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Evaluation of the methods based on triglycerides and sterols for the detection of hazelnut oil in olive oil

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RESUMEN

Evaluación de los métodos basados en triglicéridos y esteroles para detectar la presencia de aceite de avellana en aceite de oliva.

Dos métodos analíticos, basados en la diferencia entre triglicéridos teóricos y experimentales y razones entre algunos esteroles libres y esterificados, se han evaluado para determinar su utilidad detectando la presencia de pequeñas cantidades de cualquier tipo de aceite de avellana en aceite de oliva. La validación de los métodos se llevó a cabo mediante validación interna y externa, la última llevada a cabo con 21 laboratorios diferentes en tres estudios colaborativos. La información resultante sugiere un valor de corte en la detección de la adulteración del 8% para el método basado en triglicéridos y del 10% para el basado en la cuantificación de esteroles. El primero también muestra mejores valores de los parámetros de fiabilidad en reproducibilidad; por ejemplo, número de falsos positivos, selectividad (90% vs. 82%) e índice de Youden (0.81 vs. 0.77).

PALABRAS-CLAVE: Aceite de avellana – Aceite de oliva – Esteroles – Estudios colaborativos – Parámetros cualitativos – Triglicéridos.

SUMMARY

Evaluation of the methods based on triglycerides and sterols for the detection of hazelnut oil in olive oil.

Two analytical methods, based on the difference between theoretical and empirical triglycerides and the ratios between some free and esterified sterols have been checked to determine their usefulness in detecting the presence of low quantities of any kind of hazelnut oil in olive oil. The methods were confirmed by means of internal and external validations, the latter carried out in 21 different laboratories in three inter-comparison trials. The resulting information suggests a cut-off at 8% for the method based on triglycerides and 10% for that based on the quantification of sterols. The former also shows better reliability measures in reproducibility; i.e., number of false positives, efficiency (90% vs. 82%) and Youden index (0.81 vs. 0.77).

KEY-WORDS: Hazelnut oil – Inter-laboratory studies – Olive oil – Qualitative parameters – Sterols – Triglycerides.

1. INTRODUCTION

Although the metrological approaches are mainly focused on quantitative aspects at present, a careful analysis of the information typically delivered by analytical laboratories shows that a growing proportion is of a qualitative nature (Ríos *et al*., 2003). However, while the analytical performances of qualitative methods are well documented and the protocols and algorithms to implement them are described in the bibliography (Cardenas and Valcárcel, 2005), the metrological approach to the binary yes/no response in qualitative chemical analysis is scarcely considered in classical methodology (Valcárcel and Cárdenas, 2005). The mathematical algorithms that express concepts such as robustness or uncertainty, which qualify quantitative methods, cannot be applied in qualitative methods. It is necessary to reformulate the classical chemical metrology in order for it to be applied in qualitative methods (Ellison and Fearn, 2005).

Qualitative methods can be based on instruments that provide the binary response in a direct way, without any data pre-treatment, or on analytical techniques that produce raw data requiring further mathematical treatment to be converted into a binary response. Whichever the analytical tool used, the response usually possesses quantitative information. Thus, three quantitative reference levels are involved in a binary response, the limit of detection that is inherent to the analytical method, the cut-off level that is given by the analyst in the light of a probability value (fixed at a percentage 100% of reliable with respect to the threshold limit when possible), and the threshold level, which is usually established by legislation (Kateman and Buydens, 1993).

This work analyzes the quality characteristics of two quantitative methods with qualitative results that are used to detect the presence of hazelnut oil in olive oil. The yes/no binary response of the methods is based on two different mathematical algorithms that use sterols and triglycerides as input variables. The quantitative quality parameters (relative standard deviations in repeatability and reproducibility) of the input variables together with the qualitative quality parameters (selectivity, sensitivity and reliability) of the binary responses are presented.

2. MATERIALS AND METHODS

2.1. Samples

The samples were collected from several oil producing countries (France, Greece, Italy, Morocco, Spain, Tunisia, and Turkey). The oils were obtained from single varieties or mixtures of several varieties. Some of them were refined as the main challenge is the detection of refined hazelnut oil in refined olive oil.

98 samples (35 blinds) were analyzed by the proposer of the method based on the quantification of free and esterified sterols (Mariani *et al*., 2006) while 175 samples (35 blind) were analyzed by the laboratory that proposed the method based on the differences between empirical and theoretical trilglycerides (Cert and Moreda, 2000). The olive oil samples were spiked at percentages that varied from 2% to 20%.

The validation process of the methods was carried out with a new set of 29 samples that were selected and delivered to the laboratories that participated in three successive inter-comparison studies by the International Olive Oil Council (IOOC). The samples were mainly from Turkey and all of them were refined. 11, 16 and 14 laboratories participated in the three successive inter-comparison studies respectively. A sample of genuine olive oil and of each one of the samples spiked with 8%, 10%, 15% and 20% hazelnut oil were delivered in duplicate to the participants in the first study. Eight samples of the second study were a genuine olive oil and a sample of each one of the olive oils spiked with 5%, 10% and 18% hazelnut oil, all of them in duplicate. The laboratories that participated in the third group received, in duplicate, a sample of genuine olive oil and of the olive oil sample spiked with 5% and 10% hazelnut oil plus the other three genuine olive oil samples. All the samples were coded so that the percentages of genuine olive oils in the samples were unknown to the participants.

2.2. Methods

The quantitative analysis of sterols is one of the most promising methodologies that has been set up and validated recently (Mariani *et al*., 2006). The method involves the separation of the apolar fraction (containing sterols esterified with fatty acids) from the polar fraction (containing free sterols) by silica-gel column chromatography and the independent quantification of these series of compounds by gas-chromatography. Four minor compounds (esterified Δ^7-stigmastenol esterified Δ^7-avenasterol esterified campesterol and free Δ^7-stigmastenol) are responsible for the differentiation between hazelnut oils and olive oils. Then, two simple and understandable mathematical equations (R1 & R2) determine the authenticity of any sample in accordance with the established cut-off values for the decision rules (Scheme 1).

The difference between theoretical and empirical triglycerides has been proven useful in detecting this recalcitrant adulteration (Cert and Moreda, 2000; Christopoulou *et al*., 2004; Fedeli, 2001; Aparicio-Ruiz and Aparicio, 2000). The method combines Analytical Chemistry with Mathematics to produce a binary response which determines whether the sample is or is not genuine. Fatty acids, quantified by the official GC method, are used to rebuild the triglycerides (theoretical) by means of a mathematical algorithm based on the Hilditch theory. The empirical triglycerides are quantified by particular HPLC analytical conditions (Moreda *et al*., 2003), propionitrile mobile phase being the most remarkable. The mathematical algorithm is based on six decision rules, or criteria, which were built with numerous linear equations (Aparicio, 2004). The decision rules (Scheme 2)

$$R1 = Campesterol \cdot ester \frac{(\Delta^7 - stigmastenol \cdot ester)^2}{\Delta^7 - avenasterol \cdot ester}$$

$$R2 = \Delta^7 - stigmastenol \cdot free \cdot (mg/kg) \frac{\Delta^7 - stigmastenol \cdot free}{\Delta^7 - stigmastenol \cdot ester}$$

If the concentration of esterified sterols is lower than 200mg/kg and:

$R1 < 1.6$,	then the sample is a genuine olive oil, or
$R1 \geq 1.6$,	then the sample is a non-genuine olive oil

If the concentration of esterified sterols is higher than 200mg/kg and lower than 600mg/kg and:

$R1 < 1.0$,	then the sample is a genuine olive oil, or
$R1 \geq 1.0$	then the sample is a non-genuine olive oil

If the concentration of esterified sterols is higher than 600mg/kg and:

$R1 < 1.0$,	then the sample is a genuine olive oil,
$R1 > 1.0$ & $R2 \leq 0.5$,	then sample is a genuine olive oil
$R1 > 1.0$ & $0.5 < R2 < 0.6$,	then it cannot be determined if the sample is or is not a genuine olive oil
$R1 > 1.0$ & $R2 \geq 0.6$,	then the sample is a non-genuine olive oil.

Scheme 1
Mathematical equations and decision rules to determine the olive oil genuineness based on sterols according to the method's proposer (Mariani *et al*., 2006)

$$K1 = \frac{(LLL_{exp} + OLLn_{exp}) \times (OLL_{theor} + OOLn_{theor})}{(LLL_{theor} + OLLn_{theor}) \times (OLL_{exp} + OOLn_{exp})}$$

If $K1 \leq$ its lower limit, the oil is genuine
If $K1 >$ its upper limit, the oil is not genuine
If $K1$ is between its lower and upper limits, check the next criterion

$$\Delta R1 = \left[\frac{LLL}{OLLn}\right]_{exp} - \left[\frac{LLL}{OLLn}\right]_{theor}$$

If $\Delta R1 \leq$ its lower limit, the oil is genuine
If $\Delta R1 >$ its upper limit, the oil is not genuine
If $\Delta R1$ is between its lower and upper limits, check the next criterion

$$\Delta R3 = \left[\frac{OLL}{OOLn}\right]_{exp} - \left[\frac{OLLL}{OOLn}\right]_{theor}$$

If $\Delta R3 \leq$ its lower limit, the oil is genuine
If $\Delta R3 >$ its upper limit, the oil is not genuine
If $\Delta R3$ is between its lower and upper limits, check the next criterion

$$L4 = \frac{(LLL_{exp} - LLL_{theor}) \times (OLLn_{exp} + OLLn_{theor})}{LLL_{theor}}$$

If $L4 \leq$ its lower limit, the oil is genuine
If $L4 >$ its upper limit, the oil is not genuine
If $L4$ is between its lower and upper limits, check the next criterion

$$L3 = \frac{(LLL_{exp} - LLL_{theor}) \times (OLLn_{exp} + OLLn_{theor})}{OLLn_{theor}}$$

If $L3 \leq$ its lower limit, the oil is genuine
If $L3 >$ its upper limit, the oil is not genuine
If $L3$ is between its lower and upper limits, check the next criterion

$$R2 = \frac{(OLL_{exp} - OLL_{theor}) \times LLL_{theor}}{(LLL_{exp} - LLL_{theor}) \times OLL_{theor}}$$

If $R2 \leq$ its lower limit, the oil is genuine
If $R2 >$ its upper limit, the oil is not genuine

Scheme 2
Mathematical equations and decision rules to determine the olive oil genuineness based on triglycerides according to the method's proposer

follow a sequential process from the first to the sixth criterion. A decision is made by the program at each node (criterion) in function of the resulting value of the criterion.

2.3. Statistical procedures

Cochran and Grubbs' tests were applied to detect the outliers by examining the laboratory variances and, then, to determine the relative standard deviation in repeatability and reproducibility (Unknown, 1995).

The mathematical algorithms proposed to determine the presence or absence of the adulterant (Schemes 1 & 2) can only be seen as a binary test or qualitative method and, in consequence, its performance characteristics have to be evaluated according to qualitative algorithms.

The basic information of the qualitative methods are two, so-called "false positives" and "false negatives". A false positive arises when a genuine sample is classified as adulterated by the algorithm. A false negative, on the contrary, corresponds to the classification as genuine of an adulterated sample. These kinds of errors, which are strongly related to the concept of "null hypothesis", allow for the evaluation of the main properties of qualitative methods: selectivity, sensitivity and reliability. Selectivity is defined as the method ability to produce results which are exclusively dependent on the mesurand. Selectivity is quantified as the ratio between the number of true negative tests and the sum of it and the number of false positive tests. Specificity is defined as the ultimate level of selectivity, which is the absolute absence of interferences. Sensitivity is formulated as the ratio between the number of true positive tests and the sum of it and the number of false negative tests. Efficiency is defined as the ratio between the sum of true positive and negative tests and this figure plus the sum of the false positives and false negatives. Youden index is the consequence of the previous analytical properties and it is formulated as the complement to the sum of probabilities of error of the first and second kind (Ríos *et al*., 2003). Table 1 shows the formulas of the reliability measures in the qualitative analysis just described.

The parameter that determines the method's usefulness is the method cut-off since it is defined as the minimum percentage of adulterant (hazelnut oil) needed to ascertain the adulterant detection with a stated probability. But the relative percentage of the adulterant in relation to the cut-off percentage affects the type of error: if the percentage of adulterant is slightly higher than the cut-off, then false negatives are expected while for percentages lower but close the cut-off value, false positives are

Table 1
Reliability measures in qualitative analysis

Reliability measure	Expression
False positive rate	$\frac{FP}{TP + FP}$
False negative rate	$\frac{FN}{TP + FN}$
Sensitivity	$\frac{TP}{TP + FN}$
Specificity	$\frac{TN}{TN + FN}$
Efficiency	$\frac{TP + TN}{TP + TN + FP + FN}$
Youden index	$100 \times (\text{Sensitivity} + \text{Specificity} - 1)$
Likelihood ratio	$\frac{1 - FNr}{FPr}$

Note: FP, Number of False Positives; FN, Number of False Negatives; TP, Number of True Positives or genuine olive oil samples; TN, Number of True Negatives or spiked samples; FNr, False Negative rate; FPr, False Positive rate.

to be expected. In this study, the threshold is determined as the minimum concentration of the adulterant that reduces the risk of a false negative, once we have stated the minimum percentage of the adulterant that can be detected. Thus, for instance, if the minimum percentage of detection is 10% then we cluster all the samples with percentages of adulteration lower than 10% as genuine olive oils, and with this new grouping we proceed to recalculate the false positives and false negatives.

An in-house statistical program was used to implement the statistical tests and the quality variables.

3. RESULTS AND DISCUSSION

The response expected from a qualitative analysis is binary but the information supporting this answer is not so simple. Although the qualitative methods are usually thought to be based on analytical processes that do not need any kind of mathematical treatment (i.e. pH indicator test strips), many of the qualitative methods are, in fact, quantitative methods whose results have been mathematically processed to produce a binary response (e.g. presence/absence of adulterants). While the analytical variables can be evaluated by means of quality parameters for quantitative methods (bias, precision, robustness, repeatability, reproducibility et cetera) (Boqué *et al*., 2002), the resulting binary response has to be evaluated by other statistical qualifiers (Ríos *et al*., 2003).

The adulteration of olive oil with refined hazelnut oil is a challenge for antifraud institutions as the current official standards are unable to detect the presence of the adulterant at percentages lower than 20% (García-González and Aparicio, 2006). The main interest is not to detect the amount of the adulterant in olive oil, which is very difficult due to the enormous amount of different olive and hazelnut oils, but the presence or absence of the adulterant that is a binary response. Thus, the most promising analytical methods produce a binary response although their results are based on the quantification of chemical compounds (Bowadt and Aparicio, 2003). It means the first part of the whole analytical process can be evaluated by the classical parameters for quantitative methods while the second part of the process needs to be evaluated by qualitative qualifiers.

3.1. Detection of hazelnut oil in olive oil by sterols

A previous step to validating the method with external laboratories was the validation of the method by analyzing a set of 98 samples in the laboratory that proposed the method. Thus, five sets of samples in duplicate (10 samples) were used to inform on the method repeatability. The second column of Table 2 shows the range of the relative standard deviation (%RSD_r) in repeatability of the method's proposer. The maximum values of %RSD_r were too high for free Δ^7-Stigmastenol (41.0%) and esterified Δ^7-Stigmastenol (20.6%) compounds. The poor repeatability is not the only flaw of this excellent method but the variability range of all %RSD. The sterol compounds of the equations (R1 & R2 in Scheme 1) are minor components of the sterol fraction, Δ^7-compounds in particular (Firestone and Reina, 1996), and it influences on %RSD_r of the resulting values of the equations: 21.0% for R2 and 40.2% for R1. The consequence is obviously the number of false positives and false negatives when samples are qualified by the method.

The qualitative analysis of the whole set of samples (98 with 35 being blinds) has been used to establish the detection of the percentage of hazelnut oil in olive oil at several values (2%, 5%, 7%, 8%, and 10%) from the whole range of spiked samples analyzed (2%-20%). Table 3 shows not only the number of false positives and false negatives but also the percentages associated to

Table 2
Ranges of the relative standard deviation in repeatability and reproducibility of the chemical parameters used to detect the presence of hazelnut oil of the method based on sterols. The results are of the method's proposer (RSD_r) and of the three inter-comparison studies.

Parameter	Method's Proposer	1st Inter-comparison		2nd Inter-comparison		3rd Inter-comparison	
		RSD_r (min-max)	RSD_R (min-max)	RSD_r (min-max)	RSD_R (min-max)	RSD_r (min-max)	RSD_R (min-max)
Campesterol (esterified)	0.5-6.2	0.8-2.9	3.3-4.8	2.1-6.2	5.2-8.8	5.3-7.0	5.3-7.1
Δ^7-Stigmastenol (esterified)	2.4-20.6	3.4-10.7	12.6-34.8	7.3-27.5	14.7-27.5	7.8-9.5	8.0-15.0
Δ^7-Avenasterol (esterified)	0.0-14.0	3.5-14.8	9.6-41.7	4.5-43.0	20.7-43.6	3.9-8.2	6.1-22.3
Total sterol esters	1.5-12.0	2.5-5.0	8.3-9.9	2.6-4.5	8.7-26.0	1.1-3.0	24.4-27.4
Δ^7-Stigmastenol (free)	1.5-4.9	5.8-41.0	19.3-42.5	7.2-22.7	14.8-37.6	5.4-13.9	13.3-18.8
Total free sterols	0.1-7.5	1.9-3.4	3.1-5.0	1.3-4.9	1.3-4.9	1.9-3.6	3.2-6.4
R1	2.0-40.2	6.0-13.0	34.0-41.0	10.1-44.0	12.9-47.3	7.9-16.1	20.2-30.2
R2	0.0-21.0	14.0-42.0	37.0-64.0	8.6-28.3	15.1-52.3	9.3-22.0	19.0-27.6

Note: RSD_r, Relative Standard Deviation in repeatability; RSD_R, Relative Standard Deviation in reproducibility.

Table 3
Results of the parameters and qualifiers of the sterol method, applied by the method's proposer, using different cut-offs.

Percentage of addition	TN	TP	FN	FP	FN ratio	FP ratio	Sensitivity	Selectivity	Efficiency	Youden index
> 0%	22	76	25	2	8.3%	24.8%	75.3%	91.7%	78.4%	0.67
> 2%	26	72	22	2[a]	7.1%	23.4%	76.6%	92.9%	80.3%	0.70
> 5%	34	64	15	5[a]	12.8%	19.0%	81.0%	87.2%	**83.1%**	0.68
> 7%	45	53	14	9[a]	16.7%	20.9%	79.1%	83.3%	81.0%	0.62
> 8%	50	48	10	14[a]	21.9%	17.2%	82.8%	78.1%	80.3%	0.61
>10%	61	37	5	21[a]	25.6%	11.9%	88.1%	74.4%	79.0%	0.63

Note: TN, True Negatives or genuine olive oil; TP, True Positives or spiked samples; FN, False Negative; FP, False Positive; [a], two are genuine olive oil classified as adulterated (FP) while the others are samples spiked at percentages lower than the figure of the first column (hypothetical FN).

sensitivity, specificity, efficiency, and the Youden index. The values show that the cut-off value would be approx. 5% when the efficiency reaches the maximum value, 83.05%, with two false positives as three of the total number (5) (Table 3) are samples spiked with percentages of hazelnut lower than 5%.

3.2. Detection of hazelnut oil in olive oil by triglycerides

The previous step to validating this method with external laboratories was also the validation of the method by analyzing a set of 175 samples in the laboratory that proposed the method. Thus, five sets of samples in duplicate (10 samples) were used to inform on the method repeatability. The second column of Table 4 shows the range of the relative standard deviation (%RSD_r) in repeatability of the method mastermind. The maximum value of %RSD_r of the compounds involved in the equations of Scheme 2 is lower than 8%, which is much lower than the method based on sterols.

The qualitative analysis of the whole set of samples (175 with 35 being blinds) has been used to establish detection of the percentage of hazelnut oil in olive oil at several values (2%, 5%, 7%, 8%, and 10%). Table 5 shows not only the number of false positives and false negatives but also the percentages associated to selectivity, sensitivity and reliability. The values show that the cut-off value would be approx. 5%, which corresponds to an efficiency value of 83.3% with only one false positive since fifteen of the total number (16) (Table 5) are samples spiked with percentages of hazelnut lower than 5%.

3.3. Validation process of the methods

Three inter-comparison studies were carried out to determine the basic quality parameters of the method. 11, 9 and 9 laboratories participated in

Table 4
Ranges of the relative standard deviation in repeatability and reproducibility of the chemical parameters used to detect the presence of hazelnut oil by means of the method based on triglycerides. The results are of the method's proposer (RSD_r) and of the three inter-comparison studies.

Triglycerides	Method's Proposer	1st Inter-comparison		2nd Inter-comparison		3rd Inter-comparison	
		RSD_r (min-max)	RSD_R (min-max)	RSD_r (min-max)	RSD_R (min-max)	RSD_r (min-max)	RSD_R (min-max)
C16:0	0.1-1.8	0.4-1.9	4.0-5.6	0.7-2.6	1.8-3.7	1.2-1.8	2.5-3.2
C16:1	0.8-2.3	1.0-2.4	2.9-4.3	1.2-2.6	3.5-4.8	3.0-5.5	4.5-6.5
C18:0	<0.1-1.1	0.5-1.5	1.2-2.9	0.6-2.0	1.8-3.8	0.6-1.8	3.6-3.8
C18:1	0.1-0.3	0.1-0.6	0.5-1.6	0.1-0.4	0.3-0.6	0.2-0.5	0.5-0.7
C18:2	<0.1-1.8	0.3-1.0	0.9-2.2	0.4-1.8	1.4-2.5	0.5-0.6	1.3-1.7
C18:3	0.7-4.0	1.4-3.5	6.4-10.9	2.6-4.3	8.6-12.6	2.5-3.6	5.9-7.9
C20:0	1.4-3.0	1.5-17.3	5.1-17.3	3.4-7.8	4.3-9.5	2.4-4.1	5.4-5.7
C20:1	<0.1-4.3	2.3-5.8	5.2-9.0	4.8-10.1	7.5-12.1	4.1-4.3	5.8-7.4
LLL	<0.1-5.2	2.7-5.8	4.3-8.4	3.6-9.4	4.4-10.2	3.5-7.0	6.1-10.1
OLL	0.3-1.8	0.8-2.8	1.7-3.1	1.5-2.5	2.2-4.9	1.0-1.6	2.8-3.3
OLLn+PoLL	1.8-7.8	7.0-9.8	12.4-24.8	11.9-18.5	21.4-25.7	6.9-15.0	12.3-15.3
OOLn+PoOL	<0.1-7.8	1.6-3.1	2.8-7.4	2.1-4.3	6.3-7.3	1.6-3.7	6.2-7.4
PLL+PoPoO	0.8-7.5	1.7-4.8	3.2-10.5	6.3-11.3	7.8-16.2	3.8-5.0	9.1-10.4
ECN42	1.0-5.7	5.0-8.0	11.8-20.7	4.5-12.8	8.33-16.5	2.4-8.7	7.8-10.9
ECN44	0.4-2.9	1.8-4.0	2.3-5.0	1.9-5.2	3.3-5.5	1.4-2.5	2.1-3.6

Note: RSD_r, Relative Standard Deviation in repeatability; RSD_R, Relative Standard Deviation in reproducibility.

Table 5
Results of the parameters and qualifiers of the triglyceride method, applied by the method's proposer, using different cut-offs.

Percentage of addition	TN	TP	FN	FP	FN ratio	FP ratio	Sensitivity	Selectivity	Efficiency	Youden index
> 0%	21	154	48	1	23.8%	4.6%	76.2%	95.5%	78.1%	0.72
> 2%	31	144	40	3[a]	21.7%	8.8%	78.3%	91.2%	80.3%	0.69
> 5%	64	111	19	16[a]	14.6%	20.0%	85.4%	80.0%	**83.3%**	0.65
> 7%	85	90	12	29[a]	11.8%	25.4%	88.2%	74.6%	81.0%	0.63
> 8%	102	73	6	39[a]	7.6%	27.6%	92.4%	72.3%	79.6%	0.65
>10%	117	58	4	51[a]	6.5%	30.4%	93.6%	69.6%	76.1%	0.63

Note: TN, True Negatives or genuine olive oil; TP, True Positives or spiked samples; FN, False Negative; FP, False Positive; [a], one is genuine olive oil classified as adulterated (FP) while the others are samples spiked at percentages lower than the figure of the first column (hypothetical FN).

each one of the studies. The statistical studies were focused on the detection of outliers by Cochran and Grubb tests and the subsequent determination of the relative standard deviation in repeatability and reproducibility of the free and esterified sterols selected for the equations. The results were worse when quantifying free sterols than esterified sterols, the exception was the total concentration of free sterols. The RSD value of each sterol was, however, similar to the obtained quantifying total sterols in another inter-comparison study (Cert *et al.*, 1997).

The errors associated with the quantification of the chemical compounds increased the RSD associated with the equations (so-called R1 and R2). RSD values of these equations were high enough although they were slightly lower in the third inter-comparison study when the laboratories were more familiarized with the method application. These poor values of repeatability and reproducibility were responsible for the high number of false positives and false negatives of the method to detect the presence or absence of hazelnut oil in olive oil over the course of the three inter-comparison studies. Table 6 shows the percentage of false positives (FP) and false negatives (FN) detected in each one of the inter-comparison studies classified by the percentages of addition of the adulterant to olive oil. The number of FP was unacceptable in the first two inter-comparisons and high enough in the third. However, this number was acceptable when the proponent of the method applied it. This result can only be interpreted to the fact that the laboratories that participated in the trials did not apply this complex method properly.

The number of laboratories that participated in the inter-comparison study of triglycerides was higher (11, 14, and 14). Table 7 shows the number and percentage of false positives (FP) and false negatives (FN) detected in each one of the inter-comparison studies. The number of FPs was unacceptable in the first trial but acceptable in the other two. The results of these trials are sufficient as only four FP (6.7%) were detected working with 14 different laboratories in each trial. The results of the method's proposer are much better since only one FP was detected in 175 samples (Table 5).

A comparison of the results attained by both methods after the inter-comparative studies reflects the method based on the quantification of sterols as showing poorer efficiency than that based on triglycerides. Thus, the sterol method shows the maximum efficiency (81.8%) at a cut-off of 10% while the maximum efficiency of the triglyceride method (90.3%) is already at a cut-off of 8%. In consequence, the method based on the differences between theoretical and empirical triglycerides has shown much better values of the qualitative and quantitative parameters. Furthermore, its main advantage is that the protocol for identifying and quantifying triglycerides, and obviously FAMEs, is extensive and also precise and well-documented. Furthermore, all the laboratories have routinely applied methods for FAME and TAG quantifications. Its main disadvantage is the structure of the

Table 6
Number and percentage of false positives (FP) and false negatives (FN) of the three trials of the sterol method. Results classified in accordance with percentage of adulterant added to olive oil.

Trial	1st Inter-comparison		2nd Inter-comparison		3rd Inter-comparison	
Laboratories	11		9		9	
Type of error	FP	FN	FP	FN	FP	FN
Samples	11×1×2	11×4×2	9×1×2	9×3×2	9×5×1	9×2×2
Number of errors	16	3=1+1+0+1	14	2 =1+1+0	17	5=4+1
Error percentages	72.7%	4.6% at 8% 4.6% at 10% 0% at 15% 4.6% at 20%	77.8%	5.5% at 5% 5.5% at 10% 0% at 18%	37.7%	22.5% at 5% 5.5% at 10%

Note: Number of samples per inter-comparison = laboratories × % of adulteration × replicates; FP, False Positive; FN, False Negative.

Table 7
Number and percentage of false positives (FP) and false negatives (FN) of the three trials of the triglyceride method. Results classified in accordance with percentage of adulterant added to olive oil.

Trial	1st Inter-comparison		2nd Inter-comparison		3rd Inter-comparison	
Laboratories	11		14		14	
Type of error	FP	FN	FP	FN	FP	FN
Samples	11×1×2	11×4×2	14×1×2	14×3×2	14×5×1	14×2×2
Number of errors	11	17=10+4+2+1	4	9=5+3+0	4	13=12+1
Error percentages	50%	45.5% at 8% 18.2% at 10% 9.1% at 15% 4.5% at 20%	14.3%	17.9% at 5% 10.7% at 10% 0% at 18%	6.7%	42.8% at 5% 3.3% at 10%

Note: Number of samples per inter-comparison = laboratories × % of adulteration × replicates; FP, False Positive; FN, False Negative.

mathematical algorithm - which makes the decision on the genuineness - which is absolutely opaque and has not allowed for the determination of qualitative and quantitative parameters of each node of the decision system (Scheme 2).

Regardless of the cited disadvantage, the TAG method has shown to be superior to the sterol method. This is more evident, in particular, when considering the number of false positives despite the fact that the samples were selected to be as problematic as possible.

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