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

PART 2: Sterols, aliphatic alcohols and triterpene dialcohols

Current EU legislation provides for objective criteria aimed at classifying the various types of olive oils (virgin, clear, 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 for:

- guarantee the commercialization 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 relating to the quantification of the parameters of interest; many of these involve laborious sample preparation, large quantities of solvents and consumables, as well as the use of qualified operators for a long time.

SRA Instruments, in collaboration with the Centro Analisi Biochimiche, conducted an in-depth study of the methods in use, in order to offer a series of analytical solutions capable of fully automating the sample-prep phases, 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 high number of QCs within a batch; in this way, high productivity and extremely reliable final data in terms of precision and accuracy are guaranteed.

The robotic 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 sterols, triterpene dialcohols and aliphatic alcohols.

Fig. 1
Principle of operation:
1- Injection of the sample in HPLC
2- Collection of the fraction of interest
3- Storage in vials
4- Dry evaporation and derivatization
5- Injection into GC

Method automation

The guiding principle of the solution consists in the elimination of off-line preparative techniques (LC, TLC, LLE), replacing them with an automated separation of the fractions of interest via HPLC, as shown in Figure 1.
Specifically, the careful optimization of the chromatographic parameters makes it possible to make the elution times of the various classes (aliphatic alcohols and sterols / triterpene dialcohols, in this case) extremely repeatable.
The versatility of the MPS RoboticPRO platforms also allows you to extend the automation of the workflow by including the necessary steps of dry evaporation and recovery with derivatizer. The subsequent injection in SSL-FID mode immediately provides the analytical data, limiting the operator's intervention to the simple start of the analysis sequence.

Effectiveness of automation

The automation of the sample-prep starts from the unsaponifiable fraction prepared in accordance with the official method (see Annex XIX Part 1).

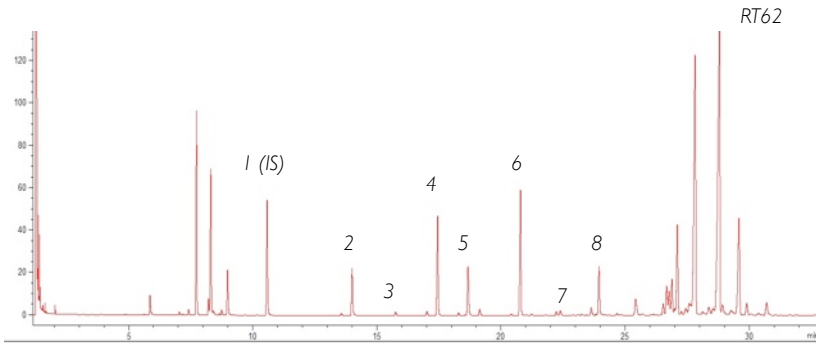
This fraction is placed on the sampler and processed automatically, completely avoiding the separation procedure via TLC. To highlight the increase in productivity, with a simultaneous drastic reduction of solvent and necessary consumables, table 1 shows the comparison between the traditional method (Annex XIX to the EEC regulation No. 2568/91) and the one implemented on the platform proposal.

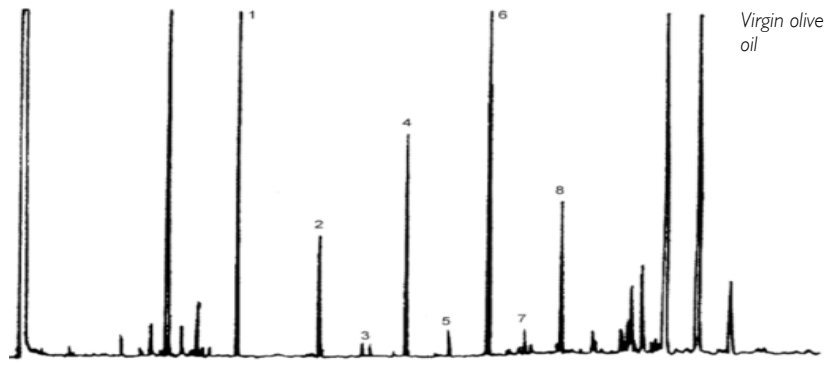
Complete analysis of sterols and alcohols	Traditional method	HPLC / GC automation
preparation by the operator	TLC separation of the unsaponifiable fraction Evaporation to dryness Recovery with derivatizer	Positioning of the unsaponifiable fraction on the autosampler
volume of solvent per sample	> 1000 ml, in relation to the size of the developing chamber, TLC plate	~ 15 ml
volume of solvent for a batch of 10 samples	~ 3000 ml, 10 TLC plates	~ 150 ml
time required to process a single sample	~ 3 h	< 1,5 h
time required to process a batch of 10 samples	~ 10 h, of which ~ 4 needed for batch preparation	7 h, of which ~ 30 minutes required for batch preparation

It is important to specify that also Annex XIX (part 2-6) actually reports an alternative procedure, based on separation via HPLC, to obtain the two fractions of interest. This method, however, in addition to providing an HPLC run of about half an hour, requires manual intervention for the collection of the fractions and their subsequent evaporation to dryness and recovery with derivatizer. The proposed platform, with an HPLC separation conducted in less than 5 minutes, automates the entire process up to the GC injection. The automatic management of analytical times also allows an overlap of GC and HPLC runs; in fact, the time required for the analysis is reduced to just the GC run.

Analytical performance

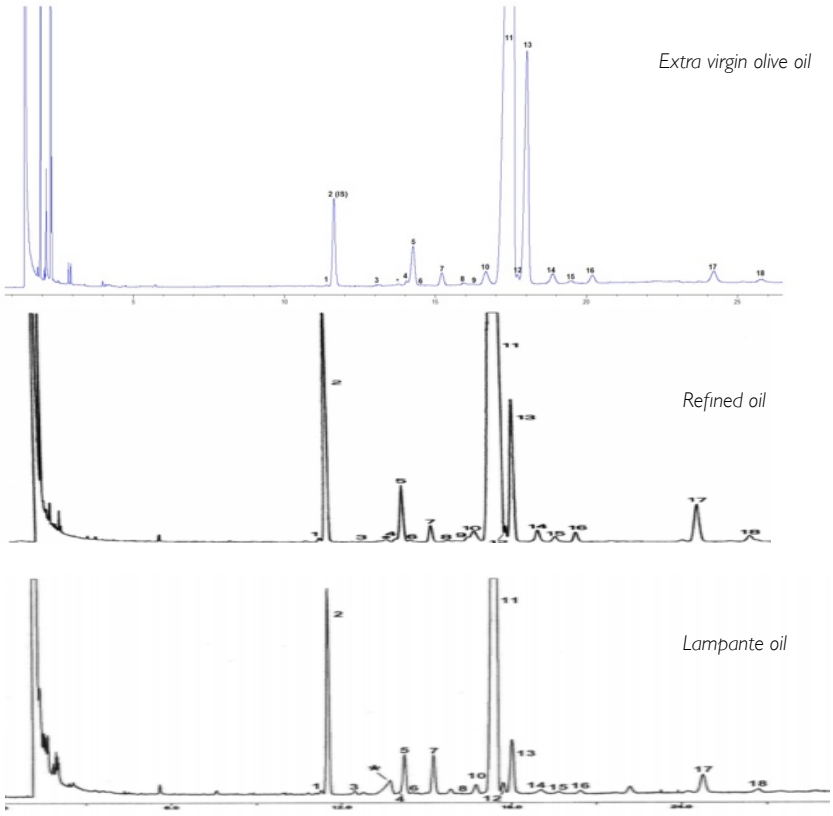
Once the undoubted advantages related to the automation of the preparation process have been highlighted, it is important to verify that the final data is characterized by a level of analytical reliability equal to or greater than the one guaranteed by the traditional method. About this, a series of experimental tests were carried out using a reference virgin oil as a control sample, and whose content in aliphatic alcohols, sterols and triterpene dialcohols is certified by the interlaboratory circuit of the Rome Chamber of Commerce - Company Special Samer Ring Test. No. 62 (RT62). Different aliquots of the same sample, interspersed with process blanks, were processed by the analytical platform automatically and without any operator supervision.
The standard chromatograms (in red for aliphatic alcohols, in blue for sterols and triterpene dialcohols), compared with those reported in the EEC regulation (in black), are shown in figures 2 and 3.





Aliphatic alcohols
1. *Eicosanol (IS)*
2. *Decosanol*
3. *Tricosanol*
4. *Tetracosanol*
5. *Pentacosanol*
6. *Hexacosanol*
7. *Heptacosanol*
8. *Octacosanol*

Fig. 2



Sterols and triperpenic dialcohols
1. *Cholesterol*
2. *2. Cholestanol (IS)*
3. *3. Brassicasterol*
* *Ergosterol*
4. *24-methylene cholesterol*
5. *Campesterol*
6. *Campestanol*
7. *Stigmasterol*
8. *Δ-7-campesterol*
9. *Δ-5,23-stigmastadienol*
10. *Clerosterol*
11. *ß-sitosterol*
12. *Sitostanol*
13. *Δ-5-avenasterol*
14. *Δ-5,24-stigmastadienol 15.*
15. *Δ-7-stigmastenol*
16. *Δ-7-avenasterol*
17. *Erythrodiol*
18. *Uvao*

Fig. 3

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

Validation of aliphatic alcohols

#	mg/kg (sum)
1	258.5
2	265.5
3	267.0
4	263.2
5	262.4
6	259.0
7	261.9
8	256.1
9	269.3
10	262.0

Average	St. dev.
262.5	4.0
Reference value (as per RT62) alcohols = 258.7	
Bias%	CV_r%
1.46%	1.53%

Validation of sterols and triterpene dialcols

#	mg/kg total sterols
1	1464
2	1383
3	1419
4	1398
5	1408
6	1428
7	1409
8	1439
9	1453
10	1420

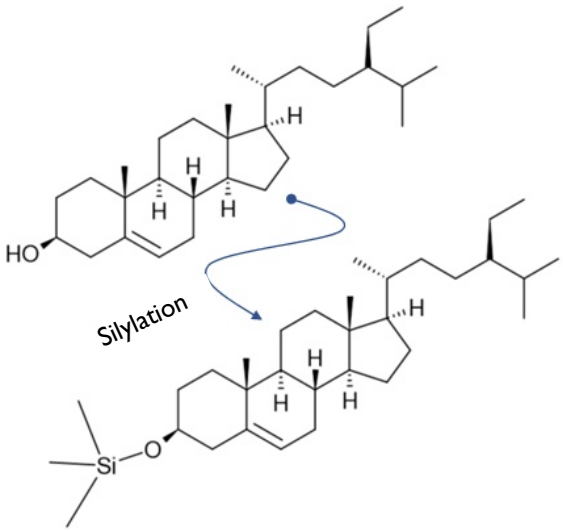
Average	St. dev.
1422	25
Reference value (as per RT62) Sterols = 1447	
Bias%	CV_r%
1.73%	1.74%

The quantification of the single analytes, in particular for the sterol fraction, also yields excellent results for all analytes, including the sterols present at the lowest concentration (brassicasterol, Δ-7-stigmastenol):

#	Cholesterol	Brassicasterol	Campesterol	Stigmasterol	Δ-7-stigmastenol	Erythrodiol Uvaol	ß-sitosterolo (total)
1	0.125	0.047	4.22	1.69	0.610	5.76	92.7
2	0.139	0.045	4.21	1.76	0.537	5.40	92.7
3	0.125	0.047	4.18	1.76	0.596	5.75	92.7
4	0.138	0.044	4.15	1.77	0.566	5.42	92.8
5	0.139	0.045	4.14	1.75	0.570	5.44	92.7
6	0.128	0.056	4.20	1.75	0.585	5.86	92.7
7	0.121	0.053	4.17	1.76	0.579	5.52	92.7
8	0.131	0.057	4.24	1.86	0.553	5.96	92.6
9	0.118	0.044	3.91	1.76	0.597	5.42	93.0
10	0.125	0.047	4.20	1.71	0.573	5.75	92.7
RT62	0.136	0.045	3.95	1.81	0.552	5.368	92.5
Avg	0.129	0.048	4.16	1.76	0.577	5.628	92.7
CVr%	5.98%	10.27%	2.25%	2.47%	3.76%	3.72%	0.11%
BIAS%	5.10%	7.67%	5.31%	3.02%	4.45%	4.83%	0.28%

Conclusions

The proposed solution allows to fully automate the sample prep procedure, with consequent reduction of costs per sample in terms of lower consumption of solvent and accessory materials.
Analysis times are drastically reduced, almost completely eliminating operator intervention. In addition, the fact of working automatically limits the incidence of random errors enormously, ensuring long-term precision, accuracy and robustness of the measurements.



The proposed preparatory station is part of a wider range of solutions, developed by SRA Instruments in collaboration with Gerstel GmbH and Biochemical Analysis Center Sas, aimed at automating specific applications such as:
INANOIL series analyzers:

- characterization of olive oils in accordance with EEC regulation No. 2568/91: analysis with exhaustive automation of sample preparation
  - Part 1: Methyl / ethyl esters and waxes
  - Part 3: Stigmastadienes
- MOSH / MOAH analysis in accordance with the DIN EN 16995: 2017-08 method, including AlOX purification and epoxidation. https://www.srainstruments.com/s/mosh-moah-gerstel-sample-prep-solution/
- determination of 2 & 3 MCPD and GE according to the official AOCS Cd 29 (a & b & c) -13 methods. https://www.srainstruments.com/s/determination-of-3-mcpd-and-glycidol-in-edible-oils-by-gc-ms/

Similar systems capable of automating:

- the analysis of additional parameters included in the EEC regulation No. 2568/91
- online saponification of olive oil
- the determination of Polycyclic Aromatic Hydrocarbons (IPA) pursuant to EC regulation 1881/2006 and subsequent changes.

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