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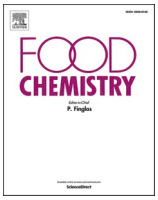

Revealing adulterated olive oils by triacylglycerol screening methods: Beyond the official method

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ABSTRACT

Official control methods to detect olive oil (OO) adulteration fail to provide satisfactory consumer protection. Thus, faster and more sensitive screening tools are needed to increase their effectiveness. Here, the official method for adulterant detection in OO was compared with three untargeted screening methods based on triacylglycerol analysis using high-throughput (FIA-HESI-HRMS; HT-GC–MS; HPLC-RID) and pattern recognition techniques (PLS-DA). They were assayed on a set of genuine and adulterated samples with a high natural variability (n = 143). The sensitivity of the official method was 1 for high linoleic (HL) blends at ≥2 % but only 0.39 for high oleic (HO) blends at ≥5 %, while specificity was 0.96. The sensitivity of the screening methods in external validation was 0.90–0.99 for the detection of HL and 0.82–0.88 for HO blends. Among them, HT-GC–MS offered the highest sensitivity (0.94) and specificity (0.76), proving to be the most suitable screening tool for OO authentication.

1. Introduction

According to the latest report from the EU Food Fraud Network (European Union, 2021), olive oil (OO) tops the list of reported adulterated food products. One of the most common frauds is mixing OO with cheaper vegetable oils (Casadei et al., 2021; The Food Integrity Project, 2018). The official methods to assess OO purity and detect the presence of extraneous vegetable oils include the analysis of fatty acids (FAs), triacylglycerols (TAGs) and sterols (Regulation (EU) No 2568/91 and its amendments). Adulterants may be masked by the removal of minor compounds, as occurs with desterolised seed oils, but major constituents such as FAs and/or the corresponding TAGs appear to be more robust parameters. As not only the amounts of FAs but also their arrangement to form TAGs are genetically determined, the official method based on TAGs rather than FA composition has proved to be more effective in detecting low levels of vegetable oils in OO (Christopoulou et al., 2004). Nonetheless, the current official method for TAG analysis is time- and reagent-consuming because it entails a double analysis of the samples. Firstly, High Performance Liquid Chromatography coupled to a Refractive Index Detector (HPLC-RID) is used to determine the experimental amount of TAGs and calculate the equivalent carbon number 42 (ECN42). Then, the theoretical ECN42 is determined from the FA content analysed by Gas Chromatography coupled to a Flame Ionization Detector (GC-FID), and both values are compared to calculate the ΔECN42 parameter. The lengthiness of this procedure limits the annual conformity checks to one per thousand tons of the OO marketed in European Union member states (Regulation (EU) No 2568/91 and its amendments), which does not guarantee a satisfactory level of consumer protection (European Commission, 2020). Additionally, this method shows low sensitivity when high linoleic (HL) seed oils are present at low levels or when the adulterants have a similar TAG composition to OO, as is the case of high oleic (HO) vegetable oils (Conte et al., 2020; Mailer & Gafner, 2020). Considering these drawbacks, and that the illegal blending of OO with both HL and HO vegetable oils is reported to be a common practice (Casadei et al., 2021; Everstine et al., 2013; Mailer & Gafner, 2020; Yan et al., 2020), more efficient methods

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are needed to detect extraneous vegetable oils in OO.

In this context, the development of fit-for-purpose screening tools would allow a more rapid assessment of a higher number of samples for a more efficient purity control of OO (Aparicio et al., 2013; Ruiz-Samblás et al., 2015). Screening methods featuring a binary qualitative output (compliant/non-compliant) obtained by time-efficient analytical methods, and combining different analytical and classification techniques, have been developed to overcome limitations in food authentication (López et al., 2014). Their advantages include immediacy in decision-making and reducing the time and cost of routine analysis (Muñoz-Olivas, 2004). The main goal of these screening methods is to achieve high sensitivity values for an efficient food fraud alert. Thus, qualitative screening can identify all suspicious samples, and subsequent control checks can confirm whether they have been adulterated or not (Magnusson & Örnemark, 2014). This is of particular importance when the fraudulent products pose a threat to food safety (López et al., 2014), in which case adulteration not only implies a loss of traceability due to blending with substances from unknown sources, but also carries the risk of allergenic or toxic effects related to the nature of the adulterants (Arlorio et al., 2010; Gelpí et al., 2002).

Most of the screening methods proposed as alternatives to the official method for adulterant detection in OO are also based on the measurement of the TAG fraction, due to its advantages as an authenticity marker (Meenu et al., 2019). Approaches based on high-throughput analytical techniques coupled to multivariate classification methods have proved to be efficient in fraud detection and have a short analysis time (Cavanna et al., 2018; Esslinger et al., 2014). Among them, untargeted profiling and fingerprinting methods have been applied as an alternative to the classic targeted approach of the current official method (Ballin & Laursen, 2019). Besides involving an effortless data treatment, untargeted approaches can consider more analytical information than conventional targeted methods, which may be valuable for authentication (Quintanilla-Casas et al., 2020). Although some of the alternative methods have provided promising results, comparison with the official method is difficult, as they differ in sample sets, variability, and analytical conditions. A further challenge to assessing the performance of the new methods is that multivariate validation is not as well defined as univariate validation (López et al., 2014).

In the present study, three promising screening methods for adulterant detection in OO based on TAG analysis and the official method based on ΔECN42 (Regulation (EU) No 2568/91 and its amendments) were compared. The selected screening methods are all based on an untargeted approach. One involves TAG profiling by Flow Injection Analysis-Heated Electrospray-High Resolution Mass Spectrometry (FIA-HESI-HRMS), an extremely fast high-throughput instrumental method requiring minimal sample preparation, which had already been tested for the analysis of OO adulteration with satisfactory results (Quintanilla-Casas et al., 2021). The other two methods were developed in the present study and follow a fingerprinting approach, a cutting-edge strategy that uses the whole analytical signal to find specific patterns characteristic of a given food category. Thus, TAG fingerprinting by High-Temperature Gas Chromatography coupled to Mass Spectrometry (HT-GC–MS) was developed by adapting the chromatographic conditions of (Ruiz-Samblás et al., 2012) to MS detection, which allowed us to build an unfolded matrix from Selected Ion Monitoring (SIM) chromatograms (Torres-Cobos et al., 2021). The third method, TAG fingerprinting by High Performance Liquid Chromatography coupled to Refractive Index Detector (HPLC-RID), was developed by applying chemometrics to the raw HPLC-RID data obtained by the official method, but without the need for any further TAG identification or quantitation. Finally, unlike in previous studies, the performance of each method could be directly compared because the same sample set (genuine samples and their blends with HO and HL adulterants) was analysed in each case.

2. Material and methods

2.1. Samples and experimental design

A set of 150 samples was analysed by the four methods described above. The set was composed of 30 traceable genuine extra virgin olive oils (EVOO) and their blends (n = 120) with 36 adulterant oils of different botanical origin, including HL oils (refined sunflower oil [SFO, n = 14] and refined soybean oil [SBO, n = 10]), as well as HO oils (virgin and refined hazelnut oils [HZN, n = 10] and high-oleic refined sunflower oils [HOSFO, n = 12]). The blends were in-house prepared at 2 % and 5 % for the HL (n = 60) and at 5 % and 10 % for the HO (n = 60) adulterants, following a balanced incomplete Latin squares experimental design. Seven of the chromatographic or FIA runs had to be discarded due to analytical problems, and these samples were eliminated from the datasets of the four methods to avoid any bias between the different models tested. The final dataset (n = 143) is provided in Table S1 of Supplementary Information.

All samples were obtained directly from reliable producers in the framework of the Autenfood project (ACCIÓ-Programa Operatiu FEDER Catalunya 2014–2020). The EVOO samples represented a range of geographical production regions, olive cultivars and technological conditions. The natural variability of the samples was further ensured by including several adulterant oils of each type in the sampling.

2.2. Method I. Official method to determine ΔECN42 according to Regulation (EU) No 2568/91

The difference between the experimental and theoretical ECN42 values of TAGs was calculated and compared with the limit established for EVOO, i.e., $\Delta ECN42 < |0.20|$.

2.2.1. Material and reagents

Methanol (>99.5 %), HPLC grade hexane (99 %), HPLC grade heptane and diethyl ether stabilized with 7 ppm of BHT were purchased from Scharlau (Sentmenat, Spain). Potassium hydroxide was purchased from Panreac (Castellar del Vallès, Spain), and propionitrile (>99 %) from Sigma-Aldrich (St. Louis, USA). Methanolic potassium hydroxide solution (2 M) was prepared according to the official method. Extra Bond® solid phase extraction cartridges (6 mL), packed with 1 g of silica phase, and 13 mm-0.45 μm nylon filters, both from Scharlau (Sentmenat, Spain), were used for sample purification and preparation.

2.2.2. TAG analysis by reverse phase (RP)-HPLC-RID and assessment of experimental ECN42

Once the samples were prepared and purified according to Annex XVIII of Regulation (EU) No 2568/91 and its amendments, TAGs were determined by HPLC-RID using an Agilent Technologies 1100 instrument (Agilent Technologies, Santa Clara, California, USA). Analytes were separated on a Luna C18 column (250 × 4.6 mm, I.D., 5 μm) from Phenomenex (Torrance, California, USA) at 40℃. The analysis was performed in isocratic mode with propionitrile as the mobile phase at 1 mL/min. The injection volume was 20 μL. The peak areas of TAGs with an ECN between 42 and 52 were integrated and normalized, obtaining the experimental TAG content with ECN42.

2.2.3. Analysis of FA methyl esters (FAME) by GC-FID and calculation of theoretical ECN42

After obtaining FAME by transmethylation according to Annex X of Regulation (EU) No 2568/91 and its amendments, they were analysed by GC-FID using an Agilent 4890D chromatograph, coupled to an Agilent Technologies 7683B automatic sampler (Agilent Technologies, Santa Clara, California, USA). The injection volume was 1 μL, with a split injection ratio of 1:100. The temperature of the injector and detector was 250 ℃. Analytes were separated on a SP-2380 capillary column (60 m × 0.25 mm I.D., 0.2 μm) (Supelco, St. Louis, USA). The initial column

temperature was 165 ℃ and it was held for 8 min; it was then increased to 210 ℃ at 2 ℃ /min and held for 15 min. Hydrogen (99.995 %) was the carrier gas, and the flow rate was 1 mL/min. The areas of the FAME with 16 and 18 carbon atoms were integrated and normalized. The theoretical content of ECN42 TAGs was calculated according to Annex XVIII of Regulation (EU) No 2568/91 and its amendments.

2.3. Method II. Screening method based on TAG profiling by FIA-HESI-HRMS

2.3.1. Material and reagents

Dichloromethane (SupraSolv® for GC-ECD/FID), methanol (SupraSolv® for gas chromatography) and NaCl (ACS reagent, ≥99.0 %) were purchased from Merck (Darmstadt, Germany). Nitrogen (Alphagaz, purity 99.999 %, Air Liquid) was used in the Orbitrap-Exactive as nebulization gas.

2.3.2. Sample preparation

As described by Vichi et al. (2012), 30 mg of oil sample was dissolved in dichloromethane:methanol (70:30, v/v) and diluted 1:100 using the same solvent mixture. The solution was saturated with NaCl as the cationization agent and vortex-mixed for 30 s. The supernatant was then further diluted to 1:50 with the same solvent mixture and analysed.

2.3.3. TAG profile by FIA-HESI-HRMS

Untargeted profiling of TAGs was done according to Vichi et al. (2012), using an Orbitrap Exactive instrument (Thermo Fisher Scientific, Bremen, Germany) equipped with an electrospray source (H-ESI II) and coupled to a Surveyor MS pump with an Accela Open automatic sampler (Thermo Fisher Scientific, San Jose, USA).

The selected mass peaks were single positive-charged molecular ions forming adducts with sodium. These peaks were exported to peak lists and feasible elemental formulae attributable to TAGs were generated. Different restrictive criteria were set to generate reliable elemental formulae: C ≤ 200, H ≤ 400, O ≤ 15, Na = 1 and RDB: 2.5–19.5 (Quintanilla-Casas et al., 2021). Mass error tolerance was set at 5 ppm. Signal intensities of TAGs were expressed as a relative intensity, the total TAG profile being equal to 100 %. The molecular formulae calculation was performed with Xcalibur 2.1 (Thermo Fisher Scientific, Bremen, Germany), and the posterior data analysis was done using excel files, as shown in Fig. 1a.

2.4. Method III. Screening method based on TAG fingerprinting by HT-GC–MS

2.4.1. Material and reagents

Dichloromethane (SupraSolv® for GC-ECD/FID) was purchased from Merck (Darmstadt, Germany).

2.4.2. Sample preparation

A small amount (60 mg) of oil sample was dissolved in 3 mL of dichloromethane to a final concentration of 2 % (w/v).

2.4.3. TAG fingerprinting by selected ion Monitoring (SIM) HT-GC -MS

HT-GC–MS analysis was carried out on an Agilent Technologies 6890 N gas chromatograph coupled to an Agilent 5973 Network quadrupolar mass selective analyser (Agilent Technologies, Santa Clara, California, USA). A total of 2 μL of sample was injected with a split ratio of 1:20. Analytes were separated on a Rtx®-65TG column (Restek, Bellefonte, PA, USA) (30 × 0.25 mm I.D., 0.10 μm). The initial column temperature was 315 ℃, which was increased to 350 ℃ at 2 ℃ /min and held for 15 min. Helium was the carrier gas and the flow rate was 1.5 mL/min. The temperature of the injector was 370℃ and the transfer line, 350℃. Electron impact mass spectra were recorded at 70 eV ionization energy. Mass spectra were acquired in SIM mode.

A fingerprinting approach was followed using extracted ion chromatograms (EIC) obtained for *m/z* signals attributable to 11 TAG fragment ions, according to Barber, Merren & Kelly (1964): acyl ions corresponding to a FFAA (Po: palmitoleic acid, C16:1; P: palmitic acid, C:16:0; O: oleic acid, C18:1; L: linoleic acid, C18:2; Ln: linolenic acid, C18:3) molecule (RCOOH) with a loss of an OH group $[RCOOH - OH]^+$ (*m/z* 237, $[Po - OH]^+$; *m/z* 239, $[P - OH]^+$; *m/z* 260, $[Ln - OH]^+$; *m/z* 262, $[L - OH]^+$; *m/z* 264, 265, $[O - OH]^+$) and to a FFAA acyl ions attached to a residual of the glycerol skeleton $[RCOOH - C_3H_5O]^+$ (*m/z* 311, $[Po - C_3H_5O]^+$; *m/z* 313, $[P - C_3H_5O]^+$; *m/z* 335, $[Ln - C_3H_5O]^+$; *m/z* 337, $[L - C_3H_5O]^+$; *m/z* 339, $[O - C_3H_5O]^+$). A data matrix was built for each ion with the 3159 scan intensities of each EIC (columns) for all samples (rows) (143 samples × 3159 variables) and it was aligned by the COW algorithm in Matlab® (Nielsen et al., 1998) to solve the retention time shifts among samples. The optimal COW parameters for each case

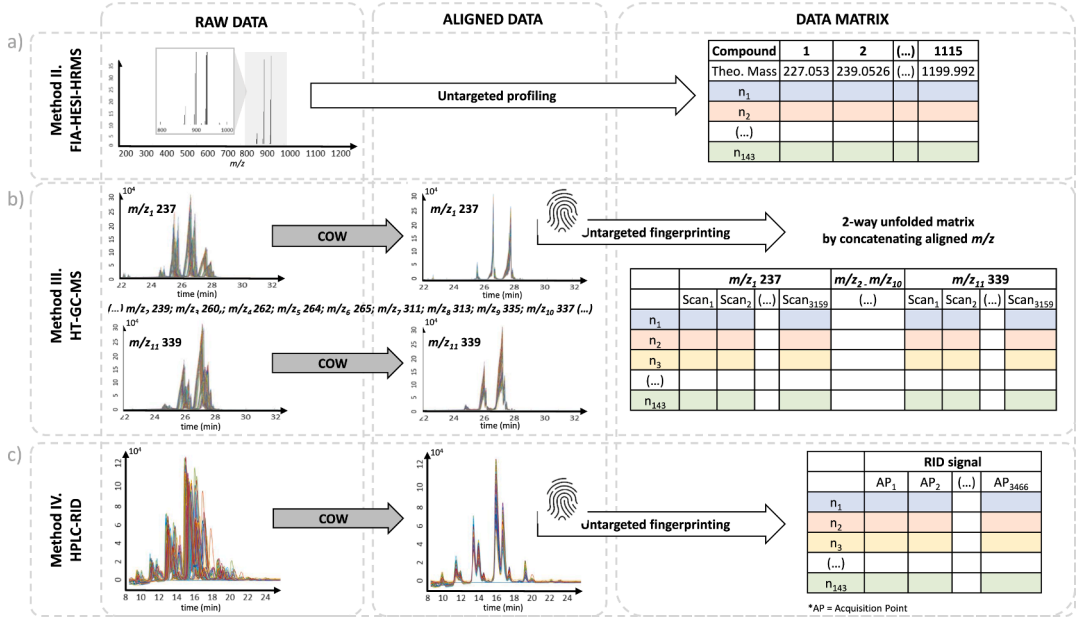

Fig. 1. Scheme of the data matrices building for each screening method: a) Method II: FIA-HESI-HRMS, b) Method III: HT-GC–MS fingerprinting and c) Method IV: HPLC-RID fingerprinting.

were calculated upon the starting parameters provided in Table S2, Supplementary information.

Then, the 11 aligned matrices – one for each EIC - were concatenated to obtain a two-way unfolded matrix (143 samples × 34749 variables) (Fig. 1b).

2.5. Method IV. Screening method based on TAG fingerprinting by RP-HPLC-RID

2.5.1. Sample preparation

Samples were prepared as described for Method I – Official method (section 2.2.2).

2.5.2. TAG fingerprinting by RP-HPLC-RID

Separation and detection of compounds were as described in section 2.2.3 for the official method (Method I). Using a fingerprinting approach, a data matrix was built with the chromatographic intensities obtained for each acquisition point from minute 8 until minute 25 for each sample (143 samples × 3466 variables). Chromatograms were aligned by the COW algorithm in Matlab® to solve the retention time shifts among samples (Nielsen et al., 1998) (Fig. 1c). The optimal COW parameters for each case were calculated upon the starting parameters provided in Table S2, Supplementary information.

2.6. Data processing and chemometrics

Data obtained by the official method (method I) were processed according to Regulation (EU) No 2568/91 and its amendments. Regarding the data matrices provided by each of the tested alternative screening methods (II-IV), classification models were developed and validated with SIMCA v13.0 (Umetrics AB, Sweden).

First, a Principal Component Analysis (PCA) was performed to explore the data and identify potential outliers. In no case were outlier samples detected by PCA, according to the Hotelling's T^2 range and distance to the model parameters.

Then, the whole sample set (n = 143) (Table S1, Supplementary information) was randomly split 7 times (7 iterations) into: i) a training set (80 % of the samples, n = 114; genuine EVOOs, n = 22; HL blends, n = 46; HO blends, n = 46) and ii) a validation set (20 % of the samples, n = 29; genuine EVOOs, n = 6; HL blends, n = 12; HO blends, n = 11). For each screening method and each iteration, two independent binary Partial Least Squares Discriminant Analysis (PLS-DA) models were built: one to discriminate between genuine and HL adulterated samples (n = 68; HL blends, n = 46; genuine EVOOs, n = 22) and the other to discriminate between genuine and HO adulterated samples (n = 68; HO blends, n = 46; genuine EVOOs, n = 22). In PLS-DA binary models, classes are expressed as PLS dummy variables (here 0 for the non-adulterated and 1 for the adulterated class). Then, the PLS predicted value of each sample was used for its classification into one class or the other according to a classification threshold (predicted value = 0.5). PLS-DA models were calibrated by leave 10 %-out cross-validation, selecting the optimal number of Latent Variables (LV) according to the lowest Root Mean Squared Error of Cross Validation (RMSEcv). A permutation test and ANOVA were carried out on the cross-validated predictive residuals (p-value) to assess overfitting. The Q^2 values and efficiency, expressed as the % of correct classification, were assessed to evaluate the suitability of each PLS-DA model.

After testing multiple pre-processing treatments, the optimal one for the HL and HO models based on FIA-HESI-HRMS profiling data was found to be logarithm 10, mean centring and scaling to unit of variance; for the HL model based on HT-GC–MS fingerprinting, it was mean centring and scaling to unit of variance, and for the HO model based on HT-GC–MS fingerprinting, logarithm 10. Finally, the pre-processing applied for the HL model based on RP-HPLC-RID fingerprinting was logarithm 10, mean centring and scaling to unit of variance and for the HO model, a first derivative was also needed. The optimal pre-processing treatments for the HL and HO models were applied to training sets of each iteration.

2.6.1. External validation of the authentication strategy

Samples in the abovementioned validation sets (20 %, n = 29 for each of the 7 iterations) were not included in the previous calibrated models but used to externally validate the authentication tool following a combined strategy according to Quintanilla-Casas et al. (2021) (Fig. 2). Thus, validation samples were classified as non-adulterated (with HL oils at ≥ 2 % or HO oils at ≥ 5 %) only when identified as such by both models, whereas they were considered as adulterated when identified as such by at least one of the authentication models (HL or HO).

External validation results for each screening method were compared according to the % of correct classification of each class, and the sensitivity (true positives/[true positives + false negatives]) and specificity (true negatives/[true negatives + false positives]) values, positive samples being those containing the adulterant.

2.6.2. Exploration of regression coefficients

For the three screening methods, the regression coefficients of the binary HL and HO PLS-DA models developed with the full sample set were explored to tentatively identify the variables that most contributed to the discrimination between adulterated and genuine samples. Regression coefficients were considered as significant when a jack-knife standard error of cross-validation (SEcv) was lower than the given coefficient value.

3. Results

3.1. Method I. Official method to determine ΔECN42 according to Regulation (EU) No 2568/91

The results obtained with the official method based on ΔECN42 showed high percentages of correct classification for genuine (96.4 %) and HL adulterated samples (100 %), whereas only 38.6 % of the blends with HO oils were classified as adulterated (Table 1).

3.2. Screening methods (II-IV) based on untargeted TAG profiling and fingerprinting

For all the screening methods, the cross-validation results of models built upon training sets (7 iterations) were successful. Mean overall sensitivity and specificity were 0.96–1 and 0.95–1, respectively. Each of the PLS-DA models (7 per screening method) were then used to predict the class of the samples in the corresponding validation set. The process was run seven times to evaluate the effect of the sample set composition and to increase the robustness of the external validation. The results achieved by the external validation of each screening method (sensitivity and specificity) (Table S3, Supplementary information) were expressed as mean values of the seven iterations (Table 2) and showed a sensitivity above 0.82 for all the developed methods, the HT-GC–MS method standing out with an average sensitivity for HL and HO adulterated samples of 0.94. Remarkably, the detection performance for HO blends was not far below the results obtained for the HL adulterants (Table 2). The three tested methods had a sensitivity close to 0.9 (mean value of the 7 iterations) and a specificity between 0.50 and 0.76 (mean values of the 7 iterations).

As explained in section 2.6.2, the coefficients of models were studied to tentatively identify the variables that contributed most to the discrimination between HL adulterated and genuine samples, or between HO adulterated and genuine samples. For FIA-HESI-HRMS, the relevant coefficients agreed with those reported by Quintanilla-Casas et al. (2021). In both models, the highest regression coefficients corresponded to several minor TAG species distributed within the entire experimental m/z range. Specifically, for the HL model, the most

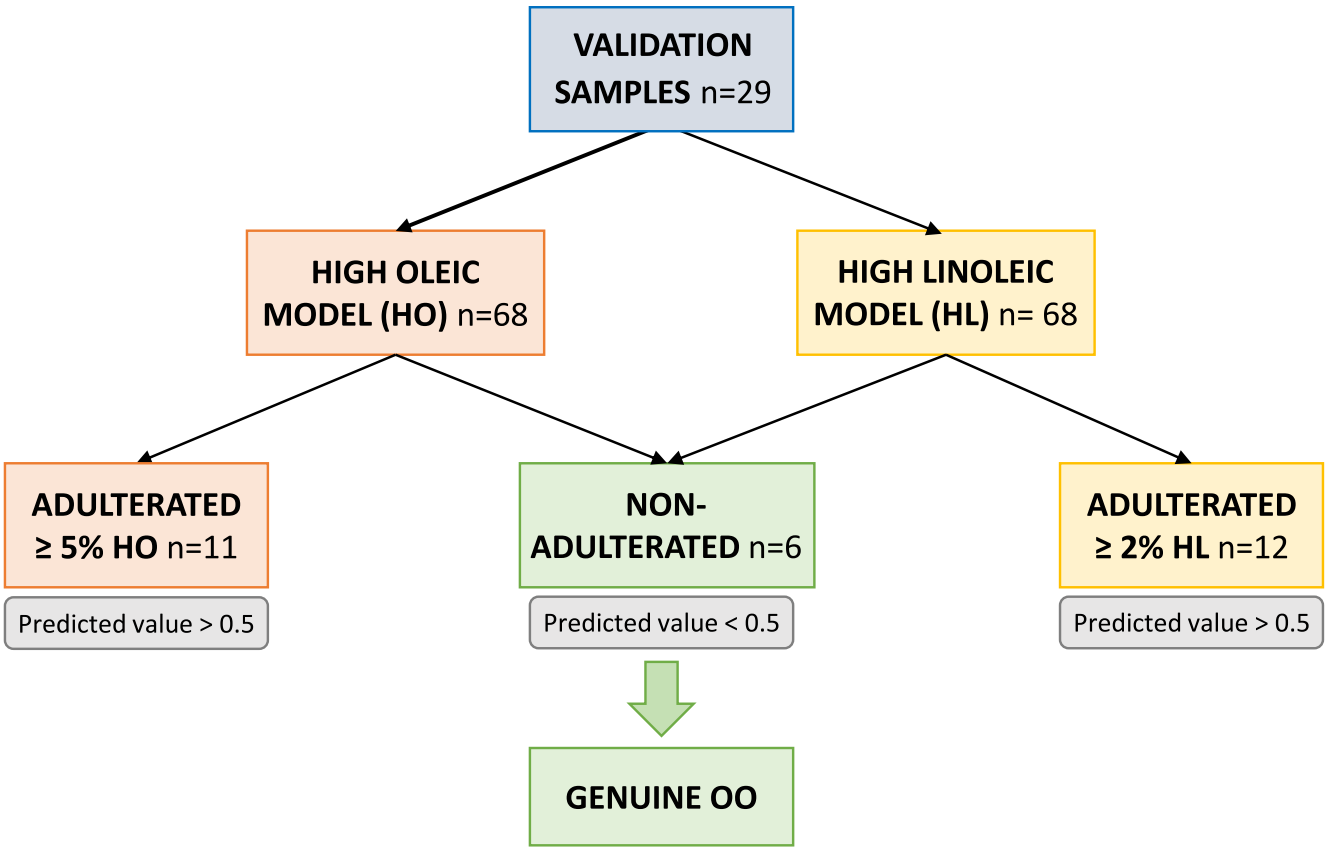

Fig. 2. Classification diagram based on two combined binary PLS-DA models to predict the presence of high linoleic (HL) (≥2%) and high oleic (HO) (≥5%) oil adulterants in olive oil (OO) using the tested untargeted screening methods.

Table 1
Classification results according to the official method for OO adulteration detection.

	n	Adulterated (n)	Genuine (n)	Correct classification (%)	Sensitivity	Specificity
Adulterated	115	80	35	**69.6**	**0.70**	
HL blends	58	58	0	**100**	**1**	
HO blends	57	22	35	**38.6**	**0.39**	
Genuine	28	1	27	**96.4**		**0.96**
Total	143					

relevant coefficients for the HL adulterated class belonged to TAGs containing L and Ln acids, such as C54 TAGs LLL and LLLn, and C52 TAGs such as PoLL/PLLn. On the other hand, for the HO model, the most discriminant variables associated with the HO adulterated class were signals corresponding to long-chain TAGs, C54 and C58, which could contain arachidic (A), behenic (B) or lignoceric acids (Li). Additionally, some TAGs containing L and short-chain TAGs C23, C24 and C26 also played a significant role in detecting HO adulterated samples (Quintanilla-Casas et al., 2021).

The study of the coefficients of models based on the HPLC-RID method (Fig. 3) produced results consistent with the FIA-HESI-HRMS findings, as the most relevant coefficients also belonged to minor TAGs. Specifically, to detect the HL adulterated samples, the highest coefficients were found in the first part of the fingerprint, covering the region of TAG clusters with ECN40, ECN42 and ECN44. According to the elution order within each cluster (Regulation (EU) No 2568/91 and its amendments), the most discriminant TAGs could corresponded to species containing L and Ln acids, such as LLLn (ECN40), LLL, OLLn, PoLL, PLLn (ECN42), PLL, PoPoO, and POLn (ECN44). Although ECN42 TAGs (tentatively identified as LLL, OLLn, and PoLL) and ECN44 TAGs (tentatively identified as OOLn, PoOL, PoPoO, and PLL) were also relevant for the detection of HO adulterated samples, the highest coefficients of the HO model also include TAGs in the ECN46 region (OOL, PPLn, PPoO, PoSL, SOLn, PoPoS), or even higher (Fig. 3), which agree with the relevant coefficients found in the FIA-HESI-HRMS model corresponding to long chain TAGs.

Finally, for the HT-GC–MS method (Fig. 3), some of the most relevant ions for the detection of samples adulterated with HL oils were found in the C54 TAG cluster and corresponded to glyceryl fragments with acyl species such as Ln, L and O. According to the fragmentation (Barber et al., 1964) and the expected elution order (Ruiz-Samblás et al., 2010), these relevant ions would agree with TAGs such as OLLn (*m/z* 335), LLLn (*m/z* 260, *m/z* 262, *m/z* 335 and *m/z* 337) and OLnLn (*m/z* 260, *m/z* 335 and *m/z* 339). In addition, some relevant coefficients for the *m/z* 262 and *m/z* 337 were found in chromatographic regions where the eluted TAGs were of a higher carbon number, such as C56 TAG, and could contain L and A. Several high coefficients corresponded to other glyceryl species of lower molecular weight that eluted prior to the main TAG clusters, which could be C38 diglycerides (DAG) such as OA (*m/z* 264) and LA (*m/z* 262) and C40 diglycerides such as OB (*m/z* 264, *m/z* 265 and *m/z* 339), and LB (*m/z* 262 and *m/z* 337).

For the HO model, some of the highest regression coefficients to

Table 2
External validation of PLS-DA models (HL blends vs genuine olive oils; HO blends vs genuine olive oils) developed by FIA-HESI-HRMS, HT-GC–MS and RP-HPLC-RID screening methods. Results are mean values obtained from seven randomly selected validation sets. Results of individual sets are reported in Supplementary information (Table S3).

FIA-ESI-HRMS[a]						
	n	Adulterated (n)	Genuine (n)	Correct classification (%)	Sensitivity	Specificity
Adulterated	23	20.6 ± 1.7	2.4 ± 1.7	**89.4 ± 7.5**	0.89 ± 0.07	
HL blends	12	10.9 ± 0.7	1.1 ± 0.7	90.5 ± 5.8	0.90 ± 0.06	
HO blends	11	9.7 ± 1.4	1.3 ± 1.4	88.3 ± 12.6	0.88 ± 0.13	
Genuine	6	3.0 ± 0.8	3.0 ± 0.8	**50.0 ± 13.6**		0.50 ± 0.14
Total	29					
HT-GC-MS[b]						
	n	Adulterated (n)	Genuine (n)	Correct classification (%)	Sensitivity	Specificity
Adulterated	23	21.6 ± 0.5	1.4 ± 0.5	**93.8 ± 2.3**	0.94 ± 0.02	
HL blends	12	11.9 ± 0.4	0.1 ± 0.4	98.8 ± 3.2	0.99 ± 0.03	
HO blends	11	9.7 ± 0.5	1.3 ± 0.5	88.3 ± 4.4	0.88 ± 0.04	
Genuine	6	1.4 ± 0.8	4.6 ± 0.8	**76.2 ± 13.1**		0.76 ± 0.10
Total	29					
HPLC-RID[c]						
	n	Adulterated (n)	Genuine (n)	Correct classification (%)	Sensitivity	Specificity
Adulterated	23	20.6 ± 0.8	2.4 ± 0.8	**89.4 ± 3.4**	0.89 ± 0.03	
HL blends	12	11.6 ± 0.5	0.4 ± 0.5	96.4 ± 4.5	0.96 ± 0.04	
HO blends	11	9.0 ± 0.8	2.0 ± 0.8	81.8 ± 7.4	0.82 ± 0.07	
Genuine	6	2.4 ± 0.5	3.6 ± 0.5	**59.5 ± 8.9**		0.60 ± 0.09
Total	29					

For all models HL and HO models: n = 143 and ANOVA p-value < 0.05.
[a] HL model: mean LVs = 6, $Q^2 > 0.4104$, RMSEcv < 0.3626; HO model: mean LVs = 5, $Q^2 > 0.3594$, RMSEcv < 0.3972.
[b] HL model: mean LVs = 8, $Q^2 > 0.7404$, RMSEcv < 0.2898; HO model: mean LVs = 8, $Q^2 > 0.5679$, RMSEcv < 0.3292.
[c] HL model: mean LVs = 5, $Q^2 > 0.4551$, RMSEcv < 0.3473; HO model: mean LVs = 5, $Q^2 > 0.2674$, RMSEcv < 0.4069.

detect HO adulterated samples were also found in the C54 TAG cluster and included acyl species such as L, Ln, O and Po (Fig. 3). In agreement with the fragmentation and the expected elution order (Ruiz-Samblás et al., 2010), these glyceryl species would correspond to PoOA (*m/z* 237), OLL (*m/z* 262 and *m/z* 339), OOLn (*m/z* 335), LLL (*m/z* 262), OLLn (*m/z* 335), LLLn (*m/z* 335 and *m/z* 337), and OLnLn (*m/z* 335). Moreover, some other relevant coefficients were found in the C48 and C50 TAG clusters that included P and Po as acyl species, and that would agree with PPoPo (*m/z* 237) and PPoS (*m/z* 237) TAGs, respectively. In addition, some fragments that could correspond to DAG also appeared as relevant coefficients: PoL (*m/z* 311), SO (*m/z* 339), SLn and LLn (*m/z* 335), LA (*m/z* 262), LB (*m/z* 262) and OB (*m/z* 264 and *m/z* 265).

These relevant coefficients agree with those from the HPLC-RID and FIA-HESI-HRMS models, as all of them found C54 TAGs conformed by Ln and L (OLLn, LLLn), which are relevant for detecting HL adulterated samples. For the HO model, both HT-GC–MS and FIA-HESI-HRMS detected relevant variables corresponding to C54 TAGs conformed by longer chain FAs such as A, and for the HPLC-RID coefficients, C54 TAGs containing O and Ln (OOLn) were also significant to detect HO adulterated samples in the HT-GC–MS method.

4. Discussion

The high rate of correct classification achieved by ΔECN42 for genuine OO samples demonstrated the high specificity of the current reference method (Table 1), which guarantees that genuine samples will not be misclassified as adulterated. The specificity achieved by the official method in the present study was higher than that reported by Beccaria et al. (2016), who obtained between 16 and 19 % of false positives when analysing genuine EVOOs from very different geographical locations. This may denote a high dependence of the ΔECN42 parameter on OO characteristics or factors not included in the official analytical protocol. The official method also showed a high sensitivity for HL adulterants even when present in low amounts (≥2%). However, it proved unsuitable for the detection of adulteration with HO oils at 5–10 % as they have a similar TAG and FA composition to OO. This is a worrisome result, given that HO oils are emerging adulterants for the illegal blending of OO. While higher sensitivity values have been reported for the official method in HZO detection (García-González et al., 2007), our results agree with those of another study, that set the minimum detectable amount of HZO in OO at 20 % by this method (Moreda et al., 2003). The present results can be considered as representative of the efficacy of the method, as HO oil from different sources (HZO and HOSFO) and suppliers were included in the modelling and validation. Poor sensitivity towards HO adulterants, also reported by other authors (Beccaria et al., 2016), can thus be added to the other drawbacks of the official method. As well as being a time-consuming analytical procedure, requiring two different analyses per sample, it involves the use of highly contaminating organic solvents such as propionitrile, and lacks robustness due to the low resolution of the HPLC-RID peaks, specifically the very minor ECN42 cluster that includes the LLL peak (Beccaria et al., 2016).

In contrast, the new screening methods proved more suitable for the detection of adulterated samples, providing high sensitivity values regardless of the adulterant composition (Table 2). Among the developed screening tools, the HT-GC–MS method performed the best in terms of sensitivity (0.99 for HL and 0.88 for HO models). High sensitivity and a relatively short analysis time, allowing the rapid analysis of a high number of samples, are of paramount importance for a screening method (López et al., 2014). HT-GC–MS was also the screening tool that showed the highest specificity value (0.76). Although this value was lower than the one of the official method, it is an acceptable value for a screening method, given that confirmatory analyses or checks can be carried out afterwards to identify false positives (Magnusson & Örnemark, 2014). Moreover, it is important to highlight that these values were obtained with a sampling of a high natural variability as it included a high number of genuine olive oils of different varieties, regions and harvests, and various samples of various adulterant types, blended at low adulteration levels. These results demonstrate that a fingerprinting approach applied to multidimensional data such as

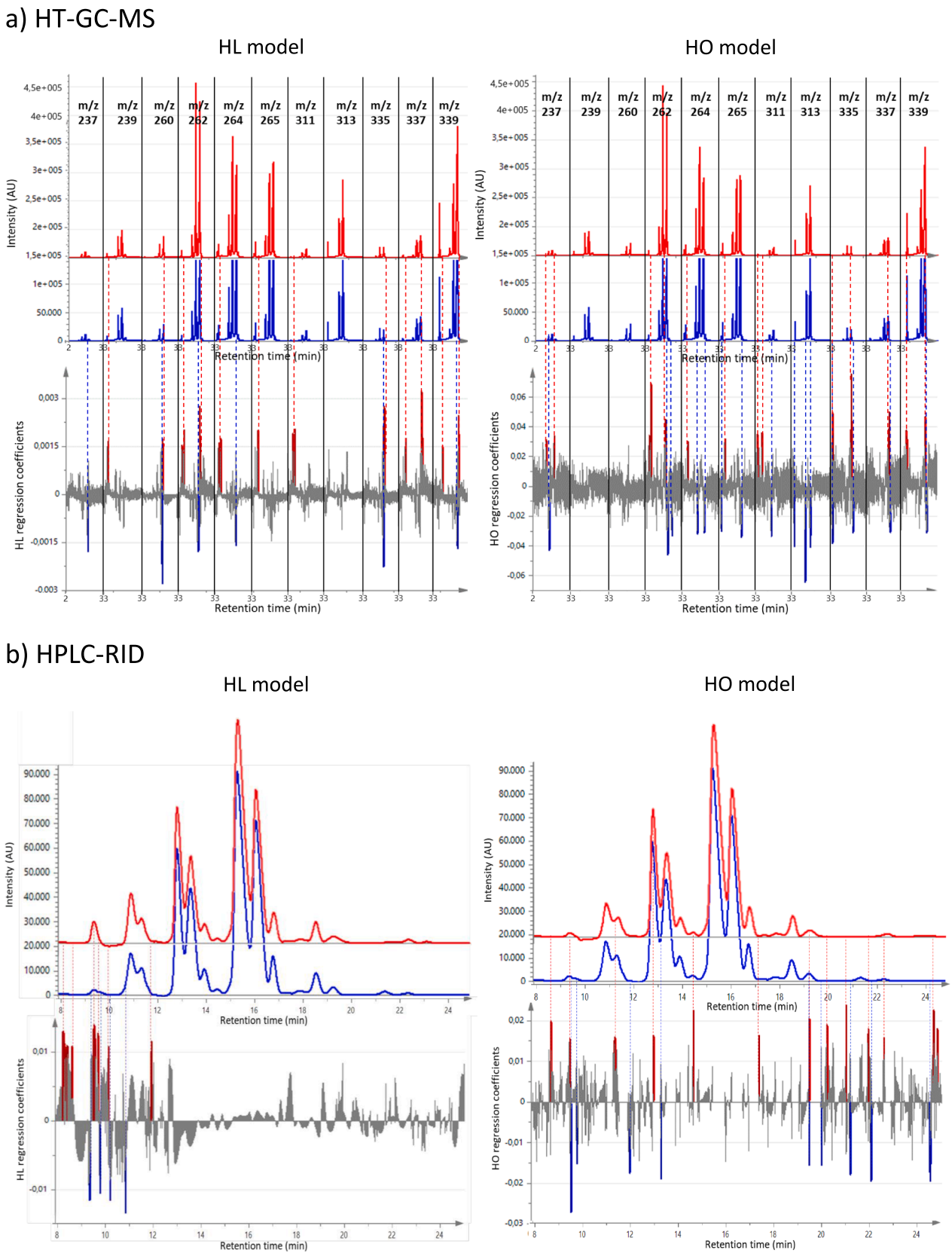

Fig. 3. Regression coefficients of the high linoleic (HL) and high oleic (HO) PLS-DA models on the a) HT-GC–MS fingerprint, plotted against the concatenated EICs of an adulterated sample (red EICs) and a genuine olive oil (blue EICs); and b) HPLC-RID fingerprint, plotted against the chromatographic profile of an adulterated sample (red chromatogram) and a genuine olive oil (blue chromatogram). For each model, relevant coefficients for the prediction of the adulterated class are highlighted in red (positive coefficient) and blue (negative coefficient).

GC–MS, can exploit the valuable specific information about authentication markers in the unfolded matrix built with specific TAG ions.

The method based on HPLC-RID also followed a fingerprinting approach, but its performance was lower compared to HT-GC–MS. This can be attributed to the RID-chromatographic data, which did not provide any specific information on these markers or distinguish unresolved or overlapping TAGs. Nonetheless, the HPLC-RID fingerprint effectively detected adulterated samples (sensitivity of 0.96 for HL and 0.82 for HO models) (Table 2) and is the most affordable and easily applicable of all the methods tested here. Considering that it is based on part of the data produced by the official method, the implementation of both in parallel could improve the detection of HO blends.

Finally, the FIA-ESI-HRMS method proved as successful as HPLC-RID in terms of overall sensitivity (0.89), showing a similar ability to detect both HL (0.90) and HO (0.88) adulteration adulterants (Table 2). The specificity value of this method (0.50) was low compared with that of previous models developed with the same method with a larger sample set (0.80) (Quintanilla-Casas et al., 2021). This fact indicates that the model developed upon this untargeted TAG profiling data seems to be highly dependent on the size of the sample set. Nonetheless, in view of the high sensitivity and extremely short analysis time (<2 min per sample) of this high-throughput method, its use with large-scale sampling could still be considered as a screening tool to be used in parallel with the current control practices.

Exploring the regression coefficients of the models developed for each screening method allowed us to tentatively ascertain the variables that contributed most to discriminate between adulterated and genuine samples. In all models, the highest regression coefficients corresponded to minor TAG species distributed within the entire experimental mass range. Although the structural elucidation of discriminant markers was not the scope of the present comparative study, some TAG species were tentatively identified in correspondence with the most relevant coefficients. Several of them were highly discriminant in models generated for different screening methods, demonstrating the consistency of the results. Particularly, TAGs that could correspond to PoLL/PLLn, OLLn, LLLn and LLL were highly discriminant for the HL models, whereas species that may match TAGs such as OOLn and OLLn and various other TAGs including Po, A or B were significant for the HO models.

In view of these results, the developed screening methods, especially HT-GC–MS TAG fingerprinting, could represent useful tools to facilitate inspections by official control bodies, improving the risk analysis on which they are currently based (Cugat & Biel, 2016). In particular, the high sensitivity of HT-GC–MS, an affordable and green analytical technique, indicates it has potential as a fit-for-purpose screening tool able to process a high number of samples and enhance the effectiveness of current official controls. Nonetheless, to confirm its high performance, this promising tool needs further testing with an enriched model, including a greater number of both genuine and adulterated samples.

5. Conclusion

Three different screening methods were tested as tools to support and improve official controls, being able to detect ≥2 % of HL and ≥5 % of HO adulterants in OO with sensitivity values >0.90 and >0.82, respectively. Among them, the HT-GC–MS method was the screening tool showing the best performance, with a 0.99 and a 0.88 sensitivity values for HL and for HO adulterants, respectively, and a specificity of 0.76, being the most promising screening tool tested for OO authentication.

The FIA-ESI-HRMS and HPLC-RID methods provided lower specificity values. However, these methods deserve to be further explored as possible supporting screening tools given that they still provide the high sensitivity desired for screening methods. FIA-ESI-HRMS is a fast technique proved to be suitable when assayed in large scale studies, and the HPLC-RID method can be run in parallel with the official OO analysis by using the same raw data.

The present study allowed to compare three screening methods among them and with the official method providing relevant information about their performance when applied to a dataset of genuine and adulterated samples with a high natural variability. On these bases, optimal models should be further developed and evaluated using a large scale dataset.

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CRediT authorship contribution statement

Berta Torres-Cobos: Formal analysis, Investigation, Methodology, Validation, Data curation, Visualization, Writing – original draft. **Beatriz Quintanilla-Casas:** Formal analysis, Investigation, Methodology, Data curation, Validation, Writing – review & editing. **Giulia Vicario:** Formal analysis, Investigation. **Francesc Guardiola:** Supervision, Writing – review & editing. **Alba Tres:** Conceptualization, Methodology, Resources, Supervision, Project administration, Funding acquisition, Writing – review & editing. **Stefania Vichi:** Conceptualization, Methodology, Resources, Supervision, Project administration, Funding acquisition, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

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