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

Publisher rights
Copyright 20__ Elsevier.
This manuscript is distributed under a Creative Commons Attribution-NonCommercial-NoDerivs License (https://creativecommons.org/licenses/by-nc-nd/4.0/), which permits distribution and reproduction for non-commercial purposes, provided the author and source are cited.

General rights
Copyright for the publications made accessible via the Queen's University Belfast Research Portal is retained by the author(s) and / or other copyright owners and it is a condition of accessing these publications that users recognise and abide by the legal requirements associated with these rights.

Take down policy
The Research Portal is Queen's institutional repository that provides access to Queen's research output. Every effort has been made to ensure that content in the Research Portal does not infringe any person's rights, or applicable UK laws. If you discover content in the Research Portal that you believe breaches copyright or violates any law, please contact openaccess@qub.ac.uk.

Open Access
This research has been made openly available by Queen's academics and its Open Research team. We would love to hear how access to this research benefits you. – Share your feedback with us: http://go.qub.ac.uk/oa-feedback

Download date:02. May. 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

Corresponding Author: Dr. Ewa Wielogorska,

Corresponding Author's Institution: QUB

First Author: Ewa Wielogorska

Order of Authors: Ewa Wielogorska; Olivier Chevallier, PhD; Connor Black; Pamela Galvin-King ; Marc Delêtre; Colin T Kelleher; Simon Haughey, PhD; Christopher Elliot , Prof.

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.

*Manuscript
Click here to download Manuscript: Manuscript_final corrections_notrack.docx Click here to view linked References

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

Ewa Wielogorska [a,*], Olivier Chevallier [a], Connor Black [a], Pamela Galvin-King [a], Marc Delêtre [b], Colin T. Kelleher [b], Simon A. Haughey [a], Christopher T. Elliot [a]

[a] Institute for Global Food Security, Advanced ASSET Centre, School of Biological Sciences, Queen's University Belfast, Northern Ireland, United Kingdom

[b] DBN Plant Molecular Laboratory, National Botanic Gardens of Ireland, Glasnevin, Dublin 9, Ireland

*Corresponding author. Address: Institute for Global Food Security, Queen's University Belfast, 18-30 Malone Road, Belfast BT9 5BN, United Kingdom; e-mail: e.wielogorska@qub.ac.uk; phone number: +44(0)28 90974155.

Remaining authors' e-mail addresses: Olivier Chevallier o.chevallier@qub.ac.uk; Connor Black cblack38@qub.ac.uk; Pamela Galvin-King p.galvin-king@qub.ac.uk; Marc Delêtre deletrem@tcd.ie; Colin T. Kelleher colin.kelleher@opw.ie; Simon A. Haughey s.a.haughey@qub.ac.uk; Christopher T. Elliott chris.elliott@qub.ac.uk;

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 (R^2>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 (R^2>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.

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

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

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

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

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

2. Materials and methods

2.1.Reference plant material and identity confirmation

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

2.1.1. DNA extraction and PCR conditions

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

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

2.1.2. DNA sequence analysis

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

2.2.Sample preparation

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

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

2.3.Untargeted LC-HRMS analysis

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

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

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

2.4.Targeted LC-MS/MS analysis

2.4.1. Experimental conditions

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

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

2.4.2. Biomarker selection and method development

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

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

2.4.3. Method validation

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

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

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

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

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

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

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

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

2.5.1. Sample preparation of adulterated oregano

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

2.5.2. Spectral Data Acquisition using FTIR

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

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'

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

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

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

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

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

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% (25^{th} percentile at 31% and 75^{th} 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.

529 References

530

531 American Spice Trade Association (1999). American Spice Trade Association's Cleanliness
532 specifications for Spices, Seeds and Herbs. http://www.astaspice.org/food-safety/cleanliness-
533 specifications/ Accessed 05.10.16
534 Black, C., Haughey, S. A., Chevallier, O. P., Galvin-King, P., & Elliott, C. T. (2016). A
535 comprehensive strategy to detect the fraudulent adulteration of herbs: The oregano approach.
536 *Food Chemistry, 210*, 551-557.
537 Bononi, M., & Tateo, F. (2011). Lc-Esi-Ms/Ms Identification of Oleuropein as Marker of Olea
538 Europaea L. Leaves Used as a Bulking Agent in Ground Oregano and Sage. *Italian Journal of*
539 *Food Science, 23*(3), 245-251.
540 De Falco, E., Mancini, E., Roscigno, G., Mignola, E., Taglialatela-Scafati, O., & Senatore, F. (2013).
541 Chemical Composition and Biological Activity of Essential Oils of Origanum vulgare L.
542 subsp vulgare L. under Different Growth Conditions. *Molecules, 18*(12), 14948-14960.
543 European Commission (2002). Commission Decision of 12 August 2002 implementing Council
544 Directive 96/23/EC concerning the performance of analytical methods and the interpretation
545 of results (2002/657/EC). *Official Journal of the European Communities, L 221/8-36*.
546 European Commission (2016). Food Fraud.
547 http://ec.europa.eu/food/safety/official_controls/food_fraud/index_en.htm Accessed 08.10.16
548 European Food Safety Authority (2002). Regulation (EC) No 178/2002 of the European Parliament
549 and of the Council of 28 January 2002 laying down the general principles and requirements of
550 food law, establishing the European Food Safety Authority and laying down procedures in
551 matters of food safety. *Official Journal of the European Communities, L 31/1-24*.
552 Ellis, D. I., Muhamadali, H., Haughey, S. A., Elliott, C. T., & Goodacre, R. (2015). Point-and-shoot:
553 rapid quantitative detection methods for on-site food fraud analysis - moving out of the
554 laboratory and into the food supply chain. *Analytical Methods, 7*(22), 9401-9414.
555 European Parliament (2013). EP Report 2013/2091(INI). Report on the food crisis, fraud in the food
556 chain and the control thereof. European Parliament - Committee on the Environment, Public
557 Health and Food Safety
558 European Spice Association (2015). European Spice Association Quality Minima Document.
559 http://www.esa-spices.org/index-esa.html/publications-esa Accessed 05.10.16
560 Esslinger, S., Riedl, J., & Fauhl-Hassek, C. (2014). Potential and limitations of non-targeted
561 fingerprinting for authentication of food in official control. *Food Research International, 60*,
562 189-204.
563 Johnson, R. (2014). Food Fraud and "Economically Motivated Adulteration" of Food and Food
564 Ingredients. DC: Congressional Research Service.
565 Kress, W. J., Wurdack, K. J., Zimmer, E. A., Weigt, L. A., & Janzen, D. H. (2005b). Use of DNA
566 barcodes to identify flowering plants. *Proceedings of the National Academy of Sciences of the*
567 *United States of America, 102*(23), 8369-8374.
568 Marieschi, M., Torelli, A., Poli, F., Sacchetti, G., & Bruni, R. (2009). RAPD-Based Method for the
569 Quality Control of Mediterranean Oregano and Its Contribution to Pharmacognostic
570 Techniques. *Journal of Agricultural and Food Chemistry, 57*(5), 1835-1840.
571 Marieschi, M., Torelli, A., Poli, F., Bianchi, A., & Bruni, R. (2010). Quality control of commercial
572 Mediterranean oregano: Development of SCAR markers for the detection of the adulterants
573 Cistus incanus L., Rubus caesius L. and Rhus coriaria L. *Food Control, 21*(7), 998-1003.
574 Marieschi, M., Torelli, A., Bianchi, A., & Bruni, R. (2011). Detecting Satureja montana L. and
575 Origanum majorana L. by means of SCAR-PCR in commercial samples of Mediterranean
576 oregano. *Food Control, 22*(3-4), 542-548.Moore, J. C., Spink, J., & Lipp, M. (2012).
577 Development and Application of a Database of Food Ingredient Fraud and Economically
578 Motivated Adulteration from 1980 to 2010. *Journal of Food Science, 77*(4), R118-R126.
579 Moore, J. C., Spink, J., & Lipp, M. (2012). Development and Application of a Database of Food
580 Ingredient Fraud and Economically Motivated Adulteration from 1980 to 2010. *Journal of*
581 *Food Science, 77*(4), R118-R126.

582
583 Parveen, I., Gafner, S., Techen, N., Murch, S. J., & Khan, I. A. (2016). DNA Barcoding for the
584 Identification of Botanicals in Herbal Medicine and Dietary Supplements: Strengths and
585 Limitations. *Planta Medica, 82*(14), 1225-1235.
586 Sanzini, E., Badea, M., Dos Santos, A., Restani, P., & Sievers, H. (2011). Quality control of plant
587 food supplements. *Food & Function, 2*(12), 740-746.Kress, W. J., Wurdack, K. J., Zimmer,
588 E. A., Weigt, L. A., & Janzen, D. H. (2005). Use of DNA barcodes to identify flowering
589 plants. *Proc Natl Acad Sci U S A, 102*(23), 8369-8374.
590 Sumner, L. W., Amberg, A., Barrett, D., Beale, M. H., Beger, R., Daykin, C. A., Fan, T. W. M.,
591 Fiehn, O., Goodacre, R., Griffin, J. L., Hankemeier, T., Hardy, N., Harnly, J., Higashi, R.,
592 Kopka, J., Lane, A. N., Lindon, J. C., Marriott, P., Nicholls, A. W., Reily, M. D., Thaden, J.
593 J., & Viant, M. R. (2007). Proposed minimum reporting standards for chemical analysis.
594 *Metabolomics, 3*(3), 211-221.
595 Taberlet, P., Coissac, E., Pompanon, F., Gielly, L., Miquel, C., Valentini, A., Vermat, T., Corthier, G.,
596 Brochmann, C., & Willerslev, E. (2007). Power and limitations of the chloroplast trnL (UAA)
597 intron for plant DNA barcoding. *Nucleic Acids Research, 35*(3).
598 United Nations (1992). United nations' Convetion on Biological Diversity, 1992,
599 https://www.cbd.int/doc/legal/cbd-en.pdf Accessed 05/01/2017
600 U.S. Pharmacopeial Convention (2016). USP's Food Fraud Database. https://www.foodfraud.org/
601 Xie, P. S., Chen, S. B., Liang, Y. Z., Wang, X. H., Tian, R. T., & Upton, R. (2006). Chromatographic
602 fingerprint analysis - a rational approach for quality assessment of traditional Chinese herbal
603 medicine. *Journal of Chromatography A, 1112*(1-2), 171-180.
604 Yu, J., Xue, J.H., Zhou, S.L. (2011). New universal *matK* primers for DNA barcoding angiosperms.
605 *Journal of Systematics and Evolution* 49 (3): 176–181.
606 Yuan, J. J., Wang, C. Z., Chen, H. X., Ye, J. Z., & Zhou, H. (2016). Identification and Detection of
607 Adulterated Camellia Oleifera Abel. Oils by Near Infrared Transmittance Spectroscopy.
608 *International Journal of Food Properties, 19*(2), 300-313.

609

610

611

612

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

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

Click here to download Figure(s): Figure 1.docx

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		

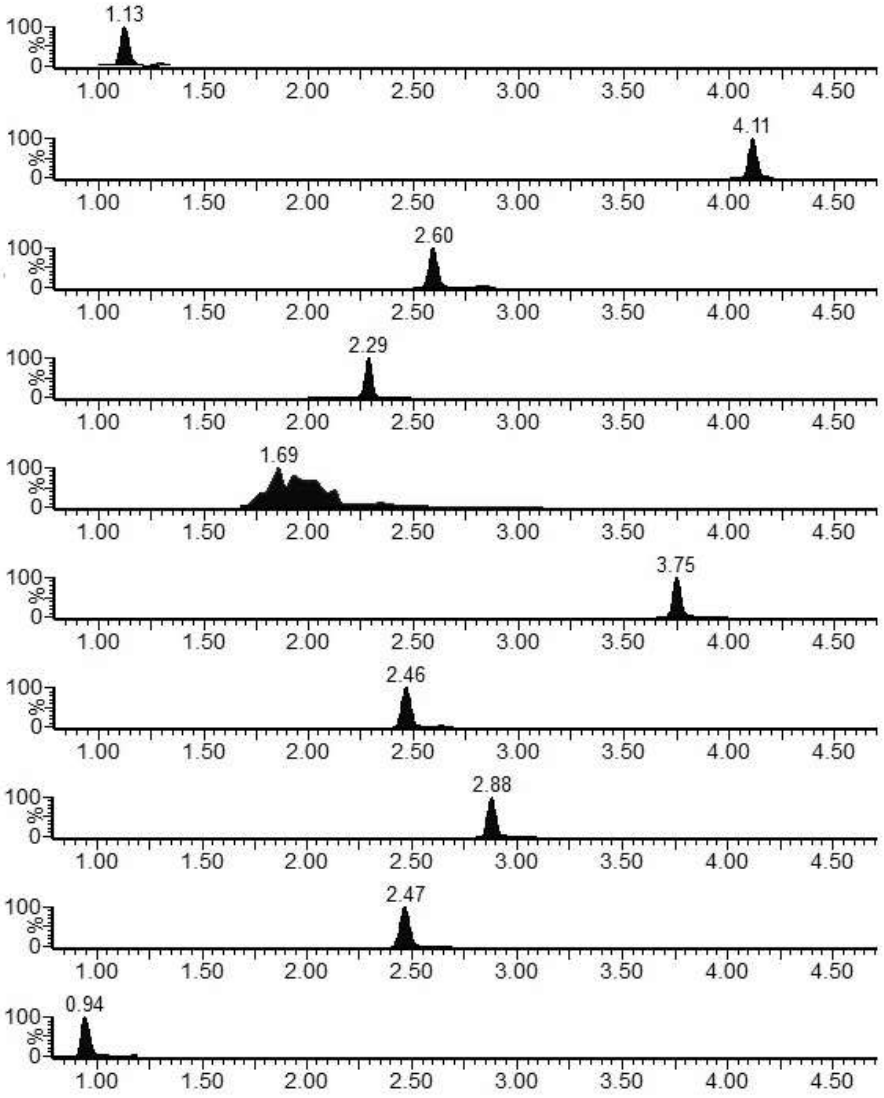


Figure2

Click here to download high resolution image

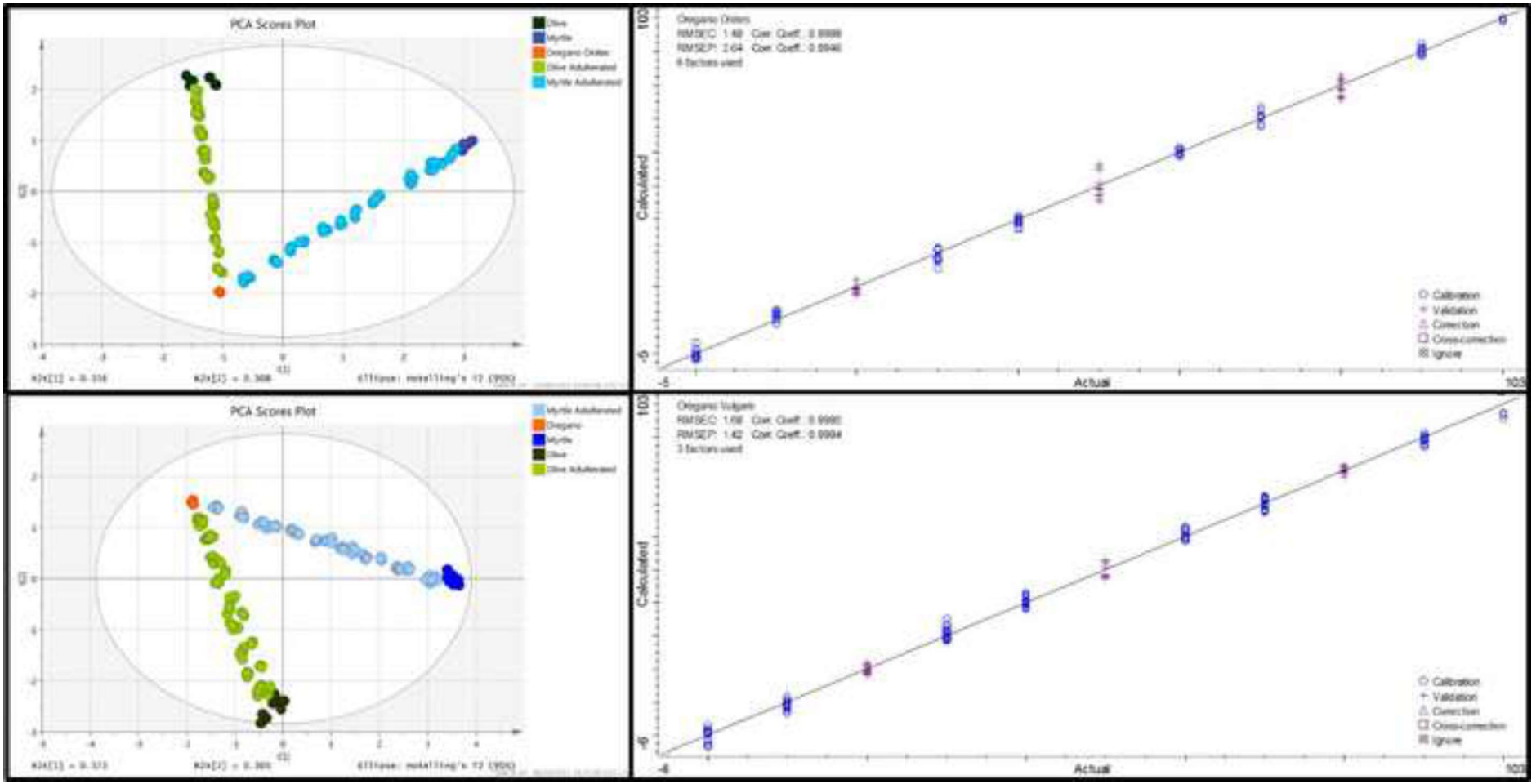
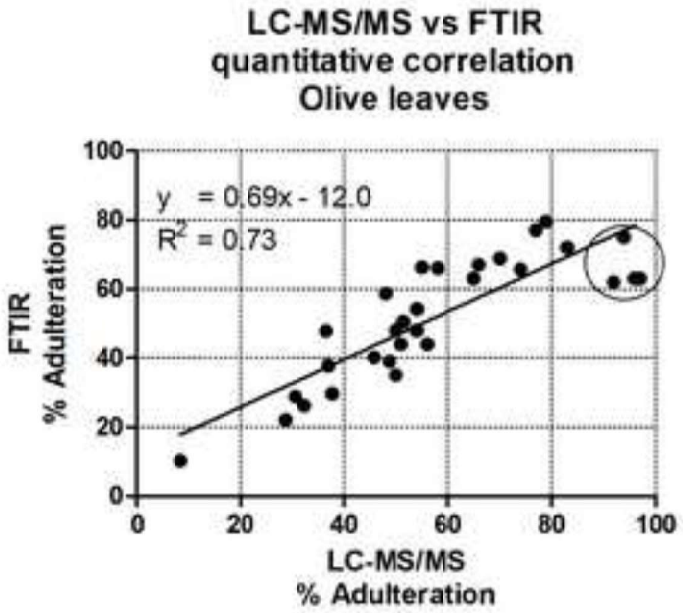
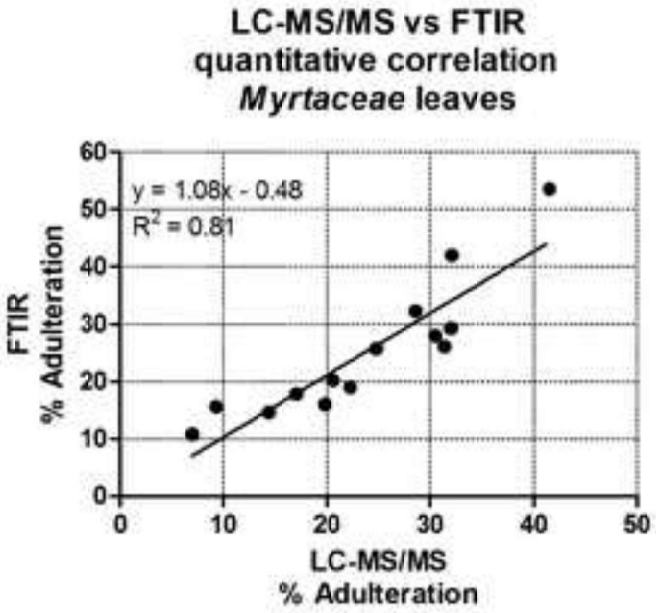


Figure3
Click here to download high resolution image





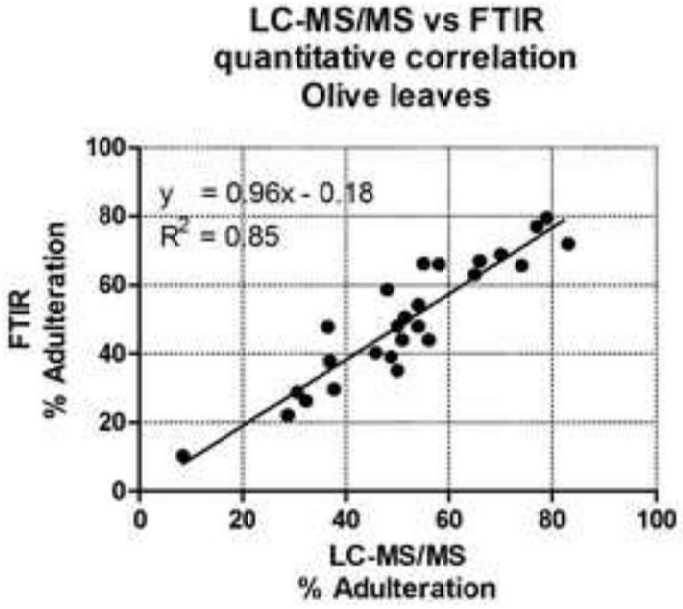
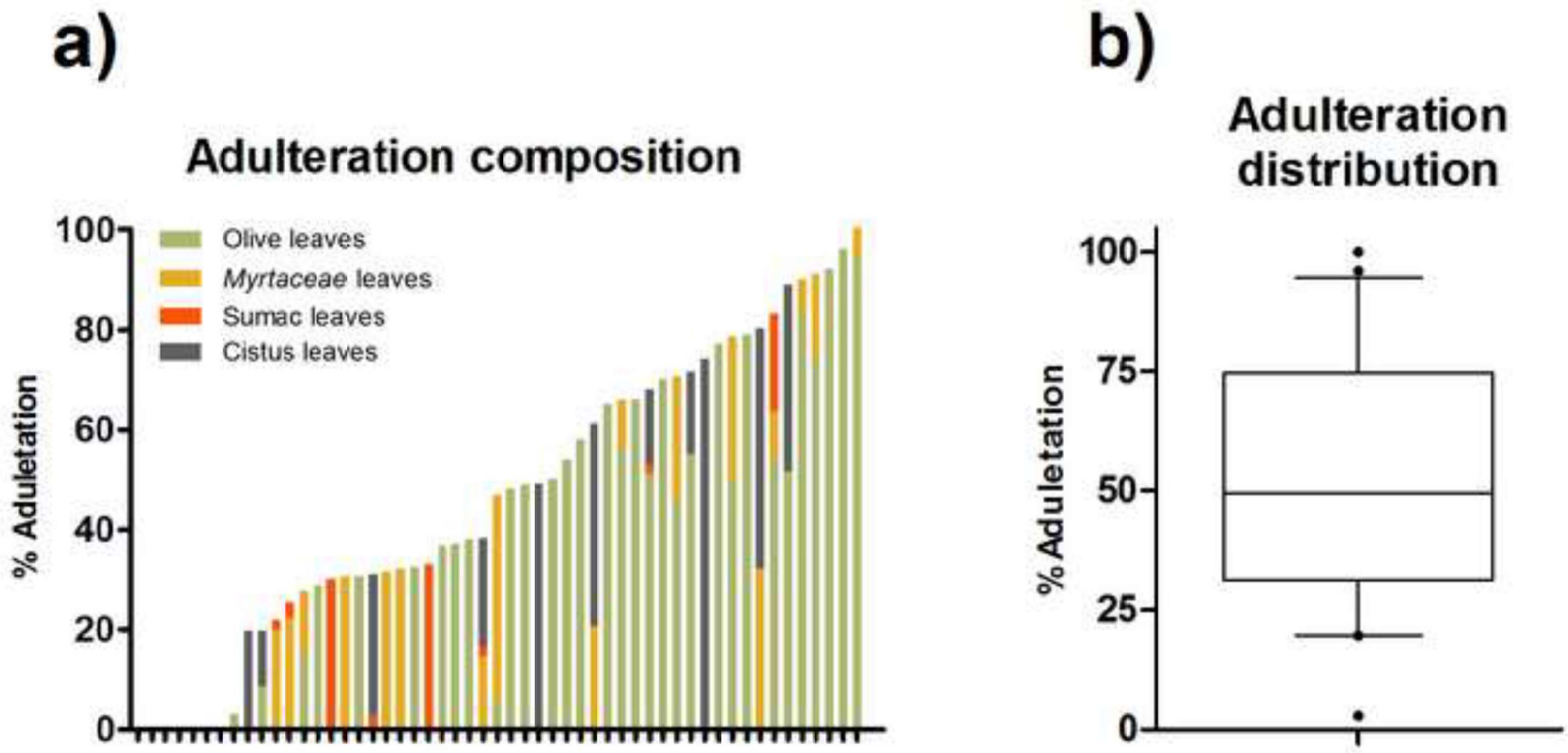


Figure4
Click here to download high resolution image



Supplementary Material

Click here to download Supplementary Material: SUPPLEMENTARY MATERIAL.docx

***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.