International Organization for Chemical Sciences in Development

Working Group on Plant Chemistry

CHEMISTRY, BIOLOGICAL AND PHARMACOLOGICAL PROPERTIES OF AFRICAN MEDICINAL PLANTS

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African traditional healer and *Harpagophytum procumbens* (Pedaliaceae)

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19. Quantitative and qualitative analysis of the saponins from berries of cultivated *Phytolacca dodecandra* by LC/UV and LC/MS

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Introduction

The dried berries of Endod, *Phytolacca dodecandra* L'Hérit (Phytolaccaceae), are used in Ethiopia as a soap substitute. The molluscicidal properties of their constituents were discovered by Lemma in 1965 and this plant became rapidly of great importance for the local control of bilharzia or schistosomiasis (Lemma, 1970). This parasitic disease affects more than 200 million people in over 70 countries in Africa, South America and in the Far East. As shown in Fig. 19.1. (Marston and Hostettmann 1985), the use of molluscicides affects dramatically the life cycle of the parasitic nematode *Schistosoma* species.

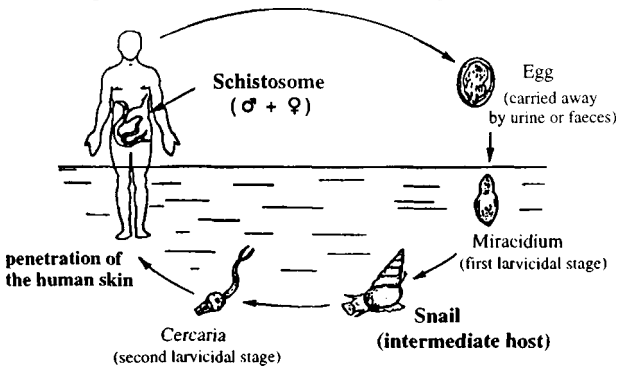

Fig. 19.1. Life cycle of *Schistosoma* species.

Eggs from infected individuals are carried away with urine and produce miracidia which locate snails as intermediate host. They multiply into thousands of cercariae which can penetrate the intact skin of humans who come into contact with the infested water. Once in the body they gradually change into schistosomes and pass into veins and bladder.

The destruction of the snails - intermediate hosts of the parasit - represents a very interesting alternative in the struggle against this tropical disease, and the use of snail-killing compounds of plant origin for the local control of schistosomiasis is attractive due to the economic advantage of cultivating the plants locally, instead of importing costly synthetic chemicals.

However, *Phytolacca dodecandra* is not grown in Swaziland and there was the need to develop a large scale cultivation. To improve the yields of berries and their saponin content, an experimental cultivation plan was undertaken. The growth of three phenotypes was tested under four conditions of field treatment, including the use of a new polymer developed in Slovenia called Eco-agrogel (see Fig. 19.2.).

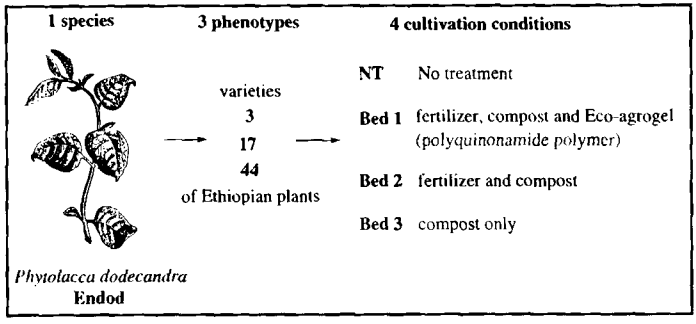

Fig. 19.2. Cultivation plan undertaken for the study of the growth of *Phytolacca dodecandra*.

The number of leaves and average height of each cultivated plant were recorded at fortnightly intervals and the berries harvested. These measurements have shown that a higher growth rate could be obtained with the first field treatment (Bed 1). The effect of the addition of Eco-agrogel to the soil is nevertheless variable for the different phenotypes (Makhubu *et al.* University of Swaziland, unpublished resuls). But the aim of the cultivation was not only to obtain a high yield of berries at maturity stage, but also to maintain a high saponin content. Thus, the study of the growth had to be completed by a quantitation of the saponin content of the berries to evaluate the effect of cultivation conditions on molluscicidal potency.

Extraction procedure

The molluscicidal activity of saponin containing extracts is strongly dependant of the extraction solvents used. Indeed, extraction with methanol provides principally inactive bidesmosidic saponins (glycosylated at positions C-3 and C-28), while water gave active monodesmosidic saponins (glycosylated at position C-3 only) (Glasenapp *et al.* 1988). Temperature and water quality also influence the extraction

process and often, differences between field and laboratory extracts can be observed. Thus, in order to evaluate the real content of the berries, the extractions were carried out with methanol as follows: the dried berries were powdered and 10 g of powder were extracted with 200 ml of methanol during 24 hours at 20°C. After filtration, the solvent was evaporated and the extracts were lyophilised and weighed.

In Fig. 19.3., the extraction yields of berries produced in different cultivation conditions are compared. It can be observed that these yields vary between 20 and 30% and that non-treated berries give higher extraction yields than those grown with special field treatment.

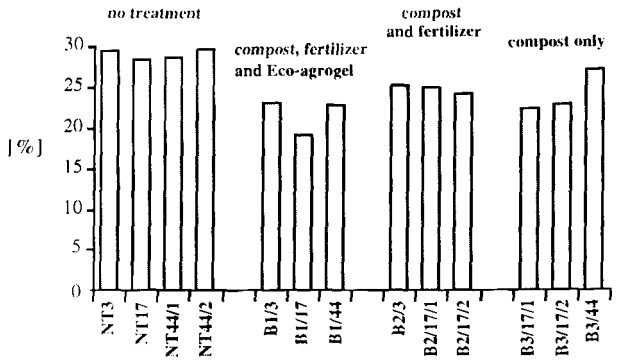

Fig. 19.3. Comparison of methanolic extract yields.

Quantitative determination

The quantitation of the saponin content of all these extracts was then realised by HPLC-UV analysis, with five pure bidesmosidic saponins as standard mixture. These saponins were previously isolated from the plant and were chosen for the quantitative determination because they are representative of the more abundant aglycones present in *P. dodecandra*. These aglycones are oleanolic acid, bayogenin and hederagenin. The structures of the five standards are presented in Fig. 19.4. They present all glucosyl moieties at position C-28 and different sugar chains at C-3.

In Fig. 19.5. are shown the two UV chromatograms of the standard mixture and of a methanolic extract of dried berries. These HPLC analyses were performed on a Waters NovaPak C_{18} (4 µm, 150 x 3.9 mm i.d.) column under the following conditions: MeCN-H_2O 15:85 to 50:50 in 30 min at a flow rate of 1 ml/min. Detection was realised at 206 nm.

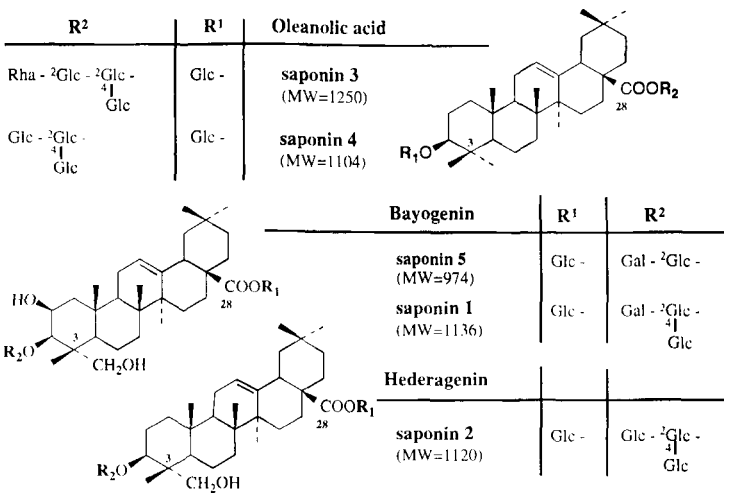

Fig. 19.4. Structures of the five standards representative of the more abundant aglycones present in *P. dodecandra*: oleanolic acid, bayogenin and hederagenin.

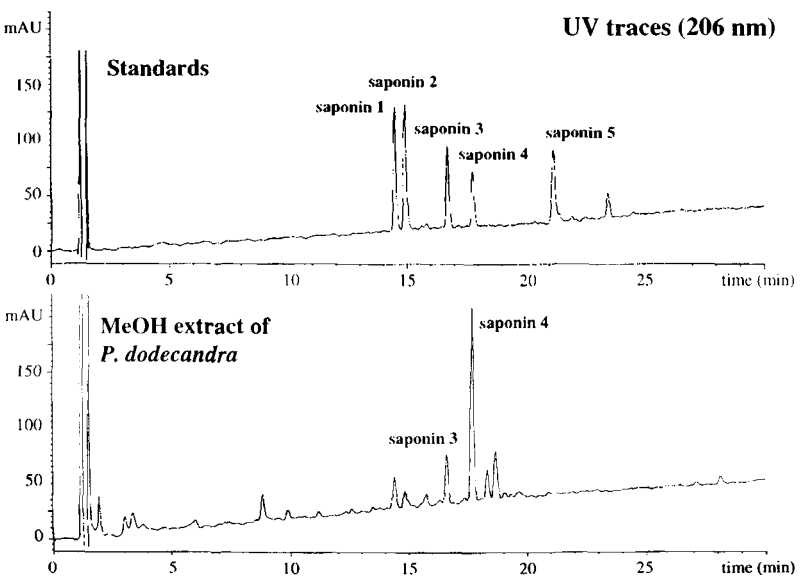

Fig. 19.5. LC/UV analyses of the standard mixture and of a methanolic extract of dried berries.

It can be observed that the methanolic extract is mainly composed of saponins. Of these, glycosides of oleanolic acid (compounds **3** and **4**) represent the major components.

These LC/UV analyses showed that the composition of the extracts did not vary significantly from one sample to another, but differences were observed in the total saponin content of the extracts as shown in Fig. 19.6.

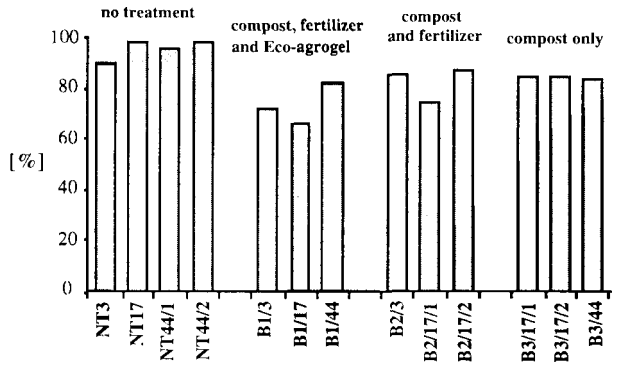

Fig. 19.6. Comparison of the saponin content of the methanolic extracts.

Again, extracts of non-treated berries present higher saponin contents than those obtained with berries grown with special field treatment. Thus, it seem that the increased growth rate obtained by the use of compost, fertiliser or Eco-agrogel is accompanied by a diminution in the saponin content in the berries. Field and laboratory measurements have now to be carefully analysed to determine if these two phenomena are linked.

It can also be observed that the saponin content in the extract is directly proportional to the mass of the extracts. Thus, by simply weighing the standardised methanol extracts, it is possible to get a good idea of the saponin content of the dried berries. This is an important point for future cultivation experiments, because it means that the measurements could be realised without sophisticated material.

Qualitative determination

While LC/UV is an adequate technique for quantitative determination, it does not give any structural information as to the type of the aglycones or the sugar chain sequence of the saponins. Some qualitative experiment have been performed by the hyphenated technique LC/MS with two different ionisation modes, thermospray (TSP) and electrospray (ES).

In each case, the analyses were performed under the same chromatographic conditions as with the methanolic extract NT44/1. For each ionisation mode, the characteristic MS spectrum of the pentaglycosylated saponin **3** obtained on-line will be presented. This bidesmosidic saponin is one of the five standards and is present in all the extracts. Its molecular weight is 1250 u and its aglycone is oleanolic acid (MW: 456 u). This molecule is an interesting example due to its branched sugar chain at position C-3 and its terminal rhamosyl unit. Its structure is shown in Fig. 19.7.

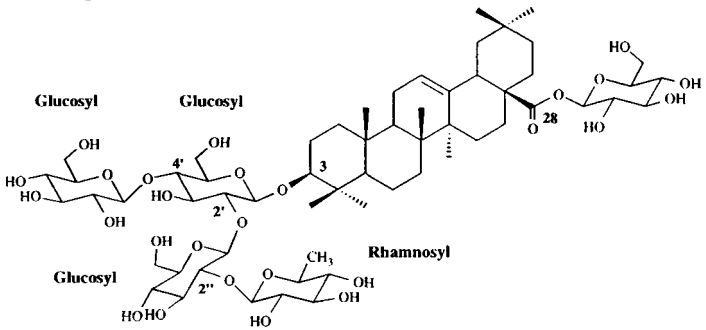

Fig. 19.7. Structure of the pentaglycosylated saponin **3.**

LC/TSP-MS analysis

The LC/TSP-MS analyses were performed on a Finnigan MAT TSQ-700 instrument with a TSP 2 interface.

In Fig. 19.8., are presented the LC/TSP-MS traces of the standard mixture, together with the on-line TSP-MS spectrum of saponin **3**.

A buffer (NH_4OAc 0.5 M, 0.2 ml/min) was added post column and the conditions were as follow: source 280-310°C, vaporiser 90, aerosol 280-360°C, positive ion mode, filament off.

LC/TSP-MS analyses permitted the identification of the aglycones, due to strong and characteristic dehydrated aglycone ions in the TSP spectra. Oleanolic acid presented only one $[(M-H_2O)+H]^+$ at *m/z* 439, whereas bayogenin and hederagenin exhibited $[(M-H_2O)+H]^+$ and $[(M-2H_2O)+H]^+$ ions at *m/z* 455, 437 and *m/z* 471, 453, respectively, due to the presence of more than one hydroxyl group. By displaying the single ion traces corresponding to each dehydrated aglycone moiety, it is possible to obtain a chromatogram for each type of aglycone, in this case the three aglycones oleanolic acid, bayogenin and hederagenin. However, due to the variations of the thermospray response, quantitative analyses are not highly reproducible. Weak sodium adducts were also observable and allowed the determination of the molecular weights of the most abundant saponins in the extracts. Indeed, the $[M+Na]^+$ ions at *m/z* 1273 are

clearly visible in the case of saponin **3**. Nevertheless, TSP analyses do not give supplementary information about the sugar chain.

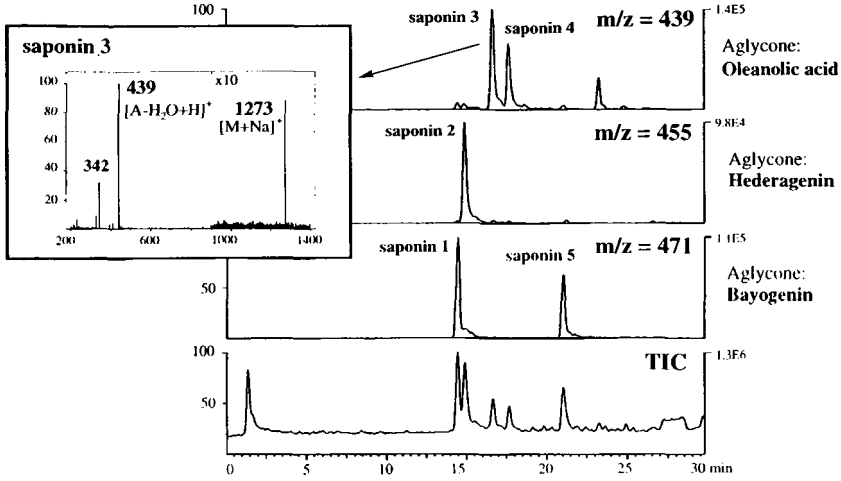

Fig. 19.8. LC/TSP-MS analysis of the saponin standards.

LC/ES-MS analysis

The electrospray interface (ES) was also tested for the analysis of the methanolic extracts. These analyses were performed in the negative ion mode on a Finnigan MAT TSQ-7000 instrument with a capillary temperature of 220°C. The electrospray interface appeared to be the most suitable technique for the analysis of extracts of *P. dodecandra*. In addition to its relatively easy use, electrospray allowed a good ionisation of saponins and was much more sensitive than TSP. Indeed, intense pseudomolecular ions $[M-H]^-$ were exhibited in the ES-MS spectra of all the saponins present in the extracts (see Fig. 19.9.).

With this interface, some information of the sugar sequence were obtained. Indeed, the fragmentation level can be adjusted by choosing an adequate voltage in the entrance of the optic system. The ions accelerated this way are fragmented by collision induced dissociation (up-front CID). It was thus possible to observe differences of 162 or 146 u between fragmentation peaks in the ES-MS spectra. These were characteristic for the loss a hexosyl moiety such as glucosyl or a desoxyhexosyl moiety such as rhamnosyl.

Thus, in the case of saponin **3**, the molecular weight was deduced from the $[M-H]^-$ ion at *m/z* 1249. The strong $[(M-Glc)-H]^-$ ion at *m/z* 1087 was due to the loss of the glucosyl moiety attached at position C-28 with an ester linkage. Peaks at *m/z* 941, 925 and 779 were also observable, suggesting the loss of the glucosyl

and rhamnosyl units of the sugar chain.

However, due to the number of peaks, the electrospray spectra may be sometimes difficult to interpret.

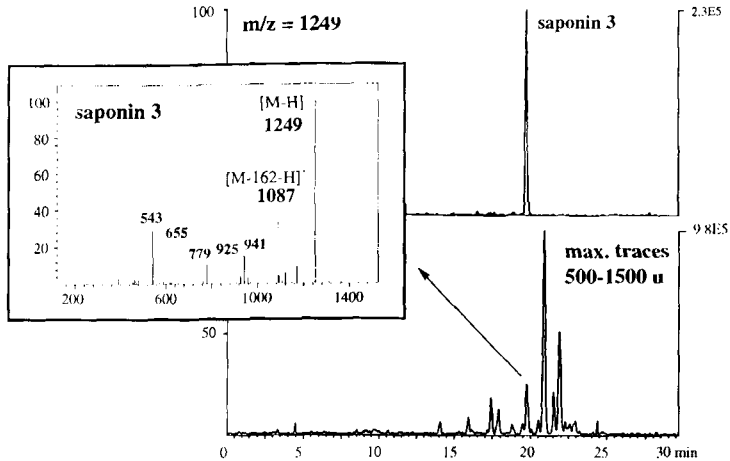

Fig. 19.9. LC/ES-MS analysis of the methanolic extract of *P. dodecandra* berries.

MS^n electrospray analysis

An early prototype of a new ion trap mass spectrometer was tested for the determination of sugar sequence. With this MS-MS instrument, it was possible to isolate and excite only one ion of interest and thus, to decrease the amount of consecutive reactions (Wolfender *et al.* 1995). The sugar sequence information was obtained by successive decomposition of the main ion, as shown in Fig. 19.10.

The first step was the fragmentation of the strong TFA adduct at *m/z* 1363 (Fig. 19.10a.), giving a deprotonated molecular ion $[M-H]^-$ (Fig. 19.10b.). This latter ion yielded a first fragment at *m/z* 1087 (Fig. 19.10c.). This first loss of 162 u corresponded to the departure of the glucosyl moiety at position C-28. This sugar was particularly sensitive due to the ester linkage. Then, the $[(M-Glc)-H]^-$ ion cleaved into two fragments at *m/z* 941 and *m/z* 925 (Fig. 19.10d.), showing the simultaneous loss of a rhamnosyl or a glucosyl unit, respectively. These losses were characteristic for a branched sugar chain. In Fig. 19.10e., the ion at *m/z* 779 issuing from the fragmentation of *m/z* 941 (-Rha) or *m/z* 925 (-Glc) was observed. This ion corresponded to the diglucoside moiety, which gave finally the monoglucoside $[(A+Glc)-H]^-$ at *m/z* 617 (Fig. 19.10f.) and the aglycone ion characteristic of oleanolic acid at *m/z* 455.

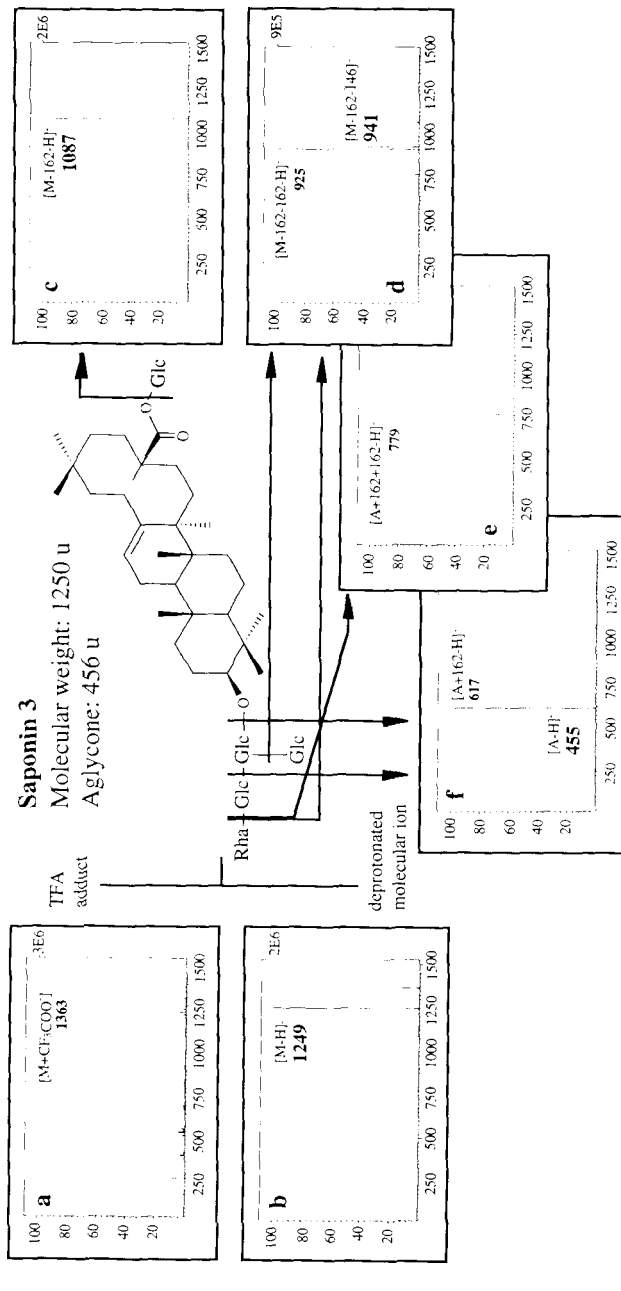

Fig. 10. MS_n electrospray experiment on the pentaglycosylated saponin **3**

Thus, this MS^n analysis showed that it was possible to cleave only one sugar at a time by adjusting the collision energy, making the interpretation simpler. This type of experiment was found to be very useful for clarifying the sugar sequence of saponins.

Conclusion

The HPLC/UV analyses carried out on the different methanolic extracts of the *Phytolacca dodecandra* berries harvested showed that the cultivation conditions had only a weak influence on the qualitative composition of the extracts. However, extraction with methanol demonstrated that these conditions can affect significantly the total saponin content of the berries. It seems that the increased growth rate obtained by the use of compost, fertiliser or Eco-agrogel is accompanied by a diminution in the saponin content in the berries. Other measurements have now to be done to determine if these two phenomena are effectively linked. For example parameters such as the maturity or the time of collection of the berries have to be considered (Ndamba *et al*, 1994).

LC/UV remains a rapid and efficient technique for the quantitation of the saponin content of the different samples of cultivated *P. dodecandra*. However, weighing the standardised methanolic extracts is sufficient for comparing the saponin content of dried berries. Indeed, as the extracts are mainly constituted of saponins, this simple method gives a good idea of the yield in saponin in the dried berries and could thus be used for the further measurements.

Extraction with water under the same conditions as those encountered in the field have now to be performed to obtain active extracts. These will have to be tested in order to evaluate and compare their molluscicidal properties.

LC/TSP-MS analyses gave supplementary information on the composition of the extracts, or more precisely, the type of the aglycones and the molecular weights of the major peaks. However, due to the variation of the thermospray response, this method is not ideal for the precise quantitation of molecules as large as bidesmosidic saponins.

LC/ES-MS, combined with MS-MS experiments appears to be perfectly adapted for qualitative analyses of triterpene glycosides. Indeed, it allows the determination of the molecular weight, even for minor peaks and gives essential structural information on the sugar chain. The use of an electrospray interface for quantitation purposes has still to be tested, but might be promising.

Acknowledgements

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