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1 Atmospheric Pressure Photolonization *versus* Electrospray for
2 the dereplication of highly conjugated natural products using
3 molecular networks

4 Teo Hebra, Véronique Eparvier, David Touboul*

5 Université Paris-Saclay, CNRS, Institut de Chimie des Substances Naturelles, UPR 2301,
6 91198, Gif-sur-Yvette, France.

7 * Corresponding author : David Touboul, david.touboul@cnrs.fr , phone number : +33 169 82
8 30 32

9 Abstract :

10 Natural products are sources of inspiration and reservoir of high valuable molecules.
11 Recently, analytical tools based on liquid chromatography coupled to tandem mass
12 spectrometry to generate molecular network became widely employed for dereplication. This
13 strategy greatly accelerates the identification of known and structural hypothesis of unknown.
14 Despite the availability of different ionization sources, alternatives to classical electrospray
15 ionization (ESI), such as atmospheric pressure chemical ionization (APCI) or photoionization
16 (APPI), have been neglected. In particular, APPI has been described for its ionization
17 efficiency on non-polar molecules bearing no acid or basic groups. For that reason, we
18 investigated APPI potential to generate molecular network and compare it to ESI on several
19 criteria that are generation of ion species, sensitivity and signal-to-noise ratio (SNR) for
20 different extracts rich in highly conjugated natural products. We first optimized APPI
21 experimental conditions on crude extract from a fungus, *Penicillium sclerotiorum* (SNB-
22 CN111), producing polyketones belonging to the azaphilone family. Then we compared APPI
23 and ESI on different fractions of the fungus and on two plant extracts, French Guyanese
24 *Swartzia panacoco* (Aubl.) R.S. Cowan (arial parts) and Indian *Cassia auriculata* L. (leaves)
25 containing phenolic compounds, such as flavonoids. While ESI generated more ion species
26 and displayed a better sensitivity, APPI generated only protonated adduct and better SNR.
27 Comparing ESI and APPI generated species on molecular network reveal that both
28 strategies overlap for the majority of protonated ions.

29 Keywords: APPI, Molecular Network, Metabolomic, Azaphilone, Flavonoid

30 Natural products are a source of inspiration as well as an important reservoir of molecules of
31 high values in many economic fields such as medicine [1], nutrition [2], materials science [3]
32 or cosmetics [4]. The analytical strategies used in metabolomics become gold standard in
33 natural product research [5,6,7]. In particular, metabolomics accelerates the dereplication of
34 complex mixtures, i.e. rapid annotation of already published compounds, for the discovery of
35 undescribed products. One of the most widely used dereplication tool is the strategy based
36 on liquid chromatography coupled to tandem mass spectrometry to generate molecular
37 networks (LC-MS/MS-MN) [5]. Briefly, a data-dependent analysis (DDA) is performed
38 throughout the chromatographic run. Cyclically, a MS1 scan is performed, then a fixed
39 number of charged species, typically from 3 to 5, will be sequentially fragmented and their
40 respective MS2 spectra will be acquired. These fragmentation patterns will then be
41 compared leading to the calculation of a cosine-score related to a structural homology
42 distance. The generated distance matrix is finally used to generate the molecular networks
43 (MNs). T-SNE algorithm can be finally implemented to improve the visualization of the MN.
44 (gnps.ucsd.edu) [8].

45

46 LC-MS/MS experiments to produce data for MN are usually performed with an electrospray
47 ion source. More recently, methodologies to generate MNs from GC-EI-MS data have been
48 introduced [9], (gnps.ucsd.edu). Nevertheless, alternative ion sources such as Atmospheric
49 Pressure Chemical Ionization (APCI) or Photoionization (APPI) have been largely neglected
50 for such dereplication approaches. In particular, APPI has been previously described to be
51 efficient for detection of non-polar or highly conjugated molecules bearing no acid or basic
52 groups [10].

53 During APPI process, direct photoionization can occur if the ionization energy of the analyte
54 is lower than the energy of photons emitted by the discharge lamp, typically 10.0 and 10.6 eV
55 for krypton. This phenomenon can be efficient on aromatic compounds for example.
56 However, the major part of the analytes are not ionized or not sufficiently ionized by APPI
57 leading to the introduction of a dopant molecule into the eluent. This electron or proton donor
58 is usually a small organic molecule showing an ionization energy below 10 eV such as
59 anisole, toluene or acetone whereas [10] the common solvents, i.e. water and acetonitrile,
60 are not photoionized due to ionization potential at 12.6 and 12.2 eV, respectively [11].

61 Some families of natural products have already been studied by APPI such as pentacyclic
62 triterpene [12], mycotoxins [13], lipids [14,15,16], chlorophyll derivatives [17], flower
63 absolutes [18] or polyphenols [19]. However, these studies were performed only from a
64 diagnostic or quantification perspective and never in an unsupervised way. We choose to

65 investigate two families of molecules likely to be studied with APPI: first,. highly conjugated
66 and colored polyketides (azaphilones and derivatives), with biological properties such as
67 antiparasitic [20] and antiviral [21] and proposed as potential natural food colorant [22,23]
68 and second, flavonoids, a molecular class with well-described antioxidant properties [24].
69 Therefore, extracts from a microscopic fungus *Penicillium sclerotiorum* SNB-CN111, which
70 species is mentioned to produce azaphilone and derivatives [25] and two plants, *i.e.* French
71 Guyanese *Swartzia panacoco* (arial parts) and Indian *Cassia auriculata*, (leaves), both from
72 the *Fabales* order, were investigated. *Cassia auriculata* is used in traditional Indian medicine
73 and its extract possesses antioxidant properties [26]. We thus propose to compare APPI and
74 ESI for the acquisition of MS2 data to generate MN in terms of number and type of detected
75 species, sensitivity or MN information.

76

77

78 2. Material and method

79 2.1.1 Fungus material

80 The fungus strain *Penicillium sclerotiorum* SNB-CN111 of strain library collection of ICSN
81 (Sequencing allowed for strain identification by NCBI sequence comparison (Blastn®); the
82 sequence has been registered in the NCBI GenBank database (<http://www.ncbi.nlm.nih.gov>)
83 under the registry number KJ023726) was cultivated on a large scale using 330 Petri dishes
84 (14 cm diameter) at 28 °C for 15 days on potato dextrose agar (PDA) medium (Dominique
85 Dutscher SAS, Brumath, France). The culture medium containing the mycelium was cut into
86 small pieces and macerated three time at room temperature with ethyl acetate (EtOAc) on a
87 rotary shaker (70 rpm) for 48h, 24h and 48h, respectively. The contents were extracted each
88 time with 10 L of EtOAc using a separatory funnel. Insoluble residues were removed *via*
89 filtration and the organic phase was washed three times with an equivalent volume of water
90 (H₂O), dried with anhydrous solid Na₂SO₄ and evaporated using a rotary evaporator under
91 reduced pressure to yield a crude extract (6.5 g). The whole crude extract was fractionated
92 by reversed-phase flash chromatography (Grace Reveleris, Grace, Maryland, USA) using a
93 120 gram C18 column and ultraviolet (UV) and evaporative light scattering detector (ELSD)
94 detectors. A linear gradient of H₂O/formic acid (99.9/0.1) - acetonitrile/formic acid (99.9/0.1)
95 (v/v, 95:5 to 0:100 over 40 min, flow rate at 80 mL.min⁻¹) followed by another gradient of
96 acetonitrile - methylene chloride (v/v, 50:50 to 0:100 over 15 min, flow rate at 80 mL.min⁻¹)
97 was set up to generate 11 fractions that were labeled F1 to F11.

98 2.1.2 Plant material

99 *Swartzia panacoco* stems and leaves were collected on the Couy River near Kourou (N
100 05°01.523', W 052.43.490'), French Guiana, in April 2014. This species is not a protected
101 plant and can be collected without restriction at this location. A herbarium voucher (XC27)
102 was deposited in Guyane IRD Herbarium (CAY). Plant samples were dried (room
103 temperature, 10% air relative humidity) and finely powdered. Two sample of 300 g of stems
104 and leaves were extracted at room temperature by maceration using 3 × 1 L of EtOAc or
105 MeOH providing four different extracts after evaporation of the solvents.

106 *Cassia auriculata* leaves (1 g, Aroma-Zone, Paris, France) from India were macerated during
107 24 h at room temperature on a rotary shaker (70 rpm) with either methanol, ethyl acetate or
108 methylene chloride (200 mL). Insoluble residues were removed *via* filtration and the organic
109 phase was washed three times with an equivalent volume of H₂O using a separatory funnel,
110 dried with anhydrous solid Na₂SO₄ and evaporated using a rotary evaporator under reduced

111 pressure to yield a crude mixture. The first equivalent of water from EtOAc extract was kept
112 to be dried leading to 6 mg for H₂O fraction, 82 mg for MeOH, 62 mg for EtOAc and 56 mg
113 for CH₂Cl₂.

114 2.2 Data acquisition

115 Fractions from *Penicillium sclerotiorum* SNB-CN111 or crude extract from *Cassia auriculata*
116 leaves and *Swartia panacoco* leaves and stems were prepared at 1 mg.ml⁻¹ in methanol and
117 filtered on 0.45 µm PTFE membrane. LC-MS/MS experiments were performed with a 1260
118 Prime HPLC (Agilent Technologies, Waldbronn, Germany) coupled with an Agilent 6540 Q-
119 ToF (Agilent Technologies, Waldbronn, Germany) tandem mass spectrometer. LC
120 separation was achieved with an Accucore RP-MS column (100 × 2.1 mm, 2.6 µm, Thermo
121 Scientific, Les Ulis, France) with a mobile phase consisting of H₂O/formic acid (99.9/0.1) (A)
122 and acetonitrile with 0.1% formic acid (B). The column oven was set at 45 °C. Compounds
123 were eluted at a flow rate of 0.4 mL.min⁻¹ with a gradient from 5% B to 100% B in 20 min and
124 then 100% B for 3 min. Injection volume was fixed at 5 µL for all analyses. For electrospray
125 ionization source, mass spectra were recorded in positive ion mode with the following
126 parameters: gas temperature 325 °C, drying gas flow rate 10 L.min⁻¹, nebulizer pressure 30
127 psi, sheath gas temperature 350 °C, sheath gas flow rate 10 L.min⁻¹, capillary voltage 3500
128 V, nozzle voltage 500 V, fragmentor voltage 130 V, skimmer voltage 45 V, Octopole 1
129 RFVoltage 750 V. The collision energy was fixed at 30 eV for all the MS/MS experiments.
130 For ESI, internal calibration was achieved with two calibrants (*m/z* 121.0509 and *m/z*
131 922.0098) providing a high mass accuracy better than 3 ppm. For atmospheric pressure
132 photoionization source mass spectra were recorded in positive ion mode with the following
133 starting parameters for the optimization: gas temperature set at 325 °C, vaporizer set at 300
134 °C, capillary voltage set at 2500 V, fragmentor voltage at 130 V. The dopant flow was set at
135 30 µL per minute. For the analysis once optimized, APPI parameters were set to gas
136 temperature set at 325 °C, vaporizer set at 300 °C, capillary voltage set at 2000 V,
137 fragmentor voltage at 130 V. The dopant was acetone and its flow was set at 10 µL.min⁻¹.
138 External calibration was achieved with LC/MS tuning mix, for APCI/APPI (Agilent
139 Technologie, Massy, France) and mass accuracy was better than 5 ppm at *m/z* 383. The
140 data-dependent MS/MS events were acquired for the five most intense ions detected by full-
141 scan MS (maximum of five precursors per cycle of 1.2 s) above an absolute threshold of
142 1000 counts. Selected parent ions were fragmented at a fixed collision energy of 30 eV and
143 with an isolation window of 1.3 amu. The mass range of the precursor and fragment ions was
144 set as *m/z* 200–1000.

145 2.3 Data processing and analysis

146 The data files were converted from the .d (Agilent Technologies, Massy, France) standard
147 data format to .mzXML format using the MSConvert software, part of the ProteoWizard
148 package 3.0 [28]. All .mzxml were processed using MZmine2v51 as previously described
149 [29]. The mass detection was realized with MS1 noise level at 1000 and MS2 noise level at
150 0. The ADAP chromatogram builder was employed with a minimum group size of scans of 3,
151 a group intensity threshold of 1000, a minimum highest intensity of 1000, and m/z tolerance
152 of 0.008 (or 20 ppm). Deconvolution was performed with the ADAP wavelets algorithm
153 according to the following settings: S/N threshold=10, minimum feature height=1000,
154 coefficient/area threshold=10, peak duration range 0.01-1.5min, RT wavelet range 0.00-
155 0.04min. MS2 scans were paired using an m/z tolerance range of 0.05 Da and RT tolerance
156 range of 0.5 min. Isotopologues were grouped using the isotopic peak grouper algorithm with
157 an m/z tolerance of 0.008 (or 20 ppm) and a RT tolerance of 0.2 min. Peaks were filtered
158 using Feature list row filter, keeping only peaks with MS2 scan (GNPS). Adduct identification,
159 i.e. sodium- or potassium-cationized species, was performed on the peak list with a retention
160 time tolerance of 0.1 min, an m/z tolerance of 0.008 or 20 ppm, and a maximum relative peak
161 height of 150%. Complex search, such as dimers, was performed with a retention time
162 tolerance of 0.1 min, an m/z tolerance of 0.008 or 20 ppm and a maximum relative peak
163 height of 150%. Peak alignment was performed using the join aligner with an m/z tolerance
164 of 0.008 (or 20 ppm), a weight for m/z at 20, a retention time tolerance of 0.2 min and a
165 weight for RT at 50. The MGF file and the metadata were generated using the export/submit
166 to GNPS option.

167 Cosine score were calculated on MetGem 1.3 software [8], MetGem parameters. MS2
168 spectra were window-filtered by choosing only the top 6 peaks in the ± 50 Da window
169 throughout the spectrum. The data was filtered by removing all peaks in the ± 17 Da range
170 around the precursor m/z. The m/z tolerance windows used to find the matching peaks was
171 set to 0.02 Da and cosine score were kept in consideration for spectra sharing 2 matching
172 peaks at least. The number of iterations, perplexity, learning-rate and early exaggeration
173 parameters were set to 5000, 25, 200 and 12, respectively.

174 Annotations of molecules in MN were made using “Find standards in library” or “Find analogs
175 in library” functions of MetGem. Queries were made against Massbank NA
176 (<https://mona.fiehnlab.ucdavis.edu>), GNPS Public Spectral Library (<https://gnps.ucsd.edu>),
177 NIST14 Tandem (<https://www.nist.gov>), NIH Natural Product (<https://www.nih.gov>) and MS-
178 Dial (<http://prime.psc.riken.jp/compms>) libraries with absolute m/z tolerance of 0.02, 4
179 minimum matched peaks and minimal cosine score of 0.5.

180 To annotate molecules with high confidence in MN using standard search in library, both
181 exact mass, comparison of MS² spectra and previous report of molecules within genera or
182 species are taken into account.

183 Ion intensity and signal-to-noise ratio (SNR) for diagnostic ions were extracted with
184 MassHunter software (AgilentTechnologie, Massy, France).

185 Figures were generated using R and related packages (ggplot2, VennDiagram, Rcolorbrewer
186 and gridextra).

187

188 3. Results and discussion

189 3.1 Parameter optimization

190 In order to optimize APPI conditions, several parameters were investigated such as vaporizer
191 temperature, the capillary voltage, the dopant chemistry and flow rate. The influence of these
192 parameters on ionization and signal-to-noise ratio was then evaluated. Therefore, we
193 selected several ions belonging to the azaphilone family from a crude extract from
194 *Penicillium sclerotiorum* SNB-CN111. Five of them were the most abundant species, *i.e.*
195 ochrephilone ($[M+H]^+$, m/z 383.1860); sclerotioramine ($[M+H]^+$, m/z 390.1472); sclerotiorin
196 ($[M+H]^+$, m/z 391.1312); geumsanol B ($[M+H]^+$, m/z 417.1914); isochromophilone VI ($[M+H]^+$,
197 m/z 434.1735)) and three were less abundant ones (unknown($[M+H]^+$, m/z 353.1967),
198 isorotiorin($[M+H]^+$, m/z 385.2015) and unknown($[M+H]^+$, m/z 451.1525). Formation of radical
199 ion $[M]^{*+}$ is expected on APPI experiment but none were observed with acetone as dopant,
200 and less than 2% with toluene (supplementary figure 1). It can be explained by several of our
201 experimental condition: protic solvent like water are known to support apparition of $[M+H]^+$
202 [30], acetone as dopant is described to enhance the formation of protonated species [31]. As
203 for toluene, working with acetonitrile inhibit toluene $[M]^{*+}$ formation, thus leading to major
204 $[M+H]^+$ population [32]. Optimizations of drying gas temperature, capillary voltage and dopant
205 flow are illustrated below with acetone as a dopant.

206

207 3.1.1 Effect of the vaporizer temperature

208 Because ionization under APPI happens in a vapor like state, vaporizer temperature is a key
209 factor in order to maximize the ion intensity or the signal-to-noise ratio (SNR). A low
210 vaporizer temperature could not vaporize enough mobile phase leading to signal dilution
211 whereas too high temperature could lead to molecular degradation. A temperature range
212 between 200 to 450°C with 50°C step was thus investigated for acetone at a flow rate of 10
213 $\mu\text{L}\cdot\text{min}^{-1}$.

214 Two types of behavior can be described based on figure 1. For 5 over 8 diagnostic ions (m/z
215 353.1967, 390.1472, 417.1914 434.1735 and 451.1525), both intensities and SNR are the
216 lowest at 200°C then reach a plateau between 250 and 350°C then dramatically decrease.
217 For the other 3 diagnostic ions (m/z 383.1860, 385.2015 and 391.1312), a slight intensity
218 decrease or a plateau is occurring from 200 to 450 °C. Therefore, a vaporizer temperature at
219 300°C was chosen for the following optimization steps because this source condition led to

220 the best intensity and SNR for 6 over 8 diagnostic ions while being acceptable for the two
221 last ones (figure 1a, a1b).

222

223 3.1.2 Effect of the capillary voltage

224 The capillary voltage can affect the transmission efficiency of the ions at the entrance of the
225 mass spectrometer and is rather independent to the vaporizer temperature. A voltage range
226 from 2000 to 3500V by 500V step was scanned. As depicted on Figure 2, this parameter has
227 no significant effect on the final intensity or SNR of the diagnostic ions and was optimal at
228 2000 V. This value was chosen for the following optimization steps.

229

230 3.1.3 Effect of the dopant flow rate

231 When direct photoionization of compound is not efficient, the addition of a dopant is
232 necessary. The dopant will exchange its charge or radical hydrogen to the analyte [33]. In the
233 case of acetone, the main ionization process is the formation of protonated acetone followed
234 by a proton transfer to the analyte. One important reported parameter in the literature is the
235 flow rate of the dopant [34,35]. If the dopant quantity is too low, the ionization process will not
236 be optimal whereas a too high quantity will lead to increasing noise signals. The dopant flow
237 rate has been empirically established to 5-10% of the total flow rate. Therefore, typical
238 dopant flow rates from 10 to 50 $\mu\text{L}\cdot\text{min}^{-1}$ were investigated (figure 3a, 3b).

239 Without dopant, proportion of $[\text{M}]^{++}$ significantly increase (Supplementary figure 2) suggesting
240 that highly conjugated azaphilone are subject to direct photoionization but both signal
241 intensity and SNR for all diagnostic ions are drastically increased when introducing the
242 dopant. The dopant flow rates had no significant impact on the intensity of the 8 diagnostic
243 ions (figure 3a) whereas increasing flow rate of the dopant decreases the SNR (figure 3b).
244 Thus the optimal value is 10 $\mu\text{L}\cdot\text{min}^{-1}$ for acetone representing 2.5 % of the eluent flow rate.
245 This value is slightly under described ones [34] 5 to 10 % of eluent flow rate but it could be
246 related to the use of acetone instead of toluene.

247

248 3.1.4 Effect of the dopant chemistry

249 Acetone, anisole and toluene are classical dopants handled to increase APPI efficiency. We
250 excluded anisole as a dopant from the analysis due to several intense ions at m/z 206.1666,
251 230.0937, 263.0917, 530.4691 and 647.4576 throughout the chromatographic time
252 saturating the signal. The same optimization procedure as for acetone was operated for
253 toluene (supplementary figure 3). The selected parameters for toluene were as follows:
254 vaporizer temperature at 350 °C, capillary voltage at 2500 V and dopant flow at 30 $\mu\text{L}\cdot\text{min}^{-1}$.
255 The optimized flow rate of toluene was higher compared to acetone as previously described
256 in the literature [34].

257 Acetone as a dopant outperforms toluene for the intensity of 6 diagnostic ions and displays
258 same intensities for the two others (m/z 417 and 434) (figure 4a). Regarding SNR, acetone
259 fully exceeds toluene by a factor of 3 (figure 4b). Some author reported this phenomenon
260 and suggest that toluene impurities could explain this discrepancy [35]. Based on this
261 experiment, acetone was kept as a dopant for further experiments using the previously
262 optimized ionization conditions.

263

264 3.2 Comparison of ESI and APPI for the dereplication of *Penicillium sclerotiorum* SNB- 265 CN111

266 In order to evaluate APPI-MS for dereplication of complex mixture of natural products by
267 molecular networking [8,28, 36], *Penicillium sclerotiorum* SNB-CN111 crude extract has been
268 fractionated by polarity. Nine fractions were thus analyzed by reversed-phase liquid
269 chromatography couple to tandem mass spectrometry (LC-MS/MS) in ESI and APPI positive
270 modes. Then, the data have been processed by MZmine2 and MetGem software to construct
271 molecular network containing data from both ionization sources.

272

273 3.2.1 Qualitative analysis of ESI and APPI analysis

274 Chromatographic run with ESI source produce significantly more ion signals than APPI
275 (Figure 5a). Indeed, over the 9 analyzed fractions, ESI generated 3152 nodes (precursor ion
276 / retention time couples) for which a MS² was acquired. This is 4.2 time more than APPI
277 (747). About 15 % (462) of ESI signals were annotated by MZmine2 fragment search
278 algorithm as $[\text{M}+\text{H}]^+$, $[\text{M}+\text{Na}]^+$, $[\text{M}+\text{K}]^+$, $[\text{2M}+\text{H}]^+$, $[\text{2M}+\text{Na}]^+$, $[\text{M}+\text{M}'+\text{H}]^+$ or $[\text{M}+\text{M}'+\text{Na}]^+$ of the
279 same molecule M. A manual search within the most intense ions, as well as the clustering of
280 ESI nodes in the t-SNE representation, suggests that this number is largely underestimated.
281 This phenomenon is common within ESI experiment and manual inspection is often

282 mandatory to avoid misinterpretation or redundant information. On the contrary, APPI ion
283 signals were strictly annotated as protonated species. Common features between ESI and
284 APPI are estimated to be 497 corresponding to 66% of APPI signals. Moreover, these
285 common features are related to ion population of high intensity (Figure 5b, 5c) and can be
286 employed for reducing the redundant information from non-protonated species. Furthermore,
287 ESI led to the detection of 125 features present in each sample and in the blank (pure
288 methanol) whereas APPI only exhibited 7 interferences from the blank, *i.e.* pure methanol.

289 An intensity threshold value of 100 000, corresponding to two orders of magnitude between
290 the most and less intense feature, was added to simplify the reading of the molecular
291 networks (figure 5c, 6a) and get rid of very low intensity ions. The dataset is finally composed
292 of 691 ions, *i.e.* 374 APPI features, 643 ESI ones which 102 of them are non-protonated
293 species and 326 are common between the two ionization methods (figure 6b). This clearly
294 highlights the fact that major differences between ESI and APPI features are for low
295 abundant species and non-protonated species.

296 It is interesting to note that the species present in the south of the t-SNE MN is mainly
297 constituted of bimolecular species such as $[2M+H]^+$, $[2M+Na]^+$, $[M+M'+H]^+$ or $[M+M'+Na]^+$
298 (figure 6a). To understand better the differences of ionization between several scaffold of
299 azaphilone such as ochrephilone, isochromophilone VI or geumsanol B, MN annotation is
300 provided in Figure 7 and led to the annotation of 16 molecular classes using dereplication
301 tools such as MS² database search, the Natural Product Atlas [37] and a review of literature
302 on Azaphilone and *Penicillium sclerotiorum* or *multicolor*. [38,39]

303 Several signals have been directly annotated through MS² database comparison, the
304 database compound with the highest homology cosine score was used for annotation, such
305 as isochromophilone VI (cluster1, cosine score = 0.85), sclerotioramine (cluster2, cosine
306 score = 0.97), ochrephilone (cluster4, cosine score = 0.92), hypocrellone A (cluster 6, cosine
307 score = 0.76), geumsanol C (cluster 7, cosine score = 0.59), several diketopiperazine
308 (cluster 8, CycloPhe-Pro, cosine score =0.97, CycloPheLeu, cosine score = 0.92, CycloPhe-
309 HydroxyPro, cosine score = 0.80), monoelaidin (cluster 9, cosine score = 0.86),
310 isochromophilone IV (cluster 13, cosine score = 0.75), sclerotiorine (cluster 14, cosine score
311 =0.82), lauryl ethanolamide (cluster 15, cosine score = 0.87). Ergosterol derivatives (cluster
312 10) and sclerotioramine analogs (cluster 12) were found through analogs search into MS²
313 databases. Their scaffolds were common and redundant over several nodes present in their
314 respective cluster. Geumsanol C (cluster 7) annotation with a homology cosine score from
315 databases of 0.59 was low for a highly confident annotation, we decided to include it
316 because we manually found other evidence that increased our confidence: its chlorinated

317 analog is present within the same cluster and also annotated through databases search with
318 the highest compound hit of 0.66. . Finally, Peniazaphilone A (cluster 3, 0 ppm from
319 calculated mass), Geumsanol B (cluster 5, +1.7 ppm from calculated mass) are dereplicated
320 using the structural cluster search of Natural Product Atlas. Most of these molecule share a
321 common scaffold and belongs to the azaphilone family. Nevertheless, there is a diversity of
322 substructure that can impact ionization: addition of hydroxyl (Geumsanol B, Geumsanol C,
323 Hypocrellone A), a lactone ring (Peniazaphilone A, Ochrephilone, Geumsanol B), substitution
324 of the oxygen by a nitrogen (Isochromophilone VI, Sclerotioramine, Peniazaphilone A). This
325 is why we decided to make a dilution range of the fractions from 1000 $\mu\text{g}\cdot\text{mL}^{-1}$ to 25 $\mu\text{g}\cdot\text{mL}^{-1}$
326 in order to evaluate sensitivity and SNR for APPI compare to ESI (figure 8b-8k).

327 ESI outperformed APPI in term of ionization for 4 out of 6 species (Ochrephilone (I),
328 Sclerotiorine (II), Sclerotioramine (III), Isochromophilone VI (IV)) and displayed similar
329 ionization to APPI for the two others (Geumsanol B (V) and 5-chloroisorotiorin (VI)) at 25 and
330 100 $\mu\text{g}\cdot\text{mL}^{-1}$. At 250 $\mu\text{g}\cdot\text{mL}^{-1}$ ESI performed better for 3 out of 6 ions (Ochrephilone (I),
331 Sclerotioramine (III) and Isochromophilone VI (IV)). This is not the case anymore for
332 Sclerotiorine (II) as APPI and ESI equally perform, as well for Geumsanol B (V) and 5-
333 chloroisorotiorin (VI). Despite a saturation phenomenon at 1000 $\mu\text{g}\cdot\text{mL}^{-1}$, it is interesting to
334 note that only the 2 nitrogen molecules (Sclerotioramine (III) and Isochromophilone VI (IV))
335 are better ionized by ESI (figure 8a - 8g).

336 APPI leads to higher SNR for 3 molecules out of 6 (Ochrephilone (I), Isochromophilone VI
337 (IV), Geumsanol B (V)) compared to ESI, similar values for (5-chloroisorotiorin (f)) and lower
338 for 2 molecules (Sclerotiorine (II), Sclerotioramine (III)) at 25 $\mu\text{g}\cdot\text{mL}^{-1}$. The capability of APPI
339 to achieve higher SNR compared to ESI increases for the higher concentration: 5 out of
340 6 molecules (Ochrephilone (I), Sclerotiorine (II), Sclerotioramine (III), Isochromophilone VI
341 (IV) and 5-chloroisorotiorin (VI)) display better SNR value with APPI at 100 $\mu\text{g}\cdot\text{mL}^{-1}$, for
342 example (figure 8a, 8h - 8k).

343 Overall, ESI and APPI displayed different but complementary features for the analysis of the
344 specialized metabolism of *Penicillium sclerotiorum*. While ESI displays better sensitivity
345 overall, APPI showed better SNR and produces only protonated species leading to an easier
346 data interpretation. Similar results were obtained on other natural product families, indicating
347 that overall, APPI is less susceptible to matrix effect [40, 41]. Once data from ESI and APPI
348 are combined together, they allow a highly valuable molecular snapshot of a complex
349 sample.

350

351 3.3 Comparison of ESI and APPI for the analysis of flavonoid containing plants.

352 To extend the scope of this study, we have chosen to investigate another class of natural
353 product, *i.e.* flavonoids in crude plant extracts. *Swartia panacoco* and *Cassia auriculata*
354 specialized metabolites were extracted with solvent of different polarity (water, methanol,
355 ethyl acetate and dichloromethane). The extract was thus analyzed by LC-MS/MS using
356 either ESI or APPI in positive mode.

357

358 3.3.1 Qualitative analysis of ESI and APPI analysis

359 In accordance with the results previously described with *Penicillium sclerotiorum* fractions,
360 LC-MS with ESI source produces significantly more ion signals than APPI (Figure 9a, 9c).
361 Indeed, over the analyzed 8 crude extract (2 plants and 4 extraction solvents each), ESI
362 generated 3560 nodes (precursor ion / retention time couples) for which a MS² was acquired.
363 This is 5.2 time more nodes than APPI (675). About 6% (222) of ESI signals were annotated
364 by MZmine2 fragment search algorithm as [M+H]⁺, [M+Na]⁺, [M+K]⁺, [2M+H]⁺, [2M+Na]⁺,
365 [M+M'+H]⁺ or [M+M'+Na]⁺ of the same molecule M. On the contrary, APPI ion signals were
366 strictly annotated as protonated species. Common features between ESI and APPI are 384
367 corresponding to 56% of APPI signals. Moreover, these common features are related to ion
368 population of high intensity (Figure 9b) and can be employed for reducing the redundant
369 information from non-protonated species. Furthermore, ESI led to the detection of 152
370 features present in each sample and in the blank (pure methanol) whereas APPI only
371 exhibited 9 interferences from the injection solvent. All these observations are in perfect
372 accordance with results obtained on *Penicillium sclerotiorum*.

373 Following the same procedure as before, an intensity threshold was added to simplify the
374 reading of the molecular networks and get rid of very low intensity ions. This time value of
375 50 000 has been defined, to keep to two orders of magnitude between the most and less
376 intense feature. The dataset is finally combining 898 nodes, *i.e.* 295 APPI features, 870 ESI
377 ones which 49 of them are non-protonated species and 267 are common between the two
378 ionization methods (figure 10a, 10b). Once again major differences between ESI and APPI
379 are concentrated on low abundant or non-protonated species.

380 It is interesting to note that the west of the t-SNE MN is mainly constituted of high intensity
381 nodes produced by both APPI and ESI. The nodes specific to APPI are concentrated into two
382 clusters (in the south west and the south east) and one isolated node in the north. MN
383 annotation is provided in Figure 11 to better understand the specificity of APPI and ESI, and
384 to verify if our hypothesis considering APPI as a technic of interest to search for flavonoid in
385 a complex extract. It led to the annotation of 14 clusters using MS² database search
386 dereplication tools. Cluster1 was annotated as glycosylated flavonoids (quercetin-4O-

387 glucoside, m/z 465.0988, cosine score = 0.79), cluster 2 as flavonoids (3-O-methylquercetin,
388 m/z 317.0628, cosine score = 0.84, luteolin, m/z 287.0724, cosine score = 0.88 and catechin,
389 m/z 291.0848, cosine score = 0.87), cluster3 as Pheophorbide A (m/z 593.275, cosine score
390 = 0.75), cluster 4 as Pheophytin A analogs (Pheophytin A, m/z 871.5748, cosine score
391 =0.69), cluster 5 as ceramide (analog search on the whole cluster), cluster 6 as
392 phytosphingosine (m/z 318.2977, cosine score = 0.73), cluster 7 as phosphatidylcholine
393 (Lyso-PC(16:0), m/z 496.3387, cosine score = 0.87), cluster 8 as adenosine and derivatives
394 (adenosine, m/z 268.1211, cosine score = 0.83), cluster 9 as phtalates (dioctyl phtalate,
395 m/z 391.2822, cosine score = 0.80), cluster 10 as monoglyceride (glycerol 1-stearate, m/z
396 359.313, cosine score = 0.89), cluster 11 as fatty acids (octadecadienoic acid, m/z 295.225,
397 cosine score = 0.66), cluster 12 was pentacyclic triterpene (glycyrrhetic acid, m/z 471.3433,
398 cosine score = 0.71) , cluster13 as diglyceride/triglyceride (analog search on the whole
399 cluster) and cluster 14 as fatty acid amide (linoleyl-ethanolamide, m/z 324.287, cosine score
400 = 0.79).

401 A single cluster at the extreme east, and annotated as polyethylene glycol (PEG) derivatives,
402 has been cut off because it was too far away and took away legibility. From the two clusters
403 constituted of APPI-only feature, one is constituted of ceramide analogs while the other
404 seems to be a subclass of pentacyclic triterpene. Moreover, the APPI generated ion intensity
405 of pentacyclic triterpenes is directly depending to their polarity (Supplementary figure 4); the
406 less polar, the better the intensity was. As previously described in the literature, highly apolar
407 pentacyclic triterpenes are well suited for APPI analysis in the positive mode [12].

408 Because our aim was to characterize and compare APPI performances over ESI on highly
409 conjugated molecules, we extracted both intensity and SNR and intensity for a panel of 6
410 molecules representative of flavonoid clusters. Two molecule from the purple sub-cluster (3-
411 O-methylquercetin, cosine score = 0.84, and formononetin, cosine score = 0.84, cluster 2), 1
412 molecule from the north pink sub-cluster (luteolin, cosine score = 0.88, cluster 2), 2 molecule
413 from the brown sub-cluster (Epigallocatechin, cosine score =0.7 and Catechin, cosine score
414 = 0.87, cluster 2) and one molecule from the glycosylated flavonoid green sub-cluster
415 (Quercetin-4O-glucoside, cosine score = 0.79, cluster 1) of the t-SNE representation (Figure
416 11).

417 For 3-o-methylquercetin (I), APPI outperforms ESI both in intensity and SNR. ESI and APPI
418 displays equal sensitivity for Formononetin (II) and Luteolin (III) and similar SNR for
419 Formononetin (II) while SNR of Luteolin (III) is better for ESI. For the last 3 diagnostic
420 molecules (Epigallocatechin (IV), Catechin (V) and Quercetin-4-o-glucoside (VI)) ESI
421 operates much better than APPI for both intensity and SNR. The global behavior is following

422 the retention times and thus the polarity as described for pentacyclic triterpenes. This results
423 confirmed that APPI can be highly performant for the ionization of apolar molecules, as
424 described within several lipids classes [14], leading to highly informative molecular networks.

425

426 Conclusion

427 APPI-MS parameters were optimized for the analysis of highly conjugated and apolar
428 compounds from a fungus crude extract with an emphasis on sensitivity and SNR. While
429 ESI-MS generates more species and displayed a better sensitivity overall, APPI-MS only
430 generates protonated species, displays better SNR and generated less interfering species.
431 These conclusions were extended by the study of arial parts extracts from French Guyanese
432 *Swartzia panacoco* and Indian *Cassia auriculata*, especially for flavonoid and pentacyclic
433 triterpene families. Using complementarity between APPI-MS and ESI-MS, a strategy for the
434 analysis of complex natural extracts by molecular network could start with an APPI-MS
435 analysis to characterize and annotate ions. This will avoid redundant signals because APPI-
436 MS generate only protonated species with high SNR. Then a second batch of analysis could
437 be performed using ESI-MS in order to detect and annotate low abundance ions thanks to
438 the better sensitivity of ESI-MS.

439

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449 Figure legends:

450 Figure 1: Effect on (a) intensity or (b) signal-to-noise ratio (SNR) of vaporizer temperature for
451 8 ions from *Penicillium sclerotiorum* SNB-CN111 crude extract. Logarithmic scale.

452 Figure 2: Effect on (a) intensity or (b) signal-to-noise ratio (SNR) of capillary voltage for 8 ions
453 from *Penicillium sclerotiorum* SNB-CN111 crude extract. Logarithmic scale.

454 Figure 3: Effect on (a) intensity or (b) signal-to-noise ratio (SNR) of acetone dopant flow for
455 8 ions from *Penicillium sclerotiorum* SNB-CN111 crude extract. Logarithmic scale.

456 Figure 4: Effect on (a) intensity or (b) signal-to-noise ratio (SNR) of dopant chemistry for 8
457 ions from *Penicillium sclerotiorum* SNB-CN111 crude extract. Logarithmic scale.

458 Figure 5: (a) Ion distribution from *Penicillium sclerotiorum* SNB-CN111 fractions between ESI
459 and APPI; (b) their respective intensity their (c) t-SNE representation constructed on MS²
460 homology the size of nodes is related to their intensity.

461 Figure 6: *Penicillium sclerotiorum* SNB-CN111 fractions: (a) t-SNE representation
462 constructed on MS² homology and (b) Ion repartition between ESI and APPI. An intensity
463 threshold value of 100 000 is applied to the dataset, the size of nodes is related to their
464 intensity.

465 Figure 7: Structural annotation of the molecular network related to *Penicillium sclerotiorum*
466 SNB-CN111 fractions. Contaminants are composed of polydimethylsiloxane (PDMS) from
467 vial septum (hit on databases).

468 Figure 8: (a) Diagnostic molecules (Ochrephilone (I), Sclerotiorine (II), Sclerotioramine (III),
469 Isochromophilone VI (IV), Geumsanol B (V) and 5-chloroisorotiorin (VI)). Ion counts (b, c, d,
470 e, f, g) and SNR (h, i, j, k) for the respective protonated species. Logarithmic scale.

471 Figure 9: (a) Ion distribution of specialized metabolites from *Swartia panacoco* and *Cassia*
472 *auriculata* crude extracts analyzed by ESI and APPI, (b) their respective intensity (logarithmic
473 scale), and (c) t-SNE representation constructed on MS² homology, the size of nodes is
474 related to their intensity.

475 Figure 10: (a) t-SNE representation –from *Swartia panacoco* and *Cassia auriculata* crude
476 extracts– constructed on MS² homology, the size of nodes is related to their intensity. (b) Ion
477 repartition between ESI and APPI. An intensity threshold value of 50 000 is applied to the
478 dataset.

479 Figure 11: Annotation of the molecular network (t-SNE view) from 8 extracts of two plant
480 leaves (*Swartia panacoco* and *Cassia auriculata*). 1: Glycosylated flavonoid, 2: Flavonoid,
481 3: Pheophorbide A, 4: Pheophytin A analogs, 5: Ceramide, 6: Phytosphingosine,
482 7: Phosphatidylcholine, 8: Adenosine and derivative, 9: Phtalate, 10: Monoglyceride, 11:
483 Fatty acid, 12: Pentacyclic triterpene, 13: Diglyceride/Triglyceride, 14: Fatty acid amide.

484 Figure 12: (a) Diagnostic molecules (3-O-methylquercetin (I), Formononetin (II), Luteolin (III),
485 Epigallocatechin (IV), Catechin (V) and Quercetin-4-O-glycoside (VI)), Ion counts (b) and
486 SNR. (c) for the respective protonated species Logarithmic scale.

487

488

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