Characterization of olive oils in accordance with EEC Regulation No 2568/91 and subsequent amendments. Fully automated sample preparation and analysis.

PART1:
Methyl/Ethyl Esters and Waxes

Current EU legislation provides for objective criteria aimed at classifying the various types of olive oils (virgin, lampante, refined, etc.). These criteria are explained by the EEC regulation No. 2568/91, with reference to the latest revision of 20/10/2019.

Assigning each oil to the correct class it belongs to is of fundamental importance in order to:

- guarantee the marketing of olive oils that meet the characteristics declared on the label
- avoid potential adulteration, even of a malicious nature
- ultimately protect the health and interests of the final consumer.

Annexes II ÷ XX of the regulation define the analytical methods suitable for the parameters of interest; many of these methods involve laborious sample preparation that requires the use of large amount of solvents and consumables, as well as requiring the use of skilled operators for a long time.

SRA Instruments, in collaboration with Centro Analisi Biochimiche, has conducted an in-depth study of the methods in use, coming today to offer on the market a series of analytical solutions capable of fully automating the sample-prep steps, with consequent savings in terms of time, solvent and materials.

The use of robotic stations also allows you to greatly limit the possibility of incurring random errors, as well as keeping the process under control by inserting a large number of QCs within a batch; in this way not only high productivity is guaranteed, but also an extremely reliable final data in terms of precision and accuracy.

The **robotic sample-prep station** object of this application note is able to fully automate the sample preparation process and the subsequent analysis aimed at **determining the content of alkylesters and waxes** in olive oils.

Fig. 1
Principle of operation:
1- HPLC sample injection
2- Fraction withdrawal
3- Storing into vial
4- GC fractions injection

Method automation

The guiding principle of the solution consists in the elimination of any off-line preparative techniques (LC, TLC, LLE), replaced by a separation of the fractions of interest conducted via HPLC, as schematized in fig. 1. Specifically, the careful optimization of the chromatographic parameters allows to make the elution times of the various classes (methyl/ethyl esters and waxes, in this case) extremely repeatable. The withdrawal of this fraction, and the subsequent injection in LVI-COC-FID, immediately provides the analytical data, limiting the operator's intervention to simple starting of the analysis sequence.

Automation effectiveness

To highlight the increase in productivity, with a simultaneous drastic reduction in solvent and necessary consumables, tab. I shows the comparison between the traditional method (Annex XX to the EEC regulation No. 2568/91) and the one implemented on the proposed platform:

Alkyl esters & Wax analysis	CEE N. 2568/91 Method	HPLC/GC automation
manual sample prep required steps	LC column preparation Sample loading and eluition Evaporation to dryness Solvent reconstitution	Sample dilution
solvent amount required for a single sample	~ 300 ml (and 15 g of pre-conditioned silica)	~ 50 ml
solvent amount required for a batch of 10 samples	~ 3000 ml (and 150 g of pre-conditioned silica)	~ 500 ml
time required to process a single sample	~ 2 h	< 1 h
time required to process a batch of 10 samples	~ 12 h, of which ~ 4 needed for sample prep	~ 8 h

A further advantage of the system is the chance to selectively collect one of the two fractions, in order to:

- standardize the method to the provisions of Annex IV to EEC Regulation No. 2568/91, with similar advantages in terms of time and consumption of solvents and silica (collection of the "wax" fraction only)
- eliminate the problem related to the presence of high concentrated matrix interferences, which could prevent the correct dosage of ethyl esters (collection of only the "alkylesters" fraction).

In the case of the analysis of alkylesters only, a further reduction of the analysis times is obtained up to less than 30 minutes for the single sample (approximately 6 hours for a batch of 10).

Analytical performance

Once the undoubted advantages related to the automation of the preparation process have been highlighted, it is equally important to verify that the final data are characterized by a level of analytical reliability equal to or greater than that guaranteed by the traditional method.

In this regard, a series of experimental tests were carried out using a reference virgin oil as control sample, whose content in alkylesters and waxes is certified by the interlaboratory circuit. Different aliquots of the same sample, interspersed with process blanks, were processed by the platform automatically and totally unattended. The standard chromatogram (in blue), compared with that reported in the EEC regulation (in black), is shown fig. 2.

Alkylesters RT interval

1 - Methyl C16; 2 – Ethyl C16; 3 – Internal Standard methyl heptadecanoate; 4 - Methyl C18
5 - Ethyl C18; 6 - Squalene; 7 - Internal standard lauryl arachidate (waxes)

Waxes RT interval

I.S. - internal standard (lauryl arachidate); 1 – Diterpen esters; 2+2' - Esters C40;
3+3' - Esters C42; 4+4' - Esters C44; 5 - Esters C46; 6 – Sterol esters and triterpen alcohols
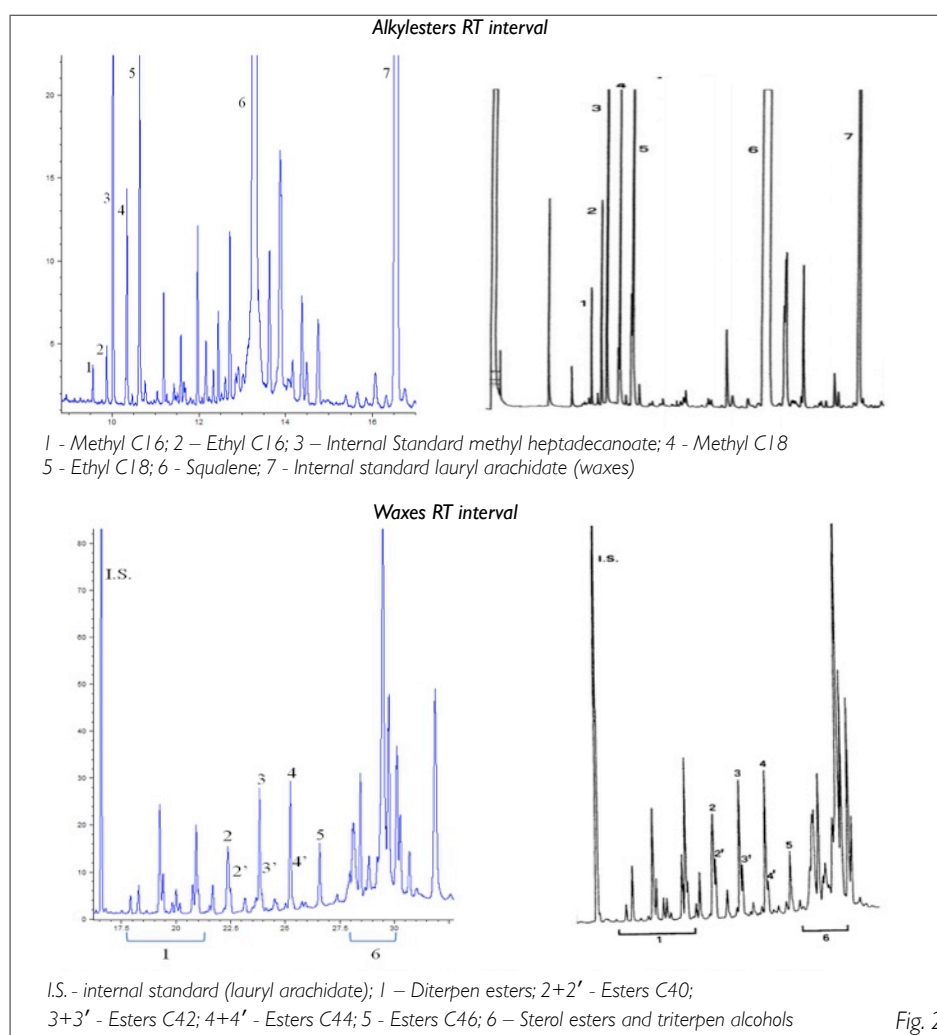
Fig. 2

The result of the validation batch on a series of 10 repetitions, shown in fig. 3, highlights the absolute reliability of the data in terms of accuracy and precision:

Waxes validation

#	mg/kg (As sum)
1	225.80
2	216.94
3	215.20
4	215.54
5	227.31
6	215.14
7	224.17
8	224.67
9	215.78
10	224.05

avg	dev. St.
220.46	**5.10**

Reference value
221.027

bias%	CV_r%
0.26%	2.31%

Alkylesters validation

#	mg/kg FAEE (As sum)
1	36.56
2	35.65
3	36.04
4	35.50
5	35.00
6	35.18
7	35.07
8	35.05
9	35.16
10	35.06

avg	dev. St.
35.43	**0.52**

Reference value
FAEE = 36.253

bias%	CV_r%
2.28%	1.47%

A further strength of the system is its long-term reliability.
In fig. 3. the overlay of two control analyses, conducted on the reference used for the validation of the system and obtained two months apart, is shown. Between the two analyses, more than 1000 chromatographic runs were conducted (real samples, QCs, blank).
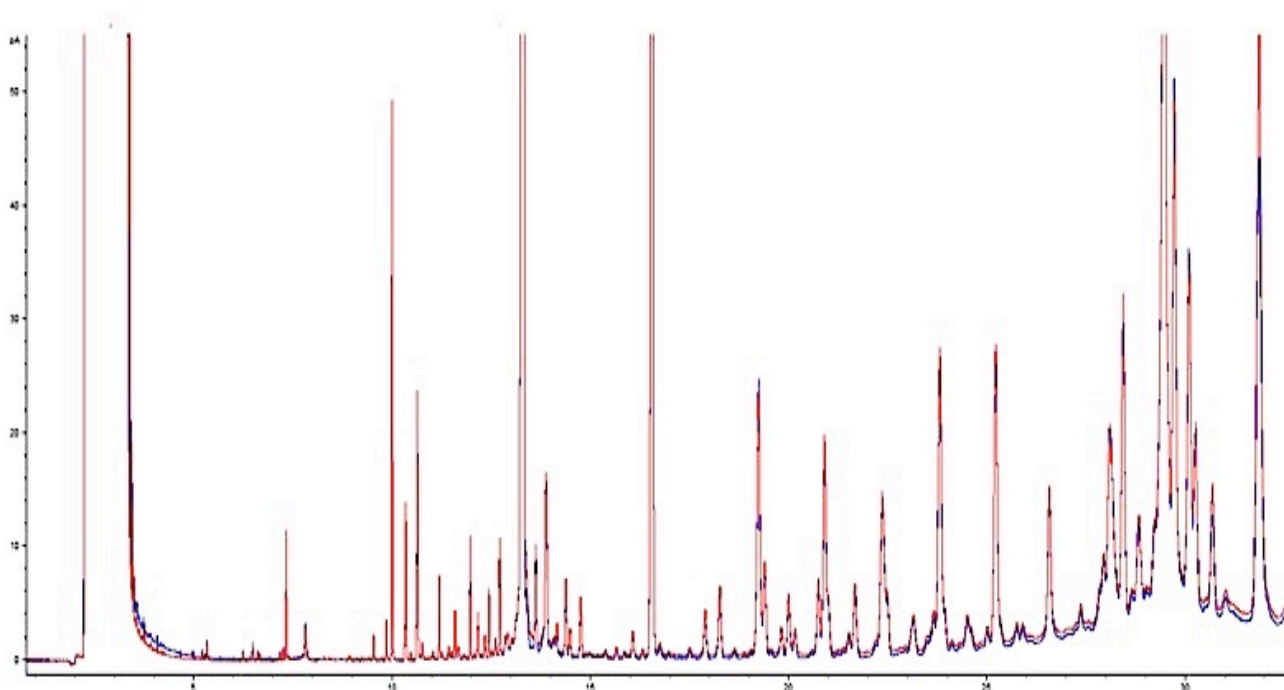

Fig. 3

Conclusions

The proposed solution allows to fully automate the sample prep procedure, with a consequent reduction in costs per sample in terms of lower consumption of solvent and accessory materials. Analysis times are significantly reduced, bringing operator intervention to almost zero. In addition, the very fact of operating automatically drastically lowers the incidence of random errors, ensuring precision, accuracy and robustness of the measurements over the long term.

The proposed sample-prep station is part of a wider range of solutions, developed by SRA Instruments in collaboration with Gerstel GmbH and Centro Ricerche Biochimiche Sas, aimed at automating specific applications such as:

- MOSH/MOAH analysis according to DIN EN 16995:2017-08, including Epoxidation and AlOX purification https://www.srainstruments.com/s/mosh-moah-gerstel-sample-prep-solution/
- 2&3 MCPD and GE in edible oils according to AOCS Cd 29(a&b&c)-13. https://www.srainstruments.com/s/determinazione-di-3-monocloro-propandiolo-3-mcpd-e-di-glicidil-esteri-degli-acidi-grassi-in-matrici-alimentari/

Are also being validated:

- The automations for the analysis of alcoholic and sterol fractions and stigmastadienes (Annexes XIX and XVII to EEC No. 2568/91 and subsequent amendments)
- Automated sample-prep stations for other analyses of olive oils in accordance with EEC regulation No. 2568/91 and subsequent amendments.
- Olive oil saponification process
- PAH analysis according to Regulation (EC) N°1881/2006 and subsequent amendments.

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