

LC/MS analysis of neoflavonoids: application for biodiversity investigation and bioactive molecule screening

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Abstract

The chemical composition investigation of the leaves extracts of *C. inophyllum* from French Polynesia led to isolation and identification of neoflavonoids compounds (Calophyllolid, Inophyllums B, P and C) including a new compound named Inophyllum F as a minor component.

To determine neoflavonoid content in leaf crude extracts at a microscale level, a new analytical method had been developed to allow the separation of target compounds and their characterization by mass spectrometry. LC-MS 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. The HPLC neoflavonoid profile was then used as a routine analysis method of crude leaf extract for a bioactive molecule screening.

Neoflavonoid contents of the crude leaves extracts of *C. inophyllum* collected from five Polynesian archipelagos were determined. The compiled data were submitted to multivariate statistical analysis (PCA, FDA). Three distinctive groups were thus established, according to their archipelago origin : Society, Tuamotu, and Marquesas.

Keywords: *Calophyllum inophyllum*, neoflavonoids, NP LC-MS, sheath liquid interface, biodiversity

Introduction

Calophyllum inophyllum is an evergreen tree mainly found in tropical parts of the indo-pacific area (1). Different parts of this plant have been widely used in traditional and folk medicines for many centuries (2, 3). Plants for the genus *Calophyllum* (Clusiaceae) are known as a rich source of bioactive secondary metabolites such as xanthenes, coumarins, flavonoids and triterpenes. In particular, neoflavonoids from *C. inophyllum* have been shown to exhibit significant biological effects such as anti-HIV activities (4).

Biodiversity investigation of *C. inophyllum* from French Polynesia was carried out using the neoflavonoid leaf content as biochemical marker for biodiversity and bioactive screening. To this purpose, the major neoflavonoid constituents of *C. inophyllum* leaf extracts were isolated and their structures determined.

Optimal chromatographic resolutions were obtained while HPLC analysis of neoflavonoid mixture were performed using normal phase column. In order to determine the neoflavonoid composition of *C. inophyllum* leaf extracts at a microscale level, a new interface to perform

on-line coupling of normal-phase LC with MS that allows the ionisation conditions have been developed. This analytical method was then applied to a series of samples collected from the five polynesian archipelagos for a bioscreening.

Results and discussion

The study of the chemical composition of the leaves extracts of *C. inophyllum* from French Polynesia led to isolation and identification of neoflavonoid compounds (Calophyllolid, Inophyllums B, P and C) including a new compound named Inophyllum F as a minor component (figure 1). Their structures were determined from 1D and 2D NMR techniques as well as HR-ESI-MS and tandem mass spectrometry.

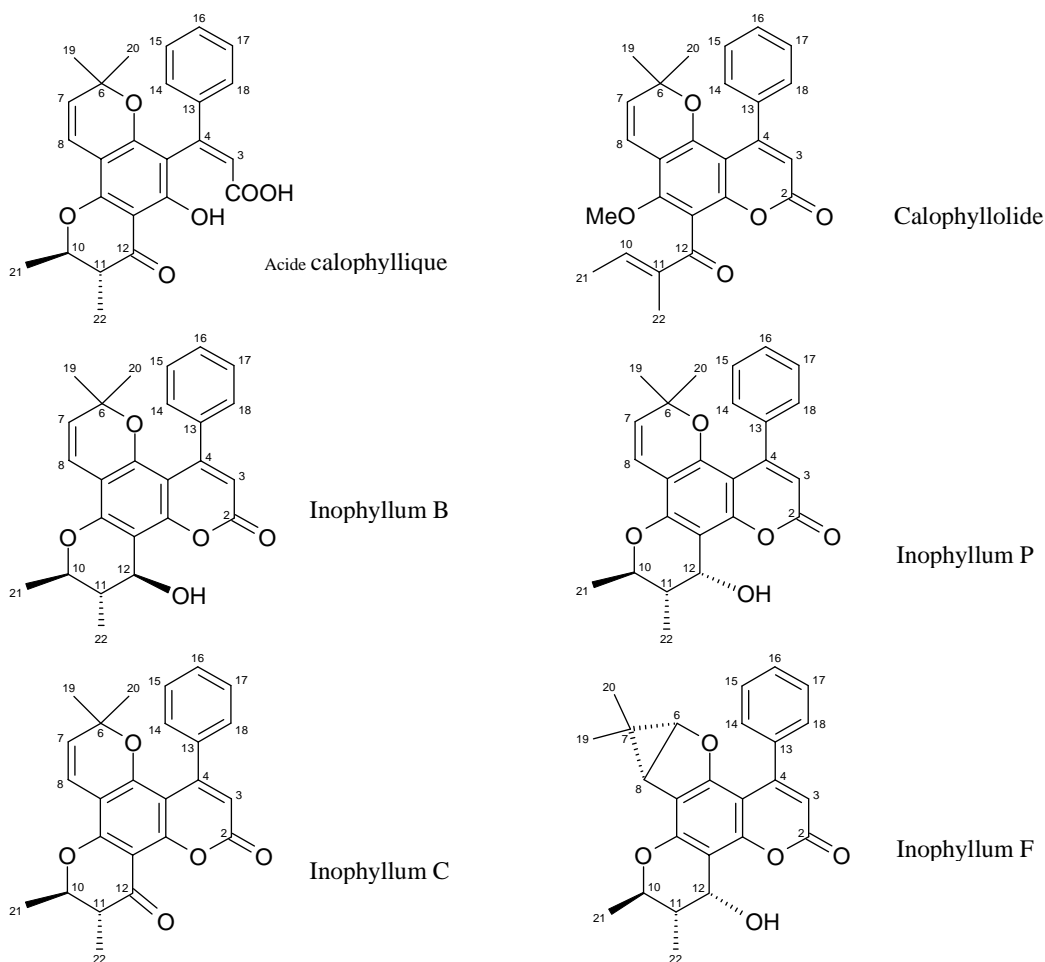


Figure 1: Neoflavonoid compounds isolated from *Calophyllum inophyllum* leaf extract.

Inophyllum F contains not only a cyclopropane ring as well as the rare inophyllums G1 and G2, but in addition a chromanol ring having the three sequential (R, S) stereochemistries at the 10 and 11 positions which is involved in the antiviral activity such as anti-HIV-1 and potent inhibitor of HIV-1 reverse transcriptase (5).

Separation of neoflavonoids 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. 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 (6). This interface utilizes a triaxial flow arrangement where the chromatographic effluent, split down to 50 $\mu\text{L}/\text{min}$ 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 (NH_4OAc in solution with methanol) to the capillary exit; a third concentric tube delivers a gas flow to assist the spray formation (figure 2).

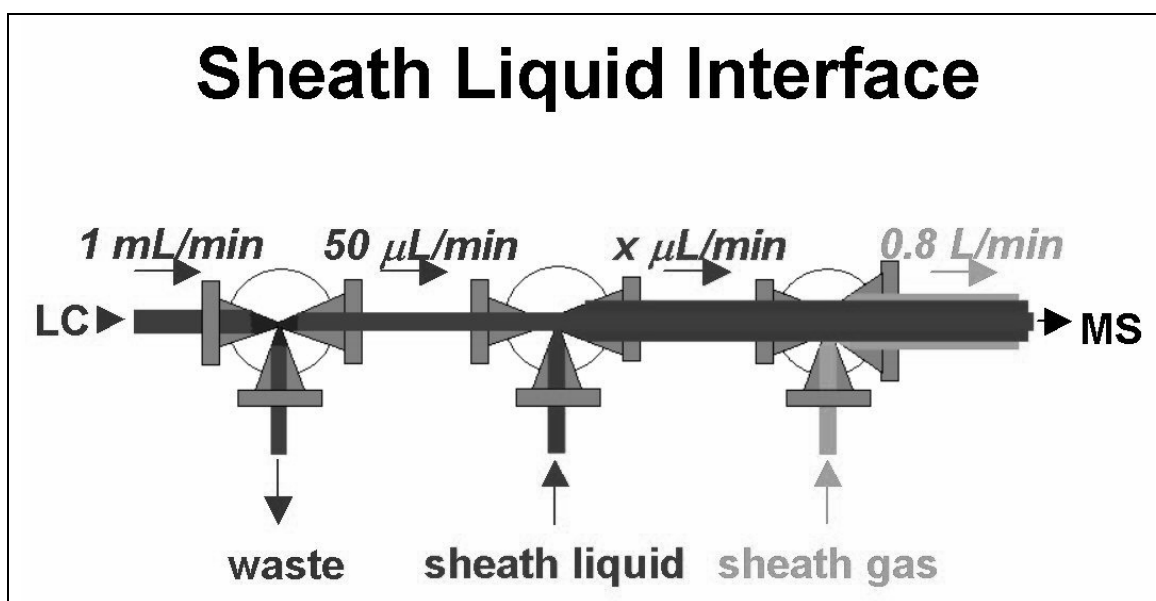


Figure 2: Sheath liquid interface

Flow injection analysis (FIA) experiments were performed to investigate the effects of sheath liquid composition and flow rate on MS detectability. Optimization experiments were carried out with inophyllum P as a model compound. The flow-rate from which the signal discrepancy is obtained varies as a function of ammonium acetate content in the sheath liquid: the lower the concentration, the higher is the flow-rate required for sensitivity to drop off. The optimum sheath liquid system is reached at $5 \mu\text{l min}^{-1}$ for a 60mM NH_4OAc in methanol and was therefore implemented in NPLC/MS coupling (figure 3).

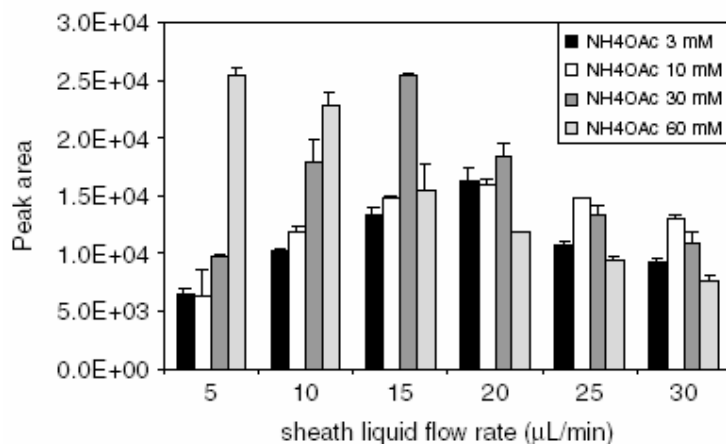


Figure 3: Effects of sheath liquid composition and flow-rate on inophyllum P signal, monitored as the selected MS/MS reaction m/z 405 \rightarrow 387

The performance of the method is demonstrated for the analysis of isolated neoflavonoids used as standards (calophyllolide, inophyllum B, inophyllum C and inophyllum P) by LC-MS/MS. Protonated molecules generated in the electrospray source were submitted to CID in the collision cell of the triple-quadrupole mass spectrometer. MS/MS experiments showed that the major fragmentation route of protonated molecules formed from compounds containing a hydroxy function, i.e. inophyllum B and inophyllum P, consists of the loss of a water molecule. Accordingly, the mass spectral transition m/z 405 \rightarrow 387 was selected to monitor these two isomers. A 56 u neutral loss was found to be the main fragmentation reaction of $[M+H]^+$ ions of both inophyllum C and calophyllolide. This reaction was interpreted as the loss of a but-2-ene molecule in both cases. The mass spectral transition m/z 403 \rightarrow 347 was therefore selected for the specific detection of inophyllum C whereas m/z 417 \rightarrow 361 was used in selected reaction monitoring of calophyllolide. A neoflavonoid standard mixture was injected on to the column and, by monitoring the MS/MS transition defined for each compound, the total ion current (TIC) chromatogram presented in figure 4 was obtained. All compounds are baseline resolved and assignment of the chromatographic peaks was based on both the extracted chromatograms and the retention times (t_R) obtained from individual standard injections. The high sensitivity of the method was shown by the measures of limits of detection and quantification given in Table 1.

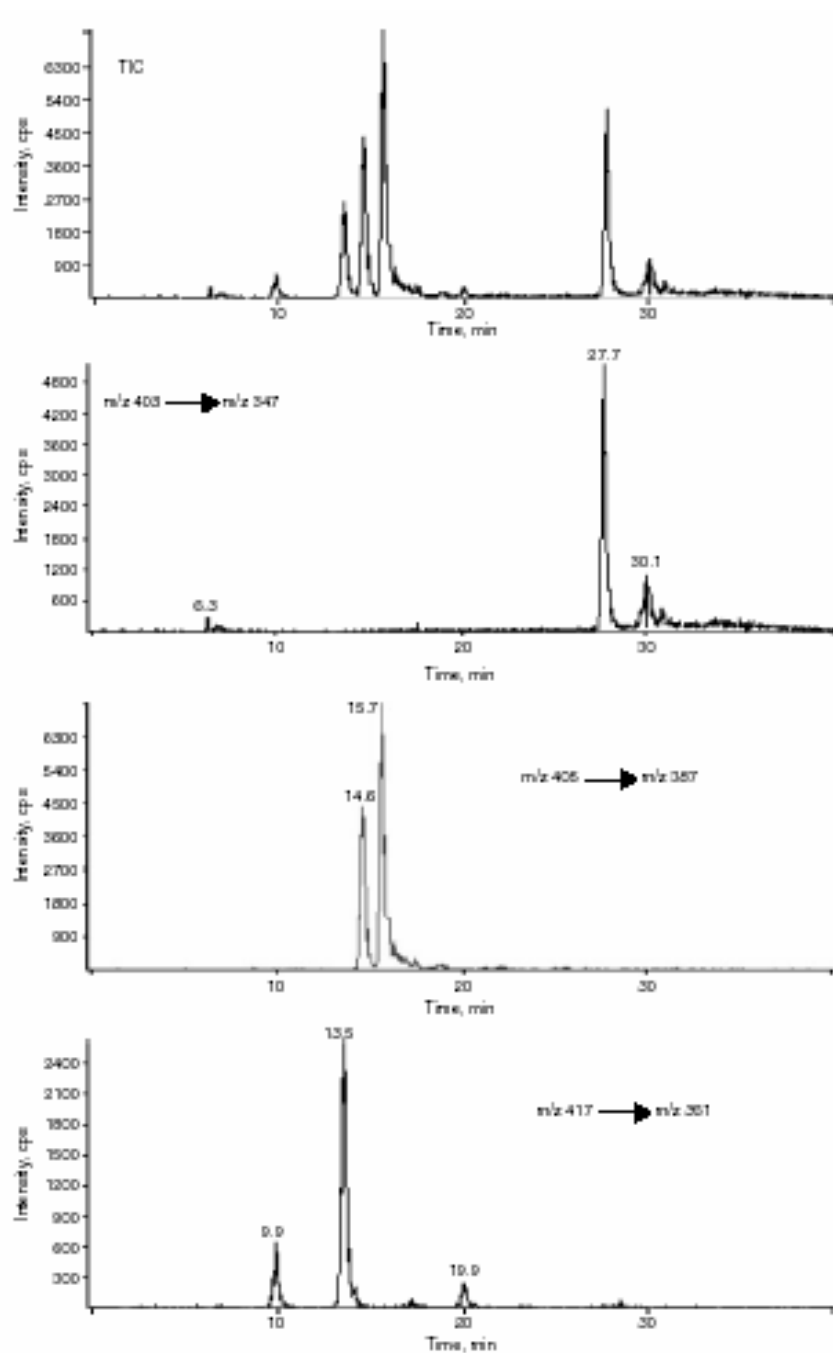


Figure 4: Top: TIC chromatogram of a neoflavonoid standard mixture recorded in the MRM detection mode and using a 60 mM NH_4OAc methanolic sheath liquid infused at $5\mu\text{l min}^{-1}$ in the electrospray source. Analytes are identified in the extracted chromatogram associated with their specific MS/MS transition: inophyllum C at t_R 27.7 in the m/z 403 \rightarrow 347; inophyllum B at t_R 14.6 in and inophyllum P at t_R 15.7 in the m/z 405 \rightarrow 387 and calophyllolid at t_R 13.5 in the m/z 417 \rightarrow 361.

Table 1: Analytical performance of the NPLC/ESI-MS/MS coupling for the analysis of four neoflavonoid compounds.

Compound	Mass spectral transition (m/z)	LOD (ng/ml)	LOQ (ng/ml)
Calophyllolid	417 → 361	100	250
Inophyllum B	417 → 361	25	60
Inophyllum P	405 → 387	15	50
Inophyllum C	403 → 347	100	400

Limits of detection (LOD) and limits of quantification (LOQ) were calculated as 3σ and 10σ of a blank signal, respectively.

This NPLC/MS method was then used in routine analysis of a series of *C. inophyllum* leaf extracts collected from the five archipelago in French Polynesia to establish their neoflavonoid compositions. For all the collected samples, the major neoflavonoids identified were bearing the two methyl groups respectively at C-10 and C-11 in trans-diaxial positions such as calophyllolide, calophyllic acid and inophyllums (B, P, C and F). This fact may suggest an original biosynthetic pathway leading mainly to these stereoselected compounds and could be considered as one characteristic point of French Polynesia *C. inophyllum* biodiversity.

The compiled data of neoflavonoid compositions of the 138 leaf samples collected in French Polynesia were submitted to a multivariate statistical analysis. Factorial Discriminant Analysis (FDA) showed geographical segregation of samples according to their archipelago origin and three distinctive groups were thus established: the Society, the Tuamotus, and the Marquesas (figure 5).

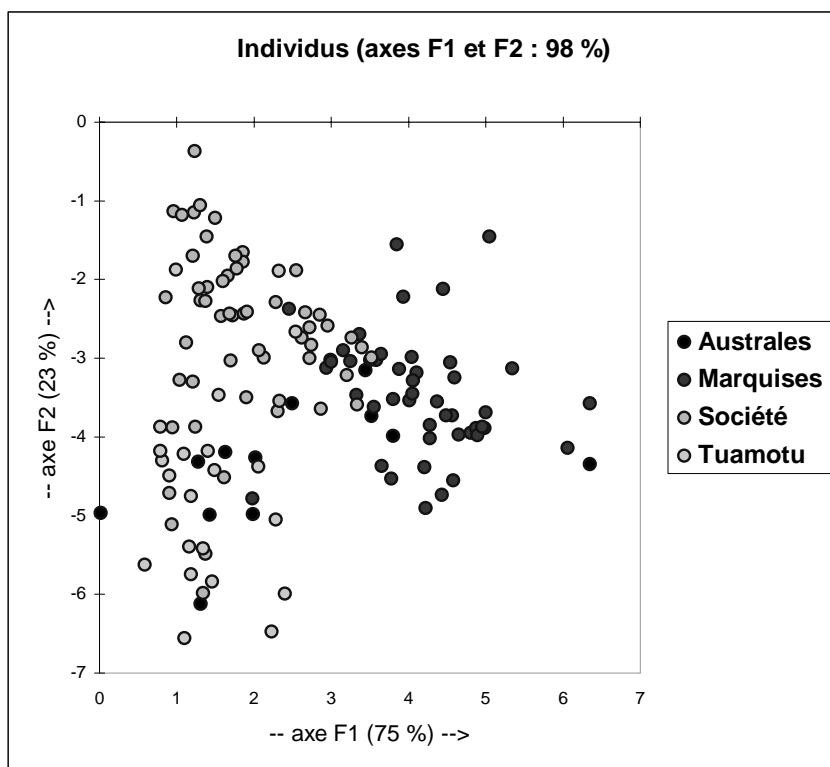


Figure 5: Factorial Discriminant Analysis showing the geographical segregation of the leaf samples within their neoflavonoid content.

Due to their antiviral potentiality (including anti-HIV), three neoflavonoids (inophyllum B, inophyllum P and inophyllum F) content were checked for a bioactive screening among all collected samples. This screening allowed to establish that:

- the highest inophyllum B content (22-23%) are found in samples collected from Rurutu island (Australes archipelago) and Rangiroa island (Tuamutu archipelago)
- the highest inophyllum P content (9-10.5%) are found in samples originated from Marquesas archipelago mainly from Hiva Oa and Ua Uka islands.
- Inophyllum F (2-3%) is mostly found in samples from Tetiaroa island (Society archipelago) and Hao island (Tuamotus archipelago).
- Samples containing well-balanced content of these three neoflavonoids are from Moorea (Society archipelago) with approximatively 15% of inophyllum B, 5% of inophyllum P and 1.5% of inophyllum F.

Conclusion

A unique method has been developed to couple NPLC with MS via an ESI interface modified to accommodate the introduction of a sheath liquid solution at the tip of the electrospray probe. This method was successfully applied to the analysis of neoflavonoid content of *C. inophyllum* leaf extracts for biodiversity and bioactive screening investigations. It should be possible to extent this methodology to the analysis of any complex natural extracts requiring normal phase separation.

Experimental

Plant material

A 3kg leaf sample was collected in one stand at Moorea island for neoflavonoid standards isolation. 138 samples of *C. inophyllum* leaves were collected from 19 islands scattered in the 5 French Polynesia archipelagos (Marquesas, Tuamutus, Society, Australes and Gambier). For each sample, about 10 leaves was collected for biodiversity screening.

Extractions and isolation

Three extractions were performed in a soxhlet apparatus for 8h on a 2kg sample of *C. inophyllum* leaves using respectively 2l of n-hexane, ethyl acetate and methanol, respectively. Ethyl acetate extract (140g) was further triturated in 1l of dichloromethane and the soluble part was then evaporated to yield a very viscous dark green oil (52g) which was further purified. Three fractions were successively eluted from the purification column (0.5m x 5cm i.d.) using n-hexane, ethyl acetate and methanol. The ethyl acetate fraction (14g) was further fractionated into 150 parts using a second silica gel column (1.2m x 2cm i.d.), performing a gradient eluent from n-hexane-ethyl acetate (85:15, v/v) to ethyl acetate. A total of 11 fractions were selected after TLC analysis and were submitted to preparative LC. Compounds were eluted isocratically at 10 ml min⁻¹ using isooctane-ethyl acetate (80:20, 70:30 or 60:40, v/v), depending on fraction polarity. Isolated neoflavonoid standards were monitored by NMR and MS analysis.

Each sample of air dried and chopped leaves (10 g) was extracted with 100ml of ethyl acetate giving crude extract which was further analysed by LC-MS.

LC-MS

All experiments were performed using an API III Plus triple-quadrupole mass spectrometer (Perkin-Elmer SCIEX), equipped with an atmospheric pressure ionisation (API) source. Positive mode ESI was performed at 5kV and the orifice voltage was set at 70V. Data acquisition was done in the multiple reaction monitoring (MRM) mode. LC analyses were carried out using a QS LiChrosorb Si column (250 x 4.6 mm i.d., 5µm). Solvent A was pure isooctane and solvent B was isooctane-propan-2-ol (90:10, v/v). The following linear gradient was performed at 1ml min⁻¹ flow-rate: 0 min, 90% A (held for 5 min); 20 min, 60% A; 25 min, 10% A (held for 5 min); 35 min, 90% A (held for 5 min).

Statistical analysis

Statistical analysis were processed on the software Addinsoft XL-Stat 6.1 and included: Principal Component Analysis (PCA) using a varimax rotation and Discriminant Analysis (DA).

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