Olive oil between scientific research and need for regulation

Lanfranco Conte [1] ✉

[1] SISSG - Società Italiana
per lo Studio delle Sostanze Grasse,
Via Giuseppe Colombo, 79
20133 Milano - Italia

Science, by its nature, is often driven by curiosity with the aim of describing and explaining the surrounding world; this is also true for foods, in which safety issues also play a significant role. Olive oil has also been an important area for food chemistry in developing experimental approaches to describe its different characteristics. Results of scientific research were therefore adopted by legislation to guarantee the purity of olive oils; the adoption of a method required the assessment of its reliability and, of course, the time needed to carry out this process depends on the complexity of the experimental procedure. Over a number of decades, analytical methods for edible oil quality and purity assessment were carried out according to the so-called "chemistry of indexes", consisting of a number of different tests that verified whether the behaviour of a sample, under established conditions, was similar to that of a selected oil. Results were reported as "positive" or "negative", according to the evaluation of the analyst; sometimes the result was also reported as "slightly negative" or "slightly positive", and no numerical results were provided. Nevertheless, many of these tests were adopted in some national legislation (we must bear in mind that for many decades no international standard existed).

After the development of instrumental analytical techniques, the scenario dramatically changed and numerical data became available: spectrophotometric and chromatographic methods were developed. Spectrophotometric UV analysis was developed in the early 1960s in Italy to assess the purity and quality (freshness) of olive oils; when it was adopted as an international standard, some problems arose because of different normative sources (EU vs IOC). UV analysis, however, still belongs to a family of measurements that does not identify the single compound responsible for selected results; the development of separation techniques, mainly chromatographic ones, made this possible.

Early quantitative data available dealt with the composition of fatty acids and later of sterols; however, the adoption of related mandatory limits is a procedure that is still not complete: as olive cultivation expands into new areas, some of them must be modified. International standards, of course, cannot accept any modification of well-established limits without careful checks, and this requires long periods that scarcely meet the requirements of producers. Another example of improvement in analytical techniques that required modification of international standards was the generalized adoption of capillary gas chromatography, whose higher separation power compared with packed columns highlighted the presence of previously unknown compounds (mainly sterols); however, the management of this information did not actually improve the "quality" of the information obtained. While current standards establish limits for single compounds, recent developments in analytical approaches consider a "holistic" evaluation of sets of data, usually with the aim of chemometric analysis; this, of course, requires a different approach to the establishment of legal limits, and at present this still seems difficult to achieve.

✉ CORRESPONDING AUTHOR
Lanfranco Conte
Formerly full professor of Food Chemistry
at University of Udine, retire in 2018

lanfrancoconte@gmail.com
Via Alessandro Volta, 30 – 33100 Udine IT

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Since the early 1960s, the quality and, mainly, the identity of oils and fats were assessed according to the so-called "chemistry of indexes" (Table I), consisting of a number of different tests that verified whether the behaviour of a sample, under well-established conditions, was similar to that of a known selected oil; results were reported as "positive" or "negative", according to the evaluation of the analyst; sometimes the result was also reported as "slightly negative" or "slightly positive", and no numerical results were provided.

Nevertheless, many of these tests were adopted in some national legislation (we must bear in mind that for many decades no international standard existed), while some of them provided the basis for developing more effective evaluations thanks to the development of instrumental analytical techniques.

The development of these techniques dramatically changed the scenario, and numerical data became available.

According to international standards (legislation in the case of the EU [1], the Trade Standard in the case of the IOC [2], or Codex Alimentarius [3], Table II), olive oil quality is described by free fatty acids, peroxide value, UV absorption, ethyl esters and sensory assessment, while identity/purity is described according to fatty acid and sterol composition, as well as by the concentration of waxes and the evaluation of triacylglycerols.

This protocol, although generally considered very

Table I - The "Chemistry of indexes"

Index	Nowadays
Refractive index	Purity Unsaturation degree still used
Halphen reaction sterculic acid (Malvaceae oils)	Purity No more used, GC of FAMEs and sterols
Villavecchia-Fabris and Isodoro Pavolini reactions (in Italy, for sesame oil)	Purity No more used
Saponification Index	Purity Amount of fatty acids, no more used
Esters index	Purity Amount of TAGs, no more used
Iodine number	Purity Unsaturation degree still used
Thermosulphuric index	Purity Unsaturation degree no more used
Acidity index	Quality, still used
Peroxide value	Quality, still used
Kreiss test	Rancidity no more used
Fitelson test (tea test in olive oil)	Purity no more used
Bellier test (research of solvent extracted olive oil)	Purity no more used, substituted by measurement of waxes
Vizern test (research of solvent extracted olive oil)	Purity no more used, substituted by measurement of waxes

Table II - Olive oil composition according to normative standards

Compound	Limit	Quality/Purity	Normative source ***
Myristic acid	≤ 0,03 %	Purity	UE, IOC, Codex
Linolenic acid $C_{18:3}$	≤ 1,0 % *	Purity	UE, IOC, Codex
Arachic acid $C_{20:0}$	≤ 0,60 %	Purity	UE, IOC, Codex
Eicosenoic $C_{20:1}$	≤ 0,50 %	Purity	UE, IOC, Codex
Behenic $C_{22:0}$	≤ 0,20 %	Purity	UE, IOC, Codex
Lignoceric $C_{24:0}$	≤ 0,20 %	Purity	UE, IOC, Codex
Cholesterol	≤ 0,5 %	Purity	UE, IOC, Codex
Brassicasterol	≤ 0,1 %	Purity	UE, IOC, Codex
Campesterol	≤ 4,0 % *	Purity	UE, IOC, Codex
β Sitosterol («apparent»)**	≥ 93,0 %	Purity	UE, IOC, Codex
Δ^7 Stigmastenol	≤ 0,5 %*	Purity	UE, IOC, Codex
Waxes (C42+C44+C46) ****	≤ 150 mg/kg	Purity	UE, IOC, Codex
Ethyl esters	≤ 75 mg/kg	Quality	UE, IOC

* Limits are not always mandatory, some tolerance can be admitted for selected oils

** β Sitosterol" apparent" = sum of Colerosterol, $\Delta^{5,23}$ stigmastadienol (if present), β Sitosterol, sitostanol, Δ^5 avenasterol,

(sometimes excessively) complex and time-consuming, does not adequately consider a number of compounds whose presence, precise identification and concentration could improve the effectiveness of the control; however, these have never been considered as candidates for inclusion in standards.

As Shakespeare said:

"There are more things in heaven and earth, Horatio than are dreamt of in your philosophy. (W. Shakespeare, Hamlet, Atto I scena I)".

On the other side, we must bear in mind that the presence of Δ^5 avenasterol was identified by Itoh in 1973, [4], using packed column with OV17 stationary phase instead of less polar ones (e.g., JXR or SE30), (Figure 1).

If we consider only sterols, whose composition obtained by capillary GC has already been included in international standards for a number of years (in Italian legislation since 1967, more or less), some observations could suggest useful improvements.

The presence of $\Delta^{5,23}$ stigmastadienol, e.g., bears witness to the presence of refined oils (Figure 2).

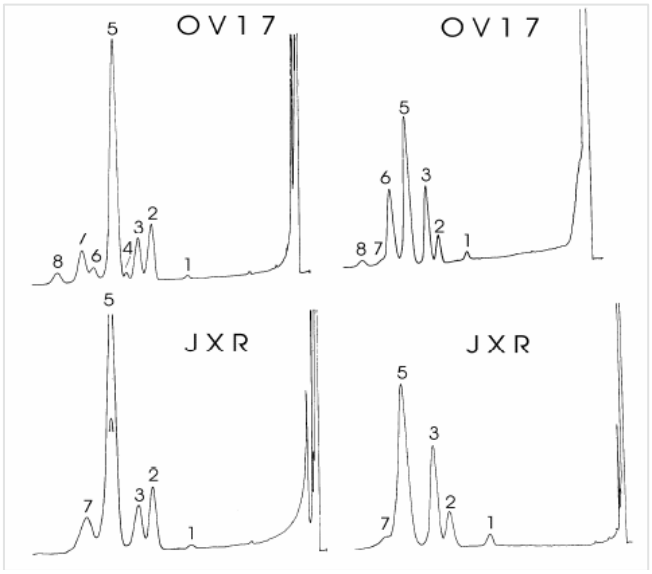


Figure 1 - GLC separation on packed columns with different stationary phases [4]

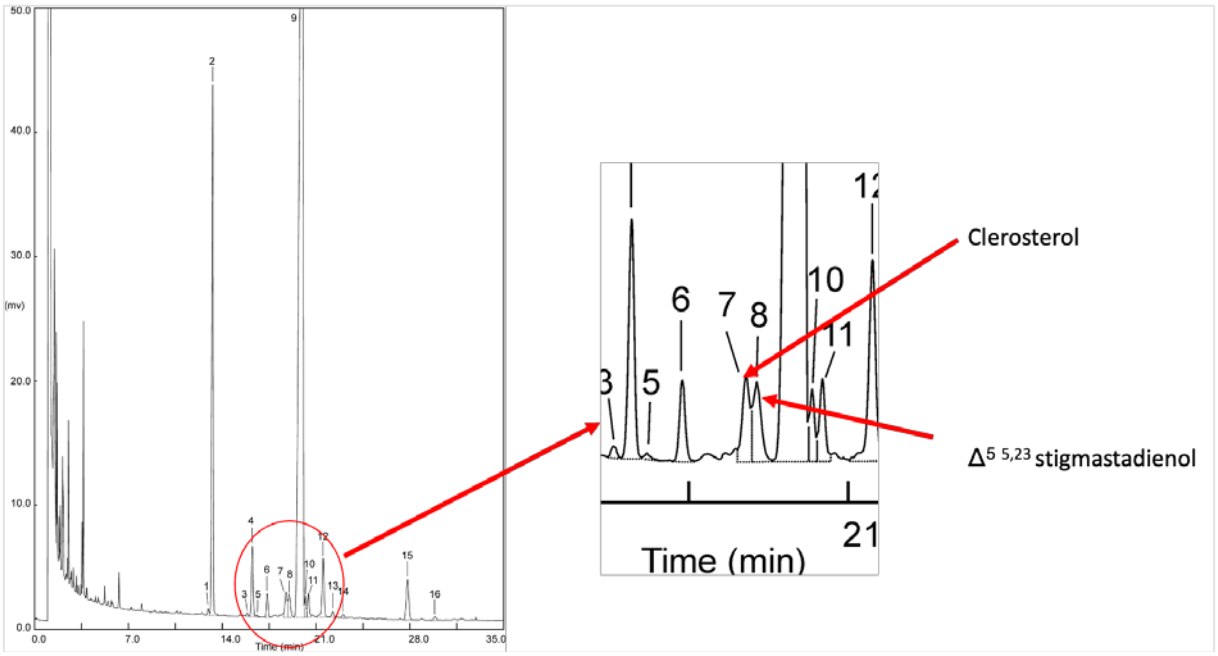


Figure 2 - GLC (capillary) analysis of sterols of a refined olive oil (source www.internationaloliveoil.org COI /T.20/DOC.No26Rev 5 June 2020) [5]

Of course, the establishment of a limit for a selected parameter involves the collection of a huge amount of data in order to comprise any possible value within a probably wide range; in this case, additional factors can affect the amount of the compound considered, e.g., the conditions of the refining process.
Anyway, bearing this in mind, the presence/absence of $\Delta^{5,23}$ stigmastadienol would be a suitable factor for assessing whether any technological process had been applied: its presence should be excluded in any virgin oil, whether edible (extra virgin and virgin) or not edible (lampante).Nowadays, so-called "cold-pressed" seed oils are also gaining market share; in consumers' perception, these oils should be obtained simply by pressing seeds after crushing, without any further process (maybe just filtering them), so for these oils, too, the presence of $\Delta^{5,23}$ stigmastadienol could be adopted in standards as a purity parameter (purity meaning useful for discriminating between crude oils and oils subjected to any technological treatment).
Standards should report a limit, maybe not "zero" but, e.g., ≤ 0.01%: if no limit is reported or if the compound is not listed, it could be considered as having "no limit at all".
At this point, it could be useful to summarize the procedure that must be used to adopt an analytical method (Figures 3 and 4).
The main hurdles to obtaining good results from a ring test devoted to method standardization concern the widespread variability in the composition of natural products; furthermore, the less a product undergoes processing, the more variable its composition can be.
It is rather difficult to have five samples representing the whole range of different concentrations for every analyte; this can lead to the need for further amendments after the method has been adopted with the aim of establishing standard limits.
Nowadays, analytical methods have been standardized and validated for every analyte listed in Table II; however, other classes of compounds have been studied in the past. In the early to mid-1990s, a fraud involving the mixing of high-oleic sunflower oil depleted of sterols (so-called "de-sterolised" oils) was carried out, and several analytical approaches were developed to detect it. It must be mentioned that when these oils were obtained using acid bleaching earths, trans fatty acids were formed; because of this, those committing fraud moved to non-acid bleaching methods.
The main goal of de-sterolising high-oleic sunflower oil was to remove Δ^7 stigmastenol, which was present in very high amounts; among several approaches, the group involving Koni Grob and Carlo Mariani highlighted the presence of $\Delta^8(14)$ stigmastenol [6], which was hidden by Δ^7 stigmastenol when the analytical conditions reported in the official method were applied. However, the proposed approach involved the use of an on-line LC-GC system or a very long (100 m) capillary column; the use of a 50% phenyl stationary phase then allowed the separation of Δ^5 and Δ^{14} sterols from $\Delta^8(14)$ and Δ^7 sterols.
Because of difficulties in separation, the authors proposed a limit for the sum of Δ^7 and $\Delta^{5,8}(14)$ stigmastenol (≤ 75 mg/kg) and a limit of ≤ 2 mg/kg for $\Delta^8(14)$ stigmastenol [7].
The previously mentioned separation problem, however, prevented this interesting approach from undergoing further examination as a possible international standard; in the meantime, the measurement of sterenes was adopted as a tool to assess technological treatment (mainly thermal) applied to crude oils.
Sterols are often considered a well-known topic; however, even among commonly determined sterols, something new can be considered.
In 1997, the maximum permitted relative concentration of brassicasterol in olive pomace oil within the EU and IOC was increased from 0.1% to 0.2% according to data obtained from the analysis of many olive oil samples, even though several doubts remained about the actual identification of compounds with the RT of brassicasterol.
In 1983, Morchio and De Andreis [8] reported the presence of an unknown peak that appeared as a shoulder of the brassicasterol peak; in 1998, Mariani [9] achieved full separation of this peak using a T.A.P. column and identified it by GC-MS as ergosterol. It was present in "lampante" olive oil with a high content of free fatty acids, and its presence was related to microbial (specifically mould) growth on poor-quality olive fruits. In this case, the only difference from the EU/IOC standard methods is the use of a more polar column (T.A.P.).
The use of stationary phases of different polarity also allowed the separation of an unknown peak very close to campesterol, which in some seed oils was about 25–40% of it [10] (Table III); it was identified as $\Delta^{22,23}$-dihydrobrassicasterol, which is an epimer at C24.

Table III - concentration of $\Delta^{22.23}$ dihydrobrassicasterol in different oils

Oil	% $\Delta^{22.23}$ dihydrobrassicasterol
Olive	25 - 30
Sunflower	30 - 35
Peanut	35 - 40
Rapeseed	35 - 40
Soybean	35 - 40
Corn	60 - 65
Castor	50

Considering the very similar values for different oils, these results could certainly not be included in official standards.
Sterols are usually evaluated after alkaline hydrolysis; however, in this way, some information is lost, while

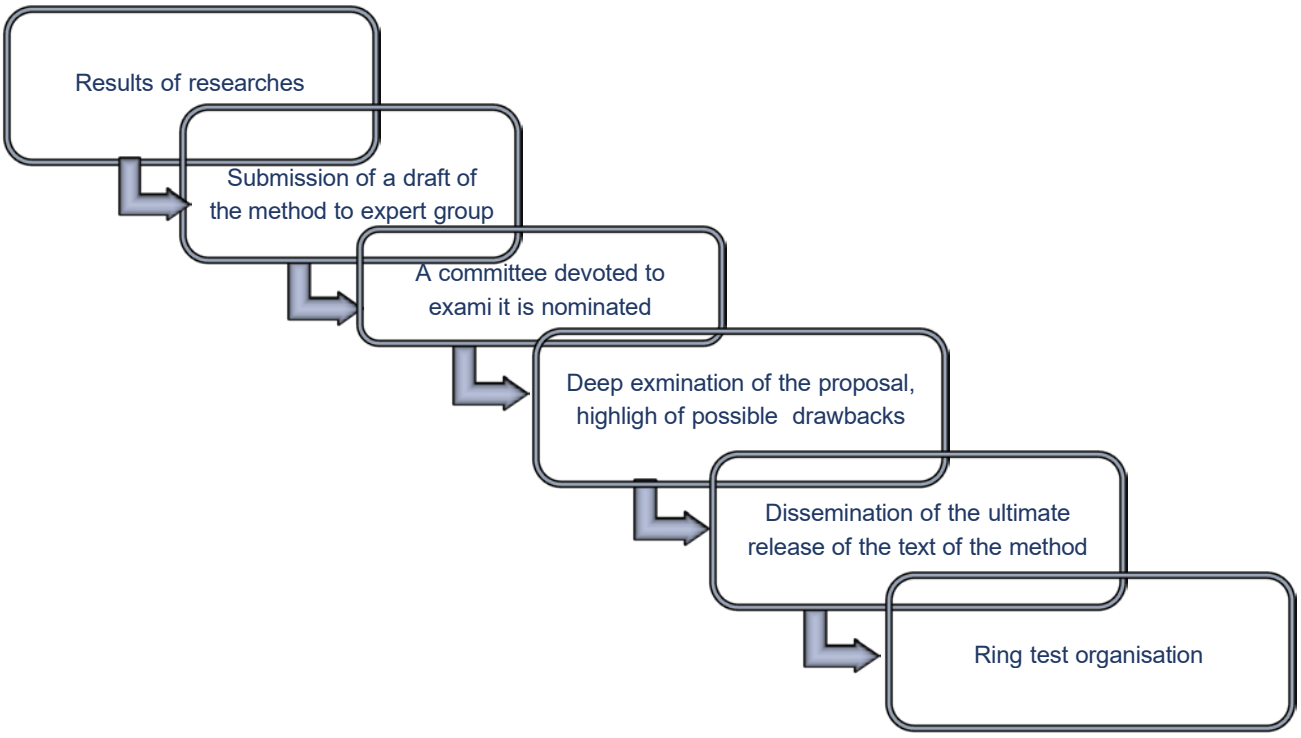


Figure 3 - Scheme of a ring test organization

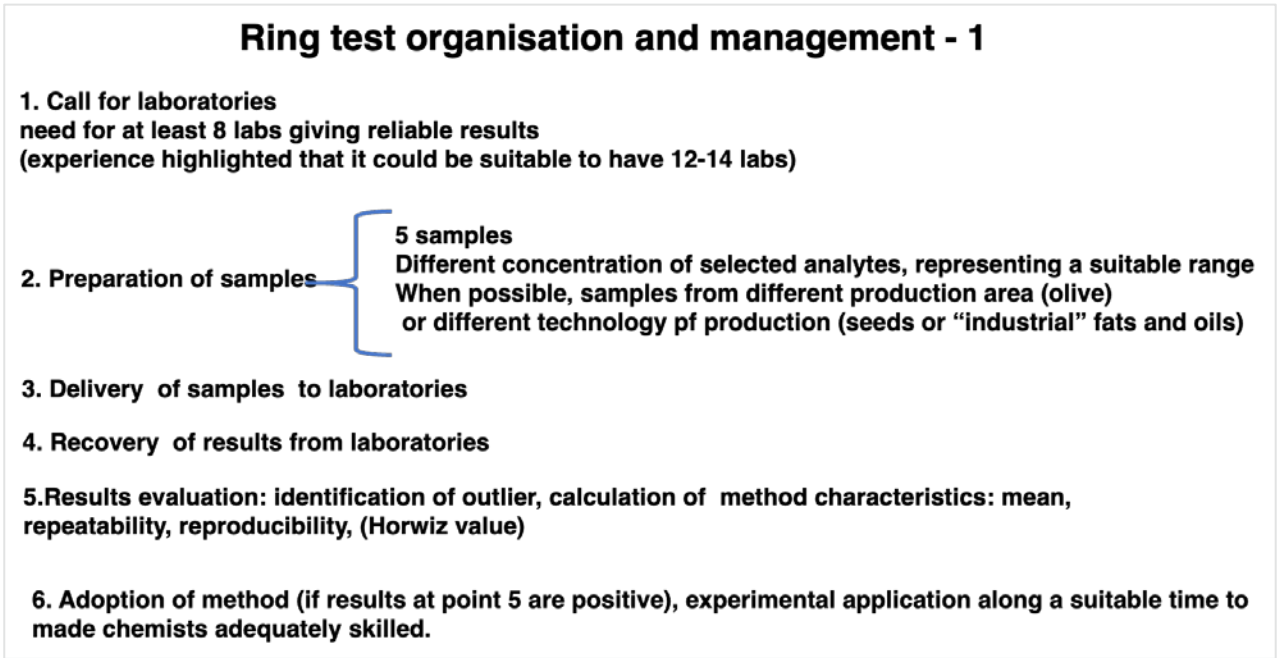


Figure 4: Ring test organization and management

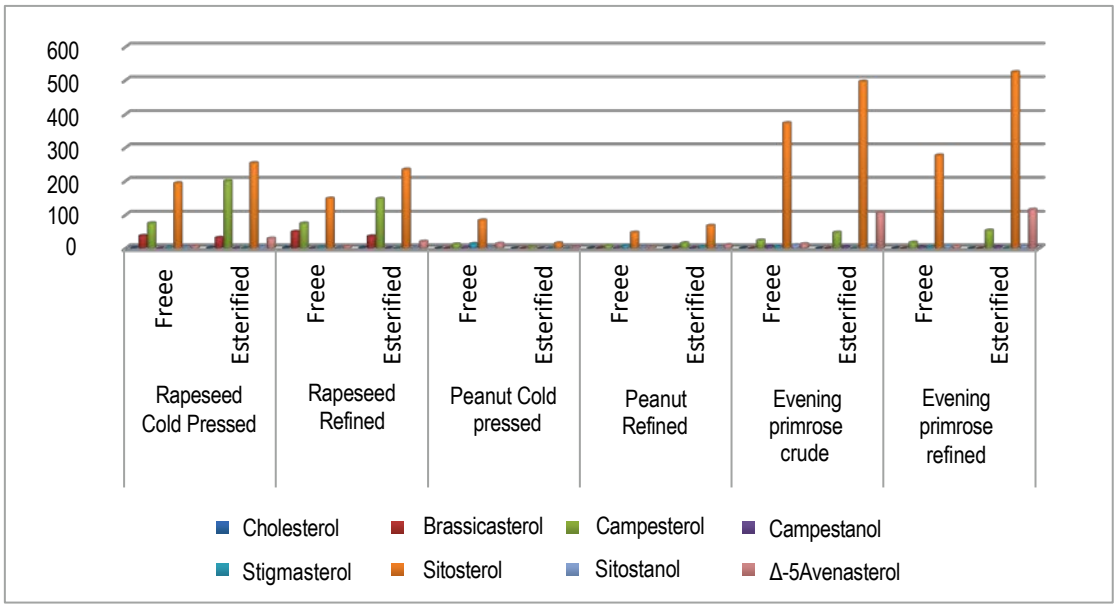


Figure 5 - Free and esterified sterols distribution in some vegetable oils (Elaboration from data of K. Phillips et Al. , 2002) [11]

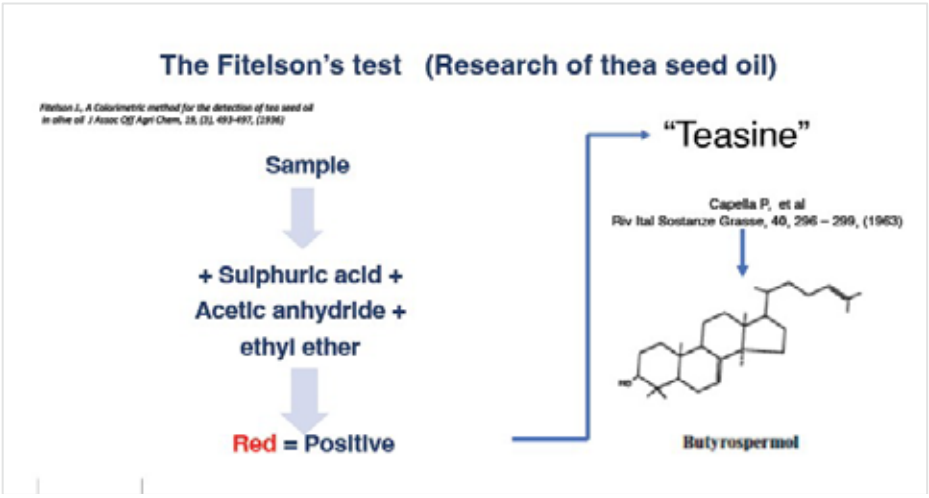


Figure 6 - The experience of identify the compound responsible of positive result of the "Fitelson test"

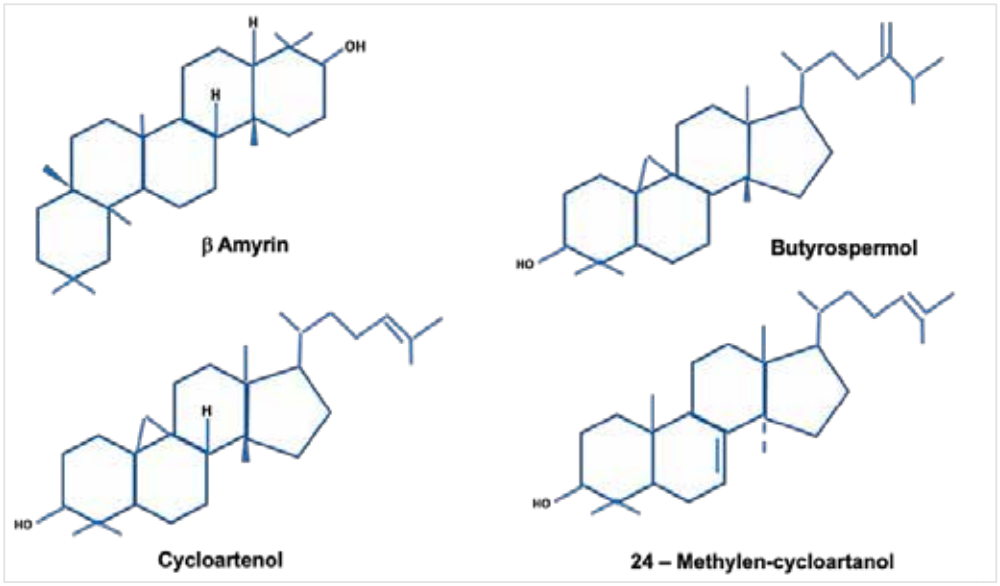


Figure 7 - Triterpenic alcohols

the determination of esterified sterols can preserve much more information (Figure 5).
The composition of the unsaponifiable fraction involves not only sterols but also triterpenic alcohols and methyl sterols; however, these classes of compounds have remained a research topic [12–17].
Coming back to the "chemistry of indexes", in 1963 tea seed oil was used to adulterated olive oil. The analytical method used to assess its presence was the "Fitelson test" (Figure 6), whose positive result was attributed to the presence of a compound named "teasin", which was isolated in the unsaponifiable fraction and identified as butyrrospermol [18], belonging to the class of triterpenic alcohols, previously identified in the unsaponifiable
fraction of karité-shea (Butyrospermum parkii) oil [19]. This study is of particular interest as it represents the connection between the chemistry of indexes and modern separation techniques.
Within this research, other compounds were identified, such as β-amyrin, which at that time was identified on the basis of elemental analysis, optical rotation, and I.R. spectrum. Within olive oil, four compounds were identified; further studies identified several other triterpenic alcohols in different seed oils (rapeseed, linseed) [11, 12, 14, 15, 17, 20] (Figure 7).
According to the data reported in Figure 8, as well as by many other authors, the composition of triterpenic alcohols, if adequately studied within a systematic approach, could probably prove as useful for classifying oils and fats (which means, of course, establishing purity standards) as sterols.
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The same consideration could apply to the fraction of 4-methylsterols (Figure 9): gramisterol (24-methytenelophenol), obtusifoliol and citrostadienol, which were detected in most oils. Gramisterol is 67% in palm oil, while it drops to 20% in palm kernel oil; in olive oil, obtusifoliol is 12%, while in sunflower oil it is 30%.
A systematic approach means collecting a huge amount of compositional data obtained by analysing samples from different areas of production and different crop years. Even in the case of olive oil, whose composition seemed well known, these two sources of variation have caused serious problems that have led to changes in international standards. It is worthwhile highlighting that, in the most recent release of the method for the determination of sterols adopted by the IOC [5], Figure 10 of the method reports a GLC trace identifying the area of the chromatogram containing triterpenic alcohols (Figure 10 of this paper).
Triterpenic alcohols (cycloartenol and 24-mehylencycloartanol) were also identified by Camera [21] in a proposed method useful for assessing the presence of olive pomace oil in olive oil; the method was based on determining the concentration of aliphatic alcohols. Camera proposed the calculation of the so-called "alcoholic index", using the formula:

$$\text{Alcoholic index} = \frac{C_{22}}{C_x} \times \frac{C_{22}+C_{24}+C_{26}+C_{28}}{\text{Cycloartenol}+24-\text{methylencycloartanol}}$$

The proposed method was based on the presence of waxes in pomace oils, which was also the basis of the Vizern index (see Table I), which, however, often gave false-positive results. Aliphatic alcohols derived from the hydrolysis of these compounds, which, because of their high MW, cannot be analysed by GC on packed columns.
Even though the results were very good for the purpose for which the method was proposed, it was not adopted as an official method because a term in the formula was not identified (Cx); only later, in 1985, was complete validation of the method achieved [22], and the method with the corresponding limit was adopted within EEC Regulation [23].
After some years of use of this method, however, some difficulties emerged because the concentration of alkanols in some high-quality extra virgin olive oils from selected areas of production (Greece, southern Italy) exceeded the legal limit.
An in-depth investigation of these oils, carried out thanks to the availability of LC-GC instrumentation, led to the identification of high concentrations of alcohols without "corresponding" concentrations of waxes, meaning that no extraction oil was present.
According to this result, the determination of waxes replaced the determination of alkanols in olive oils. Validation of the method for waxes presented some difficulties because of the mandatory use of the on-column injection port, which initially was not widely available in every laboratory, as well as limited experience in its use.
Even though adequate skills were acquired by most olive oil chemists, some separation problems were reported, leading to an overestimation of the concentration of waxes; indeed, Mariani [25] highlighted that some peaks identified as waxes were related to esters of fatty acids with fatty alcohols, but esters of diterpenes.
The solution adopted by the IOC (and consequently the EU) was to remove C40 from the calculation.
In conclusion, we can say that despite the long history of research devoted to the composition of olive oils, mainly with the aim of developing analytical methods, some scope remains for new approaches, perhaps by reconsidering topics already studied.
The evaluation of triacylglycerols adopted by the IOC and EU combines the results of two different

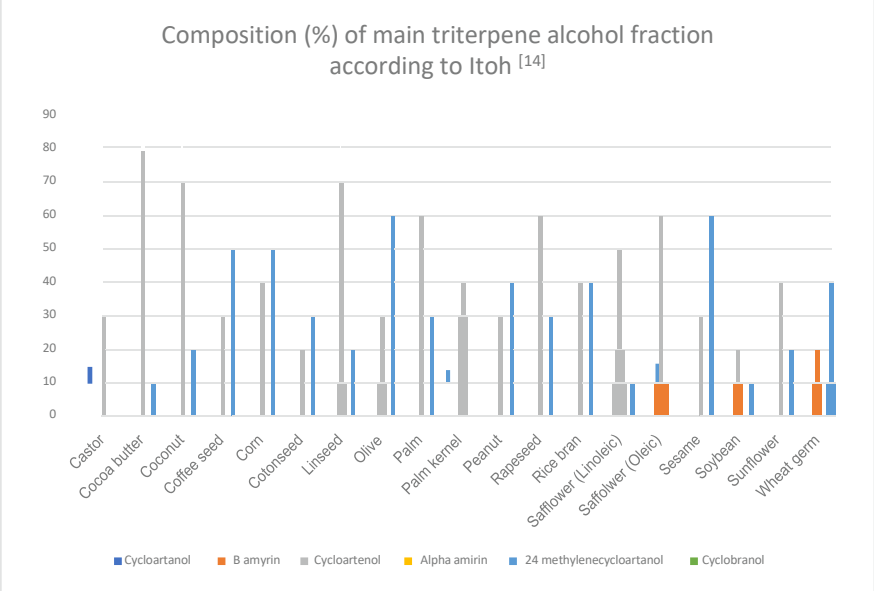


Figure 8 - Triterpenic alcohols (approximate %) according to Itoh et al, 1973 [14]

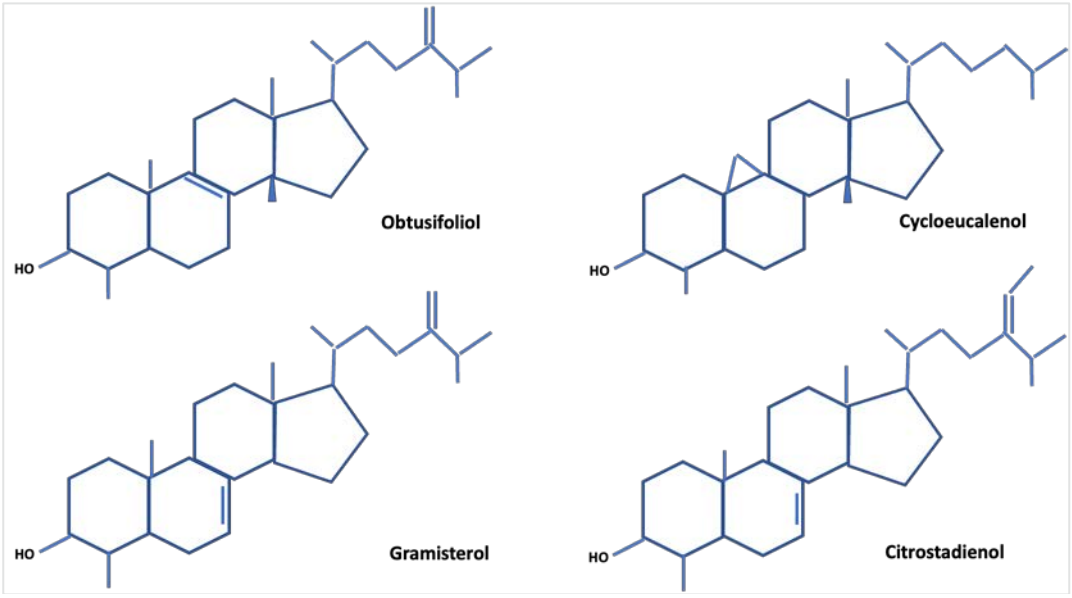


Figure 9 - 4-methylsterols

methods: fatty acid composition (by GLC) and triacylglycerol analysis (by HPLC); an algorithm then verifies the compliance of the former with the latter. Equally reliable results can be obtained by direct GLC analysis of triacylglycerols using a short capillary column, as reported by Mariani and Fedeli in 1993 [27] (Figure 11); on this basis, Conte & Moret obtained different distributions of TAGs in different oils (Figure 12).

A different topic that, however, presents some problems in its application, even though a validated method exists [29], concerns the so-called "polyphenols", which are a cornerstone in promoting extra virgin olive oil as a health-promoting food but face some hurdles in being used by producers as a suitable tool for this purpose.

The main drawback concerns the formulation of the health claim adopted by EFSA and the EU [30], which states: "The claim can be used only for olive oil which contains at least 5 mg of hydroxytyrosol and its derivatives (e.g. oleuropeine complex and tyrosol) per 20 g of olive oil…".

This sentence is incorrect by a chemical point of view, because hydroxytyrosol derives from oleuropein, which is a more complex molecule, while tyrosol is a different molecule.

Furthermore, when the claim was adopted, no standardized and validated method was available and, in many cases, a colorimetric one (Folin-Ciocalteu) was applied.

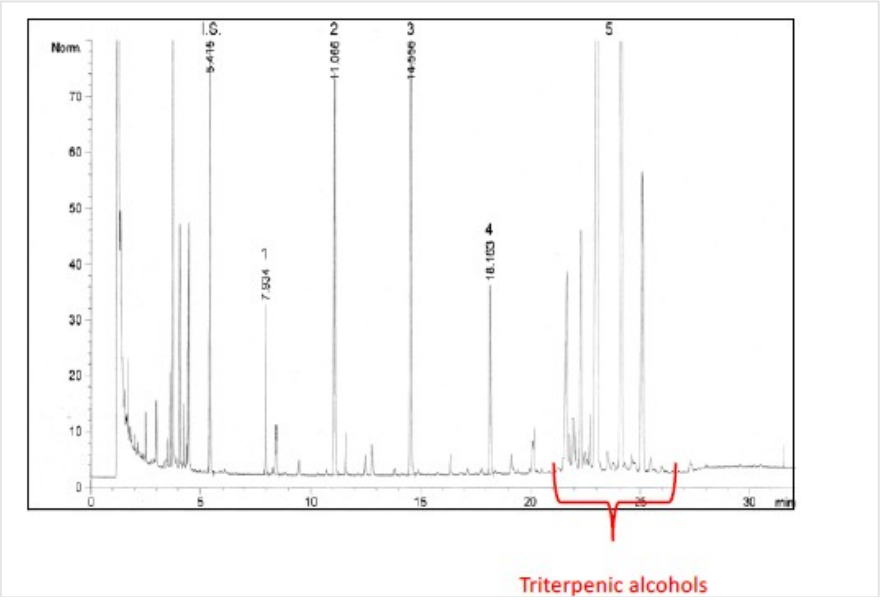


Figure 10 - GC-FID chromatographic profile of aliphatic alcohols and triterpenic alcohols of olive oil (I.S.) C20-o, (1) C22-ol, (2) C24-ol, (3) C26-ol, (4) C28-ol, (5) triterpenic alcohols [5]

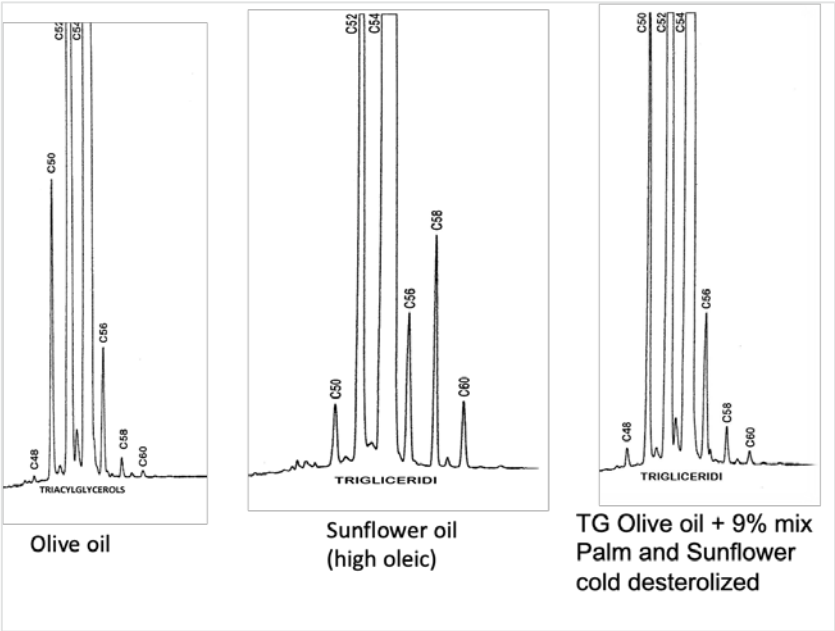


Figure 11 - GC analysis of triacylglycerols [27]

Later, within the IOC, a method was validated and adopted; however, the scope of the method is declared to be the measurement of the total concentration of polyphenols, and only later was a new version of the method developed that was suitable for measuring each individual polyphenol.

The method was adopted by the IOC, but not by the EU to date, because the EU usually adopts methods for parameters for which a limit has been established. In the case of polyphenols, the establishment of a limit, even though the statement by EFSA provided indications in this regard, is rather problematic because there is a risk of penalising high-quality oils with "sweet" sensory characteristics that do not reach the suggested concentration of polyphenols.

The main difference between the approach that leads to the establishment of standards (e.g., fatty acids, sterols) and what research does nowadays is that proposing the use of some molecules as markers requires the acquisition of a huge amount of data; despite the great improvement in the speed of analysis nowadays, it seems rather difficult to obtain them.

Experience with methods already adopted, which highlighted several non-compliant cases, also suggests the need for a wide range of samples, comprising as many areas of production as possible, bearing

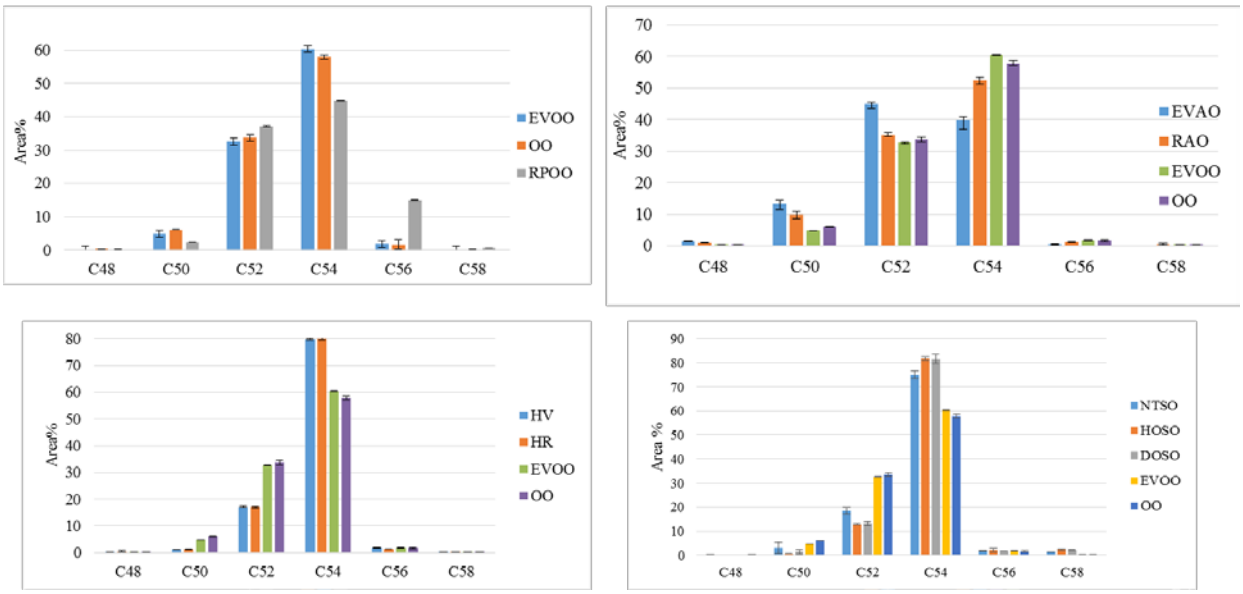


Figure 12 - Triacylglycerols composition of some oils obtained by capillary GC (Conte L, Moret E, unpublished results)

in mind that changes in the environmental conditions of cultivation can apparently produce substantial changes in composition.

The availability of a huge number of samples poses some problems: probably the main problem is that, e.g., a systematic study on triterpenic alcohols aimed at proposing a new parameter/limit would be carried out by applying routine, relatively non-innovative methods (TLC or HPLC for unsaponifiable fractionation), so it could be unattractive to young researchers because scientific journals generally ask for innovative approaches using innovative instrumentation and methods that are as little time-consuming as possible. Current trends in analytical chemistry applied to oils and fats focus on systems suitable for capturing large amounts of information that require chemometric or A.I. processing, often without allowing precise measurement (a measurement with the possibility of calculating measurement uncertainty) for each individual compound; this is probably a very effective approach, but it seems rather difficult to transpose it into international standards/legislation, which usually require a single compound with a single concentration value in order to allow simple interpretation of the data suitable for legal decision-making processes; perhaps the future could accept this approach.

This lecture is dedicated to the memory of Dr Carlo Mariani (who passed away on September 8th, 2025), who spent his life studying lipids in depth and developed many of the methods now adopted as official methods for olive oil quality and purity control.

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