Characterization of olive oils in accordance with EEC regulation No. 2104-2105/2022. Fully automated analysis of sample preparation.

Current EU legislation provides for objective criteria aimed at classifying the various types of olive oils (virgin, clear, refined, etc.).
These criteria are listed in the above mentioned EEC regulation No. 2104-2105/2022.
Assigning each oil to the correct class it belongs to is of fundamental importance for:

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

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, the use of qualified operators for a long time.

SRA Instruments, in collaboration with the Centro Analisi Biochimiche Sas, has conducted an in-depth study of the methods in use, coming to offer the market **a series of analytical solutions based on the Gerstel GmbH platform, able to fully automate the sample-prep phases, resulting in savings in terms of time, solvent and materials.**
The robotic stations developed are able to fully automate the sample preparation process and subsequent analyzes aimed at determining, respectively, the content of:

1. **alkyl esters and waxes**
2. **sterols, triterpene dialcohols and aliphatic alcohols**
3. **stigmastadienes.**

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.

Courtesy of Dr Carmine Ventre, Centro Analisi Biochimiche Sas - Rizziconi - Italy)

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. Specifically, the accurate optimization of the chromatographic parameters makes it possible to make the elution times of the various classes.

Alkylesters and waxes: full automation of the sample preparation and analysis process

Off-line preparative techniques (LC, LLE), are replaced with an automated separation of the fractions of interest via HPLC, as shown in Figure 1.
The careful optimization of the chromatographic parameters makes it possible to make the elution times of methyl / ethyl esters and waxes extremely repeatable.
The withdrawal of this fraction and the subsequent injection in LVI-COC-FID mode immediately provides the analytical data, limiting the operator's intervention to the simple start of the analysis sequence.

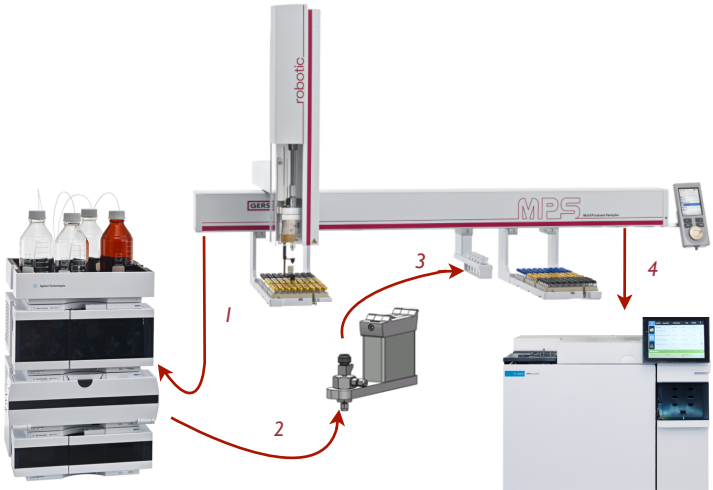

Fig. 1
Principle of operation:
1- Injection of the sample in HPLC
2- Collection of the fraction of interest
3- Storage in vials
4- Injection into GC

Effectiveness of automation

To highlight the increase in productivity, with the simultaneous drastic reduction of solvent and necessary consumables, the table alongside shows the comparison between the traditional method and the one implemented on the INANOIL platform.
Another advantage of the system is the possibility of selectively collecting one of the two fractions in order to:

- standardize the method by current legislation, 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 concentrations of matrix interferents, which could prevent the correct dosage of alkyl esters (collection of the "alkylester" fraction only).

In the case of the analysis of alkylesters alone, a further reduction in analysis times is obtained (less than 30 min. for the single sample).

Complete analysis of alkylesters & waxes	Traditional Method	HPLC/GC Automation
preparation by the operator	Column preparation for LC Sample loading and elution Evaporation to dryness Recovery with solvent	Dilution of the initial sample
volume of solvent per sample	~ 300ml (plus 15g pre-conditioned silica)	~ 20 ml
volume of solvent for a batch of 10 samples	~ 3000ml (plus 150g of pre-conditioned silica)	~ 200 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 batch preparation	~ 8 h of which ~ 30 minutes required for batch preparation

Analytical performance

A series of experimental tests were carried out using a virgin reference oil as control sample, whose content in alkylesters and waxes is certified by the interlaboratory circuit of the Bari Chamber of Commerce - Ring Test. N. 62 (RT62) .
The result of the validation batch on a series of 10 repetitions, highlights the absolute reliability of the data in terms of accuracy and precision.

Waxes validation

Average 3.70	St.: Dev.: 5.1
Reference value (as per RT62) waxes= 221.0 mg/Kg	
BIAS = 0.25 %	CV_r%= 2.32%

Alkylesters validation

Average: 3.70	St.: Dev.: 0.5
Reference value (as per RT62) FAEE = 36.2 mg/Kg	
BIAS = 2.18%	CV_r% = 1.51%

Sterols and alcohols: full automation of the sample preparation and analysis process (including saponification)

Off-line preparative techniques (LC, TLC, LLE), are replaced with an automated separation of the fractions of interest via HPLC, as shown in Figure 2.

Specifically, the accurate 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).

The versatility of the MPS RoboticPRO platforms also allows you to extend the automation of the workflow by including the necessary steps of evaporation to dryness 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.

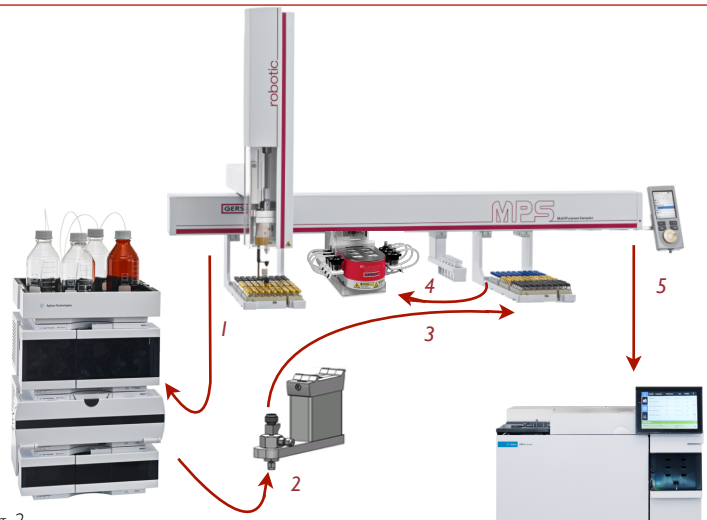

Fig. 2

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

Saponification included

Effectiveness of automation

The automation of sample preparation starts with saponification included in the workflow. The unsaponifiable thus obtained is immediately processed automatically, completely avoiding the TLC separation procedure. To highlight the increase in productivity, with a simultaneous drastic reduction in solvent and necessary consumables, the table alongside shows the comparison between the traditional method and the one implemented on the INANOIL platform (the additional, important, savings related to the automation of the saponification process are not considered).

The SRA solution, 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.

Complete analysis of sterols and alcohols	Traditional Method	HPLC/GC Automation
preparation by the operator	Saponification TLC separation of the unsaponifiable fraction Evaporation to dryness	Dilution of the initial sample
volume of solvent per sample	Recovery with derivatizer	~ 15 ml
volume of solvent for a batch of 10 samples	> 1000 ml, in relation to the size of the developing chamber, TLC plate	~ 150 ml
time required to process a	~ 3000 ml, 10 TLC plates	<1.5 h
single sample time required to process a batch of 10 samples	~ 3 h	7 h, of which ~ 30 minutes required for batch preparation

Analytical performance

A series of experimental tests were carried out using a virgin reference oil as a control sample, whose content in aliphatic alcohols, sterols and triterpene dialcohols is certified by the interlaboratory circuit of the Rome Chamber of Commerce - Ring Test 62 (RT62). The result of the validation batch on a series of 10 repetitions, highlights the absolute reliability of the data in terms of accuracy and precision.

Alifatics alcohols validation

Average: 3.70	St.: Dev.: 4.0
Reference value (as per RT62) alcohols = 258.7 mg/Kg	
BIAS = 1.46%	CV_r% = 1.53%

Sterols and e Triterpenic Dialcohols validation

Average: 3.70	St.: Dev.: 25
Reference value (as per RT62) Sterols = 1447 mg/Kg	
BIAS = 1.73%	CV_r% = 1.74%

Stigmastadienes: full automation of the sample preparation and analysis process. Direct injection mode

Off-line preparative techniques (LC, LLE) are replaced with an automated separation of the fractions of interest via HPLC, as shown in Figure 3. **This analytical approach also allows to eliminate the saponification step.**

The optimization of the chromatographic parameters makes it possible to reliably separate stigmastadienes from the interference constituted by squalene. The fraction of interest is directly routed int the GC injection port. Once more: placing the sample on the tray is the only manual operation required.

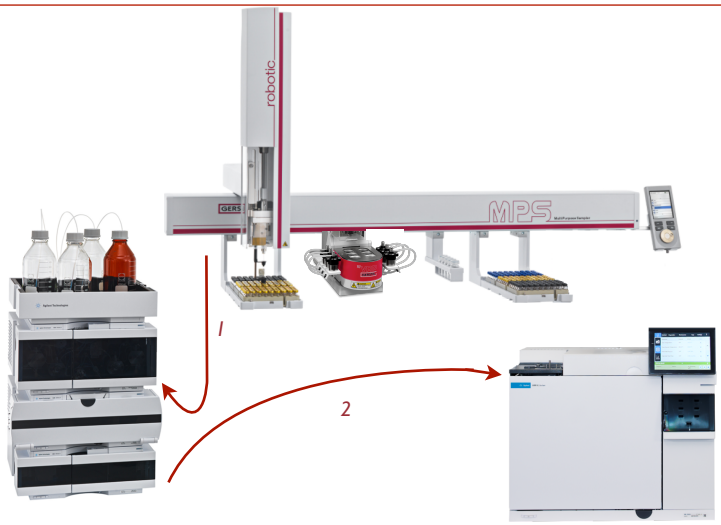

Fig. 3
Principle of operation:
1- Injection of the sample in HPLC
2- Direct transfer of the fraction containing stigmastadienes

Effectiveness of automation

To highlight the increase in productivity, with the simultaneous drastic reduction of solvent and necessary consumables, table 1 shows the comparison between the traditional method and the one implemented on the platform INANOIL.

The SRA solution, 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.

Complete stigmastadiene analysis	Traditional Method	HPLC/GC Automation
preparation by the operator	Saponification Column preparation for LC Sample loading and elution Evaporation to dryness Recovery with solvent	Dilution of the initial sample
volume of solvent per sample	> 400 ml (plus 15 g of preconditioned silica)	<20 ml
volume of solvent for a batch of 10 samples	> 4000 ml (plus 150 g of pre-conditioned silica)	~ 200 ml
time required to process a single sample	~ 3 h	~ 1 h
time required to process a batch of 10 samples	~ 15 h, of which ~ 10 needed for batch preparation	<10 h of which ~ 30 minutes required for batch preparation

Analytical performance

A series of experimental tests were carried out using a virgin reference oil as control sample, whose concentration of stigmastadienes is certified by the interlaboratory circuit of the Rome Chamber of Commerce - Ring Test No. 62 (RT62). The result of the validation batch on a series of 10 repetitions, highlights the absolute reliability of the data in terms of accuracy and precision.

Stigma RT62 validation

Average: 3.70	St.: Dev.: 0.04
Reference value (as per RT62) Stigma = 3.70 mg/Kg	
BIAS = 1.66%	CV_r% = 1.16%

Stigma cut-off validation

Average:0.061	St.: Dev.: 0.004
Reference value (as per RT62 dil 1/65) Stigma = 0.057 mg/Kg	
BIAS = 6.03%	CV_r% = 6.48%

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