Workshop on olive oil authentication

Madrid, Spain
10 & 11 June 2013

Organised by
European Commission
Directorate General Agriculture and Rural Development &
European Commission Joint Research Centre
Institute for Reference Materials and Measurements

With the participation of the International Olive Council
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What’s a Certified Reference Material (CRM)?

A material, sufficiently homogeneous and stable with respect to one or more specified properties, which has been established to be fit for its intended use in a measurement process, and has been characterized by a metrologically valid procedure for one or more specified properties, accompanied by a certificate that provides the value of the specified property, its associated uncertainty, and a statement of metrological traceability (ISO/IEC Guide 99:2007).

Aim of the study

Hereewith we present the results of a series of studies for the elaboration, certification and distribution of several CRMs of olive oil, which could be used in olive oil quality control laboratories.

Structure of the project

1st stage: InterOLEO-CRM

The certification of all the analytical parameters is related to the physicochemical characteristics (updated Regulation EU 2568/91).

2nd stage: SensOLEO-CRM

We focused on the certification of several sensory properties of olive oils.

Further steps: Once the materials have been produced, we consider as necessary:

- to assure the homogeneity of the units of the lot,
- to characterize the values of the analytical parameters to be certified, and
- to evaluate stability of the materials, under the recommended storage conditions.

References

INTRODUCTION

There are several approaches that can be applied to the olive oil authentication. They differ in the scientific foundation, according to the information obtained. The most common techniques are: (i) chemical composition; (ii) stable isotopes; and (iii) DNA.

In order to practical consequence of the chemical-based approach, it could be used three analytical methods: chemical markers, compositional profile, and instrumental fingerprinting. Our research focuses on fingerprinting methodologies. They consider the entire analytical signal, which is acquired and recorded by the analytical instrument, directly from olive oil or from a previously isolated fraction, i.e. a spectrum or a chromatogram. The shape and intensity of the recorded signal constitutes the instrumental fingerprint from the whole olive oil sample, or from the considered fraction, because it is characteristic and reflects implicitly its chemical composition. Therefore, the methodology is based on the existence of the chemical composition and they can be applied when the compositional methods are suitable.

METHODOLOGY

Edible oil (mainly olive oil) → Pre-processing → Models

- TGAs TRIACYLGlycerols
- STs STEROLS
- VOCs VOLATILE ORGANIC COMPOUNDS

Signal acquisition

GC, LC, PTR-MS → GC-MS chromatograms of vegetable oil blends samples after peak shifting pretreatment (iCoshift) (5)

OBJECTIVES

- Categories, varieties
- Geographical origin
- Identity, characterisation
- Adulteration

AUTHENTICATION

REFERENCES


(*) Currently under study
INTRODUCTION

Extra virgin olive oil (EVOO) is a fundamental landmark of the Mediterranean diet which has noticeable nutritional and organoleptic characteristics. European Regulation (EEC) 2568/91 has been setting the minimum requirements in order to allow labelling of an oil as extra virgin. These general requirements, are based on physical-chemical and organoleptic parameters directly linked to freshness and quality of the product (free acidity, peroxide index and ultraviolet absorption, panel test and recently alkyl esters) and other purity parameters, intended to prevent fraudulent mixing with cheaper oils (refined, pomace or seed oils) otherwise, however, EVOOs products exhibit great differences, either in organoleptic or nutritional or functional characteristics (e.g. polyphenols and tocopherols content, aroma, etc.), which are related to cultivar of olives, production techniques and geographical origin.

The lack of official methods of analysis for assessing the origin implies that official control of special quality regulated production, such as PDO or PGI, must rely only on checking the accompanying paper documentation. The use of Isotope Ratio Mass Spectrometry (IRMS) has demonstrated as a tool that can improve geographical discrimination of unknown samples, because this technique presents the advantage of giving results which are almost independent from cultivar employed and production technique (1-3).

In this work the evaluation of the composition of Fatty Acids Methyl Esters (FAME) alongside with the determination of stable isotope ratio of C in bulk oils and in main FAME constituents has been made.

EXPERIMENTAL

Samples: Sampling of authentic extra virgin olive oil was made by ICRQF inspctive personnel at oil mills. Selected olive oils of known origin and designated to achieve the Protected Designation of Status. For each PDO/PGI three oil samples, related to three different maturation stages, were collected at a distance of about 20-30 days from each other. For each sample additional information regarding agronomical and technological features were also collected.

Oils samples were collected in 250 ml dark glass bottles covered with a black plastic envelope and then shipped to the laboratory. Upon receive oils were filtered by means of a 0.45 µm barrel type nylon filter in order to remove any sediment eventually formed which may deteriorate the product. Samples were kept at 8°C until the analysis. An individual set of three samples was taken for each geographical mention for every cultivar.

Fatty acid analysis by GC/FID: samples were transesterified with methanolic potassium hydroxide according to official method reported in annex XI(A) of Regulation (EC) 702/2007 - analysis by gas chromatography of methyl esters of fatty acids.

C Isotope analysis by bulk/EA/IRMS: The carbon isotope ratio (13C/12C) of bulk oils was determined by flash combustion on a Thermo (Bremen, Germany) elemental analyzer (EA) connected to a Thermo Fisher Delta V Plus (Bremen, Germany) isotope ratio mass spectrometer (IRMS) by analyzing the ratio of ionic currents of m/z 44 ([12C]O2), m/z 45 ([13C]O2) ed m/z 46 coming from carbon dioxide arising from samples, which were in the same assumption in the elemental analyzer.

The stable isotope composition of carbon, is calculated in delta (δ) notation as the per thousand (%), as deviations of the isotope ratio relative to known standards (‰ C = R sample - R standard / R standard). For 13C/12C ratio the standard utilized is Vienna Pee Dee Belemnite (VPMDB).

Determination of isotopic ratio of individual FAME: the determination was realized with a GC Isotlink device constituted by a GC Thermo Trace FID coupled to a ThermoFinnigan Delta V Plus isotope ratio mass spectrometer by means of a combustion interface GC/C/IRMS. Results were corrected for the contribution of δ 13C of the methanol employed for trans-esterification. Esterification procedure was the same as for the determination of the fatty acid composition.

RESULTS

In order to carry out the statistical analysis, samples were divided into four categories defined according to the area of origin.

The distribution of samples within each category was first evaluated by univariate analysis through representation of mean box and whisker diagrams which show the dispersion of the samples within each class in function of the measured values for the isotopic ratio (Figure 1b-a, b, c, d).

Samples were divided into the following four macro-regions (figure 2): Nord: including samples produced in the regions of Lombardy, Veneto, Trentino, Liguria and Emilia Romagna; Center: including samples produced in Tuscany, Latium, Abruzzo Umbria, Marche, Sardinia; South: including samples produced in Campania, Apulia, and Calabria; Sicily: including samples produced in Sicily.

Composition data has demonstrated that there is a certain degree of correlation among main constituents fatty acids with geographical area of proven origin according to latitudine. The values of δ 13C of δ 13C in both bulk and FAME also have indicated a similar evidence of correlation. However overlapping zones are very wide.

MULTIVARIATE ANALYSIS

The use of multivariate analysis represent a tool that can improve geographical discrimination of unknown samples. Principal component analysis (PCA), applied in the first stage of the data processing, represents one of the most frequently used chemometric tools, because allows to project in an easy way data from high dimensional space.

In the preliminary analysis of the olive oil data, 3 distinct preliminary PCA were performed to investigate clustering of samples on the basis of the area of provenience (Figure 3).

The PCA shows that the geographical discrimination of the samples improves by making of appropriate combinations of the parameters up to now considered individually. As one can see the introduction of the measure of the carbon isotope ratio on bulk oil and of the individual components fatty acids makes a gives better separation of the four classes. The diagrams have been built after an autoscaling pretreatment of the data.

PLS-DA techniques as well shows (Figure 4) that when the whole set of data is considered (isotopic and composition), there is a substantial discrimination of the origin of the oil. As it can be seen, a clear separation between the data related to geographical origin can be observed.

REFERENCES

On-line HPLC-GC-FID for the evaluation of the quality of olive oils though the methyl, ethyl, and wax esters

Maurus Biedermann
Official Food Control Authority of the Canton of Zürich, Zürich, Switzerland

Introduction
Increased ester contents in olive oils indicate degraded olives; methanol and ethanol formed during fermentation are transesterified with fatty acids from the triglycerides. Wax esters are more readily extracted from the soft skin of overripe olives. A promising correlation of chemical analysis and sensorial evaluation was confirmed: oils with bw concentration of these esters were of high sensory quality, whereas many of the oils with high contents not even met the requirements for extra virgin olive oils. Limits on the alkyl and wax ester content in olive oils are specified in the Commission Regulation 61/2011.

Analytical method
Diluted oils are pre-separated by normal phase HPLC and the ester fraction on-line transferred into GC via the Y-interface (fig. 1) by fully concurrent solvent evaporation (fig. 2). The method includes several verification standards to monitor proper performance in the critical aspects for each analysis: wax and methyl esters are eluted at different times from HPLC; standards were introduced to monitor the edges of the fraction window. Fully concurrent eluent evaporation may cause losses of the most volatile fatty acid methyl esters through the vapor exit. A volatile verification standard was added to optimize the transfer conditions [1].

Verification
The performance of each analysis is checked by built-in tools:
- Losses of fatty acid esters by co-evaporation with the eluent is critical applying concurrent solvent evaporation. The internal standard (IS1, ethylcholesterate, E20:20) is less volatile than the methyl and ethyl oleate. A more volatile verification standard is added, methyl heptadecanoate (VS1, Me-17:0). The ratio Me-17:0/IS1 = 10% is monitored.
- Another critical point is shifting retention times in NPLC, the analytes may be eluted of the LC fraction window. The wax ester 21:0/14:2 (IS1) is eluted at the beginning of the fraction. Methyl enolic acids (VS2, Me-20:2) are added as second verification standard, its elution determines the end of the fraction (fig. 4). The ratio between IS2, IS2 and IS1 is monitored.

Results
100 olive oils from the Swiss marked asd extra virgin were analyzed chemically. A selection of these (with low and high contents of methyl and ethyl esters) were also sensorial evaluated.

Conclusions
Methyl and ethyl esters of fatty acids are useful indicators for determining the quality of olives and the oil produced from these. Online HPLC-GC provides a largely automated method for routine analysis. The high stability of FID as well as the large volume on-column transfer facilitates the method for routine use. A more detailed analysis of the wax ester fraction is described in reference [3].

References
ABSTRACT

• Extra virgin olive oil is frequently subjected to adulterations with addition of oils obtained from plants other than olive. DNA analysis is a fast and economic tool to identify plant components in oils.

• Extraction and amplification of DNA by PCR was tested in olives, in milled seeds and in oils, to investigate its use in olive oil traceability.

  DNA was extracted from different oils made of hazelnut, maize, sunflower, peanut, sesame, soybean, rice and pumpkin. Comparing the DNA melting profiles in reference plant materials and in the oils, it was possible to identify any plant components in oils and mixtures of oils.

  Real-Time PCR (RT-PCR) platform has been added of the new methodology of High Resolution Melting (HRM), both were used to analyze olive oils mixed with different percentage of other oils. Results showed HRM a cost effective method for efficient detection of adulterations in olive oils.
DNA EXTRACTION FROM OILS

Yields of DNA (ng/mL of starting volume of oil) after extraction based on NucleoSpin Plant kit and CTAB. The yields are expressed as averages of three independent extractions, with standard deviation.

REAL-TIME PCR ANALYSIS OF OILS

Melting curves analysis of amplicons obtained by Real Time PCR conducted on DNA extracted from leaves (or seeds) and oils. (A) olive; (B) hazelnut; (C) maize; (D) sunflower; (E) sesame; (F) rice; (G) pumpkin; (H) peanut.
**SEQUENCE CHARACTERIZED AMPLIFIED REGION**

- The use of a SCAR marker, derived from multilocus markers, such as AFLPs or RAPDs, can allow to find the adulteration of an olive oil with a non olive oil, such as hazelnut oil. Moreover, a SCAR marker can be used in a high-throughput platform to assess and quantify the contribution of a single cultivar in commercial multivarietal oils. In Real-Time PCR, a SCAR marker, named CP-rpl16T, derived from an AFLP fingerprint of olive oil, was applied either on DNA extracted from (1) leaves and from a (2) 100% olive oil and (3) 90% olive oil and 10% hazelnut oil.

**DETECTION OF PLANT OIL DNA USING HIGH RESOLUTION MELTING (HRM) POST PCR ANALYSIS: A TOOL FOR DISCLOSURE OF OLIVE OIL ADULTERATION**

A. Red curve: HRM of DNA extracted from olive oil; Blue : HRM of DNA extracted from maize seed oil; Green : HRM of DNA extracted from an olive and maize oil mix (90%-10%).

B. Red : HRM of DNA extracted from olive oil; Blue : HRM of DNA extracted from sunflower seed oil; Green : HRM of DNA extracted from an olive and sunflower oils mix (90%-10%).

C. Red : HRM of DNA extracted from olive oil; Blue : HRM of DNA extracted from hazelnut seed oil; Green : HRM of DNA extracted from an olive and hazelnut oils mix (90%-10%).
Acknowledgements

This study has been carried out with financial support from the Commission of the European Communities, specific RTD programme “Quality of Life and Management of Living Resources” project,QLK1-CT-2002-02386, “Traceability of origin and authenticity of olive oil by combined genomic and metabolomic approaches (OLIV-TRACK)” coordinated by N. Marmiroli. The content of this paper does not necessarily reflect the Commission of the European Communities views and in no way anticipates the Commission’s future policy in this area. This paper had also the contribute of the Italian Minister of University and Research special program PRIN “Rintracciabilità della composizione e dell’origine di oli d’oliva DOP, IGP e 100% Italiani attraverso metodiche genomiche, proteomiche e metabolomiche” coordinated also by N. Marmiroli and a contribute from the University of Parma (fund FII, 2002, 2003, 2004, 2005, 2006). This work was also supported financially by Emilia-Romagna (IT) Regional project SQUAL within the research framework PRRIII, Misure 3.4.

References


Introduction

Virgin olive oil (VOO) freshness has become a matter of concern among consumers in relation to VOO exceptional nutritional and sensory characteristics. As loss in the latter are observed in the oil mill transportation or at sale points upon storage due to practices that accelerate oxidation and hydrolysis, scientific support is required in cases of dispute. Discussions are on the way for updating the relevant EU regulations on VOO quality characteristics. Toward this direction modernization of methods of analysis is expected.

Aim of the study

The exploitation of the multiple information provided in situ by Fourier Mid-IR spectroscopy equipped with an Attenuated Total Reflectance (ATR) cell as a means to extract information for loss of VOO freshness under mild storage conditions for a period of 12 months.
Results

Table 1. Value ranges of quality indices determined for the test samples \((n = 11)\) at different storage periods in the dark

<table>
<thead>
<tr>
<th>Quality Index</th>
<th>t=0</th>
<th>t=6</th>
<th>t=12</th>
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<tbody>
<tr>
<td>Acidity (% oleic acid)</td>
<td>0.32-0.67</td>
<td>0.34-0.70</td>
<td>0.35-0.76</td>
</tr>
<tr>
<td>PV (meq O₂/ kg oil)</td>
<td>6.5-8.6</td>
<td>9.0-13.1</td>
<td>13.3-20.0</td>
</tr>
<tr>
<td>(K_{232})</td>
<td>1.54-1.72</td>
<td>1.76-2.01</td>
<td>2.02-2.42</td>
</tr>
<tr>
<td>(K_{270})</td>
<td>0.10-0.12</td>
<td>0.12-0.15</td>
<td>0.13-0.20</td>
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Figure 1. Frequency distribution of A) peroxide and B) \(K_{232}\) values for EVOO samples \((n = 11)\) stored in the dark for 0, 6 and 12 months

Discussion

For all test samples, regardless storage time, the measured values for each index (Table 1) were within the official limits (EU 2568/91 and amendments) for extra virgin olive oil (EVOO)

Thus, upon storage up to 12 months, oxidation of samples was still at early stages

Frequency distribution analysis for e.g. peroxide (Figure 1A) and \(K_{232}\) (Figure 1B) values showed a progressive increase which can be justified by the presence of oxygen (10% headspace of the bottles)

Changes in acidity were slight
**Figure 2.** Typical FT-IR/ATR spectra of EVOO samples stored in the dark and frequencies of bands of selected functional groups.

Changes in the intensity values at 3470 cm\(^{-1}\) (hydroperoxides) and in values of intensity ratios (\(A_{3006/2924}\), \(A_{3006/2853}\), \(A_{3006/1746}\), \(A_{3006/1465}\), \(A_{3006/1163}\), \(A_{1118/1097}\), \(A_{2853/1746}\), \(A_{2853/1417}\), \(A_{2853/1163}\), \(A_{2853/1118}\), \(A_{2853/1097}\), and \(A_{2853/723}\)) relevant to the degree of oil unsaturation did not offer more information than the physicochemical criteria when examined one by one.

**Principal component analysis (PCA)** applied to the intensity values of the frequencies used in the above-mentioned ratios (**Figure 3**) showed that samples stored for 1 year (excluding one as an outlier) clearly differed from the rest. Those stored up to 6 months were grouped together with fresh ones.

**Discriminant analysis (DA)** after leave one out cross-validation gave a 81.3% correct classification: [6/11 (t=0), 10/11 (t=6), and 10/10 (t=12)]
<table>
<thead>
<tr>
<th>Spectral region</th>
<th>Number of PCs</th>
<th>Total variance explained</th>
<th>Classification</th>
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<tr>
<td></td>
<td></td>
<td></td>
<td>Original</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>t=0</td>
</tr>
<tr>
<td>550-4000 cm(^{-1})</td>
<td>15</td>
<td>91.1%</td>
<td>10/11</td>
</tr>
<tr>
<td>715-2935 cm(^{-1})</td>
<td>14</td>
<td>91.3%</td>
<td>9/11</td>
</tr>
<tr>
<td>1020-1260 cm(^{-1})</td>
<td>3</td>
<td>92.1%</td>
<td>10/11</td>
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Figure 4. Score plot of the first three PCs obtained by PCA applied to the second derivative of the spectral region 1020–1260 cm\(^{-1}\) from the spectrum of EVOO samples stored in the dark for 0 (n= 11), 6 (n= 11), and 12 (n= 11) months

Examination of the discriminant activity of the 2\(^{d}\) derivative of almost the whole spectral region (550–4000 cm\(^{-1}\)) and two narrower (715–2935 and 1020–1260 cm\(^{-1}\)) recently used on accelerated oxidation studies of olive oil at 60 and 180 °C\(^{3,4}\) showed that using the narrowest region:

- Classification using a small number of components (only three PCs) was achieved
- A better separation among oils stored at 0, 6, and 12 months was obtained on the plane PC1-3
- Successful classification of samples by 94% was achieved even after cross-validation

The frequencies contributing the most in PC3 according to scatter plot were 1229, 1175 (negative loadings), 1065, and 1053 cm\(^{-1}\) (positive loadings) which should be related to –C–O group
Conclusions

- FT-MIR/ATR is a sensitive technique providing useful information for the early stages of EVOO oxidative status even when physicochemical indices of oil remain within the official limits
- Only the samples stored for 12 months were easily discriminated from the rest
- The most useful approach in checking freshness of an EVOO was the statistical treatment of the 2\textsuperscript{d} derivative of the spectral region 1020-1260 cm\textsuperscript{-1}.

Present findings add to the usefulness of IR spectroscopy as a rigorous and low cost technique for internal quality control in the olive oil industry but systematic inter-laboratory studies are required before it can be considered as a robust tool in VOO analysis.

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<td>N.N. thanks Ms A. Androulaki for tutorial in SPSS software use.</td>
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<td>P.X. acknowledges the Union of Agricultural Cooperatives of Sitia for financial support in terms of oil sampling, storage, and physicochemical analyses that were carried out in its installations.</td>
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Experimental part

Oil samples and storage conditions

Extra virgin olive oil (EVOO) samples (500 mL) from Koroneiki cv olives and representative of the production in the harvest year 2009-2010 were collected directly from the three phase decanter of 11 olive oil mills in the regional union of Lassithi (Crete, Greece). Portions were transferred in transparent glass bottles (10% headspace) and sealed hermetically. Those at t=0 were immediately frozen and kept at -18°C till analysis. The rest were stored in the dark at RT (23 ± 3°C) for t=6 and 12 months, respectively, and then kept frozen till analysis.

Chemical and FT-IR analyses

Acidity, peroxide values and K\textsubscript{232/270} indices were measured according to EU Regulation 2568/91 and amendments.

FT-IR spectra were acquired (64 scans/sample or background) in the range of 4000-400 cm\textsuperscript{-1} at a resolution of 4 cm\textsuperscript{-1} with the aid of an IRAffinity-1 spectrometer (Shimadzu Corporation Kyoto Japan) using 0.8 ml of sample.

Data processing

The intensities of selected wavenumbers were collected from the untreated spectra recorded for the samples. For chemometrics, all spectra were baseline corrected with the aid of the IR-solution software.
Challenges of U.S. Enforcement Increase with Conflicting Standards & Methods

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As the world’s largest volume importer of olive oil and third-largest consuming country, U.S. regulators and importers historically relied on expertise from the IOC and producing supplier countries. Today, some rapidly growing domestic producers are exploiting the lack of public knowledge and limited in-country technical expertise and pursuing a mass media campaign aimed at discrediting all imported olive oil along with existing global standards.

Enforcement in the U.S. is already a challenge because there is not a national mandatory standard for grade levels of olive oils and olive-pomace oils. If the FDA established a standard of identity it could be enforced anywhere along the supply chain by either government or private action. The USDA published grade standards in 2010, but compliance is voluntary. Mandatory compliance might be partially achieved through a USDA marketing order, although marketing orders are limited to testing only at time of local production or import. Enforcing marketing orders delays imports and is costly to both domestic and imported suppliers, and will not offer complete insurance against adulteration as ultimately there is no oversight to what is blended or sold after lot testing occurs. There are also four U.S. states that have established olive oil standards (CT, CA, NY and OR) but enforcement is limited within state borders.

When the industry was unified in support of the IOC standard, progress toward a path to enforcement was happening – all of the state standards and the new USDA standard were implemented from 2008 – 2010. Progress began falling apart when the Australian AOA and U.S. UC Davis Olive Center began promoting research based on new methods claimed to be superior to the IOC methods. As the new methods were not being accepted into global standards, promotion campaigns based on these tests have been targeted to the consumer market instead and have become the basis for urging consumers to purchase only domestic olive oils. Of course, consumers (and even many industry personnel) don’t understand the meaning of the various authenticity and quality measures. They also don’t have access to confirm authenticity directly. The result is consumers that fear purchasing olive oil cut with olive-pomace oil or seed oils are given a false sense of security by certifications like the COOC Seal, which in reality doesn’t include authenticity analysis or even use the newly proposed testing methods.

The disagreement on which methods or standard is appropriate has created major barriers in the advancement of enforcement opportunities in the U.S. Any effective standard will need to ensure both quality AND authenticity. In an attempt to reduce testing time and costs, divergent standards are now being proposed at various levels of state and federal government. Ultimately, divergent programs create trade issues and further confuse the marketplace. It is critical for the global industry to come together and agree on a common standard so it can be used as the basis for enforcement in countries like the U.S.

References