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Determination of salvinorins and divinatorins in *Salvia divinorum* leaves by liquid chromatography/multistage mass spectrometry

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Salvinorin A is the most potent naturally occurring hallucinogen known and rivals synthetic LSD in potency. Structurally it belongs to the neoclerodane diterpenoids, and it is the only known non-nitrogenous kappa-opioid-selective agonist. *Salvia divinorum* (Diviner's sage) is a member of the mint family that was used in ancient Mexican traditional practices. Today it is widely cultivated in Europe as a recreational marijuana substitute; it is illegal to buy, sell or possess the plant or the active principle in some countries. Six different salvinorins and three divinatorins have been isolated from *Salvia divinorum* leaves. The ion fragmentation, separation and quantitation of these diterpenes by liquid chromatography/electrospray ionization multistage mass spectrometry (LC/ESI-MS^n) are described. The importance of LC in herbal extract determination and the chemical diagnostic power of MS^n in the analysis of classes of natural organic products are discussed. Copyright © 2005 John Wiley & Sons, Ltd.

Salvia divinorum Epling & Jativa (*Lamiaceae*) is a rare member of the mint family, used for many centuries by the Mazatec Indians of Oaxaca, Mexico, in traditional medicine and spiritual practices because of its hallucinogenic properties. This particular sage, also called 'magic mint', is known by many other names, including *ska Maria*, *ska Pastora*, *hierba de Maria*, *hojas de la Pastora*: all reflect the Mazatec belief that *S. divinorum* is the incarnation of the Virgin Mary.

Recently, the use of this plant as a legal hallucinogen has spread throughout the world, especially through e-commerce. It is widely cultivated in Europe as a recreational marijuana substitute;[1] at present, only in Australia, Belgium, Denmark, Finland, South Korea, Spain and Italy it is illegal to buy, sell or possess the plant or the active principle.

S. divinorum leaves may be taken by the following routes: (1) mastication and swallowing; (2) swallowing the juice obtained by crushing; and (3) smoking.

The molecule involved in the psychoactive effect is salvinorin A,[2] similar in potency to the synthetic hallucinogen lysergic acid diethylamide. This compound is a neoclerodane diterpenoid, and is the only known non-nitrogenous selective kappa-opioid receptor (KOR) agonist.[2,3]

This study reports the identification, separation and quantitation of six salvinorins[4–7] and three divinatorins[8] (Fig. 1) by liquid chromatography/electrospray ionization multistage ion trap mass spectrometry (LC/ESI-IT-MS^n) and the analysis of *Salvia divinorum* leaves of different geographic origins (Mexico and Hawaii). Members of this family of compounds show different pharmacological properties: KOR affinities of salvinorins[9,10] and divinatorins[11] have been extensively described.

Quantitative gas chromatography/mass spectrometry (GC/MS)[12] and liquid chromatography/ultraviolet (LC/UV)[13] methods have been proposed for the analysis of salvinorin A, but these techniques are affected by their extraction requirement and their low sensitivity. Moreover, extraction at the high pH values typically used in forensic alkaloid analysis can lead to hydrolysis of the ester groups present in many natural terpenoid derivatives; LC/MS allows these problems to be bypassed as it permits the direct analysis of aqueous or alcoholic solutions of plant extracts. Furthermore, the presence of plant biomarkers is more easily detected when investigating the adulteration of plant products. The ion trap (IT) mass analyzer has allowed us to obtain spectra up to MS^4 (in one case up to MS^6) that are rich in information and has produced selective analytical methods for each member of this class of compounds, showing typical losses.

EXPERIMENTAL

Standard and sample preparation

Salvinorins and divinatorins, isolated from leaves of *Salvia divinorum*, were kindly provided by M. A. Rizzacasa and T. A. Munro (University of Melbourne, Australia). Standard compounds were dissolved in methanol and then diluted with methanol/water (50:50, v/v). HPLC-grade water was obtained from a MilliQ Academic system

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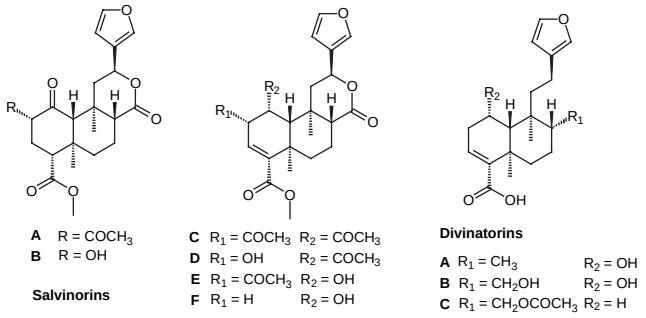

Figure 1. Structures of the six salvinorins and three divinatorins studied.

(Millipore, Vimodrone, Italy). HPLC-grade methanol was filtered through a 0.45 μm filter before use. All solvents and reagents were from Aldrich (Milan, Italy).

Salvia divinorum leaves (30 mg; Sierra Mazateca and Hawaiian varieties, obtained from D. J. Siebert[14]) were extracted with 150 mL of acetonitrile/water (50:50, v/v) and, after filtration (0.45 μm), were analyzed by LC/MS.

HPLC/UV-MS conditions

A ThermoFinnigan Surveyor instrument (Thermo Electron, Rodano, Italy), equipped with autosampler and PDA-UV 6000 LP detector, was used. Mass spectrometric analyses were performed using a ThermoFinnigan LCQ Deca XP plus ion trap mass spectrometer, with ESI interface.

The chromatographic separations were run on a Phenomenex Luna C-18 (2) column (150 × 2 mm, 3 μm particle size; Chemtek, Anzola Emilia, Italy). The injection volume was 10 μL and the flow rate 200 μL min^{-1}. A gradient mobile phase composition was used: 80/20 to 0/100 v/v water with 0.05% of formic acid/acetonitrile in 40 min.

The LC column effluent was delivered to the UV detector (200–400 nm) and then to the ion source using nitrogen as both sheath and auxiliary gas (Claind nitrogen generator apparatus, Lenno, Italy). The source voltage was set at 4.5 kV in the positive ion mode and at 4.0 kV in the negative ion mode. The heated capillary was maintained at 300°C. The acquisition method used was previously optimized in the tuning sections (capillary, magnetic lenses and collimating octapole voltages) for the $[M+H]^+$ ion of salvinorin, in order to maximize sensitivity. The collision energy (CE) was generally chosen in order to maintain about 10% abundance of the precursor ion. The tuning parameters adopted for the ESI source were the following: source current 5.00 μA, capillary voltage 39.00 V, tube lens −20 V; for ions optics, multipole 1 offset −6.75 V, inter multipole lens voltage −16.00 V, multipole 2 offset −10.50 V. Mass spectra were collected in full-scan positive ion mode in the range *m/z* 50–700 and in the following tandem MS modes: MS^2 of (+) *m/z* 433 with 25% CE in the range *m/z* 115–440 (salvinorins A, D, E); MS^2 of (+) *m/z* 391 with 20% CE in the range *m/z* 105–400 (salvinorin B); MS^2 of (+) *m/z* 475 with 20% CE in the range *m/z* 130–480 (salvinorin C); MS^2 of (+) *m/z* 375 with 25% CE in the range *m/z* 100–385 (salvinorin F); MS^2 of (−) *m/z* 331 with 25% CE in the range *m/z* 90–340 (divinatorin A); MS^2 of (+) *m/z* 363 with 27% CE in the range *m/z* 90–370 (divinatorin B); MS^2 of (−) *m/z* 373 with 40% CE in the range *m/z* 90–380 (divinatorin C); MS^3 of (+) *m/z* 373 with 25% CE from *m/z* 433 with 20% CE in the range *m/z* 100–380 (salvinorin A); MS^3 of (+) *m/z* 415 with 30% CE from *m/z* 433 with 25% CE in the range *m/z* 105–440 (salvinorins D, E); MS^3 of (+) *m/z* 373 with 27% CE from *m/z* 391 with 20% CE in the range *m/z* 100–380 (salvinorin B); MS^3 of (+) *m/z* 415 with 30% CE from *m/z* 433 with 20% CE in the range *m/z* 110–480 (salvinorin C); MS^3 of (+) *m/z* 357 with 27% CE from *m/z* 375 with 25% CE in the range *m/z* 95–385 (salvinorin F); MS^3 of (−) *m/z* 269 with 38% CE from *m/z* 331 with 35% CE in the range *m/z* 70–340 (divinatorin A); MS^3 of (+) *m/z* 345 with 25% CE from *m/z* 363 with 27% CE in the range *m/z* 90–370 (divinatorin B); MS^3 of (−) *m/z* 329 with 40% CE from *m/z* 373 with 40% CE in the range *m/z* 70–340 (divinatorin C).

MS^4, MS^5 and MS^6 spectra were collected only by flow injection analysis, using the mass spectrometer syringe pump in the range between the ion trap cut-off and the precursor ion mass. Mass width accuracy was in all cases ±1.5 Th.

GC/MS conditions

An Agilent Technologies 6890N instrument (Agilent Technologies, Cernusco sul Naviglio, Italy), equipped with autosampler and 5793 mass spectrometric detector (EI interface, quadrupole analyzer) was used for the GC/MS studies.

The chromatographic separations were run on a Restek (Superchrom, Milano, Italy) 5Ms column (30 m × 0.25 mm). The injection volume was 1 μL (splitless injection) and the injector temperature 250°C. The temperature gradient was: 40–300°C over 15 min then held at 300°C for 12 min. Mass spectra were collected in full-scan mode in the range *m/z* 100–650.

RESULTS AND DISCUSSION

Fragmentation of salvinorins

Each compound was injected into the ESI-MS system with a syringe pump and then fragmented in the ion trap up to MS^4 using the CID (collisionally induced dissociation) method. All the salvinorins and divinatorin B were studied in the positive ion mode, while divinatorins A and C were studied in the negative ion mode.

MS^2, MS^3 and MS^4 spectra of salvinorin A are shown as examples in Fig. 2. The main product ions for all the compounds are shown in Tables 1 and 2, while hypotheses about fragmentation pathways are shown in Schemes 1–4.

As can be seen from each fragmentation pathway, the main losses are of acetic acid or methyl formate (60 Da), water, methanol, carbon monoxide and formic acid.

Salvinorin A ($[M+H]^+$, *m/z* 433) loses CH_3COOH (60 Da), giving the product ion at *m/z* 373, the most abundant ion in the MS/MS spectrum. The *m/z* 373 ion then follows different fragmentation mechanisms: it eliminates $HCOOCH_3$ (60 Da) or CH_3OH (32 Da), giving ions at *m/z* 313 and 341, or alternatively it loses one water molecule giving the ion at *m/z* 355. The elimination of a water molecule may be at first sight more difficult to explain. However, if we assume that the protonation site is very likely located on the ether oxygen of the lactone ring of the molecule, characterized by a more favourable proton affinity value in respect to the oxygen

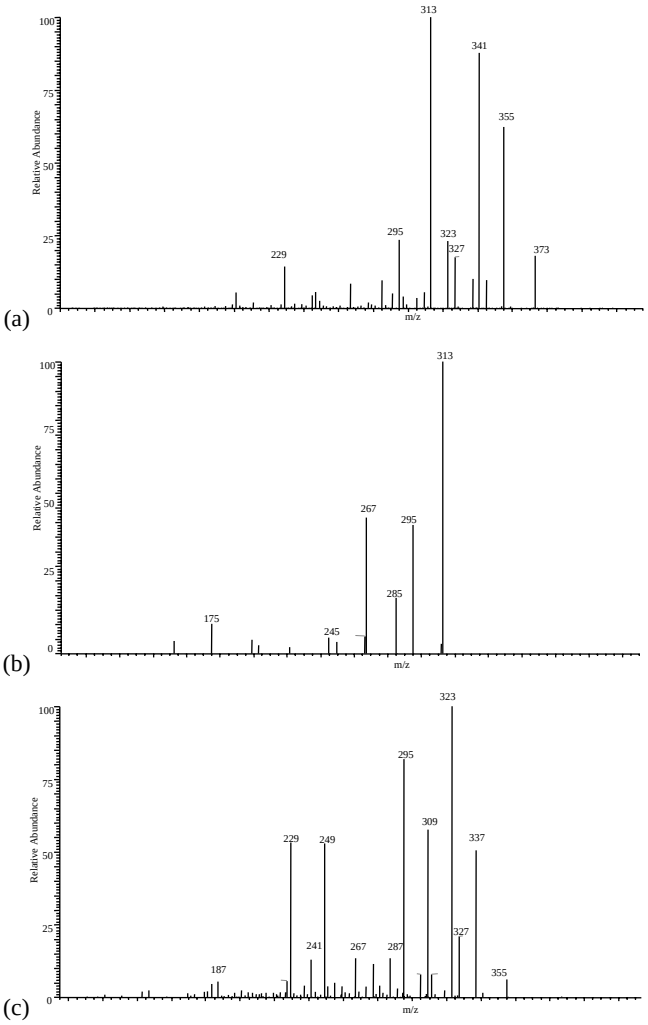

Figure 2. Examples of ESI-MS^3 and ESI-MS^4 spectra of salvinorin A: (a) MS^3 fragmentation of *m/z* 373 with 25% CE from ion *m/z* 433 with 20% CE; (b) MS^4 fragmentation of *m/z* 313 with 20% CE from *m/z* 433 (20% CE) and *m/z* 373 (25% CE); and (c) MS^4 fragmentation of *m/z* 355 with 30% CE from *m/z* 433 (20% CE) and *m/z* 373 (25% CE).

Table 1. The main product ions (*m/z* values) observed in the ESI-MS^n positive ion spectra. For MS conditions, see Experimental section

Compound	Precursor ion *m/z* $[M+H]^+$	MS^2	MS^3	MS^4
Salvinorin A	433	373	355	337, 323, 309, 295
			341	313
			313	295, 285, 267
Salvinorin B	391	373	355	337, 323, 309, 295
			341	313
			313	295, 285, 267
Salvinorin C	475	415	355	323, 337
			261	
		457	415	
Salvinorin D	433	415	—	
		373	341	
			313	267, 285, 295
Salvinorin E	433	415	397	
			295	277
Salvinorin F	375	357	325	251, 297
Divinatorin B	363	345	327	267, 295

Table 2. The main product ions (*m/z* values) observed in the ESI-MS^n negative ion spectra. For MS conditions, see Experimental section

Compound	Precursor ion *m/z* $[M-H]^-$	MS^2	MS^3
Divinatorin A	331	287	269, 207
Divinatorin C	373	329	

atoms present in the other substituent groups of the molecule,[15] a rearrangement involving the donation of an hydrogen of the ring to the charge site allows the subsequent water elimination and a concerted ring contraction (rH and rD mechanisms following the nomenclature of McLafferty[16]).

All ions obtained by MS^3 fragmentation gave further product ions: the ion at *m/z* 313 generates *m/z* 267, 285 and 295 through the losses, respectively, of HCOOH, CO and H_2O; the ion at *m/z* 355 generates *m/z* 295, 323 and 337 through the losses, respectively, of $HCOOCH_3$, CH_3OH and H_2O; and the ion *m/z* 341 generates *m/z* 313 through the loss of CO. The product ions of the elimination reactions involving hydrogen are written out using the H atom nearest to the eliminated group.

Salvinorin B ($[M+H]^+$, *m/z* 391) only loses one water molecule in MS/MS fragmentation, giving the sole ion product ion at *m/z* 373, which follows the salvinorin A pathway up to MS^4.

The $[M+H]^+$ ion of salvinorin C at *m/z* 475 gives *m/z* 457 due to elimination of H_2O, with the same mechanism as described for salvinorin A. Ions at *m/z* 415, generated by the loss of $HCOOCH_3$ or CH_3COOH (60 Da), isobaric with the one derived from *m/z* 457 by the loss of ketene, H_2CCO (42 Da), show multiple fragmentations involving elimination of the same molecules (water, methanol and CO) in different sequences up to MS^6. Each one of the ions *m/z* 415 can also lose another molecule of 60 Da, giving the ion *m/z* 355, from which elimination of water (18 Da) or methanol (32 Da) by MS^4 fragmentation can occur. If the fragmentation continues to MS^5 we see water loss from the ion that eliminated methanol, and methanol loss from the ion that eliminated water with convergence to the ion at *m/z* 305, which may lose CO (28 Da) in MS^6 to yield the product ion at *m/z* 277.

Both salvinorin D and salvinorin E, isobaric with salvinorin A, generate the ion at *m/z* 415 by losing water from the $[M+H]^+$ ion at *m/z* 433, while the loss of 60 Da is only important for salvinorin D. The *m/z* 415 ion of salvinorin E fragments with the loss of water and acetic acid/methyl formate; the *m/z* 373 ion, as in salvinorins A and B, principally loses water and methanol.

The hydroxyl group on salvinorin F (Scheme 3) allows the MS^2 loss of water from the $[M+H]^+$ ion at *m/z* 375, with generation of the ion at *m/z* 357. The ensuing fragmentation shows principally methanol elimination to *m/z* 325, while its MS^4 breakdown leads to *m/z* 297 by the loss of CO and to *m/z* 251 by the simultaneous loss of CO and HCOOH.

The divinatorins were studied in different polarity modes. Because of the low sensitivity of the positive ions generated by compounds A and C, which both possess a carboxylic acid group, they were run in negative ion mode with a good yield of the $[M-H]^-$ ion. Both $[M-H]^-$ ions fragment by

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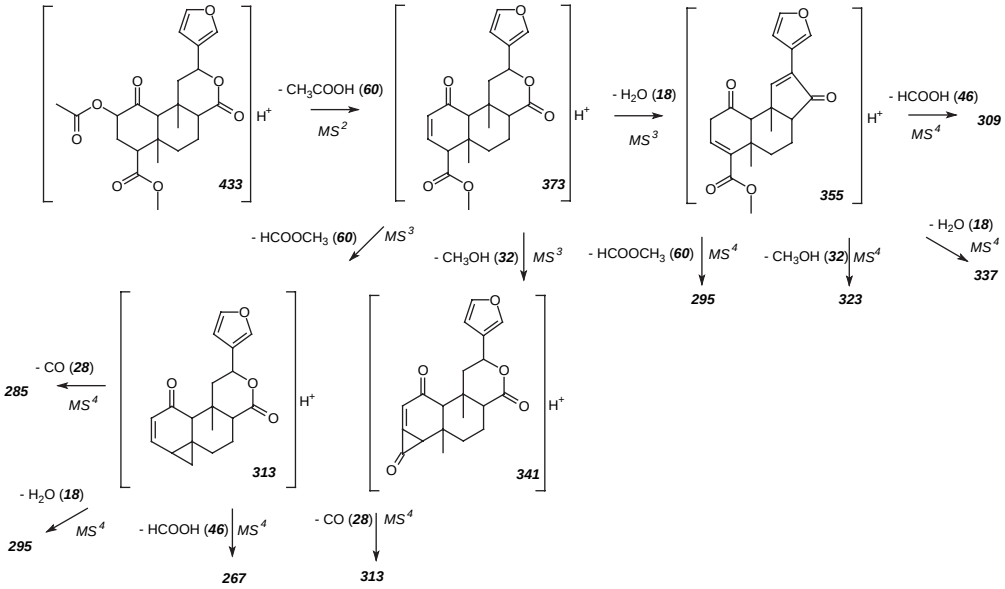
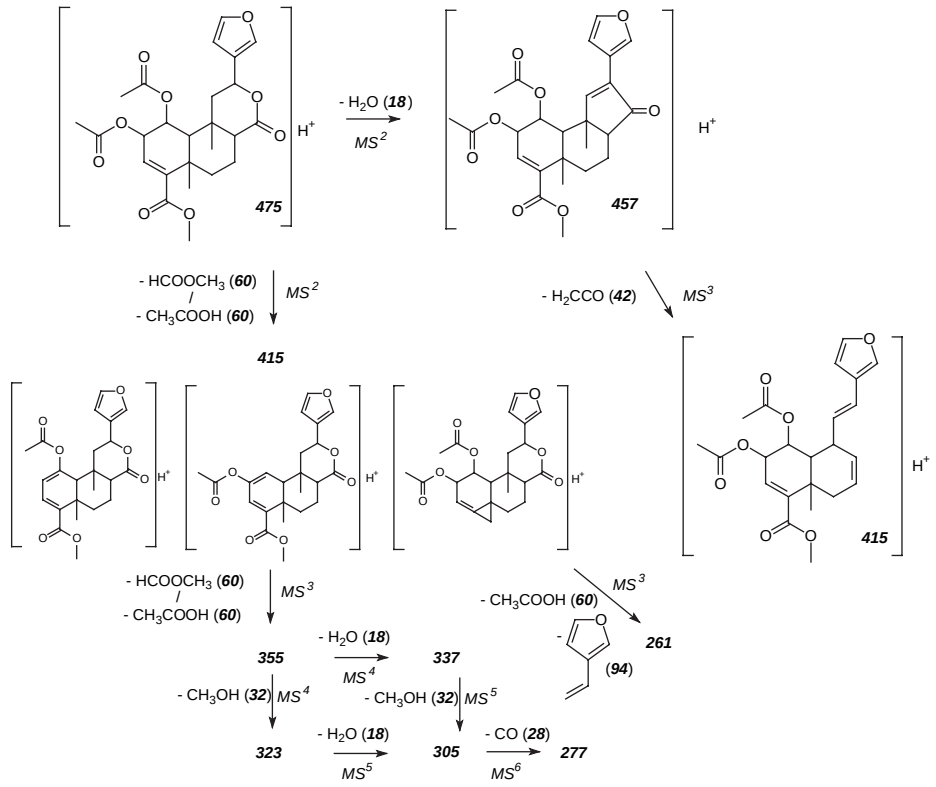
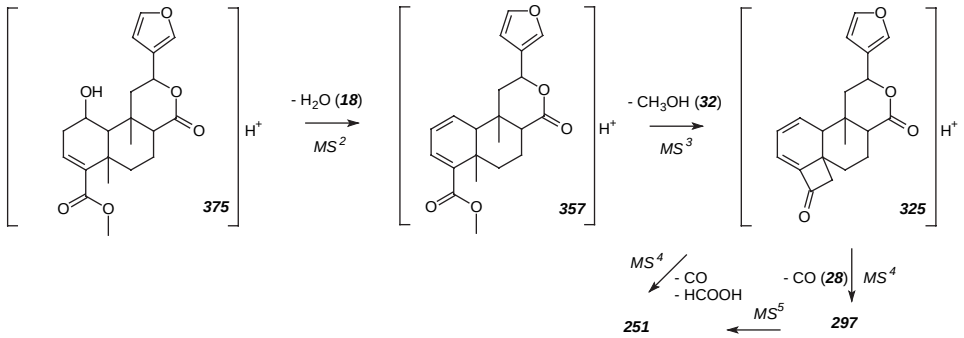


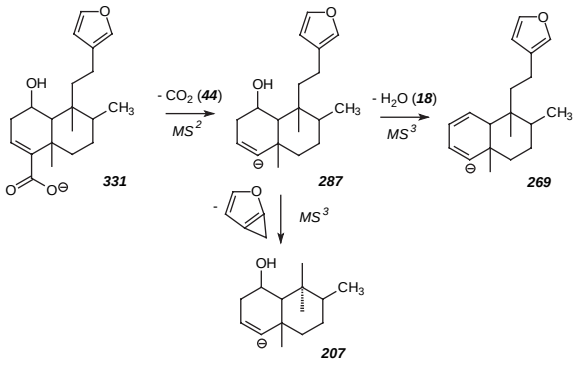
Scheme 1. Fragmentation pathways of salvinorin A.



Scheme 2. Fragmentation pathways of salvinorin C.



Scheme 3. Fragmentation pathways of salvinorin F.



Scheme 4. Fragmentation pathways of divinatorin A.

decarboxylation; in the case of divinatorin A the product ion $[M-H-44]^-$ fragments further by losing water (18 Da) and methylenefuran (80 Da).

Separation and quantitative analysis of salvinorins and divinatorins

Selected compounds were also quantified by LC/MS^n to compare analytical performances using different techniques. The chromatographic separation is quite satisfactory using the UV detector (Fig. 3); using MS detection, selective determination is possible (Fig. 3). LC/MS^2 and LC/MS^3 were compared with GC/MS and LC/UV determinations. In order to detect salvinorin A and its isomers, salvinorins D and E, in a selective way, we exploited the MS^3 capability of the ion trap to follow different fragmentation pathways: fragmentation of *m/z* 433 to *m/z* 373 for salvinorin A and of *m/z* 433 to *m/z* 415 for its isomers. In a similar way we followed all the other salvinorins by both MS^2 and MS^3 methods and divinatorins by MS^2 methods (see Table 1 and Experimental section for *m/z* values).

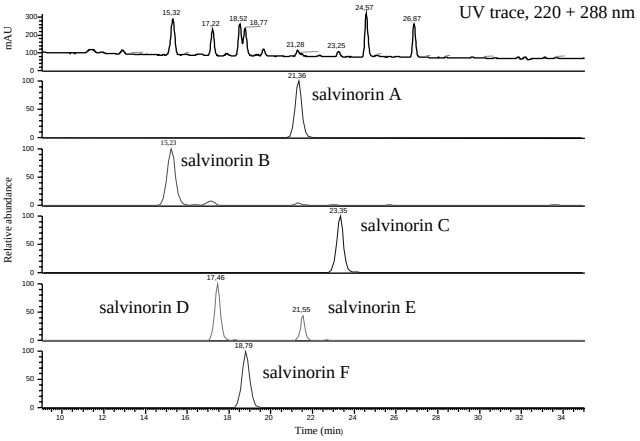


Figure 3. Chromatographic separation: trace 1: standard mixture of salvinorins and divinatorins, UV detection; trace 2: salvinorin A (MS^3 of *m/z* 373 with 25% CE from *m/z* 433 with 20% CE); trace 3: salvinorin B (MS^3 of *m/z* 373 with 27% CE from *m/z* 391 with 20% CE); trace 4: salvinorin C (MS^3 of *m/z* 415 with 30% CE from *m/z* 475 with 20% CE); trace 5: salvinorins D (first eluted) and E (MS^3 of *m/z* 415 with 30% CE from *m/z* 433 with 20% CE); trace 6: salvinorin F (MS^3 of *m/z* 357 with 27% CE from *m/z* 375 with 25% CE).

Table 3. Limits of detection (LODs) in ng mL^{-1} for selected compounds with different techniques

Compound	LC/MS^2	LC/MS^3	GC/MS	LC/UV (220 + 288 nm)
Salvinorin A	3	9	40	367
Salvinorin B	7	12	51	1144
Salvinorin C	2	8	46	221
Salvinorin D	9	17	36	128

The limits of detection (LODs) for salvinorin standard compounds are reported in Table 3 and show a marked improvement in sensitivity using LC/MS^n acquisition vs. GC/MS and LC/UV methods. The LODs were calculated as three times the standard deviation of analytical background on LC/UV, LC/MS^2, LC/MS^3 and GC/MS calibration curves, obtained in the range 10–5000 ng mL^{-1}. The selectivity shown by the LC/MS^3 method enables each compound to be easily measured in mixtures such as total plant extracts. For forensic purposes, many commercial herbal preparations containing salvinorin A could be evaluated with this method. The presence of salvinorin A alone, rather than of all the salvinorins, may be a marker of the artificial addition of the active principle to the plant. Decreased quantities of all the salvinorins indicate 'dilution' of the original plant with other inactive plant species. Using a 50:50 mixture of water and acetonitrile as extraction medium, authentic *Salvia divinorum* leaves of different geographical origins were evaluated, precisely Sierra Mazatecan and Hawaiian samples. Levels of salvinorins are reported in Table 4. These data show that salvinorin A is the most abundant compound in the Sierra Mazatecan variety. The Hawaiian variety contains more salvinorin B than A. For all other compounds the relative quantities are very similar.

CONCLUSIONS

In this study the LC/MS behavior of nine neoclerodane diterpenoids was investigated. Using electrospray ionization, the majority of compounds produce abundant $[M+H]^+$ ions in positive ion mode; two of the substances studied, bearing carboxylic acid substituents, generate abundant $[M-H]^-$ ions in the negative ion mode. All the fragmentation patterns up to MS^4 are easily explained by the elimination of small molecules. MS^n acquisition mode is thus useful for the selective analysis of each compound. The LC/MS technique

Table 4. Levels of salvinorins in *Salvia divinorum* samples of different geographical origins

Compound	Sierra Mazatecan sample (% w/w)	Hawaiian sample (% w/w)
Salvinorin A	0.76	0.78
Salvinorin B	0.42	1.04
Salvinorin C	0.59	0.62
Salvinorin D	0.05	0.05
Salvinorin E	0.02	0.03
Salvinorin F	0.16	0.15

has been demonstrated to be extremely useful for the analysis of herbal preparations.

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