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Development and validation of a Partial Least Squares-Discriminant Analysis (PLS-DA) model based on the determination of ethyl glucuronide (EtG) and fatty acid ethyl esters (FAEEs) in hair for the diagnosis of chronic alcohol abuse

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Authors: E. Alladio, L. Giacomelli, G. Biosa, D.Di Corcia, E. Gerace, A. Salomone, M. Vincenti

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Development and validation of a Partial Least Squares – Discriminant Analysis (PLS-DA) model based on the determination of Ethyl Glucuronide (EtG) and Fatty Acid Ethyl Esters (FAEEs) in hair for the diagnosis of chronic alcohol abuse

E. Alladio[1,2*], L. Giacomelli[2], G. Biosa[2], D. Di Corcia[1], E. Gerace[1], A. Salomone[1], M. Vincenti[1,2]

[1]*Centro Regionale Antidoping e di Tossicologia "A. Bertinaria", Regione Gonzole 10/1, 10043 Orbassano (TO), Italy*

[2]*Dipartimento di Chimica, Università degli Studi di Torino, via P. Giuria 7, 10125 Torino, Italy*

[*]**Corresponding author**: Eugenio Alladio, PhD

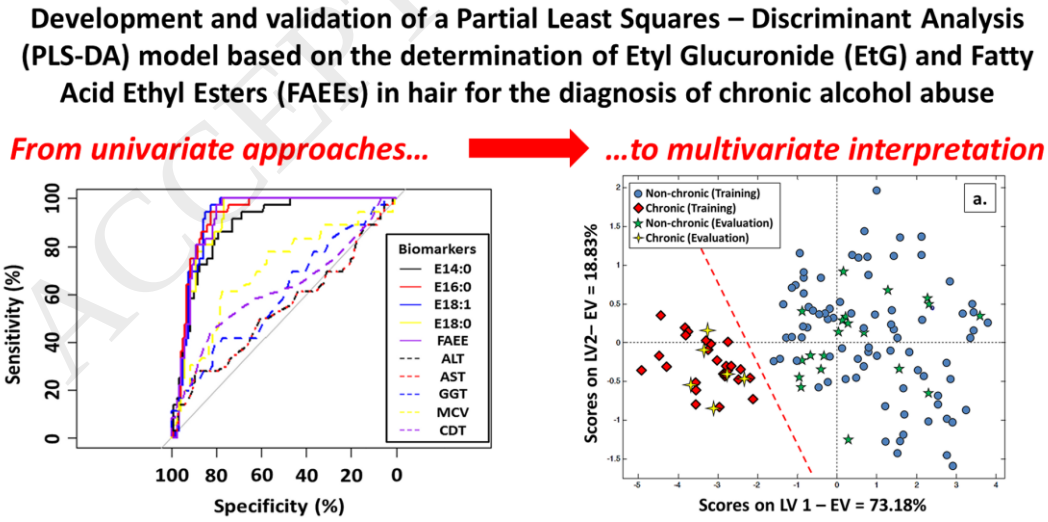
Università degli Studi di Torino, Dipartimento di Chimica

Via Pietro Giuria 7 - 10125 Torino, Italy

Mobile: +39 3460171979 Phone: +39 0116705255

E-mail: ealladio@unito.it

Graphical abstract



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Highlights

- Superior reliability of FAEEs over indirect biomarkers was demonstrated once more;
- Chemometrics turns effective for the identification of chronic alcohol drinkers;
- PLS-DA combined the predictive capabilities of both EtG and FAEEs parameters;
- This model yielded a classification decision based on probabilistic foundation;
- PLS-DA overcomes most of the drawbacks related to the use of single cut-off values.

Abstract

The chronic intake of an excessive amount of alcohol is currently ascertained by determining the concentration of direct alcohol metabolites in the hair samples of the alleged abusers, including ethyl glucuronide (EtG) and, less frequently, fatty acid ethyl esters (FAEEs). Indirect blood biomarkers of alcohol abuse are still determined to support hair EtG results and diagnose a consequent liver impairment. In the present study, the supporting role of hair FAEEs is compared with indirect blood biomarkers with respect to the contexts in which hair EtG interpretation is uncertain. Receiver Operating Characteristics (ROC) curves and multivariate Principal Component Analysis (PCA) demonstrated much stronger correlation of EtG results with FAEEs than with any single indirect biomarker or their combinations. Partial Least Squares Discriminant Analysis (PLS-DA) models based on hair EtG and FAEEs were developed to maximize the biomarkers information content on a multivariate background. The final PLS-DA model yielded 100% correct classification on a training/evaluation dataset of 155 subjects, including both chronic alcohol abusers and social drinkers. Then, the PLS-DA model was validated on an external dataset of 81 individual providing optimal discrimination ability between chronic alcohol abusers and social drinkers, in terms of specificity and sensitivity. The PLS-DA scores obtained for each subject, with respect to the PLS-DA model threshold that separates the probabilistic distributions for the two classes, furnished a likelihood ratio value, which in turn conveys the strength of the experimental data support to the classification decision, within a Bayesian logic. Typical boundary real cases from daily work are discussed, too.

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Introduction

The abuse of alcohol has an impact on different aspects of the consumers' life and generates multiple physical and psychological damages together with an increased rate of road and work accidents. For these reasons, it is necessary to adopt appropriate procedures for the recognition of individuals with alcohol-related problems, and also to monitor them during recovery programs. The current toxicology state of the art identifies a person who falls into the category of excessive alcohol consumer through the analysis of direct biomarkers in hair, i.e. ethyl glucuronide (EtG) and fatty acid ethyl esters (FAEEs) [1–9]. Although traditional indirect biomarkers (i.e., not formed by alcohol metabolic processes), including aspartate transferase (AST), alanine transferase (ALT), gamma-glutamyl transferase (GGT), mean corpuscular volume of the erythrocytes (MCV) and carbohydrate-deficient-transferrin (CDT) on blood/serum are still utilized to evaluate chronic excessive alcohol intake [5,10–12], they rather reveal the damaging effects of alcohol on target organs, but exhibit unsatisfactory sensitivity and specificity [11,13]. In fact, indirect effects largely depend on the inter-individual variability, resulting in a high rate of false positive and false negative outcomes.

Among direct biomarkers, the EtG concentration in 3-6 cm hair samples is currently used as the reference parameter for the assessment of both chronic alcohol abuse (cut-off 30 pg/mg) and abstinence (cut-off 7 pg/mg), because of its excellent diagnostic sensitivity and specificity [11,14–20]. Alongside EtG, the determination of FAEEs in hair has been largely investigated in recent years in order to support the interpretation process by adding a second trustworthy biomarker useful in doubtful situations [5,8,9,21–24]. FAEEs are a group of more than twenty compounds, formed by

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non-oxidative metabolic esterification of fatty acids as a result of ethanol consumption [25]. Traditionally, the four most abundant FAEEs are quantified, namely ethyl myristate (E14:0), ethyl palmitate (E16:0), ethyl oleate (E18:0), ethyl stearate(E18:1) [25]. More recently, the sole ethyl palmitate (E16:0) has been proposed for interpretation instead of the sum of the four FAEEs [26,27]. New cut-off values for E16:0 have been established by SoHT, namely 0.35 ng/mg for 0-3 cm proximal hair segment and 0.45 ng/mg for 0-6 cm proximal hair segment [26].

FAEEs and EtG absorption in hair is potentially influenced by several factors [28] including cosmetic treatments [29,30], seasonality [31], hair crumbling method and extraction pre-treatment procedures [32,33] among others. Consequently, the correlation observed between alcohol consumption and biomarkers' concentrations is not exact and partly depends also on their hydrophilic (EtG) or lipophilic (FAEEs) nature. Other sources of bias are the use of alcohol-based hair care products [34] and lipophilic hair waxes [13], respectively leading to FAEEs increase or decrease. False positive EtG findings had been sporadically associated with the application of EtG-containing hair care products [35]. Beside specific sources of bias, the intrinsic data variability makes sometimes the interpretation of ethanol biomarkers challenging, especially when the measured EtG and FAEEs values are close to their cut-off values or provide contradictory results. This drawback is labelled as "fall-off-cliff" problem and it is strictly embedded with the use of cut-off values [36]. It refers to the fact that small variations of the detected concentration around the cut-off value may reverse the final decision. For these reasons, combining the results from FAEEs and EtG analysis represents a valuable approach to decrease the number of misleading conclusions [21–23,37–39].

In previous studies, we proposed to use multivariate data analysis for the detection of chronic alcohol abuse [11,40–41], where the supporting role of indirect biomarkers initially chosen to strengthen hair EtG results was subsequently taken by FAEEs. In the present study, the relative synergistic contributions of FAEEs and indirect biomarkers was preliminarily evaluated on a large dataset using Receiver Operating Characteristic (ROC) curves and Principal Components Analysis

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(PCA). Then, Partial Least Squares - Discriminant Analysis (PLS-DA) was selected as the most effective statistical tool [43–45] to objectively identify chronic alcohol abuser on a multivariate basis. A PLS-DA model based on concordant hair EtG and FAEEs was developed and then validated on a large range of real caseworks. The coordinates obtained from each subject under investigation within the PLS-DA model provided the ideal score to ascertain the occurrence of chronic alcohol abuse, by means of a Bayesian approach, where the calculated likelihood ratio defines the probability and support strength for the chronic vs. non-chronic classification [41]. The apparent limitation of the present study, namely the modelling dataset was necessarily built from real casework results, not from subjects consuming controlled (both moderate and excessive) amounts of alcohol due to obvious ethical reasons, is overcome by the proven usefulness of the classification model in a group of 81 clinically-classified patients.

Materials and methods

Reagents and reference substances

Ethyl myristate (E14:0), ethyl palmitate (E16:0), ethyl oleate (E18:1), ethyl stearate (E18:0), n-heptane and dimethyl sulfoxide (DMSO) were obtained from Sigma-Aldrich (Milan, Italy). The deuterated standards D_5-ethyl myristate, D_5-ethyl palmitate, D_5-ethyl oleate, D_5-ethyl stearate were provided by Toronto Research Chemicals (TRC). Stock solutions of FAEEs, as well as the deuterated analogues, were prepared in n-heptane (1 mg/mL). A working solution containing all four D_5-FAEE at concentration of 1 µg/mL was prepared by dilution and used as internal standard (ISTD). A solution containing the non-deuterated FAEEs at concentration of 1 µg/mL for E14:0 and E18:0 and 4 µg/mL for E16:0 and E18:1 was also prepared. All solutions were stored in a refrigerator at -20°C.

Sample preparation

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The preparation of hair samples for FAEEs detection was performed in analogy to Pragst et al. [6], Suesse et al.[9] and Albermann et al.[46]. The proximal segment 0-3 cm was analyzed for each sample and 50 mg of hair was washed twice with n-heptane (3 mL, vortex mixing for 5 min). After the removal of the washing solvent, the hair aliquot was dried at room temperature overnight and then cut into 1-2 mm segments. The resulting hair samples were fortified with 30 µL ISTD, to yield a final D_5-FAEEs concentration of 0.6 ng/mg, followed by the addition of 2 mL n-heptane and 0.5 mL DMSO. Then, the samples were shaken in a multimixer for 16 hours at room temperature. Afterwards, they were cooled at -20°C (freezing of DMSO) for 30 mins and the organic phase was transferred into a 20 mL headspace vial, dried at 70°C by nitrogen stream and reconstituted with 1 mL of phosphate buffer. Then, the vials were closed with magnetic caps and placed into the vial rack of the MultiPurpose Sampler Flex.

Hair EtG analysis was executed and validated as reported previously [31]. The determination of indirect biomarkers was performed as described elsewhere [41].

Instrumentation

Headspace-Solid Phase Micro Extraction (HS-SPME) experiments were performed using a MultiPurpose Sampler Flex A05-FLX-0001 (Est Analytical, West Chester Township, OH, USA) equipped with a 65 µm Stableflex™ polydimethylsiloxane/divinylbenzene fiber (PDMS/DVB) from Supelco (Sigma-Aldrich, Milan, Italy). For HS-SPME, the following conditions were used: fiber conditioning 10 mins at 250°C; preheating 5 mins at 90°C and 250 rpm agitation; headspace adsorption 30 mins at 90°C; desorption in the GC injection port 1 min at 250°C. The injector was set in splitless mode. GC/MS determinations were performed using a 6890N GC (Agilent Technologies, Milan, Italy) equipped with a 50 m fused-silica capillary column (J&W Scientific DB 5-MS), i.d. of 0.25 mm and film thickness of 0.25 µm, used for GC separation. Helium was employed as the carrier gas at a constant pressure of 20.16 psi. The GC oven temperature was set at 140°C for 1 min and then

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raised to 265°C with a 25°C/min heating rate and further to 300°C with a 15°C/min heating rate. The total run time was 10 min. The GC injector was maintained at 250°C while the transfer-line, the ion source and the quadrupole were maintained at 280°C, 230°C and 150°C, respectively. The chromatograph was coupled to a 5975-inert MSD from Agilent Technologies (Milan, Italy) with EI at 70 eV. FAEEs and their deuterated analogues were detected by operating the mass spectrometer in Selected Ion Monitoring acquisition. The fragment ions monitored were **93**, 162, 218, 261/88, **101**, 157, 256 (D_5-ethyl myristate/ethyl myristate); **93**, 106, 162, 246/**88**, 101, 157, 241 (D_5-ethyl palmitate/ethyl palmitate); 93, **106**, 315/**88**, 101, 310 (D_5-ethyl oleate/ethyl oleate); and 93, **106**, 274, 317/88, 101, 269, **312 (**D_5-ethyl stearate/ethyl stearate). The ions used for the quantitation are marked in bold.

Validation

The procedure was validated in accordance with ISO/IEC 17025:2005 requirements. According to the expected ratio of the four esters in real samples [9], 6-point calibration curves were built at the concentrations of 0.04, 0.08, 0.12, 0.24, 0.36, 0.60 ng/mg for E14:0 and E18:0 and 0.16, 0.32, 0.48, 0.96, 1.44, 2.40 ng/mg for E16:0 and E18:1 The calibration curves proved linear over the whole range for all monitored biomarkers. Several tests were performed to verify the features of the calibration curves calculated with least-squares linear regression, according to Raposo [47]. ANOVA, lack-of-fit, back-calculation and Mandel's tests showed satisfactory results in terms of linearity and homoscedasticity in the calibration ranges considered. Limits of detection (LOD) and quantification (LOQ) values reported in Table 1 were determined with the Hubaux-Vos' method [48], and were successfully verified with experiments. Selectivity, specificity, precision and accuracy, intra- and inter-day reproducibility, carryover and thickness parameters resulted eligible in the ranges of validation too.

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Cohort description

236 individuals (225 males and 11 females) were investigated in this study. Their toxicological analyses were commissioned by Local Committees for Driving Licences and Alcohol Abuse Treatment Services (SerD) located in Piedmont (northern Italy). For 155 individuals (148 males and 7 females) the following parameters were collected: AST (μg/L), ALT (μg/L), GGT (μg/L), MCV (fl) and CDT (%), EtG (pg/mg), E14:0 (ng/mg), E16:0 (ng/mg), E18:1 (ng/mg), E18:0 (ng/mg) and ∑FAEEs (i.e. sum of E14:0, E16:0, E18:1 and E18:0 - ng/mg). On the remaining 81 individuals (77 males, 4 females) only the direct biomarkers were measured. The latter cohort was used as a models verification set. The study was accepted and granted by the Ethical Committee of the Azienda Ospedaliero-Universitaria San Luigi Gonzaga of Orbassano (Protocol Number 0012756).

Univariate and Multivariate Data Analysis

A 155×11 data matrix (Supplementary Material) was prepared to perform descriptive statistics, correlation studies and Receiver Operating Characteristics (ROC) curves by means of R software version 3.2.4 (*pROC* package was employed for this purpose) [49,50]. A few missing data (i.e. concentration levels lower than LOD values) were substituted with a value equal to one half of their corresponding LOD concentrations reported in Table 1. All the data were log-transformed to make their distribution closer to normality (according to quantile-quantile (QQ) plots and violin plots, before and after the log-transformation). PCA was applied after data autoscaling. Hotelling T^2 vs. Q Residuals plots were evaluated to identify and remove the outliers, i.e. samples that exhibited rare features [51] or anomalous concentration levels [52] with respect to the reference population.

For the development of PLS-DA models, the first-set of 155 individuals was prearranged into two categories of chronic and non-chronic alcohol drinkers on the basis of the coherence of EtG and E16:0 values with respect to the SoHT cut-offs [27]: chronic alcohol drinkers – i.e., “positive” – exhibited coherent values of EtG ≥ 30 pg/mg and E16:0 ≥ 0.35 ng/mg (29 subjects), while non-

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chronic alcohol drinkers –i.e., "negative" – had coherent values of EtG < 30 pg/mg and E16:0 < 0.35 ng/mg (101 subjects); 25 individuals out of 155 exhibited uncoherent EtG and E16:0 results with respect to the cut-off values, yielding a third category of "unlabelled" subjects. The data relative to the 130 classified individuals were arranged into multiple training sets (randomly composed by 104 subjects, i.e. 80% of 130) and evaluation sets ("outer loop", composed by the remaining 26 subjects, i.e. 20% of 130) in order to perform a repeated double cross-validation procedure [53,55]. In turn, the training set was split into calibration and internal validation samples ("inner loop") by applying the cross-validation venetian blinds design to a number of data splits equal to 5. PLS-DA models [44,56,57] were developed on EtG and FAEEs values, using both single FAEEs and their sum. The coherence of biomarkers output was considered a reasonable criterion to assemble the training sets on which a robust classification model for chronic alcohol drinkers recognition could be built. The final PLS-DA model was subsequently tested on the external 81 subjects dataset which was split into two sub-sets of 49 and 32 individuals, respectively. The first group of 49 individuals (labelled "Test") were provisionally classified according to their self-declaration of alcohol consumption. The remaining 32 individuals were provisionally classified according to the physicians of the Alcohol Abuse Treatment Services (SerD) collaborating to this study (labelled "SerD"). The physicians' judgement was based on the clinical history traced during the admission medical examination of patients and their indirect biomarkers of ethanol consumption, whenever available.

Multivariate models were carried out on MATLAB software version 7.13.0 with PLS_Toolbox version 8.2.1 [56] and the Classification Toolbox for MATLAB from Milan Chemometrics and QSAR Research Group [44]. Repeated double cross-validation strategies were performed with the help of R *chemometrics* package [55].

Results and discussion

Univariate and Multivariate Exploratory Data Analysis

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The simplest approach used to assess chronic excessive alcohol consumption is to determine hair EtG alone, and compare the measured level with the 30 pg/mg EtG cut-off value established by SoHT [27]. Following this criterion, 36 out of 155 individuals (23%) initially considered could be labelled as chronic alcohol abusers, while 119 out of 155 subjects (77%) were included in a single category comprising teetotallers and social drinkers, according to the EtG cut-off value. In order to verify how the results of other biomarkers compare with EtG data, ROC curves (Figure 1) were built for various indirect (i.e. ALT, AST, CDT, GGT, MCV) and direct (i.e. E14:0, E16:0, E18:1, E18:0 and FAEEs) biomarkers of ethanol consumption, using EtG results as the "true" classification rule. Figure 1 shows that direct biomarkers compare significantly better with EtG results than any one of the indirect biomarkers. In particular, E16:0 biomarker provided the highest value of Area Under the Curve (AUC) equal to 0.92, thus corroborating the recent update of SoHT consensus document [27], that asserts the reliability of E16:0 as a biomarker for the discrimination of non-chronic from chronic excessive alcohol drinkers.

Keeping the same simplified classification rule based on EtG cut-off, a principal component analysis (PCA) was conducted to verify how much information was added by considering various combinations of other biomarkers within a multivariate strategy. The first PCA model was calculated considering all the biomarkers simultaneously (Figure 2a-b), with the obvious exclusion of EtG; a cumulative variance (CV) of 87.72% was explained with four principal components (PCs). A rough distinction between chronic (red diamonds) and non-chronic (blue circles) individuals was observed along PC1 direction, as is evident in the PC1 vs. PC2 scores plot (68.31% CV) reported in Figure 2a. The loadings plot (Figure 2b) confirms that the highest contribution to PC1 is provided by FAEEs (both singularly considered and summed), followed by CDT, while the other indirect biomarkers contribute to the explained CV mainly along the PC2 direction, that provides no distinction between the two categories of individuals.

The second PCA model (Figure 2c-d) was built only on the indirect biomarkers; a CV of 72.18% was

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obtained from three PCs. The scores plot reported in Figure 2c, shows no distinction between the two categories of subjects, with chronic and non-chronic data points completely mixed up. The third PCA model (Figure 2e-f) was calculated on FAEEs only, yielding 95.34% CV from the first two PCs. Due to the high correlation among FAEEs, the PC1 explains more than 90% variance and provides the direction along which discrimination between chronic from non-chronic drinkers can be established. As anticipated from the ROC curves, the four FAEEs had similar loading values (Figure 2f) for PC1 and their limited variance is expressed in the PC2 exclusively. From the three PCA models, it can be concluded that effective support to EtG data in doubtful, uncertain, and biased situations is provided only by the determination of hair FAEEs, not by indirect biomarkers whose practical worth is restricted to the evaluation of health conditions of the examined subjects.

The PCA model represented in Figure 2e, shows that low PC1 values (corresponding to low FAEEs concentrations) are exclusively associated with non-chronic drinkers, but high PC1 values may correspond to both chronic and non-chronic drinkers, confirming that FAEEs determination has high sensitivity but limited specificity. To obtain a better representation for the 155 individuals of the training set, E16:0 and EtG results were plotted in a bivariate Cartesian diagram (E16:0 on the abscissa, EtG on the ordinate (Figure 3). Using both biomarkers for the evaluation of chronic excessive alcohol drinking instead of only EtG, most individuals were clearly classified either as non-chronic alcohol drinkers in Figure 3 (blue circles) – 101 out of 155 (65%) with coherent values of EtG < 30 pg/mg and E16:0 < 0.35 ng/mg – or chronic alcohol drinkers (red diamonds) – 29 out of 155 subjects (19%) with coherent values of EtG ≥ 30 pg/mg and E16:0 ≥ 0.35 ng/mg –. As a consequence, this bivariate approach classified 130 over 155 individuals (84%) with concordant biomarkers results, while 25 individuals (16%) showed contradictory EtG and E16:0 results with respect to their respective cut-off values and were marked as “unlabelled” in Figure 3 (grey triangles). In detail, 7 subjects showed EtG ≥ 30 pg/mg, but E16:0 < 0.35 ng/mg, while 18 subjects presented EtG values lower than the cut-off limit of 30 pg/mg, but E16:0 concentrations higher than 0.35 ng/mg.

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The use of concordant EtG and E16:0 results as a criteria to classify the subjects that compose the modelling dataset represents an arguable choice, somehow necessary due to the fact the ideal setting (i.e., controlled administration of alcohol) is prevented by ethical reasons, as long as the condition of excessive consumption has to be included.

Partial Least Squares-Discriminant Analysis

A linear discriminant analysis (LDA) approach was initially tested to classify the subjects on a multivariate basis. However, the developed models proved not to be sufficiently robust, due to the strong correlation existing among the direct biomarkers. Therefore, the PLS-DA technique was applied to develop more robust models for the identification of chronic alcohol drinkers with respect to the single marker approach currently used in most situations. The combinations of EtG with four FAEEs and their sum were considered for this purpose. The subjects forming the training and the evaluation matrixes were categorised as non-chronic (101), chronic (29) and unlabelled (25), in agreement with the definition provided above. The 130×6 dataset of "classified" individuals was used to develop a PLS-DA model, which was cross-validated using several randomly-built 104×6 training sets and 26×6 internal evaluation sets, consisting of 20 negative and 6 positive individuals (see Materials and Methods). The first two latent variables (LV) of the PLS-DA model described a CV of 92.01%. The corresponding scores plot (Figure 4a) showed complete discrimination between chronic and non-chronic alcohol drinkers, as is evidenced by the PLS-DA threshold (red dashed line) which separates the two classes. Thus, optimal results in terms of sensitivity (100%) and specificity (100%), on both cross-validated training (red diamonds and blue circles, in Figure 4a) and evaluation (yellow 4-point and green 5-point stars, in Figure 4a) datasets were obtained.

The new PLS-DA model based on EtG and FAEEs was tested on the group of the 25 individuals with contrasting EtG and E16:0 outcomes and defined as "unlabelled" in the bivariate approach. The biomarkers concentration levels and PLS-DA responses for these subjects are reported in Table 2.

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According to the PLS-DA threshold reported in Figure 4a, 10 out of 25 unknown subjects (40%) were identified as chronic excessive alcohol drinkers, while the remaining 15 individuals (60%) were classified as non-chronic drinkers (the scores of the individuals defined as “unlabelled” are denoted by grey stars in Figure 4b). In particular, all seven subjects (19-25) with EtG results exceeding the 30 pg/mg cut-off were classified as chronic excessive drinkers by the model, in agreement with the fact that their FAEEs concentrations were relatively high (sum of FAEEs range: 0.46-0.92 ng/mg; E16:0 range: 0.20-0.27 ng/mg). For the remaining three subjects classified as chronic excessive drinkers, E16:0 levels exceeding the cut-off value were recorded along with relatively high EtG concentrations (EtG range: 22-26 pg/mg). On the other side, the 15 subjects classified as non-chronic by the PLS-DA model showed high E16:0 results in conjunction with EtG values far from the 30 pg/mg cut-off. As an example, subject 14 had a E16:0 concentration equal to 2.15 ng/mg, but only 6 pg/mg for EtG.

Although the proposed PLS-DA model indicates the most probable classification for each of these individuals, this model still suffers somehow from the “fall-off-cliff” problem - typical of univariate approaches - that we described in a previous study and was overcome by introducing a likelihood-ratio Bayesian method to alcohol biomarkers evaluation [41]. For example, individuals 13 and 17 exhibited similar EtG, E16:0, and FAEEs concentrations (all of them are slightly higher for subject 17, see Table 2), but were classified in opposing ways because they are close to the PLS-DA threshold on opposite sides. Further caution should be addressed also to subjects 12 and 15, whose EtG level is 18 pg/mg, significantly below the cut-off, but in conjunction with extremely high FAEEs levels. For all these situations, the present PLS-DA model can be used to provide the optimal probabilistic background on which the cited likelihood ratio method is built, which in turn is used to express the support to the chronic vs. non-chronic classification with an appropriate scale (inconclusive, weak, moderate, moderately strong, strong, very strong support) [41]. Accordingly, a likelihood ratio (LR) model was built on the PLS-DA scores of the subjects of the training set (showed in Figure 4a), in agreement with our previous work [41]. LR values and their relative verbal translation describing the

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strength of the support associated with the classification prediction are reported in the last two columns of Table 2. Following LR values, the aforementioned individuals 13 and 17 are no more classified in opposing ways, since a weak support to the non-chronic hypothesis (LR = 8.55E+01) is expressed for subject 13, whereas an inconclusive result (LR = 1.15E-01) is obtained for subject 17. This LR approach intrinsically takes into account the variability of biomarkers values associated with potential influencing factors and helps the forensic expert during the decision process, by providing him a confidence level for the conclusion drawn from the PLS-DA model. With reference to the subjects 12 and 15 cited above, whose LR values turned to be equal to 1.62E+02 and 3.26E+03, respectively. In the present case, LR values suggested moderate (subject 12) and moderately strong (subject 15) supports to the "non-chronic" hypotheses, in accordance with the PLS-DA model.

Real caseworks

The PLS-DA model was tested on 81 subjects from real caseworks, among which 49 subjects provided a self-declaration of their drinking habits (1-49, group defined as "Test"). The remaining 32 individuals (50-81, group defined as "SerD") were tentatively classified by the physicians of Alcohol Abuse Treatment Services (SerD), according to their clinical history, whose records ranged from a single visit to years-long care-taking. The biomarkers concentrations for the examined individuals of the "Test" group, their self-declared classification, the prediction of the PLS-DA model, the LR values, and their verbal conversion are reported in Table 3a. Figure 5a shows the PLS-DA model scores plot in which the data-points for these subjects were inserted, respectively as green stars (i.e. self-declared non-chronic drinkers) and yellow stars (i.e. self-declared chronic excessive drinkers). Complete agreement between the self-declared classification (42 non-chronic, 7 chronic) and PLS-DA model prediction was observed.

The PLS-DA model was subsequently tested on the 32 individuals of the "SerD" group. The

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corresponding biomarkers results, the classification suggested by the physicians and the prediction of the PLS-DA model are reported in Table 3b, together with the corresponding LR results. In this group, 19 out of 32 subjects were *a-priori* classified as non-chronic drinkers by SerD physicians (green stars in Figure 5b), while 13 were defined as chronic excessive alcohol drinkers (yellow stars in Figure 5b). The PLS-DA model confirmed the provisional classification for 24 subjects only (50-73) whereas the alternative classification was suggested by the PLS-DA model for 8 subjects (74-81) with respect to the SerD proposal. Among the latters, the PLS-DA model indicates subjects 74-80 as alleged chronic excessive drinkers. As a matter of fact, relatively high EtG, E16:0, and FAEEs levels were recorded for all of them, even if E16:0 and FAEEs concentrations were below the cut-off values for subjects 78 and 79. Indeed, the clinical history for all subjects 74-80 proved to be extremely limited, as all of them had been previously visited only once, and the physicians preliminarily classified them just after they took on responsibility of these patients. An opposite situation is represented by individual 81, who was a long-term patient of SerD: the physicians still classified him as a chronic excessive drinker due to the fact that relatively high values for his blood biomarkers were recorded, especially CDT and GGT, despite the subject declared that he considerably reduced his drinking habits in the last 40 days, till complete abstinence. Direct alcohol biomarkers (e.g. 19 pg/mg for EtG and 0.073 ng/mg for E16:0) and the PLS-DA model strongly supported the patient's statement, reversing the SerD judgement, as suggested by the LR model too.

In conclusion, the adoption of our PLS-DA model based on hair analysis of direct chronic alcohol consumption biomarkers (EtG and FAEEs) proved to represent a valuable tool to assist the SerD physicians during their clinical evaluation process. This validated approach, combined with the Bayesian method recently described [41,42], represents a further development our previous research activity [1,11,40] that underlined the need of introducing a multivariate interpretation of the available alcohol consumption biomarkers data in order to obtain a clearer characterization chronic excessive alcohol drinkers and their drinking habits, for clinical purposes.

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Conclusions

In a large analytical toxicology laboratory, hundreds of hair samples are processed each week to identify, by means of EtG analysis, the possible subsistence of chronic excessive alcohol intake conditions in subjects involved in workplace testing, driving license granting, and rehabilitation programs. Although hair EtG determination is generally granted with about 98% sensitivity and selectivity using the prescribed cut-off value, it turns out that 3-4% of samples risks to be incorrectly classified (several units each week), most of which have EtG values close to the cut-off. In dubious, uncertain, and borderline situations the determination of further alcohol biomarkers is recommended, in particular hair FAEEs (or the single E16:0) [26], although several laboratories still prefer indirect blood biomarkers. In the current study, the superior reliability of FAEEs with respect to indirect biomarkers to support EtG data has been demonstrated once more. However, the frequent occurrence of conflicting compliance of EtG and FAEEs results opens the data interpretation question, that can be rationally approached only on the ground of statistical reasoning.

Multivariate data analysis clearly represents the most effective approach for the identification of chronic excessive alcohol drinkers. In particular, the multivariate PLS-DA model proposed in the present study proved to combine the predictive capabilities of both EtG and FAEEs parameters with optimal relative weighting, yielding a classification decision based on probabilistic foundation, which in turn relies on the multivariate space organized from a selected reference populations of chronic and non-chronic alcohol drinkers. The borderline cases taken from the real daily workflow considered in this study confirm the effectiveness of the present classification strategy.

Although the adoption of such a PLS-DA model overcomes most of the drawbacks related to the use of single cut-off values. It still requires further data elaboration to express its probabilistic significance on a graduate scale. This objective was easily achieved by introducing the PLS-DA scores

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obtained from each subject under study within the probabilistic distributions for the two modelling classes, yielding a likelihood ratio value, which expresses –numerically and verbally - the strength of the support to the classification decision, within a Bayesian logic [41].

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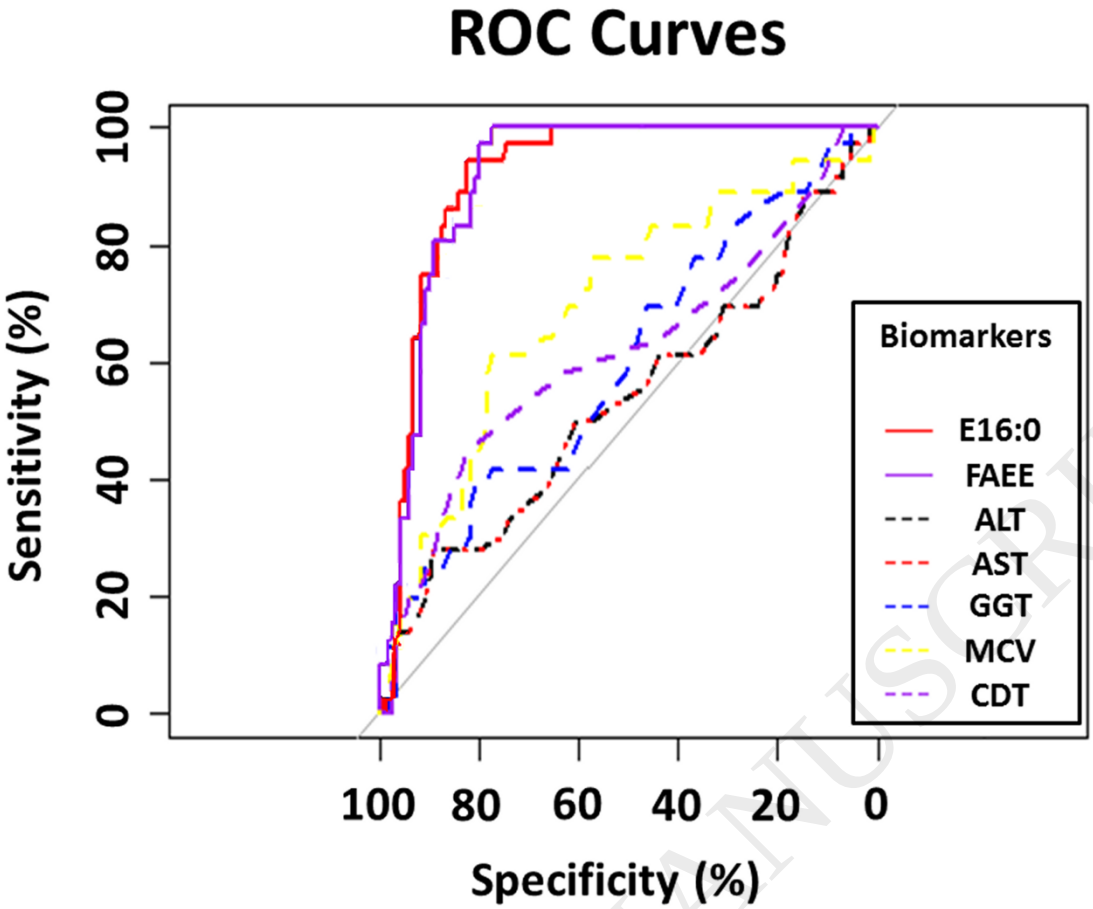

Figure 1. ROC curves for direct (i.e. E14:0, E16:0, E18:1, E18:0 and FAEEs, continuous lines) and indirect (i.e. ALT, AST, CDT, GGT and MCV, dashed lines) biomarkers of ethanol consumption, with respect to SoHT EtG cut-off value equal to 30 pg/mg, used as the classification criterium.

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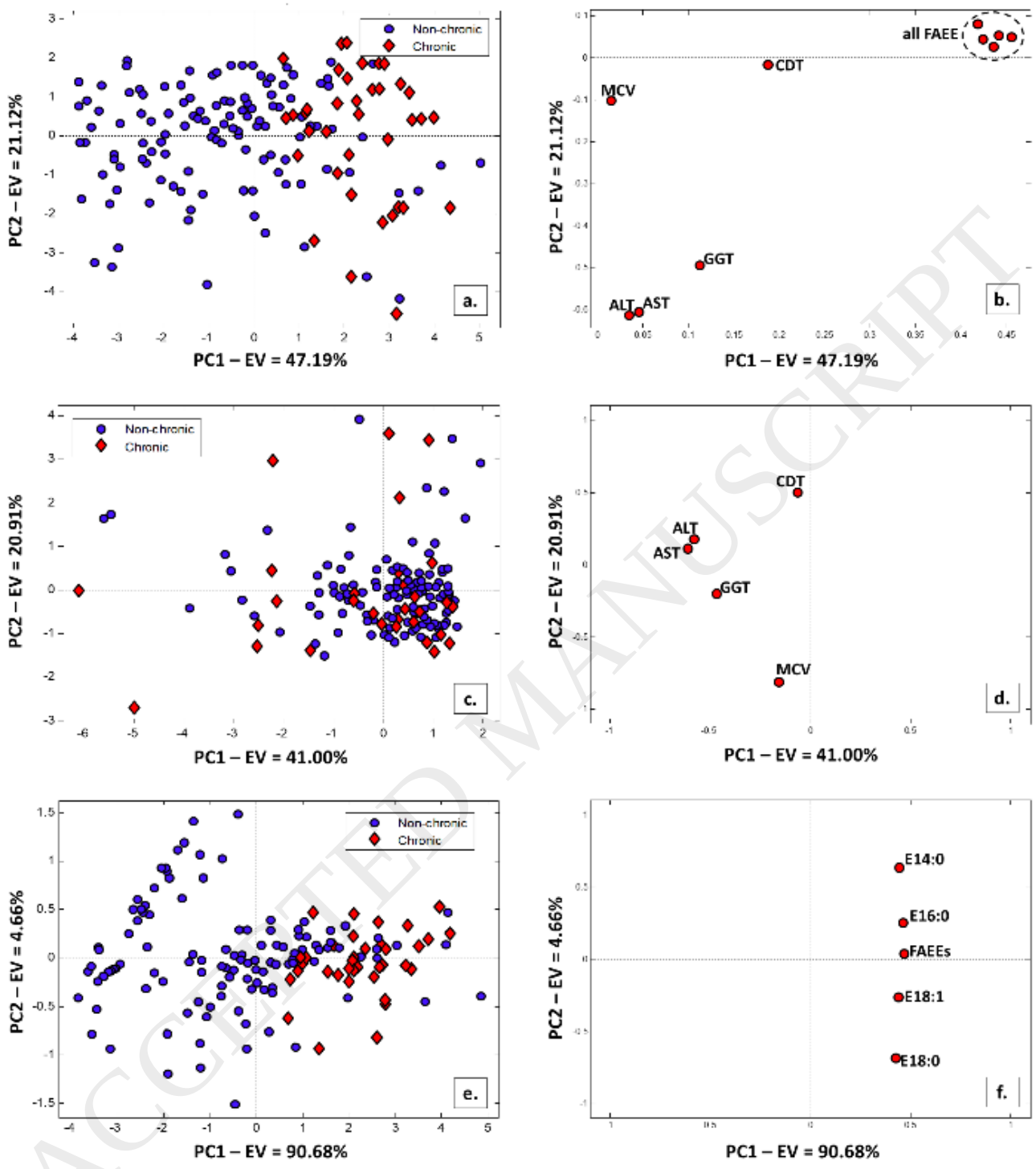

Figure 2. PCA scores plots (a, c, e) and loading plots (b, d, f) using different biomarkers as variables. The subjects are classified with reference to SoHT cut-off value of EtG (30 pg/mg). (a, b) PCA scores and loadings graphs obtained from indirect biomarkers and FAEEs; (c, d) PCA scores and loadings graphs obtained from indirect biomarkers only; (e, f) PCA scores and loadings graphs obtained from FAEEs only. Chronic excessive

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alcohol drinkers are represented by red diamonds, while social drinkers are indicated by blue circles.

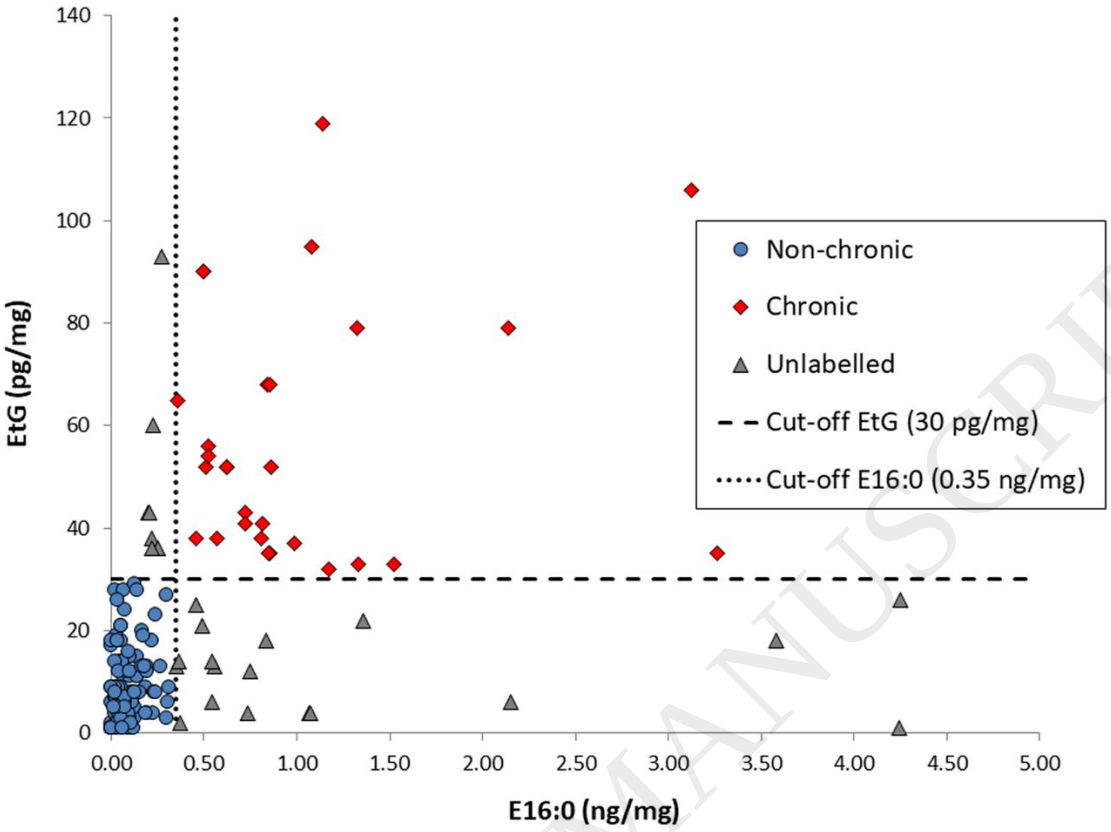

Figure 3. E16:0 vs EtG plot representing the bivariate classification criteria defined by SoHT cut-off values [26]. Chronic excessive alcohol drinkers are represented by red diamonds, while social drinkers are indicated by blue circles. Grey triangles represent the individuals (i.e. "unlabelled" category) whose EtG and E16:0 results yielded incoherent classification with respect to their cut-off values [26].

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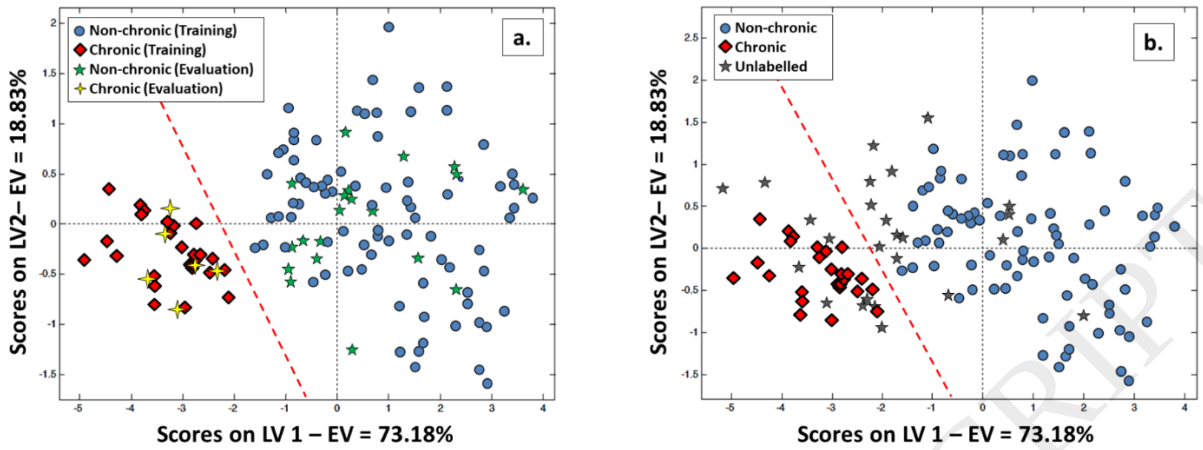

Figure 4. PLS-DA scores plots built with the use of all the direct biomarkers on (a) the training (104×6) and the internal evaluation (26×6) sets, and (b) the 25 individuals providing EtG and E16:0 values uncoherent to SoHT cut-offs (named "unlabelled"), represented by grey stars. Chronic excessive alcohol drinkers are represented by red diamonds (training set) and yellow 4-point stars (evaluation set), while non-chronic drinkers are indicated by blue circles (training set) and green 5-point stars (evaluation set). The red dashed line represents the PLS-DA delimiter.

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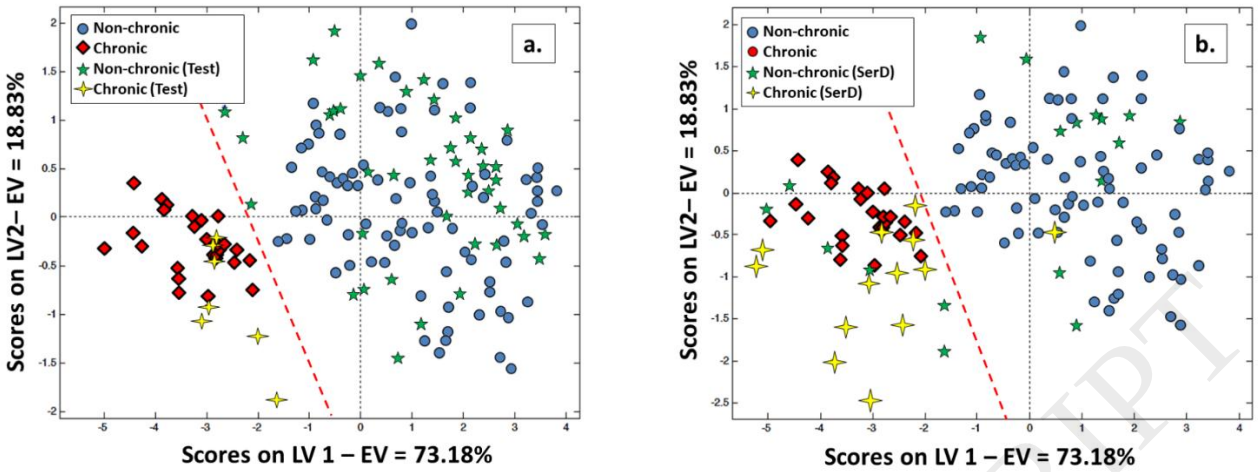

Figure 5. PLS-DA scores plots relative to the predictions of the tested datasets consisting of the real caseworks' subjects (a) that were *a-priori* classified according to their self-declared drinking habits (named "Test" group), and the ones (b) that were classified by physicians according to their clinical history (named "SerD" group). Non-chronic alcohol drinkers are represented by blue circles (training set) and green 5-point stars (Test/SerD groups), while chronic alcohol drinkers are indicated by red diamonds (training set) and yellow 4-point stars (Test/SerD groups). The red dashed line represents the PLS-DA delimiter.

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Table 1. Validation data of the determination of FAEEs by HS-SPME-GC/MS-SIM.

Fatty Acid Ethyl Esthers (FAEE)	LOD (ng/mg)	LOQ (ng/mg)
E14:0	0.003	0.005
E16:0	0.008	0.016
E18:1	0.017	0.033
E18:0	0.002	0.004

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Table 2. E14:0, E16:0, E18:1, E18:0, FAEEs and EtG concentration levels relative to the selected subjects defined as "unlabelled" according to the bivariate model considering EtG and E16:0 cut-offs only. EtG and E16:0 values higher than the cut-offs established by SoHT (30 pg/mg and 0.35 ng/mg, respectively) are reported in bold. PLS-DA classification, likelihood ratio values, and their relative verbal translation are reported [41]. Support strength: INC = Incoherent; W = weak; M = moderate; MS = moderately strong; S = strong; VS = very strong.

"Unlabelled" individuals (Evaluation set)

ID	E14:0 (ng/mg)	E16:0 (ng/mg)	E18:1 (ng/mg)	E18:0 (ng/mg)	FAEEs (ng/mg)	EtG (pg/mg)	PLS-DA Classification	Likelihood Ratio	Strength of the support
1	0.065	**0.352**	0.240	0.065	0.721	13	Non-Chronic	8.17E+02	M
2	0.093	**0.363**	0.201	0.064	0.720	14	Non-Chronic	6.26E+02	M
3	0.110	**0.369**	0.144	0.106	0.729	2	Non-Chronic	2.05E+07	VS
4	0.167	**0.748**	0.239	0.093	1.246	12	Non-Chronic	1.34E+02	M
5	0.092	**0.559**	0.231	0.079	0.962	13	Non-Chronic	5.58E+01	W
6	0.103	**0.546**	0.119	0.739	1.506	14	Non-Chronic	7.14E+04	S
7	0.259	**1.068**	0.471	0.284	2.082	4	Non-Chronic	1.26E+13	VS
8	0.253	**1.070**	0.571	0.188	2.083	4	Non-Chronic	1.54E+11	VS
9	0.166	**0.737**	0.431	0.192	1.526	4	Non-Chronic	4.28E+11	VS
10	0.911	**4.240**	1.313	0.524	6.988	1	Non-Chronic	4.82E+08	VS
11	0.131	**0.543**	0.227	0.127	1.027	6	Non-Chronic	3.43E+08	VS
12	0.600	**3.578**	1.550	0.802	6.530	18	Non-Chronic	1.62E+02	MS
13	0.141	**0.492**	0.209	0.073	0.915	21	Non-Chronic	8.55E+01	W
14	0.180	**2.155**	1.920	0.891	5.147	6	Non-Chronic	3.81E+04	S
15	0.201	**0.838**	0.771	0.207	2.016	18	Non-Chronic	3.26E+03	M
16	0.613	**4.249**	3.488	3.629	11.978	26	Chronic	8.27E-07	VS
17	0.137	**0.456**	0.200	0.115	0.909	25	Chronic	1.51E-01	INC
18	0.316	**1.356**	0.771	0.311	2.755	22	Chronic	1.07E-02	W
19	0.089	0.197	0.164	0.074	0.524	**43**	Chronic	1.31E-03	M
20	0.031	0.223	0.265	0.397	0.916	**38**	Chronic	6.47E-03	M
21	0.061	0.255	0.196	0.061	0.573	**36**	Chronic	2.23E-03	M
22	0.062	0.217	0.149	0.083	0.511	**36**	Chronic	5.88E-03	M
23	0.020	0.224	0.165	0.102	0.511	**60**	Chronic	8.21E-05	S
24	0.083	0.275	0.117	0.093	0.568	**93**	Chronic	1.73E-05	S
25	0.043	0.207	0.135	0.074	0.460	**43**	Chronic	4.46E-04	MS

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Table 3a. E14:0, E16:0, E18:1, E18:0, FAEEs and EtG concentration levels relative to the selected subjects belonging to the "Test" group. EtG and E16:0 values higher than the cut-offs established by SoHT (30 pg/mg and 0.35 ng/mg, respectively) are reported in bold. PLS-DA classification, likelihood ratio values, and their relative verbal translation are reported [41]. Support strength: INC = Incoherent; W = weak; M = moderate; MS = moderately strong; S = strong; VS = very strong.

Real caseworks individuals ("Test" group)

ID	E14:0 (ng/mg)	E16:0 (ng/mg)	E18:1 (ng/mg)	E18:0 (ng/mg)	FAEEs (ng/mg)	EtG (pg/mg)	Self-declared Classification	PLS-DA Classification	Likelihood Ratio	Strength of the support
1	0.002	0.078	0.009	0.016	0.104	3	Non-Chronic	Non-Chronic	1.04E+39	VS
2	0.002	0.004	0.009	0.007	0.021	2	Non-Chronic	Non-Chronic	3.74E+51	VS
3	0.002	0.095	0.210	0.013	0.320	2	Non-Chronic	Non-Chronic	7.00E+20	VS
4	0.002	0.238	0.111	0.016	0.366	9	Non-Chronic	Non-Chronic	4.56E+09	VS
5	0.002	0.017	0.035	0.008	0.062	2	Non-Chronic	Non-Chronic	2.87E+36	VS
6	0.268	0.056	0.009	0.029	0.361	2	Non-Chronic	Non-Chronic	2.03E+42	VS
7	0.002	0.061	0.009	0.008	0.079	2	Non-Chronic	Non-Chronic	2.36E+46	VS
8	0.015	0.131	0.136	0.009	0.292	3	Non-Chronic	Non-Chronic	3.65E+16	VS
9	0.002	0.216	0.009	0.099	0.325	3	Non-Chronic	Non-Chronic	7.23E+40	VS
10	0.002	0.112	0.009	0.004	0.126	3	Non-Chronic	Non-Chronic	3.20E+37	VS
11	0.002	0.004	0.023	0.006	0.034	3	Non-Chronic	Non-Chronic	5.11E+37	VS
12	0.002	0.004	0.009	0.001	0.015	2	Non-Chronic	Non-Chronic	3.49E+53	VS
13	0.002	0.004	0.009	0.001	0.015	3	Non-Chronic	Non-Chronic	5.83E+47	VS
14	0.002	0.027	0.009	0.003	0.041	1	Non-Chronic	Non-Chronic	1.15E+61	VS
15	0.002	0.055	0.009	0.001	0.066	14	Non-Chronic	Non-Chronic	2.40E+22	VS
16	0.002	0.067	0.009	0.004	0.081	3	Non-Chronic	Non-Chronic	5.46E+38	VS
17	0.002	0.078	0.009	0.001	0.089	2	Non-Chronic	Non-Chronic	2.93E+44	VS
18	0.002	0.051	0.009	0.001	0.062	7	Non-Chronic	Non-Chronic	3.82E+29	VS
19	0.002	0.016	0.009	0.004	0.030	3	Non-Chronic	Non-Chronic	6.39E+42	VS
20	0.002	0.004	0.009	0.004	0.018	3	Non-Chronic	Non-Chronic	2.60E+46	VS
21	0.002	0.072	0.009	0.022	0.105	2	Non-Chronic	Non-Chronic	1.48E+47	VS
22	0.002	0.004	0.009	0.039	0.053	2	Non-Chronic	Non-Chronic	4.89E+48	VS
23	0.002	0.152	0.193	0.795	1.142	3	Non-Chronic	Non-Chronic	1.88E+19	VS
24	0.002	0.186	0.148	0.655	0.990	3	Non-Chronic	Non-Chronic	1.14E+21	VS
25	0.002	**0.459**	0.043	1.207	1.711	2	Non-Chronic	Non-Chronic	1.08E+42	VS
26	0.002	0.213	0.009	0.043	0.266	2	Non-Chronic	Non-Chronic	1.82E+48	VS
27	0.002	**0.436**	4.680	1.162	6.279	3	Non-Chronic	Non-Chronic	7.30E+04	S
28	0.002	**0.500**	0.076	1.596	2.174	3	Non-Chronic	Non-Chronic	2.73E+29	VS
29	0.002	0.004	0.009	0.041	0.055	3	Non-Chronic	Non-Chronic	9.31E+41	VS
30	0.002	0.209	0.026	0.657	0.894	3	Non-Chronic	Non-Chronic	2.70E+34	VS
31	0.091	0.259	0.126	0.010	0.486	2	Non-Chronic	Non-Chronic	4.43E+21	VS
32	0.390	**1.028**	0.583	0.131	2.132	5	Non-Chronic	Non-Chronic	6.24E+07	VS
33	0.002	0.064	0.009	0.057	0.131	5	Non-Chronic	Non-Chronic	1.49E+31	VS
34	0.011	0.052	0.009	0.033	0.105	2	Non-chronic	Non-Chronic	7.96E+45	VS
35	0.002	0.022	0.009	0.046	0.077	8	Non-chronic	Non-Chronic	9.95E+26	VS
36	0.097	**0.517**	0.321	0.107	1.042	17	Non-chronic	Non-Chronic	1.03E+04	S
37	0.005	0.008	0.023	0.024	0.060	26	Non-chronic	Non-Chronic	2.96E+13	VS
38	0.012	0.044	0.009	0.028	0.093	**34**	Non-chronic	Non-Chronic	7.42E+10	VS
39	0.014	0.019	0.025	0.023	0.081	27	Non-chronic	Non-Chronic	4.00E+09	VS
40	0.015	0.045	0.094	0.024	0.178	22	Non-chronic	Non-Chronic	7.49E+04	S
41	0.013	0.054	0.047	0.024	0.138	25	Non-chronic	Non-Chronic	3.16E+06	VS
42	0.012	0.033	0.022	0.030	0.097	28	Non-chronic	Non-Chronic	1.27E+09	VS
43	0.027	0.279	0.232	0.084	0.623	**98**	Chronic	Chronic	2.64E-04	MS
44	0.140	**0.551**	0.972	0.050	1.714	**59**	Chronic	Chronic	3.97E-05	S
45	1.211	**2.218**	1.379	1.163	5.971	**201**	Chronic	Chronic	2.60E-08	VS
46	0.748	**2.525**	4.282	0.498	8.053	**252**	Chronic	Chronic	1.82E-06	VS
47	0.081	**0.415**	0.320	0.108	0.923	**153**	Chronic	Chronic	1.01E-06	VS
48	0.095	**0.447**	0.352	0.101	0.995	**147**	Chronic	Chronic	6.58E-05	S
49	0.101	**0.695**	0.424	0.171	1.391	**273**	Chronic	Chronic	5.16E-06	VS

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Table 3b. E14:0, E16:0, E18:1, E18:0, FAEEs and EtG concentration levels relative to the selected subjects belonging to the "SerD" group. EtG and E16:0 values higher than the cut-offs established by SoHT (30 pg/mg and 0.35 ng/mg, respectively) are reported in bold. PLS-DA classification, likelihood ratio values, and their relative verbal translation are reported [41]. Support strength: INC = Incoherent; W = weak; M = moderate; MS = moderately strong; S = strong; VS = very strong.

Real caseworks individuals ("SerD" group)

ID	E14:0 (ng/mg)	E16:0 (ng/mg)	E18:1 (ng/mg)	E18:0 (ng/mg)	FAEEs (ng/mg)	EtG (pg/mg)	Self-declared Classification	PLS-DA Classification	Likelihood Ratio	Strength of the support
50	1.211	**2.218**	1.379	1.163	5.971	**194**	Chronic	Chronic	2.55E-08	VS
51	0.005	0.008	0.023	0.024	0.060	23	Non-Chronic	Non-Chronic	1.10E+14	VS
52	0.012	0.044	0.009	0.028	0.094	3	Non-Chronic	Non-Chronic	3.12E+37	VS
53	0.014	0.019	0.025	0.023	0.081	29	Non-Chronic	Non-Chronic	1.69E+09	VS
54	0.748	**2.525**	4.282	0.498	8.053	**149**	Chronic	Chronic	4.51E-08	VS
55	0.015	0.045	0.094	0.024	0.178	2	Non-Chronic	Non-Chronic	1.37E+25	VS
56	0.013	0.054	0.047	0.024	0.138	2	Non-Chronic	Non-Chronic	5.65E+30	VS
57	0.078	0.211	0.328	0.141	0.757	**278**	Chronic	Chronic	1.12E-07	VS
58	0.106	0.280	0.328	0.118	0.832	**42**	Chronic	Chronic	1.63E-03	M
59	0.257	**0.552**	0.497	0.141	1.447	**266**	Chronic	Chronic	3.54E-08	VS
60	0.141	**0.548**	0.367	0.179	1.235	**136**	Chronic	Chronic	1.56E-07	VS
61	0.002	0.044	0.009	0.001	0.056	1	Non-Chronic	Non-Chronic	1.31E+59	VS
62	0.214	**0.617**	0.477	0.151	1.458	**307**	Chronic	Chronic	7.39E-09	VS
63	0.096	**0.452**	0.217	0.623	1.388	**77**	Chronic	Chronic	2.83E-05	S
64	0.081	**0.427**	0.320	0.108	0.935	**85**	Chronic	Chronic	2.86E-05	S
65	0.095	**0.447**	0.352	0.101	0.995	24	Non-Chronic	Non-Chronic	1.07E+02	M
66	0.101	**0.695**	0.424	0.171	1.391	**104**	Chronic	Chronic	2.75E-06	VS
67	0.007	0.016	0.002	0.034	0.060	2	Non-Chronic	Non-Chronic	1.53E+61	VS
68	0.049	0.242	0.252	0.092	0.637	**62**	Chronic	Chronic	1.94E-03	M
69	0.012	0.033	0.022	0.030	0.097	2	Non-Chronic	Non-Chronic	2.31E+37	VS
70	0.079	0.309	0.250	0.059	0.696	**184**	Chronic	Chronic	1.47E-05	S
71	0.005	0.028	0.012	0.020	0.066	3	Non-Chronic	Non-Chronic	4.08E+36	VS
72	0.186	0.286	0.206	0.059	0.738	1	Non-Chronic	Non-Chronic	1.11E+35	VS
73	0.037	0.154	0.127	0.047	0.365	1	Non-Chronic	Non-Chronic	1.42E+37	VS
74	1.143	**2.912**	1.743	0.343	6.141	**44**	**Non-Chronic**	**Chronic**	7.10E-06	VS
75	0.131	**0.626**	0.448	0.110	1.315	**85**	**Non-Chronic**	**Chronic**	5.79E-04	MS
76	1.429	**3.299**	1.974	0.606	7.308	**82**	**Non-Chronic**	**Chronic**	1.04E-07	VS
77	0.436	**1.126**	1.096	0.158	2.817	**81**	**Non-Chronic**	**Chronic**	1.86E-05	VS
78	0.086	0.165	0.586	0.001	0.838	**70**	**Non-Chronic**	**Chronic**	4.49E-03	M
79	0.001	0.349	0.372	0.117	0.839	**82**	**Non-Chronic**	**Chronic**	1.08E-03	M
80	0.057	**0.448**	0.672	0.362	1.539	**37**	**Non-Chronic**	**Chronic**	4.10E-03	M
81	0.005	0.073	0.027	0.028	0.132	19	**Chronic**	**Non-Chronic**	9.35E+10	VS