TITLE: Rapid and accurate identification of *Escherichia coli* STEC O157:H7 by mass spectrometry, artificial intelligence and detection of specific biomarkers peaks.

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ABSTRACT

The different pathotypes of *Escherichia* can produce a large number of human diseases. Surveillance becomes complex since their differentiation are not easy.

Particularly, the detection of Shiga toxin-producing *Escherichia coli* (STEC) serotype O157:H7 consists of stool culture of a diarrheal sample in enrichment and/or selective media, identification of presumptive colonies and confirmation by Multiplex PCR technique for the genotypic characterization of serogroup O157 and Shiga toxins (*stx*1 and *stx*2), in addition to the traditional biochemical identification.

All of these procedures are laborious, require a certain level of training, are time consuming and expensive. Among the currently most widely used methodologies, MALDI-TOF MS mass spectrometry (matrix-assisted laser desorption/ionization with time-of-flight mass detection), allows a quick and easy way to obtain a protein spectrum of a microorganism, not only in order to identify the genus and species, but also the discovery of potential biomarker peaks of a certain characteristic. In the present work, the information obtained from 60 clinical isolates was used to detect peptide fingerprints of STEC O157:H7 and other diarrheagenic *E. coli*. The differences found in the protein profiles of the different pathotypes established the foundations for the development and evaluation of classification models through automated training.

The application of the Biomarkers in combination with the predictive models on a new set of samples (n=142), achieved 99.3% of correct classifications,

allowing the distinction between STEC O157:H7 isolates from the other diarrheal *Escherichia coli*.

Therefore, given that STEC O157:H7 is the main causal agent of haemolytic uremic syndrome and based on the performance values obtained in the present work (Sensitivity=98.5% and Specificity=100%), this development could be a useful tool for diagnosis of the disease in clinical microbiology laboratories.

INTRODUCTION.

The contribution of *Escherichia coli* to human intestinal disease may be largely uncharacterized, because many types of pathogenic *E. coli* are not routinely tested in clinical microbiology laboratories.

Shiga toxin-producing *Escherichia coli* (STEC) is associated with outbreaks that causes diarrhea, hemorrhagic colitis, and hemolytic-uremic syndrome (HUS) in humans. It is part of the diarrheagenic *E. coli* (DEC) group, which also includes: enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC), enteroaggregative *E. coli* (EAEC), enteroinvasive *E. coli* (EIEC) and diffusely adherent *E. coli* (DAEC). Although, there are more than 150 serotypes **[1]** that share the same pathogenic potential, O157:H7 is the most frequent. In particular, the detection of STEC O157:H7 consists of the culture of the faecal sample in enrichment and/or selective media such as MacConkey agar with sorbitol for the identification of presumptive non-fermenting sorbitol colonies and confirmation by Multiplex PCR for the genotypic characterization of serogroup O157 and Shiga toxins (*stx*1 and *stx*2), in addition to subsequent traditional biochemical identification.

However, all this methodological complexity is very difficult to implement in a traditional laboratory **[2].**

On the other hand, mass spectrometry (MS), specifically MALDI-TOF MS (matrix-assisted laser desorption/ionization with time-of-flight mass detection), provides a simple, rapid, robust, and low-cost microbial identification. MALDI-

TOF MS is a technique based on the analysis of protein spectra containing peaks with an exactly determinable mass-charge ratio (m/z) generated by the impact of a laser on a previously crystallized isolate with an organic matrix. In recent years, MS has acquired great importance in the identification of pathogens that are clinically relevant in public health **[3-5]**. However, the potential of this methodology combined with machine learning algorithms for the detection of profiles in a wide variety of samples and its use as a screening technique is expanding, due to its low-cost and high performance **[6]**.

In this study, we wanted to verify the usefulness of MS to rapidly identify O157:H7 STEC from pathotypes other than diarrheagenic *E. coli*; then, we proposed to detect and analyse peaks in the spectra generated by MALDI-TOF MS to find possible biomarkers and thus establish differential patterns between a wide variety of *E. coli* strains.

MATERIALS AND METHODS.

Isolates collection.

The spectra obtained from 60 isolates corresponding to four different DEC categories were used for the development of predictive models and the detection of biomarkers: EPEC (n=15), ETEC (n=15), STEC NON O157 (n=20) and STEC O157:H7 (n=10). The detail of the isolates can be found in **Table S1** in Supplementary Material.

For the final validation, we used 142 different isolates of: STEC O157:H7 (n=65), non-toxigenic *E.coli* O157 (n=13), STEC NON O157 (n=17), ETEC (n= 11), EPEC (n=12), EAEC (n=15), EIEC (n=7), and *E. coli* without virulence

147 factors (n=2). All of them were obtained mainly from faecal samples from
148 different health institutions in our country and food samples subsequently
149 referred to the National Reference Laboratory- Servicio de Fisiopatogenia INEI-
150 ANLIS ¨Dr. Carlos G. Malbrán¨- for confirmation and characterization of specific
151 virulence factors **[7].**
152
153 **Acquisition of spectra.**
154 The Microflex LT mass spectrometry equipment (Bruker Daltonics) was used to
155 obtain the protein spectra from *E. coli* isolates. Subsequently, each isolate was
156 spotted in quadruplicate in the wells of the steel plate provided by the
157 manufacturer using the direct method and crystallized by adding 1 ul of HCCA
158 matrix (α-Cyano-4-hydroxycinnamic acid in 50% of acetonitrile and 2.5%
159 trifluoroacetic acid). After a few minutes of drying, the plate was introduced into
160 the equipment and once the vacuum was reached in the Flex Control v3.4
161 software, the spectra were acquired in the linear positive mode, with 30-40%
162 laser power. and in a mass range of 2 to 20 kDa. Each well was read twice, so
163 eight individual spectra were obtained for each isolate, thus minimizing the
164 variability of the technique.
165 The external calibrator provided by the manufacturer, BTS (Bruker Test
166 Standard), was used prior to each run.
167 The 142 isolates used in the subsequent validation set were processed in the
168 same way by direct method, but each isolate was spotted in duplicate and read
169 only once, simulating the routine procedure of a microbiology laboratory.
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MALDI-TOF analysis

All isolates were identified using the MALDI Biotyper RTC software. According to the manufacturer's recommendations, the identification is considered reliable at the species level when the score values greater than 2.0 are obtained. When the score values are between 1.7 and 1.99, it is considered reliable identification at the genus level; and it is considered 'No Identification' when the value of the score is ≤ 1.69 **[8]**.

Bioinformatic analysis.

To perform data analysis, ClinPro Tools software (version 3.0, Bruker Daltonik GmbH, Bremen, Germany) and Flex Analysis v3.4 software (Bruker Daltonics, Bremen, Germany) were used.

Data pre-processing.

In order to take advantage of the greatest amount of information contained in the spectra, the following data pre-processing steps were performed: baseline correction (top hat 10% of minimum width of the baseline), smoothing and calibration excluding null spectra or out of range, according to the literature **[9-11]**.

Peak Selection.

The exploration of the potential biomarkers was performed on the protein profiles generated from the 60 isolates that were part of the initial training group and which were also, used to create the classification models.

Flex Analysis v3.4 Software.

All spectra were exported as mzXML files using CompasXport CXP3.0.5 and a series of analyses were performed according to standard Bruker setup. Spectrum quality criteria for overall aspect and intensity were checked. Next, visually identifiable biomarker peaks were explored in the different views offered by the program. The mass list was exported to Excel (Microsoft, Redmond, WA) to analyse possible biomarker peaks. Values of "1" or "0" (data binarization) were assigned to the presence or absence of a peak within the tolerance interval (+/- 10Da). Based on this analysis, groups of potentially useful peaks for the diagnosis of STEC O157:H7 were found.

ClinProTools v3.0 Software

The spectra of STEC O157:H7 were assigned as class 1 and the rest of the DEC isolates were class 2. Biomarker peaks were automatically identified by class comparison using the function "Peak Statistic Table".

To select the characteristic peaks of the two classes, the following statistical tests were used: the t test/analysis of variance ANOVA (PTTA), Wilcoxon or Kruskal–Wallis test (W/KW), and Anderson–Darling test (AD). A p value of 0.05 was established as the cut-off point **[12]**:

-if p is <0.05 in the AD test, a characteristic peak is selected if the corresponding p-value in the W/KW test is also <0.05.

-if p is 0.05 in the AD test, then a characteristic peak is selected if the corresponding p value in ANOVA is also <0.05 **[13]**.

The discriminative power for each biomarker was further described by receiver operating characteristic (ROC) and area under the curve (AUC) analysis.

ROC curve indicates the relationship of the true-positive rate (TPR) and the false-positive rate (FPR). The area under the ROC curve is equal to the probability that a biomarker sorts a randomly selected positive sample higher than a randomly selected negative one.

Principal Component Analysis (PCA).

To explore and compare spectra in multidimensional space and in order to evaluate the possible distributions or clusters on the isolates of both classes, a first exploratory and unsupervised analysis was performed of all 60 samples.

Classification models.

Supervised classification models were performed using the following algorithms provided by ClinPro Tools software: Supervised Neural Network (SNN), optimized genetic algorithm combined with k-nearest neighbour classification (GA/ kNN) and a quickclassifier (QC).

For each model, the following parameters were calculated: Recognition Capability (CR) and Cross Validation Percentage (VC), both of which are indicators of the theoretical behaviour that the model will have in future classifications.

Selection of isolates for final validation.

To evaluate the robustness of the models created, an independent set of isolates different from those used to developed the algorithms was selected (N=142). For each isolate, a spectrum was presented to the selected classification model. The software then returned a result that was compared to current reference techniques.

Statistical analysis.

The parameters evaluated were: sensitivity, specificity, positive predictive value, negative predictive value **[14]**. Besides, the CLSI guide, EP12-A2, was used to compare methods that report results qualitatively. When the comparison is made with a method that is not considered a reference, the degree of similarity between the methods is measured through the percentage of negative agreement and the percentage of positive agreement. The diagnostic parameters of the methods are then compared to determine if the difference between the two of them is statistically significant.

RESULTS.

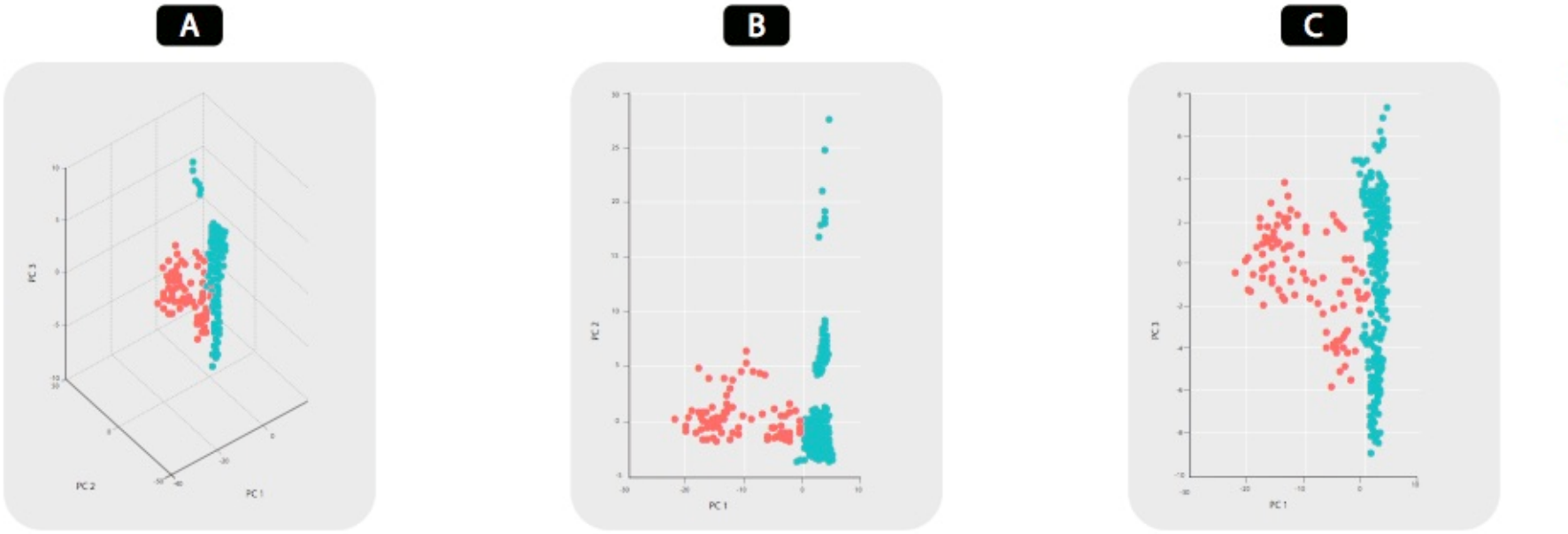
Confirmation at the genus-species level

All isolates were identified at the species level as *Escherichia coli* with a score value greater than 2.0, in agreement with the result of the reference techniques.

Unsupervised Analysis.

First, a Principal Component Analysis (PCA) was performed for the 60 isolates used as a training set. In this way it was possible to reduce all the information contained in the MALDI-TOF spectra in a few new variables.

This allows us to graphically represent all the spectra together in three and two dimensions on the Score plot. (Figure 1) The complete list of significant peaks found in ClinPro Tools can be found in **Supplementary Material S2.**



280 **Figure 1**. PCA plots results. Data originated from the external MATLAB software tool integrated into ClinPro Tools.

Graph A shows the three components plotted simultaneously in three dimensions, while in graphs B and C shows PC1 versus PC2 and PC1 versus PC3 respectively.

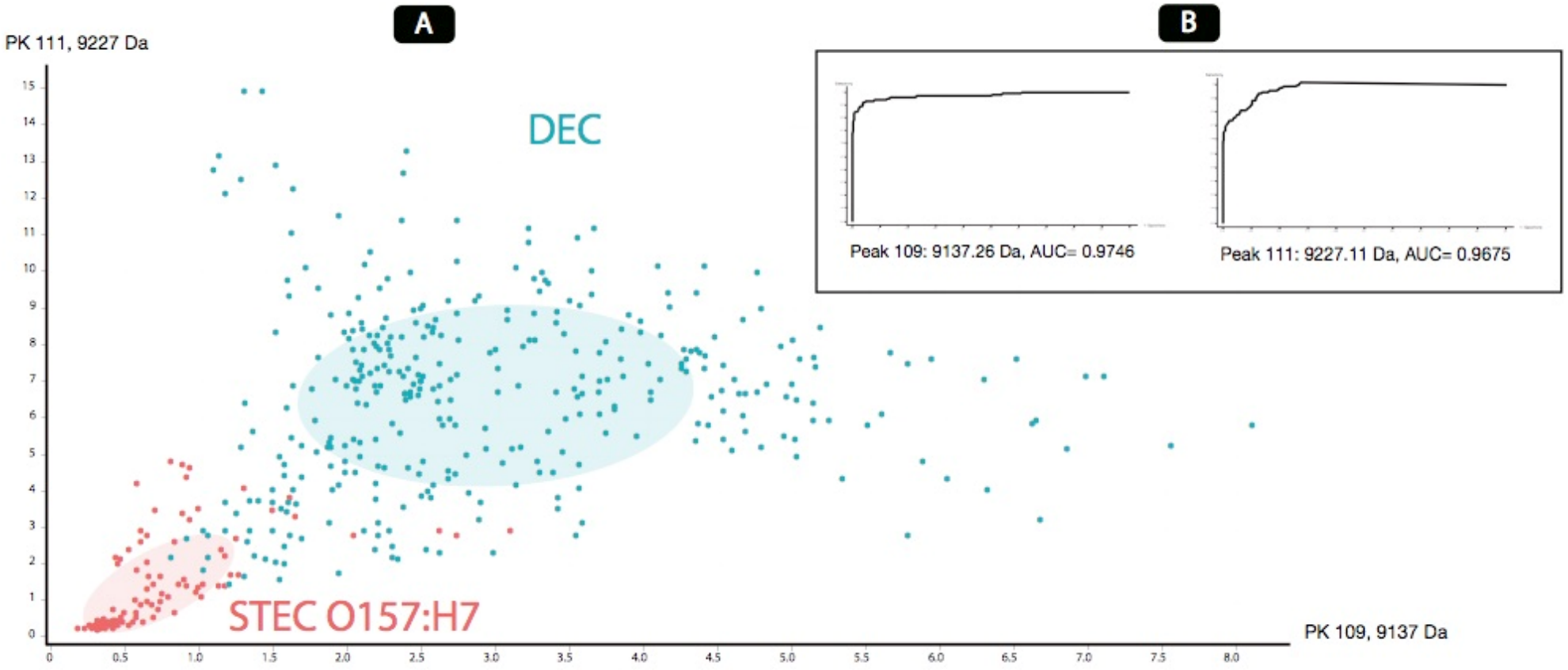
Supervised Analysis.

Subsequently, a supervised multivariate analysis was performed with the additional information of each isolate to define each class:

CLASS 1: STEC O157:H7

CLASS 2: NON STEC O157 (other DEC)

Figure 2-A shows the two-dimensional distribution plot of all the spectra of each class based on the two best peaks obtained for their classification; which correspond to the 9137.26 Da peak and the 9227.11 Da peak. The peak number and its m/z values are shown on the x and y axes respectively, while the ellipses represent the 95% confidence interval. On the other hand, Figure 2-B shows the ROC curves of the two selected peaks. The area under the curve (AUC) represents the discriminatory potential of each biomarker peak.



302 **Figure 2**. **A**- 2D graph of peak distribution of the 2-class model. **B**- ROC curves of the most discriminating peaks according to the analyses performed

303

304 **Classifier Models.**

305 The predictive models were calculated based on three available algorithms:
306 GA/kNN, SNN and QC, the results of the different parameters of each algorithm
307 are summarized in **Table 1**.
308 The SNN algorithm was discarded from the successive analyses due that the
309 results obtained were not optimal.
310
311 **Table 1**. Results of RC, CV and integration areas for each algorithm.

312

Classifiers Algorithms	RC	CV	Integration areas used by each model
GA / kNN	100.00%	100.00%	3082;4939;5080;8813;8994
QC	92.83%	92.47%	6389;9136;9226

RC= Recognition Capability; VC=Cross Validation

317
318
319 As a result of the external validation carried out with the set of 142 isolates, a
320 good performance was observed with the GA/kNN algorithm and with the QC
321 algorithm. Nevertheless, we decided to combine both models; first, using the
322 QC algorithm but applying a cut-off value >= 1.55, since from this value 100%
323 correct classifications were observed compared to the reference technique
324 (Figure 3).
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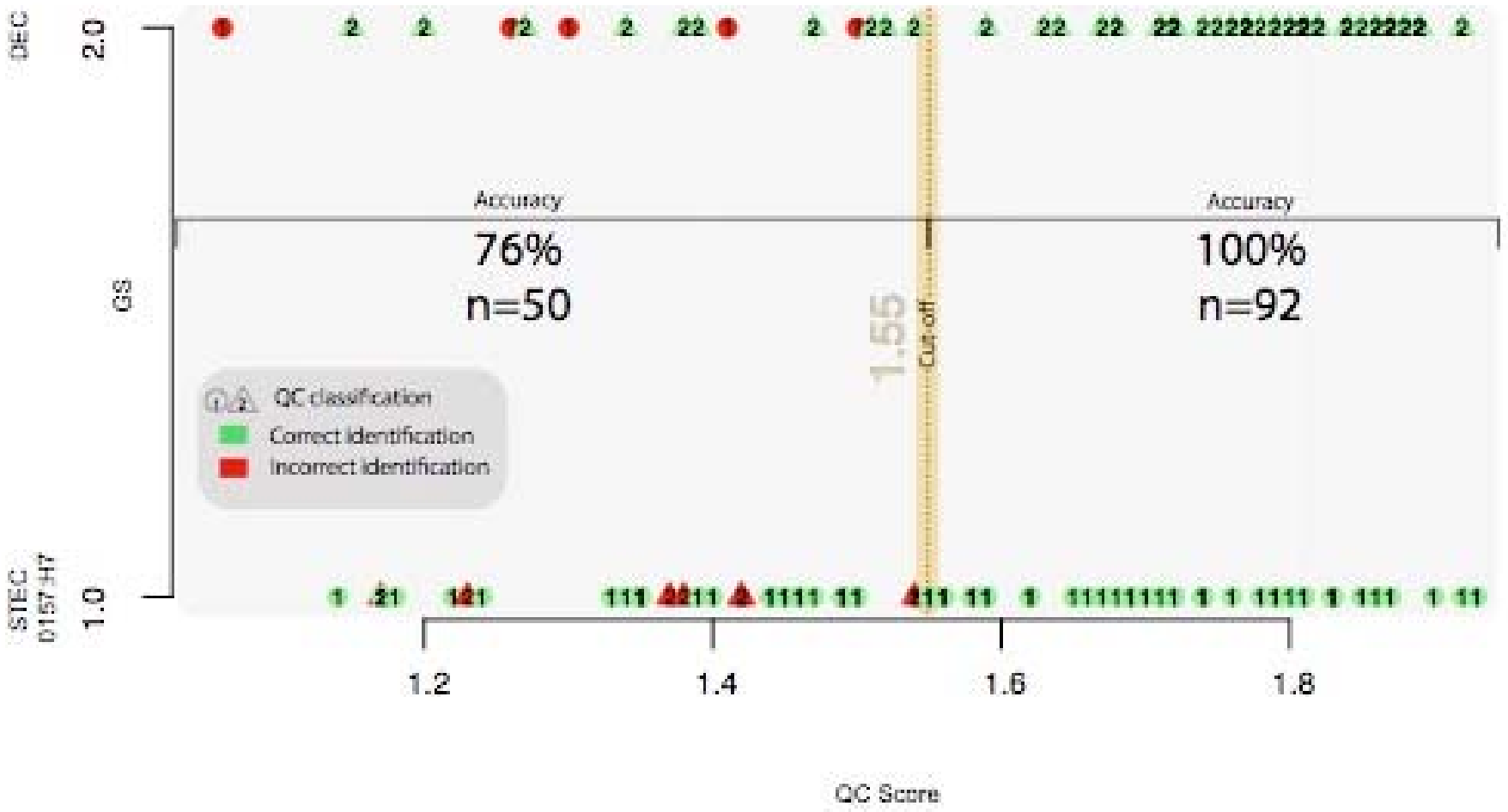
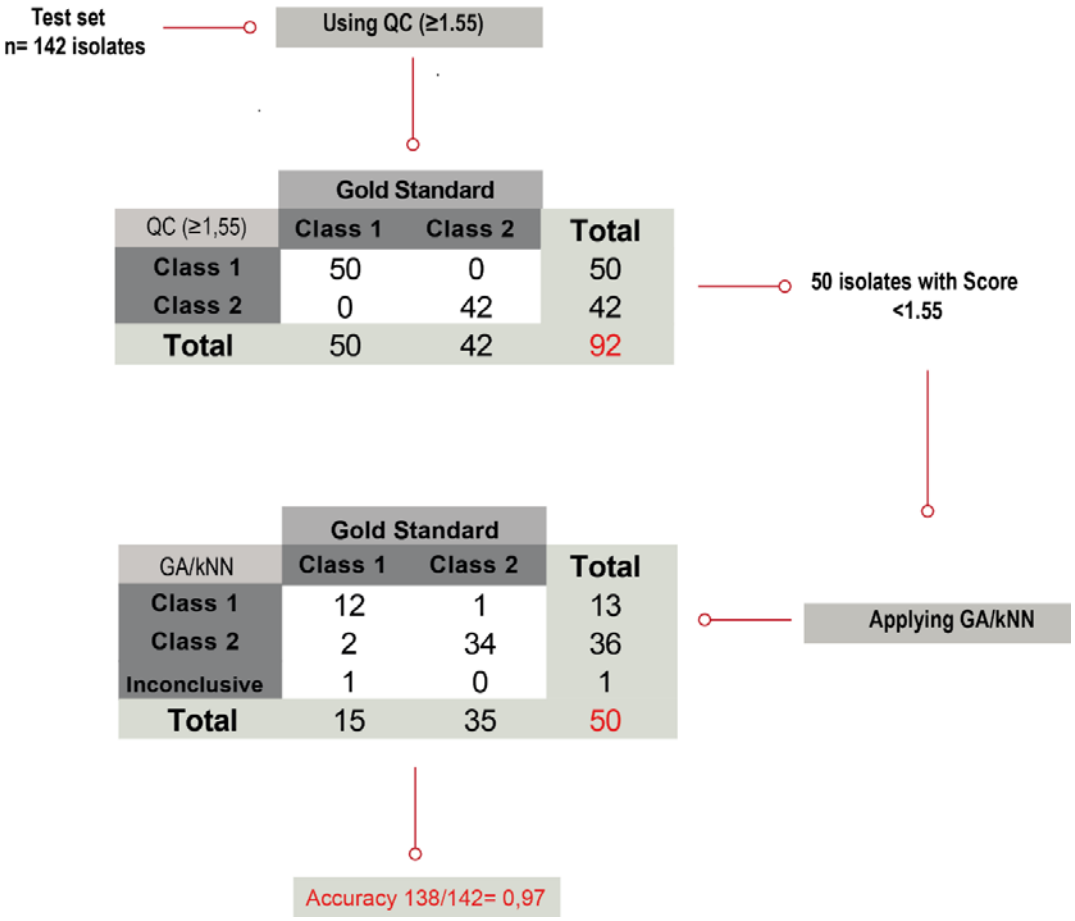


3:

331 **Figure 3**. Graph of QC scores where the 100% concordance of the QC algorithm with the reference method is observed from the standardized cut-off value of

332 1.55.

333 On the other hand, the isolates that were classified with a QC value <1.55 the
334 GA/kNN algorithm was applied (Figure 4). This combination managed to
335 increase the identification up to 97%, as detailed later in Table 3.
336 There were three isolates incorrectly classified using this scheme and one was
337 considered Inconclusive since the result of the QC algorithm was <1.55 and
338 when applying the GA/kNN algorithm dissimilar results were obtained between
339 the sample and the duplicate.
340



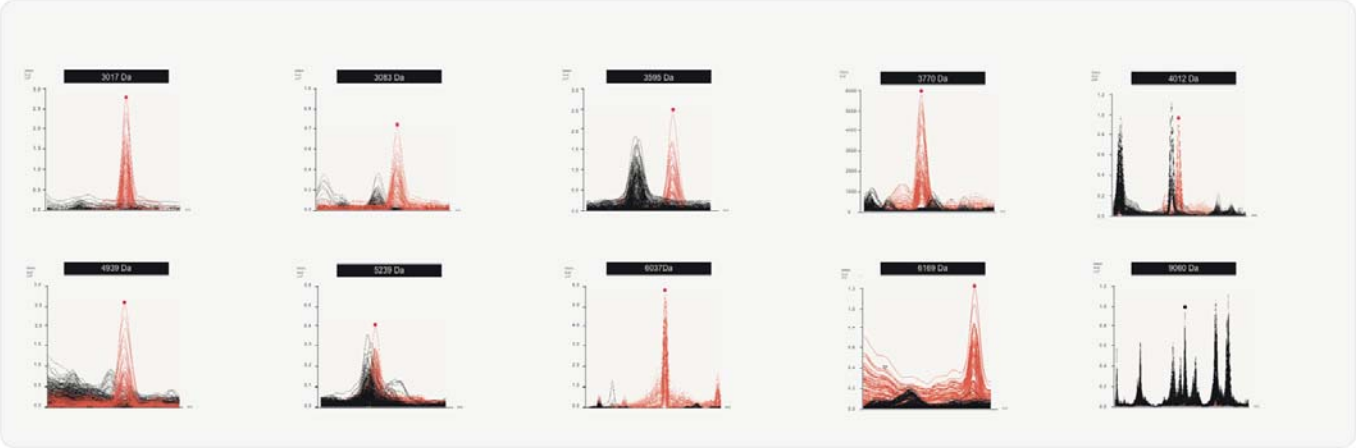
341

342 **Figure 4**. Algorithm applied for the identification of STEC O157:H7 based on predictive
343 classification models.
344
345

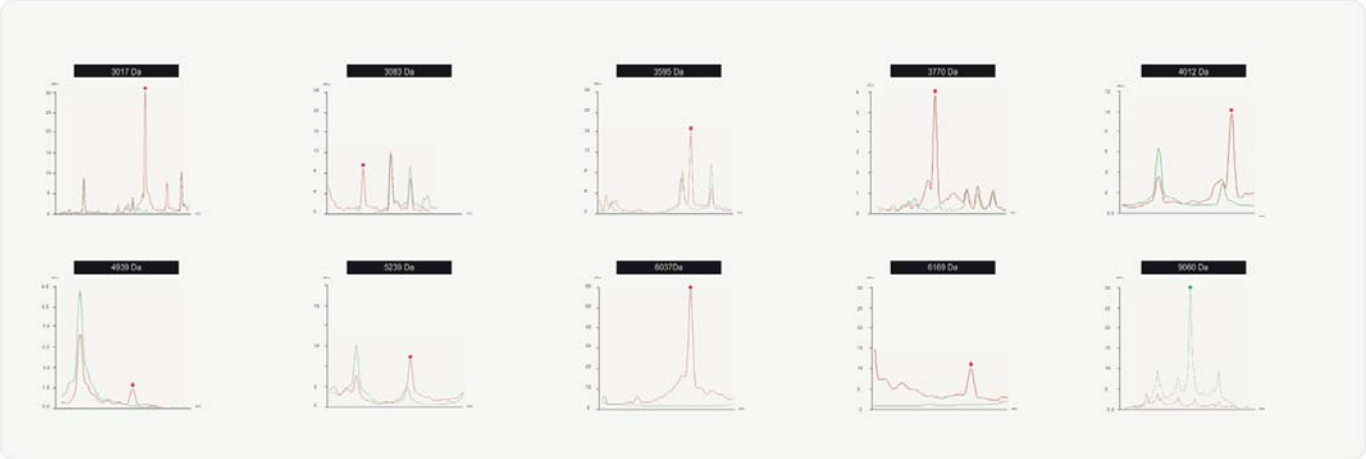
Biomarker detection.

10 differential peaks with statistical significance were found in both software; of which 9 correspond to the STEC O157:H7 pathotype and a single biomarker was present only in NON STEC O157 (Figure 5).

A



B



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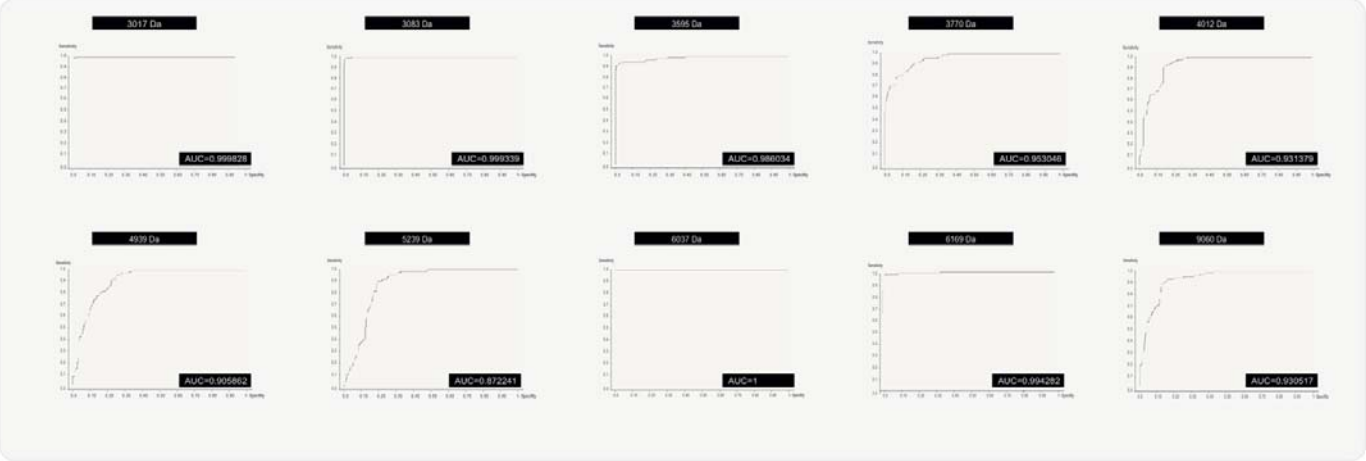

Figure. 5. **A-** Characteristic peaks (biomarkers) in individual spectra of STEC O157:H7 samples (red) versus DEC samples (black), obtained by manual analysis in Flex Analysis v3.4 software. **B-** Average spectra of the same peaks, STEC O157:H7 (red); DEC (green), obtained by ClinPro Tools v3.0. **C-** ROC curves and AUC values originated from the external MATLAB software tool integrated in ClinPro Tools.

The profile of the 10 potential biomarkers selected for the differentiation of STEC O157:H7 from DEC isolates is shown in Table 2. The description of the profiles found for all the challenged isolates is found in Table S3 of the Supplementary Material.

Table 2. Profile of the 10 potential biomarkers selected for the differentiation of STEC O157:H7 from DEC.

Classification	Biomarkers (m/z)									
	3017	**3083**	**3595**	**3770**	**4012**	**4939**	**5238**	**6037**	**6169**	**9060**
CLASS 1 (O157:H7; n=65)	72% (n=47)	45% (n=29)	51% (n=33)	85% (n=55)	40% (n=26)	38% (n=25)	97% (n=63)	86% (n=56)	55% (n=36)	100% (n=65)
CLASS 2 (DEC; n=77)	96% (n=74)	100% (n=77)	99% (n=76)	99% (n=76)	100% (n=77)	100% (n=77)	100% (n=77)	97% (n=75)	100% (n=77)	96% (n=74)

80-100 %
70-80 %
60-70 %
<60 %
Presence
90-100 %
80-90 %
Absence

Based on the analysis of the results obtained from the BM challenge on 142 new samples in duplicate, it can be confirmed that the absence of the 9060 Da peak, added to the detection of at least one of the other nine peaks described in this work, confirmed the identification of an isolate of STEC O157:H7, although most of these isolates (92%), in addition to not showing the peak at 9060 Da, had 3 or more biomarkers.

One isolate of STEC O157:H7 and 3 other isolates (STEC NON O157, ETEC, and non-toxigenic O157) did not present any of the peaks listed above, and thus could not be classified.

Regarding the NON STEC O157, 96% presented the peak of m/z 9060 Da and 93.5% none of the nine peaks previously described.

Finally, the sensitivity of the search for probable biomarker peaks was 96.9% and the specificity 100%, as shown in Table 3.

Table 3. Sensitivity, Specificity, PPV and NPV values of the different approaches evaluated and the statistical relationship between them.

Evaluated Parameter	**Approaches**				
	A	**B**	**C**	**D**	**E**
	Model GA/kNN	Model QC	Combination QC + GA/kNN	Biomarkers	Combination QC + GA/kNN + Biomarkers
Sensibility	80,00%	92,30%	95,40%	96,90%	98,50%
Specificity	96,10%	90,90%	98,70%	100%	100%
PPV	94,50%	89,60%	98,40%	100%	100%
NPV	85,10%	93,30%	96,20%	97,50%	98,70%

PPV= Positive predictive value; **NPV=** Negative predictive value

The difference between the sensitivity and specificity of the mathematical model with respect to the manual (C and D) and the two best methods (D and E) and with respect to the reference method was estimated to conclude whether these differences were statistically significant or not. In this way, if the 95% confidence interval of the differences contains the value 0, it is concluded that there are no

409 statistically significant differences, otherwise there are differences. The results
410 of this analysis are detailed in Table 4.

411

412 **Table 4.** Results of the difference between the sensitivity and specificity of the mathematical
413 and manual model and of the two best methods with respect to the reference method.

Combination of approaches	Difference sensitivity (%)	Difference specificity (%)	Confidence Intervals 95%		Conclusion
			Lower	Upper	
C vs D	-1.54	-	-8.58	6.1	The difference between the sensitivity of the methods **is not statistically significant**
	-	1.3	-3.57	7.00	The difference between the sensitivity of the methods **is not statistically significant**
D vs E	1.56	-	-4.52	9.03	The difference between the sensitivity of the methods **is not statistically significant**
	-	0	-5.63	4.87	The difference between the sensitivity of the methods **is not statistically significant**

414

415

416 Therefore, despite the fact that there were no statistically significant differences
417 between the performance values of approaches D and E, the benefits of their
418 combined use are evident, as described in numerous publications in the current
419 literature **[15]** and it follows from this work in the resolution of discordant cases.

420

421 In summary, if both developments are applied in a complementary way to
422 isolates that could not be correctly classified by automated training or did not
423 present any of the peaks considered biomarkers, accurate detection of 98.5% of
424 STEC O157:H7 isolates is achieved and the correct classification of 99.3% of all
425 the isolates studied

426

It was observed that 3/3 isolates incorrectly classified by the predictive models were correctly resolved by the BM finding method and 3/4 that could not be classified because they did not present the peaks, were resolved by mathematical models; a single case could not be resolved by either of the two methods, reaffirming the usefulness of the combined use of both approaches (Table 5).

The table of the results obtained on the total number of isolates applying machine learning and biomarker detection, in comparison with current reference techniques, can be found in Table S3 the Supplementary Material.

Table 5. Discordant cases of the different approaches compared with the gold standard results.

Samples ID	GA/kNN+QC	Biomarkers	GA/kNN + QC + Biomarkers	Gold standard
750/18	Class 2	Class 1	Class 1	Class 1
506/18	Class 2	N/D	Class 2	Class 2
385/16	Class 1	Class 2	Class 2	Class 2
504/18	Class 2	Class 1	Class 1	Class 1
329/18	Class 2	N/D	Class 2	Class 2
714/18	N/D	N/D	N/D	Class 1
493/18	Class 2	N/D	Class 2	Class 2

N/D=not determinated

CONCLUSIONS.

Due to the important analytical capabilities that MS currently has, added to the speed of results and lower-cost, the possible implementation of the MALDI-TOF MS system coupled to simple and practical artificial intelligence tools could be considered as a STEC O157:H7 diagnostic screening method.

Through the proteomic analysis of the information contained in the spectra of the different classes of *E. coli*, and applying a combination of predictive models based on machine learning, it was possible to quickly identify 94% of the STEC O157:H7 isolates and precise, starting from characteristic suspicious colonies, which implied a substantial saving of time and resources in the routine of the conventional laboratory. By combining this approach with the search for potential biomarker peaks, the percentage of correct identifications rose to 98.5%.

There were several previous attempts in the literature to detect STEC O157 by MALDI-TOF Mass Spectrometry **[16-19]**, however, no defining peaks were found in any of the previous works. and without the requirement of complex extraction techniques or equipment with greater discriminatory power, such as the TOF-TOF type, or peak readings above 10,000 Da, which are generally less detected. On the other hand, here we detect a large number of reproducible peaks in the reading range of the order of 3000 to 9000Da by direct method, without the need to make any modifications, which results in a simple, fast and easily transferable procedure to less complex clinical laboratories that have the technology.

In some cases, a difference in the presence of a peak was observed in the duplicate of the same sample, which may be due to operator errors or by using the direct method, which presents greater variability than the extraction techniques. Evidence from the literature suggests that the protein extraction method extends or improves the range of peaks identified **[16,20]**. However, in this work direct method was prioritized, because it is much simpler, faster and easily applicable in the routine of any clinical laboratory. Due to the variability of

the method evidenced on some challenged spectra, the importance of working with technical replicates and analysing the presence/absence of several characteristic peaks is highlighted, in order to reduce the risk of making errors during the classification of one class or another. It is also evident that the search for a single peak is not enough, but rather the joint analysis of a profile, either manually or automatically, in order to performed a reliable identification.

According to previous works by Mazzeo, 2006 and Teruyo, 2014, it was possible to confirm that the absence of the peak at 9060 Da is a useful indicator of STEC O157:H7, although it is not definitive per se, as could be evidenced in our work, where the finding of one or more of the nine detected peaks would confirm the presence of a STEC O157:H7 isolate, because these are not normally found in the other diarrheal types of the genus.

The total absence of biomarker peaks that occurred in 4 isolates (three of them NON STEC O157 that would generally present a single peak) may also be due to errors in the technical procedure, which, in these cases, would be convenient to repeat or confirm by other methods if only this approach is available.

A particular case occurred where the presence of 4 peaks was detected, as occurs on isolate of STEC O157, but with the presence, in addition to the representative peak of the DEC group at m/z 9060 Da, this isolate corresponded to a O157 H7 non-toxigenic type, which had been isolated from a meat processing plant, this strengthens the idea of being in the presence of a STEC isolate O157:H7 with the loss of the Shiga toxin phage, in the same way as described in other works **[21-22].** Said isolate also presented other virulence factors such as enterohemolysin and eae, typical of STEC O157:H7.

Finally, the approach based on the detection of the presence/absence of peaks, although it is a manual method that requires a longer analysis time, presented excellent performance values and the were no differences regarding in sensitivity and specificity compared to the mathematical model, added to the availability of the Flex Analysis software in the equipment, the detection of these biomarker peaks could be applied in laboratories as a rapid screening method for suspicious STEC O157:H7 isolates.

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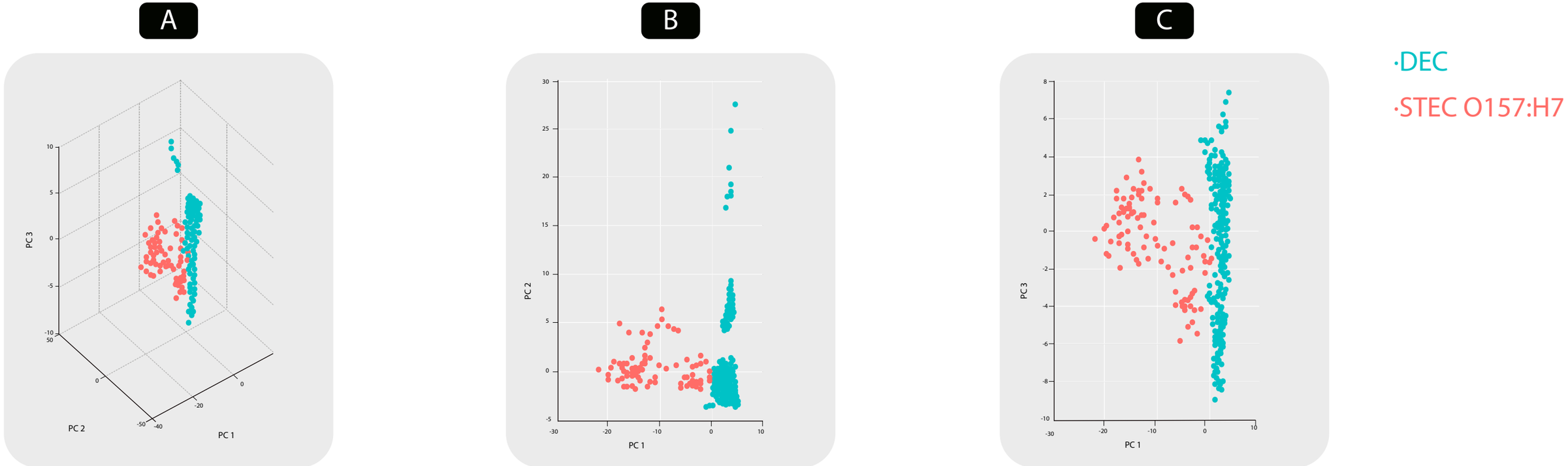


Figure 1. PCA plots results. Data originated from the external MATLAB software tool integrated into ClinPro Tools.

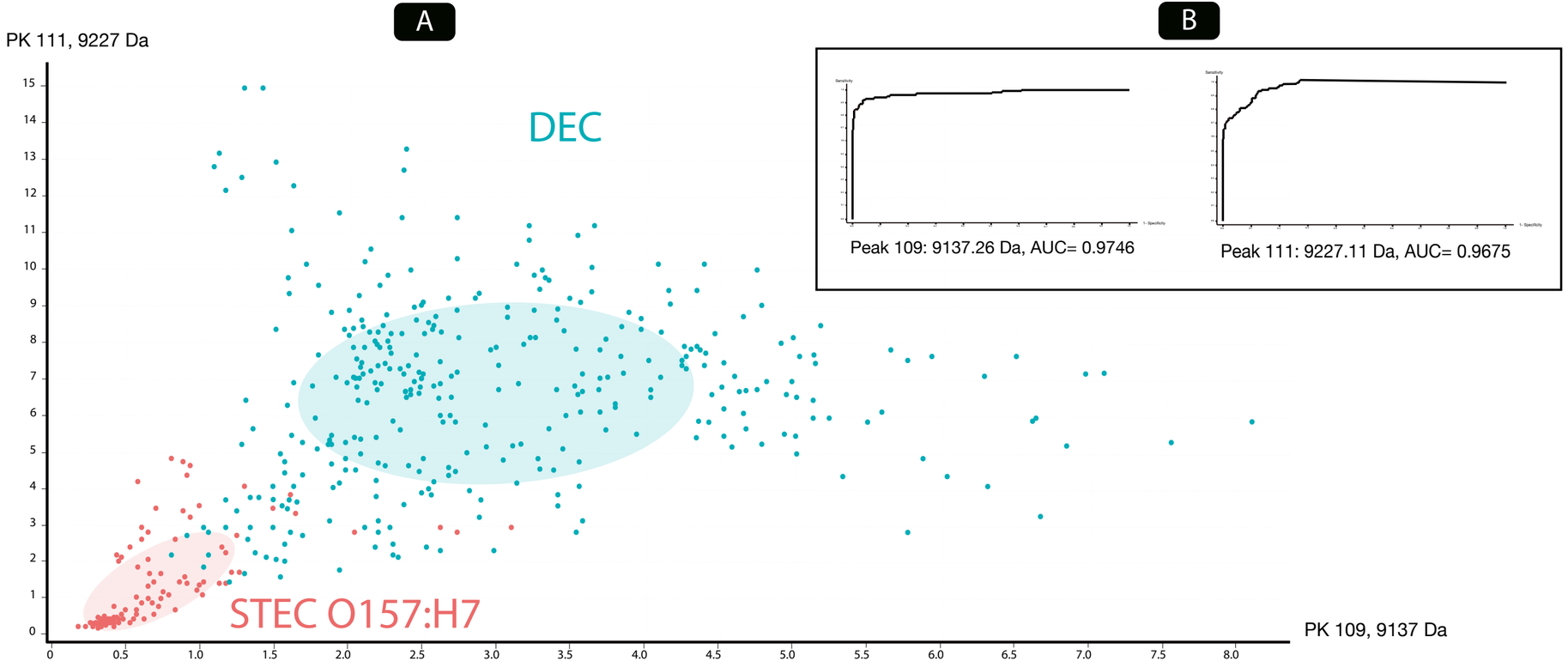


Figure 2. **A-** 2D graph of peak distribution of the 2-class model. **B-** ROC curves of the most discriminating peaks according to the analyses performed.

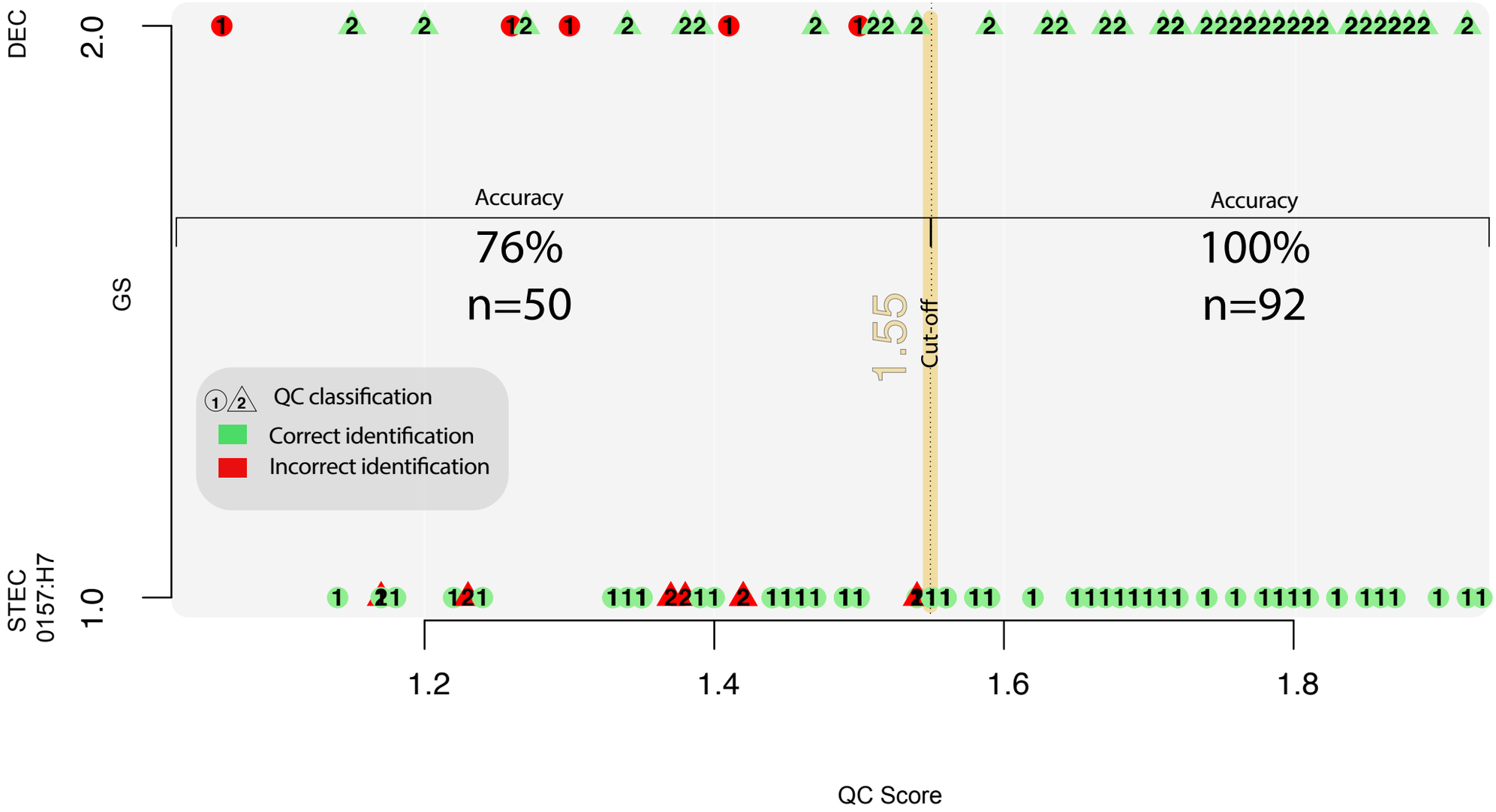


Figure 3. Graph of QC scores where the 100% concordance of the QC algorithm with the reference method is observed from the standardized cut-off value of 1.55.

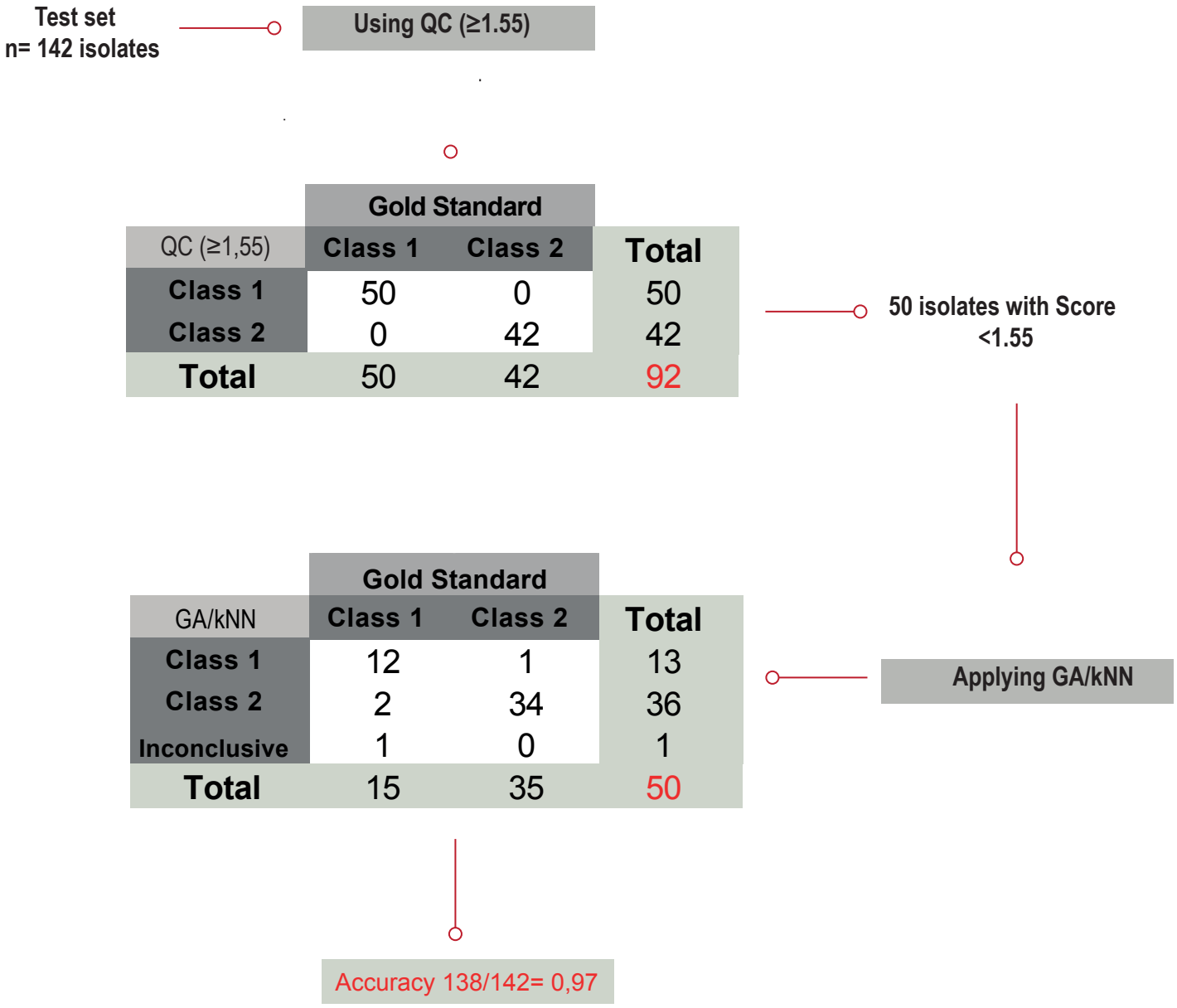
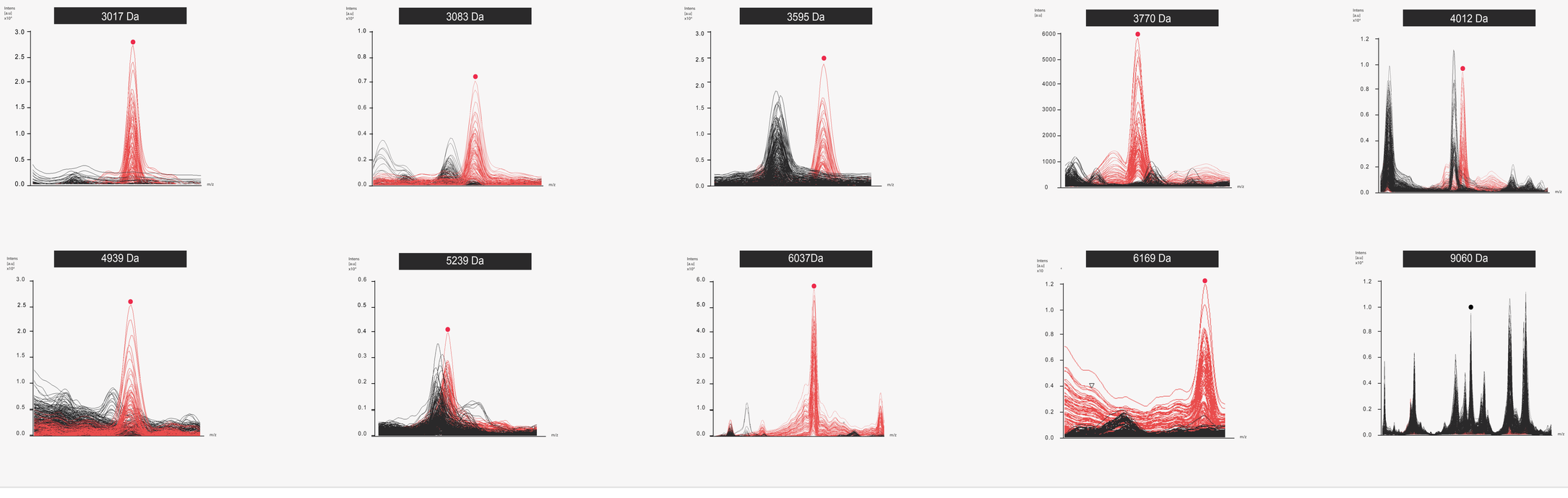
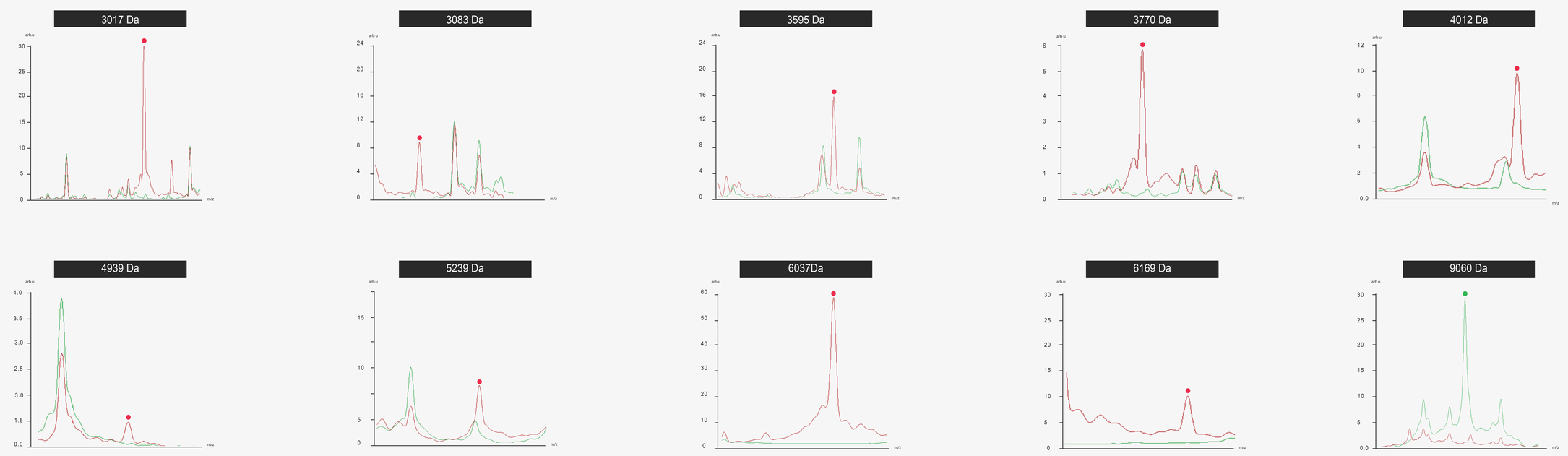


Figure 4. Algorithm applied for the identification of STEC O157:H7 based on predictive classification models.

A



B



C

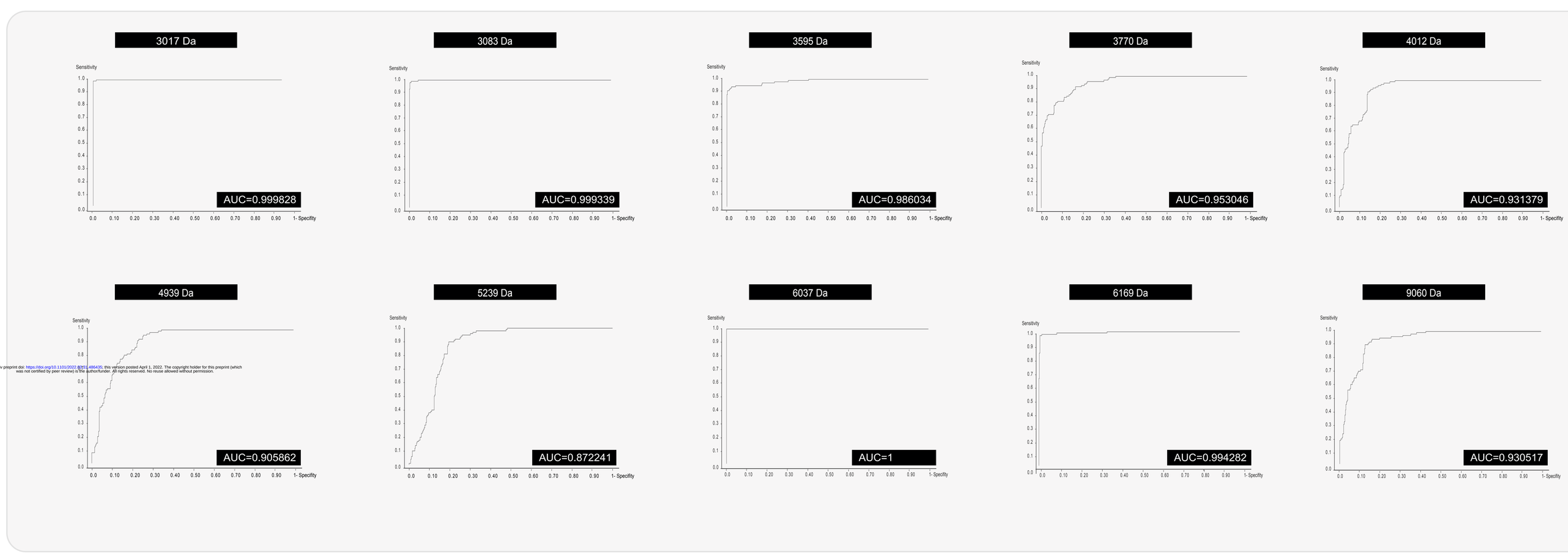


Figure 5. A- Characteristic peaks (biomarkers) in individual spectra of STEC O157:H7 samples (red) versus DEC samples (black), obtained by manual analysis in Flex Analysis v3.4 software. B- Average spectra of the same peaks, STEC O157:H7 (red); DEC (green), obtained by ClinPro Tools v3.0. C- ROC curves and AUC values originated from the external MATLAB software tool integrated in ClinPro Tools.