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Three-way models and detection capability of a gas chromatography–mass spectrometry method for the determination of clenbuterol in several biological matrices: the 2002/657/EC European Decision

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Abstract

Clenbuterol has been extracted by mixed solid-phase extraction from two biological matrices (bovine hair and urine) and detected by GC/MS (selected ion monitoring (SIM) and full-SCAN modes). The analytical signal has been modelled with univariate and three-way models, namely DTLD, PARAFAC, PARAFAC2, Tucker3 and trilinear PLS. Since clenbuterol is a banned substance a comparative study of the capability of detection (CCβ, $X_0 = 0$) has been performed as a function of the sample (hair, 74 μg kg^{-1} and urine, 0.36 μg l^{-1}), the mode in which the signals are monitored (SCAN, 283 μg kg^{-1} and SIM, 74 μg kg^{-1}) and the statistical model (univariate, 283 μg kg^{-1} and trilinear PLS, 20.91 μg kg^{-1}). The capability of detection has been calculated as stated in ISO 11843 and Decision 2002/657/EC setting in all cases the probabilities of false positive and of false negative at 0.05.

The identification of the mass spectra must be done to confirm the presence of clenbuterol and has been carried out through PARAFAC. The correlation coefficient between the spectra estimated by PARAFAC and the library spectra is 0.96 (hair, SCAN mode) and 1.00 (hair and urine, SIM mode).

The Decision 2002/657/EC advocates the use of independent mass fragments to identify banned compounds. These recommendations together with the effect of the number of ions registered on the capability of detection have lead us to select five uncorrelated fragments (86, 243, 262, 264 and 277) from the data set of 210 ions by hierarchical clustering of variables.

Keywords: Capability of detection; Decision 2002/657/EC; β_2-Agonist; Mixed SPE; Cluster; PARAFAC estimation; Trilinear PLS; GC/MS

1. Introduction

1.1. Legal framework and analytical method to detect clenbuterol

Clenbuterol is a β_2-agonist compound often fraudulently used as a promoter of growth and productivity of livestock. Due to its adverse effects on human health, the European Union (Directive 96/22/EC) has banned the administration of clenbuterol to any animal species intended for human consumption, except for therapeutic treatment for equidae and pregnant cows under veterinary supervision. Official controls for detecting its illegal administration are detailed in the Directive 96/23/EC, according to which clenbuterol is classified in Group A of Annex I.

Sampling is required both in living animals (urine [1], hair [2], etc.) and in the abattoir (retinal tissue [3], liver [1,4], feed [1], etc.) which implies different pre-treatment procedures [1] depending on the sample nature. The considerable variety of biological matrices makes the selection of the sample to be analysed an important step, not only to guarantee that the residue levels are high enough but also because the sample nature will influence the subsequent steps of the analytical method and particularly the pre-treatment. For example hair and urine are widely used for detecting clenbuterol in living animals. However the hair pre-treatment is more complex than that of urine because hair is solid so a homogenisation step will be needed to allow access of the extracting solvent to the sample. Besides clenbuterol must be isolated from the hair proteins by

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hydrolysis [5,6]. Then clenbuterol is extracted from the matrix by mixed solid-phase extraction [6,7], derivatised and detected by gas chromatography with mass spectrometry detection (GC/MS) [1,2]. Other techniques have also been found in the bibliography for detecting β_2-agonists, for example high performance liquid chromatography (HPLC) with ultraviolet [3] or electrochemical [4] detection and flow injection analysis (FIA) with fluorimetric [8] detection.

The last stage of the analytical method is the determination of the relationship between the analytical signal and the concentration of the standards. The univariate regression (peak area versus concentration) requires specific signals to be applied. However, several interferences may be registered due to the complexity of biological samples and the signals are then unspecific. These interferences are mostly unknown for the analyst and depend on the sample nature so they cannot be calibrated together with the analyte and soft calibration (for example PLS), in other words interferences can be detected but not quantified [9]. The difficulty may be overcome with second-order signals (that is mass spectra in selected ion monitoring (SIM), or SCAN mode monitored at different elution times of the chromatographic profile) and three-way models which allows one to predict the analyte concentration in a sample with uncalibrated interferences [10].

1.2. The capability of detection, CCβ

Since clenbuterol is a forbidden compound, low levels will be expected and the capability of detection must be consequently determined at a concentration level equal to zero. The inclusion of additional steps in the analytical method (for instance hair hydrolysis) will provoke a recovery decrease and a greater variability in the procedure. The capability of detection [11] is an appealing figure of merit and because including both terms, sensibility and variability, is an adequate parameter for choosing the most adequate biological matrix for the chemical analysis (high recoveries with small standard deviation will involve small detection limits).

The guideline of the International Organization for Standardization, ISO 11843 [11,12] states the methodology for evaluating the capability of detection with univariate and therefore specific signals (zero-order signals) setting the probabilities of false positive, α, and false negative, β. This methodology has been accepted by the International Union of Pure and Applied Chemistry, IUPAC [13] and Decision 2002/657/EC [14], previously draft SANCO 1085/2000, for any method of screening, identification or identification plus quantification.

With regard to multivariate (first- and higher-order) signals several methodologies have been used for estimating the detection limit with multivariate signals [9,15,16]. In this paper, the procedure described by Ortiz et al. [17,18] for setting the capability of detection has been applied with evaluation of the probabilities of false positive, α, and of false negative, β.

Here, the capability of detection has been estimated in different matrices (bovine hair and urine), for several spectrum acquisition modes (SIM, SCAN and SCAN8, eight mass fragments registered in SCAN mode) and for different statistical models (univariate and three-way models).

1.3. Identification of clenbuterol through PARAFAC

According to Decision 2002/657/EC which implements Directive 96/23/EC, the identification of the mass spectrum must be performed for the confirmation of clenbuterol. However, the coelution of several compounds with similar mass spectrum to that of clenbuterol prevents the identification of the analyte of interest. The uniqueness property of PARAFAC [10] has been used in this work for estimating the pure spectra of clenbuterol and subsequently compared with libraries. For identifying forbidden substances the decision makes use of a system of four diagnosis ions (molecular ion or its characteristic adducts, characteristic fragments and all the isotope ions). The sole condition is that fragments should not be originated from the same part of the molecule. The reason is that when signals are monitored in SCAN mode [19,20], most of the mass fragments are redundant or noise. Besides the greater the number of ions registered is the worse the signal-to-noise ratio and the capability of detection are which makes the selection of independent and non-correlated variables necessary. In this paper the hierarchical clustering of variables built with the correlation coefficient in absolute value has been applied for choosing those ions which provide independent and non-redundant information.

2. Multi-way models and the capability of detection, CCβ

GC/MS data are arranged in a three-way array, X, and analysed with DTLD, PARAFAC, PARAFAC2, Tucker3 and trilinear PLS models. A comparison between some three-way models can be consulted in Refs. [21–23]. All five models will be briefly described below.

2.1. The DLTD and PARAFAC models

The DLTD (direct trilinear decomposition) and PARAFAC (parallel factor analysis) models are two decomposition methods which decompose the original data X into triads or trilinear components [24,25]. Each component consists of three loading vectors. The structural models of DTLD and PARAFAC [10] are identical and are expressed as follows:

$$x_{ijk} = \sum_{f=1}^{F} a_{if} b_{jf} c_{kf} + e_{ijk} \quad (1)$$

where F is the number of factors, a_{if}, b_{jf} and c_{kf} are the elements of the loadings matrices and e_{ijk} the experimental error.

The main difference between the two models is that DTLD is a non-iterative procedure based on the resolution of an eigenvector and eigenvalue problem (single step) whereas PARAFAC is an iterative procedure (several steps) which minimises the sum of residual squares. That is why DTLD is frequently used as an initialisation method for the PARAFAC model estimation. The PARAFAC model is generally fitted by means of the alternating least squares (ALS) algorithm although other algorithms may be used [26]. The advantage of the ALS algorithm is the possibility of being generalised for higher-order signals and of being modified for the application of constraints depending on the a priori knowledge of the problem (non-negativity, orthogonality, etc.).

Both models have in common the uniqueness property of the solution, in other words, that the estimated component matrices cannot be rotated without loss of fit and the true underlying profiles are obtained.

2.2. The PARAFAC2 model

The PARAFAC model is highly affected by deviations from the trilinear structure [27]. Changes in the retention time from run to run are habitual in chromatography which can make the PARAFAC model fail due to the assumption of invariant profiles [10] in each sample. PARAFAC2 [28] overcomes this difficulty by modelling XX^{T} instead of X which allows some deviation in the chromatographic profiles. The PARAFAC2 model can be expressed as follows:

$$x_{ijk} = \sum_{e=1}^{E}\sum_{f=1}^{F} a_{ie}a_{jf}h_{ef}c_{ke}c_{kf} + e_{ijk} \tag{2}$$

Chromatographic data are rearranged in such a way that the first mode refers to the elution profile, the second to the mass spectra and the third to the sample concentration. The PARAFAC2 model assumes that the spectral and sample profiles together with the cross-product of the chromatographic profile are invariant in every experiment. Evidently this criterion is less strict than that of PARAFAC but it is also more difficult to determine what kind of deviations are allowed. The PARAFAC2 model is unique under the conditions indicated in Ref. [28] but in general it requires more samples to assess uniqueness.

2.3. The Tucker3 model

The Tucker3 model is the most general of those referred to in this paper. The structural model that formulates [10,24] the Tucker3 decomposition model is

$$x_{ijk} = \sum_{d=1}^{D}\sum_{e=1}^{E}\sum_{f=1}^{F} a_{id}b_{je}c_{kf}g_{def} + e_{ijk} \tag{3}$$

where a_{id}, b_{je}, c_{kf} are the elements of the loadings matrices, D, E and F the number of components of the first, second and third mode, respectively. g_{def} are the elements of the core array which define the interaction between the individual loading vectors in the different modes. Thus, the squared element $(g_{def})^2$ reflects the explained variation by the combination of the factor d from the first mode, factor e from the second and factor f in the third mode.

The Tucker3 model does not need to have the same number of components in each mode (D, E, F) and it is useful for solving problems where the analyte does not have a rank-1 signal [29]. Tucker3, contrary to PARAFAC, suffers from rotational ambiguity, that is it has rotational freedom and consequently has lost the uniqueness property.

2.4. The multilinear PLS regression

The DTLD, PARAFAC, PARAFAC2 and Tucker3 models decompose the block of independent variables X without taking into account the response. This is the main difference with the trilinear PLS regression model where the components of the independent variables, X, and the dependent variables, Y, are simultaneously built such that the covariance between them is maximum [24,30].

The model of X is

$$x_{ijk} = t_i w_j^J w_k^K + e_{ijk} \tag{4}$$

where w^J and w^K are the weight vectors generating a loading vector, t, which has maximum covariance with the unexplained part of the dependent variable. The optimisation criterion can be expressed as follows:

$$\max_{w^J w^K}\left[\mathrm{cov}(t, y)\left|\min\sum_{i=1}^{I}\sum_{j=1}^{J}\sum_{k=1}^{K}(x_{ijk} - t_i w_j^J w_k^K)^2\right.\right] \tag{5}$$

This method generates more predictive models but it has lost the uniqueness property which implies that the profiles estimated by PLS are not necessarily the true underlying ones. That is why in this paper PARAFAC estimations will be used for identifying the pure spectra.

2.5. Capability of detection, CCβ

The capability of detection or minimum detectable net concentration has been defined [11,13,14] for a given probability of false positive, α, as the true net concentration of the analyte in the material to be analysed which will lead, with probability $1 - \beta$, to the correct conclusion that the concentration in the analysed material is different from that in the blank material. The application of this figure of merit to the chemical analysis with zero-order signals and univariate calibration models is detailed in the second part of the standard ISO 11843 [12]. The capability of detection is determined for the analysis of residues for which no permitted limit has been set (forbidden compounds) by means of the Neyman–Pearson test [31] (unilateral case): null hypothesis, the concentration of the analyte in the sample is 0, $X_0 = 0$; alternative hypothesis, the concentration of the analyte in the sample is greater than 0, $X_0 > 0$. α is defined

as the probability of false positive, that is, to admit that the analyte is present when it is not true, and β, the probability of false negative or probability to accept than the analyte is not present when it is [32]

$$\mathrm{CC}\beta = \frac{\Delta(\alpha, \beta) w_{x_0} \hat{\sigma}}{\hat{b}} \quad (6)$$

In the case of first and higher-order signals modelled by two and superior-order calibrations, the capability of detection can also be determined through both probabilities α and β. The generalisation [17,18] is based on the fact that the capability of detection is not modified by linear transformations on the response. Thus the instrumental signal (first-, second- and higher-order data) can be substituted either by the loadings in the sample mode [33] or the concentration estimated by the calibration model [34] when the regression is applied to the standards of the training set. Once the instrumental signal has been transformed into univariate data, the capability of detection is estimated, as stated by ISO, through the performance of a regression between either the loadings of the sample mode or the concentration predicted and the true concentration of the standards.

The absence of outlier data in the univariate model, loadings in the sample mode or predicted concentration versus the true standard concentration was checked by the least median squares (LMS) regression [35]. Those data with an LMS standardised residual in absolute value higher than 2.5 were considered as anomalous data. Once detected, outliers are removed from the training set and the second-order model is then rebuilt. This procedure for eliminating outliers has already been applied [36,37] for estimating the capability of detection.

3. Experimental

3.1. Chemicals and reagents

Clenbuterol hydrochloride (>95%) was supplied by Sigma (Madrid, Spain). Sodium hydroxide, methanol, ammonium hydroxide, acetonitrile and hydrochloric acid were purchased from Merck (Darmstadt, Germany). Ethyl acetate, potassium hydroxide and potassium phosphate monobasic were acquired from Sigma (Madrid, Spain). Acetic acid was obtained from Panreac (Barcelona, Spain) and isooctane from Aldrich (Madrid, Spain). Deionised water was obtained by the Milli-Q Gradient A10 water purification system of Millipore (Bedford, MA, USA).

The phosphate buffer (0.1 M, pH 6) was prepared by dissolving potassium phosphate monobasic with deionised water and adjusting the pH to 6 with 1 M NaOH.

The silylating reagent is a mixture, 99:1 (v/v), of *N*,*O*-bis(trimethylsilyl) trifluoroacetamide (BSTFA) and trimethylchlorosilane (TMCS) (a silylation catalyst), from Supelco (PA, USA).

Bond Elut Certify^{TM} LRC extraction cartridges from Varian comprise a packed bed consisting of a non-polar C8 sorbent and a strong cation exchange (SCX) phase [38].

3.2. Standard solutions

Clenbuterol stock solutions at 401 mg l^{-1} (for hair samples) and 372 mg l^{-1} (for urine samples) were prepared in 0.1 M hydrochloridric acid. Stock solutions were stored in the dark at 0 °C. Diluted solutions of clenbuterol were prepared at concentrations of 4.01 and 3.72 mg l^{-1} for hair and urine samples, respectively, by 1:100 dilution of the stock solution with 0.1 M hydrochloridric acid. For the analysis of the urine samples a 372 μg l^{-1} solution was prepared by 1:10 dilution of the 3.72 mg l^{-1} solution in 0.1 M hydrochloridric acid.

Fortified hair samples were prepared daily by adding six different amounts of the 4.01 mg l^{-1} solution to 1 g of blank reference hair to get final concentrations of 20.91, 103.44, 184.00, 270.54, 352.70 and 440.30 μg kg^{-1} of clenbuterol in hair. Enriched urine samples were prepared daily by diluting the 372 μg l^{-1} solution in 10 ml of blank reference urine to get final concentrations of 0.19, 0.37, 0.96, 1.86 and 3.72 μg l^{-1} of clenbuterol.

3.3. Extraction procedures

Two different pre-treatments were performed depending on the nature of the biological samples.

3.3.1. Urine sample preparation procedure

Urine samples (blank and spiked samples) were centrifuged at 3100 rpm for 5 min. 4 ml of phosphate buffer (0.1 M, pH 6) were added to each sample.

3.3.2. Hair samples preparation procedure

Blank and spiked hair samples were digested with 6 ml of 1 M NaOH for 30 min on a water bath at 100 °C and then cooled at room temperature. Samples were washed with 5 ml of water, adjusted to pH between 5 and 7 with 4 M hydrochloric acid, centrifuged at 20 000 rpm for 5 min and then filtered. Extracts were adjusted to pH 6 with 4 ml of 0.1 M phosphate buffer.

3.3.3. Mixed solid-phase extraction procedure and pre-column derivatisation

After the pre-treatment, both urine and hair extracts were loaded and passed across Bond Elut Certify^{TM} columns from Varian (CA, USA) previously activated with 2 ml of methanol and 2 ml of phosphate buffer (0.1 M, pH 6) at a pressure of 5 mmHg. The columns were then washed with 1 ml of 1 M acetic acid, dried for 5 min, rinsed with 6 ml of methanol and again dried under vacuum for 2 min at 5 mmHg. The elution of sample extracts was carried out with 2 ml of ethyl acetate containing 3% (v/v) ammonium hydroxide. The extracts were then evaporated to dryness under a

stream of nitrogen at 60 °C. After the dried residues were dissolved with 100 μl of acetonitrile, 100 μl of BSTFA+TMCS (99:1, v/v) were added and then incubated at 60 °C for 1 h in order to obtain the trimethylsilyl derivative. The derivatised extracts were evaporated to dryness under nitrogen stream at 60 °C, reconstituted in 50 μl of *iso* octane and finally analysed by GC/MS.

3.4. Instrumental analysis

Analysis were performed using an HP 5890 series II gas chromatograph equipped with an HP 7673 automatic injector and coupled to an HP 5971A mass spectrometer. Chromatographic separation was achieved in a cross-linked HP capillary column (5% diphenyl, 95% dimethyl siloxane, 0.33 μm film thickness) with dimensions 25 m × 0.20 mm i.d. Injections were made in the splitless mode (10 min delay) and using helium as carrier gas. The injector was kept at 250 °C. The initial temperature of the oven was maintained at 70 °C for 2 min, increased from 70 to 200 °C at 25 °C min^{-1}, held at 200 °C for 6 min and subsequently raised to 300 °C at 25 °C min^{-1}, maintaining the final temperature for 12 min. Sample injection volume was 2 μl. Analyses were carried out in the electron impact (EI) ionisation mode at 70 eV operating either in scan mode (210 ions whose *m*/*z* ratio is comprised between 71 and 280) or selected ion monitoring mode, registering eight mass fragments: 86, 243, 262, 263, 264, 265, 266 and 277.

3.5. Software

Three-way models were built with the N-way Toolbox for MATLAB [39]. The detection capability, CCβ, for univariate signals was estimated with DETARCHI [32] and that for three-way data with NWAYDET which is available from the authors and it displays for a fixed α, β as a function of the capability of detection. The least median of squares regression was performed with PROGRESS [35] for the detection of anomalous data. The hierarchical clustering was built by means of PARVUS [40].

4. Results and discussion

Hair samples were registered in both data acquisition modes, SIM mode (8 *m*/*z* ratios, see Section 3.4.) and full-SCAN mode (210 *m*/*z* ratios). In addition the signal of eight mass fragments (86, 243, 262, 263, 264, 265, 266 and 277) was extracted from the data set of SCAN mode and independently analysed. In the paper this data set will be identified as SCAN8 mode. Hair GC/MS data were organised to get a three-way data set of dimensions: 7 × 8 × 22 in the SIM mode, 7 × 8 × 37 in the SCAN8 mode and 7 × 210 × 37 in full-SCAN mode. Urine samples were only recorded in the SIM mode. The dimensions of the data array were 6 × 8 × 12. In all cases the first index corresponds to the number of samples, the second to the mass spectrum and the third to the chromatographic profile. In the PARAFAC2 model data matrices need to be rearranged in order to have firstly the chromatographic profile, then the mass spectra and finally the sample. The univariate data were obtained from the peak area determined at the base peak ($m/z = 86$).

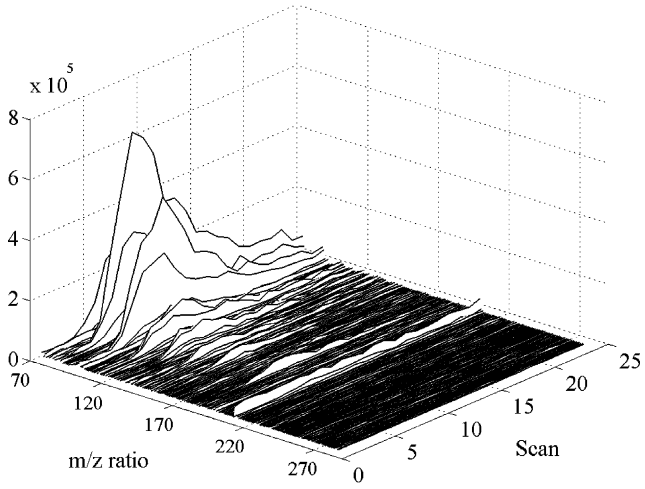

Fig. 1. Three-dimensional chromatogram (mass spectra registered in SCAN mode at several elution times) of a pure standard with 440.30 μg kg^{-1} of clenbuterol.

The landscape of a standard containing 440.30 μg kg^{-1} of pure clenbuterol is displayed in Fig. 1. GC/MS signals were recorded in the SCAN mode.

The urine signal is not specific, the clenbuterol-free sample has a peak with a retention time close to that of the clenbuterol peak. Hair signals are specific in the SIM and SCAN8 modes but in the SCAN mode (210 *m*/*z* ratios) there are unknown compounds whose characteristic ions are different from those of clenbuterol. That is why the interference is not detected in the SIM and SCAN8 modes.

Calibration standards containing 184.00 and 270.54 μg kg^{-1} of clenbuterol in hair and 1.86 μg l^{-1} in urine were eliminated from all training sets because their standardised residual is greater than 2.5 (LMS regression in Section 2.5).

4.1. Performance of three-way models

The number of factors for building the models together with the percentage of the explained variance in the block of the variables *X* are indicated in Table 1 for hair samples and in Table 2 for urine samples. Where possible the same conditions have been applied in order to compare the results (in most cases, random orthogonalised values for initialisation and unimodality and non-negativity constraints in the chromatographic and spectral modes, respectively). Only in those cases in which the restrictions worsen the qualitative (identification of profiles) and/or quantitative (CCβ) results, no constraints were imposed. This is the case of PARAFAC2 and Tucker3 in the SCAN mode in hair where the inclusion of constraints overrestricts the model.

Table 1
Results of the three-way models in hair samples: percentage of explained variance (%) in both blocks of variables X and Y, number of factors used for building the three-way models and determination coefficient (r^2) between the sample loadings and the concentration of the standards in hair samples

Mode	Model	Explained variance		No. of factors	r^2 (sample mode)
		X (%)	Y (%)		
SIM	DTLD	–	–	1	0.9954
	PARAFAC	98.84	–	1	0.9948
	PARAFAC2	98.85	–	1	0.9948
	Tucker3	98.84	–	1	0.9948
	Trilinear PLS	99.93	99.96	3	0.9993
SCAN8	DTLD	–	–	1	0.9701
	PARAFAC	98.63	–	1	0.9676
	PARAFAC2	98.35	–	1	0.9675
	Tucker3	98.64	–	1	0.9676
	Trilinear PLS	99.78	99.96	3	0.9994
SCAN	DTLD	–	–	3	0.9754
	PARAFAC	95.07	–	3	0.9726
	PARAFAC2	94.10	–	3	0.9571
	Tucker3	95.56	–	3	0.8935
	Trilinear PLS	93.36	99.99	4	0.9998

When the signals are registered in the SIM mode, the explained variance (Table 1 for hair and Table 2 for urine) is similar in both matrices, around 99%, that is the model fitting is independent of the sample nature. However significant differences appear when the variability explained by each model is compared in the different modes, SIM (99%) and SCAN (around 95%). The lower variability explained in the SCAN mode is due to the fact that there are many masses which do not contain information associated with the analyte but with noise. By selecting the characteristic ions (8 out of 210 masses, SCAN8 mode) the percentage of explained variance, 98.6%, increases because the variability not related to the analyte has been removed.

4.2. Capability of detection, CCβ

As clenbuterol is a forbidden compound the capability of detection, CCβ, or minimum detectable net concentration at zero ($X_0 = 0$) [14] must be established. CCβ was determined, Eq. (6), with a probability of false positive, α, and a probability of false negative, β, equal to 0.05. The number of replicates was fixed at 2. The capability of detection is listed in Table 3 as a function of the model, the data acquisition mode and the nature of the sample.

Table 2
Results of the three-way models in urine samples: percentage of explained variance (%) in both blocks of variables X and Y, number of factors used for building the three-way models and determination coefficient (r^2) between the sample loadings and the concentration of the standards in urine samples

Mode	Model	Explained variance		No. of factors	r^2 (sample mode)
		X (%)	Y (%)		
SIM	DTLD	–	–	2	0.9980
	PARAFAC	98.87	–	2	0.9980
	PARAFAC2	98.99	–	2	0.9974
	Tucker3	98.96	–	2	0.9892
	Trilinear PLS	99.64	99.96	3	0.9994

Table 3
Capability of detection as a function of the biological sample, the statistical model and the signal acquisition mode

	Hair ($\mu g\,kg^{-1}$)			Urine ($\mu g\,l^{-1}$)
	SCAN	SCAN8	SIM	SIM
Univariate	283.00		73.95	0.36
DTLD	–	173.0	67.02	0.46
PARAFAC	165.10	180.20	71.02	0.32
PARAFAC2	208.40	180.50	70.37	0.36
Tucker3	339.90	180.10	71.08	0.75
Trilinear PLS	20.91	23.34	26.32	0.17

The probabilities of false positive and of negative were established at 0.05.

There are two groups of models that behave differently regarding the capability of detection: the univariate and the decomposition methods (DTLD, PARAFAC, PARAFAC2 and Tucker3) and on the other hand, the trilinear PLS model. Independently of the sample nature and the mode in which the signals are registered, the best capability of detection is always obtained with the trilinear PLS model (26.32 $\mu g\,kg^{-1}$ and 0.17 $\mu g\,l^{-1}$ in hair and urine, respectively). Besides, the capability of detection of PLS in hair does not depend on the signal acquisition mode: full-SCAN mode, 20.91 $\mu g\,kg^{-1}$ and the SIM mode, 26.32 $\mu g\,kg^{-1}$. Both conclusions are linked to the manner in which the trilinear PLS model is built. The data arrays of independent and dependent variables are simultaneously decomposed such that the scores in both spaces X and Y, have maximum covariance. However PARAFAC only decomposes the block of independent variables into a trilinear model without taking into account the response. This difference usually makes the trilinear PLS model more suitable for explaining the variability intrinsic to the analyte, and consequently its capability of detection not only improves but also is not affected by the poor signal-to-noise ratios.

The univariate regression together with the decomposition models, DTLD, PARAFAC, PARAFAC2 and Tucker3, depends on the signal acquisition mode, namely on the signal-to-noise ratios. For example, the capability of detection with PARAFAC increases from 71.02 $\mu g\,kg^{-1}$ in the SIM mode to 165.10 $\mu g\,kg^{-1}$ in the full-SCAN mode. In order to test the effect of the data acquisition mode on the capability of detection, the signals of the eight ions selected in the SIM mode are extracted from the SCAN mode data set (SCAN8 mode). Its capability of detection is shown in the third column of Table 3. In all cases the capability of detection in the SCAN8 mode worsens with respect to the SIM mode and it does not always improve that of the SCAN mode. This conclusion means that those models are highly affected by the number of fragments monitored and once the signal has been recorded the intrinsic noise cannot be

avoided (difference with PLS). As the number of ions to be detected increases, the analyser must transmit them towards the detector faster which enlarges the variability and decreases the signal-to-noise ratio of the signal. The previous assertion is proved by the fact that the residual standard deviation of the regression between the PARAFAC scores and the standard concentration increases from 89344 in the SIM mode to 221205 in the SCAN mode whereas the sensitivity of the model is around 5500 in both cases.

Regarding the sample nature, the extraction procedure of clenbuterol from bovine urine ($0.17\,\mu g\,l^{-1}$) shows a better capability of detection than that from bovine hair ($26.32\,\mu g\,kg^{-1}$) because of the diverse nature of the samples which implies different pre-treatment procedures. Solid samples [6] need to be thoroughly homogenised to allow the access of the solvents and a basic digestion to release clenbuterol from hair. However liquid samples are simply centrifuged and filtrated to remove the particles in suspension. The additional step of digestion of hair samples justifies the larger variability and the subsequent worse capability of detection found in the procedure for hair. The conclusion reached points out the importance of determining the capability of detection of an analyte in several biological matrices in order to assure which sample is the most convenient one to detect either the presence or the absence of clenbuterol.

The characteristic curves of the detection limit in hair are displayed in Fig. 2 for the different statistical models and data acquisition modes. In Fig. 2 the probability of false negative, β, is represented as a function of the capability of detection, CCβ, fixing the probability of false positive, α, at 0.05. It can be observed that the best values of CCβ ($\mu g\,kg^{-1}$) are obtained with the trilinear PLS model independently of the data acquisition mode. If the univariate calibration model, DTLD, PARAFAC, PARAFAC2 or Tucker3 are used then the signals should be registered in SIM mode, that is recording few but informative mass fragments.

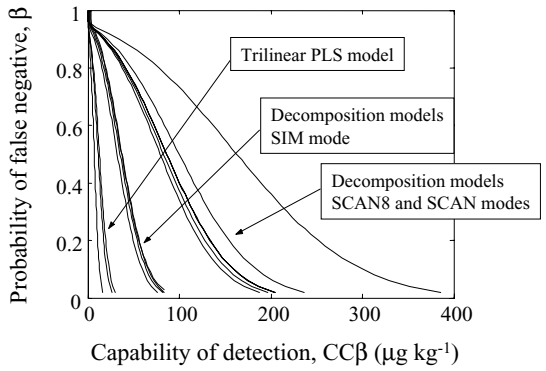

Fig. 2. Capability of detection of the GC/MS method for the analysis of clenbuterol in hair samples. Effect of the statistical model and the manner in which the mass spectra have been registered. The probability of false positive was set at 0.05 and the number of replicates at 2.

4.3. Identification of mass spectra and selection of characteristic fragments

According to Decision 2002/657/EC the mass spectrum of clenbuterol will be identified for confirmatory purposes. In some cases the steps of sample pre-treatment, extraction of the analyte from the matrix and the chromatographic separation are not sufficient for achieving specific signals and several substances with a mass spectrum similar to that of clenbuterol coelute. Therefore the identification of the mass spectrum is not possible. This problem may be solved by three-way models such us PARAFAC which has the uniqueness property [25] or property of recovering the true chromatographic and spectral profiles even in the presence of unknown interferences.

PARAFAC estimations of the mass spectra will be consequently employed for identifying clenbuterol according to Decision 2002/657/EC and are displayed in Fig. 3a (SCAN mode in hair), in Fig. 3b (SIM and SCAN8 modes in hair which are identical) and in Fig. 3c (SIM mode in urine).

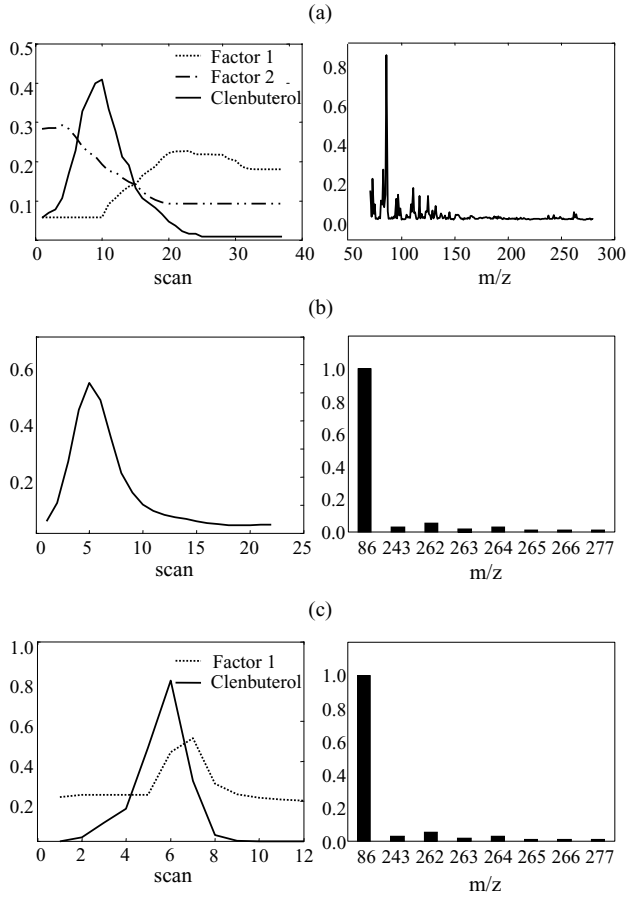

Fig. 3. Chromatographic and spectral profiles estimated by PARAFAC with signals recorded in: (a) hair in SCAN mode; (b) hair in SCAN8 and SIM modes; (c) urine in SIM mode. The graphs at the left display the chromatographic profiles of clenbuterol (solid line) and those of the interferences. The graphs at the right show the mass spectrum of clenbuterol estimated by PARAFAC.

The graphs at the left consist of the chromatographic profiles estimated. As can be observed three factors (Fig. 3a, hair in SCAN) and two factors (Fig. 3c, urine) have been employed for building PARAFAC models since the signals were not specific (see also Tables 1 and 2). The factor related to clenbuterol has been shown with a solid line. The identification of the factors was done through the correlation between the loadings of the sample mode and the true concentration (determination coefficient, r^2 in Tables 1 and 2) together with the visual inspection of the chromatographic and spectral profiles. The graphs on the right in Fig. 3 are the estimated mass spectra of clenbuterol.

Once assessed, the spectral estimates of clenbuterol can be compared with a reference spectrum (for example with libraries). In this work we have calculated the correlation coefficient between the reference and the estimated spectra (ρ in the second mode): 0.9670 in SCAN mode, 1.0000 in SCAN8 and SIM modes in hair and 0.9999 in urine. The correlation coefficient of the SCAN mode is the worst due to the large amount of variables which only provide noise.

Results obtained in Sections 4.1 and 4.2 demonstrate that the number of *m*/*z* ratios registered in the mass spectra when the spectrometer is coupled to a chromatograph influence the recorded signal. There are two main reasons, the greater number of masses are measured the greater the number of *m*/*z* ratios which do not contain useful information (noise and redundant information) and the poorer the signal-to-noise ratio is. The selection of few but informative and non-redundant mass fragments (SIM mode) is consequently advantageous for both qualitative and quantitative analysis.

For confirmation of substances listed in Group A of Annex 1 of Directive 96/23/EC (clenbuterol), a minimum of four identification points are required, the molecular ion and its characteristic adducts, characteristic fragment ions and all their isotope ions. The selected diagnostic ions should not exclusively originate from the same part of the molecule so that they will provide independent information.

The hierarchical clustering of variables has been used for identifying those mass fragments which are uncorrelated from those redundant and similar. Hierarchical clustering is a classification method characterised by the division of large groups of variables into smaller groups of more similar mass fragments [41]. The average linkage method was used here as the clustering algorithm and the similarity matrix was determined through the correlation coefficient in absolute value $(1 - |\rho|)$.

A clenbuterol standard containing 440.30 $\mu g\,kg^{-1}$ was registered in SCAN mode obtaining a data matrix with dimensions 57 × 210 (elution times × mass fragments). Fifteen ions (see Fig. 4) were previously chosen from the 210 variable set by principal components. In this way the data matrix employed for building the cluster of variables has 57 objects (elution times) and 15 variables. The cluster hierarchy can be visualised as a dendrogram in Fig. 4. The similarity is represented in the ordinate axis from 0 (minimum similarity) to 1 (maximum similarity). In the abscise axis all 15 variables have been sorted according to their similarity. All variables from a cluster are correlated and can be considered as redundant. Establishing the similarity level displayed in a broken line in Fig. 4, five uncorrelated variable groups can be found, that is, five groups of variables that provide independent information from each other. Choosing one variable from each cluster five mass fragments can be used for the identification points system. In this paper we have chosen 86, 243, 262, 264 and 277 which correspond to the fragmentation pattern shown in Ref. [1].

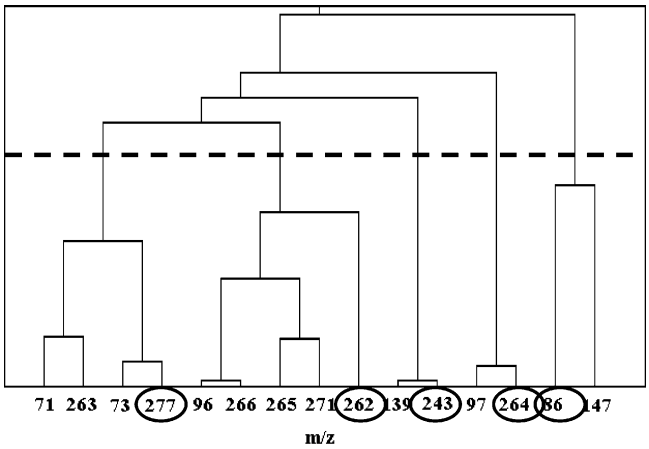

Fig. 4. Hierarchical clustering of variables (mass fragments, *m*/*z*).

5. Conclusions

The complexity of the biological samples together with the low levels of clenbuterol found make the evaluation of the capability of detection an important step in the validation method. The capability of detection has been improved by (i) analysing urine instead of hair, (ii) modelling with trilinear PLS and (iii) for the rest of the models, registering few but non-redundant mass fragments (selected ion monitoring).

The uniqueness property of PARAFAC has been successfully employed for estimating the pure spectra of clenbuterol when unidentified interferences and consequently non-specific signals exist.

Hierarchical clustering analysis of variables has been appropriate for detecting five independent ions so it can be used as an objective way to select informative variables.

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