The occurrence: a meaningful parameter to be considered in the validation of multivariate classification-based screening methods – Application for authenticating virgin olive oil

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Abstract

The development of multivariate screening analytical methods in the analytical chemistry field focused particularly on food authentication is growing in recent years, which is evidenced by the increase of scientific publications. Currently there are several guides and technical reports about how -univariate qualitative methods should be properly validated to produce reliable and accurate (fitted-for-purpose) results. Nevertheless, this is not the case when multivariate methods are considered.

Aimed at redressing this untenable disadvantage, this paper proposes some guidelines for the validation of multivariate classification-based screening methods. As an application example, the detection of adulteration of virgin olive oil with any other edible vegetal oils is showed. The analytical techniques employed are liquid chromatography coupled to diode array detector (LC-DAD) and gas chromatography coupled to flame ionization detector (GC-FID). For the correct validation of the multivariate screening method a new parameter which never considered before, named occurrence, is accounted. Also, it has been developed two new applicability indicators of the multivariate screening methods: the assignation error index (I_{ERROR}) and the index saving (I_{SAVING}) to establish the validation requirements. Then the validation parameters of the methods: precision (or target predictive value), sensitivity, non-target predictive value, specificity and accuracy were estimated. The main conclusion of the work has been the need to take accounts the occurrence value to establish the specific validation requirements to apply the multivariate screening method in a particular scenario.

Keywords

Screening analytical methods, support vector machine and random forest multivariate classification, validation, occurrence, food authentication, virgin olive oil.

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1. Introduction

Analytical methods applied in laboratories should be evaluated to ensure that the results are reliable for the specific purpose for which the method is applied, namely, these must be validated. There are several definitions of the validation of analytical methods stated by different authors and collected in written standards or guidelines. In general, the validation of a process can be defined as *the verification, where the specified requirements are adequate for an intended use* [**1**]. A more particular definition related to the analytical method is *the process of defining an analytical requirement and confirming that the method under consideration has capabilities consistent with what the application requires* [**2**]. In this way, it is verified that the analytical method produces reliable and accurate results, *i.e.*, it is fitted-for-purpose. Two steps are always required: (1) to define the initial conditions, and (2) to establish the acceptation criteria for each performance parameter, commonly named validation parameters of the method, which should be not changed to adjust with the data obtained.

Regarding the chemical analysis there are different analytical test depending on the invoked information tier [**3**]:

(a) Quantitative. Analysis in which the amount of an analyte or the level of an analytical parameter may be obtained and expressed as a numerical value with the appropriate units.

(b) Qualitative. Analysis in which a material system is qualified according to experimental evidence related to its chemical composition or structure. So, the goal of these methods is the identification of components, classification of samples or materials regarding a specific parameter whose analytical response is yes or not.

Particular types of qualitative methods are the screening methods that are aimed at detecting an attribute or to provide information of the samples on an easily and quickly way, being the most common response of these methods is binary 'yes/no' [**4**,**5**]. In this sense, Valcárcel and Cárdenas proposed to incorporate a strategy in analytical routine laboratories, which was based in the use of two analytical methods in sequence, named vanguard and rearguard analytical methods [**6**].

Depending on the kind of the method is necessary to estimate certain analytical performance parameters, which are usually: sensitivity, accuracy or decision limit for qualitative methods, and, precision (in reproducibility and repeatability conditions), detection limit, quantification limit, trueness (analytical method bias or recovery rate), robustness and measurement uncertainty for quantitative methods [**2**]. Although strictly, the measurement uncertainty is not validation parameter of the analytical methods but rather a parameter associated with the acquisition and handling of data, and to the analytical steps.

69 For all of these analytical methods there are a lot of standard protocols and procedures,
70 which are used as guide to performing the validation and quality assurance [**2,7,8,9**]. In the
71 case of multivariate analytical methods, there are some guides published, although the
72 majority of these are based on the validation of multivariate quantitative method using
73 infrared spectrometric techniques [**10**,**11**]. However, there are scarcely protocols or standard
74 criteria to estimate the validation parameters for multivariate classification methods. Some
75 authors have published recommendations to validate these methods: Marini et al., published
76 a tutorial about different approaches to validate chemometric model but this focused on from
77 a conceptual point of view [**12**]; Esbesen *et al.* performed a tutorial based on the use of
78 cross-validation how strategy for the evaluation of classification and quantification
79 multivariate methods [**13**]; Alewijn *et al.*, published a work explaining the procedure of
80 validation of a multivariable classification method based on analytical fingerprints [**14**], and
81 López *et al.*, published a tutorial focused on mainly in the estimation of the performance
82 parameters comparing univariate and multivariate methods [**15**].

83 According to our scientific knowledge, Eurolab Guide [**16**] is the first protocol published in
84 which is established a standard criteria and minimum requirements to ensure the right
85 validation of an analytical multivariate method (qualitative and quantitative), when the NMR
86 spectrometry is applied. In this guide is indicated the minimum number of samples necessary
87 to develop and then to validate the multivariate method. In this sense, the performance
88 parameters proposed are widely known: success/error contingency, sensitivity, specificity,
89 detection limit and quantification limit. This guide represents a considerable step forward
90 within the validation of multivariate analytical methods; since the use of these methods to
91 solve real analytical problems, when conventional approach (univariate approach) is not in
92 itself sufficient, is increasing. Even in the pharmaceutical field [**17**,**18**] the use of analytical
93 multivariate methods is being recommended and implemented, in contrast to food field
94 whose 'formal' application has not yet come.

95 The crucial question to solve, before developing a new method, is to determine what use will
96 be made of the results. From this use, the acceptation criteria of the method will establish
97 according to the final aim purposed. In this paper, the Eurolab Guide is followed but is has
98 been adapted for screening methods focusing on the olive oil authentication topic. The
99 current legislation [**19**] establishes that for the authentication of the olive oil purity is
100 necessary to determine around 50 analytical parameters that involves high economic cost
101 and time-consuming of the laboratories. In the most studies published the authors carried out
102 the authentication of olive oil analysing major compounds as triglycerides [**20**,**21**] or fatty acid
103 [**22**,**23**], or a specific fraction of minor compounds as sterols [**24**,**25**], phenols [**26**],
104 tocopherols [**27**] and volatiles [**28**] when are employed chromatographic techniques; this last

105 involves a prior-step of pre-treatment of sample in order to isolate the interest fraction.

106 In this sense, it is proposed two analytical multivariate screening methods using normal-
107 phase high-performance liquid chromatography coupled to a diode array UV absorption
108 detector ((NP)HPLC-DAD) and high-temperature gas chromatography-flame ionization
109 detector ((HT)GC-FID) together to support vector machine (SVM) and random forest (RF)
110 classification methods, which are correctly validated and in addition no pre-treatment of
111 sample is employed. For the validation of the methods, a new parameter never considered
112 before, named occurrence, is accounted for. Furthermore, the validation parameters of the
113 analytical methods (precision, –or target predictive value–, sensitivity, non-target predictive
114 value, specificity and accuracy or efficiency) were estimated according to this new
115 parameter. Besides, two new applicability indicators are proposed for first time: saving index
116 and assignation error index, to evaluate the profits of the analytical laboratory and the
117 screening error of the analytical multivariate method, respectively.

118

119 **2. Evaluation methodology**

120 The traditional analytical procedures applied for the evaluation of the quality or for the
121 detection of the adulteration of foods are time-consuming and expensive, since it is
122 necessary carried out a battery of analysis on all samples to assure their genuineness.
123 Usually this compulsory required to perform several analytical determinations related to
124 certain characteristic chemical components of the food (chemical markers).

125 An alternative is the application and validation of a multivariate classification-based method
126 of screening (vanguard method) before the carrying out the quantitative confirmation
127 methods (rearguard methods) to detect adulteration of virgin olive oil with other edible
128 vegetable oils. Only the samples assigned as 'adulterated olive oil' are subjected to
129 confirmatory analysis (rearguard method) while the samples declared as 'genuine virgin olive
130 oil' finish the analytical running. Figure 1 shows a graphical scheme of the applied
131 methodology.

132

Figure 1

133

134 Consequently, this would involve an economic saving for the routine analysis laboratories,
135 since the samples classified as suitable would not be submitted to all analysis. In order to
136 evaluate this economic saving, in this study we propose a practicability indicator called by us
137 as saving index, whose equation is:

138 $$I_{SAVING}\,(\%) = \frac{\text{Assig T}}{\text{TOTAL}} \times 100$$

139 Where 'Assig T' is the total number of samples assigned as belonging to the target class (the
140 class collecting the compliant objects(samples) and 'TOTAL' is the number of samples
141 analysed.

142 Moreover, another practicability indicator, named assignation error index, is proposed which
143 indicate the risk of erroneously assigning a sample as belonging to the target class, whose
144 equation is:

145 $$I_{ERROR}\,(\%) = \frac{e_{nT}}{\text{TOTAL}} \times 100$$

146 where 'e_{nT}' is the number of samples wrongly assigned to the target class. The meaning of
147 these terms could be easily understood by examining Table 3.a.

148 However, to be able to properly validate this methodology in working conditions is necessary
149 to account a parameter never considered before in multivariate analysis: the occurrence
150 (OCURR). The occurrence is a population-parameter; it means the rate of samples which
151 display a property of interest regarding to the population of samples which are subjected to
152 analysis in the laboratory. The occurrence is usually related to the samples of the target
153 class. The knowledge of this parameter is crucial to verify that the screening method works
154 correctly for the intended purpose.

155 The occurrence has a direct influence on two aspects of the analytical validation of screening
156 methods: (i) the definition of the validation requirements for the performance parameters
157 (precision, sensitivity, specificity and accuracy or efficiency), for a intended value of the
158 practicability indicators, should be established in function of the occurrence; and (ii) the
159 proportion of target and non-target samples in the validation set should be coherent with the
160 occurrence, that is to say, the following condition should be met:

161 $$\frac{\text{Tot T}}{\text{Tot nT}} \approx \frac{\text{OCURR}}{1 - \text{OCURR}}$$

162 where 'Tot T' and 'Tot nT' symbolise respectively the number of target and non-target
163 samples in the validation set.

164

165 **3. Validation requirements**

166 A real analytical application of the method involves the establishment of validation
167 requirements in order to evaluate if the method is fitted-for-purpose. In this sense is
168 necessary to decide: (i) the index saving, which means the rate of samples will not analysed

by the rearguard method considering a reliable assignation, and (ii) the assignation error index, namely the risk which it is willing to assume the presence of adulterated products in the market. Thus, it is requires estimating what precision and sensitivity values will be consistent with the applicability indexes decided. Therefore, the equations for determining the critical values of these validation parameters are:

$$\mathrm{PREC} = 1 - \frac{\mathrm{I_{ERROR}}}{\mathrm{I_{SAVING}}}$$

$$\mathrm{SENS} = (\mathrm{I_{SAVING}} - \mathrm{I_{ERROR}}) \times \frac{1}{\mathrm{OCURR}}$$

$$\mathrm{SPEC} = 1 - \mathrm{I_{ERROR}} \times \frac{\mathrm{PREC}}{1 - \mathrm{OCCUR}}$$

where PREC, SENS and SPEC denote the precision, and sensitivity and specificity of the screening method. Notice that the specificity is a validation parameter which is habitually stated, but it is linked to PREC and does not provide significant information for validation purpose of screening methods and could be left out.

For the application example of this study, an occurrence of 0.90 could be considered as representative of the target class (genuine virgin olive oil) in the worst case, that is, a maximum of ten adulterated samples (i.e., fraudulent blend of olive oil whit other edible vegetal oils) are found for every hundred analysed samples (10%). Therefore, the 90% of total of number of samples, which composed the validation set of the screening method, were genuine virgin olive oil samples and the rest were adulterated olive oil samples (10%).

Then, the validation requirements selected by us for the screening classification methods involved in this study were: 5% for $\mathrm{I_{ERROR}}$ and 66.7% for $\mathrm{I_{SAVING}}$. This means that is assumed the risk of misclassification of adulterated samples of one in twenty and as compensation, only one in three samples requires confirmation by a rearguard method. Thus, the critical values of precision and sensitivity, calculated from the previously defined equations, were 0.93 and 0.68 respectively.

4. Materials and methods

4.1 Chemicals

HPLC-grade solvents (n-hexane and isopropanol) were purchased from PANREAC Química, (Barcelona, Spain).

199

200 **4.2 Samples**

201 A total of 207 vegetable oils samples were analysed: 87 single-variety extra-virgin olive oil
202 samples (EVOO) of different regions from Spain and olive fruit varieties, 8 coupage-EVOO
203 samples and 112 blends of olive oil with other vegetable edible oils from ten botanical
204 origins: avocado (4), corn (4), palm (2), flax (2), peanut (2), pomace olive oil (17), safflower
205 (2), sesame (4), sunflower (38), and non-declared seeds (21). Tables 1 and 2 show a
206 description more detailed of the different oil samples studied.

207

Table 1

208

Table 2

209

210 **4.3 Sample preparation**

211 0.1 g of oil was placed in a 4 mL tube and mixed with 1 g of n-hexane. Then, the sample
212 solutions were passed through a polytetrafluoroethylene (PTFE) membrane syringe filter
213 (0.22 µm) and the solution stored at –20ºC until analysis. For the chromatographic analysis
214 the samples were diluted with n-hexane again at a 1:1 ratio.

215

216 **4.4 Chromatography**

217 (NP)HPLC-DAD analysis was performed with an Agilent 1260 series liquid chromatograph
218 (Santa Clara, USA) equipped with a column thermostat (Eppendorf CH30), a quaternary
219 pump and degasser auto sampler. Detection was performed with a diode-array detector
220 (DAD). Agilent ChemStation OpenLab CDS software (rev. C.01.09) for LC systems was used
221 to collect and process data. Lichrospher® 100 CN column (250 $\times$ 4 mm i.d, 5 µm) was
222 employed for the analysis and the column temperature was set at 30 ºC during the entire
223 operation. The composition of the mobile phase was n-hexane/isopropanol (96:4, v/v) at a
224 flow rate of 1.2 mL min^{-1}. The volume of injection was 20 µL and the run time was only 8 min
225 at a column temperature of 30ºC. The DAD collected spectra every 2 s in the range 190-400
226 nm, each 1 nm.

227 (HT)GC-FID analysis was carried out with a VARIAN GC 3800 gas chromatograph (PA,
228 USA) equipped with a split/splitless injector and a flame ionization detector (FID). A capillary

229 column coated with 65% diphenyl-35% dimethylpolysiloxane stationary phase (Rtx-65TG,
230 30m×0.25mm i.d. × 0.1 μm film thickneses, maximum; Restek Corp., Bellefonte, PA, USA)
231 was used. The GC oven temperature was programmed from 300 to 360°C at 10°C/min.
232 Scan control, data acquisition, and processing were performed by a MS Workstation
233 software (VARIAN, PA, USA) data system. The injection port was held isothermally at 310°C.
234 Helium (99.995%, Air Liquide, Madrid, Spain) was used as the carrier gas and the flow rate
235 was 1.5 ml/ min. The samples were introduced using a CombiPAL robotized autosampler
236 module (CTC Analytics, Zwingen, Switzerland). The volume injection was 1 μL and the split-
237 ratio injection was 1:10.

238

239 **4.5 Chemometrics**

240 The raw data files from each chromatogram were exported in 'comma separated value'
241 (CSV) format, and then converted to MATLAB format (version R2017b).

242 The dimension of the chromatogram-spectrum obtained from (NP)HPLC-DAD for each
243 sample was 2399 × 211 where 2399 is the number of rows corresponding to the number of
244 elution times and 211 is the number of absorbance spectra recorded. On the other hand, the
245 data vector collected from (HT)GC-FID was composed of 10189 variables.

246 The data pre-processing was done with a home-programmed MATLAB function. This
247 function implemented several algorithms from the MATLAB Bioinformatics Toolbox™ and
248 'icoshift' algorithm to align the peaks of the chromatograms. The pre-processing steps were:
249 (i) grouping and overlay of the chromatograms, (ii) selection of the region of interest (ROI)
250 (iii) elimination of the noise, (iv) correction of the baseline, (v) alignment of the peaks, and (vi)
251 autoscaling of the data set.

252 The original data set collected for both analytical techniques was split into two groups:
253 training and validation sets. The division of group was performed considering the occurrence
254 parameter for the validation set. Thus, the training set was made up of 147 samples (50
255 EVOO and 97 adulterated-EVOO samples) and validation set was composed by 50 samples
256 (45 EVOO plus 5 adulterated-EVOO samples). The split of the samples between training and
257 validation set was carried out by application of the Kenard-Stone algorithm [**29**].

258 The SVM and RF models were developed using PLS_Toolbox (version 8.6.1, Eigenvector
259 Research, Wenatchee, WA) and perClass Toolbox ver 4.7 (Deft, Netherlands), respectively.
260 Both software were applied under Matlab environment (Mathworks Inc., Natick, MA, USA). A
261 summarised description of how these machine learning classification methods woks may be

262 found in a previous paper of the authors [**30**]; for a more detailed explications, the readers
263 are requested to consult the specialized references quoted in that paper.

264

265 **5. Results and discussion**

266 Figure 2a and 2b shows the 2D analytical signal (heat map) collected using (NP)HPLC-DAD
267 of an genuine extra-virgin olive oil sample from 'arbequina' cultivar and an adulterated olive
268 oil sample (60% non-olive oil plus 40% EVOO).

269

Figure 2

270

271 As the figure 1 illustrated there are significant differences between the 2D analytical signals
272 of both samples, in the signal associated with adulterated olive oil sample is showed two time
273 intervals, from 2 to 3 min and from 5 to 7.5 min, in which appear new characteristic signals
274 from the non-olive oil sample (marked in red rectangles).

275 On the other hand, figure 3a and 3b displays the chromatograms from a genuine virgin olive
276 oil sample from 'cornicabra' cultivar and an adulterated olive oil sample (55% non-olive oil
277 plus 45% EVOO). The region of interest was found from 7.3 to 13 min.

278

Figure 3

279

280 Differences exist also between the chromatograms of both samples. In the adulterated olive
281 oil sample (fig 3b) turn up three new peaks at the end of the chromatogram.

282

283 **5.1 Dimensionality reduction**

284 The data collected from (NP)HPLC-DAD were three-way data, consequently, was necessary
285 to perform a variable reduction in order to extract the relevant information. For this purpose
286 the 'decomposition and vector fusion' (DVF) strategy was applied. This methodology is based
287 on obtaining of two mean individual vectors per sample which correspond to both time and
288 spectral domains. Then, these two vectors are fused per sample. Finally, all the fused
289 vectors per sample were grouped in a matrix with as many rows as samples analysed and as
290 many columns as variables (207 × 2609; 207 samples and 2609 variables). A detailed
291 description of the DVF procedure is described elsewhere [**31**].

292

293 **5.2 Exploratory analysis**

294 Principal component analysis (PCA) was previously carried out for exploring whether there
295 were natural groupings of different oils. Two PCA models were performed considering the
296 dataset obtained from (NP)HPLC-DAD and (HT)GC-FID. Five components were selected for
297 both models which explained 78.30% and 71.67% of variance for the dataset from
298 (NP)HPLC-DAD and (HT)GC-FID, respectively. Figure 4a and 4b shows the biplot for scores
299 on the PC2-PC1 plane, corresponding to the data subset from (NP)HPLC-DAD and (HT)GC-
300 FID, respectively.

301

Figure 4

302

303 Although both scores biplots allowed to distinguish the two groups of samples (adulterated vs
304 non-adulterated olive oil), the data from (NP)HPLC-DAD showed a further differentiation. As
305 it can be observed, the first principal component in the negative quarter groups the genuine
306 virgin olive oil (green squares).

307

308 **5.3 SVM methods**

309 As in the previous section, two SVM models were developed, one for each chromatographic
310 technique. The class 'genuine virgin olive oil' was indexed with the value 1 and the class
311 'adulterated olive oil' with the value 0. The decision criterion established for the classification
312 of the samples was a threshold value of 0.5. This means that all the samples with scores
313 greater than 0.5 will be classify as 'genuine virgin olive oil' and samples with scores lowers
314 than 0.5 will be classify as 'adulterated olive oil'.

315 Classification results for the validation of the methods are showed in figure 5a and 5b for
316 (NP)HPLC-DAD and (HT)GC-FID, respectively.

317

Figure 5

318

319 The classification results obtained from (NP)HPLC-DAD were better than the results from
320 (HT)GC-FID. The model from liquid chromatography misclassified only three olive samples in
321 contrast to the model from gas chromatography where eleven genuine virgin olive oil

322 samples are wrongly classified. However, it has to be underlined that no sample of
323 adulterated olive oil is classified as genuine virgin olive oil, therefore for our purpose, to
324 develop of an analytical method for the screening of non-adulterated virgin olive oils with
325 other vegetable oils; both chromatographic techniques could be effective. Note that all the
326 samples stated as 'adulterated' by the screening methods (vanguard method) will subjected
327 to confirmatory analysis by the rearguard method and then, the false non-compliant samples
328 would probably be reclassified as compliant ones or not-adulterated. The only (but not trivial)
329 difference is that, if the model from GC data is applied, a larger number of samples will
330 require confirmation.

331

332 **5.3.1 Validation**

333 Estimated parameters to carry out the validation of the SVM methods were: (i) contingency
334 table; and (ii) performance parameters as precision (PREC), this value is also known as
335 target predictive value' (TPV), sensitivity (SENS), non-target predictive value (NPV),
336 specificity (SPEC) and accuracy (ACCU) [**32**]. Note that, the terms 'positives' or 'negatives'
337 are no used in this paper because in an authentication approach, positives should be always
338 referred to the target samples, in this instance, the compliant samples (i.e., the non-
339 adulterated samples) while negatives are the non-compliant ones (i.e., the adulterated
340 samples); caution is needed because this may lead to confusion.

341 In addition, the new applicability indicators: error index (I_{ERROR}) and saving index (I_{SAVING}),
342 which were described in the section 2, were determined. Table 3 shows how the validation
343 parameters are calculated.

344

Table 3

345

346 It should be stressed that only the performance parameters related to the target class
347 (precision and sensibility) provide useful information when the classification is used as a
348 screening method. Indeed, precision and sensibility inform on the proportion of agreements
349 in relation to all assignments of target class or to all the samples belonging to the target
350 class, respectively. On the contrary, non-target predictive value and specificity yield the same
351 information on the non-target class. The success/errors of the samples belonging to the non-
352 target class are not critical information since they all are subjected to the rearguard method.

353 Table 4 shows the performance parameter from the SVM methods. The results found confirm
354 that the best method is obtained from (NP)HPLC-DAD. Although as stated above, no

355 sample of adulterated olive oil is classified as genuine virgin olive oil for both
356 chromatographic techniques, the sensitivity and accuracy of SVM method from (HT)GC-FID
357 is too low (SENS = 0.75 & ACCU = 0.78) in comparison with another method (SENS = 0.93
358 & ACCU = 0.94).

359

Table 4

360

361 Table 5 displays the new performance indicators propose by us. Both methods show an error
362 index of 0%, but the most important consideration is that the laboratory would have a saving
363 index of the 84% using the (NP)HPLC-DAD. Therefore, it would be only 8 samples should be
364 analysed using the confirmation method (rearguard method) to verify the adulteration of
365 these, which imply, in the case of the olive oil.

366

Table 5

367

368 **5.4 RF methods**

369 Selection of number of trees was carried determining the estimates errors on the training set
370 by comparing true labels and decisions. Thus, eight and seven trees were selected and
371 combined to perform the RF methods from the dataset recorded for both chromatographic
372 techniques, respectively. The classification is performed by a majority of assignation from the
373 different decision trees [**30**].

374 For both methods the classification results were similar, one adulterated olive sample was
375 classified as genuine virgin olive oil sample and fourteen and fifteen genuine virgin olive oil
376 samples were identified as adulterated-olive samples from (NP)HPLC-DAD and (HT)-GC-FID
377 data, respectively.

378 Comparing SVM and RF methods, the first methods are more reliable than the second ones.
379 Besides, this fact was verified in the method validation.

380

381 **5.4.1 Validation**

382 Similarly, the performance parameters and applicability indicators were calculated according
383 to 5.3.1 section (table 3) and are showed in tables 6 and 7, respectively.

384

Table 6

385

Table 7

386

387 Although for both methods the precision value approximately or equal to 1, meaning that the
388 majority of adulterated olive oil samples are detected (only one sample is misclassified by
389 (HT)GC-FID), the rest of validation parameters are low in comparison with SVM. Both RF
390 methods are making low error indexes (0% and 2% for (NP)HPLC-DAD and (HT)GC-FID,
391 respectively). However, in the case of (NP)HPLC-DAD the method does not satisfy the
392 remaining of the validation requirements, since the saving index is 60.0% instead of 66.7%
393 and the sensitivity value is 0.66 rather than 0.68. Consequently, this methodology could not
394 be valid for the purpose of this study.

395

396 **6. Conclusions**

397 This work shows a fast and easy methodology to validate a multivariate analytical method of
398 screening (vanguard method) considering the real conditions and the scenario where the
399 method must work. Besides, two applicability indicators have been proposed for first time in
400 order to assess economic saving for the routine analysis laboratories. Although, this
401 methodology has been employed in the detection of adulteration of virgin olive oil, it could be
402 employed in other scenario about adulteration in the food field. Considering, the occurrence
403 value necessary in order to validate of the analytical method in real conditions. In addition the
404 validation parameters (precision and sensitivity) should be established after deciding about
405 the main goal and results which the analysis or laboratory would like to obtain, namely first
406 the applicability indicators should be specified. It should however be highlighted that the
407 occurrence value is an imposed value and its value should be previously know, though this is
408 sometimes difficult.

409

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413

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415 **Tables**

416

Table 1. Olive oil samples analyzed.

Sample	Cultivar	Nº
Monovarietal-EVOO	Alfafarenca	1
	Arbequina	28
	Arbosana	2
	Blanqueta	1
	Cornicabra	5
	Empeltre	1
	Farga	1
	Frantoio	3
	Hojiblanca	5
	Koroneiki	3
	Lechín	1
	Loaimes	3
	Lucio	3
	Manzanilla	3
	Negrete	1
	Ocal	1
	Oliana	1
	Picual	12
	Picudo	4
	Royal	3
	Serrana	1
	Sikitita	1
	Tosca	1
	Verdial	1
	Vidueña	1
Coupage-EVOO	Royal + Cornezuelo	1
	Arbequina + Cornicabra	2
	Arbequina + Picual	2
	No declared	3
	Total	95

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Table 2. Percentage and composition of the olive oil and other vegetable edible oil in the oil blend samples.

Nº		Composition
1	90% nOO + 10% EVOO	90% sunflower#1 oil + 10% EVOO#1
2	90% nOO + 10% EVOO	90% sunflower#2 oil + 10% EVOO#2
3	90% nOO + 10% EVOO	90% sunflower#3 oil + 10% EVOO#3
4	90% nOO + 10% EVOO	90% sunflower#4 oil + 10% EVOO#4
5	80% nOO + 20% EVOO	80% sunflower#5 oil + 20% EVOO#5
6	80% nOO + 20% EVOO	80% seeds#1 oil + 20% EVOO#6
7	80% nOO + 20% EVOO	80% sunflower#6 oil + 20% EVOO#7
8	80% nOO + 20% EVOO	80% seeds#2 oil + 20% EVOO#8
9	70% nOO + 30% EVOO	70% pomace#1 oil + 30% EVOO#9
10	70% nOO + 30% EVOO	70% sunflower#7 oil + 30% EVOO#10
11	70% nOO + 30% EVOO	70% pomace#2 oil + 30% EVOO#11
12	70% nOO + 30% EVOO	70% sunflower#8 oil + 30% EVOO#12
13	70% nOO + 30% EVOO	70% pomace#3 oil + 30% EVOO#13
14	70% nOO + 30% EVOO	70% pomace#4 oil + 30% EVOO#14
15	70% nOO + 30% EVOO	70% sunflower#9 oil + 30% EVOO#15
16	70% nOO + 30% EVOO	70% sunflower#10 oil + 30% EVOO#16
17	70% nOO + 30% EVOO	70% sunflower#11 oil + 30% EVOO#17
18	70% nOO + 30% EVOO	70% sunflower#12 oil + 30% EVOO#18
19	67% nOO + 33%EVOO	67% avocado#1 oil + 33% EVOO#19
20	67% nOO + 33%EVOO	67% avocado#2 oil + 33% EVOO#20
21	66% nOO + 34%EVOO	66% pomace#5 oil + 34% EVOO#21
22	66% nOO + 34%EVOO	66% pomace#6 oil + 34% EVOO#22
23	66% nOO + 34%EVOO	66% seeds#3 oil + 34% EVOO#23
24	66% nOO + 34%EVOO	66% seeds#4 oil + 34% EVOO#24
25	65% nOO + 35% EVOO	65% seeds#5 oil + 35% EVOO#25
26	65% nOO + 35% EVOO	65% pomace#7 oil + 35% EVOO#26
27	65% nOO + 35% EVOO	65% seeds#6 oil + 35% EVOO#27
28	65% nOO + 35% EVOO	65% pomace#8 oil + 35% EVOO#28
29	60% nOO + 40% EVOO	60% sunflower #13 oil + 40% EVOO#29
30	60% nOO + 40% EVOO	60% sesame#1 oil + 40% EVOO#30

31	60% nOO + 40% EVOO	60% sunflower #14 oil + 40% EVOO#31
32	60% nOO + 40% EVOO	60% sesame#2 oil + 40% EVOO#32
33	60% nOO + 40% EVOO	60% sunflower #15 oil + 40% EVOO#33
34	60% nOO + 40% EVOO	60% sunflower #16 oil + 40% EVOO#34
35	58% nOO + 42% EVOO	58% seeds#6 oil + 42% EVOO#35
36	58% nOO + 42% EVOO	58% seeds#7 oil + 42% EVOO#36
37	57% nOO + 43% EVOO	57% seeds#8 oil + 43% EVOO#37
38	57% nOO + 43% EVOO	57% seeds#9 oil + 43% EVOO#38
39	55% nOO + 45% EVOO	55% sunflower#17 oil + 45% EVOO#39
40	55% nOO + 45% EVOO	55% seeds#10 oil + 45% EVOO#40
41	55% nOO + 45% EVOO	55% sunflower#18 oil + 45% EVOO#42
42	55% nOO + 45% EVOO	55% seeds#11 oil + 45% EVOO#42
43	55% nOO + 45% EVOO	55% sunflower#19 oil + 45% EVOO#43
44	55% nOO + 45% EVOO	55% sunflower#20 oil + 45% EVOO#44
45	55% nOO + 45% EVOO	55% pomace#9 oil + 45% EVOO#45
46	55% nOO + 45% EVOO	55% pomace#10 oil + 45% EVOO#46
47	54% nOO + 46% EVOO	54% sunflower#21 oil + 46% EVOO#47
48	54% nOO + 46% EVOO	54% sunflower#22 oil + 46% EVOO#48
49	50% nOO + 50% EVOO	50% sunflower#23 oil + 50% EVOO#49
50	50% nOO + 50% EVOO	50% seeds#11 oil + 50% EVOO#50
51	50% nOO + 50% EVOO	50% sunflower#24 oil + 50% EVOO#51
52	50% nOO + 50% EVOO	50% seeds#12 oil + 50% EVOO#52
53	50% nOO + 50% EVOO	50% pomace#11 oil + 50% EVOO#53
54	50% nOO + 50% EVOO	50% pomace#12 oil + 50% EVOO#54
55	50% nOO + 50% EVOO	50% sunflower#25 oil + 50% EVOO#55
56	50% nOO + 50% EVOO	50% sunflower#26 oil + 50% EVOO#56
57	48% nOO + 52% EVOO	48% pomace#12 oil + 52% EVOO#57
58	48% nOO + 52% EVOO	48% pomace#13 oil + 52% EVOO#58
59	47% nOO + 53% EVOO	47% avocado#3 oil + 53% EVOO#59
60	47% nOO + 53% EVOO	47% avocado#4 oil + 53% EVOO#60
61	46% nOO + 57% EVOO	46% sunflower#27 oil + 57% EVOO#61
62	46% nOO + 57% EVOO	46% sunflower#28 oil + 57% EVOO#62
63	45% nOO + 55% EVOO	45% pomace#14 oil + 55% EVOO#63
64	45% nOO + 55% EVOO	45% corn#1 oil + 55% EVOO#64
65	45% nOO + 55% EVOO	45% pomace#15 oil + 55% EVOO#65
66	45% nOO + 55% EVOO	45% corn#2 oil + 55% EVOO#66

67	40% nOO + 60% EVOO	40% sunflower#29 oil + 60% EVOO#67
68	40% nOO + 60% EVOO	40% seeds#12 oil + 60% EVOO#68
69	40% nOO + 60% EVOO	40% sunflower#30 oil + 60% EVOO#69
70	40% nOO + 60% EVOO	40% seeds#13 oil + 60% EVOO#70
71	40% nOO + 60% EVOO	40% safflower#1 oil + 60% EVOO#71
72	40% nOO + 60% EVOO	40% safflower #2 oil + 60% EVOO#72
73	35% nOO + 65% EVOO	35% sunflower#31 oil + 65% EVOO#73
74	35% nOO + 65% EVOO	35% sunflower#32 oil + 65% EVOO#74
75	34% nOO + 66% EVOO	35% seeds#13 oil + 65% EVOO#75
76	34% nOO + 66% EVOO	35% seeds#14 oil + 65% EVOO#76
77	30% nOO + 70% EVOO	30% sunflower#31 oil + 70% EVOO#77
78	30% nOO + 70% EVOO	30% pomace#16 oil + 70% EVOO#78
79	30% nOO + 70% EVOO	30% sunflower#32 oil + 70% EVOO#79
80	30% nOO + 70% EVOO	30% pomace#17 oil + 70% EVOO#80
81	27% nOO + 73% EVOO	27% flax#1 oil + 73% EVOO#81
82	27% nOO + 73% EVOO	27% flax#2 oil + 73% EVOO#82
83	25% nOO + 75% EVOO	25% sesame#3 oil + 75% EVOO#83
84	25% nOO + 75% EVOO	25% sesame#4 oil + 75% EVOO#84
85	24% nOO + 76% EVOO	24% seeds#15 oil + 76% EVOO#85
86	24% nOO + 76% EVOO	24% seeds#16 oil + 76% EVOO#86
87	22% nOO + 78% EVOO	22% corn#3 oil + 78% EVOO#87
88	22% nOO + 78% EVOO	22% corn#4 oil + 78% EVOO#88
89	22% nOO + 78% EVOO	22% sunflower #33 oil + 78% EVOO#89
90	22% nOO + 78% EVOO	22% sunflower #34 oil + 78% EVOO#90
91	21% nOO + 79% EVOO	21% palm#1 oil + 79% EVOO#91
92	21% nOO + 79% EVOO	21% palm#2 oil + 79% EVOO#92
93	21% nOO + 79% EVOO	21% seeds#15 oil + 79% EVOO#93
94	21% nOO + 79% EVOO	21% seeds#16 oil + 79% EVOO#94
95	20% nOO + 80% EVOO	20% sunflower#35 oil + 80% EVOO#95
96	20% nOO + 80% EVOO	20% seeds#17 oil + 80% EVOO#20
97	20% nOO + 80% EVOO	20% sunflower#36 oil + 80% EVOO#10
98	20% nOO + 80% EVOO	20% seeds#18 oil + 80% EVOO#6
99	20% nOO + 80% EVOO	20% peanut#1 oil + 80% EVOO#36
100	20% nOO + 80% EVOO	20% peaut#2 oil + 80% EVOO#50
101	17% nOO + 83% EVOO	17% seeds#18 oil + 83% EVOO#74
102	17% nOO + 83% EVOO	17% seeds#19 oil + 83% EVOO#82

103	15% nOO + 85% EVOO	15% sunflower#37 oil + 85% EVOO#10
104	15% nOO + 85% EVOO	15% sunflower#38 oil + 85% EVOO#8
105	10% nOO + 90% EVOO	10% sunflower#3 oil + 90% EVOO#23
106	10% nOO + 90% EVOO	10% seeds#20 oil + 90% EVOO#15
107	10% nOO + 90% EVOO	10% sunflower#9 oil + 90% EVOO#90
108	10% nOO + 90% EVOO	10% seeds#21 oil + 90% EVOO#24
109	100% nOO	50% sunflower#2 oil + 50% sesame#2 oil
110	100% nOO	50% sunflower#10 oil + 50% sesame#8 oil
111	100% nOO	50% sunflower#21 oil + 50% seeds#8 oil
112	100% nOO	50% sunflower#2 oil + 50% sesame#2 oil

EVOO: Extra virgin olive oil; nOO*: Non-olive oil*

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Table 3. Parameters of validation of a classification method.

a) Contingency table

		Tot T	**Tot nT**	**TOTAL**
Assignation	**nT**	e_T	a_{nT}	**Assig nT**
	T	a_T	e_{nT}	**Assig T**
		T	**nT**	
		Real		

b) Estimating of parameters

Parameter	**Equation**
Performance parameters related to the target class	
Precision (PREC) or target predictive value (TPV)	$\mathrm{PREC} = \frac{a_T}{a_T + e_{nT}} = \frac{a_T}{\mathrm{Assig\ T}}$
Sensitivity (SENS)	$\mathrm{SENS} = \frac{a_T}{a_T + e_T} = \frac{a_T}{\mathrm{Tot\ T}}$
Performance parameters related to the non-target class	
Non-target predictive value (NPV)	$\mathrm{NPV} = \frac{a_{nT}}{a_{nT} + e_T} = \frac{a_{nT}}{\mathrm{Assig\ nT}}$
Specificity (SPEC)	$\mathrm{SPEC} = \frac{a_{nT}}{a_{nT} + e_{nT}} = \frac{a_T}{\mathrm{Tot\ nT}}$
Overall performance parameters	
Accuracy (ACCU) or Efficiency (EFFIC)	$\mathrm{ACCU} = \frac{a_T + a_{nT}}{a_T + a_{nT} + e_T + e_{nT}} = \frac{a_T + a_{nT}}{\mathrm{TOTAL}}$

a_T: number of samples correctly assigned to the target class; a_{nT}: number of samples correctly assigned to the non-target class; e_T: number of samples wrongly assigned to the target class; e_{nT}: number of samples wrongly assigned to the non-target class; T: target class (genuine virgin olive oil); nT: non-target class (adulterated olive oil).

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Table 4. Results of validation of the SVM classification methods.

a) Contingency table

(NP)HPLC-DAD

Assignation		Actual T	Actual nT	
		45	**5**	**50**
Assignation	**nT**	3	5 (100%)	**8**
	T	42 (93.3%)	0	**42**
		T	**nT**	
		Actual		

(HT)GC-FID

Assignation		Actual T	Actual nT	
		45	**5**	**50**
Assignation	**nT**	11	5 (100%)	**16**
	T	34 (75.5%)	0	**34**
		T	**nT**	
		Actual		

b) Estimating of parameters

Parameter	**(NP)HPLC-DAD**	**(HT)GC-FID**
Precision	1.00	1.00
Sensitivity	0.93	0.76
Non-target predictive value	0.63	0.31
Specificity	1.00	1.00
Accuracy (or Efficiency)	0.94	0.78

T: target class (genuine virgin olive oil); nT: non-target class (adulterated olive oil)

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Table 5. Applicability indicators of SVM classification methods (an occurrence value of 0.90 is considered).

Indicator	**(NP)HPLC-DAD**	**(HT)GC-FID**
Error index (%)	0%	0%
Saving index (%)	84%	68%

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Table 6. Results of validation of the RF classification methods.

a) Contingency table

(NP)HPLC-DAD

Assignation \ Actual	T	nT	
	45	**5**	**50**
nT	15	5 (100%)	**20**
T	30 (66.6%)	0	**30**

(HT)GC-FID

Assignation \ Actual	T	nT	
	45	**5**	**50**
nT	11	4 (80%)	**15**
T	34 (75.5%)	1	**35**

b) Estimating of parameters

Parameter	**(NP)HPLC-DAD**	**(HT)GC-FID**
Precision	1.00	0.97
Sensitivity	0.66	0.76
Non-target predictive value	0.25	0.27
Specificity	1.00	0.80
Accuracy (or Efficiency)	0.70	0.76

T: target class (genuine virgin olive oil); nT: non-target class (adulterated olive oil)

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Table 7. Applicability indicators of RF classification methods (an occurrence value of 0.90 is considered).

Indicator	(NP)HPLC-DAD	(HT)GC-FID
Error index (%)	0%	2%
Saving index (%)	60%	70%

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437 **FIGURE CAPTIONS**

438

439 **Figure 1.** Graphical scheme of the analytical methodology based on the application of
440 vanguard-rearguard methods

441

442 **Figure 2.** Time-wavelength landscape of (a) an extra virgin olive oil from 'arbequina'
443 cultivar and (b) from an adulterated olive oil sample.

444

445 **Figure 3.** Chromatograms obtained from (HT)GC-FID: (a) extra virgin olive oil
446 (cornicabra) sample and (b) adulterated olive oil sample.

447

448 **Figure 4.** PC2 vs PC1 plots from the data obtained by (a) (NP)HPLC-DAD and (b)
449 (HT)GC-FID of the 207 samples analysed.

450

451 **Figure 5.** Classification plots of SVM methods: (a) data collect from (NP)HPLC-DAD
452 and (b) data recorded from (HT)GC-FID.

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456 **<Figure 1>**

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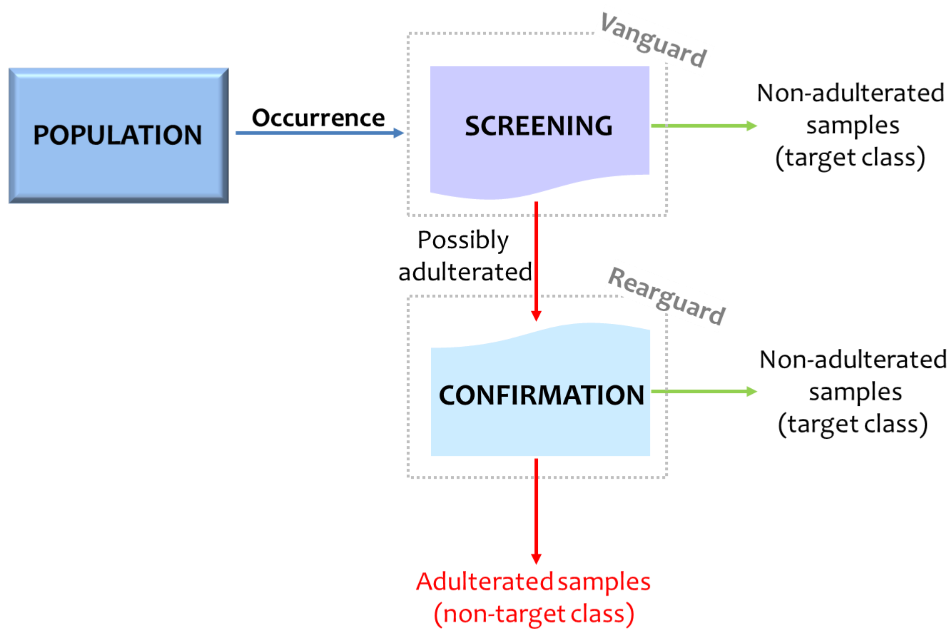

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459 **<Figure 2>**

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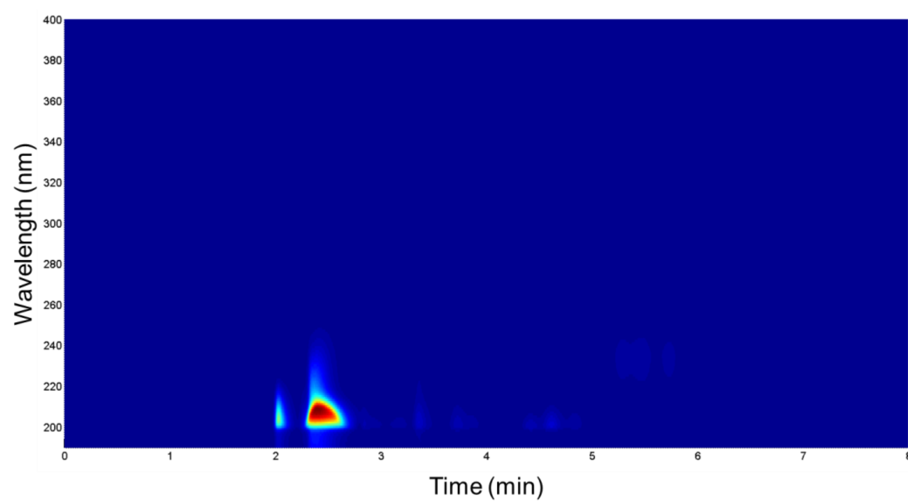

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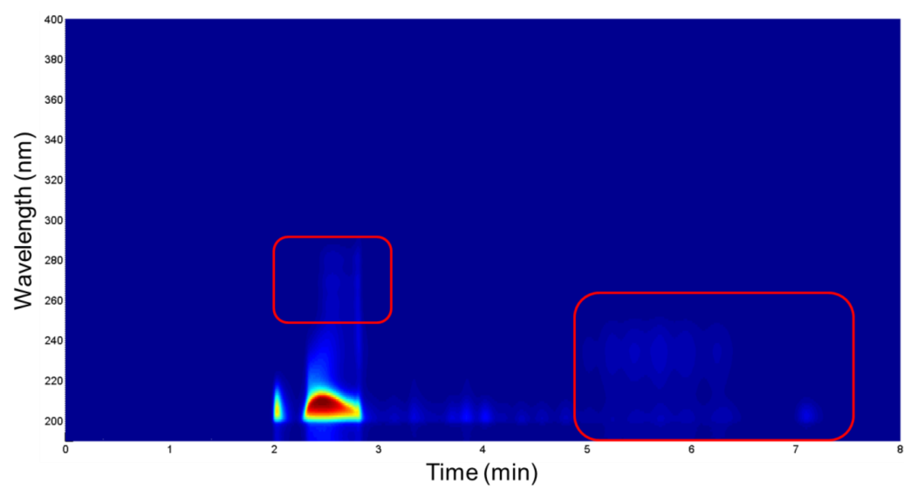

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463 **<Figure 3>**

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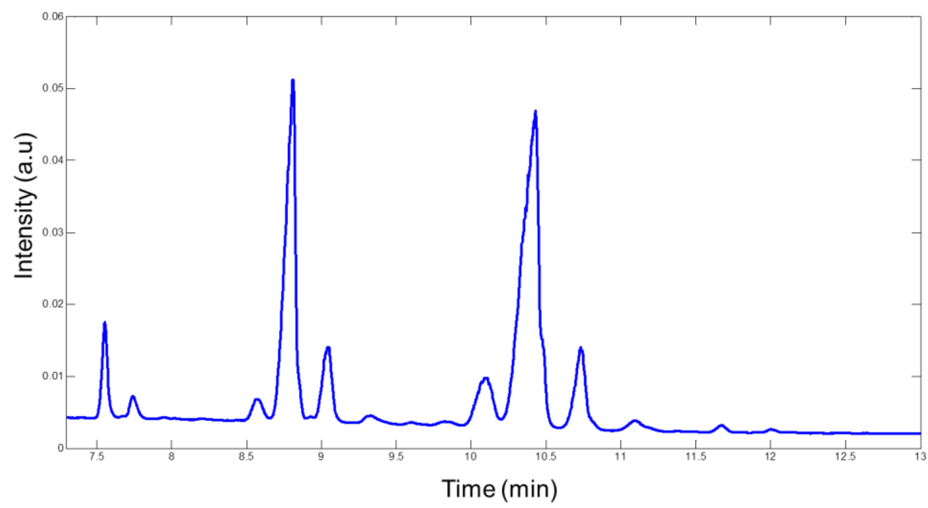

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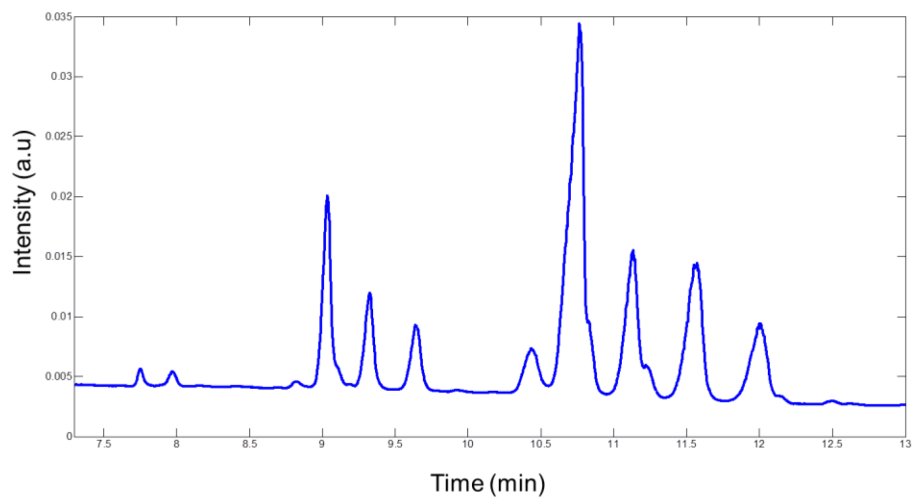

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467 <Figure 4>

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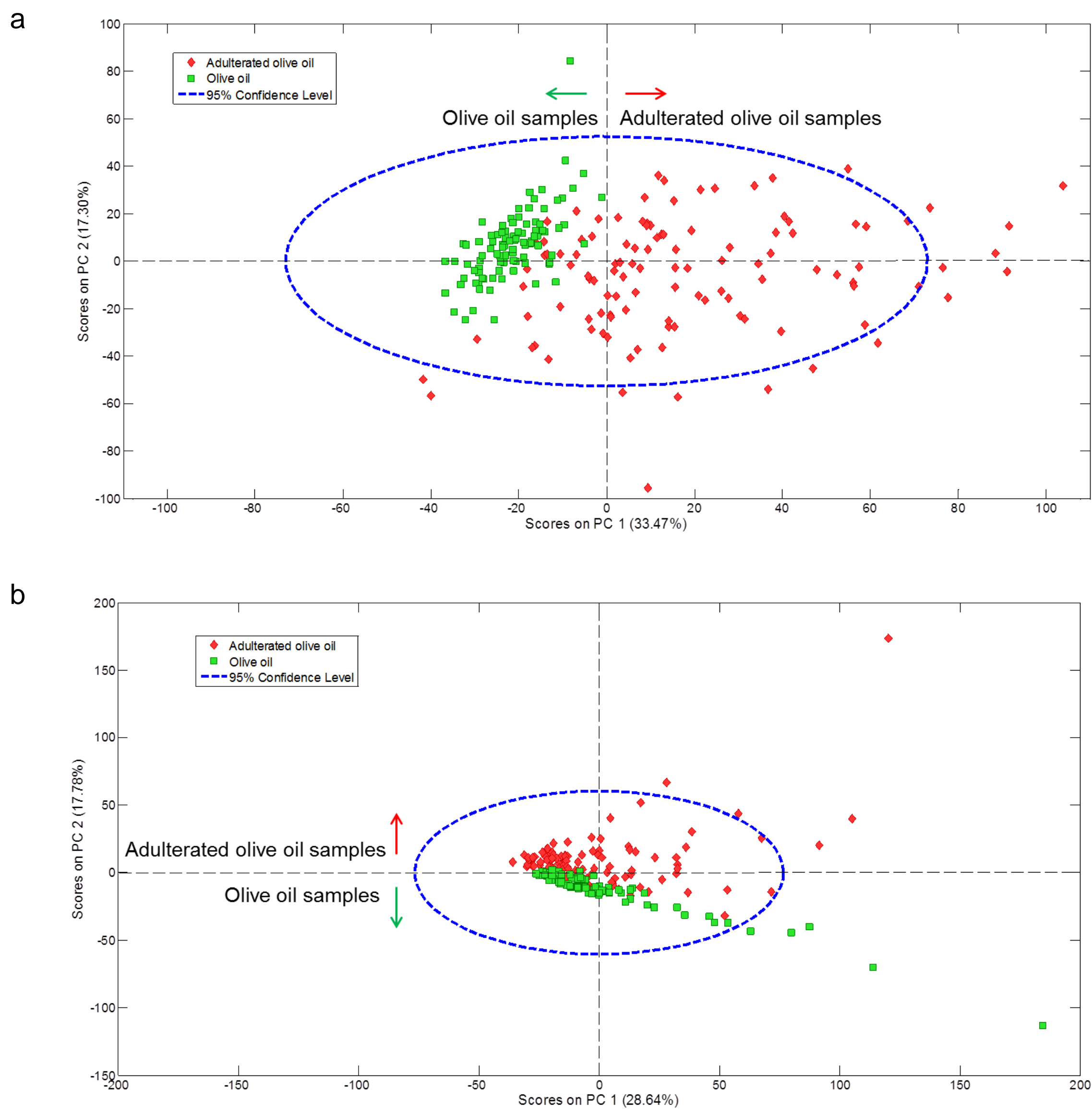

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471 <Figure 5>

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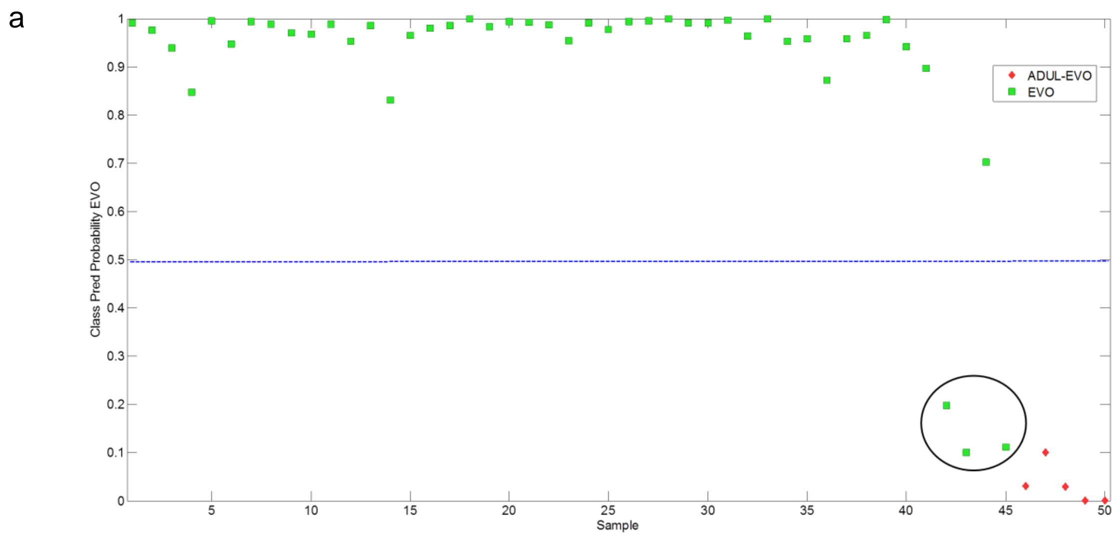

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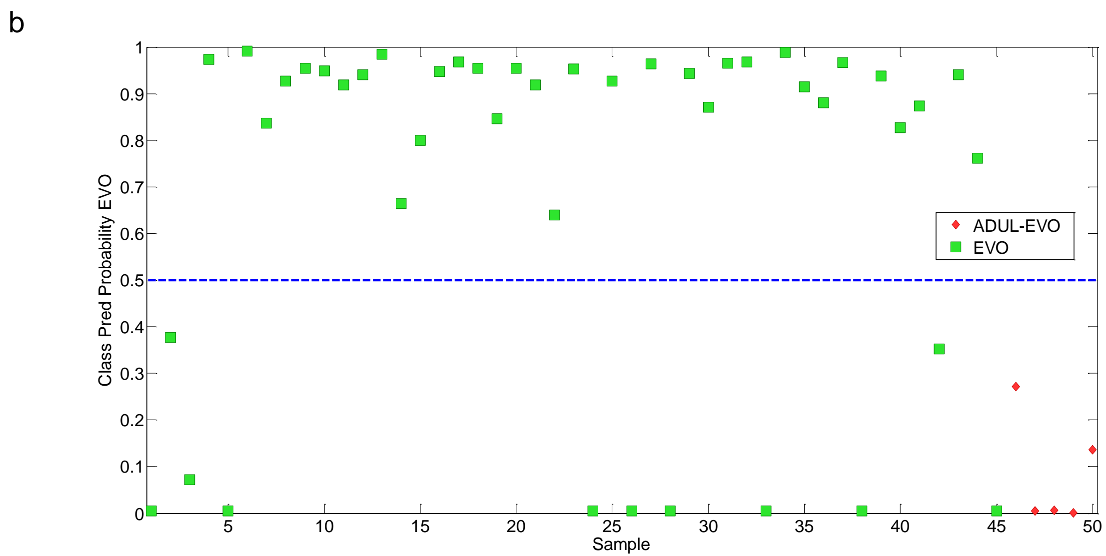

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