MASS SPECTROMETRY APPLICATIONS IN NATURAL PRODUCTS MEDICINE ANALYSIS

A number of Natural products have been proved to offer an alternative to synthetic drug substances in preventing and treating some Chronic and mild diseases, provided they are of adequate quality and properly used. Many factors influence the quality of herbs, including species variation, environmental conditions, time of harvesting, storage and processing. Moreover, plant extracts may be falsified with similar plants or added with other plants devoid or exhausted of active constituents. For example, different Echinacea species in commercial preparations can be distinguished on the basis of their caffeic acid derivative patterns (Pietta et al, 1998). Similarly, the falsification of Arnica drugs by Heterotheca inuloides can be evidenced evaluating the flavonoid pattern (Pietta et al, 1994a). A substantial difference between Korean and American ginseng extracts (van Breemen et al, 1995) can be proved assaying the amounts of each ginsenoside.
For these reasons, the quality control of plant standardized extracts is a very important step. Unfortunately, this is not simple, because plant extracts are complex mixtures of different compounds and often their characterization is sketchily known. Among the active principles present in plant extracts, terpenes such as sesquiterpenes lactones in Arnica species (Leven and Willuhn, 1987, Willuhn ef al, 1994) and ginkgolides in Ginkgo biloba (Van Beek et al, 1998), flavonoids, such as fiavonol glycosides (Pietta ef a/, 1997) and isoflavones (Pietta et al, 1990), alkaloids, such as indole in Catharanthus roseus (Favretto ef al, 1998) and isoquinoline alkaloids in Chelidonium majus (Pietta ef al, 1995) and caffeic acid derivatives (Bauer and Wagner, 1991) have attracted great interest.
Different analytical methods have been applied, including high-performance liquid chromatography (HPLC), capillary electrophoresis (CE) and gas chromatography coupled to mass spectrometry (GC-MS) (Stenke ef a/, 1993, Biber and Koch, 1999).
In particular, HPLC coupled with diode array detection has proved to be the method of choice, since it allows an efficient separation of flavonoids (Pietta et al, 1994b), terpenes (Willuhn ef a/, 1994), phenolic acids (Gocan ef al, 1996) and alkaloids (Henion et al, 1994).
CE has been proved to be a valuable alternative to HPLC (Mauri ef a/, 1996, Merfort ef a/, 1997).
Mass spectrometry (MS) has been firstly applied to study isolated plant compounds using off-line approach, such as fast atom bombardment (FAB), plasma desorption (PD), chemical ionization (Cl) and electron impact (El).

The introduction of the moving belt (MB) and thermospray (TSP) interfaces allowed direct coupling of mass spectrometry to liquid chromatography. The last interface has been widely applied to couple mass spectrometry with liquid chromatography (LC-TSP/MS), and this permitted obtaining from a single run, data on the chromatographic, UV and MS behaviour of the analytes. Thermospray mass spectrometry has been used to analyze flavonoids and terpenes in different plant extracts, such as Arnica montana (Schroder and Merfort, 1991), Gentiana species (Wolfender ef al, 1992), Ginkgo biloba (Camponovo ef al, 1995; Pietta ef al, 1994c), Camellia sinensis (Lin ef al, 1993) and Hypericum perforatum (Brolis ef al, 1998).
Unfortunately, TSP-MS fails in the Mass Spectrometry.Applications in Herbal Medicine Analysis,
Case of thermolabile compounds, in fact, flavonol glycosides [M=A+glycoside] analyzed by TSP-MS yield mainly the aglycone fragment [A+H]+, while the molecular ion [M+H]+ is present at lower abundance.
On the contrary, Electrospray Ionization Mass Spectrometry (ESI-MS) involves a low level of fragmentation, and it is particularly suitable for a rapid screening of molecular species present in herbal extracts. In addition, the ESI interface may be coupled to a triple/quadruple or to an ion trap analyser. This promotes mass fragmentation, which provides structural information on the components present in the extracts, and may help in identifying the compounds of interest.

This blog aims to describe (1) the ESI-MS and the Ion Trap-MS (ITMS) techniques and
(2) the currently applied approaches for the analysis of selected constituents of medicinal plant extracts. Specific examples will be considered. In addition, a list of recent applications of MS for medicinal plant analysis will be reported.

INSTRUMENTAL

The features of ESI-MS and ITMS will be briefly described in this section. Actually, there are other possible MS approaches. One of these is the MALDI approach (Matrix Assisted Laser Desorption Ionization) In this case, monocharged ions are produced from a matrix irradiated with laser light, which causes excitation of the analytes and their ejection. The m/z. values of MALDI ions are measured from the time required for ions to travel over a flight tube (Time-of-Fly, TOP analyser). However, MALDI-TOF doesn't permit to work in liquid phase. It is mainly used to analyze large molecules, such as proteins and polymers (Whittal era/, 1997), and its application in medicinal plant analysis is limited.

ESI-MS

ESI-MS produces ions due to the application of a potential to a flowing liquid causing the liquid to charge and spray. Eiectrospray forms very small droplets of solvent containing the analytes. Usually, the solvent is removed by heat and multi charged ions are produced (Kebarle and Tang, 1993). ESI permits the coupling of HPLC instruments directly with the mass spectrometer, and it is characterized by an ionization softer than that achievable by TSP ionization.

Sample preparation

The herb is usually extracted with methanol or aqueous methanol at room temperature or at 40-50°C. depending on the stability of its components. The resulting crude extract may be purified to remove undesired constituents, such as lipids, chlorophyll, sugars, organic acids and salts. In the case of commercial extracts, which are normally enriched in specific compounds, this step may be avoided. Similarly, the purification may be not necessary in the case of LC-MS, since the analytes of interest are separated from the interfering compounds during the chromatographic elution. By contrast, the purification of the sample is recommended in the case of the infusion or direct injection approaches. Indeed, the presence in the herbal matrix of different molecular species at concentrations ranging from 1 to 10 mm can determine ion suppression, i.e. the MS analyzer fails in detecting the ions. In some cases the matrix effect may be reduced by diluting the sample and/or lowering the flow-rate. These expedients appear to be successful when highly sensitive and salt compatible MS instruments are used.

Alkali adducts

In positive ESI-MS the molecular species can form adducts with alkali cations (sodium and potassium). In particular, potassium adducts are typical of unpurified herbal samples, because vegetable matrices are rich in potassium salts. Alkali adduct formation may be diminished by desalting the samples through solid phase extraction (SPE). Diluting the sample solutions is a simple way to replace potassium ions with sodium ions. The latter are the most common in commercial extracts of herbs.

Alkali adduct formation may be useful for obtaining structural information on the analytes. Specifically, alkali adducts of flavonoids allowed to discriminate between various flavonoid classes and provided information on the glycosylation position (Mauri etal, 1999a). Different flavonols and flavones, either as glycosides or aglycones, were examined, and it has been possible to observe a different behavior in ESI/MS in relationship of their structures. The spectrum of isoquercitrin (quercetin-3-O-rutinoside, MW610 Da) is characterized by the presence of m/z 633 ion, which is the sodium adduct, whereas the molecular ion ([MHf m/z 611) is almost absent. The abundance of aglycone residue (m/z 302) is low, indicating that the cleavage of the glucose residue is limited. Same behaviour was observed for other flavonol-3-O-glycosides. On the contrary, flavone-glycosides (lacking of the 3-OH group) yielded mainly the molecular ion and the sugar moiety was easily cleaved. For example, rhoifolin (apigenin-7-O-neohesperidoside, M.W.=578) produced the m/z values 579 ([MH]+) and 270, corresponding to molecular and aglycone residue ions, respectively. The low abundance of sodium adduct (m/z 601) suggests that the presence of the hydroxyl group at position 3 is crucial. However, this presence is not sufficient, since flavonol aglycones did not yield sodium adducts. In fact, flavonol aglycones, such as quercetin, kaempferol and isorhamnetin, didn't produce sodium adducts. Then, the presence of the 3-O-glycosyl residue appears to be important. To confirm this hypothesis, spiraeoside was subjected to ESI-MS. This compound is a quercetin derivative with glucose linked at position 4' on ring B, while the

hydroxyl in position 3 is free. In this case, no adduct was formed; the spectrum presented mainly the molecular ion ([MH]+-465) and a relevant fragmentation occurred. On the contrary, quercetin-3-O-glucoside (isoquercitrin) showed mainly the sodium adduct.

QUANTITATIVE ANALYSIS

The content of bioactive compounds present in herbs may be determined by HPLC or CE coupled with UV detection. However, in some cases (e.g. for compounds not absorbing in the UV) this approach is not recommended. GC-MS may be of value, particularly for volatile components that do not require derivatization. Recently, advanced electrospray interfaces have been developed; these devices permit to obtain accurate and reproducible quantitative results. Accordingly, the bio active components of various plant extracts are increasingly assayed by coupling separation systems (HPLC and, less commonly, CE) to ESI-MS. In addition, the possibility to perform quantitative assays by means of direct injection in ESI-MS without separation step will be also considered.

Mass Spectrometry Coupled to Separation Systems

As mentioned, LC-MS is the preferred method for quantitative analysis, and it has been applied to assay different compounds, such as synthetic drugs and their metabolites, nutrients, phytochemicals, peptides, nucieotides, and bioactive compounds present in different medicinal plants. Thus, flavonoids in Ginkgo biloba (Watson et a/, 1998), ginsenosides in Panax ginseng (Wang et a/, 1999) and naphthodianthrones in Hypericum perforation extracts have been assayed by means of LC-MS. By contrast, CE-MS quantitative analysis is less applied, as evidenced by the limited number of papers dealing with this technique.

Direct Injection

This approach, based on direct injection of the sample into the mass spectrometer without a previous separation by HPLC, it is not as accurate and reproducible as the LC-ESI-MS method. However, it requires short analysis times (about 2-3 min for each injection), and permits to assay a great number of samples.

MEDICINAL PLANT ANALYSIS BY MASS SPECTROSCOPY

In the following table recent MS applications in medicinal plant analysis are listed. MS is applied mainly for compound identification, using the new ITMS instruments. The IC-ESI-MS approach is preferred as compared to direct infusion, and this is due to the fact that this procedure permits both quantitative and structural analysis. Up to now only few examples of quantitative and CE-MS analyses are published.

List of Recent MS Applications in Medicinal Plant Analysis

Classes of	Matrices	MS Mode	Reference
Alkaloids	*Senecio* species	LC-TSP-MS	Ndjoko et a/, 1999
	Aconitum napellus	ITMS	Ying et a/, 1999
	Psychotria species	LC-MS	Verotta et a/, 1999
	Aconitum carmiechaeli	ESi-MS/MS	Weixing *et al,* 1999
	Chinese medicine	CE-ITMS	Chen et a/, 2000
		MS/MS	Garraffo et a/, 1 999
	STD and extracts	CE-MS	Stockigt et a/, 1998
	Chelidonium majus	ESI-MS	Pietta et a/, 1995
	Habropetalum dawei	*LC-ESI-MS/MS*	*Bringmann* etal, *1999*
	Different extracts	*CE-MS*	*Stockigt, 1998*
	Catharanthus roseus	*LC-ES1-MS*	*Favretto et a/, 1998*
Terpenes	Vernonia fastigiata	*LC-MS*	*Vogler et a/, 1998*
	Ginkgo biloba	*LC-ESI-MS*	*Mauri et a/, 1999b*
	Imulanthera nuda	*LC-MS*	*Gafner et a/, 1998*
	Baccharis pingrae	*LC-MS*	*Wolfender et a/, 1998*
	Different plant extracts	*LC-MS*	*Theodoridis et a/, 1998*
	Taxus brevifolia	*LC-MS*	*Kerns et a/, 1998*
	Achillae species	*LC-MS*	*Glasl et a/, 1999*
	Green tea	*LC-MS/MS*	*Ney et a/, 1996*
	Different extracts	*LC-MS*	*Hostettmann and Wolfender, 1999*
	Centella asiatica	*ESI-MS*	*Mauri and Pietta, 2000a*
Ginsenosides	Panax ginseng	*LC-ESI-MS*	*Fuzzati et a/, 1999*
	P. ginseng and quinquefolius	*LC-MS/MS*	*Wang et fa/, 1999*
	P. ginseng and quinquefolius	*LC-ESI-MS*	*Van Breemen* et al, *1995*
	Panax ginseng	*ESI-MS*	*Mauri and Pietta, 2000a*
Saponins	Panacis Japonic!	*LC-MS*	*Kanamori et a/, 1 995*
	Standards	*ESI-MS*	*Lee et a/, 1996*
	Tribulus terrestris	*ESI-MS/MS*	*Shiping et a/, 1999*
	Phytolacca dodecandra	*LC-MS*	*Perret et a/, 1999*

	Black bean	*ESI-MS/MS*	*Lee et a/, 1999*
Hypericins	Hypericum perforatum	*LC-ESI-MS*	*Balogh, 1999*
	Hypericum perforatum	*LC-MS*	*Brolis et a/, 1998*
	Hypericum perforatum	*LC-ESI-MS*	*Piperopoulos* et al, *1997*
	Hypericum perforatum	*LC-ESI-MS*	*Mauri et a/, 2000b*
	Hypericum perforatum	*LC-MS*	*Brolis et a/, 1998*
	Hypericum perforatum	*LC-ESI-MS*	*Mauri et a/, 2000b*
Flavonoids	Polygomum salicifolium	ESI-MS	Calls et al, *1999*
	Propolis	ESI-MS	Mauri and Pietta, 2000a
	Myrtus communis	LC-MS	Roman! *et* a/, 1999
Flavonoids-flavonols	*Ginkgo biloba*	LC-ESI-MS	Watson and Pitt, 1998
	Ginkgo biloba	LC-MS	Heefa/, 1996
	Hypericum perforatum	LC-MS	Brolis *et al,* 1998
Flavonols	*Hypericum perforatum*	LC-ESI-MS	Mauri *et al,* 2000b
	Sedum telephium	LC-MS	Sturm *et al,* 1999
	Ginkgo biloba	ESI-MS	Mauri and Pietta, 2000a
Biflavones	*Ginkgo biloba*	LC-MS	Gobbato *et al,* 1996
Isoflavones	Soybean	LC-MS/MS	Doergeefa/, 1998
	Soybean	ESI-MS	Mauri and Pietta, 2000a
Isoflavones	*Trifolium pratense*	LC-MS	Balogh, 1997
Isoflavones	*Pueraria species*	LC-ESI-MS	Haojing *et al,* 1998
Coumarins	*Ammivisnagae, Scopoliae*	LC-ESI-MS	Ganzera et a/, 1997
Phenolic acids	Brazilian propolis	ESI-MS	Tazawa et a/, 1998
	Cynara scolymus	ESI-MS	Mauri and Pietta, 2000a
Anthocyanins	*Catharanthus roseus*	ESI-ITMS	Piovan *et al,* 1998
	Vitis vinifera	LC-ESI-MS	Gabetta *et* a/, 2000
	Vaccinium myrtillus	ESI-MS	Mauri and Pietta, 2000b
Glycosides	*Haplophyltum patavinum*	LC-MS, CE-MS	Favretto *et* a/, 1996

The main classes of compounds studied by MS include flavonoids, such as flavonols, catechins, anthocyanins, isofloavones, phenolic acids, terpenes, such as saponins, ginkgolides and ginsenosides, and alkaloids. Only few medicinal plants, such as Panax ginseng, Ginkgo biloba and Hypericum perforatum have been throughly investigated by MS. On the other hand, MS has been applied to specific purposes, e.g. to discriminate different species and to study the pharmacokinetics of bioactive principles from medicinal plants.

CONCLUSIONS

It may be concluded that Electrospray Mass Spectrometry allows analyzing bioactive components in complex matrices of medicinal plant extracts. Being characterized by high specificity and sensitivity (up to 10 NM), ESI-MS permits to achieve rapidly finger prints of herbal extracts without pre-purification steps. In addition, MS/MS methodology by means of triple quadruple or ion trap provides a reliable assignment of the compounds present in the extracts.

Concerning quantitative aspects, LC-MS is the approach of choice, since it permits to eliminate the ion suppression effects. Direct injection approach seems to be a possible alternative. In this case, the ion suppression can be minimized by adding different standard concentrations to the herbal sample. Surely, this methodology is less reproducible and accurate than LC-MS, but it is indicated for routine quality control. Nevertheless, more work is needed to validate the direct injection approach for quantitative analysis of medicinal herbal extracts.

The development of nano-electrospray combined with high-resolution separation systems, such as μHPLC and CE will increase resolution, sensitivity and accuracy. This will result in a deeper characterization of medicinal plant constituents, including the protein pattern (proteome analysis), and it will facilitate the pharmacokinetic studies on their active components.

REFERENCE - GMP for Botanicals