

# Untargeted analysis with GC-Orbitrap: a powerful tool for the authentication of spices and herbs

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## Introduction

Oregano is widely used as an ingredient in food and beverage products and as a flavoring ingredient for culinary purposes due to its organoleptic properties. Adulteration of oregano can be accidental or intentional with the latter driven by price and demand. Leaves from other plants (e.g., olive, thyme, marjoram, sumac, myrtle, and hazelnut)



are frequently used as adulterants as they are difficult to detect by visual inspection.<sup>1</sup> As a consequence, food manufacturers must check regularly for the quality and purity of oregano outsourced from various suppliers to ensure the quality and consistency of the end product.

Oregano is a complex matrix containing essential oils, phytosterols, and pigments; its aroma derives from a complex mixture of volatiles, mainly monoterpenes and sesquiterpenes, which can be easily extracted and concentrated in one single step using the headspace solid-phase microextraction (HS-SPME) technique.

This allows for minimal sample preparation, a critical point in non-targeted analysis since every manipulation could alter the sample composition.<sup>2</sup> The fingerprint of the oregano aroma constituents can be investigated with a multiplatform approach using isotope ratio, liquid or gas chromatography coupled with mass spectrometry (LC or GC–MS), or high resolution mass spectrometry (LC or GC–HRMS), and in combination with software tools for data reprocessing and statistical analysis. The high-resolution GC-MS approach has become very popular as it offers the advantage of full-scan data acquisition combined with high sensitivity, high resolving power (up to 240,000 FWHM), and accurate mass (< 5 ppm). Moreover full-scan data acquisition allows for targeted, non-targeted, and retrospective data analysis.<sup>2</sup>

In this study GC-Orbitrap technology coupled with solid-phase micro-extraction (SPME) with Arrow technology was used to assess the volatile profile of intentionally adulterated and native oregano samples. Data were acquired in full-scan electron ionization (EI) mode and analyzed with Thermo Scientific™ Compound Discoverer™ software. Positive chemical ionization (PCI) was used to confirm the elemental composition of the molecular ions using accurate mass information, isotopic match (measured versus theoretical), and presence of specific adducts. Additional MS/MS data were acquired and used to explain the proposed chemical structure of the compounds identified via mass spectrum matching.

## Experimental

In all experiments, a Thermo Scientific™ Orbitrap™ Exploris™ GC 240 system equipped with two Thermo Scientific™ Instant Connect Split/Splitless (SSL) Injectors—one used for SPME Arrow fiber conditioning and the second used for GC sample introduction, both equipped with SPME Arrow liner 1.7 mm i.d. (P/N 453A0415)—was coupled with a Thermo Scientific™ TriPlus RSH™ autosampler with SPME Arrow configuration. Chromatographic separation was achieved on a Thermo Scientific™ TraceGOLD™ TG-1MS capillary column, 30 m × 0.32 mm × 1.0 μm (P/N 26099-2970). Additional HS-SPME Arrow and Orbitrap Exploris GC parameters are detailed in Table 1. The triple coating phase of the DVB/CWR/PDMS fiber (P/N 36SA11T3) allowed for effective extraction of a wide range of volatiles including alcohols, aldehydes, ketones and esters.

**Table 1 (part 1). TriPlus RSH-SPME Arrow and Orbitrap Exploris GC experimental parameters used for the assessment of the volatile fraction of oregano**

TriPlus RSH – HS-SPME Arrow parameters	
Fiber	SPME Arrow DVB/CWR/PDMS (P/N 36SA11T3)
Coating phase thickness (μm)	110
Coating phase length (mm)	20
Incubation temperature (°C)	60
Incubation time (min)	15
Agitation speed (rpm)	500
Extraction temperature (°C)	60
Extraction time (min)	15
Stirring speed (rpm)	1,500
Fiber depth in vial (mm)	25
Fiber depth in injector (mm)	70
Desorption time (min)	2
Analysis time (min)	40
Inlet for fiber conditioning temperature (°C)	270
Inlet module and mode	SSL, splitless
Fiber pre-conditioning time (min)	0
Fiber post-conditioning time (min)	15
Septum purge flow (mL/min)	5, constant
Purge carrier gas, flow (mL/min)	He, 6.0
Fiber depth in injector (mm)	70
Trace 1310 GC parameters	
Inlet temperature (°C)	220
Liner	Thermo Scientific™ SPME Arrow liner 1.7 mm i.d. (P/N 453A0415)
Inlet module and injection mode	SSL, split
Split ratio	30:1
Septum purge flow (mL/min)	5, constant
Carrier gas, flow (mL/min)	He, 1.8
Oven temperature program	
Temperature (°C)	40
Hold time (min)	2
Rate (°C/min)	10
Temperature 2 (°C)	150
Rate (°C/min)	5
Temperature 3 (°C)	260
Rate (°C/min)	25
Temperature 4 (°C)	300
Hold time (min)	3

**Table 1 (part 2). TriPlus RSH-SPME Arrow and Orbitrap Exploris GC experimental parameters used for the assessment of the volatile fraction of oregano**

Column	
Thermo Scientific™ TraceGOLD™ TG-1MS	30 m, 0.32 $\mu$ m, 1.0 $\mu$ m film (P/N 26099-2970)
Vials and caps	
Vials	Thermo Scientific™ 10 mL crimp top HS vials (P/N 10-CV)
Caps	Thermo Scientific™ 20 mm magnetic crimp caps (P/N 20-MCBC-ST3)

Exploris GC mass spectrometer parameters for EI	
Transfer line temperature ( $^{\circ}$ C)	280
Ionization type	EI
Ion source temperature ( $^{\circ}$ C)	280
Electron energy (eV)	70
Acquisition mode	Full Scan
Mass range (Da)	40–450
Resolving power (FWHM)	60,000 @ $m/z$ 200
Lockmass ( $m/z$ ), column bleed	207.03235

Exploris GC mass spectrometer parameters for PCI	
Transfer line temperature ( $^{\circ}$ C)	280
Ionization type	CI
Ionization gas	Methane
Ionization gas flow (mL/min)	1.3
Ion source temperature ( $^{\circ}$ C)	190
Electron energy (eV)	90
Emission current ( $\mu$ A)	100
Acquisition mode	Full Scan
Mass range (Da)	80–450
Resolving power (FWHM)	60,000 @ $m/z$ 200

### Data acquisition, processing, and reporting

Data were acquired using Thermo Scientific™ Xcalibur™ software. This single platform integrates instrument control, method development functionality, and qualitative and quantitation-focused workflows. Compound Discoverer software, version 3.2, was used for spectral deconvolution, compound identification, and multivariate statistical analysis. Thermo Scientific™ Mass Frontier™ Spectral Interpretation software, version 8.0 was used to elucidate the chemical structure of putatively identified compounds via NIST mass spectra library matching.

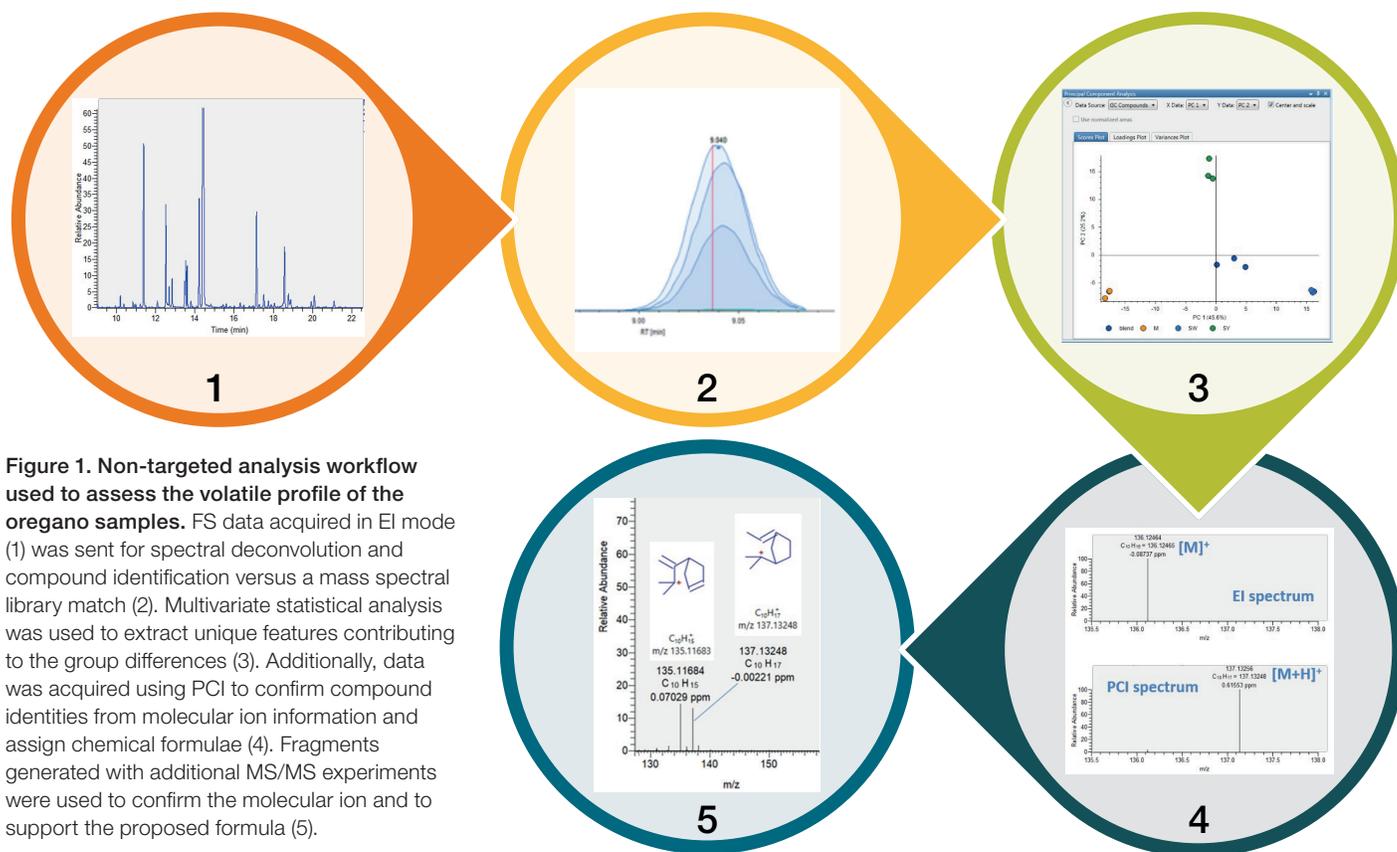
### Sample preparation

Three commercially available oregano samples were purchased from different retailers. Each oregano jar was well mixed to homogenize the matrix. Herb samples were weighed (150 mg) and transferred into 10 mL crimp top headspace vials for analysis. Samples were prepared in triplicate. In order to simulate an oregano fraud occurrence, thyme, marjoram, and olive leaves were purchased from local and online retailers and used to adulterate the oregano samples. One of the native oregano samples was randomly chosen and added with the three herbs to obtain a “fit for purpose” adulterated sample. Three bulks of fraudulent oregano samples were obtained by weighing 600 mg of native oregano and transferring into three glass containers. These containers were then adulterated by adding 10% of native marjoram, thyme, and ground olive leaves, respectively. Samples for analysis were prepared in triplicate as described above. A blend was obtained by pooling together the native and the adulterated oregano samples. In order to reduce the bias in the results, the sample vials were analyzed in a randomized order. A retention index mix (Sigma-Aldrich, C7-C30 saturated alkanes, P/N 49451-U) was injected at the beginning of the sequence and used to derive the RI of chemical components putatively identified by NIST17 library search following spectral deconvolution.

### Results and discussion

#### Workflow to assess the volatile profile in oregano samples and to identify adulterated samples

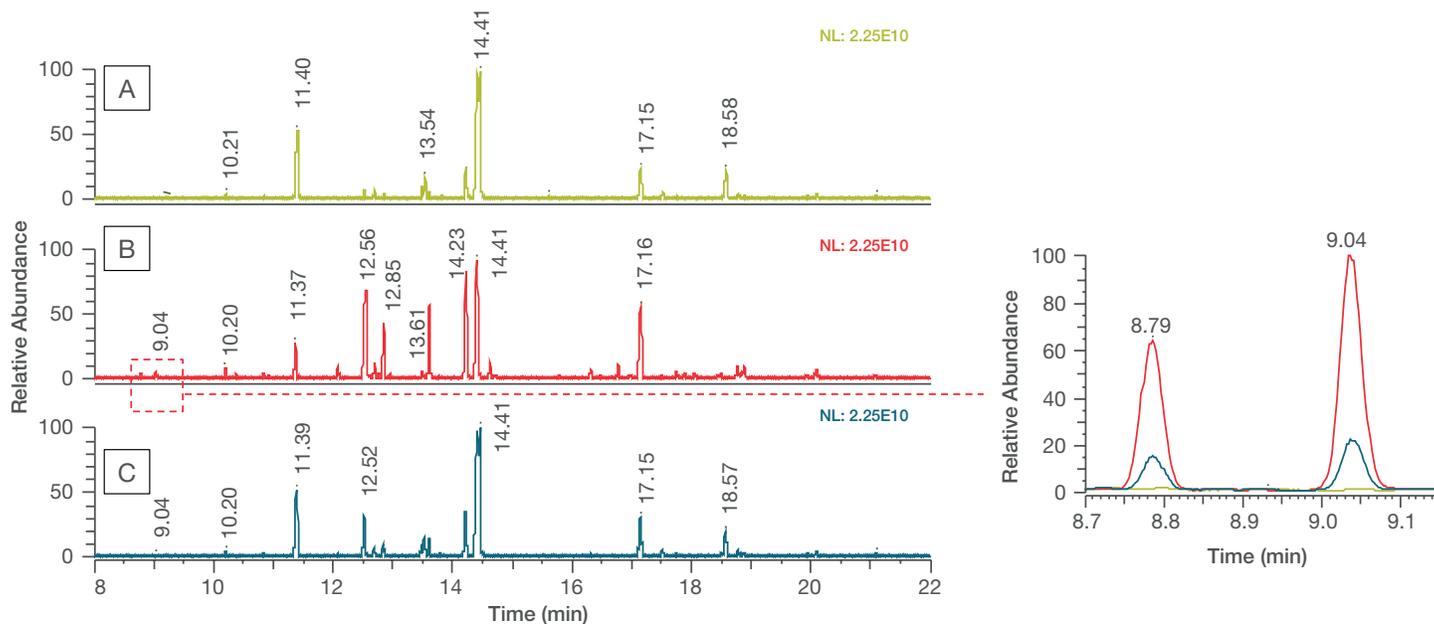
Full-scan (FS) data for native and adulterated samples were analyzed with Compound Discoverer 3.2 software for chemometric assessment and putative identification of peaks. A complete workflow was used to isolate unique components, identify compounds with a high degree of confidence, and detect the variations suggesting a possible fraud. The workflow used is reported in Figure 1. FS data was acquired using EI mode at 60,000 FWHM resolution and then imported in Compound Discoverer 3.2 software. The software was used to deconvolute, align, and filter the peaks to putatively identify the compounds using mass spectral library match (NIST 17). Multivariate statistical analysis (principal component analysis (PCA), loading and volcano plots) was used to select the significant features, defined by their  $m/z$  and retention time, contributing to the group differences. Full-scan data were acquired using PCI mode at 60,000 FWHM resolution to confirm the molecular ion and propose a chemical formula. Additional MS/MS experiments were performed to confirm that the fragments were from the molecular ion. This streamlined workflow allowed for a comprehensive characterization of the aroma components in oregano samples.



**Figure 1. Non-targeted analysis workflow used to assess the volatile profile of the oregano samples.** FS data acquired in EI mode (1) was sent for spectral deconvolution and compound identification versus a mass spectral library match (2). Multivariate statistical analysis was used to extract unique features contributing to the group differences (3). Additionally, data was acquired using PCI to confirm compound identities from molecular ion information and assign chemical formulae (4). Fragments generated with additional MS/MS experiments were used to confirm the molecular ion and to support the proposed formula (5).

### Aroma profile in adulterated samples

Differences in chromatographic profiles of the native herbs (oregano, marjoram, thyme, and olive leaves) and the fraudulent samples were visible when comparing the full-scan total ion chromatograms (TICs). Differences related to the prevalence of the common aroma components, as well as typical components of both native oregano and native adulterants, could be found in the simulated fraudulent samples. As an example, the comparison between native oregano, native thymol, and adulterated sample (oregano/10% thymol) is reported in Figure 2. Some components, putatively identified based on the spectral library (NIST 2017) SI score as  $\alpha$ -pinene (RT=8.79 min) and camphene (RT=9.04 min), originated from thyme exclusively. Thymol (RT=14.23 min) and carvacrol (RT=14.41 min) are present in both native thyme and oregano but with different abundances; thymol is predominant in thyme while carvacrol is the main constituent of oregano aroma. Although differences can be observed in the TIC comparison, all features were extracted from the data and analyzed statistically.



**Figure 2. FS TIC obtained for native oregano (A), thyme (B), and adulterated oregano sample (C).** Typical components of native thyme (e.g.,  $\alpha$ -pinene, RT=8.79 min and camphene, RT=9.04) as well as differences in the amounts of the common aroma components (e.g., thymol, RT=14.23 min and carvacrol, RT=14.41 min) or oregano unique components (e.g., aromadendrene, RT=18.58 min) could be found in the simulated fraudulent sample.

## Compound identification in fraudulent samples

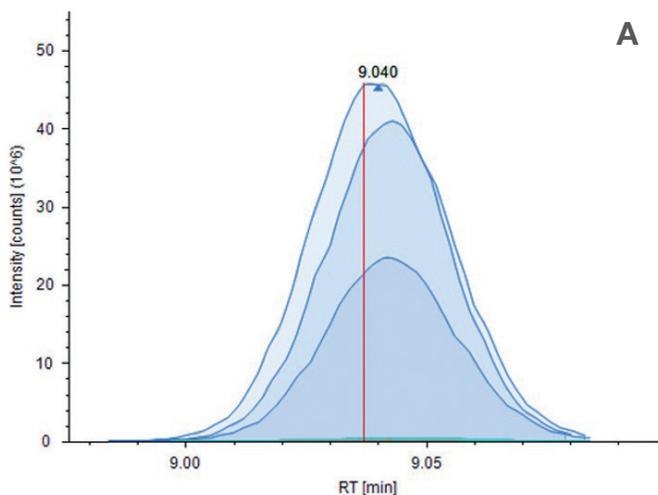
The Compound Discoverer platform includes a streamlined workflow for GC EI data allowing for extraction, deconvolution, and putative identification of the unknowns based on mass spectral library (NIST 2017).

The software first performed non-targeted peak detection within 5 ppm extraction windows. Accurate mass chromatographic deconvolution was then performed by grouping together all extracted ion peaks above a customizable signal to noise (S/N) threshold that maximize at the same retention time. The deconvoluted spectra were then searched against NIST 2017 nominal mass spectral library, and the hits were scored based on the total score derived from a combination of library search index (RSI), high-resolution filtering (HRF) value, and presence/absence of the molecular ions as well as elemental percentage. The use of a retention index acquired under the same conditions used for sample analysis helped to increase the confidence in compound identification. An example of this workflow is reported in Figure 3 with the Compound Discoverer browser

showing the overlaid XIC (extracted ion chromatogram) of the peak eluting at 9.04 min ( $m/z$  93.06982), the result table with the top hit, and the EI spectrum – deconvoluted versus NIST library. The peak was putatively identified as camphene with a total score=94.9, RSI=801, and HRF=97.53. This approach allowed putative identification of most of the detected peaks; however, for some compounds the EI spectral library match was inconclusive. In this case PCI data and accurate mass become essential to discriminate the chemical formula and provide confidence in identification.

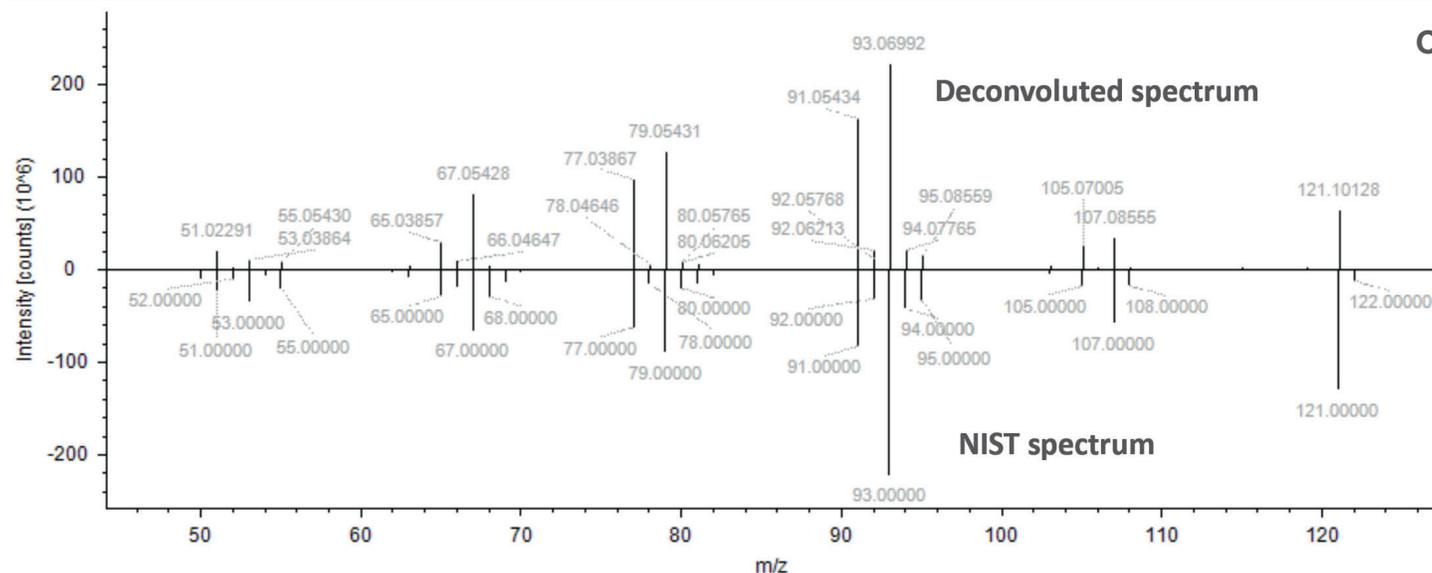
## Multivariate statistical analysis: PCA and differential analysis

Multivariate statistical analysis was carried out using Compound Discoverer 3.2 software. PCA is a well-known statistical approach that highlights variation between sample groups and allows visualization of strong patterns in complex datasets. An example of a PCA plot is reported in Figure 4. The generated PCA plot shows clear separation between the native oregano sample and the adulterated ones.



**Figure 3. Compound Discoverer results browser showing an example of compound identification for the peak eluting at RT=9.04 min, putatively (RSI 801) identified as camphene.** Overlaid XIC (base peak  $m/z$  93.06982) for camphene (A); results table with the matched compound identified based on library search and retention index (B); EI spectrum of camphene – measured vs. NIST library (C).

Matched Compound	Formula	Score	HRF Score	SI	RSI	RI Delta	Elements Found[%]	Theo Mol Mass	Observed Mol. Mass	RI Column type	RI Diff[%]
Camphene	C10H16	95.0	97.7356	794	801	4	100.0	136.12465	136.12457	StandardNonPolar	0.4



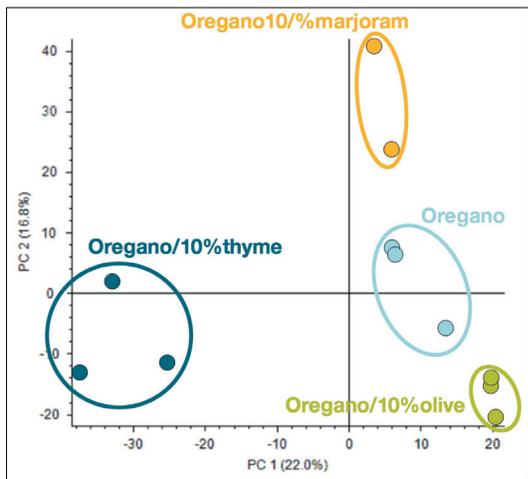


Figure 4. PCA score plot of the volatile compounds that differentiate the native oregano from the adulterated samples. A complete separation between the sample groups was observed.

Differential analysis was carried out using the volcano plots (V-plot), useful to quickly identify changes in large data sets composed of replicate data. The V-plot obtained comparing oregano adulterated with thyme and native oregano is reported in Figure 5. The main compounds responsible for differences in the analyzed groups are highlighted with light blue dots. This approach allowed the identification of suspected adulteration of native oregano, by both highlighting the changes in the amount (increase or decrease) of some compounds and the presence of compounds that are not usually found in native oregano. For oregano adulterated with 10% thyme, for example, it was possible to identify two main components such as camphene and  $\alpha$ -pinene with a content increase by a 7-fold factor and a 3-fold factor, respectively. Allyl furoate increased by 3.5-fold in oregano adulterated with olive leaves, while methyl cinnamate had a log<sub>2</sub>-fold change of 2.5 in oregano adulterated with marjoram as reported in Table 2.

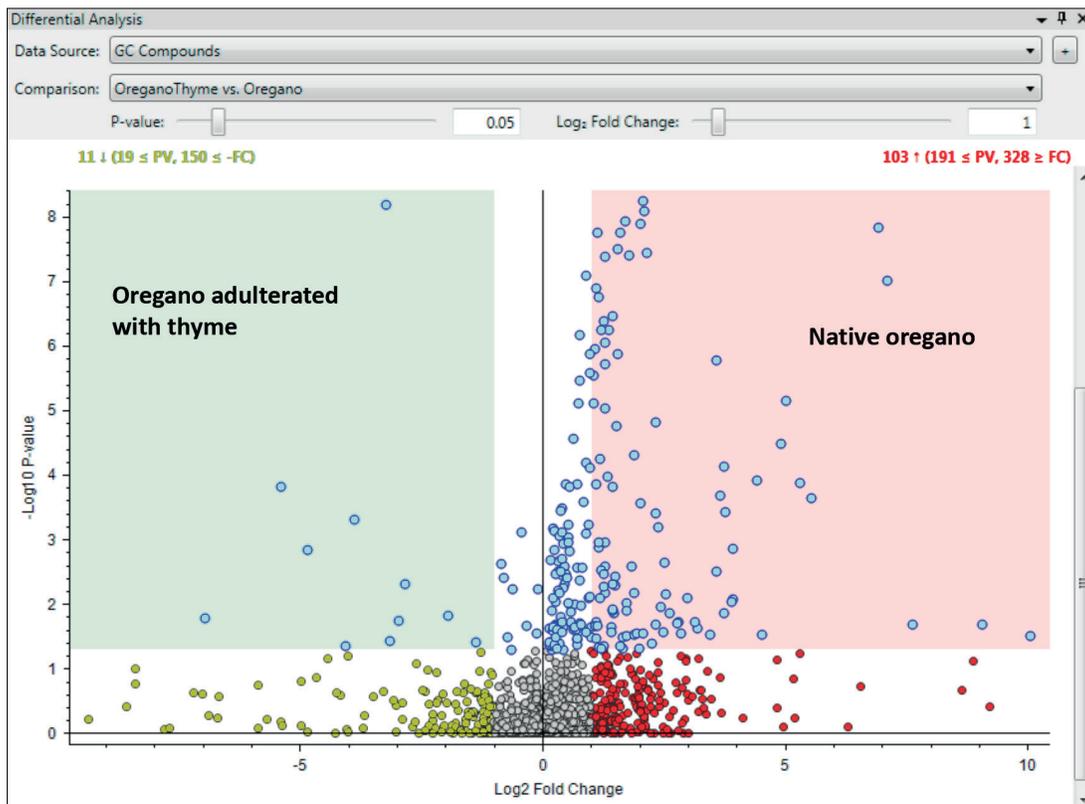


Figure 5. V-plot scatterplot showing the statistical significance (P value) versus magnitude of change (fold change) when comparing the oregano adulterated samples (with thyme) versus the native oregano. The main chemical components that are responsible for sample diversity between two sample groups are selected (light blue dots).

**Table 2. Table of fold change of main compounds that could suggest an adulteration of the native oregano.** In particular, camphene and  $\alpha$ -pinene content increased by a 7-fold and 3-fold factor, respectively, when oregano was adulterated with thyme; allyl furoate increased by 3.5-fold in oregano adulterated with olive leaves; and methyl cinnamate had a log<sub>2</sub>-fold change of 2.5 in oregano adulterated with marjoram.

Putative ID	RT [min]	Reference <i>m/z</i>	Total score	Log <sub>2</sub> fold change
<b>Oregano adulterated with 10% thyme</b>				
$\alpha$ -Thujene	8.63	77.03867	92.9	1.9
$\alpha$ -Pinene	8.79	91.05434	95.9	3.6
Camphene	9.04	93.06992	95.1	6.9
<i>trans</i> - $\beta$ -Ocimene	10.60	91.05424	93.6	-0.3
Camphor	12.08	95.08559	95.1	1.1
Pinocamphone	12.34	95.08554	94.0	1.4
cis-Ocimenol	12.85	93.06992	95.3	1.6
cis-Dihydrocarvone	12.95	67.05428	94.4	1.6
Duroquinone	13.54	117.06986	92.3	1.2
Carvacrol	14.41	135.08051	96.2	-0.1
Germacrene	20.31	133.10118	91.1	-0.3
Levomenthol	24.97	109.10131	92.8	1.5
<b>Oregano adulterated with 10% olive leaves</b>				
Furfuryl disulfide	9.41	81.03341	97.1	1.2
Allyl furoate	10.69	95.04906	92.8	3.5
Thymol	14.22	135.08049	96.0	-0.3
$\alpha$ -Ocimene	11.40	93.06971	94.1	-0.2
Sabinene	10.84	91.05434	95.3	-0.4
4-Thujanol	10.92	93.06988	95.6	-0.2
<b>Oregano adulterated with 10% marjoram</b>				
$\alpha$ -Thujene	8.63	77.03867	92.9	1.4
Sabinene	9.42	91.05424	92.6	1.3
Thujone	11.50	110.1089	92.7	-0.2
Carvacrol	14.41	135.08051	96.2	-0.1
4-Terpinenyl acetate	14.64	94.07311	94.3	1.6
Methyl cinnamate	15.75	161.0597	93.7	2.5

### Compound confirmation using PCI

Further confirmation in the identification of compounds was achieved by assessing the PCI spectra to identify the elemental composition of the parent ion by looking at common adducts. In PCI experiments using methane as the reagent gas three adducts are typically observed:  $[M+H]^+$ ,  $[M+C_2H_5]^+$ ,  $[M+C_3H_5]^+$ . As an example, EI and PCI spectra of camphene are reported in Figure 6. The observed molecular ion corresponding to *m/z* 136.12464 is present in the EI spectrum with a mass difference of -0.09 ppm from the theoretical *m/z* 136.12465 for the formula C<sub>10</sub>H<sub>16</sub>. The presence of the methane adducts in the PCI spectrum with sub-1 ppm mass accuracy confirmed *m/z* 136.12464 as the molecular ion for camphene (RT=9.04 min) and supported the elemental composition of the proposed molecule.

### Molecular ion fragment annotation using PCI MS/MS data

Additional PCI MS/MS experiments were assessed to support the proposed formula and to derive structural information. An elemental composition calculator included in the Thermo Scientific™ FreeStyle™ application was used to propose a formula for  $[M+H]^+$  ion as shown in Figure 7. The ion *m/z* 137.13252 was isolated in the quadrupole and fragmented in the HCD cell using 10 V energy to generate fragments as shown in Figure 8. Mass Frontier software was used for assignment of fragment structures to measured ions in the MS/MS spectrum. The measured fragments provided detailed information with respect to the proposed chemical formula with <1 ppm mass accuracy.

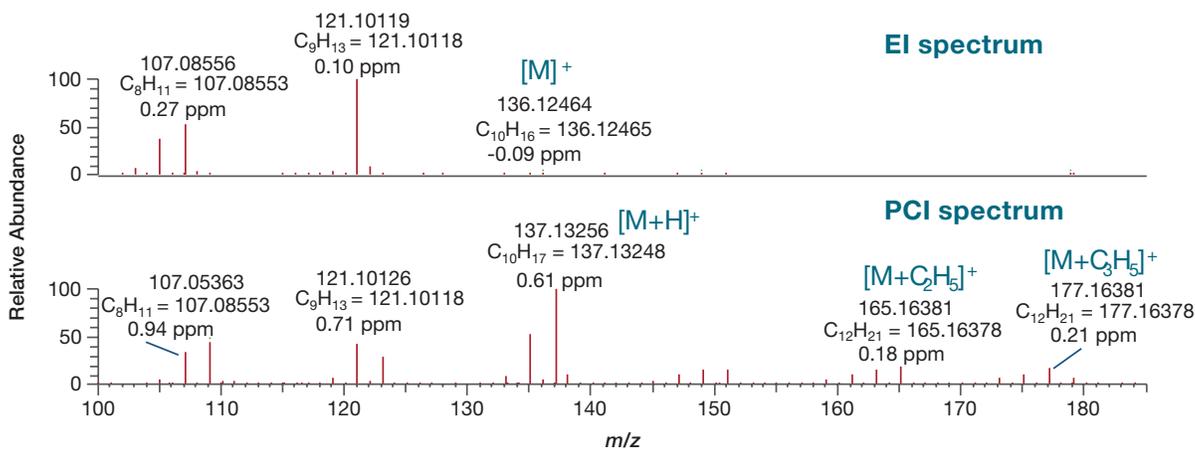
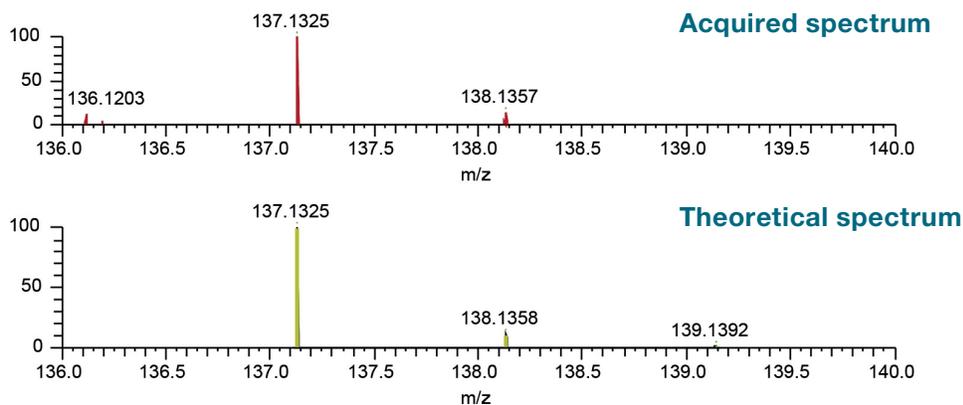


Figure 6. Comparison between EI and PCI spectrum for camphene (RT=9.04 min). The molecular ion ( $m/z$  136.12464) is visible in the EI spectrum with <1 ppm mass accuracy annotated. In the PCI spectrum the typical adducts observed when methane gas is used are clearly visible confirming the molecular ion and the proposed molecular formula for camphene.



Peak Mass	Combined Score	Display Formula	Delta [ppm]	RDB	Theo. mass
137.1325	95.87	C <sub>10</sub> H <sub>17</sub>	0.00	2.50	137.13248

Figure 7. Elemental composition calculator in the FreeStyle application proposing the chemical formula C<sub>10</sub>H<sub>17</sub> for the [M+H]<sup>+</sup> ion based on accurate mass and isotope pattern. One candidate is proposed with 0.00 ppm mass error and 96% isotopic match with the theoretical pattern.

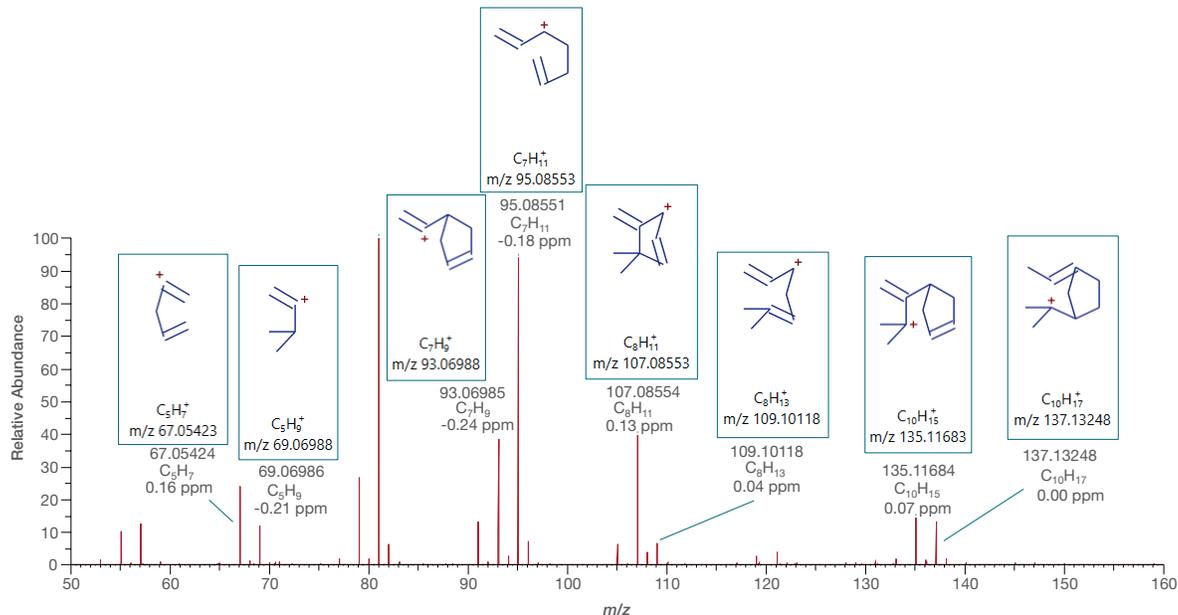


Figure 8. PCI MS/MS following  $m/z$  137.13252 fragmentation in the HCD collision cell showing the product ions annotated with measured mass, elemental composition, theoretical mass, mass accuracy (ppm), and the proposed chemical structures.

## Conclusions

The results obtained in this study demonstrate that the Orbitrap Exploris GC system represents a powerful tool for assessing food authenticity, providing an integrated omics approach for profiling complex samples and identifying unknown compounds intentionally added with fraudulent purposes.

- Automated headspace sampling with the SPME Arrow removes the need for sample preparation and speeds up the analysis.
- The high resolving power (60,000 at  $m/z$  200) and consistent sub-1 ppm mass accuracy and the wide dynamic range allow for fast and confident characterization of a large number of compounds regardless of their concentration or matrix complexity.
- The streamlined GC-EI data processing workflow in Compound Discoverer software allows for extraction, deconvolution, identification of unknown compounds, and multivariate statistical analysis.

- Principal components and differential analysis allow for the detection of differences in the composition of the aroma profile, which can identify unique compounds present in adulterated oregano samples.
- Rapid change-over from EI (for spectral library search) to softer ionization such as PCI (for molecular ion confirmation using adduct information) and the ability to perform accurate mass MS/MS experiments (for structural elucidation), combined with the use of the FreeStyle elemental composition calculator and Mass Frontier software for predictive fragmentation and structural elucidation, enable confident compound identification with unprecedented ease.

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