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Liquid chromatography coupled to different atmospheric pressure ionization sources-quadrupole-time-of-flight mass spectrometry and post-column addition of metal salt solutions as a powerful tool for the metabolic profiling of *Fusarium oxysporum*

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ABSTRACT

Fusarium oxysporum L11 is a non-pathogenic soil-borne fungal strain that yielded an extract that showed antifungal activity against phytopathogens. In this study, reversed-phase high-performance liquid chromatography (RP-HPLC) coupled to different atmospheric pressure ionization sources-quadrupole-time-of-flight mass spectrometry (API-QTOF-MS) was applied for the comprehensive profiling of the metabolites from the extract. The employed sources were electrospray (ESI), atmospheric pressure chemical ionization (APCI) and atmospheric pressure photoionization (APPI). Post-column addition of metal solutions of Ca, Cu and Zn(II) was also tested using ESI.

A total of 137 compounds were identified or tentatively identified by matching their accurate mass signals, suggested molecular formulae and MS/MS analysis with previously reported data. Some compounds were isolated and identified by NMR. The extract was rich in cyclic peptides like cyclosporins, diketopiperazines and sansalvamides, most of which were new, and are reported here for the first time. The use of post-column addition of metals resulted in a useful strategy for the discrimination of compound classes since specific adducts were observed for the different compound families. This technique also allowed the screening for compounds with metal binding properties. Thus, the applied methodology is a useful choice for the metabolic profiling of extracts and also for the selection of metabolites with potential biological activities related to interactions with metal ions.

1. Introduction

Metabolite profiling is crucial for many aspects of fundamental research and industrial applications of filamentous fungi and other microorganisms [1]. It is used to understand the small-molecule bases of biological processes such as those associated with disease pathogenesis, interactions of microbial communities, microbial biochemistry, plant physiology, drug mode of action and metabolism [2–5].

The analytical methodologies for metabolite profiling have been extensively discussed in the literature, and LC/MS is considered to be one of the most powerful approaches to identify natural product constituents as it can provide information on chemically diverse analytes which may be labile and are present at low concentrations. By using LC–MS, known and unknown compounds that are present in a biological matrix can be detected and identified without prior knowledge of their exact chemical structure [4]. Particularly, the use of a quadrupole-time-of-flight (QTOF) mass spectrometer has proved to be very suitable for this purpose due to its high mass resolution capability, which facilitates the characterization of metabolites [2,5]. Among the different ionization techniques, ESI has been the most widely used in both positive and negative ion modes [4]. Other techniques like atmospheric pressure chemical ionization (APCI) and atmospheric pressure photoionization (APPI) have also shown their ability for the ionization of medium or low polar molecules, such as lipids [6,7], cyclic peptides

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[8] including cyclosporine A (CsA) [9], terpenes [10,11] and antioxidants [12], as representative examples.

High-performance liquid chromatography combined with post-column derivatization (HPLC–PCD) is a complementary on-line technique to provide additional structural information [13,14]. In particular, post-column addition of metal chlorides was found to be a versatile technique to form metal complexes of carbohydrates for subsequent on-line MS and MS/MS analysis [15]. This methodology was recently employed for the successful differentiation of cyclosporine A and isocyclosporine A, based on the ability of CsA to form stable adducts in solution with Ca(II), Cu(II) and Zn(II) [16].

Fusarium oxysporum is one of the best represented species among the communities of soil-borne fungi. Although some strains of *F. oxysporum* are pathogenic, many others are non-pathogenic and produce antifungal metabolites that may be used for the control of phytopathogens [17]. In a previous work, the extract of a nonpathogenic strain of *F. oxysporum* (S6) isolated from suppressive soils with antagonist activity against *S. sclerotiorum* was studied, and CsA was isolated and identified. A correlation between metabolite concentration and antifungal activity was shown, and the concentration of CsA was found to be higher in the mycelium [18].

The main objective of this study was to evaluate the mycelium metabolites of *F. oxysporum* strain L11, taking into account the probable presence of CsA, by HPLC–QTOF-MS and -MS/MS. In addition to the use of different ion sources (ESI, APCI, APPI) and different ionization modes, ESI with post-column addition of a metal(II) solution was also applied. The use of different sources and ion modes is useful not only for the identification of known or suspected secondary metabolites, but also for the structure elucidation of new compounds, based on the suggested molecular formula which is obtained with high accuracy, the structural information provided by MS/MS fragmentation, and the comparison with literature data. Nevertheless, this approach is dependent on the ionization behavior of the different metabolites present in the extracts, which have diverse structures and so, its applicability may not be general. In this work, the abovementioned methodology is applied to an extract containing cyclic depsipeptides and cyclosporins (Css) and the post-column addition of metal ions is used as a screening technique for the detection of compounds with metal binding properties. This technique also allowed the discrimination of Css and isoCss in a complex mixture.

2. Experimental

2.1. Chemicals

LC/MS-grade methanol and water were purchased from Carlo Erba (Milan, Italy) and Tedia (OH, USA) respectively. Formic acid (p.a., ACS) was purchased from Merck (Merck KGaA, Darmstadt, Germany).

2.2. Fungal material and extraction of fungal metabolites

2.2.1. Fungal strain

The fungus *F. oxysporum* Schlecht strain L11 was isolated from a lettuce crop soil sample collected at Las Heras, Province of Buenos Aires, Argentina and classified by Dra M. A. Rodriguez. The strain was deposited in the BAFC Culture Collection (FCEN-UBA, CONICET) under the accession number BAFC 1647.

2.2.2. Fermentation and metabolite extraction

A well grown agar slant of *F. oxysporum* was used to inoculate 250 ml Erlenmeyer flasks containing 75 ml of malt extract medium containing malt extract 30 g and peptone 5 g per liter. After a week, one Erlenmeyer media was employed to inoculate 1 liter of the above media in 3 l Erlenmeyer flasks. The fermentation was carried out at 25 °C for 21 d under static conditions.

The fermentation broths were filtered and the mycelium was extracted with ethanol (2× 500 ml) and ethyl acetate (2× 500 ml). The organic extracts were combined. An aliquot of 1 ml of the final extract was used for the analysis without further purification steps. The remaining extract was evaporated under reduced pressure and was used for isolation purposes (supplementary data).

2.3. Analysis by RP-HPLC-ESI/APCI/APPI-QTOF-MS and -MS/MS

The mycelium extract sample was analyzed on an Agilent 1200 liquid chromatograph (Agilent Technologies, Wilmington, DE, USA) coupled to a Bruker micrOTOF-Q II mass spectrometer (Bruker Daltonics, Billerica, MA, USA) equipped with ESI, APCI and APPI sources. ESI: the instrument was operated in positive/negative ion mode at a capillary voltage of ±4.5 kV, an end plate offset of ±500 V, and a dry temperature of 180 °C. Multi-point mass calibration from *m*/*z* 100–2000 was carried out by using a sodium formate solution. APCI and APPI: the instrument was operated in positive/negative ion mode at a capillary voltage of ±4.0 kV, an end plate offset of ±500 V, and a dry temperature of 200 °C. Multi-point mass calibration from *m*/*z* 100–1500 was carried out by using an APCI/APPI tuning mix solution (Agilent Technologies, USA). N_2 as dry gas was set at 6.0 L/min and as nebulizer gas at a pressure of 3.0 bar respectively in all cases. For APCI, corona was set at 4000 nA and the APCI heater at 250 °C. Data acquisition and processing were carried out with Bruker Compass Data Analysis ver. 4.0 software supplied with the instrument. Compounds in Table 1 were obtained by dissection using the above software.

Different experiments were repeated in each case (each source in both positive and negative ion mode) with two different sets of voltages for ion funnels and collision cell (set 1: Funnels 200 Vpp, Hexapole 150 Vpp, Collision cell RF 250 Vpp; set 2: Funnel 1 300 Vpp, Funnel 2 400 Vpp, Hexapole 300 Vpp, Collision cell RF 800 Vpp) in order to enhance the sensitivity of low mass or high mass ions respectively. The second set was employed particularly for the analysis of MS2 spectra of cyclosporins. Collision energies were set from 15 to 45 eV varying proportionally to the mass.

The sample was chromatographed on a Luna C18 column (3 μm, 2.0 mm × 100 mm; Phenomenex, Torrance, CA, USA). The mobile phase consisted of water containing 0.1% formic acid (A) and methanol (B). The flow rate was 0.3 mL/min and the column temperature was set at 30 °C. Linear gradient elution was performed as follows: 55% B (0–2 min), 55–100% B (2–30 min), 100% B (30–40 min).

2.4. Post-column addition of metal ion solutions

Aqueous metal ion stock solutions (10 mM) of $CaCl_2$, $CuSO_4$ and $ZnCl_2$ were prepared. The metal ion solutions were introduced by means of a syringe pump at a flow rate of 3 μL/min, via a T-junction before entrance to the ESI ion source.

3. Results and discussion

3.1. Chromatographic separation and MS analysis

As a first step, HPLC conditions were evaluated in order to obtain well defined chromatographic peaks spread all over the run time. For this purpose, a variety of stationary phases were assessed, such as C18 (Luna and Kinetex, Phenomenex), PFP (Luna, Phenomenex), HILIC (Kinetex, Phenomenex) and C8 (Pinnacle II Restek), as well as different mobile phases (CH_3CN vs. MeOH) and gradients. The best conditions resulted from the use of Luna C18 as stationary phase and 0.1% formic acid and methanol as mobile phase. The base

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Table 1
Chemical composition of *Fusarium oxysporum* mycelium extract.

N°	R_T (min)	Observed m/z[a] ($[M+H]^+/[M-H]^-$)	Calculated m/z[a] ($[M+H]^+/[M-H]^-$)	Molecular formula[a] $[M+H]^+$	Mass accuracy (ppm)	Proposed compound	Class
1	1.3	261.1244	261.1234	$C_{14}H_{16}N_2O_3$	−3.9	*Cyc* (Tyr-Pro)	diketopiperazine
2	1.6	227.1382	227.1390	$C_{11}H_{18}N_2O_3$	3.5	*Cyc* (HPro-Leu/Ile)	diketopiperazine
3	1.6	185.1287	185.1284	$C_9H_{16}N_2O_2$	-1.4	*Cyc* (Ala-Leu/Ile)	diketopiperazine
4	1.7	219.1141	219.1128	$C_{12}H_{14}N_2O_2$	5.9	*Cyc* (Ala-Phe)	diketopiperazine
5	1.7	261.1232	261.1234	$C_{14}H_{16}N_2O_3$	0.7	*Cyc* (Phe-HPro)	diketopiperazine
6	1.8	199.1450	199.1441	$C_{10}H_{18}N_2O_2$	−4.4	*Cyc* (Val-Val)	diketopiperazine
7	2.0	211.1451	211.1441	$C_{11}H_{18}N_2O_2$	4.7	*Cyc* (Pro-Leu/Ile)	diketopiperazine
8	2.5	164.1071	164.1070	$C_{10}H_{13}NO$	0.8	phenethyl acetamide[d]	aromatic compd
9	2.7	213.1607	213.1598	$C_{11}H_{20}N_2O_2$	−4.6	*Cyc* (Val-Leu/Ileu)	diketopiperazine
10	2.7	300.1700	300.1707	$C_{17}H_{21}N_3O_2$	2.3	*Cyc* (Trp-Leu/Ile)	diketopiperazine
11	2.9	247.1444	247.1441	$C_{14}H_{18}N_2O_2$	1.2	*Cyc* (Val-Phe)	diketopiperazine
12	4.0	261.1600	261.1598	$C_{15}H_{20}N_2O_2$	0.8	*Cyc* (Phe-Leu/Ileu)	diketopiperazine
13	4.4	227.1745	227.1754	$C_{12}H_{22}N_2O_2$	4.0	*Cyc* (Leu/Ile-Leu/Ile)	diketopiperazine
14	4.9	295.1432	295.1441	$C_{18}H_{18}N_2O_2$	3.2	*Cyc* (Phe-Phe)	diketopiperazine
15	6.3	192.1378	192.1343	$C_{12}H_{17}NO$	2.6	dimethyl-phenethyl acetamide	aromatic compd
16	6.4	291.0852	291.0863	$C_{15}H_{14}O_6$	3.8	javanicin[c,d]	naphtoquinone
17	6.6	289.0703	289.0707	$C_{15}H_{12}O_6$	1.3	anhydrofusarubin or isomer	naphtoquinone
18	6.8	197.1174	197.1172	$C_{11}H_{16}O_3$	−0.8	ni	
19	7.1	163.1124	163.1117	$C_{11}H_{14}O$	−3.9	unsaturated fatty aldehyde	FA
20	7.6	172.1689	172.1696	$C_{10}H_{21}NO$	4.3	pentyl amide	aliphatic amide
21	7.7	206.1542	206.1539	$C_{13}H_{19}NO$	1.5	nd	phenethyl amide
22	8.1	158.1534	158.1539	$C_9H_{19}NO$	3.2	pentyl amide	aliphatic amide
23	8.1	218.1745	218.1751	$C_{11}H_{23}NO_3$	2.5	ni	
24	8.2	211.0855	211.0866	$C_{13}H_{10}N_2O$	5.2	ni	
25	10.1	352.2124	352.2119	$C_{19}H_{29}NO_5$	−1.5	ni	
26	10.7	309.1693	309.1697	$C_{17}H_{24}O_5$	1.1	neovasinin[c,d]	pyrone
27	11.5	343.2129	343.2115	$C_{18}H_{30}O_6$	−4.1	o-F244 [c,d]	polyketides
28	11.8	352.2126	352.2119	$C_{19}H_{29}NO_5$	−2.1	ni	
29	12.4[b]	327.2167	327.2177	$C_{18}H_{32}O_5$	2.9	TriHODE	FA
30	12.5	331.2485	331.2479	$C_{18}H_{34}O_5$	1.9	9, 10, 13 or 9, 12, 13 TriHOME	FA
31	12.7	223.1338	223.1329	$C_{13}H_{18}O_3$	−4.2	ni	
32	13.2	357.2269	357.2272	$C_{19}H_{32}O_6$	0.6	methylated o-F244	polyketides
33	13.5[a]	373.2585	373.2596	$C_{20}H_{38}O_6$	3.0	ni	FA
34[e]	13.9	331.2472	331.2479	$C_{18}H_{35}O_5$	2.2	TriHOME	FA
35	14.5[b]	311.2223	311.2228	$C_{18}H_{32}O_4$	1.7	DiHODE	FA
36	14.9	325.2009	325.2010	$C_{18}H_{28}O_5$	0.2	F244 or isomer	polyketides
37	15.0	371.2421	371.2428	$C_{20}H_{34}O_6$	2.0	dimethylated o-F244 deriv.	polyketides
38	15.1	309.1688	309.1697	$C_{17}H_{24}O_5$	2.7	ni	
39	15.2[b]	383.2089	383.2075	$C_{20}H_{32}O_7$	−3.5	acetylated o-F244	polyketides
40	15.4	293.2119	293.2111	$C_{18}H_{28}O_3$	2.7	nd	FA
41	15.5[b]	311.2243[x]	311.2228	$C_{18}H_{32}O_4$	−5.0	DiHODE	FA
42	15.6	291.1946	291.1955	$C_{18}H_{26}O_3$	2.8	nd	FA
43	16.2[b]	355.2120	355.2126	$C_{19}H_{32}O_6$	1.7	ni	
44	16.3	295.2254	295.2268	$C_{18}H_{30}O_3$	4.5	HOTE	FA
45	16.6	635.3467	635.3439	$C_{35}H_{46}N_4O_7$	−4.4	nd	cyclic peptide
46	16.8	506.3240	506.3224	$C_{27}H_{43}N_3O_6$	0.3	Table 3	peptide
47	16.9	1188.8290	1188.8330	$C_{61}H_{109}N_{11}O_{12}$	3.4	[Leu^4]CsA= isoCsb (Table 1S)	cyclic peptide
48	17.0	1188.8298	1188.8330	$C_{61}H_{109}N_{11}O_{12}$	2.6	[Leu^{10}]CsA = isoCsT (Table 1S)	cyclic peptide
49	17.1	444.3452	444.3472	$C_{28}H_{46}NO_3$	4.5	ni	
50	17.4[b]	368.2611	368.2595	$C_{24}H_{35}NO_2$	−4.4	ni	
51	17.6[b]	369.2274	369.2272	$C_{20}H_{34}O_6$	−0.6	dimethylated o-F244 deriv.	polyketides
52	17.7	1218.8378	1218.8436	$C_{62}H_{111}N_{11}O_{13}$	4.7	[Thr^2, Leu^5, Leu^{10}]CsA = isoCsh (Table 1S)	cyclic peptide
53	18.1	400.3136	400.3170	$C_{21}H_{41}N_3O_4$	2.6	ni	
54	18.2[b]	325.2385	325.2384	$C_{19}H_{34}O_4$	−0.2	DiHODE-OMe	FA
55	18.5	1202.8510	1202.8486	$C_{62}H_{111}N_{11}O_{12}$	0.8	IsoCsA (Table 1S)	cyclic peptide
56	18.5	1188.8287	1188.8330	$C_{61}H_{109}N_{11}O_{12}$	3.6	[Leu^6]CsA= isoCsU (Table 1S)	cyclic peptide
57	18.7	1188.8273	1188.8330	$C_{61}H_{109}N_{11}O_{12}$	4.8	[Ala^2]CsA = isoCsB (Table 1S)	cyclic peptide
58	18.9	1188.8297	1188.8330	$C_{61}H_{109}N_{11}O_{12}$	2.8	[Val^{11}]CsA = isoCsE (Table 1S)	cyclic peptide
59	19.1	605.3880	605.3909	$C_{32}H_{52}N_4O_7$	4.8	Table 3	peptide
60	19.2	309.2422	309.2424	$C_{19}H_{32}O_3$	0.6	HOTE-OMe	FA
61	19.3	361.2362[f]	361.2349	$C_{20}H_{34}NaO_4$	−3.5	nd	FA

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Table 1 (*Continued*)

N°	R_T (min)	Observed m/z[a] ($[M+H]^+/[M-H]^-$)	Calculated m/z[a] ($[M+H]^+/[M-H]^-$)	Molecular formula[a] $[M+H]^+$	Mass accuracy (ppm)	Proposed compound	Class
62	19.5	384.2514	384.2544	$C_{24}H_{33}NO_3$	4.9	ni	
63	19.6	1216.8584	1216.8643	$C_{63}H_{113}N_{11}O_{12}$	4.9	[Leu[5]]CsA = isoCsd (Table 1S)	cyclic peptide
64	19.7	1216.8576	1216.8643	$C_{63}H_{113}N_{11}O_{12}$	5.5	[X[2]]CsA = isoCs(D, G,M) (Table 1S)	cyclic peptide
65	19.8	323.2564	323.2581	$C_{20}H_{34}O_3$	5.3	HOTE-OEt	FA
66	20.1	321. 2417	321.2424	$C_{20}H_{32}O_3$	2.2	HODE-OEt	FA
67	20.3	619.4092	619.4065	$C_{33}H_{54}N_4O_7$	4.4	Table 3	peptide
68	20.4	337.2361	337.2373	$C_{20}H_{32}O_4$	3.7	DiHODE-OEt	FA
69	20.6	361.2220	361.2221	$C_{18}H_{32}O_7$	0.2	nd	glyceride
70	20.7[b]	295.2278	295.2278	$C_{18}H_{32}O_3$	0.1	HODE	FA
71	20.8	323.2576	323.2581	$C_{20}H_{34}O_3$	1.5	HOTE-OEt	FA
72	21.0	617.3917	617.3909	$C_{33}H_{52}N_4O_7$	−1.3	MeSan-E (Table 2)	cyclic peptide
73	21.1	401.3030	401.3010	$C_{21}H_{40}N_2O_5$	5.0	ni	
74	21.4	633.4252	633.4222	$C_{34}H_{56}N_4O_7$	−4.8	Table 3	peptide
75	21.8	573.3617	573.3647	$C_{31}H_{48}N_4O_6$	5.2	MeSan-D (Table 2)	cyclic peptide
76	21.9	1234.8331	1234.8385	$C_{62}H_{111}N_{11}O_{14}$	4.3	Cs A Hydroxiperoxide (Table 2S)	cyclic peptide
77	22.4	587.3818	587.3803	$C_{32}H_{50}N_4O_6$	2.6	MeSan-B (Table 2)	cyclic peptide
78	22.6	647.4351	647.4378	$C_{35}H_{58}N_4O_7$	4.2	Table 3	peptide
79	22.8	640.4039	640.4069	$C_{35}H_{53}N_5O_6$	4.6	MeSan-C (Table 2)	cyclic peptide
80	23.0	573.3664	573.3647	$C_{31}H_{48}N_4O_6$	3.0	San-B (Table 2)	cyclic peptide
81	23.2	1218.8367	1218.8436	$C_{62}H_{111}N_{11}O_{13}$	5.7	[Thr[2], Leu[5], Leu[10]]CsA = Csh (Table 2S)	cyclic peptide
82	23.5	601.3975	601.3960	$C_{33}H_{52}N_4O_6$	−2.5	MeSan-A (Table 2)[c,d]	cyclic peptide
83	24.2	587.3816	587.3803	$C_{32}H_{50}N_4O_6$	2.2	San-A (Table 2)	cyclic peptide
84	24.4	1188.8297	1188.8330	$C_{61}H_{109}N_{11}O_{12}$	2.8	[Leu[4]]CsA = Csb (Table 2S)	cyclic peptide
85	24.5	1188.8329	1188.8330	$C_{61}H_{109}N_{11}O_{12}$	0.1	[Leu[10]]CsA = CsT (Table 2S)	cyclic peptide
86	24.7	615.4143	615.4116	$C_{34}H_{54}N_4O_6$	4.4	MeSan-F (Table 2)	cyclic peptide
87	24.8	1174.8138	1174. 8173	$C_{60}H_{108}N_{11}O_{12}$	3.0	[Val[4]]Cs A =CsQ (Table 2S)	cyclic peptide
88	24.9	1174.8129	1174. 8173	$C_{60}H_{107}N_{11}O_{12}$	3.8	[Leu[6], Leu[10]]Cs A =CsR (Table 2S)	cyclic peptide
89	25.3	1218.8396	1218.8436	$C_{62}H_{111}N_{11}O_{13}$	3.2	[Thr[2]]CsA = CsC[c,d] (Table 2S)	cyclic peptide
90	25.6	1202.8493	1202,8486	$C_{62}H_{111}N_{11}O_{12}$	0.6	CsA (Table 2S)[c,d]	cyclic peptide
91	25.7	1188.8305	1188.8330	$C_{61}H_{109}N_{11}O_{12}$	2.3	[Gly[3]]CsA (Table 2S)	cyclic peptide
92	26.0	1188.8306	1188.8330	$C_{61}H_{109}N_{11}O_{12}$	2.0	[Ala[2]]CsA = CsB [c,d] (Table 2S)	cyclic peptide
93	26.2	1188.8379	1188.8330	$C_{61}H_{109}N_{11}O_{12}$	4.2	[Leu[6]]CsA = CsU (Table 2S)	cyclic peptide
94	26.4	443.3143	443.3156	$C_{28}H_{42}O_4$	2.9	nd	ergosteroid
95	26.5	427.3190	427.3207	$C_{28}H_{42}O_3$	3.9	nd	ergosteroid
96	26.6	1216.8607	1216.8643	$C_{63}H_{113}N_{11}O_{12}$	3.0	[Leu[5]]CsA = Csd (Table 2S)	cyclic peptide
97	26.8	459.3090	459.3105	$C_{28}H_{42}O_5$	3.3	nd	ergosteroid
98	26.9	256.2640	256.2635	$C_{16}H_{33}NO$	−2.2	C16:0 amide	FA
99	27.4	1146.8165	1146.8224	$C_{59}H_{107}N_{11}O_{11}$	5.1	[MeLeu[1]] Cs A = Csa (Table 2S)	cyclic peptide
101	27.6	1188.8283	1188.8330	$C_{61}H_{109}N_{11}O_{12}$	4.0	[Val[11]]CsA= CsE (Table 2S)	cyclic peptide
100	27.5	1188.8297	1188.8330	$C_{61}H_{109}N_{11}O_{12}$	2.8	[Gly[7]]CsA (Table 2S)	cyclic peptide
102	27.6	279.2326	279.2319	$C_{18}H_{30}O_2$	−2.6	C18:3	FA
103	27.6	282.2799	282.2791	$C_{18}H_{35}NO$	−2.8	C18:1 amide	FA
104	27.7[b]	253.2169	255.2330	$C_{16}H_{32}O_2$	1.4	C16:0 [d]	FA
105	27.7	347.2582	347.2581	$C_{22}H_{35}O_3$	−0.3	nd	C21-steroid
106	27.8[b]	279.2323	281.2486	$C_{18}H_{34}O_2$	2.5	C18:1 [d]	FA
107	27.8	393.3131	393.3152	$C_{28}H_{40}O$	5.3	nd	ergosteroid
108	27.9	279.2325	279.2319	$C_{18}H_{30}O_2$	−2.1	C18:3	FA
109	28.0	1216.8642	1216.8643	$C_{63}H_{113}N_{11}O_{12}$	0.1	[X[2]]CsA = Cs(D, G, M) (Table 2S)	cyclic peptide
110	28.2	425.3044	425.3050	$C_{28}H_{40}O_3$	1.4	nd	ergosteroid
111	28.4	395.3300	395.3308	$C_{28}H_{42}O$	2.1	DHE	ergosteroid
112	28.5	1186.8537	1186.8533	$C_{62}H_{111}N_{11}O_{11}$	0.4	[DesoxiMeBmt[1]]Cs A = CsF (Table 2S)	cyclic peptide
113	28.7	429.3362	429.3363	$C_{28}H_{44}O_3$	0.4	nd	ergosteroid
114	28.8	439.3186	439.3207	$C_{29}H_{42}O_3$	4.8	nd	ergosteroid
115	28.9	393.3170	393.3152	$C_{28}H_{40}O$	4.6	nd	ergosteroid
116	29.0	427.3196	427.3207	$C_{28}H_{42}O_3$	2.4	nd	ergosteroid
117	29.2	411.3255	411.3258	$C_{28}H_{42}O_2$	0.7	nd	ergosteroid
118	29.3	284.2948	284.2948	$C_{18}H_{37}NO$	−0.1	C18:0 amide	FA
119	29.6	409.3082	409.3101	$C_{28}H_{40}O_2$	4.6	nd	ergosteroid
120	30.2	395.3306	395.3308	$C_{28}H_{42}O$	0.6	DHE	ergosteroid

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Table 1 (*Continued*)

N°	R_T (min)	Observed m/z[a] ($[M+H]^+/[M-H]^-$)	Calculated m/z[a] ($[M+H]^+/[M-H]^-$)	Molecular formula[a] $[M+H]^+$	Mass accuracy (ppm)	Proposed compound	Class
121	30.5	307.2633	307.2632	$C_{20}H_{34}O_2$	−0.4	C18:4 OEt/C20:4	FA
122	31.1	395.3304	395.3308	$C_{28}H_{42}O$	1.2	nd	ergosteroid
123	31.4	393.3155	393.3152	$C_{28}H_{40}O$	0.7	nd	ergosteroid
124	32.0	395.3293	395.3308	$C_{28}H_{42}O$	3.9	nd	ergosteroid
125	32.5	615.4965	615.4983	$C_{39}H_{66}O_5$	2.9	DG (18:3,18:2)	DG
126	32.7	393.3154	393.3152	$C_{28}H_{40}O$	−0.6	ergone	ergosteroid
127	33.0	591.4985	591.4983	$C_{37}H_{66}O_5$	−0.3	DG (18:3/16:0)	DG
128	33.2	617.5125	617.5140	$C_{39}H_{68}O_5$	2.4	DG (18:3/18:1)	DG
129	33.5	397.3460	397.3465	$C_{28}H_{44}O$	1.3	ergosterol[d]	ergosteroid
130	33.9	754.5849	754.5828	$C_{43}H_{79}NO_9$	−2.8	cerebroside C	cerebroside
131	34.0	619.5279	619.5296	$C_{39}H_{70}O_5$	2.8	DG (18:3/18:0)	DG
132	35.2	540.5329	540.5350	$C_{34}H_{69}NO_3$	3.9	cer(d18:0/16:0)	ceramide
133	36.1	617.5136	617.5139	$C_{39}H_{68}O_5$	0.5	DG (18:2/18:2)	DG
134	37.4	619.5305	619.5296	$C_{39}H_{70}O_5$	−1.4	DG (18:1/18:2)	DG
135	37.8	645.5443	645.5453	$C_{41}H_{72}O_5$	1.4	DG (20:2/18:2)	DG
136	38.5	595.5302	595.5296	$C_{37}H_{70}O_5$	1.0	DG (16:0/18:1)	DG
137	39.1	621.5437	621.5452	$C_{39}H_{72}O_5$	2.5	DG (18:1/18:1)	DG

N°	ESI (+) ($[M+H]^+/[M+Na]^+$)	ESI (−) ($[M-H]^-$)	APCI (+) ($[M+H]^+$)	APCI (−) ($[M-H]^-$)	APPI (+) ($[M+H]^+$)	APPI (−) ($[M-H]^-$)	Ca^{2+} ($[M+(Cl/HCOO/-H)+Ca]^+$)	Ca^{2+} Dicharged adducts
1	–	–	+	–	+	–	–	–
2	+/–	–	+	–	+	–	–	–
3	+/–	–	+	–	+	–	–	–
4	–	–	+	–	+	–	–	–
5	–	–	+	–	+	–	–	–
6	–	+/–	+	–	+	–	–	–
7	+	–	+	–	+	–	–	–
8	+	–	+	–	+	–	–	–
9	+/–	–	+	–	+	–	–	–
10	+/–	–	+	–	+	–	–	–
11	+	–	+	–	+	–	–	–
12	+	–	+	–	+	–	–	–
13	+	–	+	–	+	–	–	–
14	+/–	–	+	–	+	–	–	–
15	+	–	+	–	+	–	–	–
16	+	–	+	–	+	–	–	–
17	+	–	+	–	+	–	–	–
18	+	–	+	+/–	+	–	–	–
19	+/–	–	+	–	+	–	–	–
20	+	–	+	–	+	–	–	–
21	+	–	+	–	+	–	–	–
22	–	–	+	–	+	–	–	–
23	+	–	+/–	–	–	–	–	–
24	+	–	+	–	+	–	–	–
25	+	+	+	+	+	–	–	–
26	+	+	+	+/–	+	+/–	–	–
27	+	+	+	+	+	+	+	+
28	+	+/–	+	+	+	+/–	–	–
29	–	+	–	+	–	+	–	–
30	+	+	+	+/–	+	+	+	+
31	+	+	+	+/–	+	–	–	–
32	+	+	+	+	+	+	+	+
33	+	+	–	+	–	+	–	–
34[e]	+	+	+	+/–	+	+	+	+
35	–	+	–	+	–	+	–	–
36	+	+	+	+	+	+	–	–
37	+	+	+	+	+	+	+	+
38	+	+	+	+	+	+	–	–
39	+	+	+/–	+	+/–	+	–	–
40	+	–	+	–	+	+	+	–
41	–	+	–	+	–	+	–	–
42	+	–	+	+/–	+	+/–	–	–
43	+	+	–	+	–	+	–	–
44	+/–	+	+	+	+	+/–	–	–
45	+	+/–	+	–	+	+/–	–	–
46	+	+	+	+	+	+	–	–
47	+	+/–	+	+/–	+	+/–	–	+
48	+	+/–	+	+/–	+	+/–	–	+
49	+	+/–	+	+/–	+	+/–	–	–
50	–	+	–	+	+/–	+	–	–
51	+/–	+	–	+	–	+	+	+
52	+	+	+	–	+	+	–	+
53	+	–	+	–	+/–	–	–	–
54	–	+	–	+	–	+	–	–
55	+	+	+	+	+	+	–	+

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Table 1 (*Continued*)

N°	ESI (+) ([M+H]$^+$/[M+Na]$^+$)	ESI (−) ([M−H]$^-$)	APCI (+) ([M+H]$^+$)	APCI (−) ([M−H]$^-$)	APPI (+) ([M+H]$^+$)	APPI (−) ([M−H]$^-$)	Ca^{2+} ([M+(Cl/HCOO/−H) +Ca]$^+$)	Ca^{2+} Dicharged adducts
56	+	+/−	+	−	+	+	−	+
57	+	+/−	+	−	+	−	−	+
58	+	+/−	+	−	+	−	−	+
59	+	+	+	+	+	+	−	−
60	+	+/−	+	+	+	+	−	−
61	+	−	+/−	+	−	−	−	−
62	+	−	+	−	+	−	−	−
63	+	−	+	−	+	+/−	−	+
64	+	−	+	−	+	+/−	−	+
65	+	+/−	+	+/−	+	−	−	−
66	+	−	+	−	+	−	−	−
67	+	+	+	+	+	+	+	+
68	+	−	+	−	+	−	−	−
69	+	−	+	−	+	−	+	−
70	−	+	−	+	−	+	−	−
71	+	+/−	+	+/−	+	+/−	−	−
72	+	+	+	+	+	+	+	+
73	+	−	+	−	−	−	−	−
74	+	+	+	+	+	+	+	+
75	+	+	+	+	+	+	+	+
76	+/−	+/−	+	−	+	+/−	−	−
77	+	+	+	+	+	+	+	+
78	+	+	+	+	+	+	+	+
79	+	+	+	+/−	+	+	−	−
80	+/−	+	+	+/−	+	+/−	−	−
81	+/−	+/−	+	+	+	+/−	+	+
82	+	+	+	+	+	+	+	+
83	+	+	+	+	+	+	+	+
84	+/−	+	+	+	+	+/−	+	+
85	+/−	+	+	+	+	+/−	+	+
86	+	+	+	+	+	+	−	+/−
87	+	+	+	+/−	+	+/−	+/−	+
88	+/−	+/−	+	+/−	+	+/−	+/−	+
89	+	+	+	+	+	+	+	+
90	+	+	+	+	+	+	+	+
91	+	+	+	+	+	+	+	+
92	+	+	+	+	+	+	+	+
93	+	+	+	+	+	+	+	+
94	−	−	+	+	+	−	−	−
95	−	−	+	−	+	−	−	−
96	+	+	+	+/−	+	+/−	+/−	+/−
97	−	−	+	+/−	+/−	−	−	−
98	−	+/−	+	+/−	+	−	+/−	−
99	−	−	+	−	−	−	−	+/−
101	+	+	+	+	+	+	+	+
100	+	+	+	+	+	+	+	+
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103	+	+/−	+	+/−	+	−	+/−	−
104	−	+	+/−	+	−	+/−	−	−
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126	+	−	+	−	+	−	−	−
127	+/−	−	+	−	+	−	−	−
128	+/−	−	+	−	+	−	−	−
129	+/−	−	+	−	+	−	−	−
130	+	+	+	+	+	+	+	+
131	−	−	+	−	+/−	+/−	−	−

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Table 1 (*Continued*)

N°	ESI (+) ([M+H]⁺/[M+Na]⁺)	ESI (−) ([M−H]⁻)	APCI (+) ([M+H]⁺)	APCI (−) ([M−H]⁻)	APPI (+) ([M+H]⁺)	APPI (−) ([M−H]⁻)	Ca^{2+} ([M+(Cl/HCOO/−H) +Ca]⁺)	Ca^{2+} Dicharged adducts
132	+	+	+	+	+	+	–	–
133	+	–	+	–	+/–	–	–	–
134	+/–	+/–	+	–	+	–	–	–
135	+	–	+	–	+/–	–	–	–
136	+/–	–	+	–	+/–	–	–	–
137	+/–	–	+	–	+/–	–	–	–

[a] Observed ions in APCI (positive ion mode).
[b] Observed ions in APCI (negative ion mode).
[c] Confirmed by isolation and fully NMR structural elucidation.
[d] Confirmed by comparison of retention time.
[e] Compound 34 is a mixture of two isomers with identical MS and MS2 spectra.
[f] [M+Na]⁺ in ESI (+).
ni, not identified; nd, not determined; +/–, means low relative abundance.
o-F244, open F244 (without lactonization); HODE, hydroxyoctadecadienoic acid; DiHODE, dihydroxyoctadecadienoic acid; TriHODE, trihydroxyoctadecadienoic acid; HOTE, hydroxyoctadecatrienoic acid; TriHOME, trihydroxyoctadecaenoic acid; FA, fatty acid or fatty acid derivative; DG, diglyceride; ergone, ergosta-4,6,8(14),22-tetraen-3-one.

peak chromatograms (BPC) employing different ion sources in both positive and negative ion modes are shown in Fig. 1. These chromatograms displayed a complex mixture of polar, semi-polar and non polar metabolites, and the elution order of these compounds was related to the chemical structure class as expected. Small polar compounds had short retention times while non polar lipids or high mass lipids displayed the longest retention times.

APCI in positive and negative ion modes gave account for all the detected compounds (Table 1 (and 1S in supplementary material)). APPI in positive ion mode exhibited very similar results compared to APCI even in positive ion mode albeit with slightly lower relative abundances (RA). For the screening objective both techniques exhibited the best outcomes, since they showed a higher number of components with better RA. In addition to the known capabilities of APCI and APPI to produce $[M+H]^+$ ions, which are better species for MS2 and structure elucidation than $[M+Na]^+$ adducts, these techniques allowed the observation of a larger number of ions in the case of co-eluting components when compared to ESI. This fact was especially noticeable in the case of some minor cyclic peptides which were not observed or poorly detected by ESI. BPCs in negative ion mode exhibited fewer peaks expected, while APPI in negative ion mode showed the poorest chromatogram. Mass accurate data for all the compounds and all the techniques are shown in Table 2S (supplementary material).

The BPCs using ESI with post-column addition of metal salts are shown in Fig. 2. Similar results were obtained using Ca^{2+}, Cu^{2+} or Zn^{2+}. For many compounds the RA increased and useful adducts were obtained, which helped in the structure elucidation process. Detailed comments will be described on the analysis of each type of compound present in the extract.

3.2. MS Analysis of the components

The structures of the components present in the extract were elucidated on the basis of their molecular weight/molecular formulae, the analysis of MS2 spectra and whenever possible, comparison with the retention time of a standard. In a parallel approach, the crude extract was subjected to chromatographic separation and the major components were fully analyzed and identified by 2D NMR. These compounds were chromatographed in the same conditions of the extract to obtain and compare the retention times, and are shown in Table 1.

3.2.1. Cyclic peptides

3.2.1.1. Cyclosporins. Twenty six compounds with molecular weights between *m/z* 1100 and 1300, typical for cyclosporins (Css), and with MS/MS spectra compatible with peptides, were observed in the chromatogram (Table 1). Clearly there were two groups of compounds, with retention times between 16.9 and 19.7 min for the first group, and 21.9–28.5 min for the second. It has been reported that the isomerization of Cs A to isocyclosporin A (isoCs A) can take place via a N,O-acyl migration upon acidic treatment of Cs A in aqueous solution or in organic solvents [19]. In isoCs A the carbonyl group of MeVal-11 is attached to the oxygen at C-3 of MeBmt-1 instead of the amine group of MeBmt-1. These isomers are not differentiated by MS/MS upon CID but have different retention times and show different type of adducts when LC/MS is performed using post-column addition of a metal salt solution at least for Cs A [16]. For this reason and taking into account their retention times, isoCss were assigned to the first group of compounds and Css to the second group.

The detailed MS/MS analysis [20–22] of the precursor ions from the APCI-LC chromatogram allowed the identification of 9 isoCss and 17 cyclosporins (Tables 3S and 4S, supplementary material). The identification of Cs A and isoCsA was also confirmed by comparison of their retention times with those of authentic samples. NMR data were included in the supplementary material. To the best of our knowledge, two of the compounds, [Gly^3]Cs A (R_T 25.7 min) and [Gly^7]Cs A (R_T 27.5 min) have not been previously reported.

APCI in positive ion mode was the best ionization technique, since the chromatogram showed the highest abundance of $[M+H]^+$ precursor ions. On the other hand, ESI spectra exhibited a complex mixture of $[M+Na]^+$, $[M+H]^+$ and other adduct species, while minor Css ions were absent. This fact was more evident in the case of co-eluting components. APPI showed similar results than APCI. To the best of our knowledge, there is only one previous report on the analysis of Cs A by APPI showing also similar results than APCI or ESI [9]. Most of the Css were observed in negative ion mode with lower relative abundances and for these reasons some minor Css were not detected.

By the use of post-column addition of metals(II), a remarkable difference was observed in the spectra of Css. Those Css with Bmt (2-amino-3-hydroxy-4-methyloct-6-enoic acid) as an amino acid component, like Cs A, showed single charged metal adducts $[M+(Cl/HCOO/-H)+Ca]^+$ together with doubly charged adducts (Table 1), while those without a free hydroxyl at C-3 of Bmt, like isoCs A or Cs F, only produced doubly charged adducts. As an example, the mass spectrum of Cs C (**89**), showing the adducts $[M-H+Ca]^+$, $[M+Cl+Ca]^+$ and $[M-H+HCOOH+Ca]^+$ is presented in the supplementary material (Fig. 3S). This fact is in accord with the previous results obtained with Cs A and isoCs A [16].

The presence of cyclosporins A, B and C was verified by the isolation of the compounds from the extract and NMR identification (supporting information).

Cs A is a well-known fungal metabolite with immunosuppresive and antifungal activities, that is produced by many fungal

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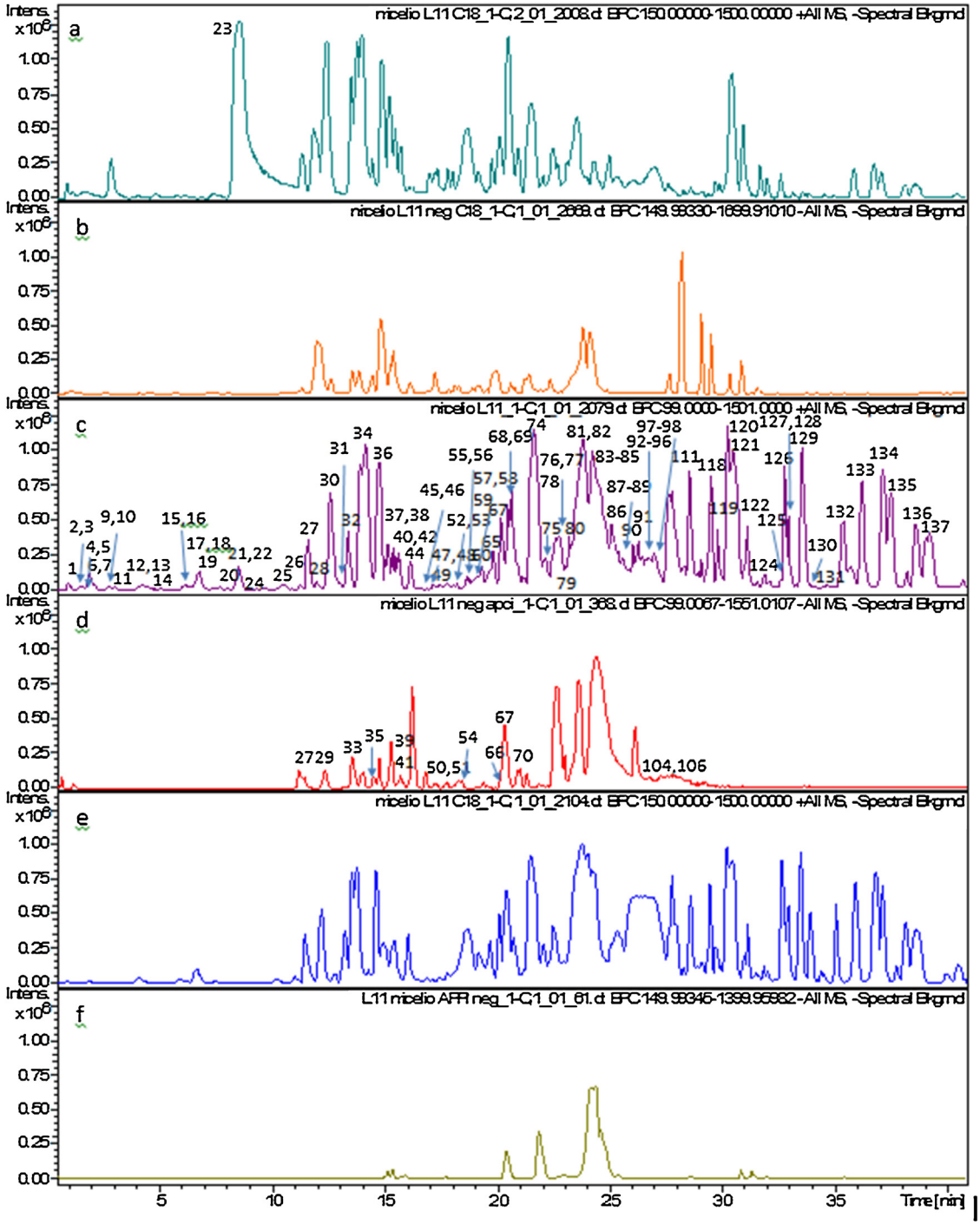

Fig. 1. Base peak chromatograms of *Fusarium oxysporum* mycelium extract employing different ion sources in both positive and negative ion modes. (a) ESI (+); (b) ESI (−); (c) APCI (+); (d) APCI (−); (e) APPI (+); (f) APPI (−).

genera and species, and is widely used during and after bone marrow and organ transplants in humans. Usually it is biosynthesized together with other minor cyclosporines with variable structures and ratios which may be overlooked or lost during the separation process.

3.2.1.2. Sansalvamides. This is another group of 14 compounds present in the extract with molecular weights near *m*/*z* 600 and with MS/MS characteristic of peptides, with retention times between 20 and 25 min (Table 1). All of the compounds showed in their MS/MS the presence of uncommon losses for amino acids. Most of them showed the loss of 114.067 u which corresponds to the molecular formulae $C_6H_{10}O_2$, indicating the presence of a hydroxy acid in the structure, and, based on this, the compounds were characterized as probable depsipeptides. Taking this fact into account, a literature search indicated a possible structural relationship with *N*-methyl Sansalvamide (MeSan A) (Fig. 3) [23], a depsipeptide isolated from a *Fusarium* strain, which contains leucic acid among its components. The MS/MS analysis of compound **82** of the extract (Table 2) showed that it was effectively MeSan A, which was confirmed by isolation and spectroscopical identification of the compound (supporting information).

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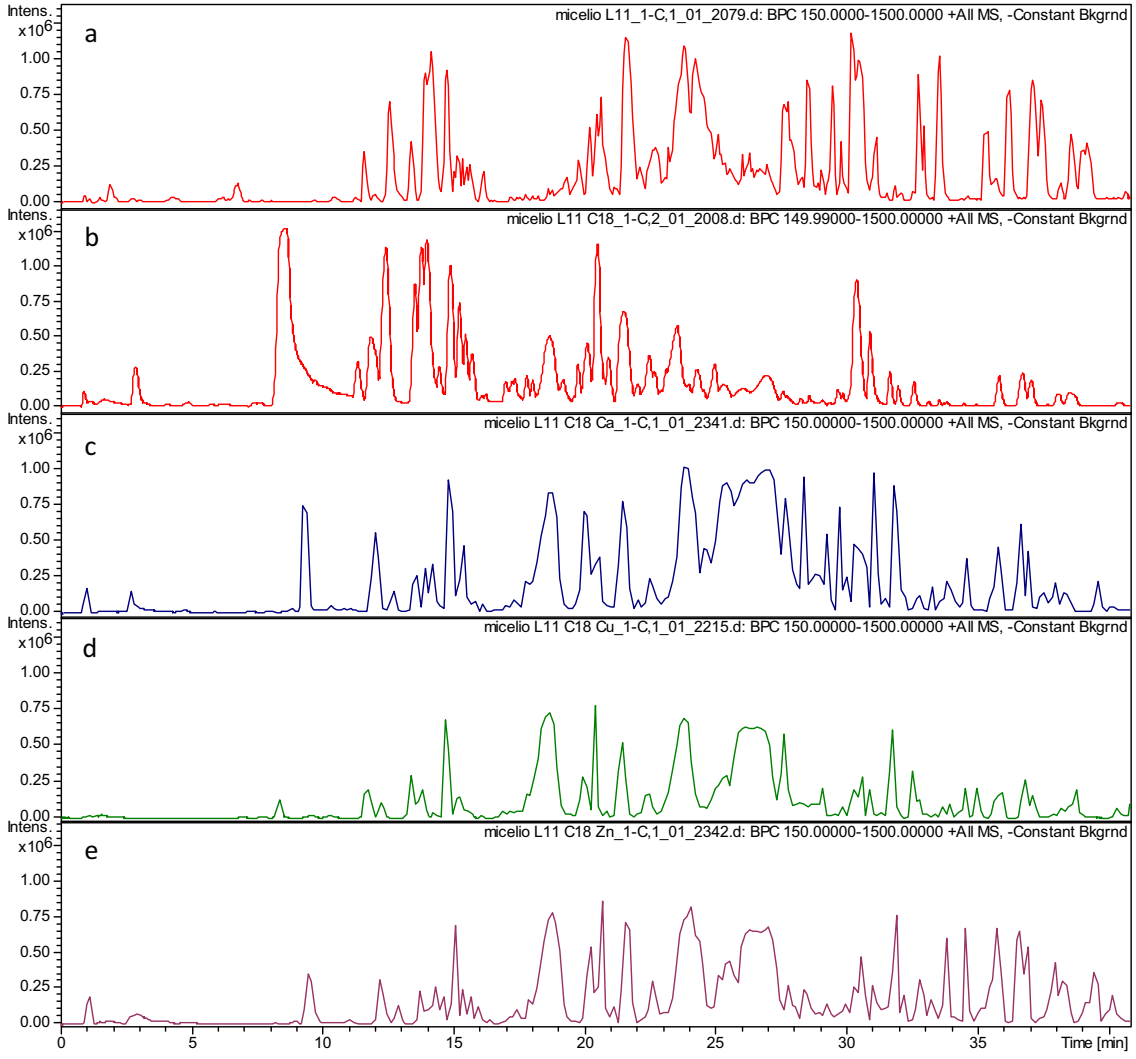

Fig. 2. Comparison of base peak chromatograms of *Fusarium oxysporum* mycelium extract employing ESI, APCI and post-column addition of metal ions. (a) APCI (+); (b) ESI (+); (c) ESI Ca^{2+}; (d) ESI Cu^{2+}; (e) ESI Zn^{2+}.

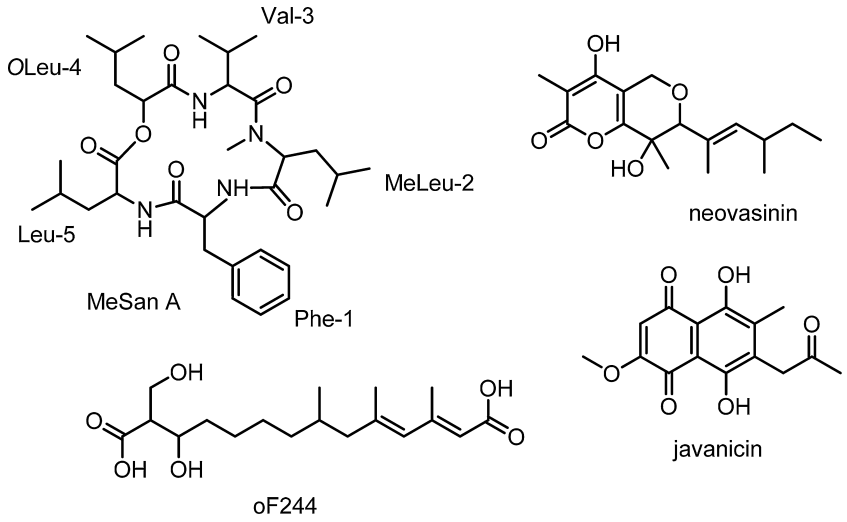

Fig. 3. Metabolites identified from *F. oxysporum*.

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Table 2
Characteristic fragment ions observed in the positive APCI MS/MS spectrum of sansalvamides **72**, **75**, **77**, **79**, **80**, **82**, **83** and **86**. Error (Δ) in ppm; RA between parentheses.

	MeSan-F (**86**)	San-A (**83**)	MeSan-A (**82**)	San-B (**80**)	MeSan-C (**79**)	MeSan-B (**77**)	MeSan-D (**75**)	MeSan-E (**72**)
	Cyc (Phe-MeLeu-Leu-OLeu-Leu)	*Cyc* (Phe-Leu-Val-OLeu-Leu)	*Cyc* (Phe-MeLeu-Val-OLeu-Leu)	*Cyc*(Phe-Leu-Val-OLeu-Val)	*Cyc* (Trp-MeLeu-Val-OLeu-Leu)	*Cyc* (Phe-MeLeu-Val-OLeu-Val)	*Cyc* (Phe-MeLeu-Ala-OLeu-Leu)	*Cyc* (Tyr-MeLeu-Val-OLeu-Leu)
R_T	24.7 min	24.2 min	23.5 min	23.0 min	22.8 min	22.4 min	21.8 min	21.0 min
$[M+H]^+$	615.4143 Δ 4.4 $C_{34}H_{55}N_4O_6$ (58.6)	587.3816 Δ 2.2 $C_{32}H_{51}N_4O_6$ (9.0)	601.3945 Δ 2.5 $C_{33}H_{53}N_4O_6$ (55.4)	573.3664 Δ 3.0 $C_{31}H_{49}N_4O_6$ (8.0)	640.4039 Δ 4.6 $C_{35}H_{54}N_5O_6$ (100.0)	587.3818 Δ 2.6 $C_{32}H_{51}N_4O_6$ (41.7)	573.3617 Δ 5·2 $C_{31}H_{49}N_4O_6$ (60.1)	617.3917 Δ −1.3 $C_{33}H_{53}N_4O_7$ (70.4)
$[M+H–CO]^+$	587.4149 Δ 3.0 $C_{33}H_{55}N_4O_5$ (100)	559.3825 Δ 5.3 $C_{31}H_{51}N_4O_5$ (59.3)	573.4000 Δ 1.8 $C_{32}H_{53}N_4O_5$ (100)	545.3682 Δ 2.8 $C_{30}H_{49}N_4O_5$ (54.4)	612.4127 Δ −1.2 $C_{34}H_{54}N_5O_5$ (95.2)	559.3830 Δ 4.3 $C_{31}H_{51}N_4O_5$ (100)	545.3692 Δ 1.0 $C_{30}H_{49}N_4O_5$ (85.5)	589.3941Δ 3.1 $C_{32}H_{53}N_4O_6$ (100)
Serie b^{1-2}								
b_4^{1-2}	488.3131 Δ −2.4 $C_{27}H_{42}N_3O_5$ (10.3)	474.2938 Δ 5.1 $C_{26}H_{40}N_3O_5$ (48.3)	474.2960 Δ −1.1 $C_{26}H_{40}N_3O_5$ (13.7)	460.2791 Δ 3.3 $C_{25}H_{38}N_3O_5$ (40.2)	513.3062 Δ 1.9 $C_{28}H_{41}N_4O_5$ (14.8)	460.2805 Δ 0.2 $C_{25}H_{38}N_3O_5$ (12.5)	446.2623 Δ 5.9 $C_{24}H_{36}N_3O_5$ (38.3)	490.2895 Δ 3.4 $C_{26}H_{40}N_3O_6$ (12.5)
$[b_4^{1-2}–CO]^+$	460.3149 Δ 4.6 $C_{26}H_{42}N_3O_4$ (71.0)	446.2992 Δ 4.7 $C_{25}H_{40}N_3O_4$ (100)	446.3003 Δ 2.4 $C_{25}H_{40}N_3O_4$ (63.7)	432.2835Δ 5.1 $C_{24}H_{38}N_3O_4$ (100)	485.3141 Δ −3.8 $C_{27}H_{41}N_4O_4$ (61.5)	432.2836 Δ 4.9 $C_{24}H_{38}N_3O_4$ (67.2)	418.2676 Δ 5.8 $C_{23}H_{36}N_3O_4$ (100)	462.2960 Δ 0.5 $C_{25}H_{40}N_3O_5$ (77.3)
b_3^{1-2}	375.2283 Δ −1.3 $C_{21}H_{31}N_2O_4$ (16.6)	375.2260 Δ 5.0 $C_{21}H_{31}N_2O_4$ (9.1)	375.2292 Δ −3.6 $C_{21}H_{31}N_2O_4$ (5.4)	361.2098 Δ 6.6[a] $C_{20}H_{29}N_2O_4$ (7.9)	414.2389 Δ −0.4 $C_{23}H_{32}N_3O_4$ (6.6)	361.2124 Δ −0.7 $C_{20}H_{29}N_2O_4$ (7.1)	375.2294 Δ −4.3 $C_{21}H_{31}N_2O_4$ (30.0)	391.2217 Δ 2.6 $C_{21}H_{31}N_2O_5$ (14.8)
b_2^{1-2}	261.1611 Δ −5.2 $C_{15}H_{21}N_2O_2$ (27.6)	261.1603Δ −1.9 $C_{15}H_{21}N_2O_2$ (37.0)	261.1606 Δ −3.1 $C_{15}H_{21}N_2O_2$ (17.4)	247.1440 Δ 0.6 $C_{14}H_{19}N_2O_2$ (19.7)	300.1687 Δ −6.5 $C_{17}H_{22}N_3O_2$ (11.8)	247.1457 Δ −6.5 $C_{14}H_{19}N_2O_2$ (24.9)	261.1597 Δ 0.3 $C_{15}H_{21}N_2O_2$ (35.2)	277.1539 Δ 2.8 $C_{15}H_{21}N_2O_3$ (25.8)
$[b_2^{1-2}+H_2O]^+$	279.1712 Δ −3.0 $C_{15}H_{23}N_2O_3$ (10.6)		279.1701 Δ 0·6 $C_{15}H_{23}N_2O_3$ (8.1)		318.1794 Δ 5.5 $C_{17}H_{24}N_3O_3$ (14.3)		279.1693 Δ 3.8 $C_{15}H_{23}N_2O_3$ (23.1)	295.1636 Δ 5.5 $C_{15}H_{23}N_2O_4$ (13.8)
$[b_2^{1-2}–CO]^+$	233.1662 Δ 1.2 $C_{14}H_{21}N_2O$ (39.1)	233.1651 Δ −1.1 $C_{14}H_{21}N_2O$ (56.6)	233.1658 Δ −4.2 $C_{14}H_{21}N_2O$ (24.0)	219.1486 Δ 2.9 $C_{13}H_{19}N_2O$ (33.8)	272.1744 Δ 5.0 $C_{16}H_{22}N_3O$ (15.1)	219.1480 Δ 5.3 $C_{13}H_{19}N_2O$ (24.5)	233.1647 Δ 0.6 $C_{14}H_{21}N_2O$ (38.2)	249.1613 Δ −6.2 $C_{14}H_{21}N_2O_2$ (33.2)
Serie b^{2-3}								
b_4^{2-3}	502.3252 Δ 4.6 $C_{28}H_{44}N_3O_5$ (4.1)			474.2941 Δ 4·5 $C_{26}H_{40}N_3O_5$ (5.7)		488.3097 Δ 4.4 $C_{27}H_{42}N_3O_5$ (3.4)		
b_3^{2-3}	388.2583 Δ 2.9 $C_{22}H_{34}N_3O_3$ (9.2)		388.2582 Δ 3.2 $C_{22}H_{34}N_3O_3$ (7.7)	360.2261 Δ 5.7 $C_{20}H_{30}N_3O_3$ (1.7)	427.2746[a] $C_{24}H_{35}N_4O_3$ (8.2)	374.2416 Δ 5.8 $C_{21}H_{32}N_3O_3$ (10.9)	388.2593 Δ 0.3 $C_{22}H_{34}N_3O_3$ (5.8)	404.2540 Δ 0.9 $C_{22}H_{34}N_3O_4$ (10.4)
$[b_3^{2-3}+H_2O]^+$	406.2721 Δ −5.2 $C_{22}H_{36}N_3O_4$ (58.7)	392.2524 Δ 5.1 $C_{21}H_{34}N_3O_4$ (4.0)	406.2688 Δ 3.0 $C_{22}H_{36}N_3O_4$ (39.3)	378.2366 Δ 5.6 $C_{20}H_{32}N_3O_4$ (3.2)	445.2809 Δ 0.1 $C_{24}H_{37}N_4O_4$ (44.5)	392.2527 Δ 4.2 $C_{21}H_{34}N_3O_4$ (39.0)	406.2679 Δ 5.4 $C_{22}H_{36}N_3O_4$ (55.1)	422.2623 Δ 6.3 $C_{22}H_{36}N_3O_5$ (53.6)
b_2^{2-3}	275.1738 Δ 5.8 $C_{16}H_{23}N_2O_2$ (63.7)	261.1603 Δ −1.9 $C_{15}H_{21}N_2O_2$ (37.0)	275.1754 Δ 0.0 $C_{16}H_{23}N_2O_2$ (48.6)	261.1601 Δ −1.2 $C_{15}H_{21}N_2O_2$ (19.2)	314.1859 Δ 1.4 $C_{18}H_{24}N_3O_2$ (91.8)	275.1763 Δ −3.3 $C_{16}H_{23}N_2O_2$ (51.0)	275.1748 Δ 2.1 $C_{16}H_{23}N_2O_2$ (48.9)	291.1690 Δ 4.5 $C_{16}H_{23}N_2O_3$ (58.2)
$[b_2^{2-3}–CO]^+$	247.1794 Δ 4.4 $C_{15}H_{23}N_2O$ (32.2)	233.1651 Δ −1.1 $C_{14}H_{21}N_2O$ (56.6)	247.1818 Δ −5.3 $C_{15}H_{23}N_2O$ (19.2)	233.1637 Δ 5.1 $C_{14}H_{21}N_2O$ (25.6)	286.1909 Δ 1.8 $C_{17}H_{24}N_3O$ (19.1)		247.1794 Δ 4.3 $C_{15}H_{23}N_2O$ (22.9)	263.1747 Δ 2.8 $C_{15}H_{23}N_2O_2$ (36.3)
Serie b^{3-4}								
b_3^{3-4}	388.2583 Δ 2.9 $C_{22}H_{34}N_3O_3$ (9.2)			360.2261 Δ 5.7 $C_{20}H_{30}N_3O_3$ (1.7)		374.2416 Δ 5.8 $C_{21}H_{32}N_3O_3$ (10.9)		
Serie b^{4-5}								
b_4^{4-5}	502.3252 Δ 4.6 $C_{28}H_{44}N_3O_5$ (4.1)	474.2938 Δ 5.1 $C_{26}H_{40}N_3O_5$ (48.3)	488.3114 Δ 1.1 $C_{27}H_{42}N_3O_5$ (2.7)	474.2941 Δ 4·5 $C_{26}H_{40}N_3O_5$ (5.7)	527.3195 Δ 6.2 $C_{29}H_{43}N_4O_5$ (2.8)	488.3097 Δ 4.4 $C_{27}H_{42}N_3O_5$ (3.4)	460.2793 Δ 2.9 $C_{25}H_{38}N_3O_5$ (8.3)	
b_3^{4-5}	355.2582 Δ 2.5 $C_{19}H_{35}N_2O_4$ (31.3)	327.2274 Δ 1.4 $C_{17}H_{31}N_2O_4$ (59.4)	341.2430 Δ 1.4 $C_{18}H_{33}N_2O_4$ (28.1)	327.2287 Δ −2.7 $C_{17}H_{31}N_2O_4$ (15.5)	341.2418 Δ 4.9 $C_{18}H_{33}N_2O_4$ (39.4)	341.2420 Δ 4.4 $C_{18}H_{33}N_2O_4$ (20.9)	313.2108 Δ 4.3 $C_{16}H_{29}N_2O_4$ (59.3)	341.2443 Δ −2.4 $C_{18}H_{33}N_2O_4$ (50.8)

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Table 2 (*Continued*)

	MeSan-F (**86**)	San-A (**83**)	MeSan-A (**82**)	San-B (**80**)	MeSan-C (**79**)	MeSan-B (**77**)	MeSan-D (**75**)	MeSan-E (**72**)
	Cyc (Phe-MeLeu-Leu-OLeu-Leu)	*Cyc* (Phe-Leu-Val-OLeu-Leu)	*Cyc* (Phe-MeLeu-Val-OLeu-Leu)	*Cyc*(Phe-Leu-Val-OLeu-Val)	*Cyc* (Trp-MeLeu-Val-OLeu-Leu)	*Cyc* (Phe-MeLeu-Val-OLeu-Val)	*Cyc* (Phe-MeLeu-Ala-OLeu-Leu)	*Cyc* (Tyr-MeLeu-Val-OLeu-Leu)
$[b_3^{4-5}-H_2O]^+$	337.2494 Δ −2.6 $C_{19}H_{33}N_2O_3$ (27.9)	309.2156 Δ 5.3 $C_{17}H_{29}N_2O_3$ (4.3)	323.2329 Δ 0.1 $C_{18}H_{31}N_2O_3$ (21.1)	309.2175 Δ −0.8 $C_{17}H_{29}N_2O_3$ (3.1)	323.2320 Δ 2.9 $C_{18}H_{31}N_2O_3$ (37.6)	323.2312 Δ 5.4 $C_{18}H_{31}N_2O_3$ (21.8)	295.2005 Δ 3.6 $C_{16}H_{27}N_2O_3$ (16.8)	323.2312 Δ 5.4 $C_{18}H_{31}N_2O_3$ (34.6)
b_2^{4-5}	228.1599 Δ −2.3 $C_{12}H_{22}NO_3$ (6.0)	214.1434 Δ 1.9 $C_{11}H_{20}NO_3$ (3.9)		214.1449 Δ −5.3 $C_{11}H_{20}NO_3$ (11.9)			<1	214.1425 Δ 6.0 $C_{11}H_{20}NO_3$ (10.3)
$[b_2^{4-5}-CO]^+$	200.1647 Δ −1.2 $C_{11}H_{22}NO_2$ (8.2)	186.1477 Δ 6.2 $C_{10}H_{20}NO_2$ (6.7)	186.1476 Δ 6.5 $C_{10}H_{20}NO_2$ (5.6)	186.1485 Δ 1.9 $C_{10}H_{20}NO_2$ (8.1)	186.1487 Δ 1·0 $C_{10}H_{20}NO_2$ (5.9)	186.1491 Δ −1.6 $C_{10}H_{20}NO_2$ (5.5)	<1	186.1493 Δ −2·2 $C_{10}H_{20}NO_2$ (15.8)
Serie b^{5-1}								
b_4^{5-1}	468.3400 Δ 6.7 $C_{25}H_{46}N_3O_5$ (27.3)	440.3108 Δ 2.6 $C_{23}H_{42}N_3O_5$ (17.7)	454.3267 Δ 1.9 $C_{24}H_{44}N_3O_5$ (25.3)	426.2951 Δ 2.8 $C_{22}H_{40}N_3O_5$ (24.1)	454.3290 Δ −3.3 $C_{24}H_{44}N_3O_5$ (59.6)	440.3099 Δ 4.4 $C_{23}H_{42}N_3O_5$ (27.9)	426.2953 Δ 2.1 $C_{22}H_{40}N_3O_5$ (38.2)	454.3256 Δ 4.4 $C_{24}H_{44}N_3O_5$ (43.6)
$[b_4^{5-1}-CO]^+$	440.3467 Δ 3.7 $C_{24}H_{46}N_3O_4$ (13.1)	412.3149 Δ 5.2 $C_{22}H_{42}N_3O_4$ (14.5)	426.3341 Δ −3.4 $C_{23}H_{44}N_3O_4$ (7.2)	398.2992 Δ 5.4 $C_{21}H_{40}N_3O_4$ (17.7)	426.3315 Δ 2.8 $C_{23}H_{44}N_3O_4$ (15.1)	412.3159 Δ 2.6 $C_{22}H_{42}N_3O_4$ (6.1)	398.3001 Δ 3.1 $C_{21}H_{40}N_3O_4$ (13.0)	426.3323 Δ 0.8 $C_{23}H_{44}N_3O_4$ (10.2)
b_3^{5-1}	341.2427Δ 2.2 $C_{18}H_{33}N_2O_4$ (17.7)	327.2274 Δ 1.4 $C_{17}H_{31}N_2O_4$ (59.4)	327.2273 Δ 1.5 $C_{17}H_{31}N_2O_4$ (4.5)	313.2107 Δ 4.6 $C_{16}H_{29}N_2O_4$ (55.7)	327.2270 Δ 2.5 $C_{17}H_{31}N_2O_4$ (5.9)	313.2127 Δ −1.5 $C_{16}H_{29}N_2O_4$ (6.3)	299.1969 Δ −1.4 $C_{15}H_{27}N_2O_4$ (16.8)	327.2274 Δ 1.2 $C_{17}H_{31}N_2O_4$ (38.5)
$[b_3^{5-1}-CO]^+$	313.2468 Δ 5.7 $C_{17}H_{33}N_2O_3$ (40.5)	299.2328 Δ 0.3 $C_{16}H_{31}N_2O_3$ (56.4)	299.2332 Δ −1.1 $C_{16}H_{31}N_2O_3$ (34.3)	285.2167 Δ 1.9 $C_{15}H_{29}N_2O_3$ (62.8)	299.2319 Δ 3.4 $C_{16}H_{31}N_2O_3$ (28.8)	285.2169 Δ 1.2 $C_{15}H_{29}N_2O_3$ (39.1)	271.2015 Δ 0.4 $C_{14}H_{27}N_2O_3$ (18.2)	299.2324 Δ 1.9 $C_{16}H_{31}N_2O_3$ (57.0)
b_2^{5-1}	228.1599 Δ −2.3 $C_{12}H_{22}NO_3$ (6.0)	228.1600 Δ 1.4 $C_{12}H_{22}NO_3$ (8.7)	228.1585 Δ 3.9 $C_{12}H_{22}NO_3$ (2.2)	214.1449 Δ −5.3 $C_{11}H_{20}NO_3$ (11.9)	228.1593 Δ 0.3 $C_{12}H_{22}NO_3$ (3.6)		228.1595 Δ −0.2 $C_{12}H_{22}NO_3$ (11.8)	228.1602 Δ −3.4 $C_{12}H_{22}NO_3$ (6.2)
ImPhe/ImTyr Im-MeLeu	120.0779 Δ 1.5 $C_8H_{10}N$ (3.1) 100.1116 Δ 4.5 $C_6H_{14}N$ (45.1)		100.1112 Δ 8.3 $C_6H_{14}N$ (27.6)	120.0782 Δ −0·9 $C_8H_{10}N$ (7.4)	100.1128 Δ −8.0 $C_6H_{14}N$ (25.3)	120.0808 Δ −0.2 $C_8H_{10}N$ (1.3)	120.0787 Δ −5·2 $C_8H_{10}N$ (4.8) 100.1117 Δ 3.5 $C_6H_{14}N$ (50.2)	136.0750 Δ 5·1 $C_8H_{10}NO$ (3.3) 100.1115 Δ 5·8 $C_6H_{14}N$ (41.9)

[a] Superimposed to $[M+1]^+$ from *m/z* 360 or *m/z* 426.

Other seven compounds, **72**, **75**, **77**, **79**, **80**, **83** and **86** exhibited a similar fragmentation pattern (Table 2) and were tentatively identified as *Cyc* (Tyr-MeLeu-Val-OLeu-Leu) (MeSan-E), *Cyc* (Phe-MeLeu-Ala-OLeu-Leu) (MeSan-D), *Cyc* (Phe-MeLeu-Val-OLeu-Val) (MeSan-B), *Cyc* (Trp-MeLeu-Val-OLeu-Leu) (MeSan-C), *Cyc*(Phe-Leu-Val-OLeu-Val) (San-B), *Cyc* (Phe-Leu-Val-OLeu-Leu) (San-A) and *Cyc* (Phe-MeLeu-Leu-OLeu-Leu) (MeSan-F) respectively. With the exception of San A and MeSan A none of these compounds were previously identified and for this reason they were named with different letters. All these compounds show a characteristic assembly of four aminoacids and leucic acid (OLeu), and in all cases an aromatic aminoacid is followed by Leu or MeLeu, then Ala, Val or Leu, then Oleu and finally Val or Leu.

The MS/MS of these compounds also showed some characteristic fragmentations. The loss of CO, typical in cyclic peptides, was the base peak for MeSans and had about 50% of relative abundance for Sans. Series b^{1-2}, b^{2-3}, b^{4-5} and b^{5-1} were almost complete and allowed a clear identification of the depsipeptides. The series b^{3-4} was almost absent and this fact suggests a preferred fragmentation of the ester linkage instead of the amide as in the other series. Ions $[b_2^{1-2}+H_2O]^+$ and $[b_3^{2-3}+H_2O]^+$ were observed for most of the MeSans. These type of adducts were previously reported for reactive acyliums [24] and may be related with the presence of leucic acid in the molecules.

Other new and related peptides **46**, **59**, **67**, **74** and **78** (OLeuValLeuPhe-OMe, OLeuValValPheLeu-OMe, OLeuValLeuPheLeu-OMe, OLeuValMeLeuPheLeu-OMe and OLeuValMeLeuPheMeLeu-OMe, respectively), were also identified (Table 3), although based on their structures they are probable artifacts derived from sansalvamides since they are linear methylated ester. The MS/MS spectra of these compounds are much more straightforward, with most of the b fragment ions present and only a few ions of the *y* type.

Compound **45** could not be identified since its MS/MS spectrum showed fragmentations corresponding to uncommon aminoacids, and this fact could not be verified by other methods.

The use of different ionization sources for the analysis of sansalvamides gave similar results to those previously obtained in the case of Css, and APCI/APPI in positive ion mode were again the most suitable techniques for the analysis. A Fig. 5S comparing absolute abundances vs. ionization technique is included in the supplementary material.

By the use of post-column addition of metal ions, most of the sansalvamides showed characteristic mass spectra with the

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Table 3
Characteristic fragment ions observed in the positive APCI MS/MS spectrum of compounds **46**, **59**, **67**, **74** and **78**.

	OLeuValMeLeuPheMeLeu-OMe 22.6 min (**78**)		OLeuValMeLeuPheLeu-OMe 21.4 min (**74**)		OLeuValLeuPheLeu-OMe 20.3 min (**67**)		OLeuValValPheLeu-OMe 19.1 min (**59**)		OLeuValLeuPhe-OMe 16.8 min (**46**)	
$[M+H]^+$	647.4351 Δ 4.2	$C_{35}H_{59}N_4O_7$	633.4252 Δ −4.8	$C_{34}H_{57}N_4O_7$	619.4092 Δ 4.4	$C_{33}H_{55}N_4O_7$	605.3880 Δ 4.8	$C_{32}H_{53}N_4O_7$	506.3240 Δ 0.3	$C_{27}H_{43}N_3O_6$
b_4	488.3098 Δ 4.3 (1.3)	$C_{27}H_{42}N_3O_5$	488.3143 Δ −4.9 (0.9)	$C_{27}H_{42}N_3O_5$	474.2944 Δ 3.8 (14.2)	$C_{26}H_{40}N_3O_5$	460.2830 Δ−5.3 (9.5)	$C_{25}H_{38}N_3O_5$	–	–
b_3	341.2427 Δ 2.4 (100)	$C_{18}H_{33}N_2O_4$	341.2419 Δ 4.6 (100)	$C_{18}H_{33}N_2O_4$	327.2269 Δ 2.9 (100)	$C_{17}H_{31}N_2O_4$	313.2106 Δ 4.9 (100)	$C_{16}H_{29}N_2O_4$	327.2277 Δ 0.4 (6)	$C_{17}H_{31}N_2O_4$
b_2	214.1424 Δ 6.5 (3.3)	$C_{11}H_{20}NO_3$	214.1430 Δ 3.6 (3.6)	$C_{11}H_{20}NO_3$	214.1426 Δ 5.5 (28.8)	$C_{11}H_{20}NO_3$	214.1428 Δ 4.3 (41.0)	$C_{11}H_{20}NO_3$	214.1448 Δ −5.0 (46)	$C_{11}H_{20}NO_3$
$[b_2–CO]^+$	186.1499 Δ −5.5 (6.8)	$C_{10}H_{20}NO_2$	186.1500 Δ −6.1 (8.7)	$C_{10}H_{20}NO_2$	186.1480 Δ 4.5 (13.5)	$C_{10}H_{20}NO_2$	186.1486 Δ 1.5 (18.2)	$C_{10}H_{20}NO_2$	186.1477 Δ 6.2 (92.4)	$C_{10}H_{20}NO_2$
y_3			420.2875 Δ −4.2 (1.0)	$C_{23}H_{38}N_3O_4$	406.2694 Δ 1.7 (4.8)	$C_{22}H_{36}N_3O_4$	392.2564 Δ−5.2 (4.5)	$C_{21}H_{34}N_3O_4$		
y_2			-		293.1848 Δ 3.8 (65.9)	$C_{16}H_{25}N_2O_3$	293.1840 Δ 6.6 (82.8)	$C_{16}H_{25}N_2O_3$	293.1846 Δ 4.8 (46)	$C_{16}H_{25}N_2O_3$
y_1							146.1173 Δ 1.8 (9.6)	$C_7H_{16}NO_2$	180.1019 Δ −0.9 (100)	$C_{10}H_{14}NO_2$
Im-Phe Im-MeLeu	100.1119 Δ 1·7	$C_6H_{14}N$	100.1113 Δ 7.5 (12.9)	$C_6H_{14}N$	120.0802 Δ 5.2 (4.8)	$C_8H_{10}N$	120.0802 Δ 4.6 (10.3)		120.0813 Δ −4.0 (4.8)	$C_8H_{10}N$
					261.1595 Δ 1.0 (14.5)	$C_{15}H_{21}N_2O_2$ LeuPhe/PheLeu	247.1428 Δ 5.2 (16.6)	$C_{14}H_{19}N_2O_2$ PheVal/ValPhe		
					233.1648 Δ 0.0 (14.5)	$C_{14}H_{21}N_2O$ (m/z 261-CO)	219.1499 Δ−3.0 (11.8)	$C_{13}H_{19}N_2O$ (m/z 247-CO)		

Error (Δ) in ppm; RA between parentheses.

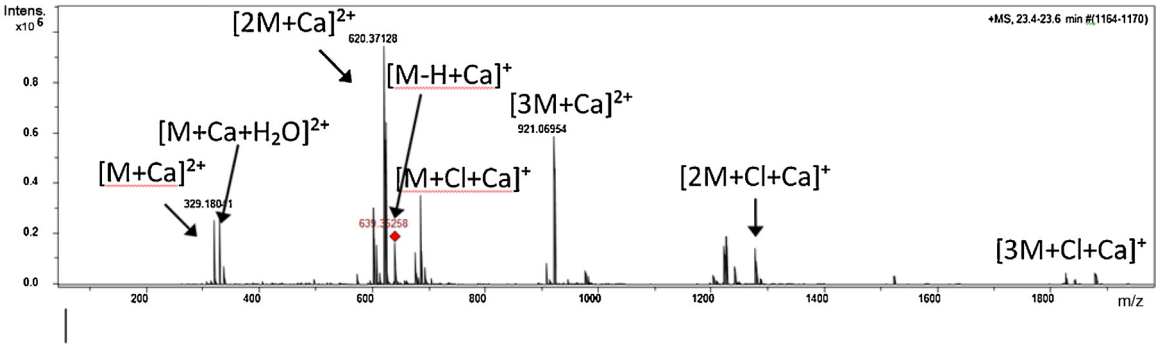

Fig. 4. ESI (post-column addition of Ca^{2+}) Mass Spectrum of MeSan A (**82**).

appearance of peaks corresponding to singly charged adducts like $[M–H+Me]^+$ and $[xM+Cl+Me]^+$ ($x = 1–3$) and doubly charged $[xM+Me]^{2+}$ ($x = 1–3$)(Me = Ca, Cu, Zn) adducts. As an example, the ESI mass spectrum of MeSan A with the addition of a solution of Ca^{2+} is shown in Fig. 4. MS Profiles are very similar with the three metals and, taking into account the absolute intensities of the adduct ions, the affinities for the metals would be $Ca^{2+} > Cu^{2+} > Zn^{2+}$. These observations of metal-binding properties for sansalvamides raise the possibility that these compounds may also bind to endogenous metal ions in biological systems, and perhaps act as ionophores. San A and MeSan A have been previously isolated only from unrelated marine *Fusarium* species [23].

In particular, MeSan A was found to have significant cancer cell cytotoxicity and acts as an inhibitor of topoisomerase I in the virus *Molluscum contagiosum* (MCV) [25] although the mechanism of the antitumor action is still not fully determined. As these compounds are privileged structures with potent activity against multiple targets in numerous cancer cell lines, the preparation of synthetic derivatives in order to enhance the bioactivity is still a very active field [26].

3.2.1.3. Diketopiperazines. Thirteen diketopiperazines (DKPs) were identified by the analysis of their MS and MS/MS spectra [27]. All of them are formed by neutral aminoacids. These compounds are widespread fungal metabolites with the ability to bind to a wide range of receptors [28], and therefore their appearance was expected. APCI/APPI in positive ion mode were the best ionization techniques for DKPs, as with the other cyclic peptide classes. These compounds did not form adducts with metals.

3.2.2. Lipids

3.2.2.1. Polyketides related to F244. Six compounds of the extract (**27**, **32**, **36**, **37**, **39** and **51**) exhibited in their MS spectra peaks corresponding to highly oxygenated molecules with 18–20 carbons. These compounds were observed employing all the previously mentioned ionization techniques although the most efficient detection was in negative ion mode, a fact which suggested the presence of an easily deprotonated functional group. The MS2 spectra of these substances showed many peaks in common, as is usual in compounds of the same family. Compound **27** was isolated and identified (2D NMR) as o-F244, the hydrolyzed form of antibiotic F244, also known as hymeglusin or A1233. This compound was previously reported as an inactive derivative of F244 [29] but was never isolated as a natural product. F244 is a specific inhibitor of eukaryotic hydroxymethylglutaryl-CoA synthase (HMGCS), a key enzyme in the cholesterol biosynthetic pathway, which inhibits mevalonate biosynthesis [30]. The interpretation of the MS2 of **27** (Fig. 1S, supplementary material) allowed a tentative identification of the other related compounds. Two of these were dehydrated forms of o-F244 (one may actually be F244), while the remaining compounds were also related to o-F244 (one of them was a methylated form of o-F244, two others were dimethylated derivatives and the remaining substance was an acetylated version of the parent compound). The possibility that the methylated derivatives may be artifacts cannot be discarded.

With the exception of compound **51** all these metabolites showed $[M+Na]^+$ ions with the highest relative abundances employing ESI in positive ion mode but also with good responses in negative ion mode using any technique. Furthermore, the MS2 data, which is important for the structure determination, could only be obtained in negative ion mode.

With the use of post-column addition of metal II solutions, most of these compounds exhibited mass spectra with characteristic peaks, in which $[2M–H+Me]^+$ was the base peak (Fig. 2S, Suppl. Material). Compound **36**, which is suspected to be F244, did not show peaks corresponding to metal adduct ions, suggesting that adduction may be related to the presence of a free carboxylic acid at position 1. It is remarkable that compound **51**, which was mainly observed in the chromatograms in the negative ion mode, showed peaks at m/z 779.4273 $[2M–H+Ca]^+$, *m/z* 802.3831 $[2M–H+Cu]^+$, and 803.4006 $[2M–H+Zn]^+$ in positive ion mode by the use of post-column addition of the corresponding metal solutions. It is clear that an advantage of this methodology is the observation of specific ion species in positive ion mode. Furthermore, as the peaks corresponding to the adducts with Cu or Zn show the characteristic isotopic mass pattern, they can be clearly identified even with low accuracy.

The metabolite F244 was originally isolated from a fungal strain believed to be a *Cephalosporium* sp. [31] and was also independently isolated from *Scopulariopsis* sp. [32] and *Fusarium* sp. [29]. To the best of our knowledge, there are no other reports about the isolation of simple natural derivatives of F244.

3.2.2.2. Fatty acids and derivatives. Different types of fatty acid derived-lipids were characterized. Most of them are normal components of cellular membranes and their presence could be anticipated. Amongst them, hydroxyoctadecanoic acids like mono, di and tri HODEs, HOTEs either in the free form or as methyl esters or ethyl esters, fatty acyl amides, glycerolipids, cerebroside C and ceramides were identified from the MS and MS/MS spectra (supporting information). All these compounds had been previously reported from fungal extracts.

All the lipids with free carboxylic acids showed similar or better responses in negative over positive ion mode, and the MS/MS spectra showed characteristic fragmentations in the negative ion mode. HODEs with a free carboxylic acid, **29**, **35**, **41**, **54** and **70** were only detected in negative ion mode.

Fatty acids exhibited in their MS spectra with the use of post-column addition of metals(II), peaks corresponding to $[M–H+Me]^+$ with Me(II) = Ca, Cu or Zn. The observation of this type of adducts

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between fatty acids and Cu(II) was previously reported and was used to identify fatty acid isomers by CID of the adducts [33].

Cerebroside C showed in the MS spectrum using post-column addition of Ca(II), only those peaks corresponding to doubly charged adducts $[M+Ca]^{2+}$ and $[2M+Ca]^{2+}$.

3.2.2.3. Steroids. Eighteen ergosteroids with retention times between 28 and 39 min were characterized in the LC/MS chromatogram on the basis of their MS/MS spectra. The analysis of these MS/MS spectra allowed the identification of ergone **126** and the determination of the relative position (either the side chain or from A to D rings) of hydroxyls and double bonds (supplementary material) although it was not possible to assign a unique structure for each chromatographic peak. The structure of ergosterol **129** was confirmed by comparison of the retention time with an authentic sample.

APCI and APPI in the positive ion mode were the best ionization methods for this type of compounds as it is already known in the analysis of compounds of low polarity such as steroids or triterpenes [11]. Ergosteroids were not observed in negative ion mode and did not form adducts with metals(II). These compounds are common metabolites of fungal membranes and their presence is expected.

3.2.3. Additional metabolites

Javanicin **16**, anhydrofusarubin or an isomer **17** and neovasinin **26** were identified from the LC/MS run. Compounds **16** and **26** were purified from the extract and their structures were confirmed by NMR analysis and comparison of retention times. None of these compounds had the capability to form adducts with metals in the corresponding spectra. These compounds have been previously isolated from several *Fusarium* strains and many other genera and rich complex culture media were found to give the greatest yields of these compounds [34].

4. Conclusion

The analysis of an extract of *F. oxysporum*, which was very rich in cyclic peptides was performed by LC/MS using different ionization sources and ionization modes. Although this combined strategy has been previously proposed, the results clearly depend on the chemical composition of the extract. In this particular case, the use of APCI in positive and negative ion modes gives a complete picture of the present metabolites without the need of other sources, with only one exception, compound **23**, observed almost exclusively by ESI (+). This compound could only be observed by APCI with very low RA, when especially looked for. APCI was not only well suited for the observation of ionic species of the components, but also for the sequencing of the cyclic peptides, since $[M+H]^+$ species were predominant. Minor components in co-eluting compounds were suppressed by ESI. The method allowed the identification of six new sansalvamides, a family of antitumoral compounds, and related peptides.

The novel proposal of the use of post-column addition of metal salts, Ca^{2+}, Cu^{2+} or Zn^{2+}, had a number of benefits. First, the method allows the possibility of screening in crude extracts for compounds with the ability to coordinate endogenous metals. Ionophores, inhibitors of Histone Deacetylases which chelate Zn^{2+}, inhibitors of metal binding enzymes, chelators of heavy metals, are just a few examples of compound classes that may have relevant biological activities because of their metal binding properties and no other methods have been reported to screen for this activity in crude extracts. Compounds that bind metals may be tested after this screening for specific bioactivities. In this sense, Css and sansalvamides showed an inherent affinity for endogenous metal ions that may be related to their biological activity.

This methodology also allows the observation of specific ion species which are characteristic of certain compound classes, permitting in this case the differentiation of Css from isoCss and also the characterization of Css that lack the amino acid Bmt.

In the case of derivatives of F244, the production of characteristic adducts $[2M–H+M_E]^+$ is another example of the usefulness of this methodology, and the structural reasons for this behavior should be explored. This methodology allows the simultaneous determination and characterization of a large number of compounds, especially cyclic peptides, and its potential utility for the analysis of other compound classes should be explored.

Finaly, the use of metal solutions for post column addition does not report any inconvenience for the instrument at least with this trade mark source.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.chroma.2015.11.073.

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