An improved electrospray interface for coupling of normal-phase liquid chromatography to mass spectrometry: application to neoflavonoid screening in *Calophyllum inophyllum* from French Polynesia.

F. Laure[1], P. Raharivelomanana[1], J.P.Bianchini[1], G. Herbette[2], L. Charles[3]

1- LCSN, Université de la Polynésie Française, BPn°6570, 98702 Faaa'a Tahiti, Polynésie Française 2- Spectropole, Fédération des Sciences Chimiques de Marseille, 13397 Marseille Cedex 20, France
3- JE TRACE, Université de Provence, 13397 Marseille Cedex 20, France

1 Abstract

Calophyllum inophyllum is an evergreen tree mainly found in tropical parts of the indo-pacific area. Different parts of this plant have been widely used in folk medicines, suggesting a rich source of bioactive secondary metabolites.
Recently, neoflavonoids from *Calophyllum inophyllum* have been shown to exhibit significant biological effects, particularly anti-HIV activities.
The screening of these molecules in crude extracts requires a robust and performant analytical method to establish the composition of the samples according to their geographical origin.
We report here the development of a new analytical method that allows the separation of targeted compounds in crude extracts and their characterization by mass spectrometry. HPLC analyses were performed on a normal-phase column and a special design of the ionisation source was implemented to circumvent the drawbacks induced by the use of non polar solvents in electrospray.

2 Introduction

Neoflavonoids isolated from *Calophyllum inophyllum* are representatives of a distinct class of non nucleoside HIV-1 specific reverse-transcriptase inhibitor under clinical trials as an AIDS chemotherapeutic[1-3].
Separation of such molecules was shown to be improved using normal-phase chromatography (NP-HPLC) as compared to reversed-phase. Detection was mainly performed by UV but lacks specificity, a key parameter in complex mixture analysis. Mass spectrometry would allow a selective screening of targeted compounds, particularly when operated in the MS/MS mode. However, the main drawback to implement a LC-MS coupling is the incompatibility of non polar solvents, used as eluents in NP-HPLC, and the mechanism of electrospray ionization. The introduction of a polar buffer in the chromatographic effluent to reach adequate conditions in the ionization source has been described[4-5] but result in dilution effects and possible loss of chromatographic resolution.
In contrast, we developed a method that uses a sheath flow interface which allows the make-up solution to be introduced at the tip of the electrospray probe. The performance of the method is demonstrated for the analysis of some neoflavonoids (calophyllolide, (+)-inophyllum B, (+)-inophyllum C and (+)-inophyllum P) by LC-MS/MS.

3 Materials and method

Extraction procedure and standard preparation

Neoflavonoid standard preparation was adapted from a methodology developed by Patil et al.[1] and is briefly described hereafter.
Three successive Sohxlet extractions were performed for 8 hours on a 2 kg sample of Tamanu leaves (*Calophyllum inophyllum* Linn. from French Polynesia), using a 2 L volume of n-hexane, ethyl acetate and methanol, respectively. Solvent evaporation of the second extract yielded a 140 g crude extract which was further triturated in 1 L dichloromethane. The soluble part of the crude extract was then evaporated and 52 g of a very viscous dark green oil was obtained.
Three fractions were successively eluted from the purification silicagel column (0.5 m x 5 cm) using n-hexane, ethyl acetate and methanol.
The ethyl acetate fraction (14 g) was further fractionated into 150 parts using a second silicagel column (1.2 m x 2 cm), performing a gradient elution from n-hexane/ethyl acetate (85:15, v/v) to ethyl acetate in 12 hours at 5 mL/min.
A total of 11 fractions could be selected after thin layer chromatography analysis and were submitted to semi-preparative HPLC chromatography. Isolated neoflavonoid standards were controlled by NMR analysis.

HPLC

Instrument: Agilent series 1100
Column: QS Lichrosorb Si column (250 x 4.6 mm, 5 µm)
Eluents: A: iso-octane, B: iso-octane/isopropanol (90:10, v/v)
Elution program:

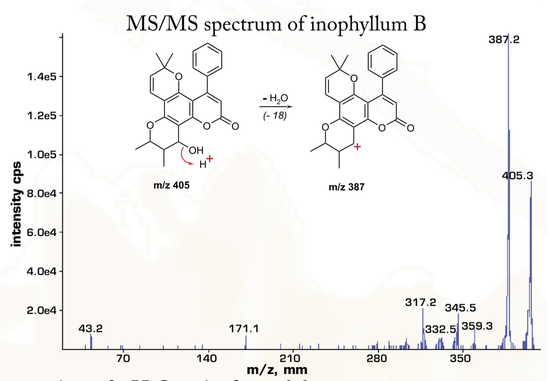
t(min)	%A	%B
0	90	10
5	90	10
20	60	40
25	10	90
30	10	90
35	90	10
40	90	10

Flow rate: 1 mL/min

Mass Spectrometry

Instrument: Sciex API III Plus triple-quadrupole mass spectrometer equipped with a pneumatically assisted electrospray source operated in the positive mode.
MS/MS signal was recorded using multiple reaction monitoring mode:
417/361 (calophyllolide), 405/387 (inophyllum B and P), 403/347 (inophyllum P).

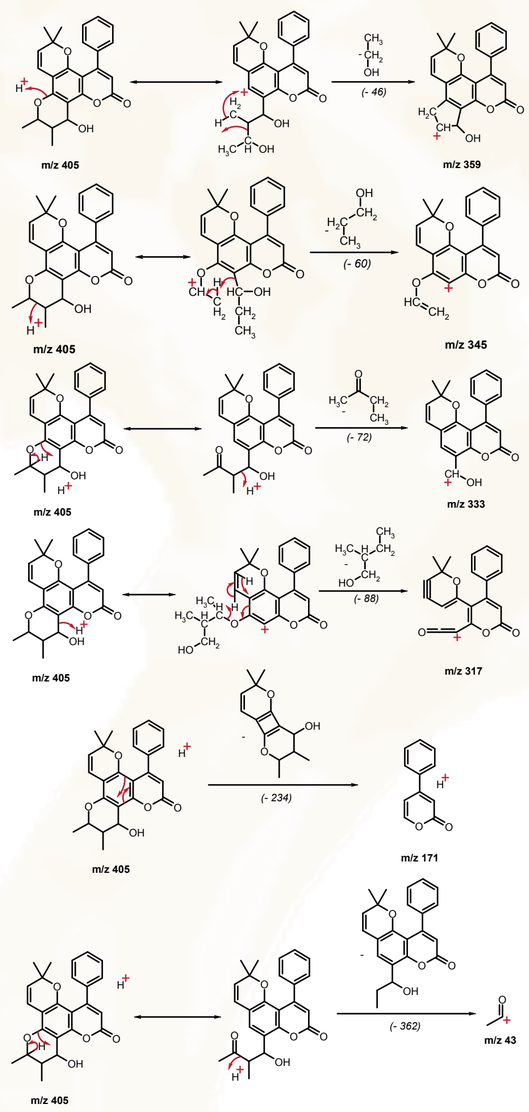
4 MS/MS spectrum



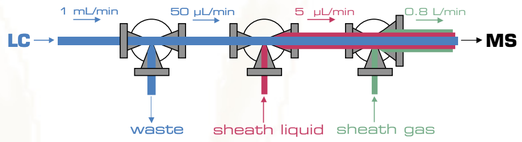
loss of a H_2O is the favored decomposition reaction

transition 405 → 387 monitored to specifically detect inophyllum B

5 Fragmentation



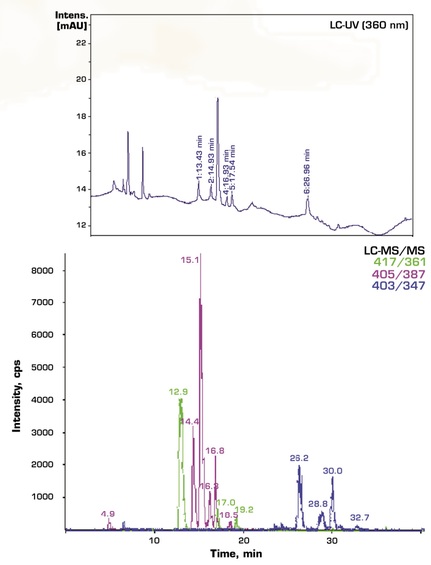
6 Sheath liquid electrospray interface



This interface utilizes a triaxial flow arrangement where the chromatographic effluent, split down to 50 µL/min using a zero-dead volume tee connector, is introduced in the atmospheric region of the electrospray source via a silica capillary inserted in a narrow metal tube which delivers the sheath liquid (methanol with 60 mM NH_4OAc) to the capillary exit; a third concentric tube delivers a gas flow to assist the spray formation.

7 LC-MS/MS of standard

8 Analysis of a crude extract



9 Discussion

- No signal observed in the absence of a sheath liquid:
  - eluents used in normal-phase HPLC not compatible with ESI
  - NH_4OAc in the sheath liquid favors $[M+H]^+$ ion formation
- MS/MS transition monitoring confers a very high specificity to the method: only ions which fragmentation fits the recorded transition are detected
  - this means that additional peaks detected in each extracted chromatogram are from isomer compounds
- Very low detection levels can be reached due to mass spectrometry high sensitivity and low background noise level associated to the MRM acquisition mode

	LOD* (ng/mL)	LOQ* (ng/mL)
calophyllolide	100	250
(+)-inophyllum B	25	60
(+)-inophyllum C	100	400
(+)-inophyllum P	15	50

*LOD and LOQ were calculated as 3σ and 10σ of a blank signal

- The method high sensitivity allows to point out the presence of trace impurities, undetected in LC-UV, in the standard solutions

10 Conclusion and Perspectives

A unique method has been developed to allow the coupling of normal-phase chromatography to mass spectrometry via an electrospray interface.
The performance of this configuration was successfully demonstrated for the analysis of neoflavonoids in complex mixtures. The method will further be used for neoflavonoid screening in biodiversity studies.
The scope of this method can also be extended to the analysis of any complex natural extracts requiring normal-phase separation.

11 References

1. Patil, A. D.; Freyer A. J.; Eggleston D. S.; Haltiwanger R. C.; Bean M. F.; Taylor P.B.; Caranfa M.J.; Breen A.L.; Bartus H.R.; Johnson R.K.; Hertzberg R.P.; Westley J. W. *J. Med. Chem*. **1993**, 36 (26) 4131-4138.

2. Ishikawa T. *Heterocycles* **2000**, 53 (2) 453-474.

3. Creagh T.; Ruckle J. L.; Tolbert D. T.; Giltner J.; Eiznhamer D. A.; Dutta B.; Flavin M. T.; Xu Z. Q. *Antimicrob. Agents Ch.* **2001**, 45 (5) 1379-1386.

4. Choudhary G.; Apffel A.; Yin H.; Hancock W. *J. Chrom.* A **2000**, 887, 85-101.

5. Shang D. Y.; Ikonomou M. G.; Macdonald R. W. *J. Chrom.* A **1999**, 849 (2) 467-482.