1 % TFA. About 1 μL of this mixture was deposited on the stainless-steel sample holder and let to dried before MALDI analysis.

MALDI analysis were performed using a MALDI-TOF Ultraflex II (Bruker Daltonics, Bremen, Germany) operating in reflectron positive and negative ion modes. Ions were formed by a pulsed UV laser beam (nitrogen laser $\lambda = 337$ nm). The instrumental parameters employed operating in reflectron positive ion mode were the following: ion source voltage 1: 25 kV; ion source voltage 2: 21.65 kV; lens: 10.5 kV; delay time: 0 ns. The instrumental parameters employed operating in reflectron negative ion mode were the following: ion source voltage 1: -20 kV; ion source voltage 2: -17.32 kV; lens: 8.4 kV; delay time: 0 ns.

External mass calibration (Peptide Calibration Standard) was based on monoisotopic values of $[M+H]^+$ of HCCA, Angiotensin II, Angiotensin I, Substance P, Bombesin, ACTH clip (1-17), ACTH clip (18-39), Somatostatin 28 at *m/z* 190.04987, 1046.5420, 1296.6853, 1347.7361, 1619.8230, 2093.0868, 2465.1990 and 3147.4714, respectively and on monoisotopic values of $[2M+H]^+$ of HCCA at *m/z* 379.09246.

RESULTS AND DISCUSSION

In a previous investigation the same commercial botanical dietary supplements (1-5) were analyzed in ESI conditions. A clear characterization was obtained on the basis of specific compounds present in the natural extracts used for the supplement preparation. However it must be considered that the obtained results gave only a partial view of the metabolic profile of the samples under investigation. In ESI conditions the detection of species already of ionic nature present in the samples is highly privileged: this aspect was proved by the posi-

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tive ion ESI spectrum of sample (1), in which the ion corresponding to Californidine is the most abundant one, even if the *Eschscholzia Californica* extract represents only a minor component of the supplement, due to the ionic nature of this compound. On the other hand all the examined compounds showed in negative ion ESI conditions the most specific spectra, indicating that the acid components of the supplements are the most characteristic ones.

A different ionization condition would lead, in principle, to a different view of the metabolic profiles and the five different commercial botanical dietary supplements (1-5) were analyzed in laser desorption/ionization conditions without and with the presence of matrices (LDI and MALDI respectively).

First of all some experiments were performed in LDI conditions, i.e. without the use of any matrix. What should be expected in these experimental set up is:

I. the desorption and detection of species already present in the sample in ionic form;
II. the direct ionization by laser irradiation of species exhibiting low ionization energy through two photon mechanism for positive ions and by electron attachment phenomena for negative ion formations;
III. the protonation of positive ions of some components by reaction with other components ionized by laser irradiation;
IV. the deprotonation through reaction with laser generated basic species in the case of negative ions.

The spectra obtained in positive ion mode of sample (1-5) are reported in Figure 1. Some ionic species are common for all the samples under study. This is the case of ion at m/z 219, $[M+K]^+$ of glucose, present in the spectra of (2), (3) and (4). At higher m/z values (m/z 381) the $[M+K]^+$ species of a disaccharide is detectable for all the samples. However, as already observed in ESI conditions, the different samples lead to LDI spectra substantially different and the most abundant ions can be assigned to molecular species already described as present in the different plant extracts constituting the samples.

In the case of Sedivitax (sample 1) the most abundant ion can be due to Californidine, present in *Eschscholzia Californica* extracts. It is interesting to observe that the *Eschscholzia Californica* extract is present in Sedivitax at very low level (0.92%) but the detection of Californidine[11] in so high yield can be well explained by its ionic nature and the consequent desorption, as previously described in point i). The metabolites of the other components of the sample are undetectable or present in very low abundance, even if their percentage in the commercial products are higher than that of *Eschscholzia*. As an example *Passiflora* percentage is three order of magnitude higher than that of *Eschscholzia*, but any trace of the related metabolites is not detectable in LDI conditions, due to ion suppression effects originating by the ionic nature of Californidine.

In the case of sample (2) (Ruscoven) two abundant peaks are present at m/z 429 and 628, undetectable in ESI conditions. On the contrary, compounds evidenced in the ESI spectra are not detectable in LDI conditions, proving the complementary data obtained in the two ionization conditions. For the species at m/z 429 the structure of [3-gallic acid-$2H_2O$-COOH] can be assigned, while the ion at m/z 628 could be due to [4gallic acid-$3H_2O$+2H]. In other words they represent tannins described as present in *Hamamelis virginiana* extracts[12].

In the case of sample (3), aside the $[M+K]^+$ of disaccharide at m/z 381, some abundant peaks are detectable in the m/z range 433-867. The most abundant species (m/z 579) corresponds to $[M+K]^+$ of Aloeresin, one of the major components of *Aloe* and the ions at m/z 395 and 457 can be assigned to $[M+H]^+$ of Aloeresin B and to $[M+K]^+$ of Aloin respectively[13, 14].

Samples (4) and (5) show only scarcely abundant peaks. Worth of noting is the presence of high mass ions in the case of Finocarbo (4) and the presence of ion at m/z 175, corresponding to $[M+Na]^+$ of Fenchone present in *Feniculum Vulgare*[15]. In the case of (5), an ion at m/z 579 is detected. It exhibits the same m/z value of Aloeresin but the related MS/MS spectra show that it is a glucoside, showing losses of a glucose residue[8].

It must be noted that the species detected in positive ion LDI conditions necessarily are due to compounds already present in ionic form as well as with low ionization energies, so that photoionization processes can occur. Aside these two possibilities a third one must be considered. The detected ions can also originate by gas phase ion-molecule reactions occur-

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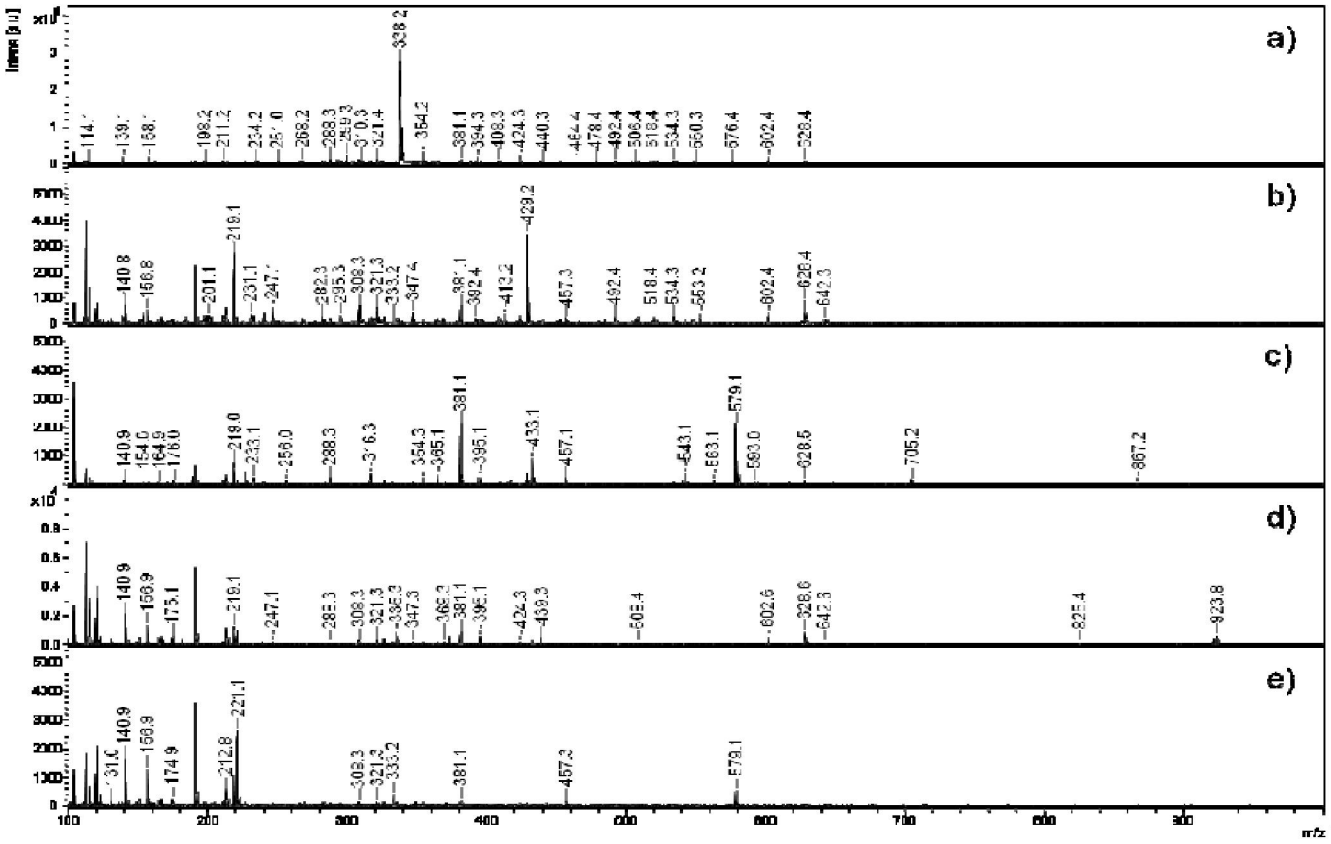

Figure 1 : LDI spectra of a)Sedivitax, b) Ruscoven, c) Sollievo, d) Finocarbo, e) MiniMas obtained in positive ion mode

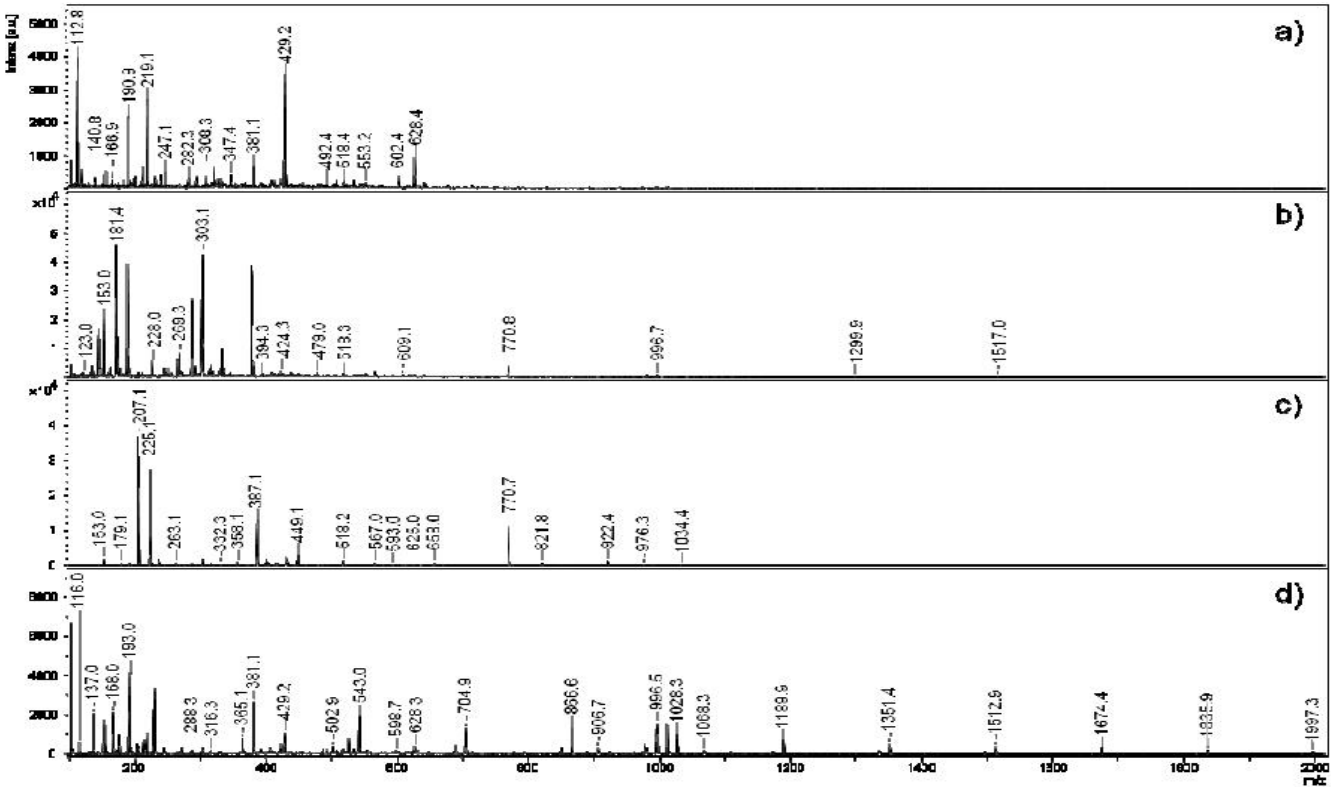

Figure 2 : Specra of Ruscoven obtained in a) LDI, and MALDI by using b) HCCA, c) DHB, d) SuperDHB as matrices, in positive ion mode

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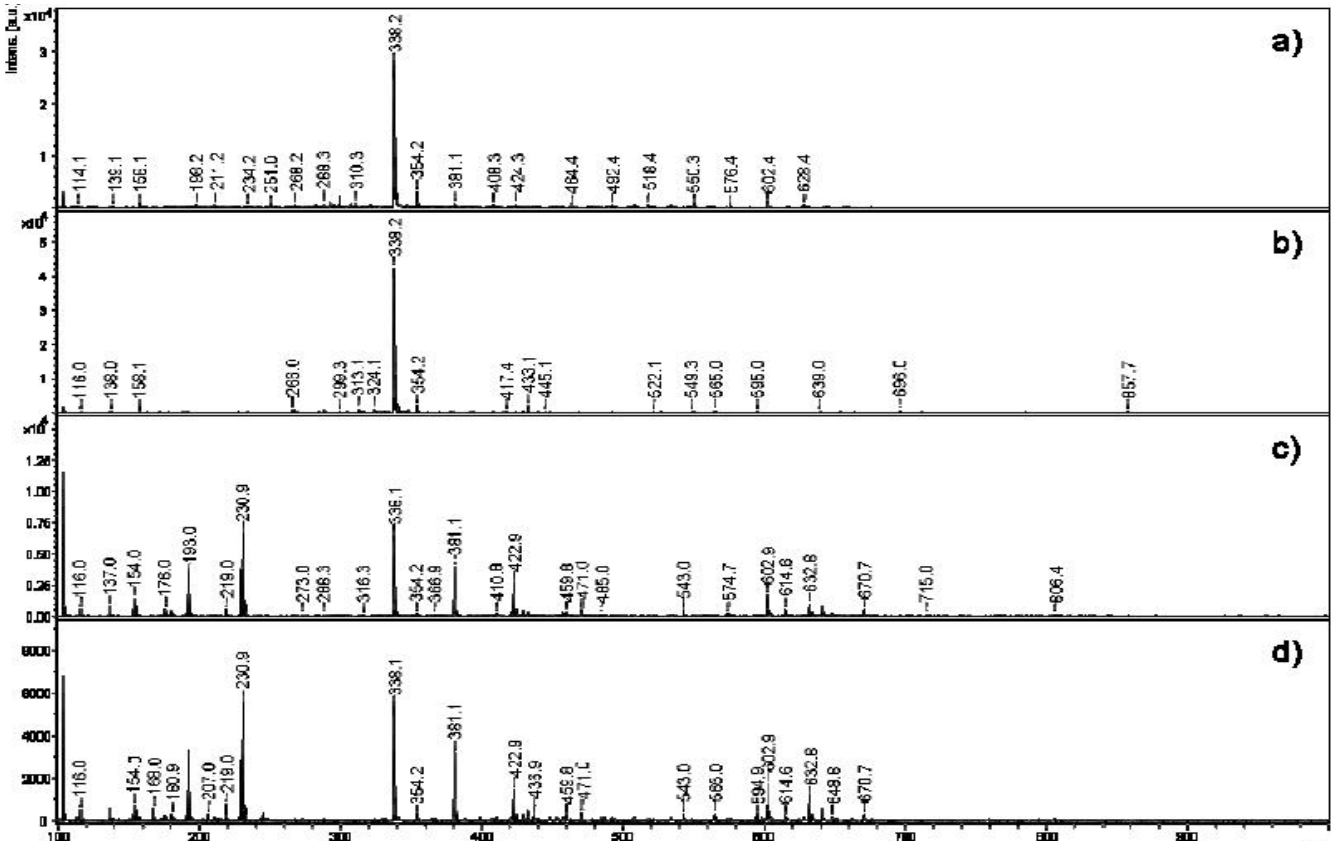

Figure 3 : Specra of Sedivitax obtained in LDI a), and MALDI by using HCCA b), DHB c), SuperDHB d) as matrices, in positive ion mode

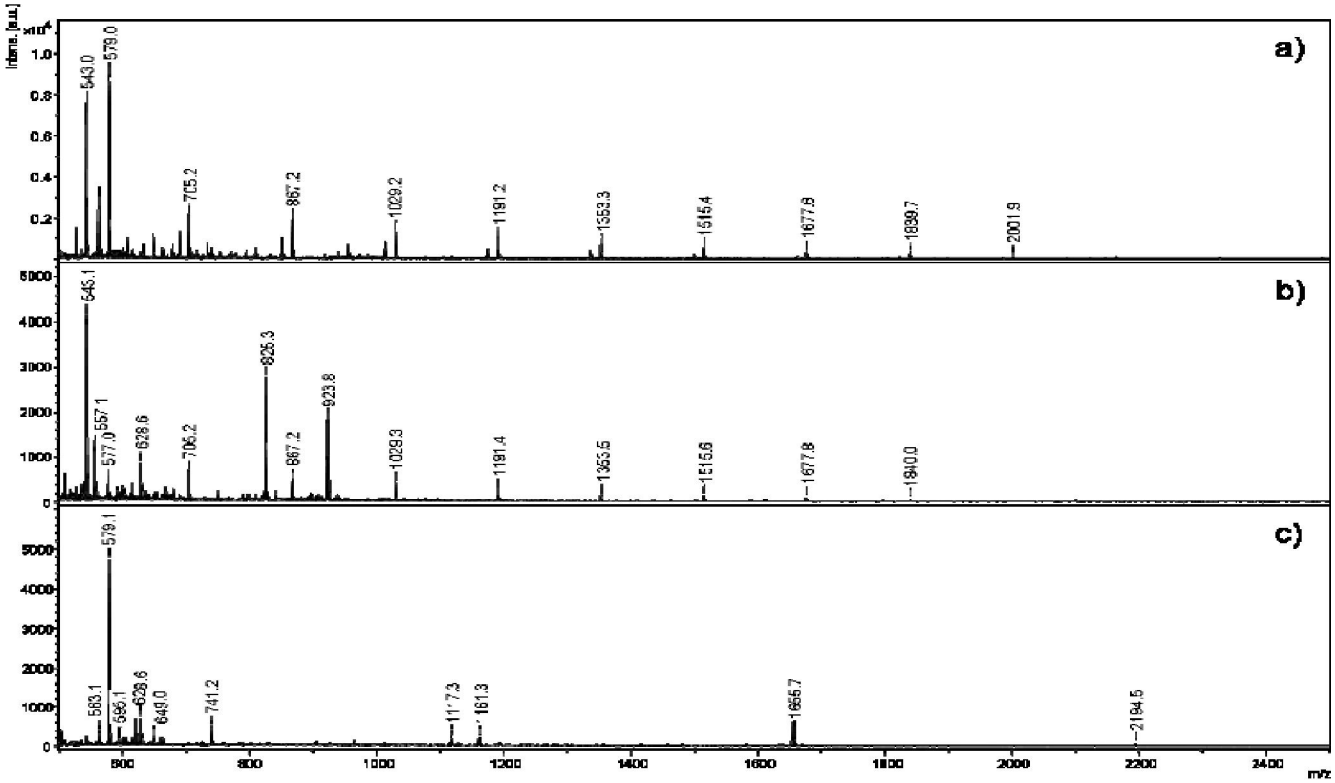

Figure 4 : MALDI positive ion spectra of a) Sollievo, b) Finocarbo, c) MiniMas obtained by using super-DHB as matrix

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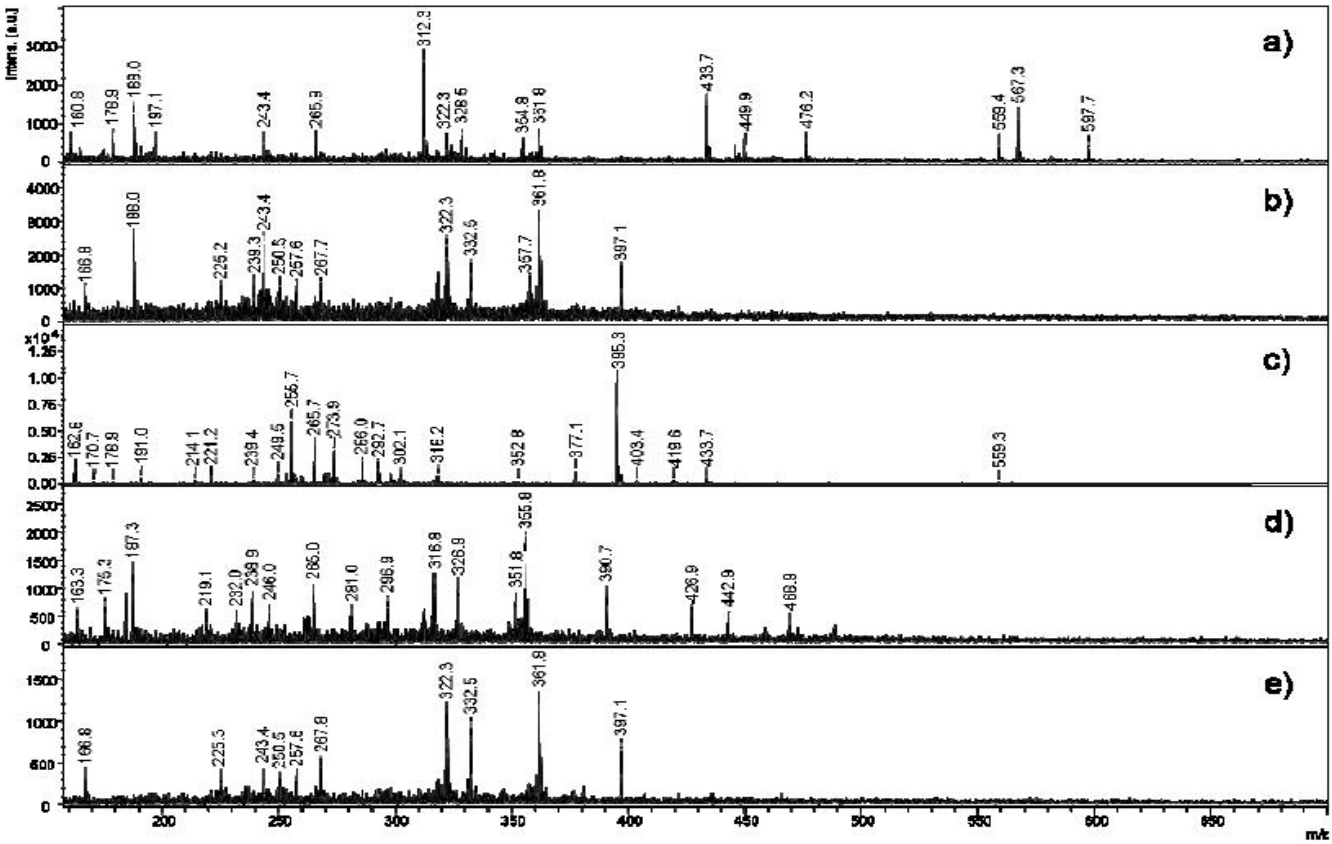

Figure 5 : LDI specra of a) Sedivitax, b) Ruscoven, c) Sollievo, d) Finocarbo, e) MiniMas obtained in negative ion mode

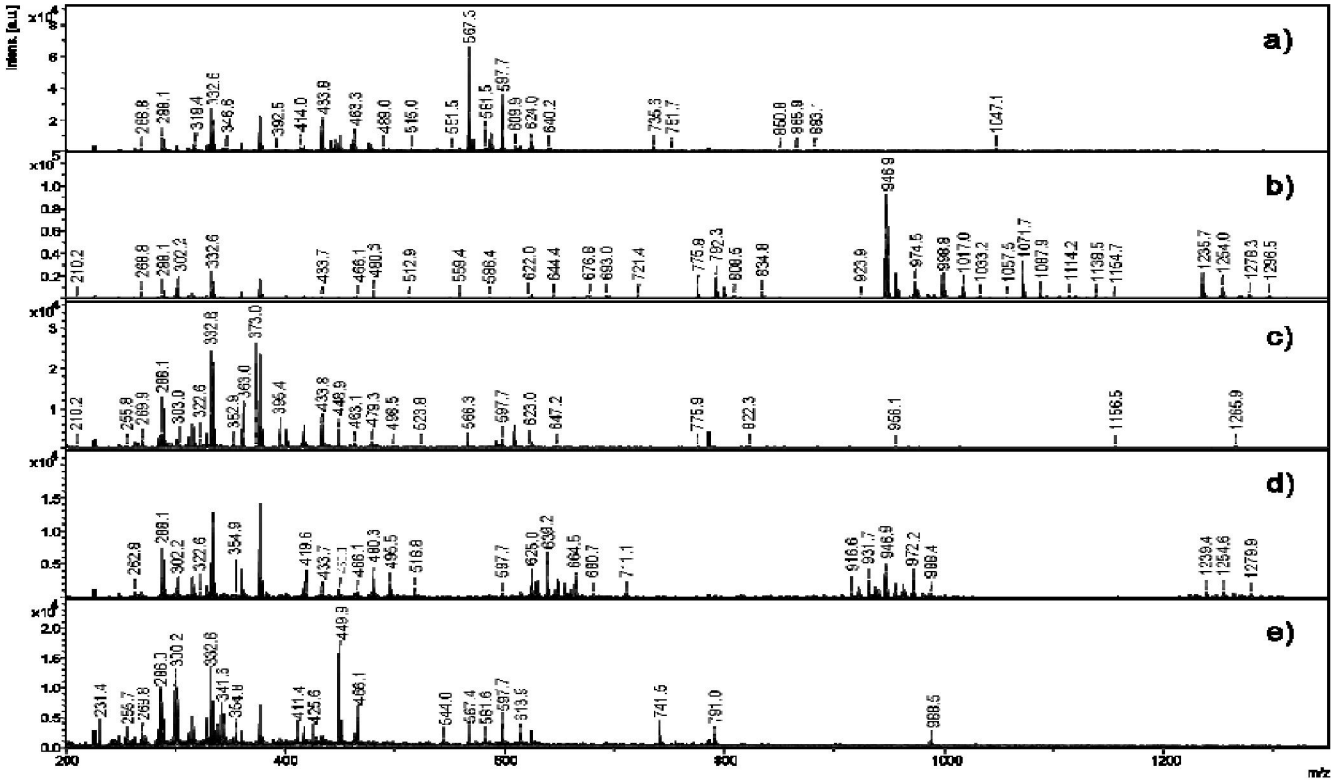

Figure 6 : MALDI negative ion spectra of a) Sedivitax, b) Ruscoven, c) Sollievo, d) Finocarbo, e) MiniMas, obtained by using HCCA as matrix.

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TABLE 2 : Data obtained from positive ion spectra for (1)-(5) in different operative conditions

Commercial Products	LDI mode	MALDI HCCA matrix	MALDI DHB matrix
Sedivitax	m/z 288 Cyanogenic glycosides passicoriacin	m/z 433 Vitexin	m/z 219 Valerenale
	m/z 338 Californidine	m/z 565 Schaftoside	m/z 423 Valtrate
	m/z 381 K^+ diglucoside	m/z 595 Vicenin	
Ruscoven	m/z 219 K^+ glucose	m/z 303 Quercetin	----
	m/z 381 K^+ diglucoside		
	m/z 429 [3-gallic acid-H_2O-COOH]		
	m/z 628 [4-gallic acid-$3H_2O$+2H]		
Sollievo	m/z 381 K^+ di glucoside	m/z 395 Aloeresin B	m/z 395 Aloeresin B
	m/z 395 Aloeresin B	m/z 541 Aloeresin E	
	m/z 457 K^+ aloina		
	m/z 579 K^+ aloeresin		
Finocarbo	m/z 219 K^+ glucose	m/z 271 Apigenin	m/z 541 Aloeresin A
	m/z 381 K^+ diglucoside	m/z 303 Quercetin	
	m/z 175 Na^+ fenchone		
Minimas	m/z 381 K^+ disaccharide	m/z 271 Apigenin	m/z 579 Luteolin-diglucoside
	m/z 579 Luteolin-diglucoside	m/z 303 Quercetin	
		m/z 433 Vitexin	
		m/z 449 Orientin	
		m/z 579 Luteolin-diglucoside	

ring in the desorbed plume between photoionized species and neutrals.

The further approach employed to obtain the positive ion fingerprinting of the samples of interest was based on MALDI experiments. In these conditions what is to be expected is the ion production through gas phase ion-molecule reactions between the neutral analytes (*A*) and protonating species generated by laser irradiation of the matrix (MaH^+). It must be emphasized that the occurrence of these reactions will depend on their hexothermicity $A+MaH^+ \rightarrow AH+Ma-\Delta H$. The $-\Delta H$ value depends by the difference of proton affinities (PA) of *A* and *Ma*. Consequently the use of different matrices, exhibiting different PA values, would lead, in principle, to different results, with the privileged ionization of different compounds.

As matter of fact samples (1- 5), when analyzed in MALDI conditions with different matrices give rise to quite different spectra. As examples, the results obtained for Ruscoven (sample 2) and Sedivitax (sample 1) using HCCA, DHB and super-DHB are compared with those obtained in LDI conditions in Figures 2 and 3 respectively. In the case of sample 2 the abundant species detected in LDI conditions (Figure 2a) are no more present in the MALDI spectra (Figure 2b, 2c, 2d). HCCA and DHB lead to quite different spectra, even if some common ions are produced in the two experimental conditions (e.g. m/z 771 and 518). With HCCA (Figure 2b) the most abundant ions are detected at m/z 181, (reasonably corresponding to $[M+H]^+$ of fructose) and at m/z 303, (corresponding to $[M+H]^+$ of flavonoid Quercetin[16]). In the case of DHB (Figure 2c) the most abundant ions are detected at m/z 207 and 225 but their structure have not been determined. Super-DHB (Figure 2d) activates the production of a series of ions differing of 162 Da units, indicating their polysaccharide nature. The spectrum shows the presence of two series of polysaccharide ions up to 14 units, originating by cationization with Na^+ and K^+.

In the case of Sedivitax the spectra obtained with different matrices are reported in Figure 3. It is to note that, even in presence of different matrices, the peak at m/z 338 due to *Californidine*[11] is always particularly abundant. Different matrices lead to the production of new ionic species. Thus in the case of HCCA (Figure 3b), the ions corresponding to Schaftoside, Vitexin and Escholtzine (m/z 565, 433, and 324 respectively) are detectable. The two ions corresponding to Shaftoside and Vitexin are characteristic of *Passiflora Incarnata*[17-19], whereas the ion Escholtzine[11] is characteristic of *Eschscholtzia Californica*. The ion at m/z 595, even if in scarce abundance, could be due to Vicenin-2. Analogous results are obtained by using DHB and super-DHB (Figure 3c and 3d), with production of abundant ions at m/z 231, 381, 423 and a quite complex peak pattern in the range m/z 540-680. The ions at m/z 423 and 219 correspond to $[M+H]^+$ species of Valtrate and Valerenal respectively, present in *Valeriana*

TABLE 3 : Data obtained from negative ion spectra for (1)-(5) in different operative conditions

Commercial Products	LDI mode	MALDI HCCA matrix	MALDI DAN matrix
Sedivitax	---	m/z 463 Quercetin-O glucoside	m/z 179 Caffeic Ac m/z 285 Luteolin m/z 359 Rosmarinic Ac. m/z 431 Vitexin m/z 447 Luteolin 6-C-glucoside m/z 563 Schaftoside m/z 609 Rutin
Ruscoven	---	----	m/z 169 Gallic Ac. m/z 285 Kampferol or Luteolin m/z 301 Quercetin m/z 325 Bilobalide m/z 447 Luteolin 6-C-glucoside m/z 463 Quercetin-O glucoside m/z 787 Tetragalloyl-glucoside m/z 939 Pentagalloyl-glucoside
Sollievo	m/z 179 Caffeic Ac. m/z 191 Quinic Ac. m/z 354 Clorogenic Ac.	m/z 301 Quercetin m/z 417 Aloin A m/z 447 Luteolin 6-C-glucoside m/z 463 Quercetin-O glucoside m/z 479 Myricetin-O glucoside m/z 608 Rutin	m/z 191 Quinic Ac. m/z 417 Aloin A
Finocarbo	m/z 164 Eugenol	m/z 625 Myricetin-O-rhamnoglucoside	m/z 167 Gallic Ac. m/z 179 Caffeic Ac. m/z 285 Luteolin m/z 301 Quercetin m/z 331 Monogalloyl-glucoside m/z 353 Clorogenic Ac. m/z 359 Rosmarinic Ac. m/z 447 Orientin m/z 463 Quercitin-O glucoside m/z 493 Myricetin-glucoronide
Minimas	----	m/z 418 Oleoside	m/z 179 Caffeic Ac m/z 285 Luteolin m/z 353 Clorogenic Ac. m/z 431 Vitexin m/z 447 Orientin m/z 463 Quercitin-O glucoside m/z 539 Oleuropina

Officinalis[20-22]. It is worth noting that Super-DHB does not put in evidence the polysaccharide sequence observed in the case of Ruscoven. Only di- and tri-saccharides, ionized by K^+ addition, are detectable at m/z 381 and 543.

Considering that super-DHB has led, in the case of (1) and (2) to the best results, the same matrix has been employed also for (3)-(5) (Figure 4). In the case of (3)

and (4) (Figure 4a and 4b respectively) the presence of polisaccharides is evidenced, with a distribution lower than that observed for (2). In the case of (5) (Figure 4c) a new series of ions, starting from m/z 579 and going up to 2193 is observed, with mass differences of 539. Considering that the ion at m/z 579 has been previously assigned by ESI MS/MS as a glucoside[8], showing the loss of 162 Da, it is reasonable to assume that the ions at m/z 1117, 1655, 2194 can be due to the condensation of 1,2 and 3 tannin units on this species. All the data obtained in positive ion conditions are summarized in TABLE 2.

In LDI conditions negative ions are usually generated by electron attachment and proton abstraction, due to the formation of basic species. The LDI negative ion spectra of (1) – (5) are reported in Figure 5.

As expected they strongly differ from those obtained in positive ion conditions (summarized in TABLE 2) and only few ionic species can be assigned to some of the components of the different commercial products. The data obtained in negative ion mode are summarized in TABLE 3. Of course these results, partially negative, are not surprising. The ionization conditions leading to negative ion production are strongly different in ESI and MALDI conditions. In the former case negative ion formation is activated in the sample solution, following the classical acid-base equilibria enhanced by the presence of the strong electrical field. In LDI and MALDI conditions the negative ions are produced in gas phase, after the formation of plume generated by laser irradiation. The possible ionization mechanism are in this case due to electron attachment (in fact slow electrons are generated by laser irradiation) or by gas phase acid-base equilibria, with kinetics 3-4 order of magnitude faster than those present in solution. Considering these differences between the two experimental conditions, different results are to be expected. As can be seen by the comparison of the data obtained by the different approaches reported in TABLE 3 a higher number of components of the extracts present in the commercial product can be detected in presence of matrices but in this case a number of ionic species lower than that obtained in ESI (-) condition is present. This result can be explained by: i) the low basicity of the matrix employed, ii) the higher yield of deprotonation reaction occurring in solution in the case of ESI either before the spraying or inside the sprayed droplet; iii) the unfavourable gas phase deprotonation in MALDI due to the absence of basic species by the solvent present in ESI conditions.

To verify these hypothesis a further series of experiments were carried out by using 1,5-diaminonaphtalene (DAN) as matrix[23], surely more basic than HCCA. The results so obtained are summarized in the third column of TABLE 3. It is at first sight evident that DAN is able to activate a high number of gas phase ion-molecule reactions leading to the $[M-H]^-$ ion production of many metabolites of the extracts present in the different dietary supplements. Even if the neutral species present in the plume generated by laser irradiation expand in the vacuum at supersonic speed, the fast kinetics present in gas phase leads, when the reactant exhibits a good basicity, to the production of $[M-H]^-$ ion in high yield.

CONCLUSION

The data obtained in LDI and MALDI conditions for five dietary supplements, constituted by natural extracts of different botanic species, allow to obtain their characterization on the basis of specific metabolites. In the case of analysis performed in positive ion conditions the presence of polysaccharides is put in evidence by using suitable matrices (super-DHB, DHB). It is to emphasized that this class of compounds is undetectable in ESI conditions, reasonably for their low ionization yield. In negative ion mode analytical results of quality lower than that obtained in ESI are achieved by using the most usual MALDI matrices (HCCA, DHB, super-DHB), reasonably due to their low basicity. If the experiments are carried out with DAN, a more basic matrix, negative ion spectra of good quality are obtained, with the detection of a high number of metabolites allowing an easy characterization of the different dietary supplements.

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