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1 Influence of regionality and maturation time on the chemical fingerprint of whisky

2

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6

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19

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21 maturation time

22

## 23 **Abstract**

24 Understanding the chemical composition of whisky and the impact of each step in the  
25 manufacturing process provides a basis for responding to the challenges of producing high  
26 quality spirits. In this study, the objective was to discriminate whiskies according to their  
27 geographical origin and authenticate the maturation time in cask based on the non-volatile  
28 profiles. The combination of FT-ICR-MS and chemometrics allowed the distinction of  
29 whiskies from four geographical origins in Scotland (Highlands, Lowlands, Speyside and  
30 Islay). Statistical modeling was also used to discriminate whiskies according to the  
31 maturation time in cask and reveal chemical markers associated with the ageing regardless of  
32 the origin or the production process. Interestingly, the flow of transfer of compounds from  
33 wood barrels to distillates is not constant and homogeneous over the maturation time. The  
34 largest transfer of compounds from the barrel to the whisky was observed around twelve  
35 years of maturation

36

## 37 **Introduction**

38 Across Scotland there are up to 100 operating distilleries producing whisky distributed in five  
39 main regions commonly recognized as Lowlands, Highlands, Speyside, Islands (including  
40 whisky from Orkney, Skye, Mull, Jura, Arran and the Campbeltown peninsula) and  
41 Islay(Barnard, 2013).

42 The composition of whisky is complex and mainly impacted by the water and the cereals  
43 used, the fermentation, the distillation, the maturation and the blending process(Garcia et al.,  
44 2013; John R. Piggott, Sharp, Duncan, & others, 1989; Reid, Swan, & Gutteridge, 1993).  
45 Traditionally, whiskies are matured in oak wood barrels for a minimum of three years before  
46 consumption, but many whiskies are matured for 12 or more years(JOHN R. Piggott, Conner,  
47 Paterson, & Clyne, 1993). As for wine, sensory properties of whiskies are known to improve  
48 during the period of maturation in the oak barrel, where the composition changes through a  
49 complex array of chemical reactions(MacNamara, Dabrowska, Baden, & Helle, 2011;  
50 Macnamara, van Wyk, Brunerie, Augustyn, & Rapp, 2001). Previous studies have attempted  
51 to describe the chemical changes during maturation, with particular emphasis on the color,  
52 the aroma, the volume, the strength and the flavor compounds(Lee, Paterson, Piggott, &  
53 Richardson, 2001a; Liebmann & Scherl, 1949; MacNamara et al., 2011; Macnamara et al.,  
54 2001).

55 Due to its large commercialization and relatively high prices, whisky counterfeiting and  
56 adulteration is quite common worldwide(Garcia et al., 2013; Wiśniewska, Dymerski,  
57 Wardencki, & Namieśnik, 2015). In case of fraudulent Whisky, maturation time and history  
58 of the oak casks can be mislabeled(Stupak, Goodall, Tomaniova, Pulkrabova, & Hajslova,  
59 2018). Authenticity is one of the major concerns for the distillers, dealers and consumers of  
60 whiskies around the world(R. I. Aylott & MacKenzie, 2010; Ross I. Aylott, Clyne, Fox, &  
61 Walker, 1994; Heller, Vitali, Oliveira, Costa, & Micke, 2011; M. MacKenzie & I. Aylott,  
62 2004; Møller, Catharino, & Eberlin, 2005; Parker, Kelly, Sharman, Dennis, & Howie, 1998;  
63 Stupak et al., 2018). Both, maturation period and history of the casks in which maturation  
64 occurred are important for the final composition(Kew, Goodall, Clarke, & Uhrin, 2016;  
65 Roullier-Gall et al., 2018). Recently, Whisky authentication has been the subject of several  
66 studies employing various analytical techniques, such as: GC and LC with different detectors  
67 (FID, AED, UV-Vis), electronic nose, atomic absorption spectroscopy and mass  
68 spectrometry(Wiśniewska et al., 2015). The aroma and flavor profiles are classically used to

69 distinguish whiskies by distillery or even to reflect their places of origin(Jack & Steele, 2002;  
70 Jackson, 2015; Lee, Paterson, Piggott, & Richardson, 2001b). Gas chromatography coupled  
71 to tandem mass spectrometry (GC-Q-ToF) was employed to distinguish malt whiskies  
72 according to the type of cask in which they were matured (bourbon versus bourbon and  
73 wine)(Stupak et al., 2018). Infrared spectroscopy with statistical analysis allowed to  
74 distinguish Scottish, Irish, and American whiskies and 2 and 3 years old beverages from 6  
75 and 12 years old whiskies(Sujka & Koczoń, 2018). Recently, two different, hypothesis-free,  
76 sensor arrays based upon three fluorophores successfully discriminated whisky samples with  
77 respect to origin (American, Irish and Scotch Whisky) and taste (rich vs. light)(Han et al.,  
78 2017). Mass spectrometry is more and more used for quality control and proof of authenticity  
79 of whisky samples. Direct infusion electrospray ionization mass spectrometry (ESI-MS) was  
80 used in order to provide a direct, rapid and sensitive method for the characterization of  
81 distilleries and authenticity of whisky samples(Møller et al., 2005). Garcia et al., highlighted  
82 the use of Fourier-transform ion cyclotron resonance mass spectrometry (FT-ICR-MS) as an  
83 approach to screen for ageing and counterfeiting(Garcia et al., 2013). FT-ICR-MS was used  
84 by Kew et al. (2016) for the discrimination of whiskies according to their blending process  
85 and type of casks used in maturation(Kew et al., 2016). More recently, non-targeted FT-ICR-  
86 MS combined with LC-MS/MS showed the impact of the wood and in particular the history  
87 of the barrel on the distillate composition during ageing and revealed the importance of the  
88 initial composition of the distillate and the distillery process(Roullier-Gall et al., 2018).  
89 The objective of this work was to go beyond our previous work and implement strategies by  
90 using statistical modeling to discriminate whiskies according to their geographical origin and  
91 authenticate the maturation time in the barrel based on the non-volatile profiles. Here, we  
92 show that the combination of FT-ICR-MS and statistical analysis of whiskies from 3 to 43  
93 years of cask maturation allowed the authentication of the geographical origin and maturation  
94 time. The combination of metabolomics and chemometrics was able to reveal chemical  
95 markers associated with the ageing, regardless of the origin or the production process.

96

## 97 **Materials and Methods**

### 98 **Whisky samples**

99 106 whisky samples from 32 different distilleries in Scotland and from 3 to 43 years of  
100 maturation were analyzed (Supplemental table 1). All samples were collected directly from  
101 the bottle and stored in 10 mL amber vials at room temperature until analysis.

102

### 103 **Direct infusion FT-ICR-MS**

104 Ultrahigh-resolution FT-ICR mass spectra were acquired with a 12 T Bruker Solarix mass  
105 spectrometer (Bruker Daltonics, Bremen, Germany) equipped with an APOLLO II  
106 electrospray source in negative ionization mode. For MS analysis, whiskies were diluted  
107 5:100 (v/v) with methanol (LC-MS grade, Fluka, Germany). The diluted samples were  
108 infused into the electrospray ion source with a flow rate of 120  $\mu\text{L h}^{-1}$ . [Operating parameters](#)  
109 [of direct infusion FT-ICR-MS](#) were carried out according to Roullier-Gall *et al.*, 2018  
110 [previous study](#). Settings for the ion source were: drying gas temperature 180 °C, drying gas  
111 flow 4.0 L  $\text{min}^{-1}$ , capillary voltage 3,600 V. Spectra were first externally calibrated by ion  
112 clusters of arginine (10 mg  $\text{mL}^{-1}$  in methanol). Internal calibration of each spectrum was  
113 conducted with a reference list including selected whisky markers and ubiquitous fatty acids.  
114 The spectra were acquired with a time-domain of 4 megawords and 400 scans were  
115 accumulated within a mass range of  $m/z$  92 to 1000 corresponding to a run time of 18 min. A  
116 resolving power of 400,000 at  $m/z$  300 was achieved. Quality control (QC) samples were  
117 prepared by pooling equal amounts of all samples. QC samples were analyzed at the  
118 beginning and after every tenth sample to monitor the reproducibility of the measurements  
119 (Roullier-Gall *et al.*, 2018).

120

### 121 **Processing of FT-ICR-MS data**

122 Raw spectra were post-processed by Compass DataAnalysis 4.2 (Bruker Daltonics, Bremen,  
123 Germany) and peaks with a signal-to-noise ratio (S/N) of at least 6 were exported to mass  
124 lists. All exported  $m/z$  features were aligned into a matrix containing averaged  $m/z$  values  
125 (peak alignment window width:  $\pm 1$  ppm) and corresponding peak intensities of all analyzed  
126 samples. Molecular formulae were assigned to the exact  $m/z$  values by mass difference  
127 network analysis using an in-house developed software tool. In total, 5979 detected features  
128 could be assigned to distinct and unique molecular formulae. More than 90% of all  
129 assignments were found within an error range lower than 0.2 ppm. All further calculations  
130 and filtering were done in Perseus 1.5.1.6 (Max Planck Institute of Biochemistry, Germany)  
131 and R Statistical Language (version 3.1.1).

132

### 133 **Repeatability of FT-ICR-MS whisky measurements**

134 The repeatability of the measurements was evaluated on QCs samples injected in triplicate at  
135 the beginning and at the end of the sequence analysis. The repeatability over time of FT-ICR-  
136 MS analyses during the entire runtime was also evaluated , by monitoring the precision of the  
137 recorded peak intensities from ten analyzed QC samples and manual comparison of the raw  
138 mass spectra (Roullier-Gall et al., 2018). In the QC samples, 92.8% of all detected  
139 monoisotopic signals showed a relative standard deviation lower than 20% (Roullier-Gall et  
140 al., 2018).

141

#### 142 **Excitation Emission Matrix Fluorescence**

143 Whisky fluorescence was acquired on a spectrofluorometer (Horiba Aqualog). Excitation  
144 ranged from 225 to 600 nm and emission was recorded from 230 to 600 nm (C. Coelho et al.,  
145 2015). The spectrofluorometer used a conventional right-angle optical setup. Excitation  
146 Emission Matrices (EEM) were corrected daily for their Rayleigh and Raman scattering, as  
147 well as inner filtering effects by using the functions provided within the FluorEssence  
148 software of the Horiba Aqualog system. The fluorescence intensity was normalized to a  
149 Starna 1 ppm quinine sulfate reference cell, measured every day.

150

#### 151 **UPLC targeted analysis of polyphenols**

152 An Acquity Waters ultra performance liquid chromatography (UPLC) with a diode array  
153 fluorescence detector (DAD) was used to separate and quantify individual polyphenolic  
154 compounds in whisky samples. The column was a BEH C18, 1.7  $\mu\text{m}$ , 2.1 mm  $\times$  150 mm.  
155 Column temperature was kept constant at 30  $^{\circ}\text{C}$ , and samples were held at 8  $^{\circ}\text{C}$ . An elution  
156 was applied, starting isocratic from 100% A (ultrapure water, 0.1% formic acid) from 0 to 6  
157 min and then the gradient was increased linearly over 56 min to 100% B (methanol, 0,1%  
158 formic acid), where it was held again until reaching 60 min, with a rate of 0.3 mL $\cdot$ min $^{-1}$ .  
159 Injection volume was 5  $\mu\text{L}$ . Detection limits (LODs), quantification limits (LOQs) and intra-  
160 and inter-day variation of the assay were determined by injecting a series of dilute solutions  
161 with known concentrations.

162

#### 163 **Total phenolic content (TPC) of whiskies**

164 Total phenolic content (TPC) in whisky was determined using the Folin-Ciocalteu assay: 0.1  
165 mL of undiluted whisky, 0.5 mL of Folin-Ciocalteu reagent, 2 mL of a 20 (w/v) sodium  
166 carbonate solution and 7.4 mL of ultrapure water were mixed and reacted for 30 min at room

167 temperature. Then, the absorbance at 750 nm was measured and calculated as  $\text{mg}\cdot\text{L}^{-1}$  of gallic  
168 acid equivalent using a calibration curve between 0 and 500  $\text{mg}\cdot\text{L}^{-1}$  of gallic acid. All  
169 samples were analyzed in triplicates.

170

## 171 **Data analysis**

172 The statistical analysis was performed with Perseus, Simca 12.0 (Umetrics, Umeå, Sweden)  
173 and R (Version 1.0.136).

174 Van Krevelen and elemental diagrams were used to visualize FT-ICR MS data (Gonsior et  
175 al., 2009; Gougeon et al., 2011; Hertkorn et al., 2008). Multivariate statistical methods (.  
176 Principal component analysis and hierarchical cluster analysis) were used to explore  
177 similarities and hidden patterns among samples (Barker & Rayens, 2003; Granato, Santos,  
178 Escher, Ferreira, & Maggio, 2018). As within-groups variability dominates the among-groups  
179 variability for the geographical origin and maturation time impact, partial least square  
180 discriminant analysis were used (Barker & Rayens, 2003). We set up diverse classification  
181 models in order to reveal the different geographical origin and maturation time for barrel  
182 specific metabolites. In order to improve the efficiency of such classification and the possible  
183 presence of overfitting and noise, we preprocessed the entire dataset applying the ReliefF  
184 algorithm (Witten, Frank, Hall, & Pal, 2016). The algorithm identified a subset of variables  
185 that was used for the next classification models maximizing the model performance in term  
186 of accuracy. The features' selection was based on the highest rank value attributed to each  
187 variable by the algorithm. Consequently, with the reduced datasets we built several partial  
188 least square discriminant analysis (PLS-DA) models. The goodness of the fit was evaluated  
189 by the coefficient of determination ( $R^2X$ ), the proportion of the variance of the response that  
190 is explained by the model ( $R^2Y$ ), the predictive ability ( $Q^2Y$ ) and validation was carried out  
191 using permutation tests. For the ageing time model, whisky samples were randomly divided  
192 into two groups: 101 samples selected to build the statistical model and the 5 remaining  
193 samples employed as external validation. A Bland–Altman plot was used to analyze the  
194 agreement between the known ageing time and the model prediction.

195

## 196 **Results and discussion**

### 197 **Geographical origin impact of Scotch whisky**

198

199 A visual comparison of FT-ICR-MS spectra of Scotch whiskies from Highland, Lowland,  
200 Speyside and Islay distilleries and from 3 to 43 years of cask maturation is presented in  
201 Figure 1. Several thousands of signals were found between  $m/z$  100 to 600. The enlargements  
202 at  $m/z$  261 exemplarily show the differences in chemical composition between the four  
203 geographical areas (Figure 1). Between  $m/z$  261.0 and 261.2, whisky spectra only differed in  
204 the intensities of the eight detected peaks. For example, signals at  $m/z$  261.04044 and at  $m/z$   
205 261.17075 correspond to the molecular formulas  $[C_{13}H_9O_6]^-$  and  $[C_{13}H_{25}O_5]^-$ , respectively,  
206 and show comparable intensities in all four whisky samples (Figure 1). The Venn and van  
207 Krevelen diagrams (Figure 1) show that 2200 formulas (out of 5979) were found in all  
208 samples of the four groups of whisky samples confirming a great consistency in the chemical  
209 composition regardless of the origin (Kew et al., 2016).

210 Despite the high similarities between whiskies (36.8% of formulas were found in all samples  
211 of the four groups of whisky samples) as shown in the Venn diagrams and the direct  
212 comparison of spectra (Figure 1), 533 (8%), 610 (10%), 401 (6.7%) and 464 (7.7%) formulas  
213 were uniquely found in at least 50% of Highland, Islay, Speyside and Lowland whisky  
214 samples, respectively. In addition to the unique formulas, differences in signal intensities  
215 between whiskies can be found which could result from particular distilleries, maturation  
216 times, geographical features or environmental impacts. As example, the signal at  $m/z$   
217 261.06158 corresponding to the molecular formula  $[C_{10}H_{13}O_8]^-$  is less intense in the whisky  
218 from Speyside ( $2 \cdot 10^7$ ) compared to the other three whiskies ( $5-6 \cdot 10^7$ ) (Figure 1).

219  
220 The impact of the type of whisky (blend or malt), the maturation wood type and the wood  
221 history on the Whisky composition has recently been studied (Kew et al., 2016; Roullier-Gall  
222 et al., 2018). However, barley, peat and water origin, malting, brewing, fermentation and  
223 distillation process, nature of the cellars, climates as well as environmental factors could also  
224 impact the complex composition of whisky. Figure 2 shows the first two principal  
225 components obtained from principal component analysis computed on the 106 analyzed  
226 whisky samples from Scotland. Whiskies showed a rough separation by their maturation time  
227 in the barrel on principal component 2. By comparison, the impact of the geographical origin  
228 - Highlands, Lowlands, Speyside and Islay - on the whisky metabolome appears less  
229 significant in the PCA compared to the maturation time (Figure 2). The samples from the  
230 Highlands and Lowlands appeared in a more discrete cluster than the Speyside and Islay  
231 samples, which are more distributed all around the plot. Interestingly, the four samples in the



232 top left corner of the PCA were from the same distillery (but with different maturation times)  
233 and the eight samples (in red) in the top right corner were from different distilleries but all in  
234 Islay and all with six years of barrel maturation (Figure 2). The great diversity in the  
235 composition of the whisky samples demonstrates the large number of environmental  
236 parameters (including production, geographical origin, maturation time...), which eventually  
237 impacts the final product.

238

239 A group separation is not easily observable in PCA models because of the high number of  
240 sample characteristics (maturation from 6 to 43 years, 4 geographical origins and 32  
241 manufacturers) and the high number of variables considered (5979 molecular formulas). A  
242 PLS regression model was used to better distinguish the geographical origins of the whisky  
243 samples. Figure 3 shows results from a PLS regression computed from FT-ICR-MS data of  
244 106 whisky samples from the four areas in Scotland, 32 different distilleries and from 3 to 43  
245 years of maturation. The first two components allowed to separate whiskies into four distinct  
246 groups according to the geographical origin (Highlands, Lowlands, Speyside and Islay) and  
247 independent of the distillery or the maturation time (Figure 3). Whiskies from Lowlands and  
248 Islay distilleries appear to be chemically more different from the samples from Highlands and  
249 Speyside distilleries (separation on the 1<sup>st</sup> component). The second component allowed to  
250 distinguish whiskies from the Highlands and Lowlands from those produced in Speyside and  
251 Islay distilleries. Based on the PLS, specific masses for each location were extracted (VIP  
252 masses) and projected into van Krevelen diagrams (Figure 3). For example, samples from  
253 Speyside clearly showed an enrichment of polyphenols or polyphenol-type compounds  
254 (Figure 3). By comparison, Highland samples seem to be characterized by higher alcohols  
255 with a profile reminiscent to the distillate (Roullier-Gall et al., 2018). Lowland and Islay  
256 appear to be characterized by carbohydrates. The schematic classification of single malts  
257 according to their origin could be based, in part, on a geological reality, of which water  
258 would be the main factor. Meier-Augenstein showed that the source water used for and during  
259 whisky production correlates with the authenticity of whisky (Meier-Augenstein, Kemp, &  
260 Hardie, 2012). Water is involved in several stages of processing: added to ground barley to  
261 produce the mash, when diluting the distilled alcohol to cask strength for maturation and  
262 when reducing cask strength whisky for bottling. Meier-Augenstein's study suggests that  
263 whisky keeps a signature of the geographic provenance of the used water (Meier-Augenstein  
264 et al., 2012). However, from all elements necessary for whisky production, water is the one

265 whose impact on the final composition is probably the most difficult to evaluate(Witten et al.,  
266 2016).

267

268 Another source of the variability in the chemical composition is the distillery brand.  
269 Compositional specificities from manufacturers can contribute to the geographical region  
270 model. Studying samples from Islay and from three distilleries including various maturation  
271 times highlights the ability of FT-ICR-MS to differentiate whiskies according to the  
272 distilleries and independently of the maturation time. Non-supervised statistics allowed to  
273 visualize the impact of the process, composition and factory on the final whisky  
274 independently of the maturation (supplementary Figure 1). According to the hierarchical  
275 cluster analysis (supplementary Figure 1), whiskies from the manufacturers W2 and W1  
276 manufacturers seem close and distinct from W3 although W2 and W3 are geographically  
277 very close to one another (less than 3 km). According to the van Krevelen diagrams in  
278 supplementary Figure S1, the whisky from distillery W1 (including whiskies from 12 to 18  
279 years in barrel) seems to be characterized by a higher concentration in polyphenolic  
280 compounds whereas whiskies from distillery W2 (12 to 21 years in barrel) are mainly  
281 characterized by carbohydrate-type compounds as well as sulfur and nitrogen containing  
282 compounds. The whisky from distillery W3 (6 to 12 years in barrel) seems to be  
283 characterized by higher alcohols and shows a profile reminiscent to distillate (Roullier-Gall et  
284 al., 2018).

285

286 The samples differing in brand and geographical origin can be properly grouped and  
287 characterized by FT-ICR-MS and chemometric analysis. These combined techniques enable  
288 the fast evaluation and authentication of Scotch whiskies independently of the maturation  
289 time or the process used. They allowed an excellent determination of whisky composition as  
290 well as distinguishing between different brands of whiskies produced in areas only 20 km  
291 apart from one another.

292

293

294

295 **Impact of maturation time on the chemical composition of Scotch whiskies**

296

297 Based on the huge impact of maturation in wood barrels on the chemical diversity of whisky,  
298 as shown in our previous study(Roullier-Gall et al., 2018), we here focused on the impact of  
299 the maturation time on the final composition. We first studied three samples from one  
300 distillery (W3) in Islay after 6, 8 and 10 years of cask maturation (Supplementary Figure 2).  
301 The samples revealed 376 mass peaks whose intensity significantly increased with maturation  
302 time, and 81 mass peaks with decreasing intensity (Supplementary Figure 2a-b). The higher  
303 number of features which showed an increase in peak intensity confirms that chemicals  
304 transfer from wood to the distillate as a function of ageing.

305

306 In order to extract age-related metabolite signatures, a PLS discriminant analysis was  
307 designed using 7 groups with different maturation times: whiskies that had been aged in casks  
308 for 6 years, 8 years, 10 years, 12 years, 14 years, 16 years and 18 years (Supplementary Table  
309 3). We selected 76 from the 106 analyzed whisky samples which ranged from 6 to 18 years.  
310 A PLS-DA model was built based on 71 of these whisky samples (Figure 4c). Five of the 76  
311 samples were used for model validation (Figure 4e). The separation of whiskies according to  
312 the ageing time (Figure 4c) showed a good predictive power with  $Q^2$  of 0.49 and  $R^2Y$  of 0.77.  
313 The scatter plot of the model group versus the predicted group for each sample confirmed the  
314 robustness of the prediction model (Figure 4 D), with a prediction average close to the  
315 maturation time and an excellent standard deviation (between 0.69 to 1.57 years,  
316 Supplementary Table 3). However, it was not possible to really differentiate groups of 12 and  
317 14 years of maturation. These samples were predicted as 12.3 years for both, the group of 12  
318 years and 14 years old whiskies (maximum standard deviation was 1 year).

319 Finally, the five whisky samples, which were not used for the model construction (maturation  
320 of 6, 10, 12, 12 and 16 years in barrel) were used to validate the model. The good predictions,  
321 achieved for these five whisky samples, confirmed the predictive ability of the maturation  
322 time model (Figure 4 C and E). After model validation, 256 discriminating and characteristic  
323 masses (VIP variables higher than 1), whose peak intensities increased with maturation, were  
324 extracted (Figure 4 F and H). As example, compounds from wine such as syringic acid and  
325 caftaric acid and from wood such as lyoniresinol, patuletin and digallic acid were found as  
326 increasing with maturation time (Figure 4 and supplementary Figure 2). In contrast, 213  
327 masses with decreasing intensity led to 54 annotations from databases including stearyl  
328 acetate and syringaledehyde (Figure 4).

329

330 ANOVA tests were applied to extract subsets of features that discriminate between whiskies  
331 with a maturation time of 6 and 8 years (supplementary figure 4A), 8 and 10 years  
332 (supplementary figure 4B), 10 and 12 years (supplementary figure 4C), 12 and 14 years  
333 (supplementary figure 4D), 14 and 16 years (supplementary figure 4E), and 16 and 18 years  
334 (supplementary figure 4F), respectively. Statistically extracted masses can be used to  
335 highlight the impact of maturation time on the whisky composition between the different  
336 ageing groups. According to the statistics, 27 molecular formulas were significantly enriched  
337 in the youngest whiskies (6 years of maturation) while 36 formulas were specific to the 66  
338 whiskies older than 6 years of maturation. In the same way, 74 molecular formulas were  
339 statistically identified as markers to differentiate between whiskies younger than 8 years of  
340 maturation (6 and 8 years of maturation) and 132 formulas were identified as markers for  
341 whiskies older than 8 years of maturation. The number of significant masses statistically  
342 extracted increased until the distinction of whiskies between 10 (121 molecular formulas) and  
343 12 (1182 molecular formulas) years of casks maturation. Then the number of significant  
344 masses starts to decrease.

345 Interestingly, the higher differences were found between whiskies after 10 and 12 years of  
346 maturation directly followed by whiskies after 14 and 16 years of maturation (supplementary  
347 Figure 4). The similarities between samples characterized by 12 and 14 years of maturation,  
348 which probably can be explained by the low ability of the model prediction to distinguish  
349 those two groups (Figure 4). The difference between young (less than 12 years of maturation,  
350 group 1) and old whiskies (more than 12 years of maturation, group 2) was further studied by  
351 Excitation Emission Matrix (EEM) fluorescence spectroscopy and analysis of polyphenols  
352 (supplementary figure 5 and 6). As illustrated in Supplementary figure 6A, PARAFAC  
353 results enabled to easily discriminate whiskies from both groups on the first component.  
354 Whiskies of group 1 (younger than 12 years of maturation) were driven by wood barrel  
355 ageing processes, conferring a relative homogeneity to this group. Whiskies from the group 2  
356 (older than 12 years of maturation) showed a higher within-group-variation (confirming  
357 results from FT-ICR-MS), which may be because of different cask finishing practices. This  
358 may include re-casking for a secondary maturation into other wood casks that were used for  
359 ageing of wines, spirits or even beers (Scotch Whisky Association, n.d.). Such practice tends  
360 to diversify Scottish whisky profiles by masking the original distillate. The fluorescence  
361 positions of the PARAFAC components suggest differences in polyphenolic compounds  
362 (Figure 6B) which could be confirmed in a TPC assay and quantification of syringic acid,

363 gallic acid and scopoletin by UPLC (Figure 6C). Scopoletin was also identified as wood  
364 marker increasing in intensity with maturation time. No significant differentiations were  
365 found for tyrosol between our two groups, surely due to a moderated retention of this  
366 compound to wood surfaces (Christian Coelho et al., 2019).  
367 Out of the 1182 molecular formulas found significantly increased for whiskies with at least  
368 12 years of maturation, only 222 annotations were found in databases: 167 were found in our  
369 home-built plant database, 154 in Metlin, 130 in KEGG and 25 in HMDB (Supplementary  
370 Figure 7 and supplementary Table 4). Interestingly, 467 of the 1182 markers could be  
371 previously identified as wood specific markers (Roullier-Gall et al., 2018) (Supplementary  
372 Figure 8). Moreover, 675 of the 1182 features could be detected in wine samples including  
373 337 compounds already identified as wood markers (Supplemental Figure 8). The higher  
374 presence of wine-based compounds in whiskies older than 12 years, could correspond to  
375 finishing practices that could take place after 10 years of ageing independently of the region  
376 of production.

377

### 378 **Conclusion**

379 In conclusion, FT-ICR-MS based metabolomics was used as a powerful tool to discriminate  
380 whiskies according to maturation time and geographical origin, regardless of the  
381 manufacturer, the barrel used and the production process. Untargeted metabolomics allowed  
382 to study a broad range of metabolites and the extraction of markers that were significantly  
383 different depending on the geographical origin and/or the maturation time. A prediction  
384 model was further developed for the comprehensive evaluation of maturation time  
385 authenticity. Interestingly, the flow of transfer of compounds from wood barrels to distillates  
386 is not constant and homogeneous over the maturation time. The largest transfer of compounds  
387 from the barrel to the whisky was observed between ten and twelve years of maturation with  
388 up to 1182 molecular formulas that showed a significant increase in their peak intensities as a  
389 function of the maturation time. 467 of these were identified as wood-specific compounds  
390 and 338 are typically also found in wine. The explanatory hypothesis for the higher presence  
391 of wine-related compounds in whiskies older than 12 years, compared to whiskies younger  
392 than 12 years could be the finishing practice that takes place after 12 years of ageing  
393 independently of the region of production.

394

395 **Figure 1:** Visualization of ESI(-) FT-ICR-MS spectra of four samples from Highlands,  
396 Lowlands, Speyside and Islay A) in the mass range  $m/z$  100 - 600. B) The enlargement at the  
397 nominal mass  $m/z$  261 shows eight ions detected and assigned to unique molecular formulas.  
398 C) The Venn diagram shows the number of detected features which are common and specific  
399 to all samples analyzed for the four regions. D) Van Krevelen diagram (H/C vs. O/C) of the  
400 common composition found in all analyzed whisky samples. Points in van Krevelen diagrams  
401 are colored according to their elemental composition: CHO in blue, CHOS in green, CHON  
402 in orange, and CHONS in red. Scaling is according to the mean peak intensity.

403

404 **Figure 2:** Principal component score plots of 106 whisky samples from Scotland, analyzed  
405 by FT-ICR-MS and colored according to A) the geographical origin and B) the maturation  
406 time (from 6 up to 18 years). The first two components explained 38.4% of the total  
407 variability.

408

409 **Figure 3:** Map of the four whisky regions in Scotland (Highland, Speyside, Lowland and  
410 Islay). PLS regression model based on 106 whisky samples and van Krevelen diagrams  
411 showing the extracted “VIP” features for each region, respectively.

412

413 **Figure 4:** A) Visualization of ESI (-) FT-ICR-MS spectra ( $m/z$  100 to 600) of four samples  
414 matured in barrel for 6, 10, 14 and 18 years. B) Enlargement of the nominal mass at  $m/z$  261  
415 showing eight masses detected and assigned to unique molecular formulas. C) PLS model  
416 colored according to maturation time, based on 71 whisky samples ( $R^2Y(\text{cum})=0.57$  and  
417  $Q^2(\text{cum})=0.53$ ). Comparison of prediction results versus the maturation time for D) the  
418 samples used to build the PLS model and for E) the 5 predicted samples. Van Krevelen  
419 diagram (H/C vs. O/C) of the significant features, which contribute to the discrimination of  
420 maturation time including F) features which showed an increase in peak intensity and G)  
421 features with decreasing peak intensity as a function of the maturation time, respectively. H)  
422 Bar chart of the absolute intensity of some biomarkers that showed a significant change in  
423 peak intensities. Points in the van Krevelen diagrams are colored according to their elemental  
424 composition, CHO in blue, CHOS in green, CHON in orange, and CHONS in red. Scaling is  
425 according to the mean peak intensity.

426

427

428 **Literature**

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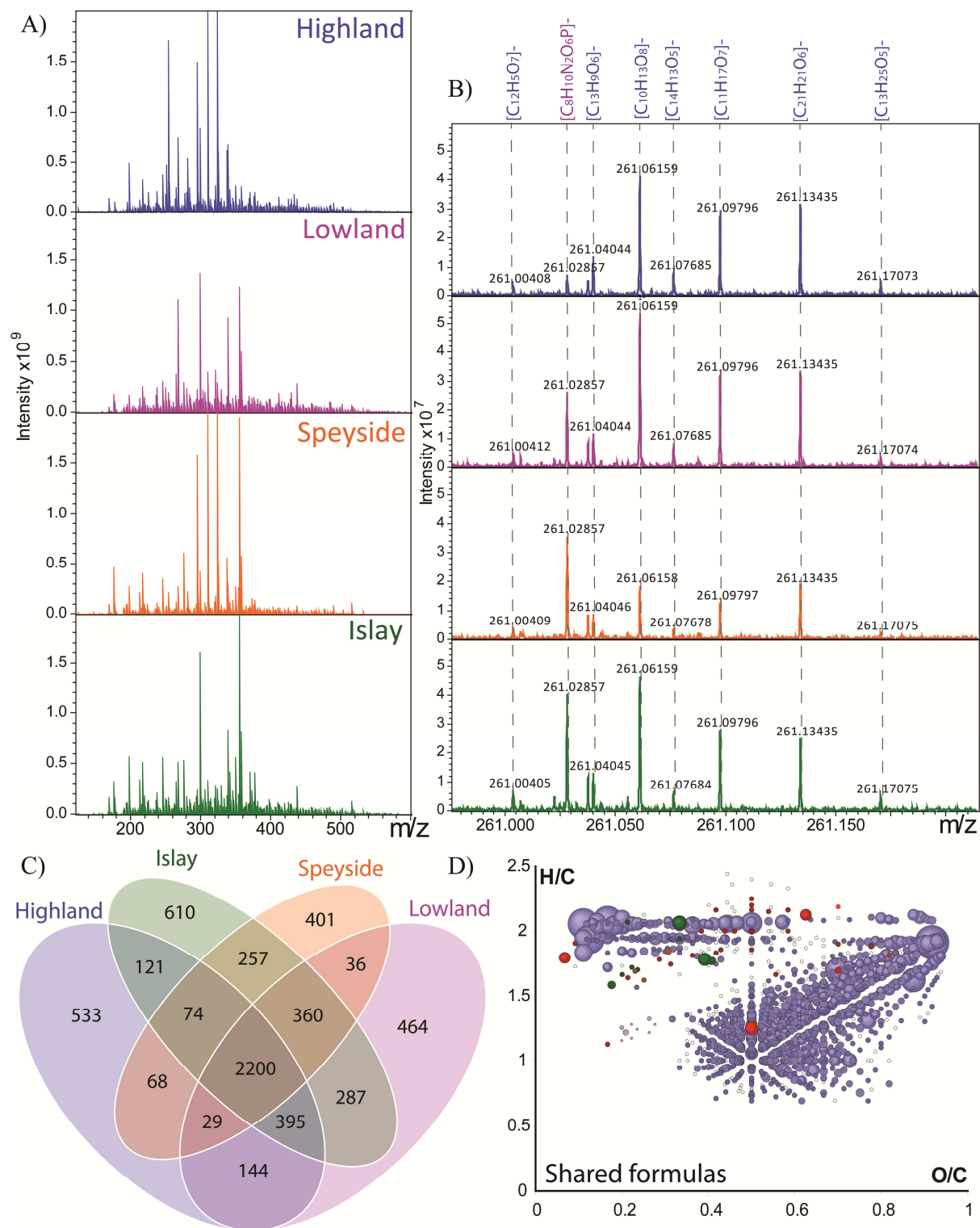
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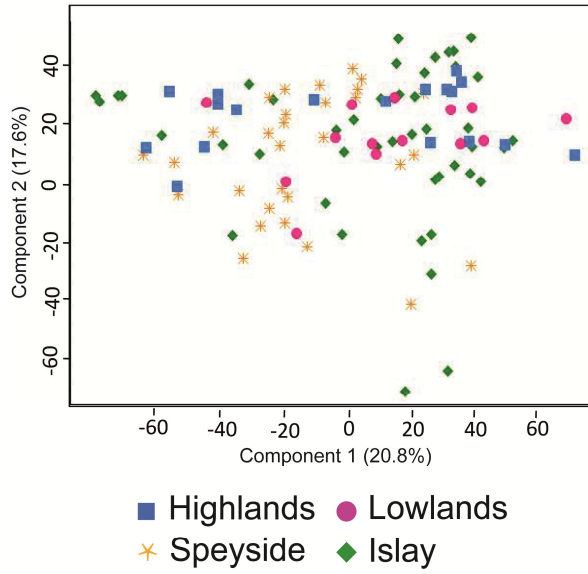


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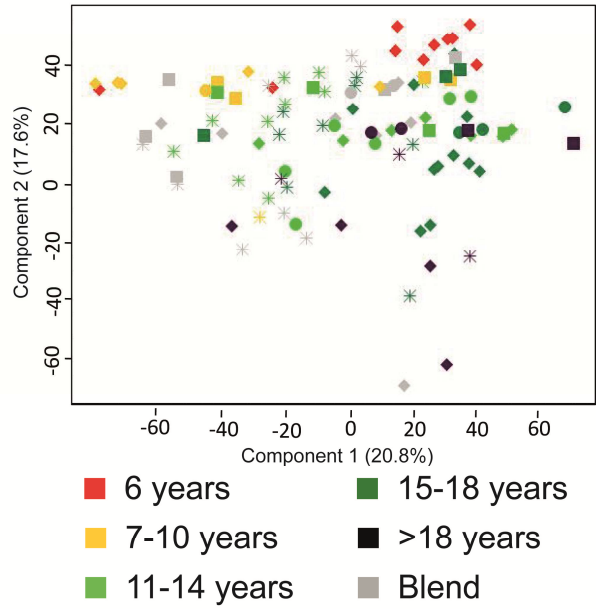
536 **Figure 1**

537

A) PCA colored by geographical origin



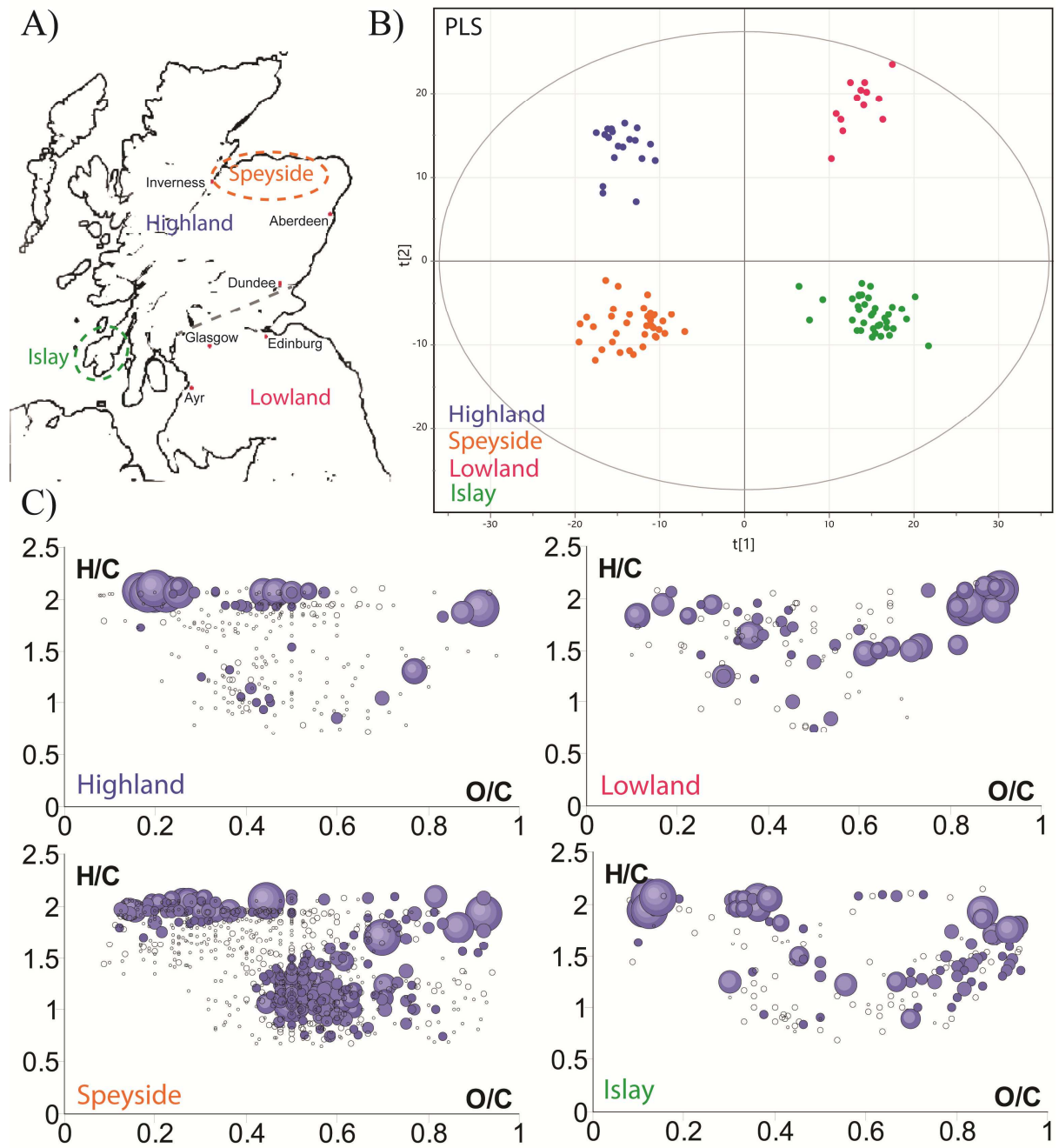
B) PCA colored by maturation time



538

539 **Figure 2**

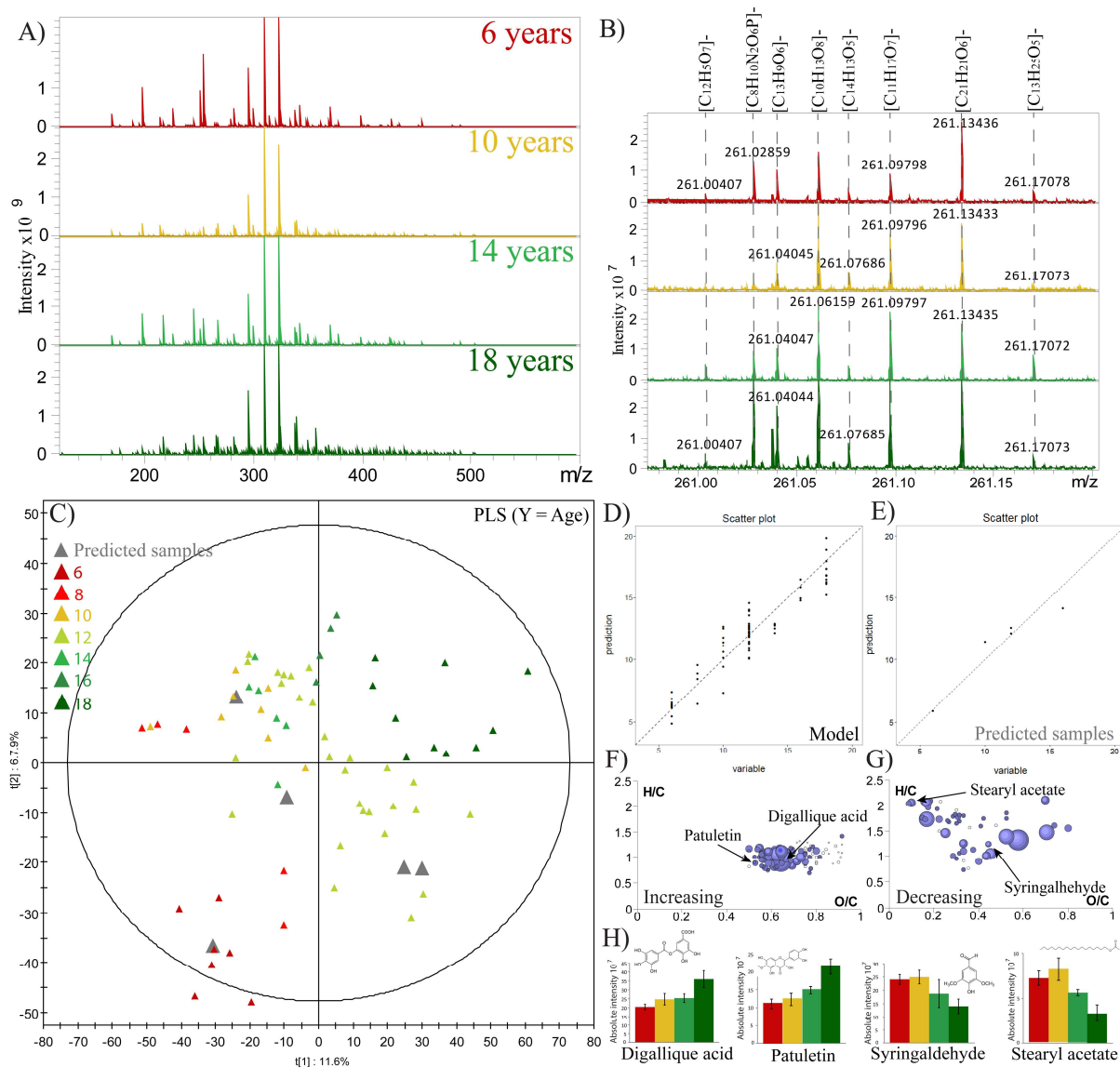
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541

542 **Figure 3**

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**Figure 4**