Development of a comprehensive analytical platform for the detection and quantitation of food fraud using a biomarker approach. The oregano adulteration case study

Wielogorska, E., Chevallier, O., Black, C., Galvin-King, P., Delêtre, M., Kelleher, C. T., Haughey, S. A., & Elliott, C. T. (2018). Development of a comprehensive analytical platform for the detection and quantitation of food fraud using a biomarker approach. The oregano adulteration case study. *Food Chemistry*, *239*, 32-39. https://doi.org/10.1016/j.foodchem.2017.06.083

Published in:
Food Chemistry

Document Version:
Peer reviewed version

Queen's University Belfast - Research Portal:
Link to publication record in Queen's University Belfast Research Portal

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Download date:19. Apr. 2024

Elsevier Editorial System(tm) for Food Chemistry
Manuscript Draft

Manuscript Number: FOODCHEM-D-16-06116R2

Title: Development of a comprehensive analytical platform for the detection and quantitation of food fraud using a biomarker approach. The Oregano adulteration case study.

Article Type: Research Article (max 7,500 words)

Keywords: food, fraud, oregano, adulteration, mass spectrometry

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Abstract: Due to increasing number of food fraud incidents, there is an inherent need for the development and implementation of analytical platforms enabling detection and quantitation of adulteration. In this study a set of unique biomarkers of commonly found adulterants became the targets in the development of a LC-MS/MS method which underwent a rigorous in-house validation. The method presented very high selectivity and specificity, excellent linearity (R2>0.988) low decision limits and detection capabilities (<2%), acceptable accuracy (intra-assay 92-113%, inter-assay 69-138%) and precision (CV<20%). The method was compared with an established FTIR screening assay and revealed a good correlation of quali- and quantitative results (R2>0.81). An assessment of 54 suspected adulterated oregano samples revealed that almost 90% of them contained at least one bulking agent, with a median level of adulteration of 50%. Such innovative methodologies need to be established as routine testing procedures to detect and ultimately deter food fraud.

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1 # Development of a comprehensive analytical platform for the
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3 approach. The oregano adulteration case study.

4

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Keywords: food, fraud, oregano, adulteration, mass spectrometry

1. Introduction

In 2002 the European Union recognised the necessity for introducing the Food Law (European Food Safety Authority, 2002) to protect consumers' health and provide improved confidence that the EU, as a major global trader in food, has the most stringent food safety laws in the world. In order to facilitate consumers' informed choices, 'the *food law should aim at the prevention of fraudulent or deceptive practices; the adulteration of food; and any other practices which may mislead the consumer*' (European Food Safety Authority, 2002). Nevertheless, to date EU legislation does not provide a definition of food fraud. In a European Parliament report 2013/2091(INI), following the horsemeat scandal, the key characteristics of food fraud were outlined and were similar to the definitions already introduced in the USA (Johnson, 2014) – i.e. non-compliance with food law and/or misleading the customer which is done intentionally and for financial gain resulting from fraudulent practices such as adulteration, substitution, tampering or counterfeiting. At the same time the 'Food Fraud Network' started to collect and exchange information in order to detect and prevent food fraud in the EU (European Commission, 2016). The largest available summary of records (over 2,000) on food fraud is contained within the U.S. Pharmacopeia Food Fraud Database (U.S. Pharmacopeial Convention, 2016), which encompasses both scholarly and media reports on the subject area. According to this database, during years 2011-2012 the number of fraud records increased by 60% when compared with the period spanning 1980-2010. The first and second most affected food ingredients quoted being oils and spices respectively, with the latter accounting for 16% of all records (Johnson, 2014). Amongst these, there have been number of recent fraud incidents, potentially posing a health risk, such as addition of toxic colorants or botanic substitutes (Moore, Spink, & Lipp, 2012; U.S. Pharmacopeial Convention, 2016). In addition there have also been reports on bulking agents used for the purpose of substitution/dilution of herbs (U.S. Pharmacopeial Convention, 2016; Marieschi, Torelli, Bianchi, & Bruni, 2011; Marieschi, Torelli, Poli, Bianchi, & Bruni, 2010) which is in agreement with a recent study focusing on oregano adulteration (Black, Haughey, Chevallier, Galvin-King, & Elliott, 2016). Almost 25% of tested UK oregano samples (n=78) were found to be adulterated with bulking agents, most commonly olive and myrtle leaves. The levels of adulteration in some samples was found to be over 70%. This example of adulteration is a classic case of food fraud where the aim is to mislead consumers for purely financial gain. This type of adulteration may pose health threats due to unknown

88 status of the bulking agents, thus exposing consumers to potentially toxic compounds present
89 in the botanical adulterants employed as well as potential microbiological and/or chemical
90 contaminants.

91 Each food fraud incident has the potential to threaten consumers' well-being but also
92 undermine confidence in the EU food market in which the herbs and spices' share is worth
93 1.8 billion Euro in the EU and 2.97 billion dollars world-wide (Marieschi, Torelli, Poli,
94 Sacchetti, & Bruni, 2009). Thus, the European Parliament issued a call to develop and
95 implement technologies and methods to detect food fraud including sensor technology and
96 fingerprinting approach (European Parliament, 2013). Such methods, including Fourier-
97 Transform Infrared spectroscopy (FTIR) have been already successfully implemented in food
98 fraud detection (Ellis, Muhamadali, Haughey, Elliott, & Goodacre, 2015). DNA sequencing
99 techniques are also considered to be reliable, nevertheless, their limitations have been
100 discussed (Parveen, Gafner, Techen, Murch, & Khan, 2016) and make them unsuitable to be
101 employed as stand-alone tools in the field of authentication. Furthermore, according to
102 European legislation concerning the performance of analytical methods (European
103 Commission, 2002), those approaches would be classified as screening methods and as such
104 they are not fit for the purpose of confirming adulteration to required legal standards, due to
105 lack of chemical structure confirmation, if the legal action is to be pursued by industry or
106 regulators. High Resolution Mass Spectrometry (HRMS) based fingerprinting approaches
107 have also been employed in food authentication, however even though such methods provide
108 the possibility of detecting unusual deviations within the sample set, instrumental analysis is
109 time consuming, requires expensive equipment, data storage facilities and high processing
110 power (Esslinger, Riedl, & Fauhl-Hassek, 2014). Available targeted mass spectrometry based
111 methods designed for the purpose of adulteration confirmation usually employ food profiling
112 approach whereby sample classification is based on analysing a selected group of matrix
113 constituents such as flavonoids in *Ginkgo biloba* or ginsenosides in *Panax Ginseng* (Xie,
114 Chen, Liang, Wang, Tian, & Upton, 2006; Yuan, Wang, Chen, Ye, & Zhou, 2016). Such
115 validated analytical methods ensure accuracy of the analytical determination (Esslinger,
116 Riedl, & Fauhl-Hassek, 2014), however are prone to 'targeted designed adulteration'
117 (Sanzini, Badea, Dos Santos, Restani, & Sievers, 2011) due to increased knowledge
118 regarding chemical composition of food commodities.

119 Consequently, designing an analytical approach suitable for rapid, cheap and reliable
120 detection and confirmation of adulteration still presents a challenge. Many important issues

121 such as selectivity and specificity of chosen markers in the presence of various botanic
122 adulterants, methods' ruggedness as well as inherent biodiversity (De Falco, Mancini,
123 Roscigno, Mignola, Taglialatela-Scafati, & Senatore, 2013) must all be considered during
124 experimental design. Thus, the aim of the present study was to explore the possibility of
125 designing a holistic system allowing for both fast and reliable FTIR screening as well as cost
126 effective, simplified, liquid chromatography tandem - mass spectrometry (LC-MS/MS) based
127 confirmation for the purpose of oregano adulteration detection and quantitation. The
128 successful outcome of this study could lead to many other future applications of such a
129 system for the analysis of other types of food fraud.

130

131 ## 2. Materials and methods

132

133 ### 2.1. Reference plant material and identity confirmation

134

135 A set of 38 samples were employed in the current LC-MS/MS method development and
136 validation. Those included either certified reference materials (*Origanum vulgare* leaf and
137 *Olea europaea* leaf referenced against vouchered (AH0024) and reagent grade biomass
138 reference material respectively, both from LGC standards, Teddington, UK) or samples
139 supplied with full provenance and traceability including leaves of culinary *Origanum* species
140 such as *Origanum vulgare* and *Origanum onites* (n=11) as well as samples of potential
141 bulking agents including olive leaves - *Olea europaea subsp. europaea* (n=6), *Myrtaceae*
142 leaves including *Myrtus communis* and *Myrtus communis var. communis* (n=6), phlomis
143 leaves - *Phlomis x cytherea* (n=2), sumac leaves – *Rhus coriaria* (n=3), hazelnut leaves -
144 *Corylus avellana* (n=3), sage leaves - *Salvia officinalis* (n=3) and cistus leaves - *Cistus*
145 *creticus* (n=2). In order to experimentally confirm the identification of the samples,
146 specimens were compared morphologically against herbarium specimens and were also
147 assessed using DNA barcoding. Material has been deposited into the DBN herbarium tissue
148 bank in the National Botanic Gardens of Ireland (registration number DBN2017:04).

149 #### 2.1.1. DNA extraction and PCR conditions

150 Genomic DNA was extracted from approximately 20 mg of lyophilized leaf tissue using
151 Macherey-Nagel NucleoSpin® Plant II kits according to the manufacturer's instructions, with

152 the exception that the cell lysis incubation step at 65°C was extended to 45 min. Species
153 identity was confirmed using DNA barcoding. Four regions were used, three plastid and one
154 nuclear region. The plastid regions were *trnH-psbA* (Kress, Wurdack, Zimmer, Weigt, &
155 Janzen, 2005), *trnL-trnF* (Taberlet, Coissac, Pompanon, Gielly, Miquel, Valentini, et al.,
156 2007) and *matK* (Yu, Xue & Zhou, 2011). The nuclear region was the Internal Transcribed
157 Spacer region (ITS) (Kress, Wurdack, Zimmer, Weigt, & Janzen, 2005). Polymerase chain
158 reactions (PCR) contained 1 μL (~50 ng) of DNA template, 1 x Bioline MyTaq™ Red Mix
159 (Bioline, USA) and 0.5 μM of the forward and reverse primers (Eurofins, Germany) in a total
160 volume of 20 μL. PCR cycle conditions were: 94°C for 2 min followed by 40 cycles of 94°C
161 for 1 min, 58°C for 1 min and 72°C for 1 min, with a final 10 min extension at 72°C. PCR
162 products were purified using the ExoSAP method (New England Biolabs) and sent to
163 Macrogen Europe for direct sequencing in one direction.

164 2.1.2. DNA sequence analysis

165 DNA sequences were aligned and cleaned using Sequencher v. 4.10.1 (Gene Codes
166 Corporation). Cleaned sequences were BLASTed against sequences available in GenBank
167 and the top hits were checked for matches with the expected taxon. Results are presented in
168 Table S1.

169 2.2.Sample preparation

170 The protocol described was based on the extraction published in the previous study (Black,
171 Haughey, Chevallier, Galvin-King, & Elliott, 2016) and simplified herein by decreasing the
172 volume of the extraction solvent from 2 mL to 0.5 mL, sonication time was shortened from
173 15 min to 10 min while final solvent exchange and filtration steps were abandoned to shorten
174 the analysis time. Shortly, the samples were milled to a homogenous powder on a PM-100
175 Retsch Planetary Ball Mill (Retsch, Haan, Germany). 50 mg (+/- 1%) was weighed out on a
176 Discovery DV215CD Analytical Balance (Ohaus Europe GmbH, Nanikon, Switzerland) into
177 a 1.5 mL Eppendorf tube and 500 μL of ultra-pure water (18.2MΩ/cm)/LC-MS Chromasolv
178 methanol (1:1, v/v) solution added (Merck Millipore, Billerica, USA and Sigma-Aldrich, St
179 Louis, MO, USA respectively). Samples were then extracted by vortexing 10 min at 2,500
180 rpm with a DVX-2500 Multitube Vortexer (VWR International, Lutterworth, UK), sonicated
181 for 10 min in camSonix C1274 sonicator (Camlab, Cambridge, UK) and centrifuged at 9,500
182 x g in a MIKRO 200R centrifuge (Hettich UK, Salford, UK). 300 μL of the resulting

183 supernatant was transferred into a maximum recovery vial (Waters, Manchester, UK) and
184 analysed on the same day.

185

186 ## 2.3. Untargeted LC-HRMS analysis

187

188 An untargeted approach has been developed to detect herb adulteration and is the basis of
189 present, follow up targeted study (Black, Haughey, Chevallier, Galvin-King, & Elliott, 2016).
190 Briefly, oregano and adulterants' (olive, *Myrtaceae*, phlomis, sumac, and hazelnut leaves)
191 extracts were analysed employing Waters Acquity I-Class UHPLC. The LC column and
192 mobile phases system consisted of Waters Acquity HSS T3 column (2.1 x 100 mm, 1.8 μm),
193 maintained at 45°C while the pump was operated at a flow rate of 0.4 mL/min with mobile
194 phases A, 0.1% formic acid in water and B, 0.1% formic acid in methanol. A linear gradient
195 from 99%A to 1%A over 20 min was applied and the same LC conditions were applied
196 during initial targeted method development. Mass spectral data were acquired over the range
197 m/z 50-1200 in both positive and negative ion mode using a Waters Acquity I-Class UHPLC
198 coupled to a G2-S quadrupole time of flight (Q-ToF) mass spectrometer. The raw data
199 generated were processed following a Progenesis QI 2.0 software work flow. The processed
200 data were then subjected to multivariate analysis using SIMCA 14.0 (Umetrics, Sweden).
201 Principal component analysis (PCA) and orthogonal partial least squares discriminate
202 analysis (OPLS-DA) were used for building the qualitative models.

203 The PCA score plots generated (Figure S1 – supplementary material) showed clear separation
204 between the authentic oregano and adulterant samples. The discovery of characteristic
205 biomarkers for each adulterant was achieved by generating individual OPLS-DA models for
206 each adulterant against pure oregano, with Table S2 (supplementary material) identifying the
207 measure of fit (R^2), the measure of predicative ability (Q^2) and RMSECV values for each
208 OPLS-DA model. These individual models enabled us to create S-plots which identified a set
209 of ions in both ionisation modes for each adulterant. As a result, these ions were thoroughly
210 investigated using both the raw data and Progenesis QI 2.0 to guarantee that they were not
211 present in any of the authentic oregano samples. Overall a total of 23 adulterant markers were
212 identified and were used to generate an in-house database within Progenesis QI software. Due
213 to lack of full chemical identification all biomarkers were assigned arbitrary IDs stemming
214 from the first letter of the adulterant's name, consecutive number and ionisation mode in

215 which they were analysed. Tables with all biomarkers' arbitrary IDs, their retention times and
216 neutral masses are presented in supplementary material (Table S3 and S4).

217

218 ## 2.4. Targeted LC-MS/MS analysis

219

220 ### 2.4.1. Experimental conditions

221 Analysis was performed on an Acquity UHPLC I-Class system (Waters, Milford, MA, USA)
222 coupled to Xevo TQ-MS triple quadrupole mass analyser (Waters, Manchester, UK)
223 operating both in positive and negative electrospray ionisation modes. The following settings
224 were applied: capillary voltage was set at 2.5 and 1.0 for positive and negative mode
225 respectively. The desolvation and source temperatures were set at 500 and 150°C,
226 respectively, while nitrogen cone and desolvation flow rates were set to 50 and 750 L/hr.
227 Argon was employed as a collision gas, with a flow of 0.15 mL/min, yielding a collision cell
228 pressure of 2.5×10^{-3} mBar. Inter -scan and -channel delays were both set to 3 ms while dwell
229 times ranged from 14 to 76 ms.

230 Analytes' separation was performed on a Waters Acquity HSS T3 column (2.1 x 100 mm, 1.8
231 μm), maintained at 45°C while the pump was operated at a flow rate of 0.4 mL/min. Mobile
232 phases consisted of A, 0.1% formic acid in water and B, 0.1% formic acid in methanol. A
233 linear gradient was then adjusted to achieve more efficient separation time i.e. isocratic 0 –
234 0.8 min 65%A, linear 0.8 – 4.20 min 20%A and isocratic column equilibration 4.30 – 6 min
235 at 65%A. After each injection the needle was washed and purged with water/methanol (1:1,
236 v/v) solution.

237

238 ### 2.4.2. Biomarker selection and method development

239 Monitored biomarkers' transitions were determined during multivariate analysis of data
240 acquired during untargeted UHPLC-HRMS analysis. Each biomarker's spectrum was
241 acquired on the TQ-MS in full scan mode applying the same chromatographic conditions
242 (described in 2.3.) as in the non-targeted analysis and compared to respective spectrum
243 acquired on Q-ToF (supplementary material – Figure S4 – S8). At least three most prominent
244 fragments of each biomarker's precursor were selected for its respective MRM window and

245 chromatographic conditions adjusted, substantially decreasing analysis time. The most
246 suitable spectrometric conditions were achieved by repeated on-column injections of a pooled
247 adulterant sample. Each of the selected adulterants was found to contain at least one specific
248 molecule (specified in Figure 1) i.e. olive leaves (O1- and O2-), *Myrtaceae* leaves (M1- and
249 M1), sumac leaves (S2 and S3), hazel leaves (H1, H2 and H1-) and phlomis leaves (Ph2-).
250 Unfortunately, no fully specific marker for oregano itself could have been established. To
251 date none of the selected compounds has been successfully identified due to high number of
252 structural isomers of suspected compounds, nevertheless, the minimal requirements of
253 chemical characterization of unknown metabolites is fulfilled as outlined by Chemical
254 Analysis Working Group within Metabolomics Standards Initiative (Sumner, Amberg,
255 Barrett, Beale, Beger, Daykin, et al., 2007). To increase reliability of the method, the three
256 most selective and specific fragments for each of the biomarkers were identified during
257 selectivity study (Figure 1), yielding 5.5 identification points per compound as defined in
258 Commission Decision 2002/657/EC (European Commission, 2002), and their ion ratios
259 closely monitored.

260

261 2.4.3. Method validation

262 The method was validated based on Commission Decision 2002/657/EC (European
263 Commission, 2002).

264 Selectivity and specificity were established by assessing interferences in the UHPLC-MS/MS
265 traces between a number of authenticated samples' extracts (n=38, as described in 2.1.)
266 representing different varieties of oregano, bulking agents and other culinary herbs, including
267 cistus and sage which have not been previously assessed by HRMS, thus no markers could be
268 elucidated.

269 Linearity was assessed for two main adulterants i.e. olive and *Myrtaceae* leaves as assessed
270 previously during commercial samples' survey (Black, Haughey, Chevallier, Galvin-King, &
271 Elliott, 2016). Four different calibration curves per adulterant were prepared, using
272 authenticated samples with most varied concentrations of selected biomarkers (as assessed
273 during pre-screening) at levels 5, 10, 20, 40 and 60% (w/w) adulteration each.

274 Due to the fact the method was developed employing incurred material which composition
275 varies and is dependent on factors such as genotype, environment or developmental stage (De

276 Falco, Mancini, Roscigno, Mignola, Taglialatela-Scafati, & Senatore, 2013), the assessment
277 of resulting biodiversity within species (United Nations, 1992) in different oregano and
278 adulterant matrices (n=21) was performed by analysing composites of varying oregano and
279 either of the two main adulterants samples at adulteration level of 10% (w/w). Results of this
280 study were also employed in the intra-day repeatability assessment.

281 To assess matrix effects as well as their influence on accuracy and within laboratory
282 reproducibility (WLR), analysis of 3 levels of adulteration with 3 mixtures containing both
283 olive and *Myrtaceae* leaves was performed. Mix 1 consisted of 10% and 60%; mix 2 - 30%
284 and 30%; mix 3 - 60% and 10% (w/w) of olive and *Myrtaceae* leaves. Six replicates of each
285 mix were extracted and analysed by three different operators on three different days (n=54).

286 Decision limit (CCα) and detection capability (CCβ) – as defined in (European Commission,
287 2002) - were established by performing a linear regression employing WLR results. The
288 intercept's standard deviation was used for establishment of limits. To confirm the
289 extrapolation results, the standard deviation of measured adulteration of 20 extracts, blended
290 at 1.5% (w/w) adulteration level (composite sample containing all five adulterants), was used
291 to experimentally confirm those limits for both olive and *Myrtaceae* leaves and assess them
292 for the rest of the adulterants.

293

294 ## 2.5.Screening employing Fourier-Transform Infrared (FTIR) analysis

295

296 ### 2.5.1. Sample preparation of adulterated oregano

297 Adulterated samples were prepared by spiking authentic oregano samples in 10% increments,
298 from 0-100% w/w, with all adulterants. This range was chosen as economically motivated
299 adulteration tends to occur at concentrations at least 10% and above.

300 ### 2.5.2. Spectral Data Acquisition using FTIR

301 For FTIR, the milled samples were placed in the ATR sample area of a Thermo Nicolet iS5
302 spectrometer (Thermo Fisher Scientific, Dublin, Ireland) equipped with ATR iD5 diamond
303 crystal and ZnSe lens and DTGS KBr detector and acquired as previously described (Black,
304 Haughey, Chevallier, Galvin-King, & Elliott, 2016).

305

306 2.5.3. Multivariate Analysis of FTIR Spectral Data

307 Multivariate analysis was used for quantitative analysis FTIR spectroscopic data. The
308 analysis was carried out using the chemometric software packages TQ Analyst (Thermo
309 Fisher Scientific, Dublin, Ireland) using the partial least squares regression (PLS-R)
310 algorithm in the current study for quantitative model building as well as SIMCA 14 (MKS,
311 Sweden) for qualitative model building using principal component analysis (PCA). The
312 quantitative calibration model selected was obtained using the lowest standard error of
313 prediction to minimise the possibility of over-fitting when the model accuracy was evaluated.
314 Calibration models were developed from FTIR spectral data in the wavenumber range 600-
315 4000 cm^{-1} which included the molecular fingerprint region. The data pre-treatments examined
316 were none (raw data), 1st and 2nd derivatives calculated in conjunction with the Savitzky-
317 Golay smoothing methods and also pre-processed using the standard normal variate (SNV)
318 technique which compensates for differences in pathlengths due to light scattering effects. To
319 select the calibration and validation data, TQ Analyst uses the information in the components
320 table, related to the analyte of interest, to construct a multidimensional sample space. It then
321 uses statistical guidelines related to the spectral and/or concentration data to randomly select
322 calibration and validation standards from the space sample model which is repeated until a
323 complete set of each is available.

324

325 3. Results and discussion

326

327 Selectivity and specificity of the LC-MS/MS method were assessed by monitoring
328 chromatographic traces after injecting different extracts of oregano and the most common
329 adulterants - olive leaves and *Myrtaceae* leaves as well as less common bulking agents such
330 as sumac, hazelnut, phlomis and cistus or other culinary herbs for potential interferences such
331 as sage (n=38). No cross-talk was noted for the selected biomarkers. To further increase
332 reliability, confirmation of adulteration with a specific bulking agent was only permitted in
333 the presence of all associated biomarkers. Oleuropein, which was previously described in the
334 literature as a marker selective for oregano adulteration with olive leaves (Bononi & Tateo,
335 2011), was shown to be also present in *Myrtaceae* leaves (Figure S9 - supplementary
336 material), which is not reported in the literature, and was thus rejected in the present study.
337 Nevertheless, the efficiency of electrospray ionisation varies depending on the analytes'

338 chemical structure and lack of analytical standards for other olive leaves markers halts
339 reliable assessment of resulting differences in sensitivity. Thus, the possibility of oleuropein
340 presence in the samples due to even minimal sample contamination with olive leaves, which
341 would not be picked up during DNA analysis nor FTIR, should not be excluded and further
342 examination of *Myrtaceae* leaves for the presence of oleuropein should be performed.

343 Linearity assessment revealed that only one of two selected biomarkers specific to each olive
344 and *Myrtaceae* leaves expressed acceptable linear correlation with the level of adulteration
345 (O2- and M1, as presented in Figure S2). Employing those biomarkers yielded excellent
346 linearity in varying matrices (n=4) with goodness of fit coefficients higher than 0.988 for six
347 point curves (including zero) for both adulterants tested. Biodiversity assessment (Table 1)
348 revealed that even in varying matrices, the accuracy of method is acceptable, ranging from
349 109 – 113% for olive and 100 – 110% for *Myrtaceae* leaves. Good intra-day repeatability was
350 also achieved in this study with maximum covariance of 17 and 18% for olive and *Myrtaceae*
351 leaves. The assessed repeatability, thus accounts for natural variability of the matrix
352 constituents, markers' levels in the assessed matrices, as well as possible commercial sample
353 composition and inherent analytical within-day variability of the method. Nevertheless,
354 samples containing biomarkers at levels outside characterised range should not be ruled out.

355 During the WRL assessment (Table 2), the method presented excellent reproducibility with
356 covariance values below 10%. Nevertheless, accuracy of the method was shown to be
357 affected by the presence of other adulterants at levels higher than 30%. Samples with 10%
358 adulteration were heavily affected by the presence of the other adulterant, at 60%, revealing
359 signal enhanced by almost 40% in case of the olive leaves biomarker while the *Myrtaceae*
360 leaves biomarker was suppressed by 30%. Such interactions were not noted whenever two
361 adulterants were tested separately during the biodiversity study, thus alteration in accuracy
362 was attributed to matrix effects exerted by the other adulterant and was shown to be heavily
363 dependent on its concentration. Thus, attention must be paid whenever quantifying samples
364 adulterated with other bulking agents at levels higher than 30%. Nonetheless, a performed
365 regression presented excellent linearity (Figure S3) and yielded very low values of $CC\alpha$ -
366 1.27% and 1.18% - $CC\beta$ – 2.16% and 2.02% - for olive and *Myrtaceae* leaves, respectively
367 (Table 2). To confirm those results, 20 oregano samples were adulterated at the level of 1.5%
368 (chromatographic traces presented in Figure S10 - supplementary material) and measured
369 concentration deviation assessed to determine experimental $CC\beta$ values – 1.76% and 1.54% -
370 which were both similar to those achieved through extrapolation for olive and *Myrtaceae*

371 leaves. The values may be slightly lower due to the assessment performed at a singular
372 adulteration level and on the same day, thus limiting experimental variability. Additionally,
373 CCβs were assessed for three other bulking agents i.e. sumac, hazelnut and phlomis leaves.
374 Assessed standard deviation, based on the absolute response, was employed and following
375 detection capabilities established: 1.45%, 1.43%, 1.70% for sumac, hazelnut and phlomis
376 leaves respectively. The achieved limits are in line with 'oregano cleanliness specifications'
377 outlined by the American Spice Trade Association and European Spice Association i.e. 1%
378 and 2% (w/w) of extraneous matter respectively (American Spice Trade Association, 1999;
379 European Spice Association, 2015).

380 Regarding FTIR analysis, Figure 2 presents the PCA qualitative model and the PLS-R
381 quantitative model for both *Origanum onites* and *vulgare* adulterated with olive leaves and
382 *Myrtaceae* leaves. The PCA models, unsupervised and based purely on the differences found
383 in the spectral data, shows clearly the differences between samples spiked at various levels of
384 adulterants. In both PCA models for *onites* and *vulgare* respectively, the first three principal
385 components calculated explain the majority of the differences (92.5% and 93.7%) between
386 samples: *onites* PC1 55.6% PC2 30.8% PC3 6%; *vulgare* PC1 57.3% PC2 30.5% PC3 5.9%.
387 Using the unsupervised PCA technique, clear differences can be observed between the
388 adulterated and authentic oregano samples. The PLS-R calibration models were derived using
389 pre-processing (SNV, 1st derivative with Savitzsky-Golay smoothing) of the FTIR spectral
390 data and includes the corresponding root mean squared error of calibration (RMSEC) and
391 root mean squared error of prediction (RMSEP). These latter values give an indication of the
392 quality of the calibration models generated. The calibration models for each set of samples
393 gave excellent correlation values (R^2) of 0.99 whilst the corresponding values for RMSEC
394 and RMSEP were found to be 1.48 - 1.68% and 1.42 - 2.64%. The graphical representation
395 for each of the PLS calibration models generated for respective oregano species show the
396 actual concentration on X-axis versus calculated (predicted) concentration on the Y-axis. The
397 samples used for calibration are indicated by the circles whilst those used for validation are
398 indicated by crosses. The calibration models produced with subsequent validation data
399 indicate that this FTIR spectral data approach could potentially be used to detect fraud and
400 adulteration of oregano with an indication of the levels of adulterants present. Therefore
401 routine testing of oregano shipments with FTIR and chemometrics could potentially lead to
402 reduced levels of fraud in the herb sector.

403

Method comparison

Both methods described were compared by means of qualitative and quantitative analysis of 54, suspect, oregano samples.

Qualitative correlation of methods for adulteration determination (defined as correct classification of each of the 54 samples as adulterated or pure) for both olive leaves and *Myrtaceae* were 94%, due to 3 adulterated samples being below the FTIR's model lowest concentration (10%). Additionally, out of 9 samples containing sumac, 6 were below the FTIR's lowest concentration thus misclassified, but still yielded an 89% correct classification rate. None of the samples were found to be adulterated with hazelnut or phlomis in the study. However, 12 samples were adulterated with cistus which were detected by FTIR only, as no exclusive biomarker could be found to confirm cistus adulteration by LC-MS/MS.

In terms of quantitative correlation for olive and *Myrtaceae* leaves adulteration, both methods show good correlation (Figure 3). In the case of samples with high levels of adulteration with olive leaves (4 samples above 90%) the methods showed slightly lower correlation (R^2 = 0.73), however, if only samples with adulteration levels below 90% are taken into consideration, higher correlation was achieved (R^2 = 0.85). Keeping that limitation in mind, the estimation of the levels of adulteration for both olive and *Myrtaceae* leaves (R^2 = 0.81) achieved during FTIR screening can be relied on, due to almost neutral slope and intercept values achieved during correlation assessment (Figure 3).

The samples chosen for LC-MS/MS analysis were initially suspected of adulteration via the FTIR method as well as a portion of samples shown to be non-adulterated. The results have shown that the samples analysed were correctly predicted by each method with 87% of samples containing at least one of the bulking agent tested for (Figure 4a). Out of those, 42% contained more than one adulterant. The median level of adulteration was established to be 50% (25th percentile at 31% and 75th percentile at 75%) (Figure 4b).

4. Conclusions

The newly developed platform consisting of a screening FTIR assay and an LC-MS/MS confirmatory, semi-quantitative assay for detection, confirmation and quantitation of oregano adulteration was thoroughly assessed. Total analysis time, including sample preparation, was 30 min, with minimal sample processing costs required. The LC-MS/MS method presented

high selectivity and specificity, good repeatability and accuracy, with low limits of detection and quantitation, in line with the herb and spice industry guidelines. Validation study underlines the need of complex assessment of the tested matrices, expected adulterants and their mixtures whenever developing similar methods due to possible matrix effects, directly influencing the quantitation obtained. Even though the platform developed presented good analytical performance in detecting selected non-culinary bulking agents, oregano samples have been also reported to be substituted with other, volatile species such as marjoram and winter savory which highlights the need for further method development. Also, fraudsters are known to change their mode of action when exposed, possibly leading to abandoning the bulking agents employed which stresses the importance of further expansion and improvement of botanicals data base to increase both methods' applicability and ruggedness. The results also stress the importance of adopting a holistic approach to the detection of adulteration. Auxiliary methods, such as DNA barcoding or FTIR in this case, may reveal invaluable insights into tested sample's composition and improve their classification due to advantages of multi-variate, fingerprint analysis.

The results obtained in terms of the level and complexity of oregano adulteration clearly demonstrate a need for further development of such analytical platforms due to the increasing likelihood of adulteration for economic gain, now being discovered in many food products.

Acknowledgements

The research undertaken was funded by a UK BBSRC CASE studentship BB/M0503162/1.

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613 Captions:

614 Figure 1. Monitored transitions of the selected adulterant markers during LC-MS/MS analysis
615 with the respective chromatographic traces (presented in the respective row). Markers and
616 transitions with asterisk were employed as quantifiers.

617 Figure 2: Qualitative PCA and Quantitative PLS-R models for oregano *Origanum vulgare*
618 and *Origanum onites* adulterated with olive leaves and *Myrtaceae* leaves based on FTIR data.

619 Figure 3. Correlation of quantitation results between FTIR screening method and LC-MS/MS
620 targeted method for olive (a, b) and *Myrtaceae* leaves (c) results. Graphs a) and b) represent
621 two differing sample sets i.e. a) include all of the adulterated samples above FTIR LOD
622 (n=51) while b) only samples below 90% adulteration level.

623 Figure 4. a) Composition of the adulteration in the assessed samples (sum of adulteration
624 confirmed by LC-MS/MS and FTIR for cistus). b) Percent total adulteration distribution in
625 positive samples (whiskers 5-95 percentile).

Table1

Table 1. Results of biodiversity assessment based on measured % adulteration in 21 extracts of varying mixtures of oregano (n=7) and either olive (n=7) or *Myrtaceae* leaves (n=6) samples at adulteration level of 10%.[a] One sample was rejected due to analyst's random error during the extraction.

Oregano leaves	Olive leaves				*Myrtaceae* leaves			
Sample #	Sample #	Measured adulteration [%]	Repeatability [CV, %]	Accuracy [%]	Sample #	Measured adulteration [%][a]	Repeatability [CV, %]	Accuracy [%]
		10.5				10.4		
		9.0				10.5		
		9.2				10.6		
24	CRM	9.4	7	92	4	10.8	3	105
		8.9				10.9		
		9.1				10.1		
		8.7						
	1	13.3			3	14.5		
	2	11.5			4	10.2		
	21	11.3			19	11.8		
24	25	11.1	13	109	27	10.9	18	110
	90	10.8			89	10.5		
	123	8.6			125	7.8		
	CRM	10.0			27	11.5		
10		12.0				7.9		
11		14.5				12.2		
12		10.4				10.7		
24	CRM	10.8	17	113	4	9.5	13	100
28		11.4				8.6		
31		8.9				9.3		
CRM		13.6				9.5		

Table2

Table 2. Results of WRL assessment based on measured % adulteration in three mixtures, all containing both *Myrtaceae* and olive leaves, each extracted 6 times, on three different days (n=54).

Adulterant	Adulteration level [%]	Reproducibility [CV, %]	Accuracy [%]	CCα [%]	CCβ [%]	Experimental CCβ [%]
Olive leaves (n=54)	10%	8.3	138	1.27	2.16	1.76
	30%	7.5	107			
	60%	3.4	99			
Myrtaceae leaves (n=54)	10%	7.0	69	1.18	2.02	1.54
	30%	5.7	81			
	60%	4.2	94			

Figure1
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Adulterant	Biomarker	Molecular Ion [m/z]	Fragment Ions [m/z]	CE [V]	MRM window	ESI
Olive leaves	O1-	403.3	100.6	20	7	-
			119.0	20		
			223.2	20		
	O2- *	827.4	487.1 *	40	10	-
			529.6	40		
			781.0	20		
Myrtle leaves	M1 *	555.24	153.2 *	30	4	+
			223.0	20		
			315.0	20		
	M1-	380.9	218.2	40	6	-
			261.0	20		
			291.0	20		
Sumac	S2	1075.0	153.0	40	5	+
			305.0	30		
			457.0	20		
	S3 *	539.0	377.0 *	50	3	+
			403.0	40		
			419.0	20		
Hazelnut leaves	H1	297.2	107.0	20	2	+
			133.0	20		
			191.1	10		
	H2 *	297.1	133.0 *	20	1	+
			177.0	10		
			191.0	10		
	H1-	475.3	118.0	20	9	-
			188.9	30		
			295.0	10		
Phlomis	Ph2- *	450.8	142.7	30	8	-
			224.8 *	20		
			243.0	20		

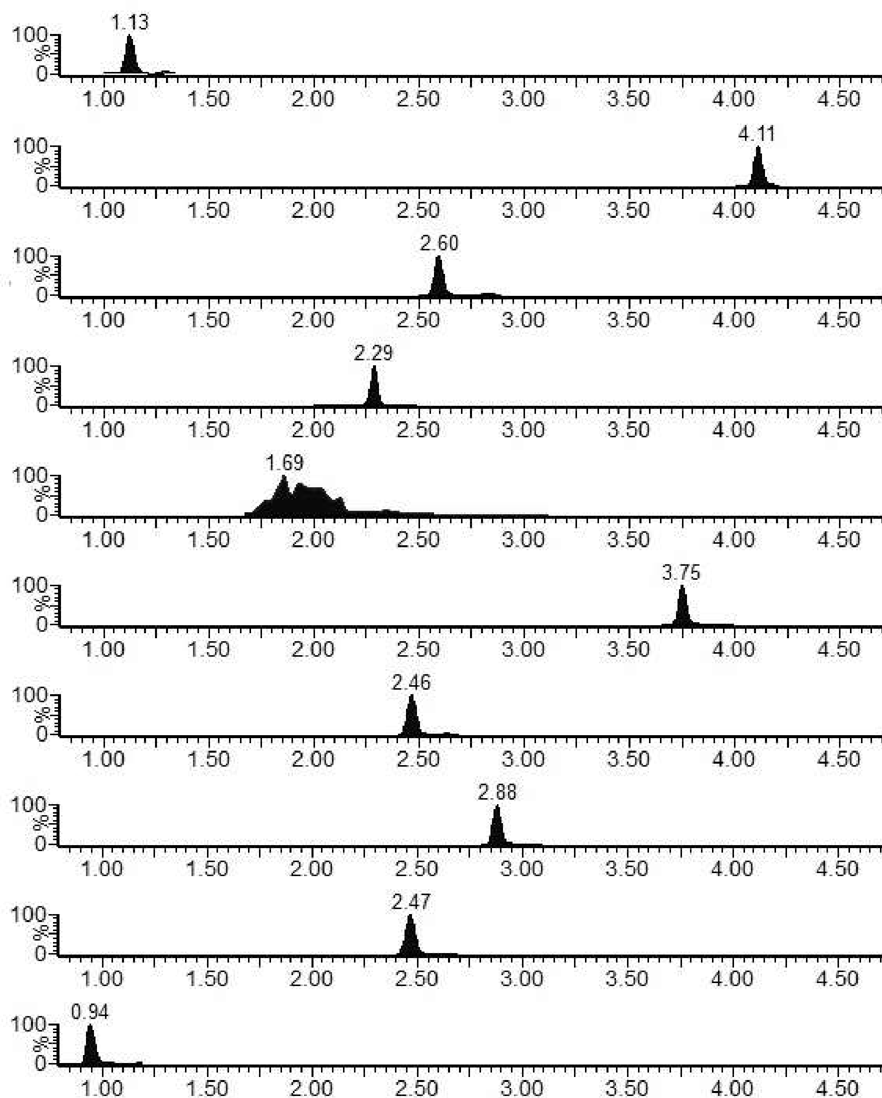

Figure2

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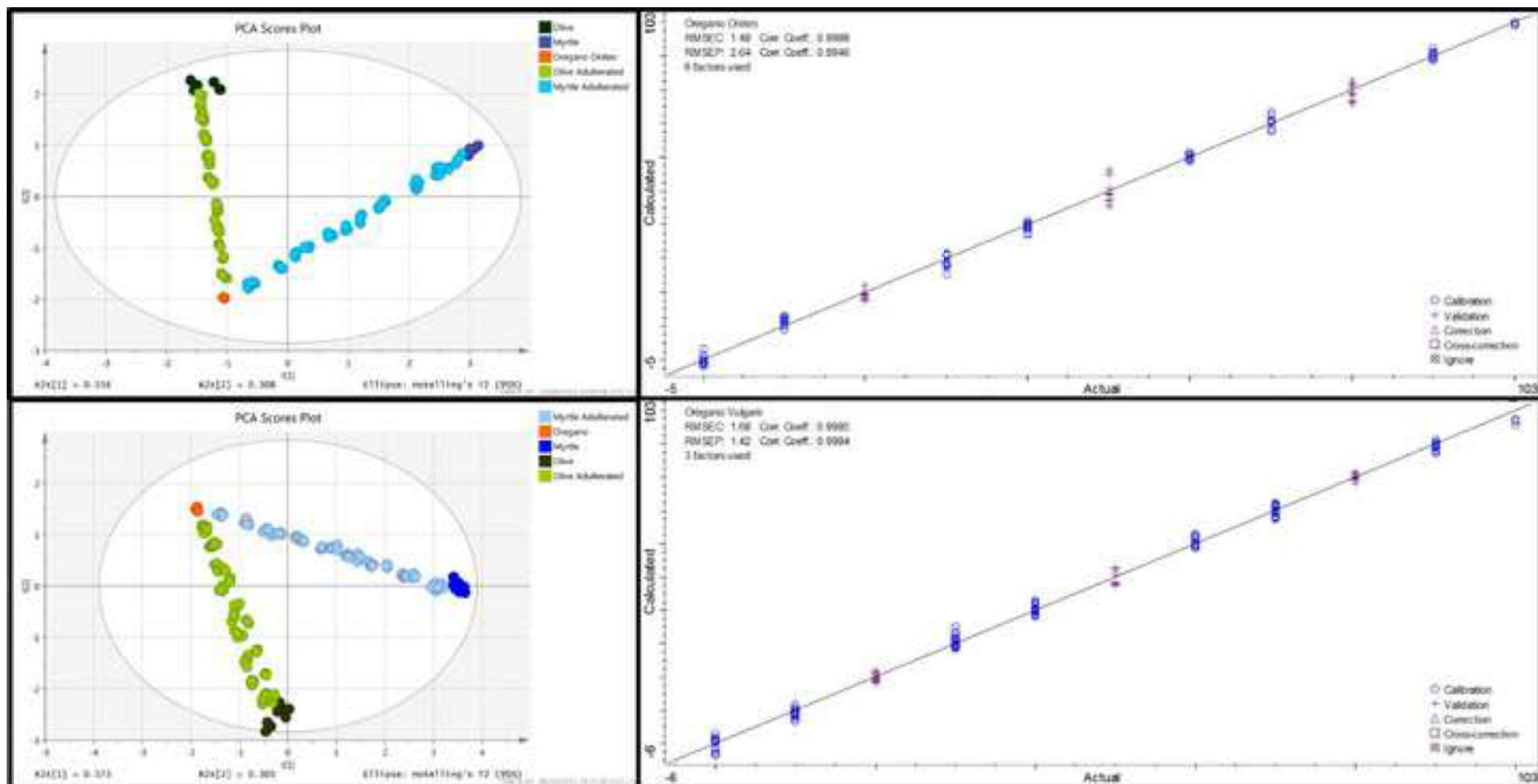

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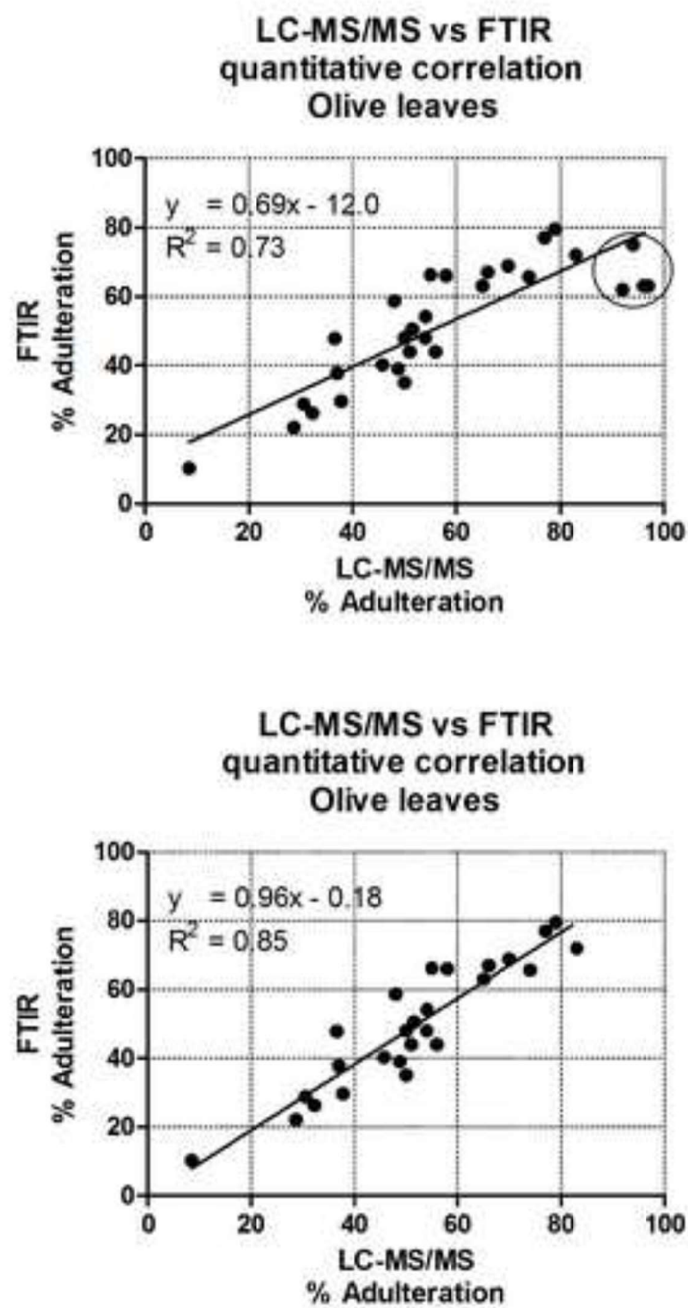

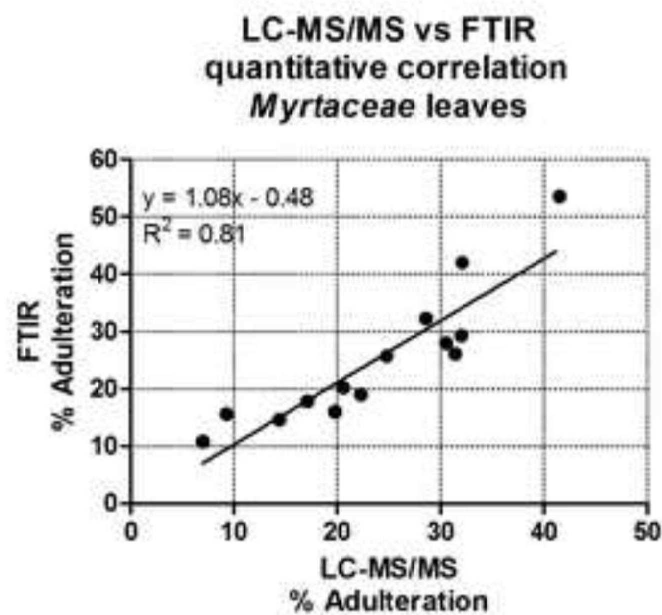

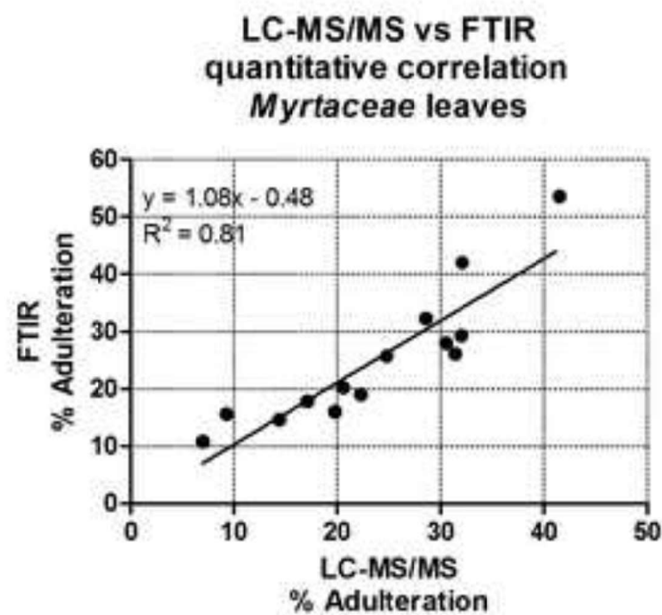

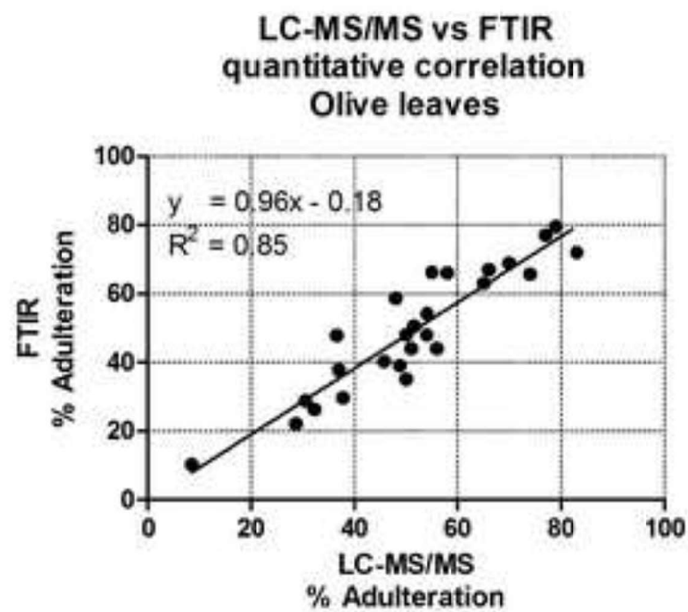

Figure4

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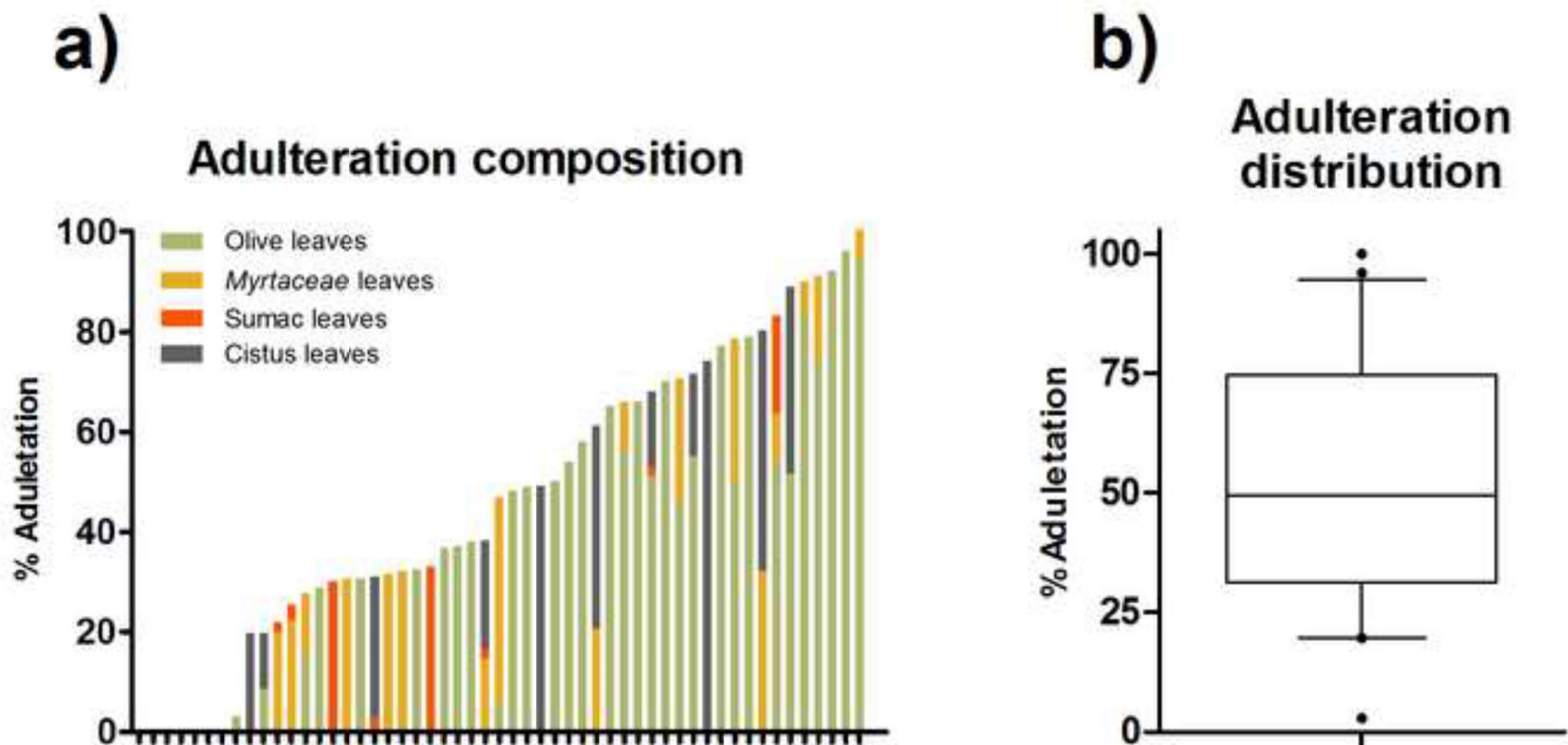

Supplementary Material
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***Highlights (for review)**

- A novel analytical platform, allowing for detection, confirmation and quantitation of herb adulteration in 30min, has been developed.
- A confirmatory, LC-MS/MS method, based on biomarker discover, has been successfully validated.
- The comparison of the LC-MS/MS method and a screening FTIR method, revealed good correlation of adulteration quantitation.
- A survey of suspected samples revealed high incident and levels of oregano adulteration.