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## Metabolic diversity conveyed by the process leading to glutathione accumulation in inactivated dry yeast: A synthetic media study

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1           **Metabolic diversity conveyed by the process leading to glutathione**  
2           **accumulation in inactivated dry yeast: a synthetic media study**

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20 **ABSTRACT**

21           Glutathione-rich inactivated dry yeasts (GSH-IDY) are purported to accumulate  
22 glutathione intracellularly and then released into the must. Glutathione is beneficial for wine  
23 quality, but research has highlighted that GSH-IDYs have a synergic antioxidant effect similar  
24 to that of molecular GSH. Combination of negative mode ultra-high-resolution Fourier-  
25 Transform Ion-Cyclotron-Resonance Mass Spectrometry ((-)FT-ICR-MS), ultra-high-  
26 performance liquid chromatography coupled to a Quadrupole-Time of Flight mass  
27 spectrometer (UHPLC-Q-ToF-MS) and HPLC/Diode Detector Array (DAD)-Fluorescence  
28 spectroscopy was applied to three inactivated dry yeasts soluble fractions, with increasing  
29 intracellular glutathione concentration, in order to explore the chemical diversity released in  
30 different synthetic media.

31 Using the mean of size exclusion chromatography/DAD and fluorescence detection we report  
32 than most of the signals detected were below the 5-75 kDa-calibrated region of the  
33 chromatogram, indicating that most of the soluble protein fraction is composed of low  
34 molecular weight soluble peptides. In light of these results, high-resolution mass spectrometry  
35 was used to scan and annotate the low molecular weight compounds from 50 to 1500 Da and  
36 showed that GSH level of enrichment in IDYs was correlated to a discriminant chemical  
37 diversity of the corresponding soluble fractions. Our results clearly show an impact of the  
38 GSH accumulation process not only visible on the glutathione itself, but also on the global  
39 diversity of compounds. Within the 1674 ions detected by (-)FT-ICR-MS, the ratio of  
40 annotated elemental formulas containing carbon, hydrogen, oxygen, nitrogen and sulphur  
41 (CHONS) to annotated elemental formulas containing carbon, hydrogen, oxygen (CHO)  
42 increased from 0.2 to 2.1 with the increasing levels of IDYs GSH content and 36 unique  
43 CHONS annotated formulas were unique to the IDY with the highest concentration of GSH.  
44 Amongst the 1674 detected ions 193 were annotated as potential peptides (from 2 to 5

45 residues), 61 ions were annotated as unique amino acid combinations and 46% of which being  
46 significantly more intense in GSH-rich IDY. Thus, the process leading to the accumulation of  
47 glutathione also involves other metabolic pathways which contribute to an increase in  
48 CHONS containing compounds potentially released in wine, notably peptides.

49 **KEYWORDS:** Mass spectrometry; Wine; Oenology; Untargeted analysis

50

#### 51 **CHEMICAL COMPOUNDS STUDIED IN THIS ARTICLE**

52 Glutathione (PubChem CID: 124886); Leucyl-arginine (PubChem CID: 3800205); Leucyl-  
53 lysine (PubChem CID: 14299197); Adenosine (PubChem CID: 60961); Glutamyl-cysteine  
54 (PubChem CID: 123938); Pantothenic acid (PubChem CID: 6613); Cysteinyl-glycine  
55 (PubChem CID: 439498); Pipecolic acid (PubChem CID: 439227); Homocitric acid  
56 (PubChem CID: 5460287); Methyl-thioadenosine (PubChem CID: 439176)

## 57 1. Introduction

58           There has been a growing interest for yeast derivatives in winemaking over the last  
59 few decades, as these products can improve fermentation processes and organoleptic  
60 properties of wine (recently reviewed by Pozo-Bayòn and collaborators (2009)). They divided  
61 enological preparations of yeast derivatives into five classes depending on the industrial  
62 process of production : the yeast hulls, yeast mannoproteins, the yeast autolysate, the yeast  
63 protein extracts and the inactivated dry yeasts (IDY). The latter are mainly *Saccharomyces*  
64 *cerevisiae* strains grown under aerobic condition in a non-limiting medium before being  
65 inactivated and dried and can then be used from the vineyards through to the fermentation and  
66 barrel aging steps of the winemaking process.

67           Although IDY have already been largely used in wineries, scientific explanations for  
68 their impact on winemaking have only recently been elucidated. IDY can release amino acids  
69 and peptides expressed in yeast assimilable nitrogen (of the order of a few mg N/L) which  
70 contribute to enrich the must in nutrients (Pozo-Bayón et al., 2009). Since the amount of  
71 nitrogen released by inactivated yeast is low (compared to yeast autolysates), these products  
72 are more generally advised for micronutrient and survival factors (notably vitamins and fatty  
73 acids). However, the extent of the fraction of assimilable nitrogen related to the presence of  
74 small peptides and proteins released by both active and IDY is still unexplored (Liu et al.,  
75 2017). Polysaccharides are also significantly released by IDY (Pozo-Bayón et al., 2009). The  
76 main component of the yeast cell wall is mannan (chains of mannose) and  $\beta$ -glucan (chains of  
77 glucose) which could be linked to proteins. Cell wall polysaccharides mainly have color  
78 stabilization properties (Escot et al., 2001; Guadalupe & Ayestarán, 2008), notably through  
79 polyphenolic interactions (Andújar-Ortiz et al., 2012). It is however acknowledged that cell  
80 walls can exhibit a potential antioxidant activity not only due to the linked proteins but also  
81 due to the polysaccharides structures themselves (Jaehrig et al., 2008; Jaehrig & Rohn, 2007).

82 Recently there has been an interest in the use IDYs during winemaking, notably with a focus  
83 on their antioxidant properties. The concept of IDY rich in glutathione (GSH), as natural  
84 antioxidant for a use in winemaking originated in 2005 with the first patent for naturally rich  
85 inactive yeast (PCT/FR2005/000115). Glutathione is a cysteine-containing peptide and is  
86 considered to be the most abundant low molecular thiol in yeast cells (Elskens et al., 1991). In  
87 winemaking, GSH has been proposed to reduce the oxidation phenomena that leads to  
88 browning, and thus improves wine preservation and varietal aroma stability during aging  
89 (Antoce et al., 2016; Kritzinger et al., 2013; Nikolantonaki et al., 2018; Webber et al., 2016).

90 IDY can have a higher GSH content by activating the GSH yeast intracellular  
91 production during processing (Li et al., 2004; Wen et al., 2004). The increase in GSH content  
92 of IDYs during processing can be achieved after modulation of the sulfur metabolic pathway  
93 when cysteine is present in the culture medium (Nisamedtinov et al., 2011). However, it is  
94 well known that sulfur-containing compounds can exert an antioxidant activity and these  
95 reactive compounds are more abundant in GSH-enriched IDY than in IDY (Elias et al., 2008;  
96 Rodriguez-Bencomo et al., 2014). Even though there is quite a lot of literature on the general  
97 metabolites which could impact the wine quality and safety, there is a lack of knowledge on  
98 the global diversity and functionality of IDY compounds released during winemaking and  
99 their impact on wines composition (Liu et al., 2016; Liu et al., 2017).

100 In this study, we combined various non-targeted analyses in order to characterize the  
101 diversity of compounds, which can be potentially extracted from GSH-enriched inactivated  
102 dry yeasts when added to wines. Three IDYs with increasing GSH enrichments were  
103 characterized for their potential molecular and macromolecular release, and the diversity of  
104 the annotated/identified compounds provides new insights on IDY functionalities under  
105 winemaking conditions, and underlines crucial IDY compositional differences in a GSH-  
106 accumulation process-dependent manner.

## 107 2. Material and methods

### 108 2.1. Sample preparation

109 Three non-commercial inactivated dry yeasts were obtained from Lallemand SAS  
110 (Blagnac, France). These products were produced at a laboratory scale process allowing  
111 optimizing chemical, physical and nutritional features of the bio-process in order to maximize  
112 the intracellular concentration of metabolites, notably glutathione. Two products were  
113 specifically produced from different yeast strains to increase the bioavailability of glutathione  
114 (G-IDY and Gplus-IDY which release 18 mg and 25 mg of glutathione per gram of IDY,  
115 respectively). The third product (N-IDY) was produced with the same strain as for G-IDY  
116 without following the specific process of GSH accumulation and releases 12 mg of  
117 glutathione per gram of IDY. At the first opening of the sealed prepared products, the three  
118 IDY products were aliquoted in pre-weighed 2 mL vials under nitrogen and stored at -18°C in  
119 dark. Three different extractions were made to obtain: the acidified Water-Soluble Fraction  
120 (WSF) from ultrapure water (18.2 MΩ, Millipore, Germany) with 0.01% (v/v) formic acid at  
121 pH 3.2, the Model Wine Soluble Fraction (MWSF), from 12% (v/v) ethanol in ultrapure water  
122 with 0.01% (v/v) formic acid at pH 3.2, and the Methanol Soluble Fraction (MSF), from  
123 methanol with 0.1% (v/v) formic acid. IDYs were resuspended at 4 g/L and soluble fractions  
124 were obtained after 1h stirring at room temperature in dark. Samples were then centrifuged  
125 (12000 g, 5 min, 4°C) and the supernatants were aliquoted and stored under nitrogen at 4°C  
126 until analysis. All samples were prepared in triplicate.

### 127 2.2. Gel Permeation Chromatography (GPC)

128 GPC was performed with a high-performance liquid chromatography (HPLC) (Elite  
129 LaChromElite, VWR, Radnor, Pennsylvanie) coupled to a Diode Array Detector  
130 (DAD)/Fluorescence detector. 50 µL of sample were injected through a column Yarra 3µ  
131 SEC-2000 300 x 7.8 mm (Phenomenex, Sartrouville, France) connected to a stationary phase

132 guard column with SecurityGuard (Phenomenex, Sartrouville, France). Elution was  
133 performed with hydro-alcoholic solution (12% (v/v) ethanol, 6 g/L of tartaric acid at pH 3.5)  
134 in an isocratic gradient (500  $\mu\text{L}/\text{min}$  which corresponds to a pressure of 75 bars) for 45 min.  
135 The temperature of the sampler and the column were kept constant at 4°C and 25°C  
136 respectively during analysis. Fluorescence detector was set at 280 nm for the excitation  
137 wavelength and 350 nm for the emission wavelength, and the DAD enabled absorption  
138 spectra acquisition from 200 to 400 nm. Data were acquired with the EZChrom Elite (Agilent  
139 Technologies, Santa Clara, California) software, exported as .dat converted into .asc and  
140 processed with CHROMuLAN v0.79 (PIKRON Ltd.). Data were calibrated against a low  
141 molecular weight standards mix (LMW Gel Filtration calibration kits, GE Healthcare,  
142 Buckinghamshire) with the following standards: Conalbumin (75 kDa), Ovalbumin (43 kDa),  
143 Ribonuclease A (13.7 kDa), Aprotinin (6.5 kDa) and Angiotensine (1.1 kDa). Elution times  
144 were converted into molecular weight masses according to the calibration curve  
145 (Supplementary information 1).

### 146 *2.3. Metabolomics analyses by Fourier Transform Ion Cyclotron Resonance Mass* 147 *Spectrometry*

148 Ultra-high-resolution mass spectra were acquired in negative mode on a Bruker  
149 SolariX Ion Cyclotron Resonance Fourier Transform Mass Spectrometer ((-)FT-ICR-MS)  
150 (BrukerDaltonics GmbH, Bremen, Germany) equipped with a 12 Tesla superconducting  
151 magnet (Magnex Scientific Inc., Yarnton, GB) and a APOLO II ESI source (BrukerDaltonics  
152 GmbH, Bremen, Germany), and operating in negative and positive ionization modes. 20  $\mu\text{L}$   
153 sample was diluted in 1mL of pure methanol and then injected at a flow rate of 120  $\mu\text{L}/\text{h}$  into  
154 the microelectrospray. Spectra were acquired with a time-domain of 4 mega words over a  
155 mass range of  $m/z$  147 to 2000. A total of 300 scans were accumulated for each sample. All  
156 samples were injected randomly in the same batch to avoid batch variability. External



157 calibration was done with clusters of arginine (10 mg/L in methanol). Internal calibration was  
158 performed for each sample by using yeast ubiquitous compounds for negative mode  
159 (Gougeon et al., 2009) (Supplementary information 2). External and internal calibration led to  
160 a day-to-day mass accuracy lower than 0.1 ppm.

#### 161 *2.4. Identification of metabolites by liquid chromatography coupled to time-of-flight mass* 162 *spectrometry*

163 Metabolite separation was performed with an ultra-high-performance liquid  
164 chromatography (Dionex Ultimate 3000, ThermoFischer) coupled to a MaXis plus MQ ESI-  
165 Q-ToF mass spectrometer (Bruker, Bremen, Germany). The non-polar and low polar  
166 metabolites were separated in reversed phase liquid chromatography (RP-LC) by injecting  
167 5  $\mu$ L in an Acquity UPLC BEH C<sub>18</sub> 1.7  $\mu$ m column 100 x 2.1 mm (Waters, Guyancourt,  
168 France). Elution was performed at 40°C by (A) acidified water with 0.1% (v/v) of formic acid  
169 and (B) acetonitrile with 0.1% (v/v) of formic acid with the following gradient: 0-1.10 min 5%  
170 (v/v) of B and 95% (v/v) of B at 6.40 min. The flow rate was set at 400  $\mu$ L/min and  
171 maintained for 5min at initial conditions before each injection. Solvent and analytes were  
172 ionized with an electrospray (Nebulizer pressure = 2 bars and nitrogen dry gas flow = 10  
173 L/min). Ions transfer was done with an end plate offset at 500 V and transfer capillary voltage  
174 at 4500 V. A divert valve was used to inject four times diluted ESI-L Low Concentration  
175 Tuning Mix (Agilent, Les Ulis, France) at the beginning of each run, allowing a recalibration  
176 of each spectrum. The mass spectrometer was calibrated with undiluted Tuning Mix before  
177 batch analysis in enhanced quadratic mode, with less than 0.5 ppm errors after calibration.  
178 Spectra were acquired on the 100 to 1500  $m/z$  mass range, both in negative and positive  
179 ionization modes. UHPLC-Q-ToF-MS quality control (mix of standard peptides and  
180 polyphenols) and experimental quality control (mix of samples) were used to guarantee the  
181 UHPLC-Q-ToF-MS system performance. All samples were injected randomly in the same

182 [batch to avoid batch-to-batch variability](#). Features (couple of  $m/z$ -values and retention times)  
183 fragmentation was performed using the AutoMS/MS function on the most intense features  
184 with a frequency of 2 Hz. The fragmentation was done at three different collision energies:  
185 15, 25 and 35 eV.

## 186 *2.5. Data analysis*

187 [Results were expressed as the average of three replicates with the associated standard](#)  
188 [deviations.](#)

189 (-)FT-ICR-MS data were handled with DataAnalysis (v. 4.3, Bruker Daltonik GmbH).  
190 Calibrated data were filtered to keep only  $m/z$  peaks with a signal to noise (S/N) ratio above  
191 10 and an absolute intensity higher than  $2.0 \times 10^6$ . Peaks alignment was made by [Matrix](#)  
192 [Generator software \(v. 0.4, Helmholtz-Zentrum Muenchen\)](#) with a mass accuracy window of  
193 1 ppm (Lucio, 2009). Peaks with intensity equal to 0 in more than 80% of samples were  
194 removed from the analysis. Finally, the in-house software NetCalc 2015 (v. 1.1a, [Helmholtz-](#)  
195 [Zentrum Muenchen](#)) was used to annotate peaks (Tziotis et al., 2011). 46% of the initially  
196 aligned peaks were annotated by NetCalc and used for this study. [Van Krevelen diagrams,](#)  
197 [which plot the H/C against the O/C ratio of annotated metabolites were generated by an Excel](#)  
198 [file, providing instantaneous chemical pictures of the metabolites diversity \(Brockman et al.,](#)  
199 [2018; Kim et al., 2003\).](#) The OligoNet webserver was also used to annotate potential peptides  
200 [with a max error of 1 ppm \(Liu et al., 2017\).](#)

201 The UHPLC-Q-ToF-MS data were calibrated internally by 1/4 diluted tuning mix with  
202 DataAnalysis (v. 4.3, Bruker Daltonik GmbH). Calibrated features were filtered to retain  
203 those with signal/noise ratio (S/N) higher than 30 and an absolute intensity of at least 1000.  
204 Before features extraction, the spectral background noise was removed. The extracted features  
205 were aligned with a [R script](#) with a  $m/z$  tolerance of 2 ppm and retention time tolerance of 0.2  
206 seconds.

207 Parent ions and fragments were submitted to different databases with the MassTRIX  
208 interface (<http://masstrix.org>) (Suhre & Schmitt-Kopplin, 2008), Metlin  
209 (<https://metlin.scripps.edu>) and YMDB 2.0 (<http://www.ymdb.ca>) (Ramirez-Gaona et al.,  
210 2017). According to the precision of UHPLC-Q-ToF-MS and (-)FT-ICR-MS, an error of 3  
211 ppm and 1 ppm was chosen respectively for the annotation of the metabolites (Roullier-Gall  
212 et al., 2014; Roullier-gall et al., 2015). The combination of these databases enabled covering  
213 the widest range of metabolites found in biological systems, and notably in yeast.

### 214 3. Results and discussion

#### 215 3.1. *IDY macromolecular profile*

216 Besides the obvious discrimination between soluble and non-soluble fractions in a  
217 specific medium, the gel permeation chromatography is dedicated to determine the global size  
218 repartition of compounds within complex samples. This first experiment was a good  
219 introduction to understand the relatively unknown diversity of compounds released by IDY in  
220 different extraction media. Acidified water and model wine media were chosen to identify  
221 what can be released by IDY in must and wine, respectively. Methanol was used to have an  
222 overview of the mildly hydrophobic soluble fraction present in our samples, independent of  
223 the oenological conditions in winemaking process. In this experiment, it was possible to  
224 separate macromolecules from 300 kDa to 5 kDa between 9.5 min and 22.5 min depending on  
225 the column specification. Separation was performed with model wine and without any  
226 denaturant to keep macromolecules in their native forms. The fluorescent detector was set up  
227 to track the presence of potent proteinaceous fluorophores among the chromophore extracted  
228 from yeast extracts (Coelho et al., 2017). Figure 1 shows GPC chromatograms of MWSF for  
229 G-IDY, Gplus-IDY and N-IDY and supplementary information 3 shows those for WSF and  
230 MSF. These chromatograms clearly revealed three different time frame windows common to  
231 all MWSF extracts. The first time-frame (I) between 9.5 and 17.3 min (*outside of the*

232 calibrated region) represents about 1 % of the total fluorescent response and had a mass range  
233 from 300 to 75 kDa. G-IDY, Gplus-IDY and N-IDY showed no significant differences in  
234 abundance of high molecular weight molecules in this region. This result is in agreement with  
235 Pozo-Bayon and collaborators (2009), who reported that high molecular weight compounds  
236 are in lower concentration than peptides and amino acids in the model wine soluble fraction of  
237 different IDY (Pozo-Bayón et al., 2009). Under our experimental conditions, the percentage  
238 of high molecular weight compounds released from G-IDY, Gplus-IDY and N-IDY was  
239 always low, whatever the solvent used (Supplementary information 3). However, methanol  
240 extracts showed limited high molecular weight compounds response, probably because of  
241 organic solvent precipitation (Chertov et al., 2004).

## 242 **FIGURE 1**

243  
244 The second time-frame window (II) in the chromatogram, between 17.3 and 22.5 min,  
245 represents medium molecular weight compounds in a mass range from 75 to 5 kDa, the latter  
246 corresponding to the exclusion limit of the column. G-IDY, Gplus-IDY and N-IDY relative  
247 concentrations in medium molecular weight compounds varied from 7 to 14% of the total  
248 fluorescence of the sample, in an extraction medium dependent manner. In detail, WSF and  
249 MWSF exhibited concentrations in medium molecular weight compounds, which were 1.5  
250 times higher for Gplus-IDY compared to those for G-IDY and N-IDY. However, MSF  
251 presented relatively low concentrations of medium molecular weight compounds, of about 3%  
252 of the total fluorescence responses (Supplementary information 3).

253 Finally, the most abundant fluorescent responses (whatever the extraction media) were  
254 observed for the third time-frame (III) from 22.5 to 45 min, where fluorescent compounds  
255 accounted for 92%, 91% and 86% of the total fluorescence response of G-IDY, N-IDY, and  
256 Gplus-IDY respectively in model wine extraction medium. This chromatographic region is

257 out of the molecular size exclusion limit of 5 kDa of the column and suggests the importance  
258 of low molecular weight compounds on the IDY soluble fraction independently of the  
259 extraction medium.

260 In addition, in order to get a more representative characterization of IDY soluble  
261 fractions, UV detection at 210 nm was also considered. UV detection is less sensitive and less  
262 specific compared to fluorescence, but allows the peptide bond, carbon to carbon and carbon  
263 to oxygen double bond detection (Stoscheck, 1990). Supplementary information 2 shows  
264 chromatograms of G-IDY, Gplus-IDY and N-IDY for the three extraction media, recorded at  
265 210 nm. UV responses were far less important than those monitored with fluorescence, and  
266 concerned specifically the third time-domain corresponding to the low molecular weight  
267 compounds (below 5 kDa).

268 On the basis of these results, and whatever the detection method or the extraction  
269 medium used, GPC could not discriminate IDY soluble fractions. The medium and high  
270 molecular weight compounds showed a similar fluorescent profile among samples which  
271 indicated that the bio-process enrichment would only impact the low molecular weight  
272 fraction of IDYs. In that respect, to further the characterization of IDY soluble fractions, ultra-  
273 high-resolution mass spectrometry was used to assess the diversity of the abundant low  
274 molecular weight compounds.

275

### 276 3.2. *IDY low molecular weight chemical diversity*

277 The ultra-high accuracy of (-)FT-ICR-MS enables the exact mass of ionizable  
278 compounds present in complex matrices to be determined, including wine, thus allowing to  
279 have a more comprehensive picture of the chemical diversity present in the sample (Roullier-  
280 Gall et al., 2017, 2014). Figure 2A gives an overview of the number of ions detected in one  
281 specific IDY (G-IDY) in the three different extraction media. Within the 1280 ions detected

282 for G-IDY, 701 (54.8%) were common to the three extraction media and only a total of 40  
283 ions (3.2%) were found to be medium specific. MWSF showed the best extraction yield with  
284 97.4% of the total extractable *m/z* ions (N-IDY and Gplus-IDY MWSF had similar extraction  
285 yields with 85.4% and 89% respectively, data not shown). The polarity of the model wine  
286 appears to lead to a better solubilization of IDY compounds than water and methanol alone.  
287 Based on this result, G-IDY, Gplus-IDY and N-IDY MWSFs were compared in order to  
288 characterise the diversity of IDY extractible compounds. For all conditions, yeast derivatives  
289 were inactivated by short term heating before being dried and sealed hermetically for storage.  
290 In that respect, compounds potentially released by IDY are a combination of bio-accumulated  
291 metabolites and bio-transformed compounds related to the production process. Since the  
292 inactivation and drying process were identical for the three IDYs, it can be assumed that the  
293 major contribution to the diversity could be mainly attributed to the bio-process.

294 As shown in Figure 2B, 37.1% of the overall 1674 extractable ions were common to  
295 each IDY and only 1.8%, 3.9% and 11.7% of the ions were unique to G-IDY, N-IDY and  
296 Gplus-IDY, respectively. We also observed that N-IDY and G-IDY, obtained from the same  
297 yeast strain, shared more common ions (62.1%) than with Gplus-IDY (46.1% and 48.6%  
298 respectively). These results show that both the intracellular accumulation of glutathione and  
299 the yeast strain are key factors modifying the metabolic signatures of IDY soluble fractions, in  
300 agreement with existing literature (Allen et al., 2003; Brauer et al., 2006; Dikicioglu et al.,  
301 2012).

## 302 **FIGURE 2**

303  
304 Network annotation of the *m/z* ions present in MWSF by NetCalc software allows  
305 access to the molecular formulas of 53% of the 1674 ions detected. The extensive chemical  
306 differences between G-IDY, Gplus-IDY and N-IDY MWSFs were clearly visible from the

307 histograms depicting the distribution of elemental compositions (CHO, CHON, CHOS, and  
308 CHONS), along with Van Krevelen diagrams of the (-)FT-ICR-MS derived molecular  
309 formulas (Figure 3). Figure 3A presents the 379 annotated  $m/z$  ions (42.7%) common to the  
310 three IDYs soluble fractions in model wine (MWSF). These ions could be considered as  
311 representative of extractable metabolites from IDYs whatever the strain or the production  
312 process. Common ions were found in different chemical spaces such as lipid-like, peptide-like  
313 and saccharide-like domains, which agrees with a glutathione accumulation bioprocess that  
314 preserves the yeast basic metabolism during the production.

### 315 **FIGURE 3**

316  
317 Figure 3B, 3C and 3D show annotated masses unique to each MWSFs, colored according to  
318 their chemical compositions and sized according to their mass peak relative intensity. An  
319 overview of the Van Krevelen diagrams reveals significant differences between the samples  
320 in terms of number of unique formulas (already visible in Figure 2) and chemical families. N-  
321 IDY (Figure 3D) appeared much richer in unique CHO containing formulas (16) than G-IDY  
322 (3) and Gplus-IDY (0). These formulas are mainly in peptide-like and lipid-like domains,  
323 which could correspond to small chain fatty acids for example. In contrast, Gplus-IDY  
324 (Figure 3B) was characterized by a significantly higher number of unique CHONS containing  
325 formulas compared to G-IDY and N-IDY (36 against 3 and 1, respectively). These formulas,  
326 mainly located in the peptide-like domain could correspond to peptides with sulfur-containing  
327 amino acid residues, such as methionine and cysteine. The high diversity of sulfhydryl  
328 containing compounds (-SH group) could explain the relative activity of these products  
329 against oxidation, as it is known than peptides and thiols could have antioxidant property in  
330 wine (Elias et al., 2008; Nikolantonaki et al., 2010, 2014). It is remarkable to note how the  
331 glutathione enrichment process, which is designed to accumulate intracellular glutathione, is

332 actually accompanied by an overall increase of the CHONS/CHO ratio when going from N-  
333 IDY to Gplus-IDY (Figure 3E), with G-IDY potentially releasing 3 times more CHONS  
334 compounds than N-IDY, and Gplus-IDY releasing more than 10 times more compounds than  
335 N-IDY. With a moderate hypothesis of 3 isomers per (-)FT-ICR-MS ion, these results show  
336 altogether that Gplus-IDYs would be discriminated by more than 100 different N,S-  
337 containing compounds, compared to G-IDY and N-IDY, thus providing an unprecedented  
338 molecular representation of the actual metabolic response of glutathione enrichment. *The*  
339 *relatively low number of unique compounds released from G-IDY is not surprising since it is*  
340 *obtained from the same strain as N-IDY, and it follows the same bio-process as Gplus-IDY,*  
341 *thus most of the released compounds are likely shared with at least one other IDY.* Although  
342 it was not the aim of this study, it is further interesting to note the strain-dependency of the  
343 glutathione enrichment process, with Gplus-IDY releasing nearly 4 times more CHONS  
344 compounds than G-IDY while the released glutathione is increased by 2.

345

### 346 *3.3. Impact of GSH enrichment process on IDYs peptides diversity*

347 The presence of cysteine in the growth medium during IDY GSH accumulation  
348 processing at industrial scale (Li et al., 2004), modifies the global metabolism of sulfur amino  
349 acids and leads to over representation of sulfur-containing metabolites (Thomas & Surdin-  
350 Kerjan, 1997). As the Van Krevelen diagrams highlight the increasing diversity of CHON and  
351 CHONS containing compounds along with the enrichment process, this diversity could be  
352 putatively attributed to peptides containing cysteine or methionine residues. Amongst the  
353 1674  $m/z$  submitted to the OligoNet webserver, 193 were annotated as potential peptides  
354 (from 2 to 5 residues) with an error below 1ppm (Supplementary information 4). Within the  
355 193 annotated ions, 132 have a Multiple Amino Acids Combination (MAAC) and 61 a  
356 Unique Amino Acids Combination (UAAC) (Figure 4A). Most of the peptides (144  $m/z$ ) are



357 common to at least two out of three IDYs, whereas Gplus-IDY presents the greatest diversity  
358 with 40 peptides against 7 and 2 for G-IDY and N-IDY, respectively. (Figure 4B). Amongst  
359 the UAAC, 26 out of 65 contain a cysteinyl residue. These unambiguous annotations of  
360 peptides allowed all possible connections between these peptides to be determined  
361 (Supplementary information 5). The Gplus-IDY clearly released more unique peptides and  
362 more peptides with a cysteinyl residue. Nevertheless, most of the UAAC are shared between  
363 the IDYs (regardless of their relative concentrations). The global similarity between our  
364 samples analyzed by (-)FT-ICR-MS allowed us to compare the absolute intensity between  
365 samples providing an indication of the abundance of each compound released. Figure 4C  
366 reveals that within the 61 UAAC released by the IDY, Gplus-IDY released 28 peptides  
367 significantly more intense than G- and N-IDY (3 peptides and 1 peptides respectively). These  
368 results are in accordance with the Van Krevelen diagram (Figure 3B) showing a higher  
369 diversity of compounds in the peptide-like domain for Gplus-IDY.

#### 370 **FIGURE 4**

371 In addition to the peptides enrichment, other metabolites could be impacted by the bio-  
372 process. (-)FT-ICR-MS enables to have access to the elemental formulas of detected ions and  
373 thus to acquire instantaneous metabolic fingerprints of what can be released by IDYs. Since a  
374 single CHONS elemental formula can be associated with numerous compounds, (especially  
375 for high  $m/z$  ions) the combination with separative UHPLC-Q-ToF-MS and UHPLC-Q-ToF-  
376 MS S/MS methods was used to access structural and quantitative information about these  
377 molecular markers (Roullier-Gall et al., 2014). The highest resolution of the (-)FT-ICR-MS  
378 (600,000 against 20,000 for UHPLC-Q-ToF-MS at  $m/z$  306) was used to annotate UHPLC-  
379 Q-ToF-MS peaks which could be aligned with (-)FT-ICR-MS peaks, using an in-house  
380 designed alignment script. Up to 128  $m/z$  peaks of the UHPLC-Q-ToF-MS spectra were  
381 found to match (-)FT-ICR-MS peaks (with an absolute error lower than 2 ppm)

382 (Supplementary information 6) . Alignment was done on the basis of the similarity of  $m/z$   
383 without consideration of the retention time, only for the negative mode of the UHPLC-Q-  
384 ToF-MS . The aim was to have both an unambiguous annotation of UHPLC-Q-ToF-MS ions  
385 with the ultrahigh resolution of (-)FT-ICR-MS, and quantitative information from UHPLC-Q-  
386 ToF-MS. Ions present UHPLC-Q-ToF-MS data but not found in (-)FT-ICR-MS data (Figure  
387 4B) were likely molecules easily suppressed in direct infusion with ESI, showing the added  
388 value of using chromatographic separation before MS analysis (Roullier-Gall et al., 2014).

389 Keeping in mind the formula annotation of  $m/z$  ions with the (-)FT-ICR-MS data, the  
390 fragmentation of 16 important ions (issued from positive and negative ionization mode)  
391 enabled to have access to molecular structural information. Fragments were submitted to  
392 metabolomic databases, and annotation of the parent's ions are summarized in Table 1.  
393 Following the new prescriptions of Sumner and collaborators, the identification point (which  
394 gives the identification confidence level) of each metabolite is 5.5 (High resolution retention  
395 time, accurate mass of parent ion, molecular formula based upon accurate  $m/z$  and isotope  
396 pattern and accurate tandem mass spectrum) (Sumner et al., 2014). Most of these compounds  
397 were already described in yeast metabolic pathways, but never so far as compounds which  
398 could be potentially extracted from IDY in winemaking conditions. Most interestingly,  
399 glutathione and its precursor (glutamyl-cysteine) were found in the three extracts, but  
400 consistently with the enrichment process, *i.e.* with a relative abundance following the Gplus >  
401 G > N trend. In contrast, lysine-leucine and arginine-leucine dipeptides appeared as GPlus-  
402 IDY markers, whereas homocitric acid appeared as N-IDY marker. Amino acids (glutamate,  
403 tyrosine, phenylalanine), which have been extensively studied in IDY (Pozo-Bayón et al.,  
404 2009) were examples of contrasted trends with relative concentrations that did not appear to  
405 be directly correlated to the GSH accumulation process. Similarly, methyl-thioadenosine, a  
406 precursors of adenosylmethionine which is a sulfur and nitrogen stock molecule in poor

407 medium (Shapiro & Schlenk, 1980), and thus a potential fermentation enhancer already  
408 putatively annotated in IDYs (Rodriguez-Bencomo et al., 2014) were identify in the three  
409 samples. Weak organic acids like citric acid have been described for their sensory impact  
410 (fresh notes), whereas homocitric acid and pipercolic are related to the lysine metabolism in  
411 yeast (He, 2006; Tucci & Ceci, 1972), but not yet described in IDYs. Finally, the pantothenic  
412 acid, which exhibits a promoting effect on yeast growth (Richards, 1936) is much more  
413 present in N-IDY than in G-IDY and Gplus-IDY. Adenosine and arginine-leucine were  
414 recently shown to possibly promote bacteria growth (Liu et al., 2016; Liu et al., 2017) and  
415 thus facilitate the malolactic fermentation. Whereas Adenosine are abundant in the three  
416 IDYs, the dipeptide Arg-Leu is specific to Gplus-IDY and almost absent in the other IDYs.  
417 Such structural identifications provide a good overview of the richness and the diversity of  
418 compounds released by IDYs, and it also highlights the yet unknown potential contribution of  
419 IDYs during fermentation.

420

#### TABLE 1

421 Most of the ions detected by (-)FT-ICR-MS and UHPLC-Q-ToF-MS are difficult to  
422 isolate and fragment, which implies that most of the compounds present in the MWSFs are  
423 still largely unknown. Supplementary Information 7 gives the list of NetCalc annotated (-)FT-  
424 ICR-MS peaks found in the database, with the relative intensity of each ion and these possible  
425 database ID with an error lower than 2ppm. In this way we can have a quick overview of  
426 specific and common compounds from each IDY and appreciate how the medium is modified  
427 by using specific IDY.

#### 428 4. Conclusion

429 In this work, the soluble fraction that can be extracted from three inactive dry yeasts  
430 by model wine, were characterized by a combination of Gel Permeation chromatography  
431 (GPC), (-)FT-ICR-MS and UHPLC-Q-ToF-MS in order to provide a comprehensive picture

432 of the diversity of potentially active compounds associated with the glutathione accumulation  
433 process. To that purpose, the extract of non-enriched IDYs (N-IDY) was compared to extracts  
434 of an GSH-rich IDY from the same strain (G-IDY) and an even more GSH-rich IDY, but  
435 from a different strain (Gplus-IDY). Consistently with the expected GSH accumulation  
436 process, Gplus-IDY exhibited higher amounts of extractable glutathione (and its glutamyl-  
437 cysteine precursor) than G-IDY, which in turn appeared richer than N-IDY. Moreover, the  
438 increase in peptides containing cysteinyl residues in Gplus-IDY shows that the glutathione  
439 accumulation bio-process also impacts the peptidome of the yeast. Modulation of the GSH  
440 pathway leads to a global modification of cysteine incorporation and thus could have  
441 important implications in wine making.

442 Our metabolomic approach provided a comprehensive molecular picture of the  
443 detailed impact of the glutathione accumulation process. It further revealed how the yeast  
444 strain can modulate the extent of the accumulation process, with the annotation of more than  
445 hundred N,S-containing potential active compounds for Gplus-IDYs, most of them being  
446 unknown yet. Altogether, our results shed important light on the potential activity of IDYs on  
447 musts and wines, in terms of organoleptic and/or stability properties.

448

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454

#### 455 **Conflicts of interest**

456 The authors wish to confirm that there are no conflicts of interest associated with this  
457 publication.

458

#### 459 **Abbreviations used**

460 IDY, inactivated dry yeast; GSH, glutathione; MS, mass spectrometry; WSF, water soluble  
461 fraction; MWSF, model wine soluble fraction; MSF, methanol soluble fraction; GPC, gel  
462 permeation chromatography; HPLC, high pressure liquid chromatography; DAD, diode array  
463 detector; LMW, low molecular weight; UHPLC-Q-ToF-MS , Ultra-High-Performance Liquid  
464 chromatography triple quadrupole time of flight mass spectrometry; RP-LC, reversed phase  
465 liquid chromatography; (-)FT-ICR-MS, Negative mode Fourier-transform ion-cyclotron-  
466 resonance mass spectrometry; S/N, Signal/Noise; kDa, kilo Dalton; MAAC, Multiple Amino  
467 Acid Combination; UAAC, Unique Amino Acid Combination; CHO, Carbon-Hydrogen-  
468 Oxygen; CHON, Carbon-Hydrogen-Oxygen-Nitrogen; CHONS, Carbon-Hydrogen-Oxygen-  
469 Nitrogen-Sulfur

470

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597

598 **Figure and Table Captions**

599 **Figure 1:** Gel permeation chromatograms of model wine soluble fractions (MWSF) of G-IDY  
600 (red), Gplus-IDY (green) and N-IDY (black) with fluorescence detection (EX/EM = 280/350  
601 nm). Dotted lines indicate the calibration areas based on standard compounds.

602  
603 **Figure 2:** Venn diagrams presenting the total counts of detected ions by (-)FT-ICR-MS, with  
604 percentages in brackets. (A) Extraction yield for the specific G-IDY in the three extraction  
605 media, and (B) unique and common ions to the model wine soluble fractions of N-IDY, G-  
606 IDY and Gplus-IDY.

607  
608 **Figure 3:** Van Krevelen representation of annotated masses from (-)FT-ICR-MS analyses of  
609 the MWSFs (Model Wine Soluble Fraction) of Gplus-IDY, G-IDY and N-IDY. (A) masses  
610 shared by Gplus-IDY, G-IDY and N-IDY MWSFs. (B) Specific  $m/z$  ions to Gplus-IDY, (C)  
611 specific  $m/z$  ions to G-IDY, and (D) specific  $m/z$  ions to N-IDY. In B, C and D common  $m/z$   
612 ions were depicted in grey. Van Krevelen plots were coloured according to molecular classes  
613 i.e CHO (blue), CHOS (green), CHON (orange), CHONS (red). Bubble sizes correspond to  
614 the triplicate averaged relative intensities of mass peaks. Bar histograms indicate the number  
615 (and corresponding percentage at the bar-top) of molecular formulae presented in the Van  
616 Krevelen diagrams. (E) Relative abundance of CHONS against CHO for the three MWSFs.  
617 For each IDY, the total number of annotated masses corresponding to CHO and CHONS were  
618 used to calculate this ratio.

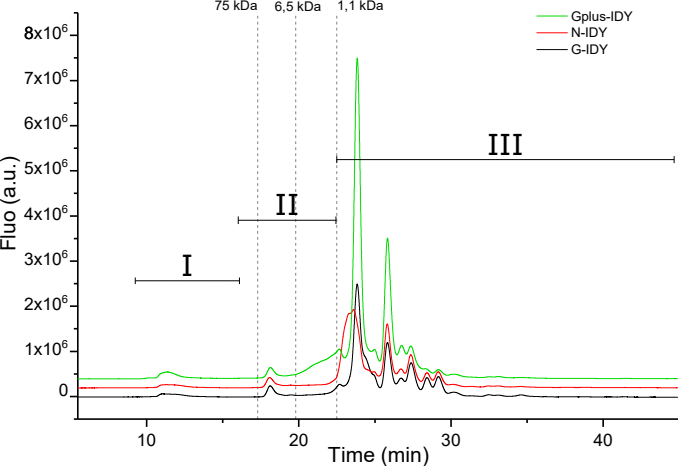
619  
620 **Figure 4:** 193 potential peptides annotation, out of the 1674  $m/z$  from (-)FT-ICR-MS  
621 submitted to the OligoNet webserver. (A) Annotations correspond to 142 Multiple Amino  
622 Acid Combination (MAAC, with 89 and 43 with and without cysteinyl residue respectively)  
623 and 61 Unique Amino Acid Combination (UAAC, with 26 and 35 with and without cysteinyl  
624 residue respectively). (B) Within the 193 potential peptides N-IDY (red), G-IDY (blue) and  
625 Gplus-IDY (green) released 2, 7 and 40 unique  $m/z$  attributed to peptides, respectively. The  
626 144 remaining potential peptides are shared (grey) by at least 2 IDYs. (C) Amongst the 61  
627 UAAC, 28 are significantly more released by Gplus-IDY, in contrast only 3 and 1 are more  
628 abundant using G- or N-IDY respectively (p-value < 0.05).

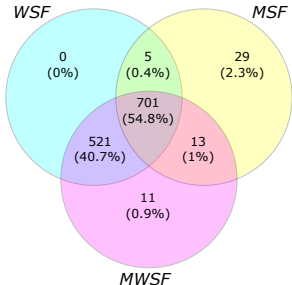
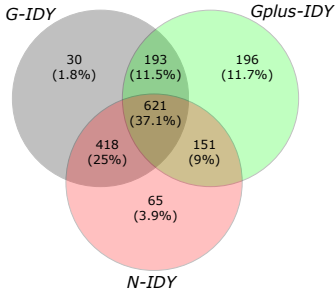
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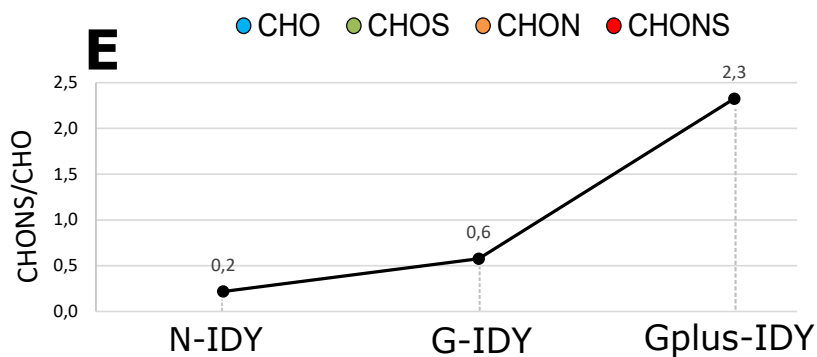
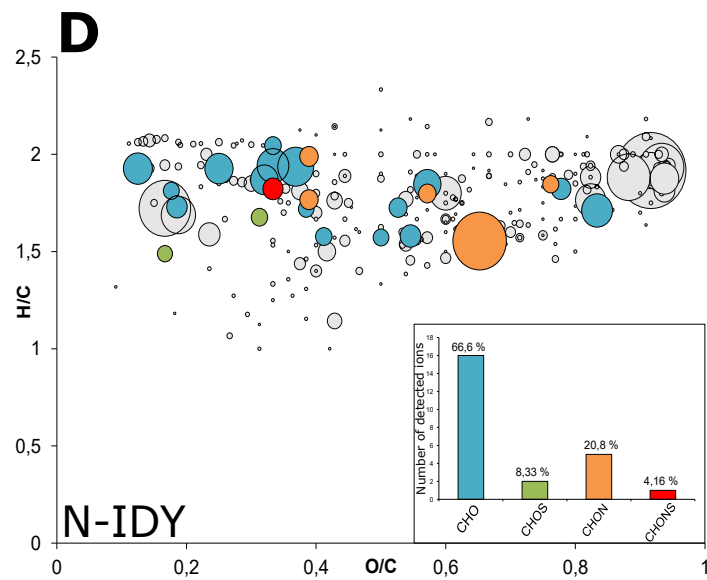
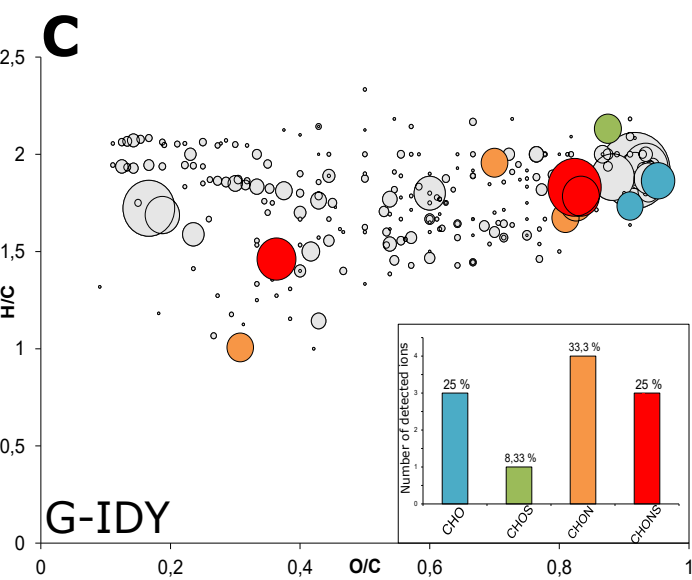
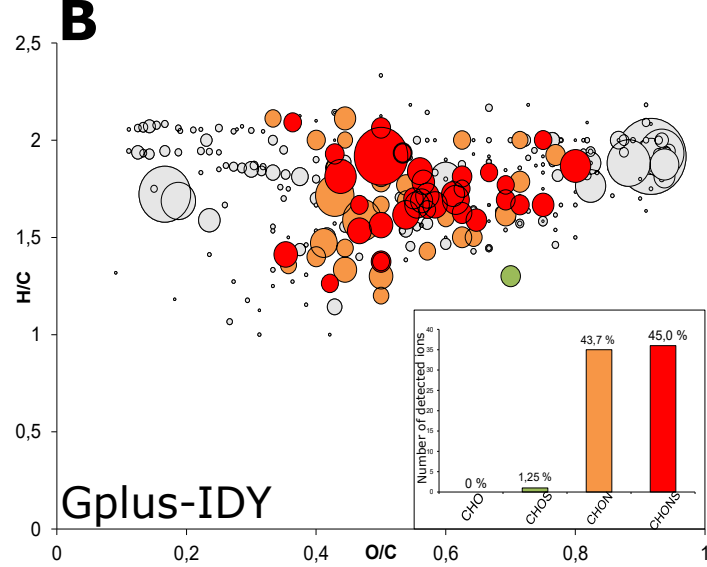
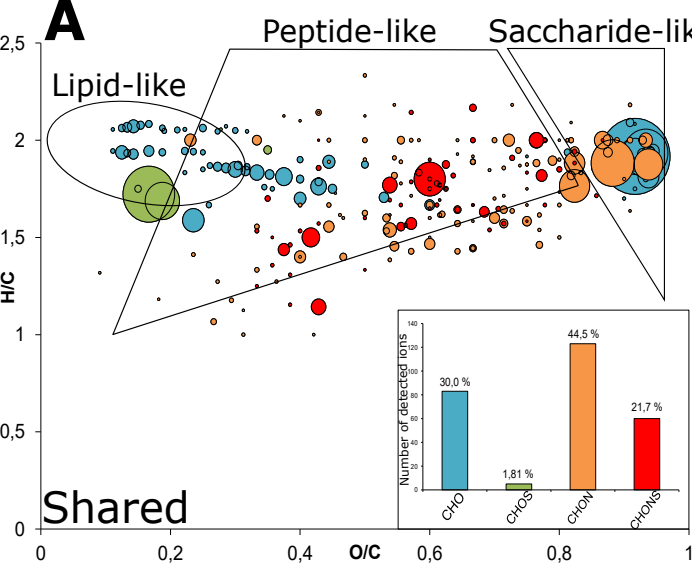
630 **Table 1:** Metabolites fragmented by UHPLC-Q-ToF-MS/MS in the MWSF for the three  
631 IDYs, and their corresponding chemical family. Main fragments used to identify features  
632 from databases are listed in the discriminant fragments column allowing a high confidence  
633 level. Relative abundance of metabolites between IDYs is expressed as percentage of the  
634 biggest area. The identification is the combination of composition elucidation by (-)FT-ICR-  
635 MS (when available) and structural elucidation by UHPLC-Q-ToF-MS /MS.  
636

m/z Adduct	Ret. time (min)	Collision energy (eV)	Discriminant Fragments	Annotation [M]	Error (ppm)	Area (%)			Identified metabolite	Database ID
						N-IDY	G-IDY	Gplus-IDY		
130.0863 [M+H] <sup>+</sup>	0.7	30	84.0808	C <sub>6</sub> H <sub>11</sub> NO <sub>2</sub>	0.35	100	43	41	Pipecolic acid	C00408 (KEGG)
148.0601 [M+H] <sup>+</sup>	0.6	15	84.0447;130.0497	C <sub>5</sub> H <sub>9</sub> NO <sub>4</sub>	-2.03	100	78	53	Glutamic acid	C00025 (KEGG)
165.0548 [M+H] <sup>+</sup>	0.7	10	147.0442;119.0492 ;91.0544	C <sub>9</sub> H <sub>8</sub> O <sub>3</sub>	1.08	47	30	100	Coumaric acid	C12621/ C00811 (KEGG)
166.0861 [M+H] <sup>+</sup>	1.0	15	166.0862;120.0806 ;131.0490	C <sub>9</sub> H <sub>11</sub> NO <sub>2</sub>	-1.20	44	36	100	Phenylalanine	C00079 (KEGG)
179.0485 [M+H] <sup>+</sup>	0.7	10	162.0222;144.0116 ;116.0164	C <sub>5</sub> H <sub>10</sub> N <sub>2</sub> O <sub>3</sub> S	0.06	36	91	100	Cysteinyl-glycine	C01419 (KEGG)
182.0811 [M+H] <sup>+</sup>	0.7	15	136.0757;123.0438 ;147.0442	C <sub>9</sub> H <sub>11</sub> NO <sub>3</sub>	-0.55	71	43	100	Tyrosine	C00082 (KEGG)
191.0199 [M-H] <sup>-</sup>	0.7	15	173.0095;129.0197 ;111.0094	C <sub>6</sub> H <sub>8</sub> O <sub>7</sub>	1.05	100	37	17	Citric acid	C00158 (KEGG)
205.0355 [M-H] <sup>-</sup>	0.8	15	143.0352;125.0248	C <sub>7</sub> H <sub>10</sub> O <sub>7</sub>	0.49	100	<1	<1	Homocitric acid	C05662 (KEGG)
218.1037 [M-H] <sup>-</sup>	1.1	15	146.082	C <sub>9</sub> H <sub>17</sub> NO <sub>5</sub>	1.38	100	63	73	Pantothenic acid	C00864 (KEGG)
251.0693 [M+H] <sup>+</sup>	0.7	15	122.0269;130.0497 ;188.0373	C <sub>8</sub> H <sub>14</sub> N <sub>2</sub> O <sub>5</sub> S	-1.19	19	71	100	Glutamyl-cysteine	C00669 (KEGG)
258.11 [M+H] <sup>+</sup>	0.6	20	184.0735;104.1070	C <sub>8</sub> H <sub>20</sub> NO <sub>6</sub> P	-0.39	18	23	100	Glycerophosphocholine	C00670 (KEGG)
260.1969 [M+H] <sup>+</sup>	0.8	30	147.1132;129.1025 ;84.0810	C <sub>12</sub> H <sub>25</sub> N <sub>3</sub> O <sub>3</sub>	0.12	<1	<1	100	Leucyl-lysine	HMDB28912 (HMDB)
268.1037 [M+H] <sup>+</sup>	0.7	15	119.0353;136.0617	C <sub>10</sub> H <sub>13</sub> N <sub>5</sub> O <sub>4</sub>	-1.12	92	100	94	Adenosine	C00212 (KEGG)
288.2030 [M+H] <sup>+</sup>	0.8	20	271.1763;175.1187	C <sub>12</sub> H <sub>25</sub> N <sub>5</sub> O <sub>3</sub>	-0.06	<1	<1	100	Leucyl-arginine	HMDB28923 (HMDB)
298.0970 [M+H] <sup>+</sup>	1.3	15	136.0616;145.0320	C <sub>11</sub> H <sub>15</sub> N <sub>5</sub> O <sub>3</sub> S	0.67	74	100	85	Methyl-thioadenosine	C00170 (KEGG)
306.0763 [M-H] <sup>-</sup>	0.7	15	272.0890;143.0464 ;210.0882	C <sub>10</sub> H <sub>17</sub> N <sub>3</sub> O <sub>6</sub> S	-0.65	33	89	100	Glutathione	C00051 (KEGG)

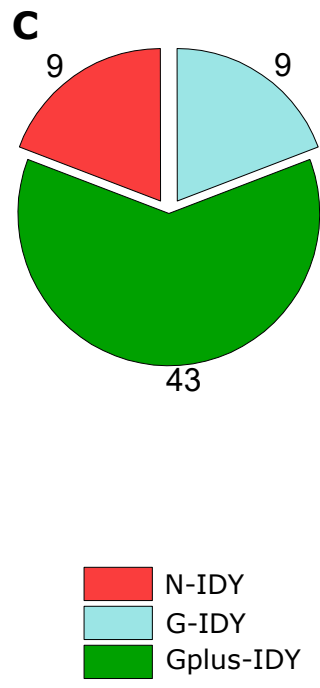
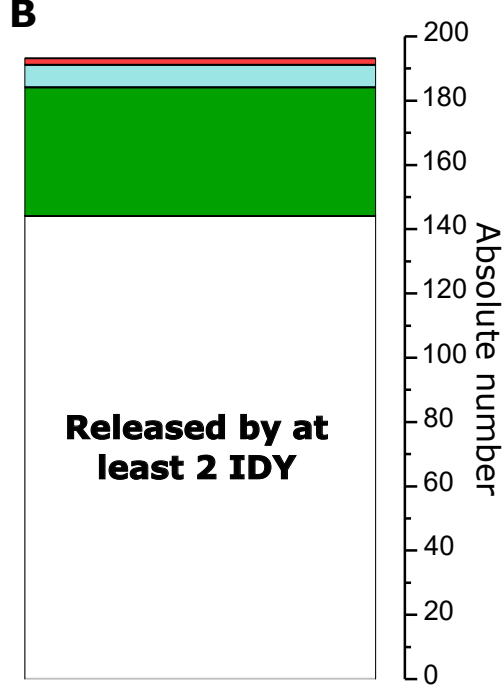
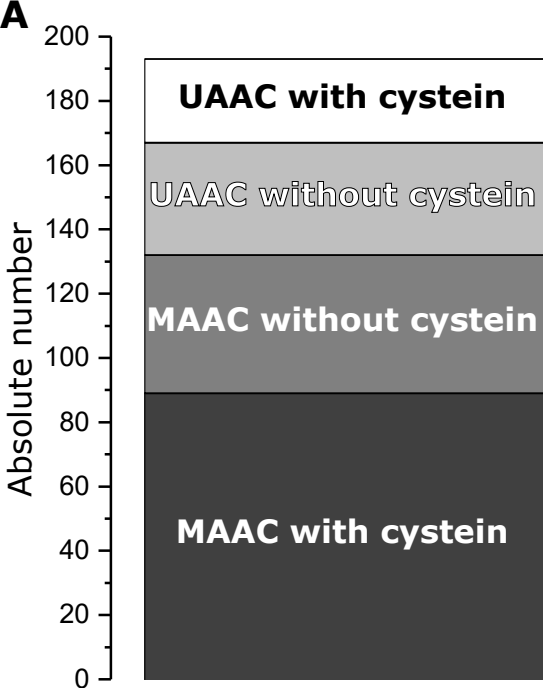
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MS-containing compounds

