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Direct characterization of isoquinoline alkaloids in a crude plant extract by ion-pair liquid chromatography–electrospray ionization tandem mass spectrometry: example of *Eschscholtzia californica*

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Abstract

An ion-pair HPLC–ESI-MS–MS method has been developed for the direct and rapid characterization of isoquinoline alkaloids in a crudely purified extract of the aerial parts of *Eschscholtzia californica* (Papaveraceae). This plant was chosen because of its increasing use in pharmaceutical industries and because its well known alkaloid composition allows the optimization of the experimental procedure through an on-line analytical sequence. Thus, 14 isoquinoline alkaloids of different types were detected and characterized. The identities of these compounds were confirmed unambigously by their fragmentation and UV spectra obtained by LC–diode-array detection. Various experiments including tandem mass spectrometry and in-orifice collision induced dissociation were performed and prove that MS–MS is a very efficient technique to identify these compounds. An explanation for each isoquinoline alkaloid type MS–MS fragmentation pattern is proposed and indicates similar neutral and/or radical losses. The order of the fragmentation depended on the type of compound but the lost fragments were similar.

Keywords: *Eschscholtzia californica*; Alkaloids; Isoquinoline alkaloids

1. Introduction

Isoquinoline alkaloids make up an extremely large and varied group of physiologically active natural products which can be divided into approximately 20 categories including the well known morphinane type [1]. Therefore, their rapid and direct characterization in crude plant extracts is crucial in pharmaceutical science.

The Californian poppy, *Eschscholtzia californica* Cham. (Papaveraceae), is an annual plant originating from California where it colonizes coastal dunes and arid areas. It was introduced in Europe in the 19th century as an ornamental plant [1]. Its chemical composition is well known and about 30 tertiary and quaternary isoquinoline alkaloids belonging to six types have been isolated from its various parts. For

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these reasons, this plant appeared to be a good model for a study of its alkaloid content by liquid chromatography–mass spectrometry (LC–MS). The main alkaloids are pavines such as californidine (**1a**) and escholtzine (**1b**) but protoberberines, benzylisoquinolines, aporphines, benzophenanthridines and protopines are also present. Although the alkaloid concentration is higher in the roots, we chose to study the aerial parts because they are the part used in medicines. The biogenesis of these alkaloids may vary with the ecological conditions and climatological zones hence the importance of a modern and powerful analytical tool for their direct characterization [2–5]. In folk medicine, the aerial parts of Californian poppy were reported to be analgesic, anodyne, diaphoretic, diuretic, soporific and spasmolytic [6]. Various pharmacological studies confirmed the sedative and anxiolytic actions of extracts of *E. californica* and the absence of toxic effects [7,8]. There is a good chance that isoquinoline alkaloids participate in these activities [9] and the complex mixture of tertiary and quaternary alkaloids present in this plant explains why many authors have studied it. Recent works reported high-performance liquid chromatography (HPLC) analysis of the main alkaloids present in the aerial parts of *E. californica* [5–10]. The detection of isoquinoline alkaloids by gas chromatography coupled to mass spectrometry was also investigated [11]. More generally, the utilization of modern ionization techniques for the study of alkaloids is now increasing [12–14], but surprisingly few works concern LC–MS studies [15,16]. To our knowledge, only one type, naphthylisoquinoline alkaloids, have been exhaustively investigated in plant extracts by various on-line coupling methods including HPLC–MS–MS, HPLC–nuclear magnetic resonance (NMR) and HPLC–circular dichroism experiments [17–19]. Different types of isoquinoline alkaloids using standard samples have been studied by capillary electrophoresis and capillary electrophoresis–MS [20,21]. However, no LC–MS studies using crude plant extracts have been reported. The interpretation of the fragmentation of alkaloids by MS remains difficult and requires the utilization of exhaustive techniques with deuterated analogues [22].

The aim of the present work is the rapid characterization of all the alkaloids present in a crudely purified methanol extract of *E. californica* by ion-pair HPLC coupled electrospray (ES) tandem mass spectrometry (MS–MS). For this, we adapted the described techniques for sample preparation and HPLC separation [23] to LC–ES-MS. Total alkaloids were prepared as dodecylsulfate salts allowing separation in one run and the direct determination of quasi-molecular cations for the tertiary alkaloids and molecular cations for the quaternary ones. Fourteen alkaloids were detected and their different families were distinguished by analyzing their degradation using in-orifice collision induced dissociation (in source-CID), MS–MS with product and precursor ion scan when possible, and by comparing their diode array detection (DAD) UV spectra. In addition, we were able to propose an explanation for the degradation spectra obtained by MS–MS, contributing to the establishment of a fragment library.

2. Experimental

2.1. Samples and general procedures

Aerial parts of *E. californica* Cham. (Papaceraceae) were obtained from Martin Bauer, medicinal plants producer (France). A voucher specimen is conserved in the herbarium of the Faculty of Pharmacy of Toulouse (OTC 3310). A 5-g amount of dried material was extracted with 100 ml of MeOH for 15 min at 60°C. The extract was evaporated to dryness under reduced pressure at a temperature of 35–40°C. The residue was dissolved in 50 ml of water acidified to pH 1 with concentrated HCl with dodecylsulfate sodium salt (0.2%). The dodecylquaternary ammonium salts were then extracted with $CHCl_3$ (50 ml×3). The combined $CHCl_3$ extracts were dried over anhydrous Na_2SO_4 and concentrated under vacuum at 35–40°C. The residue was diluted in 10 ml of MeOH and filtered before use. Authentic standards of berberine and papaverine (Sigma, St. Louis, MO, USA) were dissolved in MeOH.

2.2. General HPLC parameters

All chromatographic separations were performed using a Hypersil C_8 column (5 μm, 150×4.6 mm

I.D. for HPLC–MS and HPLC–DAD). The mobile phase consisted of: (A) acetonitrile (isocratic grade for LC; Merck, Darmstadt, Germany) and (B) demineralized water (Milli-Q quality; Millipore, Eschborn, Germany) containing 1 m*M* sodium dodecylsulfate (Aldrich) and 10 m*M* triethylamine (Prolabo, Paris, France) adjusted to pH 2.5 with phosphoric acid (Prolabo). The elution profile was: 0–40 min, linear gradient from 20 to 40% B; 40–45 min, isocratic elution with 40% B; 45–50 min, linear gradient from 40% to 100% B; 50–55 min, isocratic elution with 100% B; 55–60 min return to 20% B and the system was let to stabilize for 10 min between consecutive injections. The flow-rate was 1 ml/min and the detection wavelength 280 nm.

2.2.1. HPLC–DAD experiments

LC separation was performed using a LaChrom L-7100 quaternary gradient pump (Merck–Hitachi, Darmstadt, Germany), a Rheodyne 7725i injection valve (20 μl loop) and a LaChrom L-7450 diode array detector (Merck–Hitachi).

2.2.2. HPLC–electrospray ionization (ESI) MS–MS experiments

Analysis was performed on a Perkin-Elmer Sciex API 365 triple quadrupole mass spectrometer equipped with an ion spray source. Nitrogen served both as auxiliary and collision gas, air as nebulizer gas. The HPLC system used consisted of a PE series 200 LC pump, a Rheodyne 8125 injection valve (40 μl loop) and a PE 785 A UV–Vis detector (λ=280 nm). Both were controlled by a PE Sciex MassChrom data system (version 1.1.1). In order to determine the different MS conditions (best values for ionspray voltage IS, orifice voltage OR which determine in-source CID fragmentation, and MS–MS energy Q0-RO2), we first analyzed the two standards berberine and papaverine by direct loop injection. These values were then transposed to the various LC–MS experiments. The operating conditions for the ionspray interface were as follow: using MS mode: mass range 200–500 u, step size 0.1 u, dwell time 1 ms, MS spectra were recorded in profile mode, IS 5000 V, OR 20 V (no in-source CID) and OR 80 V (in-source CID), RNG 260 V; using MS–MS mode: the mass range varied with the ions analyzed, step size 0.5 u, dwell time 0.8 ms, MS–MS spectra were recorded in profile mode, IS 5000 V, OR 80 V, RNG 260 V, Q0 −10 V, RO2 +50 V. LC–ESI-MS (with and without in-source CID) and LC–ESI-MS–MS experiments were performed on-line directly after LC separation. The MS–MS spectra were considered to be characteristic for a signal-to-noise ratio higher than 5. The separation of the crudely purified extract solution was carried out at a flow-rate of 1 ml/min using the same conditions as described above; only 4% of the flow was introduced into the electrospray source.

3. Results and discussion

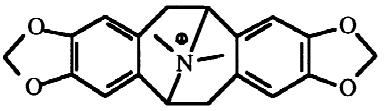
All the structures of the isoquinoline alkaloids detected in the crudely purified extract of *E. californica* are summarized in Fig. 1.

With this new ion-pair LC–MS method, tertiary and quaternary alkaloids in crude plant extracts can be directly analyzed on-line using various ESI-MS experiments: the different types of isoquinolines present in the aerial parts of *E. californica* may be distinguished by MS–MS, in-orifice CID and by comparing their UV spectra.

According to the complexity of the mobile phase (triethylamine, sodium dodecylsulfate, phosphoric acid) a blank with MeOH was first injected to identify the ions present in the eluent. The LC–MS analysis of the eluent showed ions at m/z 102 $[\text{triethylamine}+H]^+$, 197 $[2H_3PO_4+H]^+$, 295 $[3H_3PO_4+H]^+$ and 393 $[4H_3PO_4+H]^+$. These ions were suppressed in the following analysis of extracts.

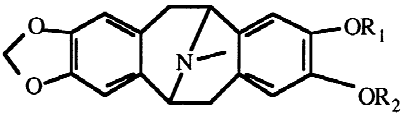
In order to demonstrate characteristic mass spectrometric data for different types of isoquinoline alkaloids, tandem mass spectra of authentic standards of berberine (protoberberine type) and papaverine (benzylisoquinoline type) were first analyzed by direct infusion (Fig. 2a and b). The same compounds analyzed using LC–ESI-MS–MS exhibited exactly the same tandem mass spectra (Fig. 2d and e).

The analysis by direct infusion of the crudely purified methanol extract exhibited only three main alkaloids (**1a**, **1b** and **4a**) (data not shown). Fig. 3a and b show the HPLC–UV and extracted ion chromatograms obtained using dodecylsulfate as ion-pairing agent giving a good separation of alkaloids



Californidine $C_{20}H_{20}NO_4$, 338 (**1a**)

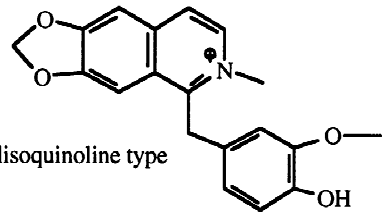
Pavine type



$R_1+R_2=CH_2$ Escholtzine $C_{19}H_{17}NO_4$, 323 (**1b**)

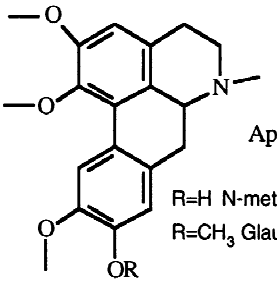
$R_1=R_2=CH_3$ O-methylcaryachine $C_{20}H_{21}NO_4$, 339 (**1c**)

$R_1=H$, $R_2=CH_3$ Caryachine $C_{19}H_{19}NO_4$, 325 (**1 d**)



Benzylisoquinoline type

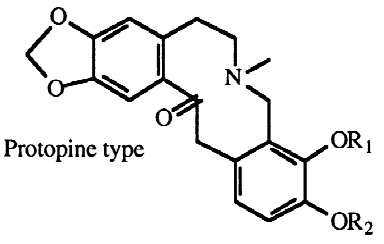
Escholamidine $C_{19}H_{18}NO_4$, 324 (**2**)



Aporphine type

R=H N-methylaurotetamine $C_{20}H_{23}NO_4$, 341 (**3a**)

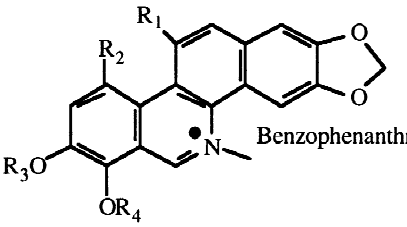
$R=CH_3$ Glaucine $C_{21}H_{25}NO_4$, 355 (**3 b**)



Protopine type

$R_1+R_2=CH_2$ Protopine $C_{20}H_{19}NO_5$, 353 (**4a**)

$R_1=R_2=CH_3$ Allocryptopine $C_{21}H_{23}NO_5$, 369 (**4b**)



Benzophenanthridine type

$R_1=R_2=H$, $R_3+R_4=CH_2$ Sanguinarine $C_{20}H_{14}NO_4$, 332 (**5a**)

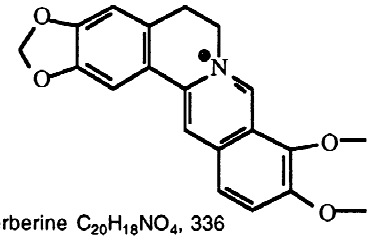
$R_1=R_2=H$, $R_3=R_4=CH_3$ Chelerythrine $C_{21}H_{18}NO_4$, 348 (**5 b**)

$R_1=H$, $R_2=OCH_3$, $R_3+R_4=CH_2$ Chelirubine $C_{21}H_{16}NO_5$, 362 (**5c**)

$R_1=H$, $R_2=OCH_3$, $R_3=R_4=CH_3$ Chelilutine $C_{22}H_{20}NO_5$, 378 (**5d**)

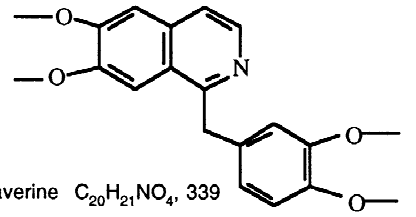
$R_1=OH$, $R_2=H$, $R_3=R_4=CH_3$ Homochelidonine $C_{21}H_{18}NO_5$, 364 (**5 e**)

Standards



Berberine $C_{20}H_{18}NO_4$, 336

(Protoberberine type)



Papaverine $C_{20}H_{21}NO_4$, 339

(Benzylisoquinoline type)

Fig. 1. Structures of isoquinoline alkaloids detected in *E. californica* and standards used.

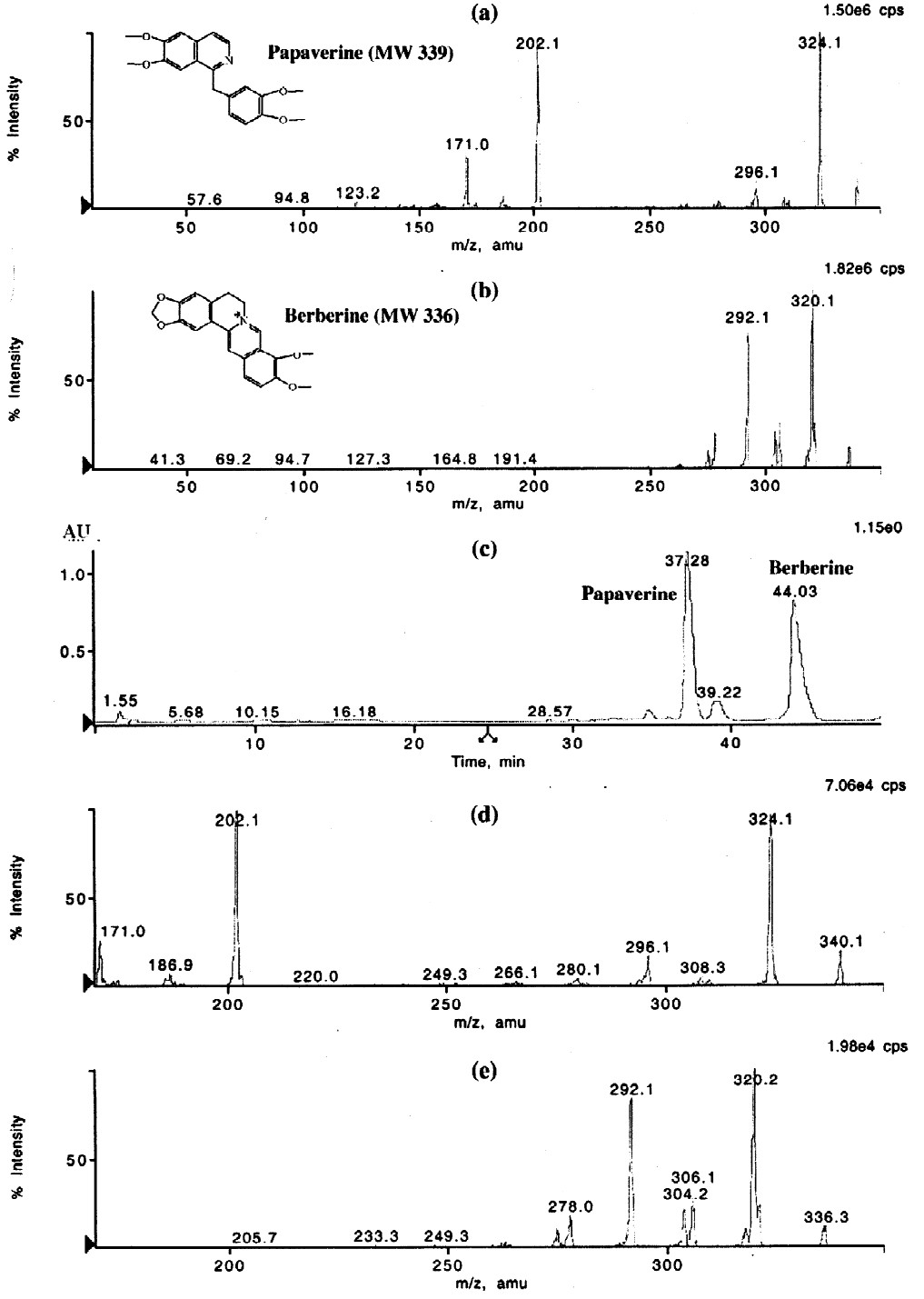


Fig. 2. (a) MS–MS product ion scan of molecular ion papaverine $[M+H]^+$ 340. (b) MS–MS product ion scan of molecular ion berberine $[M]^+$ 336. (c) HPLC–UV at 280 nm for a mixture of papaverine and berberine. (d) HPLC–MS–MS product ion scan of papaverine. (e) HPLC–ESI-MS–MS product ion scan of berberine.

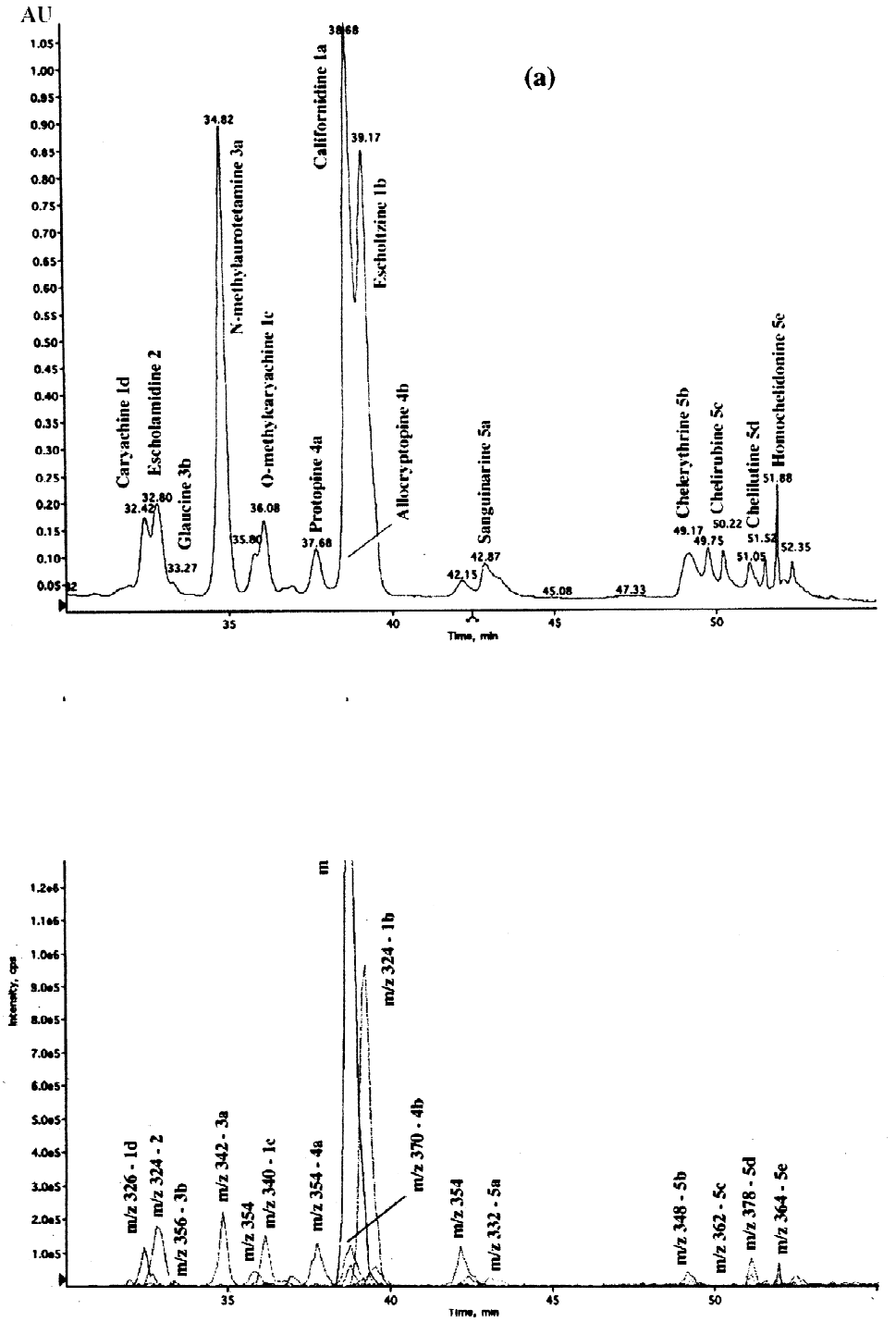


Fig. 3. (a) HPLC–UV at 280 nm of the crudely purified extract of *E. californica*. (b) Extracted ion chromatogram for the 14 isoquinoline alkaloids detected in *E. californica* (OR 80 V).

between 30 and 55 min. According to the sample preparation and the types of compounds expected, the pseudo-molecular ions were even in the positive mode, between 300 and 400 u and yielded quasimolecular ions at $[M+H]^+$ for tertiary alkaloids and $[M]^+$ for quaternary ones. No sodiated molecular ions neither other adducts were detected for tertiary alkaloids. The UV spectrum was superimposable on the extracted ion chromatogram (Fig. 3). Thus, 14 isoquinoline alkaloids belonging to five different types were detected in the crude extract of *E. californica* (Fig. 1). The two standards, papaverine and berberine, confirmed the specificity of the method. The last protoberberine-type was not detected in the aerial parts of the plant. The alkaloids were well separated using the present HPLC conditions except for the two protopine-type compounds which were not correctly eluted: the extracted ion chromatogram (Fig. 3b) showed that protopine (**4a**), detected mainly at 37.68 min, was co-eluted with *O*-methylcaryachine (**1c**) and sanguinarine (**5a**). This was confirmed by using MS–MS precursor ions scan of *m*/*z* 278 and *m*/*z* 251. Allocryptopine (**4b**) was co-eluted with californidine (**1a**).

In the present HPLC–MS method, orifice values of 20 and 80 V were investigated. Only 80 V in-source CID conditions were used to obtain the extracted ion chromatogram. Although in-source CID is a very useful method which displays molecular and fragment ions in a single HPLC–MS analysis, in the present study, the analysis of the same mixture by in-source CID and MS–MS provided similar patterns for both chromatograms only for the two main alkaloids californidine **1a** and escholtzine **1b**. For the other compounds, in-source CID generally displayed spectra with one or two degradation ions with a poor signal-to-noise ratio (data not shown).

Fig. 4 shows the LC–MS–MS product ions of the main pavine type detected in the Californian poppy. These compounds consisted of one quaternary (**1a**) and three tertiary pavines (**1b**–**1d**). For **1a** and **1b**, characteristic ion fragments were found at *m*/*z* 177, 205, 235, 283 and 293; for compounds **1c** and **1d** the two diagnostic ions at *m*/*z* 235 and 263 were observed in the spectra. In these different cases, we suggest that these fragmentations may be explained by various radical and/or neutral losses following a similar scheme illustrated in Fig. 6. The first neutral loss concerned the nitrogen group with its substituents $(CH_3)_2NH$ for the quaternary compound **1a** and CH_3NH_2 for the other tertiary pavines, involving $[M-45]^+$ and $[M+H-31]^+$, respectively. The classical neutral loss of ammonia was not observed, but the nitrogen-containing heterocycle losses may be explained by a retro-Diels–Alder (RDA) fragmentation. This type of RDA fragmentation has ever been described by Bringmann et al. [19] for naphthylisoquinolines and the fact that it occurs (or not as we will see below for unsaturated heterocycle quaternary compounds) informs about the structure of the nitrogen-containing heterocycle. The other fragment losses depended on the substituents of the compounds: in the case of methylenedioxy groups, neutral losses of CH_2O (−30) and CO (−28) were observed. For methoxy groups, radical losses of $CH_3^{\cdot}$ (−15) and/or $CH_3O^{\cdot}$ (−31) were detected. The loss of these radicals corresponds to an unusual transition from a closed-shell ion to an odd-electron ion as ever observed for methoxylated flavonoid aglycones by Ma et al. using positive ion fast atom bombardment (FAB) MS–MS [24,25]. Concerning the three tertiary pavines, a common ion at *m*/*z* 188 might be explained by the neutral losses of the disubstituted benzyl groups resulting in the protonated methylenedioxytetrahydroquinoline groups (188 u). This was confirmed by the fragmentation of compound **1c** which exhibited both neutral loss of the dimethoxybenzyl moiety (*m*/*z* 188) and of the methylenedioxybenzyl moiety (*m*/*z* 204). For the compound **1d**, exhibiting two different substituents on one side (one hydroxy and one methoxy group), neutral loss of MeOH (*m*/*z* 237 to *m*/*z* 205) was observed. This type of fragmentation is supported by the study of the MS–MS spectra of the two standards papaverine and berberine (Fig. 2) which exhibit α-disubstituted methoxy groups. These two compounds lose 16 u from the corresponding pseudo-molecular and molecular cations that may be attributed to a CH_4 loss involving the two methoxy groups and resulting in a ketone-type fragment that undergoes further CO loss leading to ions at *m*/*z* 296 and 292 for papaverine and berberine, respectively.

Fig. 5 illustrates the MS–MS spectra of the other isoquinoline alkaloid types. Concerning the benzylisoquinoline type escholamidine (compound **2**, Fig. 5) and papaverine (Fig. 2d), the loss of the

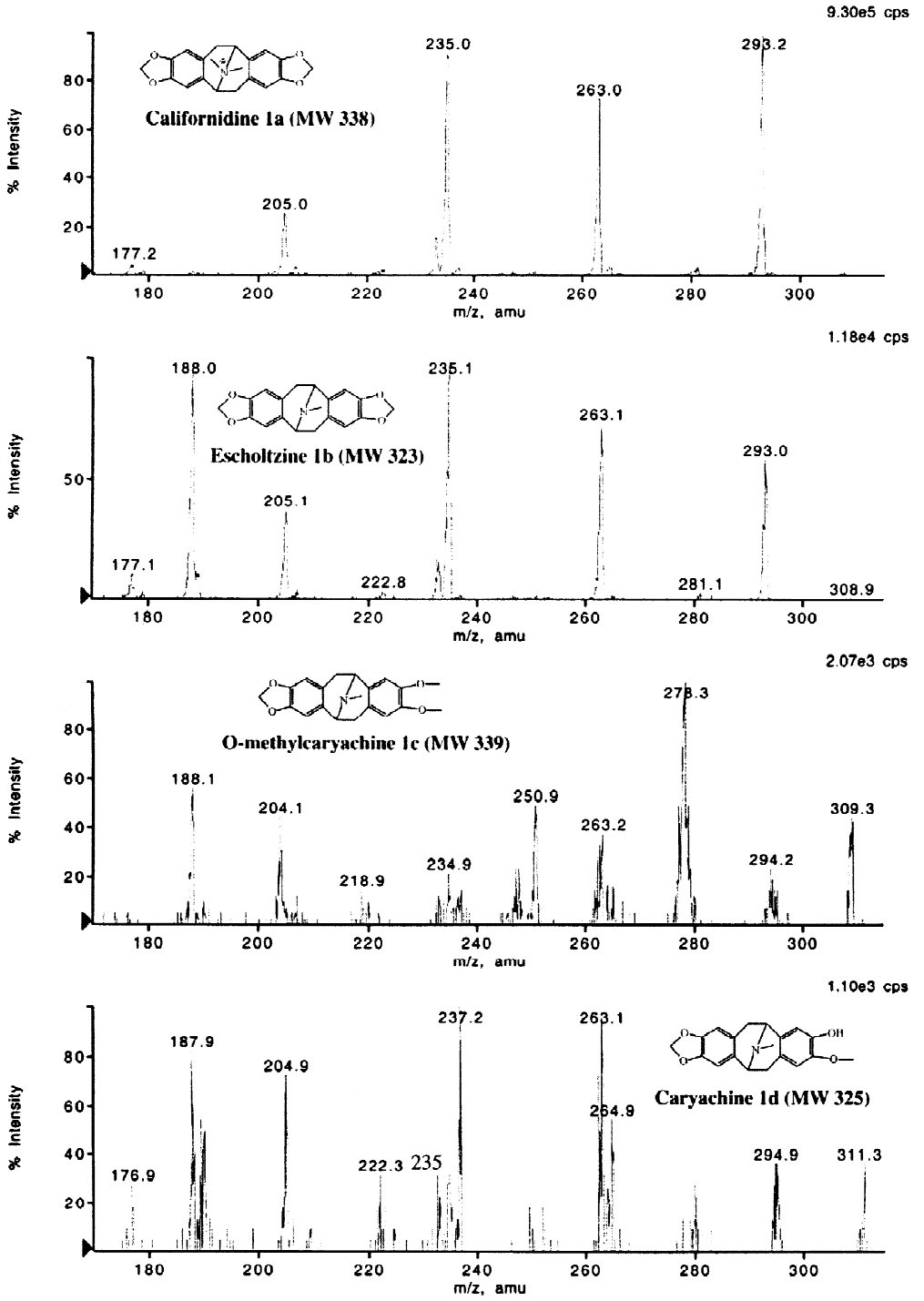


Fig. 4. HPLC–ESI-MS–MS product ion scan of californidine (*m*/*z* 338), escholtzine (*m*/*z* 324), protonated *O*-methylcaryachine (*m*/*z* 340) and protonated caryachine (*m*/*z* 326), respectively.

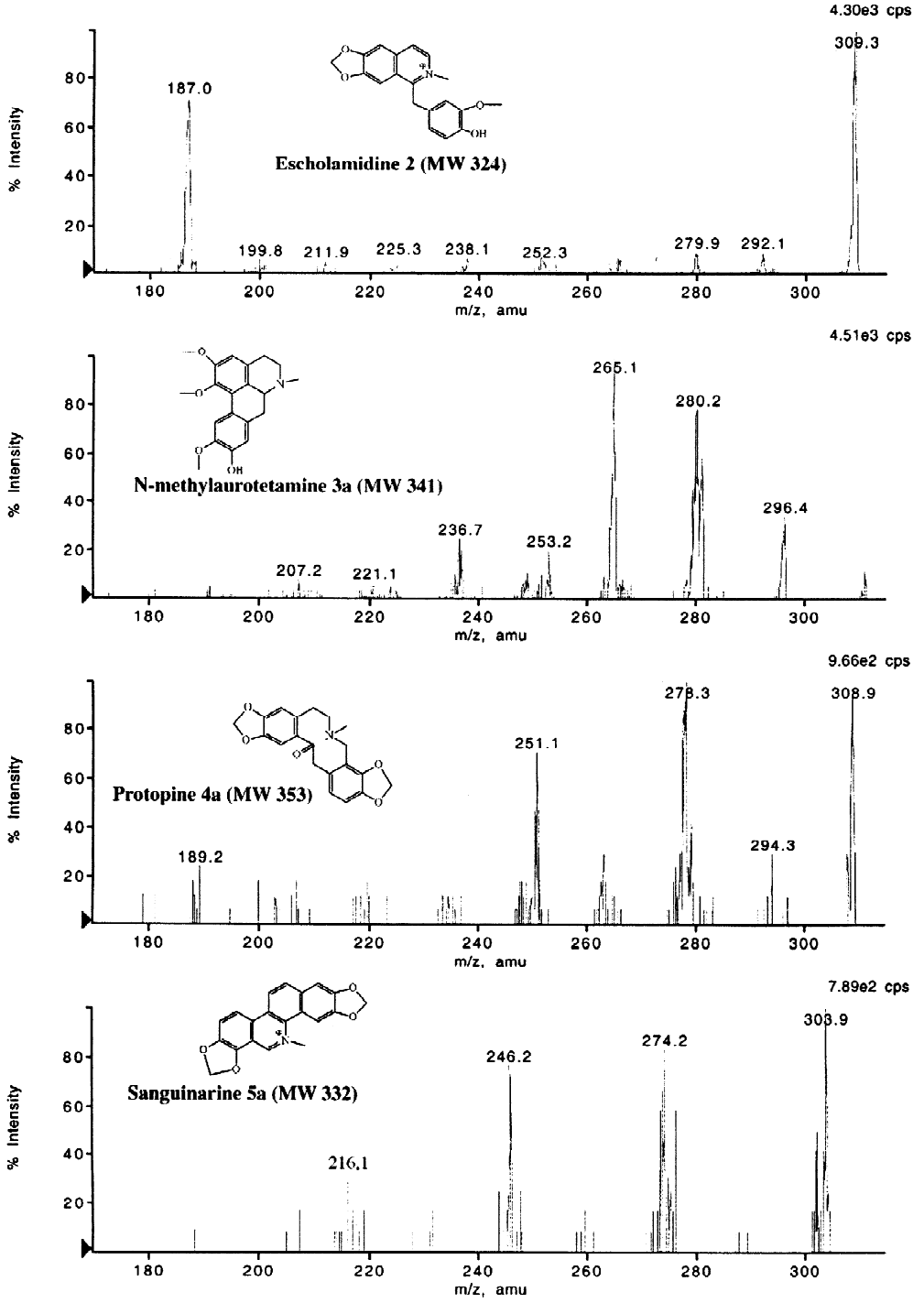


Fig. 5. HPLC–ESI MS–MS product ion scan of escholamidine (*m*/*z* 324), *N*-methylaurotetamine (*m*/*z* 342), protopine (*m*/*z* 354) and sanguinarine (*m*/*z* 332), respectively.

disubsituted phenyl groups was mainly observed (loss of methoxyhydroxybenzyl radical for **2** and neutral loss of dimethoxybenzyl for papaverine). The neutral loss of MeOH for compound **2** fragmentation confirms the previously described degradation of **1d**. For the other types, aporphines (compounds **3a** and **3b**), protopines (compounds **4a** and **4b**), benzophenanthridines (compounds **5a** to **5e**) and the standard berberine, similar fragment losses were observed but the order of departure varied with the

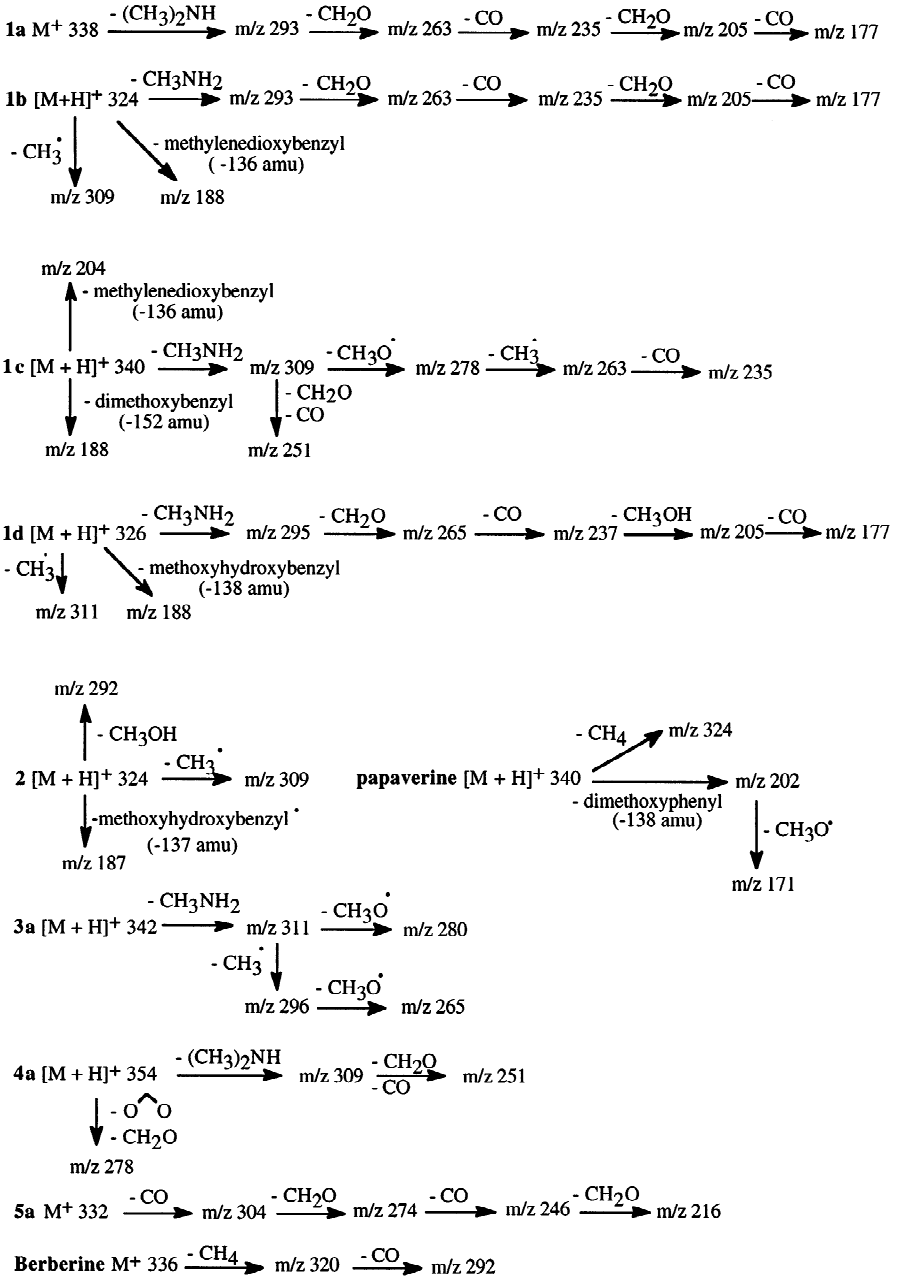


Fig. 6. Proposed fragmentation for the six isoquinoline alkaloid types analyzed in this study.

type of compound. RDA fragmentation was not observed for berberine, sanguinarine (compound **5a**) or escholamidine (compound **2**), three quaternary isoquinoline types where the nitrogen-containing heterocycle is unsaturated.

The other compounds were not sufficiently concentrated in the extract (signal-to-noise ratio lower than 5) to yield a characteristic MS–MS fragmentation. They were identified by in-orifice CID-MS, on the basis of the literature [6–8] and by comparing their UV spectra obtained by LC–DAD with the unambigously identified corresponding types.

To our knowledge it is the first time that isoquinoline alkaloid types such as pavines, protopines, benzophenanthridines or aporphines, including tertiary and quaternary ones, are studied on-line, in one run, by HPLC–ESI-MS–MS and fragmentation pathways proposed (see Fig. 6). Recently, we performed some ESI-MS^n experiments on aporphine alkaloids using an ion trap mass spectrometer (data not shown). The MS^6 or MS^7 product ion scans obtained, confirmed the sequence proposed here for the fragmentation of this type of isoquinoline alkaloids.

4. Conclusion

As demonstrated in this work, on-line coupling ion-pair HPLC–MS–MS is an efficient, rapid and direct method, for the phytochemical analysis of isoquinoline alkaloids in crude plant extracts. The experiments showed that MS–MS gave characteristic diagnostic ions for these different compounds and both the similarity and diversity of the structures detected allowed us to suggest an explanation for these degradations by crossing the data and thus enhance the specificity of the method. It is also possible, in the case of well resolved and pure chromatographic peaks, to use in-source CID-HPLC–MS as a very simple and rapid method for definitive identification of isoquinoline alkaloids using a simple quadrupole analyzer. HPLC–MS–MS is also suitable for unequivocal identification of the six types of known isoquinoline alkaloids in the crude plant extract although it requires more expensive tandem MS instrument. Similarly, rapid quantitative analysis by multiple reaction monitoring may be possible because precursor and product pairs are now available for these compounds. The application of MS–MS and the resulting diagnostic ions may also be useful, for example, in high throughput screening and in the evaluation of biological activities of little known types of compounds, such as pavines, in crudely purified plant extracts without requiring tedious and expensive isolation procedures.

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