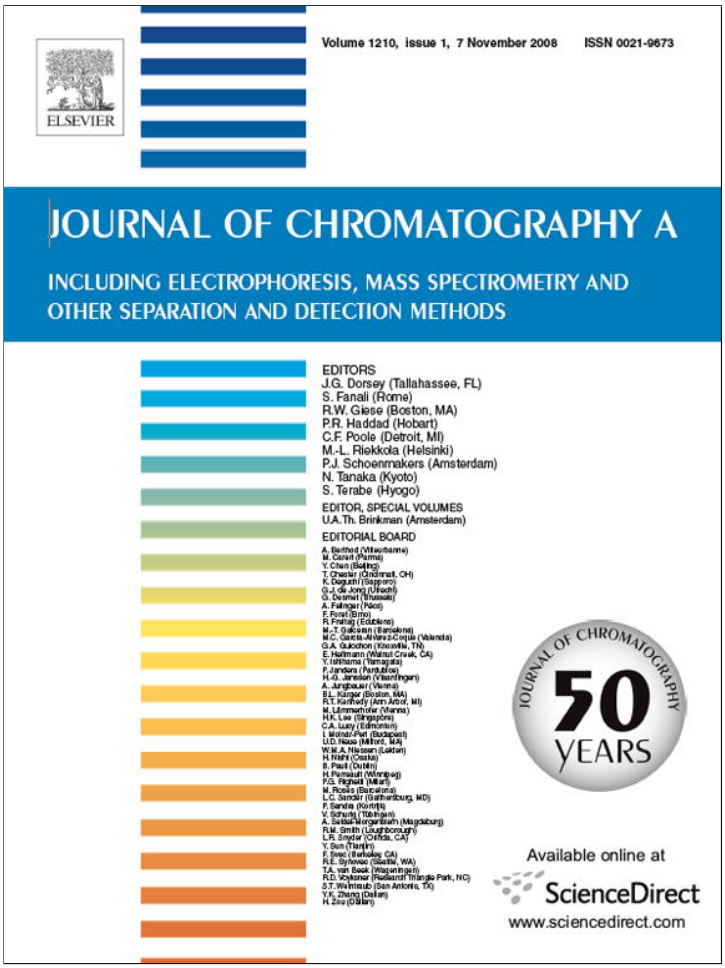

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Analysis of minor flavonoids in *Piper hostmannianum* var. *berbicense* using liquid chromatography coupled with atmospheric pressure chemical ionization mass spectrometry

Bénédicte Portet[a,*], Nicolas Fabre[a,*], Raoul Rozenberg[b], Jean-Louis Habib-Jiwan[b], Claude Moulis[a], Joëlle Quetin-Leclercq[b]

[a] *Université de Toulouse, UMR-152 IRD-UPS Laboratoire de Pharmacochimie des Substances Naturelles et Pharmacophores Redox, Faculté des Sciences Pharmaceutiques, Université Paul-Sabatier, F-31062 Toulouse cedex 09, France*
[b] *Unité CHAM 72.30, Laboratoire de Pharmacognosie, Ecole de Pharmacie, Université catholique de Louvain, Avenue E. Mounier 72, B-1200 Bruxelles, Belgium*

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ABSTRACT

The fragmentations of hydroxylated flavanones, chalcones and dihydrochalcones were investigated by direct loop injection using an ion trap mass spectrometry equipped with atmospheric pressure chemical ionization (APCI) probe. Some of them have been isolated from the leaves of *Piper hostmannianum* var. *berbicense* and standards were used to confirm their fragmentation behaviour. In negative ion mode, fragmentations of these three types of flavonoids revealed specific diagnostic ions which allowed us to identify aglycones in a crude plant extract. The major fragment ion obtained in MS/MS experiment for methoxylated chalcones is the neutral loss of a methyl radical whereas a H_2O molecule is lost in the case of methoxylated dihydrochalcones. Methoxylated chalcones and flavanones isomers could be differentiated by the relative intensity ratio of $[M–H–CH_3]^{\bullet-}$ and $[M–H–C_2H_2O]^-$ ions. Based on UV and MS data, a decision tree that includes UV λ_{max} absorptions and MS/MS diagnostic ions was built in order to obtain structural information of unknown compounds present in the extract. This tree was used to identify flavonoids in the ethyl acetate extract of *P. hostmannianum* var. *berbicense* leaves after analysis by high-performance liquid chromatography–diode array detection–atmospheric pressure chemical ionization ion trap multistage mass spectrometry. A total of 11 flavonoids were tentatively characterized based on the MS fragmentations pattern observed in MS^n experiments.

1. Introduction

Flavonoids are a group of phenolic compounds which are widely distributed throughout the plant kingdom. More than 4000 substances have been identified, many of which are responsible for the attractive colours of flowers, fruits, and leaves [1]. These natural products are known for their beneficial effects on health due to a wide range of biological activities [2]. Thus the development of analytical methods for enhanced detection and structural characterization of these compounds is an important goal.

Mass spectrometry, especially coupled with soft ionization sources (electrospray ionization: ESI and atmospheric pressure chemical ionization: APCI) generates mainly molecular ions for relatively small plant metabolites such as flavonoids [3,4]. The coupling of mass spectrometry with liquid chromatography (LC/MS) is becoming an important tool for the identification of compounds in crude plant extracts [5].

Flavanones, chalcones and dihydrochalcones are biochemically related compounds of restricted occurrence and for this reason they are described as minor flavonoids [6]. Flavanones have a saturated C-ring whereas chalcones as well as dihydrochalcones have an open structure and a carbon skeleton numbered in a different way than other flavonoids (Fig. 1).

Mass spectrometry has already been used for the structure characterization of these three classes of flavonoids. The techniques employed involved electron ionization [7,8], fast atom bombardment [9], electrospray ionization [10,11] and atmospheric pressure chemical ionization in the positive ion mode [12,13]. This is the first study on the fragmentation pathways of chalcones, dihydrochalcones and flavanones by negative APCI.

Recently, we isolated and identified by spectroscopic methods pure minor flavonoids (flavanones, chalcones and dihydrochalcones) from the *n*-hexane extract of *Piper hostmannianum* var.

* Corresponding authors at: Laboratoire Pharmacochimie des Substances Naturelles et Pharmacophores Redox, UMR 152 IRD-Université Toulouse 3, Faculté des Sciences Pharmaceutiques, 31062 Toulouse cedex 9, France.
Tel.: +33 5 62 25 68 48; fax: +33 5 61 55 43 30.
E-mail addresses: benedicte.portet@voila.fr (B. Portet), nfabre@cict.fr (N. Fabre).

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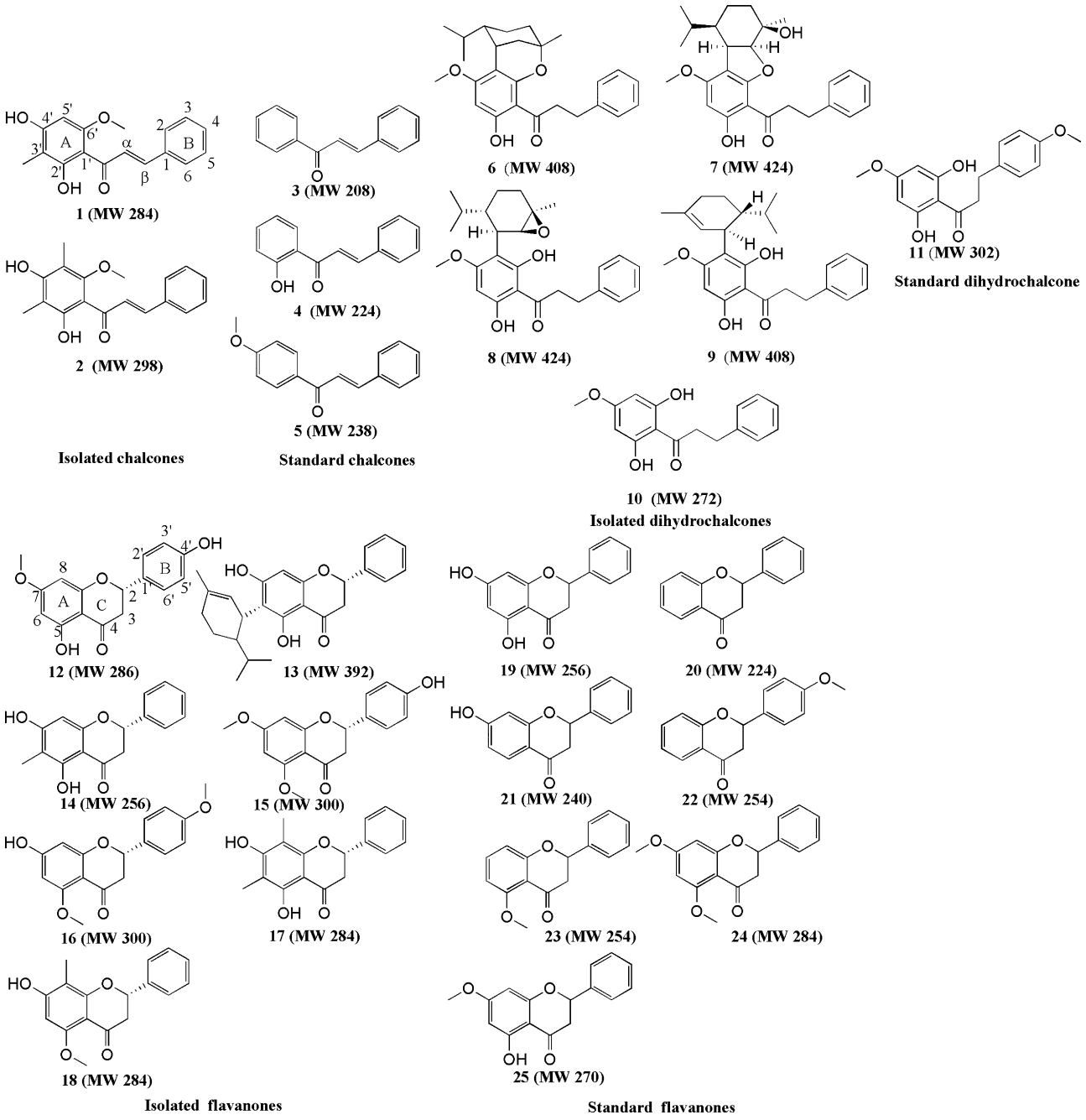

Fig. 1. Chemical structures of isolated compounds and standard flavonoids.

berbicense (Fig. 1). Some of these compounds, and particularly (−)-methyllinderatin (**9**), linderatone (**13**) and 2′,6′-dihydroxy-4′-methoxydihydrochalcone (**10**) have been reported to be antiplasmodial against two strains of *Plasmodium falciparum* [14].

In the present paper, we investigated the specific fragmentation patterns by (−)-APCI of these flavanones, chalcones and dihydrochalcones previously isolated including very unusual monoterpene-substituted derivatives. Standards were used to validate the fragmentation patterns. The negative ion mode was chosen because it appeared more selective and sensitive for further LC/MS analysis of hydroxylated flavonoids in crude plant extracts. Some fragmentation rules have been established to provide important on-line structural information. The present approach has been applied to study flavonoids in the ethyl acetate extract of *P. hostmannianum* var. *berbicense* leaves by high-performance liquid chromatography/diode array detection/negative-atmospheric pressure chemical ionization–multistage mass spectrometry (LC/DAD/(−)-APCI–MS^n). Based on the proposed identification rules, a total of 11 flavonoids, among which three are new, were tentatively characterized including flavanones, chalcones and dihydrochalcones.

2. Experimental

2.1. Standards and reagents

Chalcone (**3**), 2′-hydroxychalcone (**4**), 4′-hydroxychalcone (**5**), 2′,6′-dihydroxy-4,4′-dimethoxydihydrochalcone (**11**), pinocembrin (5,7-dihydroxyflavanone) (**19**), flavanone (**20**), 7-hydroxyflavanone

(**21**), 4′-methoxyflavanone (**22**), 5-methoxyflavanone (**23**), pinocembrin methoxy (5-hydroxy-7-methoxyflavanone) (**24**), 5,7-dimethoxyflavanone (**25**) were obtained from Extrasynthese (Geney, France). Pure chalcones (**1** and **2**), pure dihydrochalcones (**6**–**10**) and pure flavanones (**12**–**18**) were isolated from *P. hostmannianum* var. *berbicense* as previously described [14] and unambiguously identified by spectroscopic methods.

HPLC grade methanol (Peypin city, France) and ultra-pure water were used for analysis. The ethyl acetate used for plant extraction was of pure synthesis grade and purchased from SDS (Val de Reuil, France).

2.2. Plant materials and sample preparation

Standards and pure compounds were diluted in methanol to give a standard stock solutions of 1 mg/ml, and these solutions were further diluted in methanol to obtain standard work solutions at 1000 ng/ml before MS or LC/MS analysis.

The leaves of *P. hostmannianum* var. *berbicense* (100 g) were pulverized and extracted by lixiviation with ethyl acetate (2 l). A 2-g aliquot of the dried extract was dissolved in 2 ml of methanol and filtered through a micropore membrane (0.45 μm) prior to analysis. A volume of 10 μl was injected into the HPLC instrument for analysis.

2.3. HPLC conditions

The LC system consisted of a Thermo Separation Products (TSP, San Jose, CA, USA) P4000 pump, a TSP 6000LP photodiode array detector, and a TSP AS 3000 autosampler. Chromatographic conditions were as follows: column, pursuit XRs C18 (Varian), 250 mm × 4.6 mm, 5 μm; eluent: (A) water, (B) methanol. A gradient program was used as follows: elution from 60 to 100% B in A over 20 min; 100% B during 5 min; finally returning to the initial conditions in 5 min. The flow rate was 1 ml/min and the column temperature was 25 °C.

2.4. Mass spectrometry

Mass spectra were acquired using a LCQ mass spectrometer (Finnigan MAT, San Jose, CA, USA) equipped with an atmospheric chemical ionization source. The system was controlled by a Xcalibur software version 1.3. The samples were infused into the source chamber at a flow rate of 15 μl/min. Mass spectra were scanned using APCI in negative mode. MS^n experiments were carried out in *m/z* range between 100 and 1000. The collision energy was adjusted between 33 and 44% for pure compounds and at 37% for LC/MS^n analysis.

The following source settings were used for direct infusion: vaporizer temperature 370 °C, sheath nitrogen gas flow 20 units, discharge current 5 μA, capillary temperature 180 °C, and capillary voltage −7 V. For LC/MS analysis, the parameters were as follows: vaporizer temperature 420 °C, sheath nitrogen gas flow 60 units, discharge current 5 μA, capillary temperature 150 °C, and capillary voltage −17 V.

3. Results and discussion

3.1. Tandem mass spectrometry of isolated compounds and standards

Both ESI and APCI are soft ionization techniques that have ever been used for the study of flavonoids. In this work, APCI was preferred to ESI because this ionization technique is favoured for the analysis of medium to less polar compounds [15]. In our case, methyl and monoterpene substitutions decrease the polarity (Fig. 1). When coupled to HPLC, the disadvantages of ESI compared to APCI are its lower robustness to matrix interferences and changes of mobile phase conditions and especially the fact that the compatibility of ESI (a low flow (μl min^{-1}) ionization technique) with typical HPLC flow rates (around 1 ml min^{-1}) have to be achieved by pneumatically assisted electrospray ionization (also called ionspray) [5,15]. Concerning MS/MS fragment ions obtained by using both ionization techniques, previous published works dealing with the fragmentation of chalcones and flavanones by ESI and APCI and also compared to our results, show very similar structurally informative fragments ions [5,10,11]. So, APCI appeared to be the most suitable ionization probe in our conditions of analysis and equipment. Analysis in both positive and negative ionization modes were compared after direct injection of the compounds. The base peak in the negative mode was $[M-H]^-$ ions while in the positive mode, the signal of the $[M+H]^+$ ions was too low for MS/MS fragmentation to be feasible (for example, for compound **15**, 20% of relative intensity was observed for $[M+H]^+$ ion). In addition, the acidic nature of all compounds makes negative ionization a good choice for obtaining high sensibility. For all compounds, CID (collision-induced dissociation) of the $[M-H]^-$ ions were obtained and, when peak intensity was sufficient, the major MS/MS ions were selected for further MS^n analysis ($n = 3, 4$). The collision energy ranged from 33 to 44%.

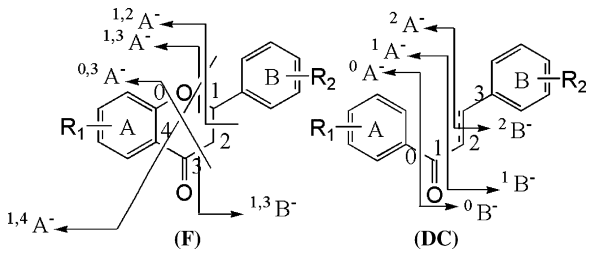

Fig. 2. Nomenclature and diagnostic ions of deprotonated flavanones (F) and (dihydro)chalcones (DC).

In the course of our mass spectral studies, we used the nomenclature adopted by Ma et al. [16] to define the various A- and B-ring fragments on chalcones, dihydrochalcones and flavanones (Fig. 2) (A- or B-type fragment means that compounds retain the A- or B-ring portion while the second ring is lost).

In addition to the cleavages due to the A- or B-ring losses, small neutral losses were observed which could be attributed to small molecules such as CO, CO_2, H_2O, H_2 or radicals ($CH_3^{\bullet}$).

3.1.1. Fragmentation of chalcones

Chalcones **3** and **5** logically give a $[M-H]^-$ ion of lower intensity due to the lack of hydroxyl groups in their structure. For hydroxylated chalcones, common fragmentation patterns could be identified. The main produced ions observed on APCI–MS^2 spectra are reported in Table 1.

3.1.1.1. A-type fragments. Product ion spectra provide informations on specific A- or B-ring fragmentation patterns (Table 1). Due to the lack of substitution on the B-ring, only two A-type fragments are observed: $^0A^-$ and $^1A^-$ types (Table 1). For methoxylated compounds, MS^n experiment provides support for an additional A-type fragment coming from the loss of 119 u (loss of a $CH_3^{\bullet}$ (15 u) and a vinylbenzene (104 u)). It could be assigned to $[^1A–CH_3]^{\bullet-}$ product ion (Table 1). This was confirmed by MS^3 experiments. The proposed structures for $^1A^-$ and $[^1A–CH_3]^{\bullet-}$ involve a cyclization between the carbonyl function and a phenol group as described by Fabre et al. [10].

Table 1
APCI–MS^2 product ions obtained from the $[M-H]^-$ ions of chalcones **1**, **2** and **4**

Fragments	Molecules		
	1	**2**	**4**
	Collision energy: 7%	Collision energy: 37%	Collision energy: 38%
Neutral losses			
$[M-H]^-$	283 (100)[a]	297 (14)	223 (35)
$[M-H-H_2]^-$	281 (12)	295 (1)	221 (42)
$[M-H-CH_3]^{\bullet -}$	268 (88)	282 (100)	/
$[M-H-H_2O]^-$	/	/	205 (14)
$[M-H-CO]^-$	255 (4)	269(1)	195 (100)
$[M-H-CH_3-CO]^{\bullet -}$	240 (6)	254 (4)	/
$[M-H-C_2H_2O]$-	241 (21)	255 (2)	/
$[M-H-C_2H_2O-CH_3]^{\bullet -}$	226 (1)	240 (1)	/
$[M-H-C_2H_2O-CH_3-CO]^{\bullet -}$	198 (1)	212 (1)	/
$[M-H-CO_2]^-$	239 (5)	253 (1)	179 (1)
A-type fragments			
$^0A^-$ (neutral loss of 130 u)	153 (8)	167 (2)	93 (4)
$[^1A-CH_3]^{\bullet -}$ (neutral loss of 119 u)	164 (38)	178 (26)	/
$^1A^-$ (neutral loss of 104 u)	179 (7)	193 (1)	119 (1)

[a] Relative intensity.

3.1.1.2. Neutral losses. In addition, small neutral losses of CO, CO_2, C_2H_2O, H_2 and $CH_3^{\bullet}$ are observed (Table 1) and have been already described in literature [10–12]. Our results confirm that the base peak for hydroxylated chalcones is due to the loss of a molecule of CO while for methoxylated chalcones, the predominant ion comes from a loss of a $CH_3^{\bullet}$ radical. Moreover, we also observed that the loss of CO_2 occurs with a low intensity for the three chalcones (Table 1).

3.1.1.3. Proposed identification rules for chalcones by UV and mass spectrometry. When HPLC–DAD–MS is employed, the combination of UV and MS spectra allows to identify chalcones in crude extracts. The UV profile of chalcones shows two maxima absorption bands at 300 and 340 nm [17,18].

All the hydroxylated chalcones studied here exhibit neutral losses of CO_2 and CO though sometimes as minor fragments. An important loss of a $CH_3^{\bullet}$ radical can be explained by the presence of methoxy group on A-ring for chalcones **1** and **2**. Combined neutral losses of 130 and 104 u indicate that the B-ring is unsubstituted. As a consequence $^0A^-$ and $^1A^-$ fragments are diagnostic ions for the A-ring substitution. For methoxylated chalcones, an additional loss of 119 u reveals the presence of the methoxy group on the A-ring (see Table 1).

3.1.2. Degradation of dihydrochalcones

The fragmentation of the dihydrochalcones **6**–**11** was studied, however, regarding the monoterpene-substituted dihydrochalcones **6**–**9**, the abundance of the fragment ions in MS/MS experiment was too low to record MS^3 spectra. Table 2 summarizes the (−)-APCI–MS/MS product ions obtained from the corresponding $[M-H]^-$ pseudo-molecular ions of dihydrochalcones **6**–**11**.

3.1.2.1. A-type fragments. As for chalcones, three A-type fragments are observed: $^0A^-$ and/or $^1A^-$ and/or $[^1A-CH_3]^{\bullet -}$ for methoxylated compounds.

Table 2
APCI–MS^2 product ions obtained from the $[M-H]^-$ ions of dihydrochalcones **6**–**11**

Fragments	Molecules					
	6 Collision energy: 44%	**7** Collision energy: 42%	**8** Collision energy: 44%	**9** Collision energy: 37%	**10** Collision energy: 33%	**11** Collision energy: 37%
Neutral losses						
$[M-H]^-$	407 (39)[a]	423 (100)	423 (100)	407 (100)	271 (82)	301(18)
$[M-H-H_2O]^-$	389 (6)	405 (26)	405 (43)	389 (99)	253 (100)	283(100)
$[M-H-CH_3]^{\bullet -}$	392 (3)	408 (1)	408 (1)	392 (3)	256 (11)	285(15)
$[M-H-CH_4-O]^-$ (M-32)	375 (10)	391 (12)	391 (5)	375 (5)	239 (3)	269(10)
$[M-H-H_2O-CH_3]^{\bullet -}$(M-33)	374 (10)	390 (3)	390 (2)	374 (20)	238 (10)	268(63)
$[M-H-H_2O-CH_3-CH_3]^{\bullet -}$	/	/	/	/	/	253(1)
$[M-H-H_2O-CH_3-CO]^{\bullet -}$	/	/	/	346 (1)	210 (1)	/
$[M-H-H_2O-CH_3-CH_3-CO]^{\bullet -}$	/	/	/	/	/	225(1)
$[M-H-C_4H_{10}]^-$(M-58)	349 (4)	365 (6)	365 (6)	349 (52)	/	/
M-72	/	351 (22)	351 (2)	/	/	/
$[M-H-monoterpene]^-$	269 ($M-C_{10}H_{18}$) (91)	270 ($M-C_{10}H_{17}O$) (13)	271 ($M-C_{10}H_{16}O$) (2)	269 ($M-C_{10}H_{18}$) (8)	/	/
A-type fragments						
$[^1A-CH_3]^{\bullet -}$	288 (100)	304 (6)	304 (6)	/	152 (9)	152 (10)
$^1A^-$	/	/	/	301 (14)	165 (7)	165 (2)
$^0A^-$	275 (49) loss of 132 u	291 (12) loss of 132 u	291 (6) loss of 132 u	275 (2) loss of 132 u	139 (1) loss of 132 u	139 (1)

[a] Relative intensity.

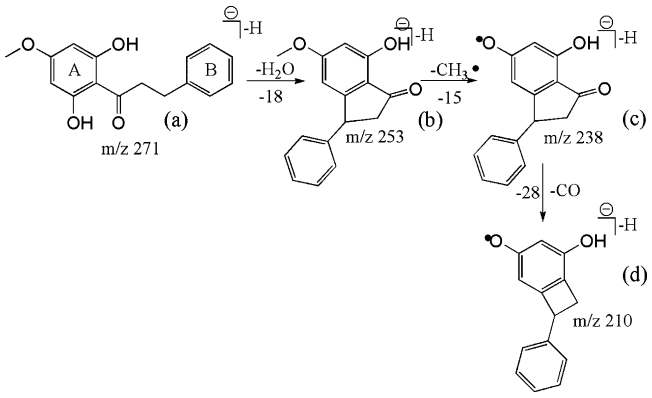

Fig. 3. Proposed structures for the dihydrochalcone **11** fragments consecutive to small neutral losses.

3.1.2.2. Neutral losses. Except for **6**, the major fragment corresponds to the neutral loss of H_2O. A fragmentation pattern of H_2O loss had already been proposed for chalcones by APCI in the positive ion mode and involved the protonated carbonyl function [12]. In the negative ion mode, we propose a five membered ring formation consecutive to the reaction between a hydroxyl group located on A-ring and a proton from the CH_2-β (Fig. 3). This is supported by the intensity of this neutral loss which is higher when A-ring is substituted by two hydroxyl groups instead of one (see fragmentations in Table 2 for compounds **8**–**11**). Moreover, the elimination of 28 u in the MS^4 spectra from fragment (c) (Fig. 3) can be explained by the loss of a molecule of CO giving rise to product (d).

3.1.2.3. Proposed identification rules for dihydrochalcones by UV and mass spectrometry. The dihydrochalcones can be first distinguished from chalcones by their UV profiles which show two maxima absorption bands at 230 and 290 nm [6,18].

All the dihydrochalcones studied here (**6**–**11**) exhibit neutral losses of H_2O whose intensity increases with the number of free OH present on A-ring.

A neutral loss of a $CH_3^\bullet$ radical (loss of 15 u) and a consecutive loss of H_2O and CH_3 (loss of 33 u) indicate the presence of methoxy group on the A- or B-ring.

In the case of monoterpene-substituted dihydrochalcones (**6**–**9**), a distinctive carbon–carbon cleavage occurs and involves the loss of the monoterpene unit and the corresponding dihydrochalcone fragment ion. Finally, the presence of the $^0A^-$ consecutive to the loss of 132 u from the $[M-H]^-$ ion, indicates an unsubstituted B-ring (Table 2).

3.1.3. Degradation of flavanones

The fragmentations of 14 flavanones among which 7 standards (Fig. 1) have been studied and the main produced ions observed on the APCI–MS^2 spectra are summarized in Table 3. It is worth noting that all the fragments obtained by APCI are identical to those obtained in ESI ionization in the negative ion mode and we confirm here the previously published data obtained by Fabre et al. for flavanone aglycones [10].

3.1.3.1. Proposed identification rules of flavanones by UV and mass spectrometry. UV spectra of flavanones show two maxima absorption bands at 230 and 290 nm as for dihydrochalcones [17,18].

In general, all the flavanones exhibit neutral losses of CO, CO_2 or C_2H_2O that may be attributed to the C-ring and an additional loss of a methyl radical is observed for methoxylated flavanones [10]. (compounds **15**, **16**, **18**, **22**, **23** and **25**). A main fragment at *m/z* 119 indicates a substitution by a hydroxy group on the B-ring ($^{1,3}B^-$ ion for compounds **12** and **15**, Table 3). For unsubstituted B-ring flavanones, a neutral loss of 104 u is observed. In UV spectroscopy, the dihydrochalcones and flavanones have the same absorption bands around 230 and 290 nm. All these observations allow to distinguish the couple "dihydrochalcone/flavanone"for which the main difference is the neutral loss of H_2O. This loss constitutes, in most cases, the major fragment for dihydrochalcones.

3.1.4. Identification rules of minor flavonoids in plant extracts

The specific fragmentation patterns of chalcones, dihydrochalcones and flavanones were investigated in order to specifically characterize them in a crude plant extract. Structural informations can be subsequently obtained by UV spectroscopy and tandem mass spectrometry. Fig. 4 reports a decision tree revealing the differences observed between chalcones, dihydrochalcones, flavanones and also flavones by UV and mass spectrometry. We introduced the flavones in the decision tree because of their UV profile similar to chalcones. Indeed, the couples chalcones/flavones and dihydrochalcones/flavanones could only be differentiated by their specific fragmentations in mass spectrometry since they have the same absorption bands in UV spectroscopy.

In addition, the differentiation between chalcone and flavanone isomers (distinguishable by UV) can be supported by their MS/MS profiles. As an example, Fig. 5 represents the MS/MS spectra of two isomers: a chalcone and a flavanone. The same fragments are observed with differences in their abundances. This phenomenon has already been observed and explained [9] by an intramolecular equilibrium between chalcone and flavanone-type molecular ions.

Indeed, the intensity ratio $[M-H-CH_3]^{\bullet -}/[M-H-C_2H_2O]^-$ is more important in the case of chalcones due to the lack of C-ring. This result allows us to distinguish the type of aglycone in the identification of unknown compounds in a crude plant extract. Therefore, the results obtained in the characterization of isolated molecules allowed us to identify minor flavonoids in a crude plant extract analysed by HPLC/DAD/(−)-APCI–MS^n.

3.2. HPLC–DAD–APCI–MS^n analysis of the plant extract

Fig. 6 shows the HPLC–UV (340 nm) and TIC (total ion current) profiles of the ethyl acetate extract of *P. hostmannianum* var. *berbicense* leaves.

The HPLC peaks were preliminary identified as flavonoids according to their on-line UV spectra. Peaks 2, 6, 9, 10 and 11 may have a chalcone or flavone aglycone (maxima absorption bands at about 290 and 340 nm) and the others a dihydrochalcone or flavanone aglycone (absorption bands at 230 and 290 nm). The structures were further elucidated based on the MS fragmentation behaviours (Table 4) with optimized LC/MS conditions. Fragmentation rules described in Fig. 4 were extensively used for the structure identifications. Thus, eleven compounds were identified in the chromatogram.

The peak 4 at 15.34 min gave a $[M-H]^-$ ion at *m/z* 271. The corresponding MS/MS spectrum yielded a predominant ion at *m/z* 253 and two others at *m/z* 238 and *m/z* 152 (Table 4). Such ions result from neutral losses of 18 u (loss of H_2O), 33 u (loss of H_2O and $CH_3^\bullet$) and 119 u (loss of $CH_3^\bullet$ and vinylbenzene). The predominant loss of H_2O suggests a dihydrochalcone aglycone (Fig. 4) confirmed by its UV profile (Table 4). The loss of $CH_3^\bullet$ (15 u) indicated a methoxylated compound and the loss of 119 u a non-substituted B-ring. The produced ion at *m/z* 152 should be the $[^1A-CH_3]^{\bullet -}$ fragment. Therefore, peak 4 was identified as 2′,6′-dihydroxy-4′-methoxy-dihydrochalcone (compound **10**) or an isomer concerning the positions of methoxy and hydroxyl groups on the A-ring. Identification was confirmed by the retention time and the fragmentation pattern which were the same as **10**, already isolated from that plant.

Table 3
APCI–MS^2 product ions obtained from the $[M-H]^-$ ions of flavanones **12–25**

Fragments	Molecules													
	12 Collision energy: 33%	**13** Collision energy: 44%	**14** Collision energy: 44%	**15** Collision energy: 33%	**16** Collision energy: 42%	**17** Collision energy: 37%	**18** Collision energy: 37%	**19** Collision energy: 44%	**20** Collision energy: 37%	**21** Collision energy: 37%	**22** Collision energy: 37%	**23** Collision energy: 40%	**24** Collision energy: 37%	**25** Collision energy: 42%
Neutral losses														
$[M-H]^-$	285 (1)[a]	391 (100)	269 (80)	299 (100)	299 (100)	283 (100)	283 (100)	255 (100)	223 (100)	239 (96)	253 (46)	253 (8)	283 (16)	269 (45)
$[M-H-H_2]^-$	/	/	/	297 (3)	/	281 (21)	281 (46)	/	221 (23)	/	/	/	/	/
$[M-H-CH_3]^{\bullet-}$	/	/	/	284 (5)	284 (61)	/	268 (61)	/	/	/	238 (100)	238 (4)	268 (18254	(100)
$[M-H-CO]^-$	/	/	241 (27)	/	/	255 (13)	255 (2)	227 (3)	195 (63)	/	225 (2)	225 (100)	255 (100)	/
$[M-H-CO_2]^-$	/	/	225 (70)	255 (2)	255 (4)	239 (7)	239 (2)	211 (37)	179 (1)	/	/	/	/	/
$[M-H-H2O]^-$	/	373 (3)	251 (11)	/	/	/	/	/	205 (10)	/	/	/	/	251 (63)
$[M-H-C_2H_2O]^-$	/	349 (31)	227 (100)	257 (6)	257 (6)	241 (20)	241 (74)	213 (64)	/	197 (100)	/	/	/	/
$[M-H-C_2H_2O-CO]^-$	/	321	199	/	/	213	213	185	/	169	/	/	/	/
$[M-H-C_3O_2]^-$	/	323 (33)	201 (7)	/	/	/	/	187 (1)	/	/	/	/	/	/
$[M-H-C_2H_2O-CO_2]^-$	/	305	183 (17)	/	/	/	/	169	/	/	/	/	/	/
$[M-H-C_2H_2O-CH_3]^{\bullet-}$	/	/	/	/	242 (3)	/	226	/	/	/	/	/	/	/
$[M-H-CH_3-CH_3]^{\bullet-}$	/	/	/	269 (1)	269 (16)	/	/	/	/	/	/	/	253(12)	/
$[M-H-CO-CH_3]^{\bullet-}$	/	/	/	/	/	/	/	/	/	/	/	210 (1)	240 (3)	/
$[M-H-CH_3-CO]^-$	/	/	/	/	256 (6)	/	/	/	/	/	/	/	/	226 (18)
$[M-CH_3-H_2O]^-$	/	/	/	/	/	/	/	/	/	/	/	/	236 (24)	
$[M-H-CH_3-CH_3-CO]^{\bullet-}$	/	/	/	/	/	/	/	/	/	/	/	/	225	/
$[M-H-CH_3-CH_3-CO_2]^{\bullet-}$	/	/	/	/	/	/	/	/	/	/	/	/	209	/
Fragments														
$^{1,3}A^-$	165 (100)	287 (11)loss of 104 u	165 (34) loss of 104 u	/	165 (19) 179	(3) loss of 104 u	179 (14) loss of 104 u	151 (23) loss of 104 u	/	135 (11)loss of 104 u	/	149 (2) loss of 104 u	179 (2) loss of 104 u	165 (26) loss of 104 u
$^{1,3}A^- - CO_2$	121 (1)	/	121 (5)	/	/	135 (1)	135 (2)	107 (3)	/	91 (1)	/	/	/	121 (2)
$^{1,2}A^-$	/	/	178 (21)	/	178 (24)	192 (7)	/	/	/	148 (3)	/	/	/	/
$^{1,4}A^-$	/	/	/	/	/	/	153 (1)	/	/	/	/	123 (3)	153 (4)	/
$^{0,3}A^-$	150 (3)	/	/	/	/	/	164 (14)	/	/	/	/	/	/	/
$[M-H-cycle\ B]^-$	191 (8)	/	/	/	/	205 (2)	/	/	145 (2)	/	/	/	/	191 (4)
$^{1,3}B^-$	119 (4)	/	/	119 (60)	/	/	/	/	/	/	/	/	/	/

[a] Relative intensity.

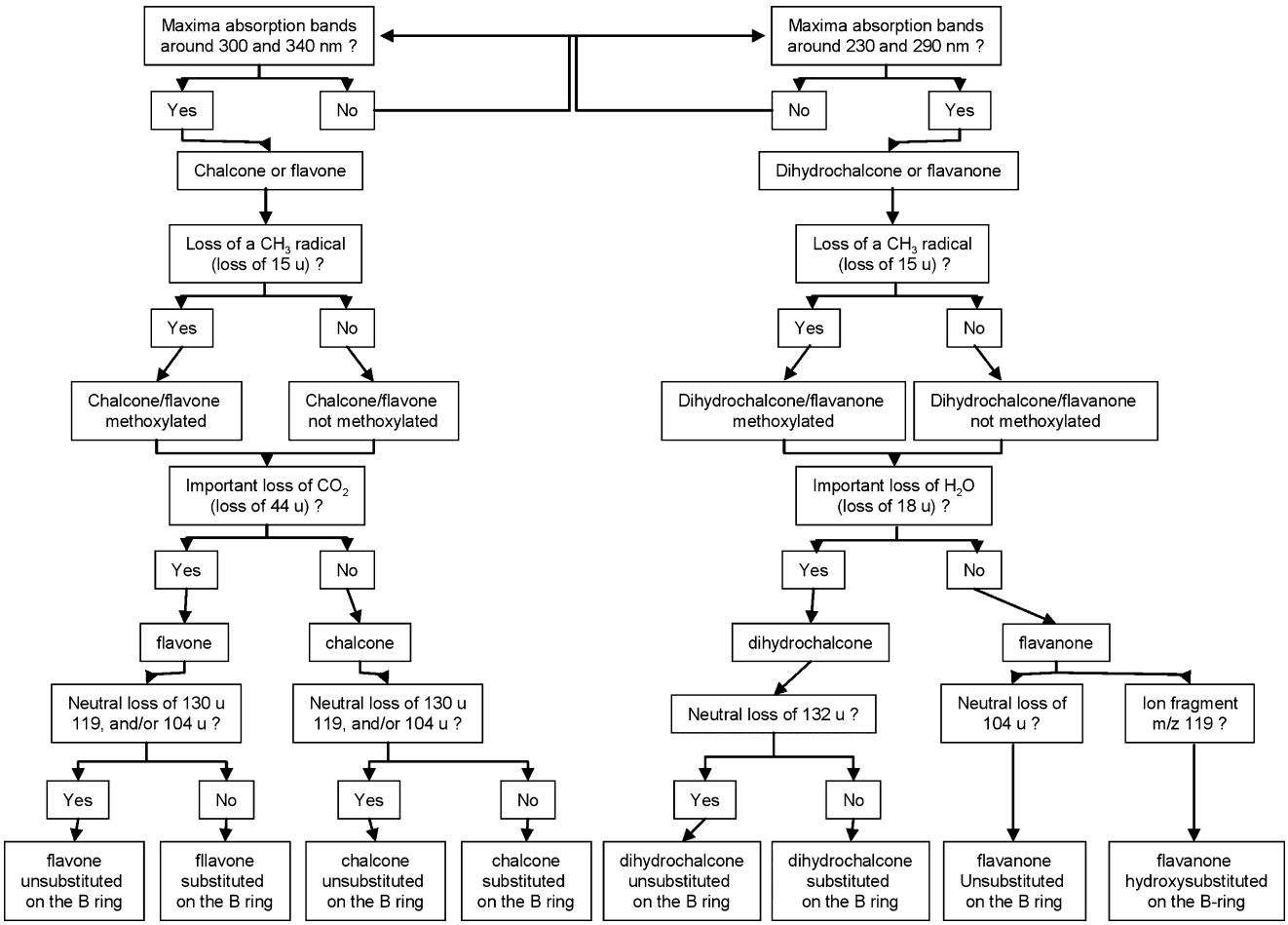

Fig. 4. Decision tree for the identification of chalcones, flavones, dihydrochalcones and flavanones by UV and negative APCI mass spectrometry.

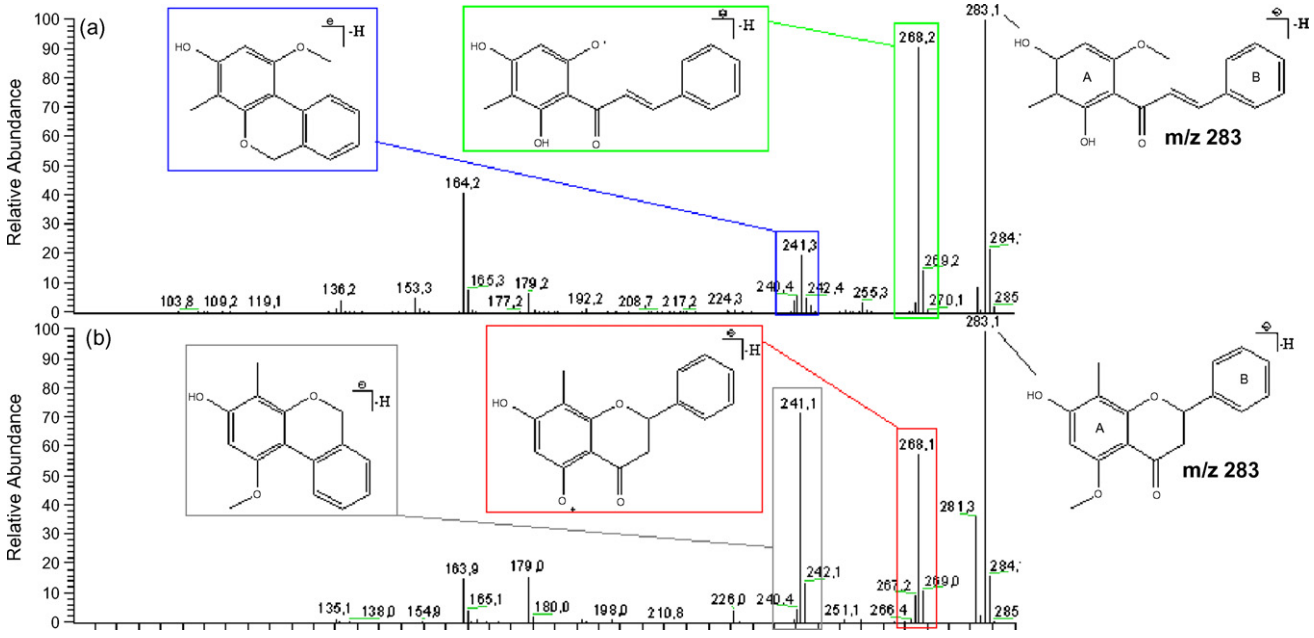

Fig. 5. (a) MS/MS of the $[M-H]^-$ ion of chalcone **1**. (b) MS/MS of the $[M-H]^-$ ion of flavanone **18**.

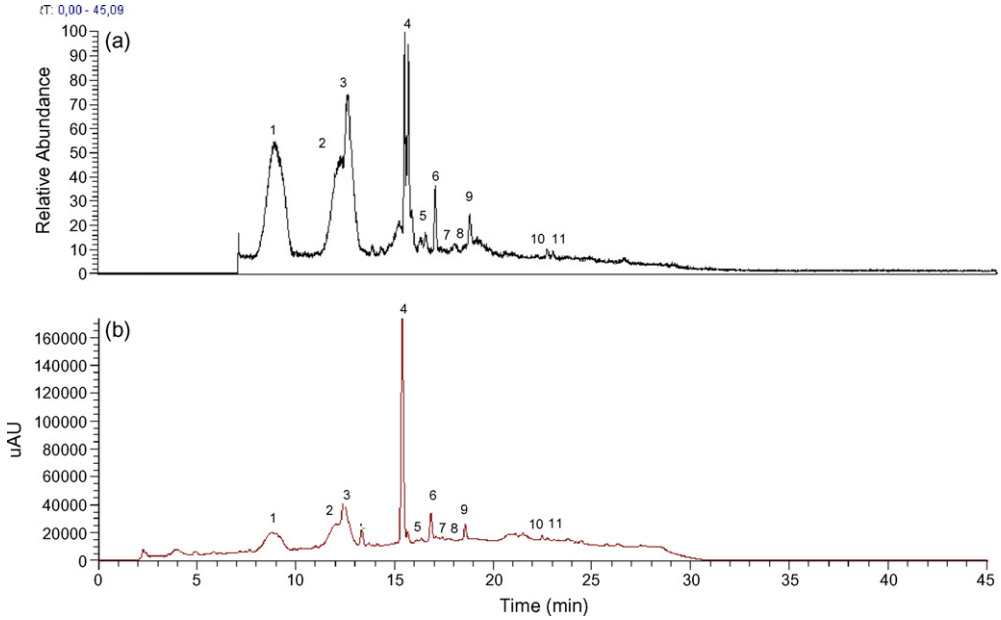

Fig. 6. HPLC–DAD–APCI–MS analysis of the ethyl acetate extract of the leaves of *Piper hostmannianum* var. *berbicense*. (a) LC-negative APCI–MS total ion current (TIC) profile and (b) HPLC–UV monitored at 340 nm.

Because of their UV profile (Table 4) peak 5 (t_R 15.73 min) may possess a flavanone or a dihydrochalcone skeleton and peak 6 (t_R 16.87 min) a chalcone or flavone one. They gave a $[M-H]^-$ ion at *m/z* 283. This pair of isomers could be distinguished by their respective MS/MS spectra (Table 4).

Peak 5 showed three main ions at *m/z* 255 (loss of CO, 28 u), *m/z* 241 (loss of C_2H_2O, 42 u) and *m/z* 192 (loss of 63 u). Based on the general fragmentation rules (Fig. 4), it should be a non-methoxylated flavanone unsubstituted on B-ring with hydroxyl groups due to the losses of CO and C_2H_2O. The product ion at *m/z* 192 may be produced after consecutive losses of B-ring and $CH_3^{\bullet}$. The $^{1,3}A^-$ ion at *m/z* 179 and the presence of another radical ion at *m/z* 164 (that may be interpreted as $[^{1,3}A^- - CH_3^{\bullet} - H]^{\bullet -}$) were consistent with a dimethyl A-ring substituted flavananone such as the known compound 6,8-dimethylpinocembrin (compound **17**) or an isomer. The structure was confirmed by the Rt value identical to one of the isolated compounds.

Peak 6 yielded five product ions at *m/z* 268 (loss of 15 u), *m/z* 241 (loss of 42 u), *m/z* 179, *m/z* 164 and *m/z* 153. The ions at *m/z* 268 and *m/z* 241 should result from neutral losses of $CH_3^{\bullet}$and C_2H_2O respectively, suggesting the presence of a methoxyl group. This compound is therefore a methoxylated chalcone or flavone. The lack of an important loss of CO $_2$ suggests a chalcone moiety (Fig. 4). The product ions at *m/z* 179, *m/z* 164 and *m/z* 153 could be attributed to A-type fragments: $^1A^-$, $[^1A–CH_3]^{\bullet -}$ and $^0A^-$ respectively indicating that the ring-A bears two hydroxyl groups and one methoxy group. Thus, peak 6 was characterized as 2′,4′-dihydroxy-

Table 4
Characterization of minor flavonoids by HPLC-DAD-APCI–MS^n from *Piper hostmannianum* var. *berbicense*

Peak no.	Retention time	$[M-H]^-$ *m/z*	UV λ_{max} (nm)	HPLC–APCI–MS^a *m/z* (% base peak)	Identification
1	8.92	327	235; 295	MS^2 [327]: 309 (1), 283 (100), 268 (1), 241 (1) MS^3 [283]: 268 (37), 241 (78), 179 (10),164 (12), 136 (2)	6-Carboxy-7-hydroxy-5-methoxy 8-Methylflavanone
2	12.34	327	295; 338	MS^2 [327]: 309 (7), 283 (100), 268 (1), 241 (1) MS^3 [283]: 26 (57), 241 (16), 179 (5), 164 (31), 136 (4)	5′-Carboxy-2′,4′-dihydroxy-6′-methoxy 3′-Methylchalcone
3	12.48	301	229; 290	MS^2 [301]: 286 (4), 269 (100), 241 (3), 225 (2) MS^3 [269]: 254 (13), 251 (9), 241 (100), 225 (39), 178 (7)	α,4′-dimethoxy-2′,6′-dihydroxy-dihydrochalcone
4	15.34	271	211; 286	MS^2 [271]: 253 (100), 238 (21), 152 (13)	2′,6′-Dihydroxy-4′-methoxy-dihydrochalcone
5	15.73	283	221; 294	MS^2 [283]: 268 (1), 255 (17), 241 (29), 239 (7), 192 (11), 179 (4), 164 (4)	6,8-Dimethylpinocembrin
6	16.87	283	305; 347	MS^2 [283]: 268 (15), 241 (30), 192 (2), 179 (8), 164 (53), 152 (15), 136 (6)	2′,4′-Dihydroxy-3′-methylchalcone
7	17.14	311	226; 278	MS^2[311]: 296 (2), 283 (100), 268 (3), 241 (22), 192 (2), 179 (2), MS^3 [283]: 268 (34) 241 (64) 179 (3) 164 (7) 136 (3)	8-Formyl-7-methoxy-6-methylflavanone
8	18.01	341	232; 287	MS^2 [341]: 323 (1) 309 (1) 283 (24) 271 (100) MS^3[271]: 253 (100), 238 (15), 152 (13)	2′,6′-Dihydroxy-4′-methoxy-3′-prenyldihydrochalcone
9	18.61	297	298; 336	MS^2 [297]: 282(100), 254(3), 178(26)	2′,4′-Dihydroxy-3′,5′-dimethylchalcone
10	22.52	311	286; 338	MS^2 [311]: 296 (12), 283 (100), 268 (15), 241 (2) MS^3 [283]: 268 (100), 241 (15), 179 (3), 164 (50), 152 (20), 136 (6)	3′-Formyl-4′,6′-dihydroxy-2′-methoxy-5′-methylchalcone
11	22.76	341	287; 337	MS^2 [341]: 326 (1), 309 (100), 297 (15), 294 (36) MS^3 [309]: 294 (10), 281 (20), 265 (100), 241 (56)	5′-Methylester-2′,4′-dihydroxy-6′-methoxy-3′-Methylchalcone

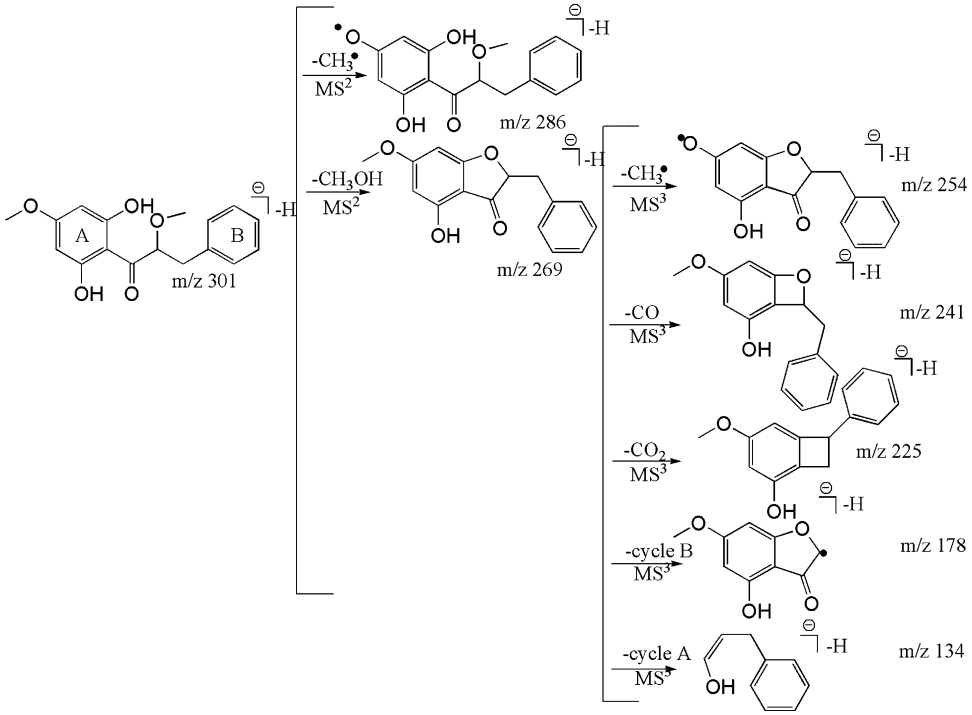

Fig. 7. Proposed MS fragmentation pattern for compound at Rt 12.48 min.

3′-methylchalcone (compound **1**) or an isomer. The structure was confirmed by co-elution with isolated compound **1**.

The UV profile of peak 9 (t_R 18.61 min) showed two absorptions at 298 and 336 nm suggesting a chalcone or flavone skeleton. The MS/MS spectrum obtained from the $[M-H]^-$ ion at *m/z* 297 yielded two product ions at *m/z* 282 (loss of 15 u) and *m/z* 178. The loss of a methyl radical indicates methoxylated chalcones and flavones. No important loss of CO_2 was observed indicating a chalcone skeleton. The presence of another fragment ion at *m/z* 178 corresponding to the loss of 119 u (consecutive losses of CH_3•and a vinylbenzene) suggests a B-unsubstituted chalcone aglycone (Fig. 4). The above fragments were consistent with the known 2′,4′-dihydroxy-3′,5′-dimethylchalcone (compound **2**) and confirmed by LC co-elution with isolated compound **2**.

The UV profile (Table 4) of peak 3 (t_R 12.48 min) suggests a flavanone or dihydrochalcone skeleton and exhibited a $[M-H]^-$ ion at *m/z* 301. Its MS/MS spectrum gave a base peak at *m/z* 269 suggesting an unusual loss of a CH_3OH molecule. Therefore, the fragmentation was further analysed by MS^3 experiment from the $[M-H-CH_3OH]^-$ ion and, therefore, does not follow the rules enacted in the decision tree. The MS^3 spectrum of *m/z* 301/269 yielded a base peak at *m/z* 241 (loss of CO) and three minor product ions at *m/z* 254 (loss of CH_3•), *m/z* 251 (loss of H_2O), and *m/z* 225 (loss of CO_2). The successive losses of a CH_3OH molecule and a CO molecule were consistent with the loss of a methoxy group in α position of a carbonyl function of a dihydrochalcone as represented in the proposed fragmentation pathway described in Fig. 7. Moreover, we could suggest the presence of another methoxy group due to the loss of a CH_3• radical (*m/z* 254). Such an unusual loss of a CH_3OH molecule in the MS^2 spectrum allowed us to detect a new compound for which the structure α,4′-dimethoxy, 2′, 6′-dihydroxy-dihydrochalcone and its fragmentation are proposed (Fig. 7). Isolation of peak 3 and extensive spectroscopic analyses would be necessary to unambiguously confirm the proposed structure of this compound.

Two other isomers were detected with a $[M-H]^-$ ion at *m/z* 311 (peaks 7 and 10), one belonging to a dihydrochalcone or flavanone skeleton (peak 7, UV absorptions at 226 and 278 nm) and the other to a flavone or chalcone skeleton (peak 10, UV absorptions at 286 and 338 nm). Their MS/MS spectra yielded a base peak at *m/z* 283 (loss of 28 u) due to the neutral loss of a CO molecule and loss of CH_3• indicating methoxylated compounds. Their MS^3 spectra gave the same ions at *m/z* 268 and *m/z* 241 but with different intensities. The comparison of the MS^3 spectrum of peak 10 with the MS^2 spectrum of peak 6 reveals similar product ions (Table 4). Thus peak 10 was plausibly identified as the known 3′-formyl-4′,6′-dihydroxy-2′-methoxy-5′-methylchalcone or an isomer concerning the position of substituents on the A- ring[19]. In the MS^3 spectra of peaks 7 and 10, the difference observed in the relative intensity ratio of $[M-H-CH_3]^{\bullet-}$ (*m/z* 268)/$[M-H-C_2H_2O]^-$ (*m/z* 241) suggests that peak 7 was the flavanone isomer of peak 10 (as shown in Fig. 5). Thus peak 7 was tentatively characterized as the known 8-formyl-7-methoxy-6-methylflavanone [19] or an isomer.

Both peak 1 (t_R 8.92 min) and peak 2 (t_R 12.14 min) gave a $[M-H]^-$ ion at *m/z* 327 but their UV profile (Table 4) shows that peak 1 has a dihydrochalcone or flavanone skeleton and peak 2 a chalcone or flavone one. This pair of isomers yielded a base peak at *m/z* 283 in their MS/MS spectra suggesting the neutral loss of a CO_2 molecule. Their MS^3 spectra (*m/z* 327/283) were respectively identical to those of peak 7 and peak 10 (Table 4). Thus peak 1 was tentatively characterized as the new 6-carboxy-7-hydroxy-5-methoxy-8-methylflavanone and peak 2 as the new 5′-carboxy-2′,4′-dihydroxy-6′-methoxy-3′-methylchalcone. Nevertheless, two-dimensional NMR experiments would be necessary to conclude on the exact position of the substituents on the A-ring.

The low retention times of these two compounds (peaks 1 and 2) are in agreement with the presence of a carboxylic acid group in the proposed structures.

Peak 8 (t_R 18.01 min) exhibited a $[M-H]^-$ ion at *m/z* 341 and two UV maxima absorptions at 232 and 282 nm suggesting a dihydrochalcone or flavanone skeleton. CID yielded a prominent ion at *m/z* 271 and another ion at *m/z* 283 resulting from neutral losses

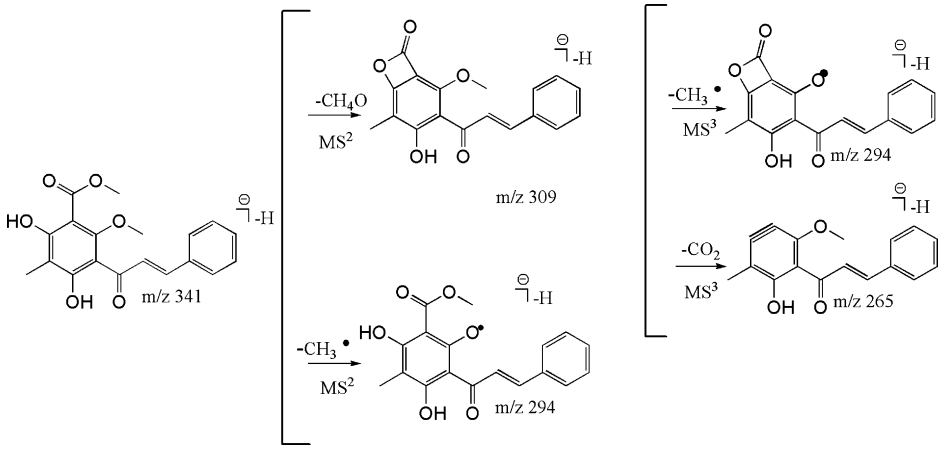

Fig. 8. Proposed MS fragmentation pattern for the compound at Rt 22.76 min.

of 70 and 58 mass units respectively. The MS^3 spectrum of the *m/z* 271 ion is similar to the MS/MS spectrum of peak 4 (identified as 2′,6′-dihydroxy-4′-methoxydihydrochalcone). The neutral loss of 70 mass units could be a loss of C_5H_{10} corresponding to a prenyl moiety frequently encountered in Piperaceae [20,21]. Thus, peak 8 was tentatively identified as 2′,6′-dihydroxy-4′-methoxy-3′-prenyldihydrochalcone (or isomers on A-ring), a new chalcone.

Peak 11 also displayed a $[M-H]^-$ ion at *m/z* 341 but its UV profile reveals a chalcone or flavone skeleton. As for peak 3, the MS/MS spectrum provided an unusual loss of a CH_3OH molecule (*m/z* 309) as base peak and two other ions at *m/z* 297 (15%, loss of CO_2) and *m/z* 294 (36%, successive loss of CH_3OH and $CH_3^{\bullet}$) (Table 4). Therefore, a chalcone skeleton (instead of a flavone, see decision tree) with two methoxy substituents was deduced for peak 11. The investigation of the MS^3 spectrum of the $[M-H-CH_3OH]^-$ ion provided a base peak at *m/z* 265 due to the loss of CO_2 instead of a CO as observed for peak 3 (Table 4). This result (successive important losses of CH_3OH and CO_2) is in good agreement with the presence of a methyl ester unit for peak 11 as drawn in Fig. 8. The loss of 15 u (*m/z* 294) from the $[M-H-CH_3OH]^-$ ion confirms the presence of a methoxy group. Based on these deductions, we plausibly identified the peak 11 as the 5′-methylester-2′,4′-dihydroxy-6′-methoxy-3′-methylchalcone or a A-ring isomer.

4. Conclusion

In the present paper, the fragmentation behaviours of chalcones, dihydrochalcones and flavanones were studied by APCI in the negative ion mode. The spectra obtained revealed specific fragmentation pathways which allowed us to increase our knowledge on the structure of these flavonoid aglycones. We demonstrated that diagnostic ions were obtained to differentiate chalcones from flavones, and dihydrochalcones from flavanones.

Based on the MS/MS fragmentation rules, the structure of eleven flavonoids was proposed from the ethyl acetate extract of *P. hostmannianum* var. *berbicense* leaves by LC/UV/(−)-APCI–MS^n. Most of the flavonoids were unambiguously identified a comparison with references, whilst identification of others would require further NMR characterization to unambiguously assign substitution positions. Some isomers and closed analogs could be distinguished from each other by comparing their UV, MS/MS and MS^3 spectra. Nevertheless, this work represents a good model for the rapid identification of bioactive minor flavonoids in crude plant extracts.

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References

[1] T. Iwashina, J. Plant Res. 113 (2000) 287.
[2] R.J. Nijveldt, E. van Nood, D.E.C. van Hoorn, P.G. Boelens, K. van Norren, P.A.M. van Leeuwen, Am. J. Clin. Nutr. 74 (2001) 418.
[3] C.M. Whitehouse, R.N. Dreyer, M. Yamashita, J.B. Fenn, Anal. Chem. 57 (1985) 675.
[4] E.C. Horning, D.I. Carroll, I. Dzidic, S.N. Lin, R.N. Stillwell, J.P. Thenot, J. Chromatogr. 142 (1977) 481.
[5] J.L. Wolfender, P. Waridel, K. Ndjoko, K.R. Hobby, H.J. Major, K. Hostettmann, Analusis 28 (2000) 895.
[6] J.B. Harborne, T.J. Mabry (Eds.), The Flavonoids: The Advances in Research, Chapman & Hall, London, 1982.
[7] Y. Itagaki, T. Kurokawa, S. Sasaki, C.T. Chang, F.-C. Chen, Bull. Chem. Soc. Jpn. 39 (1966) 538.
[8] C. Van De Sande, J.W. Serum, M. Vandewalle, Org. Mass Spectrom. 6 (1972) 1333.
[9] M. Takayama, T. Fukai, K. Ichikawa, T. Nomura, Rapid Commun. Mass Spectrom. 5 (1991) 67.
[10] N. Fabre, I. Rustan, E. de Hoffmann, J. Quetin-Leclercq, J. Am. Soc. Mass Spectrom. 12 (2001) 707.
[11] J. Zhang, J.S. Brodbelt, J. Mass. Spectrom. 38 (2003) 555.
[12] Y. Tai, S. Pei, J. Wan, X. Cao, Y. Pan, Rapid Commun. Mass Spectrom. 20 (2006) 994.
[13] F. Cuyckens, M. Claeys, J. Mass. Spectrom. 39 (2004) 1.
[14] B. Portet, N. Fabre, V. Roumy, H. Gornitzka, G. Bourdy, S. Chevalley, M. Sauvain, A. Valentin, C. Moulis, Phytochemistry 68 (2007) 1312.
[15] E. Rosenberg, J. Chromatogr. A 1000 (2003) 841.
[16] Y.L. Ma, Q.M. Li, H. Van den Heuvel, M. Claeys, Rapid Commun. Mass Spectrom. 11 (1997) 1357.
[17] J.B. Harborne, The Flavonoids: Advances in Research Since 1986, Chapman & Hall/CRC, London, 1993.
[18] T.J. Mabry, K.R. Markham, M. Thomas, The Systematic Identification of Flavonoids, Springer, New York, 1970.
[19] Y.-H.L. Chun-Lin Ye, Phytochemistry 65 (2004) 445.
[20] J.H.G. Lago, C.S. Ramos, D.C.C. Casanova, A. de Morandim, D.C.B. Bergamo, A.J. Cavalheiro, V.d.S. Bolzani, M. Furlan, E.F. Guimaraes, M.C.M. Young, M.J. Kato, J. Nat. Prod. 67 (2004) 1783.
[21] D.C. Baldoqui, M.J. Kato, A.J. Cavalheiro, V.S. Bolzani, M.C.M. Young, M. Furlan, Phytochemistry 51 (1999) 899.