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Direct introduction MALDI FTICR MS based on dried droplet deposition applied to non-targeted metabolomics on *Pisum Sativum* root exudates

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1 **Direct Introduction MALDI FTICR MS based on dried droplet deposition**
2 **applied to non-targeted metabolomics on *Pisum Sativum* Root Exudates**

3

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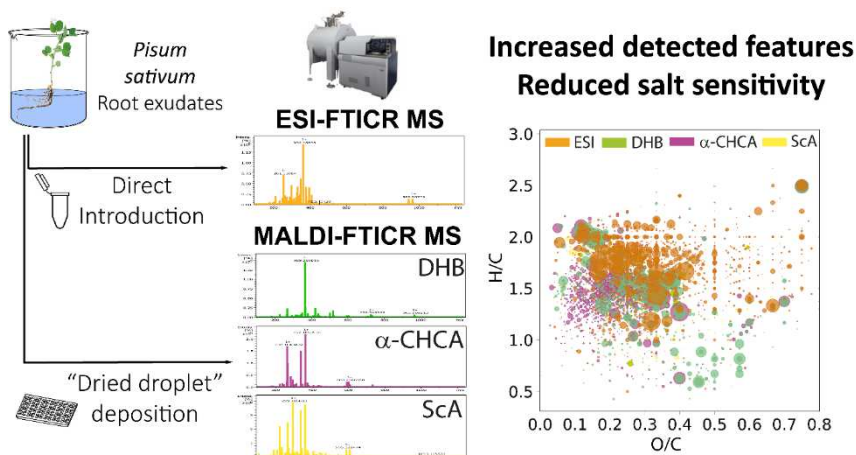
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16

17 **Key words** Root exudates, Non-targeted metabolomics, Fourier Transform Ion Cyclotron
18 Resonance Mass Spectrometry, MALDI, *Pisum sativum*

19

20 **Graphical abstract**



21

22 **Abstract**

23 Non-targeted metabolomic approaches based on direct introduction (DI) through a soft
24 ionization source are nowadays used for large-scale analysis and wide cover-up of metabolites
25 in complex matrices. When coupled with ultra-high-resolution Fourier-Transform ion
26 cyclotron resonance (FTICR MS), DI is generally performed through electrospray (ESI),
27 which, despite the great analytical throughput, can suffer of matrix effects due to residual salts
28 or charge competitors. In alternative, matrix assisted laser desorption ionization (MALDI)
29 coupled with FTICR MS offers relatively high salt tolerance but it is mainly used for imaging
30 of small molecule within biological tissues. In this study, we report a systematic evaluation on
31 the performance of direct introduction ESI and MALDI coupled with FTICR MS applied to
32 the analysis of root exudates (RE), a complex mixture of metabolites released from plant root
33 tips and containing a relatively high salt concentration. Classic dried droplet deposition
34 followed by screening of best matrices and ratio allowed the selection of high ranked
35 conditions for non-targeted metabolomics on RE. Optimization of MALDI parameters led to
36 improved reproducibility and precision. A RE desalted sample was used for comparison on
37 ionization efficiency of the two sources and ion enhancement at high salinity was highlighted
38 in MALDI by spiking desalted solution with inorganic salts. Application of a true lyophilized
39 RE sample exhibited the complementarity of the two sources and the ability of MALDI in the
40 detection of undisclosed metabolites suffering of matrix effects in ESI mode.

41

42 **1. Introduction**

43 Non-targeted metabolomics is a growing field of ‘omics’ which enables the unbiased
44 detection and annotation of a wide range of molecules at low molecular weight (< 1500 Da) in
45 biological samples [1–6]. In the case of plant science, metabolite profiling could give access
46 to the characterization of primary and secondary metabolites which are widely involved in
47 plant growth, development and health or adaptation to abiotic and biotic stress. It could be
48 drought, extremes temperatures, salinity or attack by pathogenic microorganisms [7–13].
49 Non-targeted metabolomic is typically performed through nuclear magnetic resonance (NMR)
50 [14,15] and mass spectrometry (MS) [16]. Over the past decades, MS coupled with gas (GC-
51 MS) [17] or liquid chromatography (LC-MS) [18] has been widely used as preferred
52 technique for the detection of a wide range of compounds in plant material. In the case of
53 complex matrices, LC-MS allows the separation of isomeric and isobaric species before their

54 detection as ions in gas-phase [19]. Chromatographic separation allows a reduction of sample
55 complexity but it is a highly time-consuming technique and it presents some limitations in
56 large-scale non-targeted metabolomic studies [20]. Recently, non-targeted metabolomic
57 approaches based on the direct introduction (DI) of the sample into the ion source have been
58 proved to enable faster analysis for large-scale study and wide cover-up of metabolite
59 detection [21,22]. Among these, direct introduction coupled with ultra-high-resolution
60 analyzers as in Fourier Transform ion cyclotron resonance mass spectrometry (FTICR MS)
61 [23,24] has been increasingly used in non-targeted metabolomic thanks to its advantages in
62 ultra-high resolving power, high mass accuracy and dynamic range [25–28]. Recently, many
63 studies have been published on the potentiality of FTICR MS to perform a global profiling of
64 metabolites in plants material [27–31]. FTICR analyzers enable separation according to the
65 cyclotron frequency produced by the rotation of ions in a fixed magnetic field and the
66 simultaneous detection of all m/z over a time range [32]. In this extent, when coupled with
67 FTICR MS, direct introduction allows to obtain a constant number of ions entering into the
68 ICR cell during wide time ranges, leading to ultra-high resolution and mass measure
69 precision. For this reason, direct introduction FTICR MS is normally preferred over
70 hyphenation with liquid chromatography. Direct introduction FTICR MS coupled with
71 electrospray (ESI) is routinely used as preferred source in non-targeted metabolomic studies
72 because it enables the easy and efficient ionization of a wide range of metabolites
73 characterizing many biological matrices [33–35]. However, ESI suffers from some analytical
74 limitations as matrix effects [36]. In fact, when a wide number of different analytes is injected
75 in the source, ions might compete as charge carriers (ion competition) or species at high
76 abundance can lead to reduced sensitivity of low abundance ones (ion suppression) [37–39].
77 Matrix assisted laser desorption ionization (MALDI) is an alternative ion source which
78 enables the soft ionization of a wide range of metabolites in biological samples [40]. This is
79 normally performed through the use of an organic matrix which assists the transfer of the laser
80 energy to the analyte, enabling the ionization and reducing ion fragmentation. MALDI
81 enables the performant detection of metabolites in complex biological samples without
82 preliminary clean-up or separation before analysis. However, when coupled with classical
83 mass analyzers such as time of flights, the presence of matrix ions in the low m/z zone limits
84 its applications in the analysis of low molecular weight compounds [41]. MALDI has
85 therefore been applied mostly in the analysis of large organic biomolecules such as lipids and
86 proteins[42,43], peptides [44,45] and saccharides [46] or in whole-cell samples analysis for
87 the detection of intact microorganisms [47]. Lately, coupling with FTICR MS has boosted

88 application of MALDI in low molecular weight compounds discovery thanks to improved
89 molecular formula assignment for isobaric ions [48,49]. Nonetheless, MALDI-FTICR MS is
90 nowadays mainly used in metabolomics to highlight the spatial distribution of a reduced
91 number of compounds within biological tissues [50,51]. Many studies point out on imaging
92 for biomedical applications or in plant science [52–58]. Nonetheless, to the best of our
93 knowledge, few studies points out on a non-targeted metabolomic approach based on the
94 direct introduction of a biological sample based on MALDI-FTICR MS [59]. In fact, the
95 highest drawback of mass spectrometry based on MALDI is that, besides from problems
96 connected to the noise in the low mass region [60], ionization depends strictly on the
97 homogeneity of the matrix-sample spot [61]. This can lead to issues as lack of reproducibility
98 for qualitative and mostly quantitative analysis [62].

99 In this study we present for the first time an analytical optimization of a non-targeted
100 metabolomic approach based on ultra-high-resolution mass spectrometry coupled to direct
101 introduction MALDI for improved metabolite detection and reduced ion suppression in
102 presence of salts. This method has been optimized on plant root exudates (RE), because this
103 complex biological cocktail contains a high number and diversity of molecules and residual
104 salts [11]. Indeed, RE is a mixture of primary and secondary metabolites produced by the
105 plant roots and continuously released into the narrow zone of soil surrounding roots called the
106 rhizosphere [12]. These include many compounds of different nature which take a large part
107 in the interactions between plants and their environment [63,64], especially with the
108 rhizosphere microbiota essential to plant growth, health and resistance to biotic or abiotic
109 stress [12,64–67]. Deciphering molecular signals exchanged between the root and its
110 microbiota would be an important advancement in plant science and a great hope for
111 agroecology, because a better control of interactions in the rhizosphere could allow limitation
112 of phytochemical inputs in agricultural processes [68]. These objectives could be reached
113 through the metabolomic investigation and profiling of plant RE [11,69]. Nonetheless, due to
114 the complexity of RE composition and its variability with plant growth conditions and variety,
115 performing metabolomic studies of root exudates is challenging and many efforts are required
116 for the optimization of both RE collection [70] and analysis through mass spectrometry
117 techniques [71]. RE sampling is usually divided in two steps consisting in plant growth and
118 metabolite extraction [72]. Among many plant growth methods, artificial hydroponic systems
119 are widely used as they enable an easier handling of plants and improved reproducibility with
120 low root damage and reduced metabolites absorption from matrix [70,73]. In hydroponics,
121 plant growth is obtained through immersion of seeds and roots in a nutritive medium

122 containing macronutrient [74]. Once growth completed, RE can be collected by rinsing, then
123 transferring the root system from the hydroponic medium to a solvent that is compatible with
124 analysis (i.e. water) [75,76]. It is worth to note that, in RE samples obtained from hydroponic
125 systems, metabolites occur at mM–nM concentrations; while a relatively high concentration
126 of macronutrients (mM) may be present after re-concentration of RE samples [77]. Therefore,
127 in the case of mass spectrometry-based metabolomics coupled with direct introduction,
128 metabolite extraction and salt removal are highly advised to obtain the preferential ionization
129 of the metabolites over suppression and competition effects [71].

130 Here, we evaluated the performances of MALDI-FTICR MS based on dried droplet
131 deposition to a non-targeted metabolomic approach for the detection and annotation of low
132 abundance metabolites in *Pisum sativum* RE. Literature presents many studies on *P. sativum*
133 RE showing the presence of amino acids, sugars and organic acids [78], while few
134 investigations focus on the isolation and characterization of secondary metabolites. The main
135 chemical classes have been found to be triterpenes [79], polyphenols [80,81], alkaloids [82]
136 and some classes of specialized phytohormones [83–85]. Nonetheless, to the best of our
137 knowledge, this is the first non-targeted metabolomic study on *P. sativum* RE. In this study, a
138 *P. sativum* RE desalted sample was first submitted to a classic non-targeted metabolomic
139 approach based on data dependent acquisition mass spectrometry (DDA-MS) and to ESI-
140 FTICR MS. A collection of 48 metabolites were annotated with a hypothetical structure and a
141 global profiling with an insight of different chemical classes was obtained *via* unique
142 molecular formula assignment. This information was used for the main aim of this study,
143 which attempted a development of a non-targeted metabolomic approach based on MALDI-
144 FTICR MS. A screening on the performances of six MALDI matrices and 36 different
145 conditions (sample/matrix ratio, with or without addition of acid) led to the selection of three
146 optimal combinations which enabled a satisfying non-targeted metabolite detection.
147 Moreover, a rigorous control of experimental parameters such as the laser power used for
148 MALDI measurements enabled an improvement of mass accuracy without complex sample
149 deposition, which was based on the dried droplet method. MALDI resulted a complementary
150 technique, leading to a ~60% increase of detected metabolites at both low and at high m/z
151 values. Matrix effects arising from increased salinity in ESI-FTICR MS and MALDI-FTICR
152 MS mode were evaluated on desalted *P. sativum* RE samples which were spiked with
153 increasing amounts of salts. Lastly, the optimized non-targeted metabolomic approach based

154 on MALDI was tested on a true lyophilized *P. sativum* RE sample containing a natural
155 amount of salt arising from the exudation step.

156

157 **2. Material and methods**

158 **2.1 Collection of plant root exudates.**

159 Plant seeds were purchased from RAGT Semences (Toulouse, France). Seeds were cleaned
160 and stored in containers at 5 °C in a cool room until use. Pea seeds were soaked twice for 10
161 min in an ethanol solution (96%) and then in a hypochlorite solution (26 %) under stirring.
162 After each immersion, the seeds were rinsed six times with demineralized sterile water (10 s
163 per rinse). The disinfection process was carried out in sterile conditions. For germination,
164 sterilized seeds of pea were cultivated in boxes with agar (1 %). The seeds were germinated in
165 phytotronic chamber at a day/night cycle: 16 h, 23 °C / 8 h, 20 °C for a period of 7 days. After
166 germination, seed were transferred in a hydroponic system containing ¼ Hoagland nutrient
167 solution and cultured in a phytotronic chamber in the same condition as germination. A list of
168 the oligo and micro elements with their concentration in the ¼ diluted Hoagland solution can
169 be found in table 1S. Five weeks growth seedling characterized of 6-8 leaves each plant were
170 used for exudation. Briefly, the plants roots were removed from the Hoagland solution and
171 briefly rinsed with sterile deionized water to remove the remaining nutrient solution. The
172 plants were then transferred to backers containing 500 mL sterile ultrapure water. Exudation
173 was carried out for 1h, roots were then removed and the solutions frozen at -20°C in glass
174 bottles. A blank solution was prepared using 500 mL sterile ultrapure water which was
175 incubated in the similar conditions as backers of root exudates.

176 **2.2 Metabolites extraction.**

177 Two sample treatment methods were used for metabolites extraction and concentration.
178 Desalted sample which contained an enriched fraction of *P. sativum* RE were obtained
179 through solid phase extraction (SPE). Briefly, frozen *P. sativum* RE (500 mL) were
180 completely thawed, distributed in tubes (Falcon) and solutions were centrifugated at low
181 temperature (5°C) for 15 min at 10 000 rpm (Eppendorf 5804 R) in order to remove cell
182 debris and eventual particles. Aliquots of supernatant liquid were collected to reach a final
183 volume of 420 mL. Centrifuged RE solution were then submitted to SPE on an automated
184 SPE WorkStation (6.25ws, Interchim France). A column filled with a hydrophilic modified

185 styrene-based polymer (PolyClean HLB, 3 mL x 200 mg, Interchim France) was used for the
186 extraction of metabolites. The HLB column was washed prior to sample loading with 5 mL of
187 methanol and 5 mL of water at a flow rate of 15 mL/min. RE were then passed through the
188 activated polymeric cartridge at 3 mL/min. The polymeric phase was washed with 4 mL of
189 LC-MS water at 3 mL/min and dried for 10 min using a gentle stream of nitrogen. Elution
190 was then performed by passing through 3 mL methanol at 2 mL/min. Time spent for SPE
191 extraction was around 3h for sample. The fraction was then dried completely under a nitrogen
192 stream and resuspended in 250 μ L of water. Blank was treated likewise. Samples were kept at
193 low temperature (5-7°C) during SPE and evaporation by soaking bottles in ice. This assured
194 minimal degradation of metabolites during the time-consuming sample treatment. For
195 lyophilized samples, sample treatment was similar, except that after centrifugation the final
196 volume of 420 mL RE was distributed in clean tubes, frozen at -20°C for 24h and then
197 lyophilized using a freeze dryer (BUCHI Lyovapor™ L-200). The lyophilized sample and a
198 blank were resuspended both in 250 μ L of water. SPE and lyophilized samples were stored at
199 -20°C until analysis.

200 **2.3 Sample preparation and DDA-MS measurements for metabolites annotation.**

201 *P. sativum* RE SPE extracts were thawed before analysis and solutions were directly
202 submitted to DDA-MS without further treatment. The mass spectrometer was a hybrid
203 quadrupole-time-of-flight instrument (QTOF, Synapt G2 HDMS, Waters MS Technologies,
204 Manchester, UK) [86] coupled with an ultra-high-performance liquid chromatographic
205 (UHPLC) system equipped with a diode array detector (Vanquish, Thermo Scientific, San
206 Jose, CA, USA). The travelling wave ion mobility cell included in the instrument was not
207 used during analysis. The column used for the elution was a reverse phase ACQUITY UPLC
208 HSS T3 1.7 μ m of dimension 1.0 mm x 100 mm (Waters Manchester, UK) equipped with a
209 0.2 μ m prefilter. The sample tray and column oven temperatures were set at 8 °C and 50 °C,
210 respectively. The injection volume was set at 5 μ L for MS/MS measurements in positive
211 mode and 10 μ L in negative mode. Flow rate of 0.100 mL/min was used. Mobile phase A
212 consisted of water + 0.1 % formic acid and mobile phase B of acetonitrile + 0.1 % formic
213 acid. The elution was the following: 0–2 min, 0% B; 2–3 min, 0–10% B; 3–10 min, 10–20%
214 B; 10–14 min, 20–100% B; 14–17 min, 100% B; 17–18 min, 100–0% B; 18–25 min, 0% B.
215 DDA-MS experiments were performed over a m/z 50–1200 range in resolution mode
216 (resolution 20 000 FWHM). A sodium formate solution was used for external calibration
217 while a continuous lock mass correction was applied by infusing leucine enkephalin via the

218 LockSpray™ interface (reference scan frequency 10 sec, lock spray capillary 3.0 kV and cone
219 voltage of 25 V in positive mode and respectively 10 s, 3.0 kV and 50 V in negative mode).
220 Source in positive mode was operated as following: capillary 3 kV, sampling cone 25 V,
221 extraction cone 5 V. The source temperature was 120°C, the cone gas flow and the
222 desolvation gas flows were set respectively to 20 L/h and 600 L/h; desolvation temperature
223 300 °C. For negative ion mode, parameters were the same excluded for the capillary which
224 was set at 2.2 kV. Metabolites were fragmented automatically through a survey scan function
225 when the precursor threshold peak intensity exceeded 1000 counts in positive mode and 800
226 scans in negative mode. For each MS scan, the 3 most intense MS/MS ions were fragmented
227 using 4 MS/MS scans accumulation. Then, each fragmented ion was excluded for 6 seconds
228 with a scan time of 0.200 sec and an interscan time of 0.024 sec. Experiments were performed
229 in the transfer cell which was filled with argon (6.10^{-3} mbar), using collision energy ranging
230 from 10-20 eV for low m/z values to 10-30 eV for high m/z values. A linear variation was
231 used along the whole range. The trap cell of the instrument was operated using a background
232 collision energy of 4 eV.

233 For metabolites annotation, raw data were submitted to accurate mass measure on MassLynx
234 (v. 4.2, Waters Corporation) and then converted in mzXML open format through the
235 MSCConvert tool of ProteoWizard (v. 3.0.9992) using MS1 and MS/MS levels as filters. Raw
236 data were then uploaded on the Global Molecular Networking Platform (GNPS,
237 <https://gnps.ucsd.edu>) [87,88] and experimental spectra were queried against library search
238 using the spectral library search in order to find possible matches. Ions corresponding to
239 contaminants or matrix were subtracted automatically through the GNPS analysis. Metabolite
240 annotation was performed on manual literature search for some abundant metabolites for
241 which no match was found on GNPS databases.

242

243 **2.4 Sample preparation and ESI-FTICR MS measurements.**

244 For ESI measurements, 100-fold diluted solutions in water/methanol (50/50, v/v) for both
245 SPE and lyophilized samples were prepared. For measurements in positive ion mode, 0.1 %
246 formic acid was added to the final diluted solution in order to favor protonated forms.
247 Solutions were introduced directly in the electrospray source of a high-field FTICR mass
248 spectrometer (SolariX MRMS, Bruker Bremen, Germany), equipped with a 12 T
249 superconducting magnet and a dynamically harmonized ICR cell, in both positive and
250 negative ion modes. Samples were introduced to the ion source through a syringe pump at a

251 flow rate of 90.0 $\mu\text{L min}^{-1}$. Spectra were recorded at a transient length of 1.12 s (4 million
252 points) with an accumulation time of 0.050 s in positive mode and 0.100 s in negative mode.
253 A sodium trifluoroacetate solution at 0.1 mg/mL in acetonitrile/water (50/50, v/v) was used
254 for external calibration by the acquisition of 30 accumulated scan spectra. Mass spectra were
255 acquired over a m/z 98–1500 range. A mass resolving power of around 700,000 at m/z 200
256 was achieved. A complete list of parameters used for ESI measurements can be found in
257 Table 2S.

258 2.5 Sample preparation for MALDI-FTICR MS measurements

259 For MALDI measurements, six matrices were chosen among the most used in MALDI
260 literature. These were: 2,5-Dihydroxybenzoic acid (DHB), alpha-Cyano-4-hydroxycinnamic
261 acid (α -CHCA), Sinapinic acid (ScA), trans-2-[3-(4-tert-Butylphenyl)-2-methyl-2-
262 propenylidene] malononitrile (DCTB), 1,5-Naphthalenediamine (DAN) and 9-Aminoacridine
263 (9-AA). Regarding matrix preparation: DHB and ScA were prepared at a concentration of 10
264 mg/mL in water/methanol (50/50 v/v) and 9AA at a concentration of 9 mg/mL; α -CHCA at
265 10 mg/mL in MeOH/ACN (50/50 v/v), DCTB and DAN at 10 mg/mL in MeOH. The same
266 solutions were prepared in duplicate and added of 0.1 % formic acid on the final volume. SPE
267 and lyophilized samples were then mixed without further dilution with the seven matrices
268 with a ratio sample:matrix of 1:1, 2:1 and 1:10. Then, for each mixture, two additional
269 conditions were tested, namely with or without the addition of 0.1 % of formic acid (FA). The
270 dried droplet method was used for sample deposition on a target plate (MTP 384 target plate
271 ground steel BC, Bruker Daltonics, Germany) and for each sample, 5 μL were deposited for
272 each spot, on three different positions and samples gently dried under vacuum. For external
273 calibration in positive mode, a mixture of the 3 peptides bradykinin, leucine enkephalin and
274 angiotensin (each at a concentration of 10 pmol/ μL in water) was prepared at a proportion
275 1:1:1:3 with DHB at 10 mg/mL. In negative ion mode, a solution of 9-AA at 10 mg/mL was
276 used for external calibration. For direct introduction MALDI-FTICR MS measurements, laser
277 shots value was fixed to 200 for all matrices while laser power was optimized in order to
278 reach a 10% ionization threshold. Laser power was set as follow: in positive mode for DHB at
279 20, for α -CHCA and DCTB at 14 and for ScA at 28. In negative mode for DCTB at 11, for
280 DAN at 14 and for 9AA at 26. Values of the laser power were set as following for
281 optimization in positive mode: for DHB at 12, 15, 17, 18, 20, 22, 24, 26; for α -CHCA at 8, 9,
282 10, 12, 13, 14, 15, 18; for ScA at 12, 15, 18, 19, 20, 22, 25, 27, 29, 32, 35, 37; for SA at 12,
283 15, 18, 19, 20, 22; for DCTB at 6, 7, 8, 9, 10, 12, 15, 16 and in negative mode: for 9AA at 15,

284 17, 20, 23, 25, 27, 30 and 32; for DAN at 10, 13, 14, 15, 17 and for DCTB at 6, 8, 10, 11, 12,
285 13 and 15. Curves fitted to sigmoid were obtained through the Boltzmann function on
286 OriginPro 2018 (v. b9.5.0.193). Figure 1S shows the curves obtained for optimization of the
287 laser power and the 10% threshold values for the investigated matrices. Except for source
288 parameters, all other experimental parameters used to perform MALDI-FTICR MS
289 measurements were set as in ESI-FTICR MS. These, together with additional parameters of
290 the MALDI source, can be found in table 2S. Experimental design, parameters for internal
291 calibration, molecular formula attribution and data visualization were performed accordingly
292 to ESI-FTICR MS measurements.

293 **2.6 Sample preparation for salt tolerance evaluation**

294 In order to evaluate the salt tolerance of the two sources coupled with FTICR MS, series of
295 solutions spiked with increasing amount of salts were prepared and injected in the instrument.
296 ¼ diluted Hoagland solution was used for the evaluation as used nutritive solution for plant
297 growth. For ESI-FTICR MS measurements, 100-fold diluted SPE solutions in water/methanol
298 (50/50, v/v) were used, while for evaluation on MALDI-FTICR MS, the SPE solution with
299 the DHB matrix 1:10 +0.1 % FA was the only investigated. Salt solution was added at a
300 percentage of 0.01%, 0.05%, 0.1%, 0.5%, 1%, 5% and 10% on the final volume of the
301 obtained solutions for ESI- and MALDI-FTICR MS. Measurements were performed in the
302 two modes according to the experimental parameters used before and each acquisition was
303 performed on 50 accumulated scans.

304 **2.7 Data analysis for ESI-FTICR MS and MALDI-FTICR MS measurements**

305 Following data acquisition, all data arising from both ESI- and MALDI-FTICR MS
306 measurements were treated on Bruker Compass DataAnalysis software (v. 5.1). Each
307 accumulated mass spectrum was peak picked (S/N 8), then mass calibration was confidently
308 performed using a mass list of *Pisum Sativum* RE metabolites identified through DDA-MS
309 (Table 3S). Standard deviation on m/z values determined after internal calibration on a set of
310 34 points was found to be below 0.100 ppm using a linear fit. Spectra related to samples were
311 subtracted of blanks in Xpose mode with ratio 5 and molecular formula were assigned on the
312 subtracted spectra with the common boundaries: C₁₋₅₀, H₁₋₁₀₀, N₁₋₈, O₀₋₃₀, S₀₋₁ and P₀₋₁.
313 Attribution was performed considering M + H protonated forms and M + Na, K, Ca adducts
314 for positive mode, while in negative mode only M – H forms were retained. Molecular
315 formulas were assigned for peaks with relative intensity threshold of 0.2 %, within a *mSigma*

316 limit of 800 and a tolerance mass error of 0.1 ppm. RDBE limit were set from 0 to 80 and H/C
317 ratio from 0 to 3. Data containing assigned formula were exported and manually handled to
318 avoid molecular formula redundancy (molecular formula arising from protonated and adduct
319 forms). Visualization of van Krevelen and double bond equivalent (DBE) plots were obtained
320 through an in-house script. For ESI- and MALDI-FTICR MS, performance evaluation
321 measurements for both 50 and 200 accumulated spectra were acquired in triplicate. For salt
322 tolerance evaluation with the two sources triplicate analysis were acquired by accumulating
323 50 spectra. Reduced time for analysis was used to limit major salt contamination of ESI
324 source at high salt concentration.

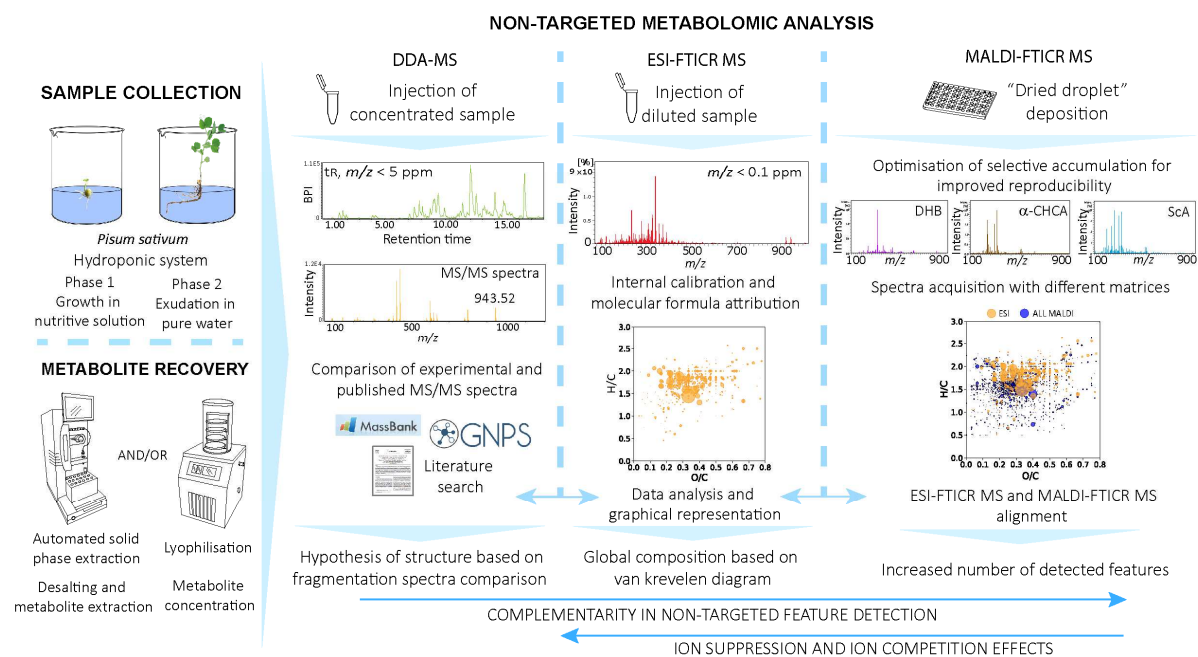
325 2.8 Solvents and reagents

326 All solvents and additives used to prepare mobile phases and sample solutions for ESI- and
327 MALDI-FTICR MS measurements were LC-MS grade and they were purchased from Fisher
328 Chemical (Pittsburgh, PA). 2,5 Dihydroxybenzoic acid, 9-aminoacridine and trans-2-[3-(4-
329 tert-Butylphenyl)-2-methyl-2-propenylidene] malononitrile were at 98% purity. 1,5-
330 Naphthalenediamine, sinapinic acid and sinapic acid were at 99% purity. All matrices were
331 purchased from Sigma-Aldrich (Saint Luis, MO, USA). LC-MS grade formic acid used for
332 sample acidification was purchased from Sigma-Aldrich (Saint Luis, MO, USA).

333

334 3. Results and discussion

335 Root exudates were submitted to preliminary DDA-MS and to ESI-FTICR MS analysis. This
336 allowed to perform at first the annotation of major compounds based on the manual
337 dereplication of fragmentation spectra and on the use of annotation tools as GNPS. Secondly,
338 thanks to the van Krevelen diagram the global metabolic profile of *P. sativum* root exudates
339 was obtained, highlighting the main chemical families. Once characterization performed with
340 the two first techniques, sample were therefore subjected to MALDI-FTICR MS. The next
341 paragraphs are dedicated to the description of the results with the different mass spectrometry
342 platforms used for the analysis of RE. A summary of the analysis, annotation and
343 interpretation workflow is showed in Figure 1.



344

345 *Fig. 1: Workflow used for the non-targeted metabolomic profiling of P. sativum root exudates*
 346 *based on DDA-MS, ESI-FTICR and MALDI-FTICR complementarity*

347 **3.1 Identification of *Pisum sativum* root exudates based on DDA-MS measurements and**
 348 **direct introduction ESI-FTICR-MS**

349 At first, DDA-MS measurements on *P. sativum* RE extracts obtained from SPE extraction
 350 were performed in ESI positive mode through a hybrid QTOF mass spectrometer.
 351 Experimental spectra were then submitted to literature manual search and database match on
 352 the GNPS website. A list of metabolites for the 48 major compounds with proposed
 353 annotation based on MS/MS spectra can be found in table 3S. The common metabolomic
 354 annotation level was used to rank confidences in proposed metabolite structures [89]. The
 355 compounds of interest belonged to seven molecular classes, namely nucleosides, amino acids,
 356 fatty acids, fatty acid amides, alkaloids, oligopeptides, phosphocholines and triterpenes. In
 357 addition to the main compound classes, other minor features presented fragmentation spectra
 358 agreeing with polyphenols, glucosyl-phenols and indoles. In this study, on a total of 48, 34
 359 features were annotated at level 2 of confidence and 14 features at a level 3. In fact, some of
 360 the most intense metabolites detected via DDA-MS experiments did not found match with
 361 literature or database search. For example, feature **19** at m/z 367.1863 and molecular formula
 362 $C_{18}H_{26}N_2O_6$ was detected together with two analogues and an isomer (features **23**, **25**, **27**)
 363 which were separated through chromatography. The fragmentation spectrum (figure 2S)
 364 presented two losses of 18 Da with fragment ions at m/z 349.17 $[C_{18}H_{24}N_2O_5+H]^+$, m/z 331.16

365 $[\text{C}_{18}\text{H}_{22}\text{N}_2\text{O}_4+\text{H}]^+$, and fragments at lower m/z corresponding to m/z 139.06 $[\text{C}_6\text{H}_6\text{N}_2\text{O}_2+\text{H}]^+$,
366 m/z 121.04 $[\text{C}_6\text{H}_4\text{N}_2\text{O}+\text{H}]^+$ and m/z 90.05 $[\text{C}_5\text{H}_4\text{N}_2+\text{H}]^+$. These metabolites presented a
367 fragmentation pathway similar to that of N-acyl homoserine lactones [90] and they were
368 therefore annotated as fatty acid amides containing histidine or urocanate connected with a
369 C18 or a C16 alkyl chain. Fatty acid amides, as N-acyl homoserine lactones, have been found
370 to have an important role as *quorum sensing* molecules for bacteria communication and
371 cooperation, especially in biofilm formation [91,92]. Moreover, it must be noted that histidine
372 and urocanic acid (a product of histidine metabolism) are found at high concentration in the
373 genus *Fabaceae*, which *P. sativum* belongs to, supporting the proposed annotation made for
374 the fatty acid amides.[93]. Nonetheless, it must be noted that the fragmentation pathway
375 showed some similarities with database spectra of some alkaloids as imidazole [94] or
376 isoquinoline alkaloids [95]. Hence, this alternative annotation should not be excluded.
377 Annotation as fatty acid amides was kept as the most trustable because the analyzed fraction
378 showed the presence of many metabolites connected to lipid and lipid-like molecules. In this
379 regard, our study pointed out on the presence of specialized metabolites, not yet isolated and
380 discovered that can be a starting point for further research. The unambiguous identifications
381 of the proposed metabolites should be performed through comparison of the experimental
382 results with standard compounds or with complementary techniques as isolation and
383 characterization through NMR. For many of the proposed metabolites, the biological role in
384 *P. sativum* root exudation process as not been yet discovered, but according to published
385 literature, *P. sativum* RE contain a high number of nitrogen-containing compounds with basic
386 functional groups that are ionized preferentially in positive mode [96]. In fact, nitrogen
387 compounds have been found to cover an important role in root-bacteria communication and
388 ecosystem functioning. Metabolites for which a hypothesis of structure was formulated (Table
389 3S) were used to create a list of reference compounds to be used as internal mass calibrants in
390 ESI-FTICR MS measurements. Chemical classes were then used to gather the additional
391 features detected through ultra-high-resolution mass spectrometry measurements and to
392 discuss the differences in ionization *via* ESI- and MALDI-FTICR MS (next paragraphs).
393 Once the first information on *P. sativum* RE obtained, 100-fold diluted RE SPE extracts were
394 then submitted to ESI-FTICR MS analysis. Mass spectra of both samples and blanks acquired
395 in positive mode accumulating 200 scans where then internally calibrated using a linear fit.
396 The standard error over a m/z 98-1500 range was lower than 0.100 ppm. After blank
397 subtraction, molecular formulas were attributed for one analytical replicate according to the
398 rules described in the materials and methods section. The wide diversity of heteroatoms (N, P,

399 S) and the presence of many adducts (Na, K and Ca) led often to multiple formula for each
400 peak, especially for high m/z values in positive mode. No other adducts (Mg, Mn) resulted in
401 preliminary formula assignment trials and they were therefore not considered. In general,
402 when a double assignment was present, peaks were checked manually to remove unlikely
403 molecular formula. This was performed when possible, based on the highest fit of the isotopic
404 fine structure distributions for proposed formulas and experimental peaks. Conversely, the
405 most logical formula based on the molecular classes that were found through DDA-MS
406 experiments was retained. Nonetheless, it must be considered that different adducts could be
407 present for the same molecule. For this reason, the obtained list was further cleaned up in
408 order to reduce data redundancy and avoid that one molecule ionized in different forms (for
409 example H^+ form and Na^+ form) could led to many different H/C ratios. Hence, molecular
410 formulas of protonated forms were subtracted of a hydrogen and molecular formulas
411 corresponding to Ca adducts were added of a hydrogen. Formula for K and Na adducts were
412 kept unaltered. The list of molecular formula was then used to plot H/C ratios in function of
413 O/C ratios and construct therefore the van Krevelen diagram (figure 3S) for ESI ionization.
414 An in-house Python script was used for data visualization. As expected, an increase of the
415 number of detected features was obtained thanks to ESI-FTICR MS measurements, with 486
416 molecular formulas assigned in positive mode for the SPE sample. At our best, resulting
417 annotation were submitted to PubChem search in order to propose insight on molecular family
418 and subclasses. Figure 3Sa shows that *P. sativum* RE was characterized by a significant
419 number of nitrogen-containing compounds, which consisted in a wide range of nucleosides
420 and cytokines, amino acids and small oligopeptides, fatty acid amides, alkaloids and
421 phosphocholines. Compounds that did not contain nitrogen or other heteroatoms were
422 confirmed to be the triterpenes soyasaponins, a wide range of fatty acids that includes some
423 plant hormones as jasmonic acid derivates or flavonoids. Among CHO compounds, a group of
424 features that were not detected in DDA-MS had a molecular formula corresponding likely to
425 glycolipids, which was taken as the best annotation. Moreover, thanks to the hypothesis of
426 structures and information on the molecular families present in the sample, some
427 unconventional molecular families were assigned for the first time to a definite zone of the
428 van Krevelen diagram (Figure 3Sb). For example, it was possible to trace back the N-
429 acylethanolamines at $H/C = 2.0$ and $O/C = 0.1$, the fatty acid amides containing histidine or
430 urocanic acid at $H/C = 1.5$ and $O/C = 0.3$ in a zone normally designed for oligopeptides, the
431 soyasaponins glucosides at $H/C = 1.6$ and $O/C = 0.4$. Similarly, oligopeptides at high N
432 number ($N=6$ or 7) were placed at $H/C = 1.3$ and $O/C = 0.2$ and glycolipids in an intermediate

433 zone between fatty acids and sugars, at $H/C = 2$ and $O/C = 0.5$. The list of metabolites found
434 through ESI-FTICR MS can be found in supplementary material. In general, all molecular
435 formula for the compounds found through DDA-MS were found in their protonated or Na^+ ,
436 K^+ and Ca^{2+} adducts and were therefore confirmed through ESI-FTICR MS, giving a higher
437 confidence level in metabolites annotation. Despite this, some compounds on a total of 48
438 annotations were not found in ESI-FTICR MS spectra (table 3S). This could be due to the
439 presence of residual salts or to an important number of ions that led likely to ion suppression
440 and ion competition effects, despite the sample desalting through solid phase extraction. In the
441 attempt to overcome these effects, experiments based on direct introduction MALDI-FTICR
442 MS based on dried droplet deposition were performed and ionization in the two sources
443 compared.

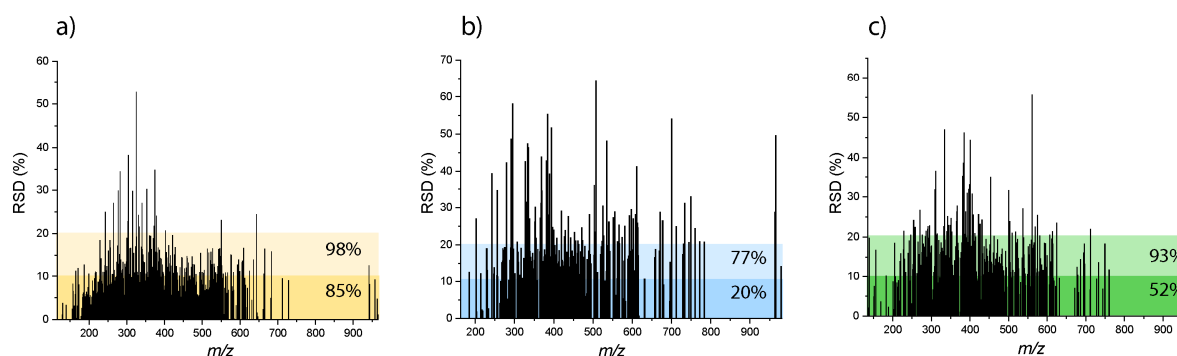
444

445 **3.2 Optimization of a direct introduction MALDI-FTICR-MS method for a non-** 446 **targeted metabolomic approach**

447 In ESI measurements samples are commonly injected with a constant flow by direct infusion.
448 This translates likely in constant scan-to-scan ion abundance transmitted to the ICR cell, to
449 reduced error on exact mass measurement and eventually, to good reproducibility of ion
450 intensity in repeated measurements. In contrast, traditional MALDI sample preparation based
451 on the “dried droplet” deposition has as main drawback an inhomogeneous distribution of
452 analytes and matrices crystals in the deposited sample [62]. This represents a problem for
453 quantitative analysis and, in the case of FTICR MS measurements, it can affect the
454 reproducibility due to a change of scan-to-scan ion abundance transmitted to the ICR cell. In
455 order to evaluate ESI and MALDI for a non-targeted metabolomic approach based on FTICR
456 MS, repeatability was evaluated by calculating the relative standard deviation (RSD) on
457 absolute signals intensity within three intra-day acquisitions of 50 accumulated scans for each
458 mass spectrum. Results in ESI-FTICR MS mode obtained for *P. sativum* RE (SPE) samples
459 showed that reproducibility of ion signals for the 85% of the features in the m/z range 98-1500
460 was found appropriate, with deviation typically below 10%. Moreover, most metabolites
461 showed RSD lower than 20% (Figure 2a). For the evaluation of reproducibility in intra-day
462 MALDI-FTICR MS measurements on dried droplet deposited samples for non-targeted
463 metabolomics, 50 scans accumulated spectra were first recorded without any supervision on
464 the scan-to-scan ion abundance transmitted to the ICR cell during acquisition. Figure 2b
465 shows the obtained RSD for MALDI features in unsupervised measurements: only 20% of the

466 features were found to have a 10% RSD and 77% below the 20%, while RSD up to 60%
467 characterized a greater number of features. In order to minimize the variation of ion
468 abundance in the ICR cell during the acquisition and to improve reproducibility in repeated
469 measurements, an optimization of the selective accumulation was performed. 50 scans
470 accumulated spectra were recorded using different ranges of selective accumulation. Selective
471 accumulation allows to set a minimum (min) and a maximum (max) of intensity [97]. Mass
472 spectra out of this range are discarded from the final average mass spectrum. Three ranges of
473 selective accumulation were tested. A first range with min intensity 5×10^7 and max 5×10^8 , a
474 second range at min 1×10^8 and max 5×10^8 and a third range at min 1×10^8 and max 3×10^8 .
475 Figure 4S shows the profiles of accumulation on the total acquisition obtained from scan-to-
476 scan total ion current entering in the ICR cell and considered for final spectrum, during a total
477 of 50 scans. In general, more the range of selective accumulation was narrow, more the total
478 ion current resulting from accumulation assumed a linear trend, reducing progressively the
479 “wavy trends” corresponding to low signal accumulation scans, as showed in figures 4Sb and
480 4Sc. Figure 4Sd shows that an ideal linear trend was obtained when selective accumulation
481 spanned from a min of 1×10^8 to a max of 3×10^8 for each accumulated scan. It is worth to note
482 that the lower limit influenced the signal threshold and its optimization allowed to discard
483 acquisition scans corresponding to low density zone on the MALDI spot in which only signals
484 of the noise were present. In this regard min value at 5×10^7 led to the accumulation of spectra
485 in which no signal corresponding to metabolites were present. While, the value of min
486 selective accumulation equal set to 1×10^8 allowed to obtain total ion signal in the mass
487 spectra in the $9 \times 10^6 - 1 \times 10^7$ range. Similarly, the optimization of the max limit avoided
488 accumulation of too many ions in “hot spots”. It is worth to note that the overall scan-to-scan
489 intensity with optimized ranges corresponded to an effective min intensity of 1×10^7 to a max
490 effective intensity of 3×10^8 in mass spectra. This range corresponded to the intensity variation
491 in one ESI mass spectrum, allowing a better comparison of final spectra acquired with the two
492 ion sources. The optimization of the selective accumulation range resulted in the overall
493 increase of mass spectra reproducibility and in a higher mass precision. In fact, RSD with
494 MALDI reached satisfying values approaching results obtained with ESI. Figure 2c shows
495 that with optimized ranges, 52% of metabolites had RSD below 10% and 93% in the 20%
496 limit. Similarly, the standard error over a m/z 98-1500 range was reduced from 0.150 ppm in
497 the unsupervised analysis to 0.080 ppm using the optimized selective accumulation ranges
498 These results represented an improvement towards a reliable analytical method based on
499 classical deposition method for MALDI-FTICR MS applied to metabolomic analysis,

500 especially for the confident formula assignment in presence of high atomic diversity
501 Nonetheless, it must be noted that, several scans were discarded yielding to an increase of
502 analysis time up to 150%. For instance, for 200 accumulated scans, the run time was around 5
503 min with unsupervised analysis and up to 12 to 15 min with the optimized selective
504 accumulation range. Optimization was performed on a same spot containing DHB mix with
505 the sample in proportion 1:10 + 0.1% FA. The laser power was optimized for all matrices in
506 order to get comparable ionization thresholds, the optimized range of selective accumulation
507 fitted with all other conditions.



508
509 *Fig. 2: Relative standard deviation of signal intensity calculated for common metabolites on*
510 *n=3 replicates found in Pisum Sativum RE for a) direct introduction ESI-FTICR MS, b)*
511 *MALDI-FTICR MS without optimization of the selective accumulation and c) MALDI-FTICR*
512 *MS under optimized conditions laying in a selective accumulation range of $min=1\times 10^8$ and*
513 *$max=3\times 10^8$.*

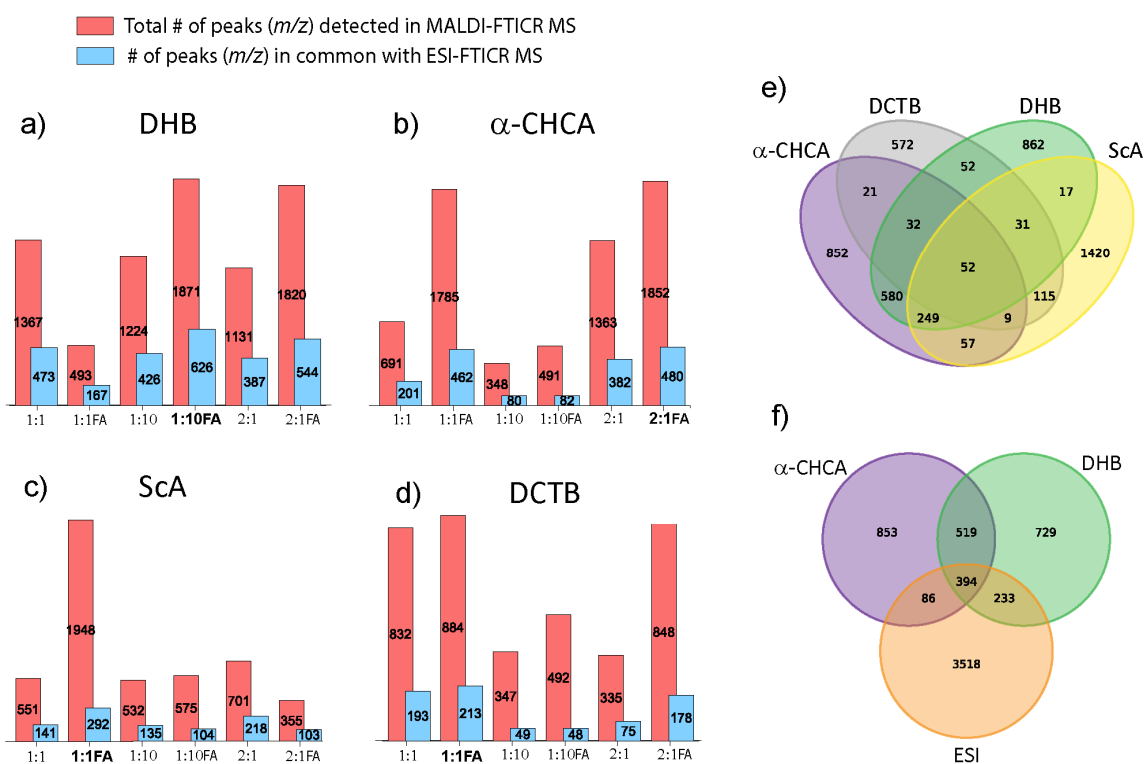
514

515 3.3 Evaluation of the efficiency of different MALDI matrices

516 In order to perform MALDI-FTICR MS analysis in best conditions for non-targeted
517 metabolomic aims, an optimization of the most suited sample/matrix combination was
518 performed. *P. sativum* RE SPE samples were mixed with the 6 matrices selected in this study
519 at a ratio 1:1, 2:1 and 1:10. As ESI-FTICR MS measurements in positive mode were
520 performed on acidified solutions to favor protonated forms against Na^+ and K^+ adducts, the
521 three sample/matrix ratio for each matrix were also tested with the addition of 0.1% FA. For
522 negative mode, the mixtures sample/matrix were used without any further addition. Solutions
523 were deposited using the “dried droplet” method, dried under vacuum and analyzed through
524 MALDI-FTICR MS. Mass spectra for the 24 conditions in positive mode and nine conditions
525 in negative mode were acquired using the optimized selective accumulation range for 50

526 accumulated scans. Mass spectra were treated as previously described based on the three-steps
527 procedure including internal calibration, blank subtraction and molecular formula assignment.
528 The optimization of the best condition for each matrix was performed at an early stage of the
529 study, the criteria for best condition selection was fixed to the higher number of common m/z
530 values found in ESI- and MALDI-FTICR MS spectra and on the highest number of features
531 obtained in order to catch the maximum of detected features for a non-targeted metabolomic
532 study (Figure 3). As alignment based on m/z values is performed before molecular formula
533 assignment, isotopic peaks were counted in the total number of peaks. Nonetheless,
534 comparative analysis for the different conditions could be still indicative of the best matrix
535 conditions. For instance, among the six conditions tested for DHB, the ratio sample:matrix
536 1:10 with the addition of 0.1% FA led to 626 common peaks with ESI-FTICR MS spectra and
537 to a higher number of detected m/z values, while the other ratios using DHB were
538 characterized by lower numbers. This led finally to select for further MALDI non-targeted
539 metabolomic investigations in positive mode, the DHB matrix the ratio 1:10 + 0.1% FA, for
540 α -CHCA the ratio 2:1 + 0.1% FA, for ScA and DCTB the ratio 1:1 + 0.1% FA. Moreover, an
541 inspection of the histograms highlighted the presence of a relatively high number of peaks
542 which were exclusively detected in MALDI spectra. These additional molecular formulas
543 could arise both from the presence of multiple adducts formed and/or to additional
544 metabolites for which detection was preferred in MALDI-FTICR MS mode (discussion in the
545 next paragraph). Based on the number of common peaks, the global ionization of the sample
546 in MALDI-FTICR MS was obtained in the order DHB > α -CHCA > ScA > DCTB in positive
547 mode. In fact, the ratio of common peaks on the total m/z detected in ESI-FTICR MS spectra
548 (4231) was respectively 14.7%, 11.3%, 6.9% and 5%. Nonetheless, it must be considered that
549 in presence of residual salts especially in ESI, the total m/z detected do not correspond
550 necessarily to a corresponding number of unique features and that one compound may be
551 present in form of multiple adducts.

552



553
 554 *Fig. 3. Evaluation of the total number of peaks and the common peaks found in common with*
 555 *ESI-FTICR MS spectra at different ratio sample/matrix for a) DHB, b) α -CHCA, c) ScA and*
 556 *d) DCTB in MALDI-FTICR MS spectra acquired in positive mode. Best ratios are highlighted*
 557 *in bold style. Venn diagram obtained from alignment of m/z lists for the four selected*
 558 *conditions (DHB 1:10 + 0.1% FA, α -CHCA 2:1 + 0.1% FA, ScA and DCTB 1:1 + 0.1% FA)*
 559 *and for the two highest ranked matrices (DHB 1:10 + 0.1% FA, α -CHCA 2:1 + 0.1% FA)*
 560 *with ESI-FTICR MS spectra are showed respectively in e) and f).*

561
 562 This can explain the surprising low percentage found for common peaks in ESI- and MALDI-
 563 FTICR MS spectra at this stage. A discussion on the number of common features obtained for
 564 ESI and MALDI after molecular formula assignment is presented in the following paragraphs.
 565 In general, a great conformity was found for all tested matrices, as the Venn diagram obtained
 566 from alignment of the four best conditions showed the presence of many common m/z values,
 567 even when two matrices of different chemical nature were compared. Only ScA was
 568 characterized by a high number of additional peaks (1420) respect to ESI which were not
 569 either in common with other matrices used in MALDI (Figure 3e). Nonetheless, DHB and α -
 570 CHCA obtained the highest score when compared with ESI and between the two, DHB
 571 seemed to be the most appropriate for non-targeted metabolomics applications. It must be

572 noted that these results considered only the number of common peaks but did not take into
573 account any information on the type of metabolites, on their intensity or on their detection in
574 form of different adducts. All these concepts will be developed more in the following
575 paragraphs.

576 MALDI in negative mode resulted in poor metabolites detection (Figure 5S) and low number
577 of common features with ESI spectra (Figure 6S) in respect to positive mode (Figure 3). In
578 fact, alignment in negative mode should be more indicative, as compounds were detected in
579 ESI spectra preferentially as deprotonated forms, removing data redundancy experienced in
580 positive mode. The best ratios sample / matrix were for 9AA 1:1, for DAN 2:1 and for DCTB
581 1:1. In these conditions, the ratio of common peaks on the total of total m/z detected in ESI in
582 negative mode (1987) was 5.2% for 9AA, 6% for DAN and 4.3% for DCTB. In conclusion,
583 spectra in ESI negative mode were very informative and easy to study thanks to the absence
584 of adducts. In comparison, MALDI negative mode did not bring substantial further
585 information in the case of RE of *P. Sativum*. As a contrary, results in MALDI positive mode
586 were highly complementary to the results in ESI positive mode. As a consequence, the
587 negative mode in MALDI was not further investigated in this study and the following
588 discussion will be focused on the non-targeted metabolomic approach in positive ion mode.
589 Nonetheless, MALDI FTICR MS in negative mode should not be discarded *a priori* and
590 further studies could be carried out with other matrices.

591

592 **3.4 Comparison of ESI-FTICR MS and MALDI-FTICR MS for non-targeted** 593 **metabolomics on *Pisum sativum* root exudates**

594 Once best conditions for MALDI ionization parameters and best ratios sample/matrix
595 obtained, experiments were repeated for the selected conditions accumulating a higher
596 number of spectra (200 accumulated scans). Measurements performed in triplicates were
597 obtained from three different spots for each condition. Selected conditions for MALDI non-
598 targeted metabolomics on *P. sativum* RE were for DHB analyte/matrix ratio of 1:10 and 0.1%
599 FA, for α -CHCA analyte/matrix ratio of 2:1 and 0.1% FA, for ScA analyte/matrix ratio of 1:1
600 with 0.1% FA. Comparison was possible with ESI spectra as, scan-to-scan accumulation, total
601 number of scans and final intensity resulted comparable (Figure 4S). Internal calibration,
602 blank subtraction and molecular formula attribution were performed as previously stated.

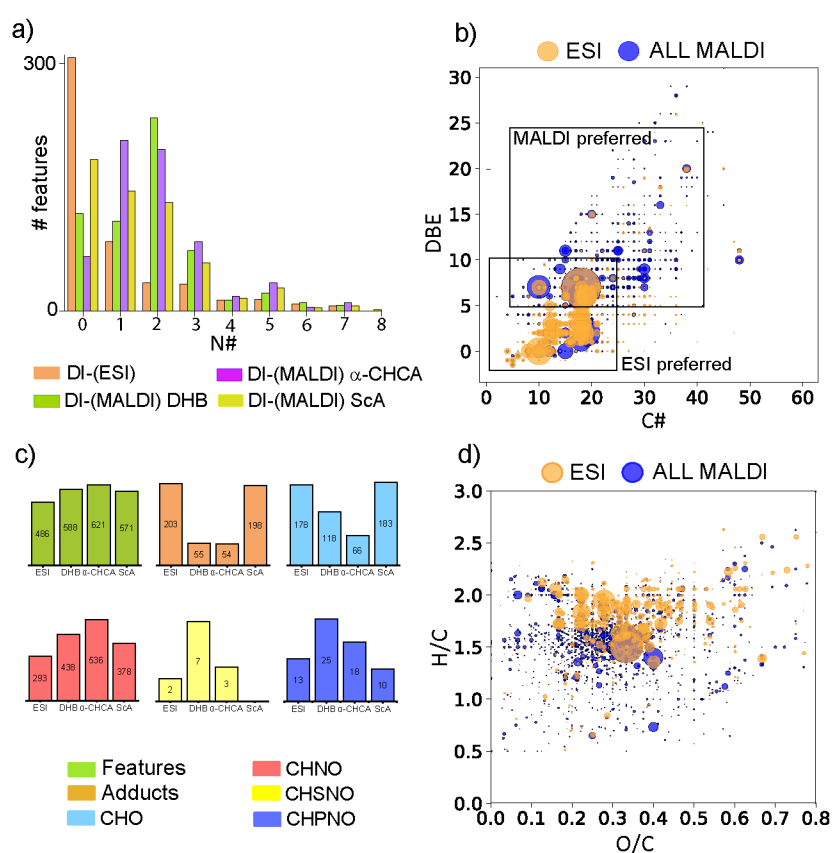
603 Figure 7S shows a comparison of the absolute intensity of selected features in ESI and
604 MALDI with the three different matrices. The ionization efficiency towards six main families

605 were then compared. The six families were nucleosides (Figure 7Sa), amino acids and
606 oligopeptides (7Sb), phosphocholines and phosphoserines (7Sc), fatty acids (7Sd), fatty acid
607 amides containing histidine (7Se) and terpenes (7Sf). For each family, a number ranging from
608 six to nine metabolites were selected according to the presence of a hypothesis of structure
609 raising from DDA-MS measurements or, for assimilation to a chemical family, according to
610 the molecular formula obtained from the ESI measurements. Error bars are relative standard
611 deviation on the three replicates intensities obtained from three different spots. An analysis of
612 the different chemical families allowed to retrieve the presence of particular ionization trends.
613 For example, for all the studied nucleotides (Figure 7Sa), a better ionization yield was
614 obtained in MALDI using α -CHCA as matrix, with an increase of absolute intensity up to
615 250% respect to the trace obtained in ESI. Conversely, DHB and ScA resulted less efficient in
616 the ionization of nucleosides, leading to lower absolute intensities for the studied metabolites,
617 when compared to ESI. For amino acids and oligopeptides (Figure 7Sb), the number of
618 nitrogen atoms (N) in the molecule resulted crucial in the intensity yield. In fact, for low N
619 number as for phenylalanine (Figure 7S-1b) or for oligopeptide with $N \leq 4$ (Figure 7S-2b, -3b,
620 -4b) ESI resulted in higher signal intensities, while MALDI with DHB matrix enabled a very
621 efficient detection of features containing $N \geq 5$. Moreover, α -CHCA led to higher absolute
622 intensity for oligopeptides containing four to five N. In general, DHB was found to be the
623 most suited matrix for oligopeptides with a signal increase respect to ESI up to 116 % and 85
624 % respect to α -CHCA. Fatty acids were preferentially ionized and detected in ESI (Figure
625 7Sd). All fatty acids features were detected in MALDI, but at relatively low intensity.
626 Triterpenes analyzed in the measurements included compounds as soyasaponin di-glucosides.
627 Soyasaponins (Figure 7Sf) showed good ionization yield in MALDI with DHB matrix and in
628 less extent also with α -CHCA. Though, it must be noted that in ESI soyasaponins were
629 ionized as protonated and Na adducts at comparable extent, while in MALDI, even with the
630 addition of a 0.1% FA in the sample/matrix solution, the only adducts were the Na forms.
631 Figure 7Sf shows therefore a comparison of intensity for Na adducts formed in the two
632 sources. In the case of phosphocholines and phosphoserines (Figure 7Sc) and of fatty acid
633 amides containing histidine (Figure 7Se), it was not possible to retrieve a global trend of
634 ionization. For these chemical classes, preferential ionization seemed to be strongly dependent
635 on the peculiar chemical structure. MALDI spectra obtained using ScA showed a preference
636 for the ionization of oligopeptides, alkaloids and for some phosphocholines, but in general,

637 signals remained of poor intensity and showed therefore no major interest in non-targeted
 638 metabolomic approaches.

639 In general, independently from the matrix, ionization in MALDI appeared to be preferred for
 640 molecules containing high number of nitrogen atoms and to increase with the presence of
 641 conjugate systems, as in the case of nucleotides. This hypothesis was first been made thanks
 642 to rationalization of ionization yield for the metabolites annotated through DDA-MS
 643 measurements. In order to deeply investigate this aspect, after molecular formula assignment,
 644 the number of features (#) containing N atoms spanning from zero to eight was counted for
 645 ESI and for MALDI with DHB, α -CHCA and ScA and plotted as histogram (Figure 4a).

646



647

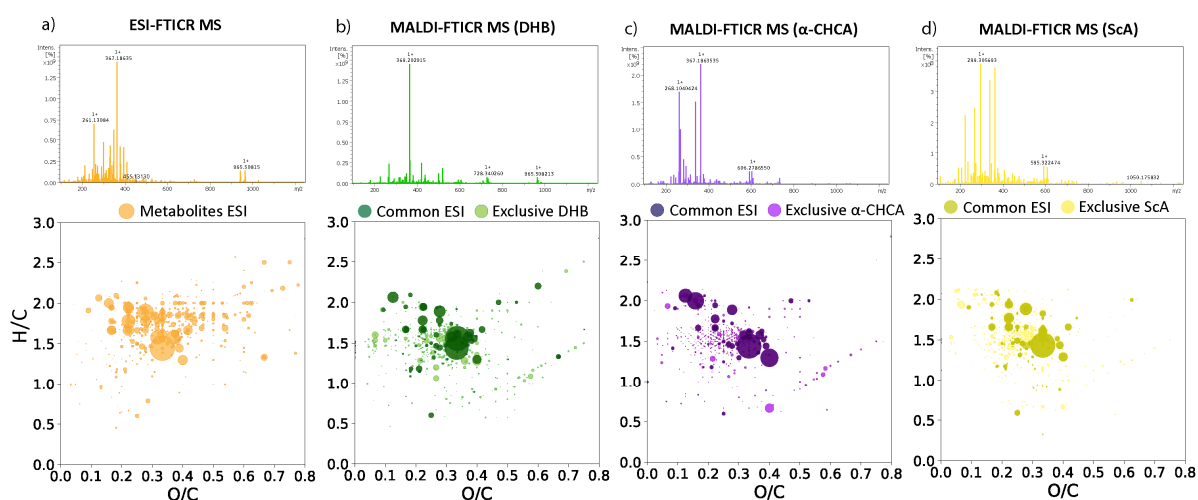
648 *Fig. 4: a) Histograms showing the number of detected features containing from one to eight*
 649 *atoms of nitrogen in ESI (orange), MALDI DHB (green), MALDI α -CHCA (purple) and*
 650 *MALDI ScA (yellow); b) and d) show respectively the DBE vs #C plot and the van Krevelen*
 651 *diagram obtained from molecular formula assignment of ESI (orange) and MALDI (blue)*
 652 *obtained from the three matrices (DHB, α -CHCA and ScA) spectra; c) total number of*
 653 *assigned molecular formulas (green) and adducts (orange) together with the distribution of*

654 features containing CHO (light blue), CHNO (red), CHNSO (yellow) and CHNPO (blue)
655 atoms.

656
657 The molecular formula assignment revealed that effectively an increase of the number of
658 features containing N was obtained with MALDI even if intensity for low number of N atoms
659 resulted lower than in ESI, as previously stated (Figure 7S). On the other side, number of
660 oxygen atoms (O#) seemed to have less effects on ionization *via* MALDI (Figure 8S), and a
661 more relevant increase of features containing O# from two to four could be experienced
662 probably as mirror of a higher N# in the molecules. These results agreed with the trends of
663 relative intensity (%) at different N# and O#, for which histograms are showed in figure 9Sa
664 and 9Sb, respectively.

665 Moreover, the double bond equivalent (DBE) and the carbon number (C#) were calculated
666 from each assigned molecular formula for both ESI and MALDI, gathering together all
667 features obtained from the three matrices (all MALDI). The resulting plot in Figure 4b shows
668 that effectively, additional features detected for the first time through MALDI were generally
669 characterized by higher DBE, up to 25, which corresponded to oligopeptides with N from six
670 to eight. This highlighted that, ionization for unsaturated and high N# molecules was
671 preferred in MALDI while ESI resulted in the preferential ionization of lower DBE
672 compounds. The presence of additional features appeared surprising because MALDI spectra
673 were characterized by simpler profiles respect to ESI spectra, while the obtained van Krevelen
674 diagram showed great similarity (Figure 5).

675



676
677 Fig. 5: Mass spectra obtained in positive mode and related van Krevelen diagrams for *Pisum*
678 *sativum* root exudates obtained from molecular formula assignment based on ultra-high-

679 resolution a) ESI-FTICR MS and MALDI-FTICR MS with b) DHB, 2,5-dihydroxybenzoic
680 acid, c) α -CHCA, α -Cyano-4-hydroxycinnamic acid and d) ScA, Sinapinic acid for the SPE
681 desalted *P. sativum* RE sample.

682
683 This mirrored both low signals for some metabolites and both a lower number of Na⁺, K⁺ and
684 Ca²⁺ adducts. In fact, after molecular formula attribution, most of the features found in
685 MALDI corresponded to protonated forms, while adducts were much less represented (Figure
686 4c). As an example, feature **35** at m/z 211.1325, corresponding to molecular formula C₁₂H₁₈O₃
687 (Table 3S) and annotated *via* DDA-MS as the plant hormone jasmonic acid (annotation level
688 2), was detected in ESI as Na⁺, K⁺ and Ca²⁺ adducts, while in MALDI only the protonated
689 form was found. Together with additional features, spectra obtained with MALDI showed
690 common metabolites with ESI. Nonetheless, because of wide differences in ionization, it was
691 difficult to evaluate the correct number of metabolites successfully ionized in both sources (as
692 protonated or adducts) and, counting only the protonated forms, for all matrices around 10%
693 of features were found to be in common. A list of metabolites found in ESI, MALDI (DHB)
694 and MALDI (α -CHCA) can be found as supplementary material. Examples of additional
695 features corresponding to potential metabolites which were detected for the first time in *P.*
696 *sativum* RE thanks to MALDI were features at m/z 152.056675 (C₅H₅N₅O, proposed
697 identification guanine), m/z 156.076770 (C₆H₉N₃O₂ proposed identification histidine), m/z
698 166.072361 (C₆H₇N₅O proposed identification methylguanine). The increase in the number of
699 metabolites detected in MALDI corresponded to small metabolites, but also to molecules with
700 higher m/z that are consistent to triterpenes and oligonucleotides as features at m/z
701 328.284593 (C₁₉H₃₇NO₃, proposed identification palmitoyl alanine), m/z 455.351968
702 (C₃₀H₄₆O₃, proposed identification moronic acid) or m/z 740.340207 (C₃₉H₄₅N₇O₃, proposed
703 identification cyclo[His-Tyr-Tyr-Leu-Tyr]). Features that were exclusively detected in
704 MALDI are highlighted in bold style in supplementary materials.

705 Figure 4d shows the van Krevelen diagram obtained from molecular formulas obtained using
706 ESI and MALDI with all matrices. It is worth to note that additional features are not likely to
707 come from MALDI in-source fragmentation as the laser power and the number of shots were
708 optimized for each matrix/sample mixture in order to be just slightly above the matrix
709 ionization threshold. Moreover, the presence of the same additional metabolites in the spectra
710 obtained from two distinct matrices represents an additional proof of the selective detection in
711 MALDI mode. Nonetheless, it should be considered that in non-targeted metabolomic, the
712 different nature of the metabolites could lead to different behaviors of chemical classes in

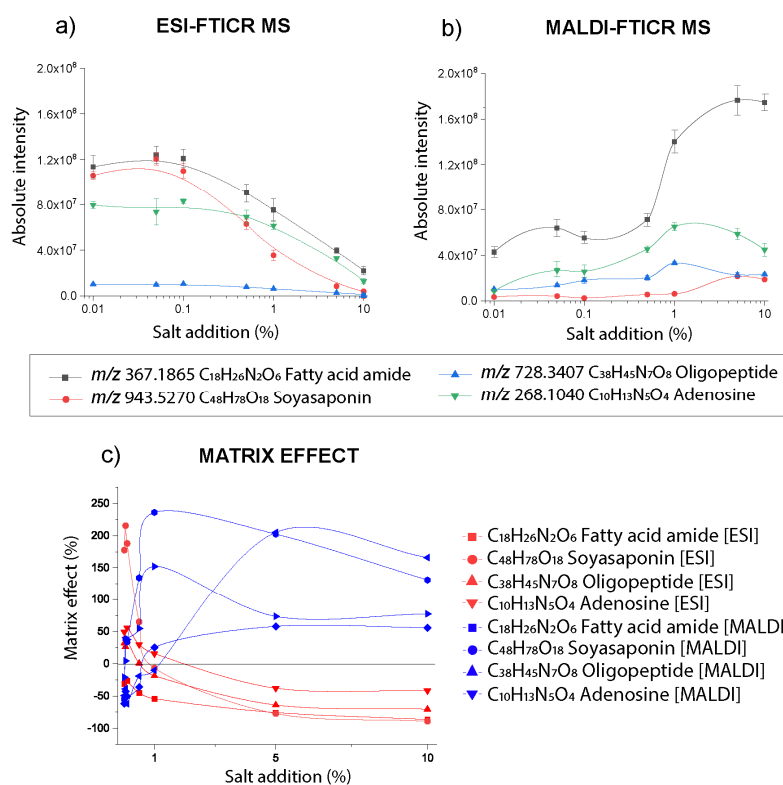
713 presence of the matrix, and a complete absence of fragmentation cannot be excluded *a priori*
714 without further investigation. Anyway, results obtained herein shows that MALDI enabled a
715 wide detection of compounds of different chemical classes, and it confirmed its potential as a
716 complementary technique to ESI for a non-targeted metabolomic approach on biological
717 samples.

718

719 **3.5 Salt tolerance and matrix effects in ESI and MALDI**

720 In order to evaluate salt tolerance of ESI and MALDI techniques for non-targeted
721 metabolomics, mass spectra were recorded for a series of *P. sativum* RE SPE solutions which
722 were spiked with various salt amounts. Indeed, in order to simulate the release of salts in
723 hydroponic cultures, solutions were spiked with aliquots of Hoagland solution [98]. The last is
724 a mixture of many salts for which the concentration is normally expressed in terms of
725 electrical conductivity (EC, dS m⁻¹), which depend on the metal content [99]. For example, ½
726 Hoagland solution has EC of ~1.1 dS m⁻¹, which for scale comprehension, corresponds to a
727 concentration of ~ 10 mM NaCl, at pH=7 [100]. It is worth to note that, during the collection
728 of the sample, a part of salts can be released together with the metabolites, but at any extent,
729 the concentration should exceed the initial Hoagland EC used for plant growth. Series of
730 solutions for measurements in the two sources were therefore prepared by adding a ¼
731 Hoagland solution at 0.01%, 0.05%, 0.1%, 0.5%, 1%, 5% and 10% on the final sample. Salt
732 ranges were chosen starting from a minimum equivalent salinity ~ 6 µM NaCl up to ~ 0.6 mM
733 NaCl corresponding to the addition of 10% ¼ Hoagland solution. Measurements were
734 performed in triplicates both for ESI and MALDI. For MALDI, the DHB matrix was selected
735 because of its suitability for non-targeted metabolomic study as demonstrated before. In fact,
736 even if α-CHCA led to higher intensity for many metabolites, DHB resulted in the
737 simultaneous detection of many chemical classes. The variation of the absolute intensity of
738 four case compounds belonging to different chemical classes, namely fatty acids,
739 oligopeptides, soyasaponin triterpenes and nucleosides, at increasing salt concentrations is
740 shown in Figure 6a for ESI and Figure 6b for MALDI.

741



742

743 *Fig. 6: Variation of the absolute intensity of four compounds of interest at increasing salt*
 744 *concentrations in a) ESI and b) MALDI. Salt concentration is expressed in terms of % of the*
 745 *Hoagland solution volume added to the sample (at log₁₀ scale). Matrix effect expressed as*
 746 *[(I_{ME}/I_{SPE})/I_{SPE} × 100] in function of the salt addition for the four case metabolites in ESI and*
 747 *MALDI are showed in panel c. Error bars calculated as standard deviation on three repeated*
 748 *measurements are also shown.*

749 The protonated form for m/z 367.1865 C₁₈H₂₆N₂O₆ (annotated as fatty acid amide containing
 750 urocanate), m/z 728.3407 C₃₈H₄₅N₇O₈ (annotated as an oligopeptide), m/z 268.1040
 751 C₁₀H₁₃N₅O₄ (annotated as adenosine) and the sodium adduct for feature at m/z 943.5270
 752 C₄₈H₇₈O₁₈ (annotated as soyasaponin) were chosen for comparison of the two sources. Two
 753 distinct trends were observed in the two sources with the increase of salt concentration. In
 754 fact, while in ESI a ~ 6-fold signal drop was measured at salinity 10% for all compounds
 755 (Figure 6a), the opposite trend was obtained in MALDI (Figure 6b). Not only the intensity
 756 yield for all the ions remained mainly constant up to 0.5% Hoagland, but surprisingly, salts
 757 addition had a positive matrix effect on ionization in the range 0.5%-5% leading to a
 758 significant increase of the absolute intensity for all ions. Nonetheless, at Hoagland
 759 concentration higher than 5%, salt tolerance in MALDI was the optimum while at higher
 760 values the ion intensity decreased.

761 Nevertheless, the mere comparison of the absolute intensity for the case metabolites in the
762 two sources can give only an idea on matrix effects at increasing salinity. For this reason,
763 matrix effects in each source separately were further assessed by comparison of the peak
764 intensity for the studied compounds in the spiked samples and in the starting SPE desalted
765 sample which was used for salt tolerance. Matrix effect was then calculated according to
766 Stahnke et al. [101] as:

$$767 \quad \text{Matrix effect (\%)} = \frac{I_{ME} - I_{SPE}}{I_{SPE}} \times 100$$

768 I_{ME} is the intensity of a metabolite in the desalted SPE sample spiked with increasing salt
769 amounts and I_{SPE} is the intensity of the same metabolite in the desalted SPE sample (with any
770 addition of salts). The intensities were retrieved from the mass spectra obtained in ESI and
771 MALDI for both desalted samples and spiked samples. In fact, assessment of matrix effects is
772 usually performed using an analytical standard of known concentration analyzed in the same
773 conditions. However, as concentration of the investigated compounds was not known in this
774 study, the starting solution obtained through SPE before spiking was considered as a standard.
775 It must be noted that this constituted an idealization as ion charge effects due to the sample
776 complexity could influence as well the ionization of compounds in the SPE sample.
777 Nonetheless, as the same SPE sample was used for spiking, the same complexity was kept
778 into account when comparing spectra obtained with the same source. With the proposed
779 formula, matrix effect values higher than 0 % described an increase of the signal in the spiked
780 samples respect to the desalted sample, and therefore they were interpreted as signal
781 enhancement. Similarly, values lower than 0 % denoted signal suppression. Figure 6c shows
782 the obtained matrix effect (%) for four metabolites belonging to the different chemical classes
783 in the two sources at increasing salinity. Signal suppression of around 50 % were obtained in
784 ESI when passing from 0.01 to 1% salt concentration and at maximum salinity (10 % salts)
785 matrix effect accounted to 70-80%. Contrarily, strong signal enhancements were obtained in
786 MALDI with matrix effects accounting up to +150%. This mean that salt concentration equal
787 to a 5% of the total sample volume would triple signal intensity for selected ions. Effectively,
788 as signal enhancement obtained in MALDI resulted to be higher than signal suppression in
789 ESI, it could be likely that for a same compound, a significative overtake of S/N in MALDI
790 respect to ESI was experienced. It is worth to note that matrix effect does not affect all the
791 compounds at the same extent. For example, in MALDI, metabolites with high N number as
792 m/z 728.3407 $C_{38}H_{45}N_7O_8$ and m/z 268.1040 $C_{10}H_{13}N_5O_4$ showed an absolute intensity drop
793 already at 1% Hoagland concentration (Figure 6a). Ionization enhancement at high salt

794 concentration has been already showed in previous studies [102,103]and it has been explained
795 as the result of the fast precipitation of salt crystals during evaporation respect to matrix-
796 sample co-crystallization, which would decrease their location on the surface and favor their
797 emplacement at the border of the spot. In the case of this study, ionization enhancement could
798 be due to the peculiar nature of the Hoagland solution and the contained metals. In this extent,
799 three different mechanisms can be considered : 1) the presence of a chelator in the Hoagland
800 solution, namely EDTA (Table 1S), which would lead to the chelation of other metals and to
801 their unavailability in forming adducts with the metabolites of the sample [104]; 2) the
802 ionization of DHB matrix ions containing two aromatic carboxylic acid as adducts, which
803 would reduce the ionization of matrix cluster and lead to an increase of solubility in water
804 (contained in the RE sample and in less extent in the matrix solution), and eventually to a
805 better analyte incorporation and homogeneity [105]; or 3) as already studied adducts can
806 competitively binds with anionic analytes, favoring their desorption and reducing chemical
807 noise [106].

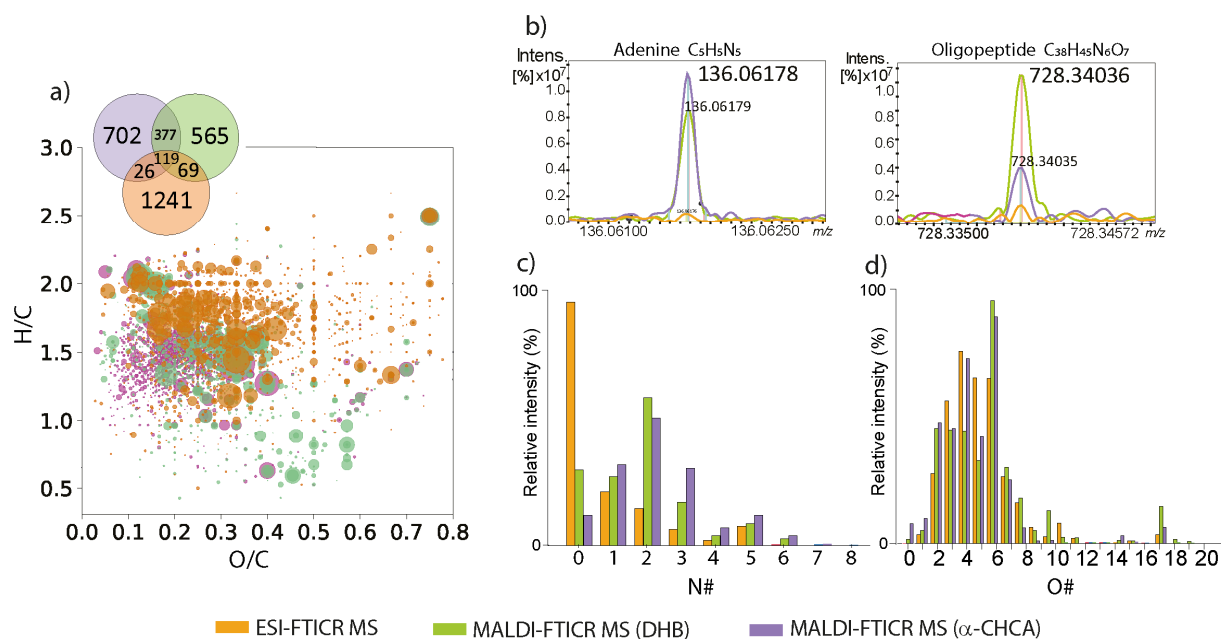
808 Likely, the improvement of the observed S/N for the metabolites in this study could be
809 explained as the combination of the three factors.

810

811 **3.6 MALDI-FTICR MS applied to a true lyophilized *Pisum sativum* RE sample**

812 The proposed method based on MALDI for non-targeted metabolomic analysis of *P. sativum*
813 RE has been optimized and evaluated on a metabolite fraction obtained through solid phase
814 extraction. This constituted a simplified sample in which, together with metabolite extraction,
815 a reduction of salts was performed. In most cases, solid phase extraction needs to be
816 optimized for each sample and it represents a long and laborious process which is time-
817 consuming. Moreover, together with salt elimination, the loss of the most polar metabolites
818 can be experienced. As RE contain normally a high number of polar metabolites,
819 lyophilization would lead to a less biased description of the real sample composition, at the
820 expense of a higher salt concentration. For this reason, in order to evaluate the performances
821 of the optimized non-targeted metabolomic approach based on MALDI on high salt
822 concentration RE sample, measurements based on MALDI and on ESI were performed on a
823 lyophilized *P. sativum* RE sample. Mass spectra were acquired, under the same experimental
824 conditions previously used. From preliminary analysis, lyophilized samples resulted in a
825 higher number of K⁺ and Na⁺ adducts, while Ca²⁺ adducts represented sporadic cases. For this
826 reason, Ca²⁺ was not considered in molecular formula assignment. Repeatability evaluated by

827 aligning the list of m/z values extracted at relative intensity threshold higher than 0.2 %, led to
 828 values of 80 % match for ESI, 78 % match for α -CHCA, 76 % for DHB in MALDI
 829 measurements. Molecular formulas were attributed accordingly to what was done for SPE
 830 desalted samples.



831
 832 Fig. 7: a) van Krevelen diagram obtained from formula assignment of ESI (orange), MALDI
 833 with DHB (green) and α -CHCA (violet) for *P. sativum* RE lyophilized samples. b) Overlap of
 834 signals traces (ESI, orange; DHB, green; α -CHCA, violet) for two m/z values corresponding
 835 to case compounds which were detected only in MALDI. c) Relative intensity of features
 836 containing from 1 to 8 atoms of nitrogen in ESI (orange), MALDI DHB (green), MALDI α -
 837 CHCA (violet) and MALDI ScA (yellow).

838 Figure 7a shows the van Krevelen diagram arising from formula assignment on spectra
 839 obtained with the two sources for the lyophilized sample. As expected, a higher number of
 840 assigned formulas was globally detected respect to the desalted sample obtained through SPE
 841 (Figure 3a), highlighting that desalting led effectively to the loss of part of the metabolites.
 842 Among these, a portion of features at O/C higher than 0.5 appeared, highlighting the recovery
 843 of a higher proportion of polar metabolites. These molecules could correspond to nucleoside
 844 phosphates, cyclic nucleotides, amino acids and glucoside flavonoids. The list of assigned
 845 molecular formula for the lyophilized sample in ESI and in MALDI measurements with DHB
 846 and α -CHCA can be found as supplementary information. In general, molecular formula
 847 assignment confirmed previous results, as a higher number of adducts was assigned in ESI
 848 compared to MALDI spectra also for the lyophilized sample (Figure 10Sa).

849 Figure 7b shows the overlap of relative signal intensity obtained for two case metabolites in
850 the two sources, namely $C_5H_5N_5$ with $[M+H]^+$ at m/z 136.06178 annotated as adenine and
851 $C_{38}H_{45}N_6O_7$ with $[M+H]^+$ at m/z 728.34036 annotated as an oligopeptide containing proline.
852 While poor intensity signals for the two metabolites characterized ESI spectra, MALDI with
853 both matrices led to higher S/N signals enabling their efficient detection and eventually,
854 confident molecular formula assignment. Effectively, high salinity leading to ion suppression
855 in ESI and ion enhancement in MALDI improved the detection of features in MALDI. This
856 was especially valid for the DHB matrix that showed a strong signal enhancement compared
857 to ESI. These findings agreed with the evolution of ion intensities at the increase of salt
858 concentration presented in the previous paragraph (Figure 6) and allowed also to retrieve the
859 approximative salinity, which was evaluated to be around 2 % (v/v) in lyophilized *P. sativum*
860 RE. At this salt concentration, some metabolites were not detected in ESI but on average a
861 higher number of detected features was obtained compared to MALDI (Figure 7a).
862 Nonetheless, as previously stated, the total number of assigned formulas was redundant in ESI
863 measurements because of the presence of several adducts. The Venn and van Krevelen
864 diagrams were then plotted considering only the protonated forms in ESI and MALDI (Figure
865 11S). In this case, similar plots were obtained, except for fatty acids and lipids ($H/C=1.5-2$
866 and $O/C=0.1-0.3$), which showed to be the most affected from adduct formation (Figure
867 10Sb). On the other side, a lower number of formulas only detected in ESI was obtained,
868 keeping the number of common formulas almost unchanged (Figure 10Sb). Trends of relative
869 intensity and number of detected features containing increasing N# and O# (showed
870 respectively in figure 7d, 7d and 11Sa, 11Sb) confirmed results obtained for the SPE desalted
871 sample. The two sources appeared to be highly complementary for a non-targeted
872 metabolomic approach on root exudate samples. MALDI enabled an increase of detected
873 features especially at relatively high salt concentration. Most of the metabolites exclusively
874 detected with MALDI corresponded to nucleosides, alkaloids, flavonoids and highly aromatic
875 compounds such as triterpenes and riboflavin analogues. For example, the exclusive detection
876 in MALDI measurements was experienced for metabolites which molecular formula that
877 could be tentatively assigned to quercetin analogues (m/z $C_{15}H_{10}O_7$), lumichrome (m/z
878 $C_{12}H_{10}N_4O_2$), lumiflavine (m/z $C_{13}H_{12}N_4O_2$), willardine (m/z $C_7H_9N_3O_4$) and anhydropisatine
879 (m/z $C_{17}H_{12}O_5$). These molecules have a biological importance and they have been found to
880 cover a principal role in *P. sativum* metabolism or to represent important mediators in plant
881 growth and interaction with microorganisms [107–110].

882

883

884 **4. Conclusion**

885 In this study, a non-targeted metabolomic approach *via* direct introduction MALDI-FTICR
886 MS based on dried droplet deposition, was optimized on *P. sativum* root exudate samples.
887 Optimized conditions of selective accumulation and best sample:matrix ratio for different
888 matrices enabled improved precision and high metabolite cover up increasing the number of
889 detected unique features compared to ESI-FTICR MS analysis. Moreover, matrix effect
890 evaluation performed on RE spiked with salt showed the presence of ion suppression in ESI-
891 FTICR MS and ion enhancement in MALDI-FTICR MS. Spectra obtained with MALDI were
892 characterized by a lower complexity and led to an increase of detected unique features as a
893 mirror of both lower number of adducts and preferential ionization of compounds at high N#
894 and high DBE. Lyophilized root exudate samples analyzed through the two sources showed a
895 good coverage of metabolite detection and annotation thanks to the ultra-high resolution, mass
896 measure precision and dynamic range of FTICR MS, demonstrating that MALDI could be an
897 alternative and complementary source for non-targeted metabolomics on biological samples.
898 The authors believe that the results of this work are relevant in the field of non-targeted
899 metabolomic and in the analysis of root exudate using a direct introduction approach based on
900 FTICR MS. Moreover, the proposed method *via* direct introduction MALDI FTICR MS
901 based on classic dried droplet deposition could be applied to other type of samples which are
902 affected by matrix effects due to the presence of salts such as DOM and NOM in seawater,
903 humic substances in fresh soil or in other biological samples as algae or blood. However,
904 future works still require addressing some unresolved questions as for example, the
905 optimization of an efficient MALDI FTICR MS method for RE analysis in negative mode or
906 the research of new and more performant matrices for improved metabolite detection, sample
907 homogeneity and high reproducibility for RE analysis. In this extent, solid-ionic or liquid-
908 ionic matrices represent potential candidates for RE analysis through MALDI FTICR MS
909 thanks to their co-crystallization properties and the possibility to be used both in positive and
910 negative mode [111]. Moreover, future investigation should be dedicated also on defining the
911 applicability and the limits of the optimized conditions found in this study on plants belonging
912 to different genus and/or family or to study the RE changes in presence of rhizobacteria in the
913 pursuit of a universal method based on MALDI FTICR MS for a comprehensive RE analysis.

914 **Author contribution: Valentina Calabrese:** Conceptualization, Data curation, Formal
915 analysis, Investigation, Methodology, Visualization, Roles/Writing - original draft **Isabelle**

916 **Schmitz-Afonso:** Conceptualization, Funding acquisition, Project administration, Resources,
917 Supervision, Validation, Writing - review & editing **Wassila Riah-Anglet:** Methodology,
918 Writing - review & editing **Barbara Pawlak:** Funding acquisition, Project administration,
919 Resources, Writing - review & editing **Isabelle Trinsoutrot -Gattin** Funding acquisition,
920 Project administration, Methodology, Resources **Carlos Afonso:** Conceptualization, Funding
921 acquisition, Project administration, Resources, Supervision, Validation, Writing - review &
922 editing.

923 **Data availability** All the data are described within the manuscript. The raw data and metadata
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943

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945

946

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