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Development of a harmonised method for the profiling of amphetamines VI Evaluation of methods for comparison of amphetamine

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Abstract

Amphetamine samples were analysed by gas chromatography–mass spectrometry (GC–MS), and the peak areas of 33 target compounds were transformed by applying various pretreatment techniques. The objective was to optimise the ability of a number of distance metrics to establish links between samples of amphetamine originating from the same batch (henceforth refered to as linked distances). Furthermore, partial least squares discriminant analysis (PLS-DA) was used to evaluate the effects of various pretreatment methods on separation of amphetamine batches synthesised by the Leuckart reaction, reductive amination of benzyl methyl ketone, and the nitrostyrene route. The most efficient way to pretreat GC–MS data varied for the different distance metrics, although best results were obtained when data were normalised to the sum of peak areas, and either the fourth root or a logarithm was applied to the normalised data. When pretreating normalised data by fourth root transformation, Pearson correlation was the distance metric that was most successful at finding linked samples. Normalisation and the use of fourth root also represented the best method of pretreating data when employing PLS-DA to separate samples synthesised by different routes. To achieve a faster and more user-friendly procedure for evaluating chromatograms, experiments were performed in which the number of target compounds used to compare samples was reduced. The effect of each compound that was removed was studied by applying PLS-DA and by using Pearson correlation to calculate linked distances as well as unlinked distances (between samples from different batches of amphetamine). Considering both links between samples from the same batch and separation of samples synthesised by different routes, the best results were obtained with the data set comprising 26 compounds. Finally, it was found that the profiling method developed in this work was superior to an existing technique with respect to separating linked and unlinked distances.
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Keywords: Amphetamine profiling; Numerical methods; Pretreatment; PLS-DA; Pearson correlation; GC–MS; Fourth root

1. Introduction

The usefulness of impurity profiling of illicit drugs is well known. The results of such profiling analysis can be used to aid police intelligence to track drug trafficking organisations and their trading routes. Moreover, the impurity profiles of different samples that are considered to be from the same batch are in some countries used in courts of law together

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with other evidence to prove that drug transactions have taken place.

Profiling analysis of amphetamine has been done primarily by gas chromatography (GC) [1–5] and the recorded chromatograms can be regarded as fingerprints, or profiles, of the contents of impurities in the amphetamine samples. These chromatograms or peak area values of specific target impurities can be stored in a database and then be used in various numerical calculations to find similarities between different samples. If necessary visual comparison of profiles can subsequently be performed to confirm the results obtained with the numerical metrics. Retrived links between samples can then be reported to law enforcement.

There are a number of numerical methods for pairwise comparison of samples available. Jonson [6] applied six different methods (i.e., Euclidean distance, Canberra distance, Pearson correlation, a similarity index, a permutation index, and a quotient method) to the peak areas of 23 target compounds to distinguish between amphetamine samples from different batches, and this author concluded that the quotient method and the similarity index were superior to the other methods tested. In another study, Krawczyk and Parczewski [7] employed the peak areas of 15 target compounds and the Euclidean distance, Manhattan distance, Pearson correlation, and Chebyshev distance capabilities to find samples with similar impurity profiles, and these authors visualised the results as dendrograms. Fischer linear discriminant analysis [8], various cosine functions, [9,10] and neural networks [11] have also been used to estimate the degree of similarity between different seizures of illicit substances other than amphetamine.

The GC peak areas of target compounds in amphetamine samples are often used as variables in the comparisons, although different methods have been used to pretreat peak area data. For example, Perkal [12] normalised GC data prior to comparison of methamphetamine samples; Klemenc [13] employed normalisation and weighting when comparing different heroin seizures; and Jonson weighted and normalised data for comparison of amphetamine samples [14]. Additional pretreatment techniques entailing the use of a logarithm [15] or fourth root [16] have been applied to data for purposes other than comparison of illicit drugs. To our knowledge, no detailed study has been performed to consider how the comparison of amphetamine profiles can be affected by applying different kinds of pretreatment techniques.

In addition to the distance methods, there are a number of techniques that provide an overview of a large number of samples at the same time. For example, dendrograms [7] can be established based on calculated distances and the multivariate technique principal component analysis (PCA) [17] can be applied to get a visual overview of a larger number of samples. The latter may also be extended to soft independent modelling of class analogy (SIMCA) [14] that enables assignation of samples to predefined groups.

This is the sixth and last report in a series of articles describing the development of a harmonised method for profiling amphetamine and the focus in this case has been on the capacity of various numerical methods to find samples with similar impurity profiles. Different procedures for pretreatment of raw data were also evaluated. Partial least squares discriminant analysis (PLS-DA) and a number of numerical metrics were employed to find the best way to pretreat the GC data. In other experiments, the importance of individual target compounds were evaluated and the number of target compounds used to compare amphetamine samples were reduced step-wise to make the method simpler in its performance. Finally, the performance of the developed amphetamine profiling method, including both data generation and evaluation, was compared to a reference method that is currently in use at some forensic laboratories. The work was carried out in three European laboratories such that the final method was collaboratively developed and tested.

2. Experimental

2.1. Samples of amphetamine used in the study

Twelve different batches of amphetamine were synthesised by three different methods: the Leuckart reaction (seven batches), reductive amination of benzyl methyl ketone (three batches), and the nitrostyrene route (two batches). Different recipes were used to synthesise the Leuckart batches (Table 1) in order to obtain dissimilar impurity profiles.

In subsequent experiments, samples of the different batches of amphetamine were used undiluted (100%) and diluted with lactose and caffeine to concen-

Table 1
Conditions used in the synthesis of amphetamine by the Leuckart route

Batch	Benzyl methyl ketone	Formamide	Formic acid	Boiling temperature (°C)	Boiling time (h)	Hydrolysis temperature (°C)	Hydrolysis time (h)	Precipitation (pH)
Leuckart 1	1[a]	4	4	150	5	110[b]	2	6
Leuckart 2	1	6	6	160–180	4	110[b]	2	3
Leuckart 3	1	2.5	2.5	140–150	6	110[b]	2	5
Leuckart 4	1	7	3.5	175–180	6	150–170[c]	2	7
Leuckart 5	1	17	0	175–180	4	150–170[c]	4	7
Leuckart 6[d]	1	10	2	175–180	3.5	150–170[c]	5	7
Leuckart 7[e]	1	6.7	3.5	160	2.5	110[b]	1.5	7

[a] The values under the headings benzyl methyl ketone, formamide, and formic acid represent the molar proportions used in the syntheses.
[b] Hydrochloric acid was used in the hydrolysis.
[c] Sulphuric acid was used in the hydrolysis.
[d] Synthesised benzyl methyl ketone was used.
[e] Purified by steam distillation before precipitated to amphetamine sulphate.

Table 2
Composition of samples of the synthesised batches of amphetamine after dilution with caffeine and lactose

Batch	Amphetamine (%)	Caffeine (%)	Lactose (%)
Leuckart 1	40	0	60
	5	0	95
Leuckart 2	40	30	30
	5	47.5	47.5
Leuckart 3	40	50	10
	5	80	15
Leuckart 4	40	30	30
	5	40	55
Leuckart 5	40	30	30
	5	40	55
Leuckart 6	40	30	30
	5	40	55
Reductive amination 1	40	30	30
	5	0	95
Reductive amination 2	40	0	60
	5	35	60
Reductive amination 3	40	0	60
	5	28	67
Nitrostyrene 1	20	0	80
Nitrostyrene 2	20	0	80

trations that could be expected in amphetamine on the illicit market in Europe (Table 2). More precisely, the Leuckart and reductive amination samples were diluted to 40% and 5%, but the nitrostyrene samples only to 20%. The prepared batches were split into three parts, which were analysed with GC–MS in three different laboratories. In addition to the amphetamine synthesised for the experiments, 383 samples from street seizures (100 from Finland, 169 from Sweden, and 114 from Switzerland) were analysed.

2.2. Sample preparation [18]

Amphetamine (200 ± 5 mg) was weighed into new 8- or 12-ml glass test tubes, after which 4 ml of Tris buffer (1.0 M, pH 8.1) was added, and the mixture was subjected to horizontal or rotary shaking for 10 min. Next, 200 μl of toluene containing nonadecane (10 μg/ml) was added, and the mixture was shaken for another 10 min. Subsequent phase separation was facilitated by centrifugation at 3000 rpm for 3 min, and a portion of the organic layer was subsequently removed and analysed by gas chromatography (GC).

2.3. Instrumentation and gas chromatographic analyses [19,20]

Quantitative analyses were performed on a gas chromatograph (Agilent Technologies 6890) coupled to a mass spectrometer (MS; Agilent Technologies 5973). The analytes were separated on a DB-35MS (35% diphenyl, 65% dimethyl silica) capillary column (30 m (L), 0.25 mm (i.d.), and d_f 0.25 μm) connected to a fused silica pre-column (2.5 m (L), 0.25 mm (i.d.) or a DB-35MS (2.5 m (L), 0.25 mm (i.d.) and d_f 0.10 μm). Splitless injection (1 μl) was done for 1 min at 250 °C, using a single tapered liner with glass wool (Agilent Technologies). The oven temperature program started at 90 °C (1 min) and was then increased by 8 °C/min to 300 °C, and that level was maintained for 10 min. The flow of the carrier gas (helium) was held constant at 25 cm/s by employing retention time locking (RTL) of the *HPChemstation software version B.01.00*. Nonadecane with a retention time of 15.00 min was used as the RTL standard. The MS analyses were performed in the full scan mode, and masses in the range 40–300 amu were monitored at a rate of 2.83 scans/s. The temperatures of the ion source and the quadropole during the analyses were 230 °C and 150 °C, respectively.

2.4. Target compounds

After an initial selection, 33 by-products (i.e., target compounds) were chosen for comparison of profiles because they fulfilled the following criteria: found in street amphetamine; stable in solutions [21]; provide reproducible peak areas in repeated gas chromatographic analyses of the same extracts [20]; and easy to identify in chromatograms. The target compounds in the MS chromatograms were identified and quantified using the QEDIT quant results function of the Chemstation software. The compounds, their GC retention times, and the ions used for identification and quantification are summarised in Table 3. The mass spectra used for identification and selection of target and qualifier ions were obtained from synthesised [22] or purchased reference materials, commercial mass spectrum libraries, or scientific publications. The target and qualifier ions of the compounds that could not be identified were chosen from mass spectra obtained in chromatograms of samples analysed in the present study.

2.5. Evaluation of methods for pretreatment of profiling data

To optimise the ability of various numerical methods to discriminate between amphetamine profiles of varying similarity, a number of pretreatment methods were tested on GC–MS data. These methods included different combinations of weighting, normalisation, and use of logarithm and fourth root. Weighting was performed by dividing the peak area of each target compound by its standard deviation (S.D.). The S.D. for each compound was determined using a dataset of 768 samples. Normalisation was accomplished by dividing the peak area of each target compound in a chromatogram by the sum of the peak areas of all target compounds in the same chromatogram.

2.6. Reduction of the number of target compounds

Thirty-three target compounds were used to compare the impurity profiles, and the effect that reducing that number had on the success of separation of different samples was studied by performing partial least squares discriminant analysis (PLS-DA) and distance calculations.

2.7. Evaluation of numerical methods

The following seven distance and similarity methods were tested and evaluated regarding their capacity to discriminate between linked (distances between samples from the same batch) and unlinked distances (distances between samples from different batches): Manhattan distance (Eq. (1)), Euclidean distance (Eq. (2)), Canberra distance (Eq. (3)), similarity index (Eq. (4)), Pearson correlation (Eq. (5)), and the squared sine (Eq. (6)). In these equations, x_{kj} and x_{lj} are the responses of impurity j in samples k and l; $\bar{x}_k$ and $\bar{x}_l$ are the mean peak areas in samples k and l; and n is the number of peak areas of target compounds used in the distance calculations. For the quotient method (Eqs. (7)–(9)) q_j represent the ratio between impurity j in samples k and l (Eq. (7)); r_{kl} is the difference between the quotients of impurities i and j in samples k and l (Eq. (8)); r_{max} is the defined maximum difference (i.e., r_{kl}) that is tolerated between two compared quotients (Eq. (9)). If the difference between two quotients r_{ij} exceeds r_{max} that quotient is not considered to be a match. The number of quotients passing this criteria is calculated and used as a measure of similarity or a tool to retrieve similar profiles in a database. A detailed description of the quotient method can be found in an article written by Jonson [24]. To enable comparison of the results of the different distance calculations, the distances obtained with each method were organised to give values in the range 0–100, where 0 indicates comparison of identical profiles.

$$\text{Manhattan distance } (D_{kl}) = \sum_{j=1}^{n} |x_{kj} - x_{lj}| \quad (1)$$

$$\text{Euclidean distance } (D_{kl}) = \sqrt{\sum_{j=1}^{n} (x_{kj} - x_{lj})^2} \quad (2)$$

$$\text{Canberra distance } (D_{kl}) = \frac{\sum_{j=1}^{n} [(|x_{kj} - x_{lj})|/(x_{kj} + x_{lj})]}{n} \quad (3)$$

Table 3
The chemical structure (when verified), retention times, and target and qualifier ions of the 33 target compounds

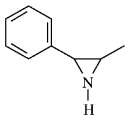
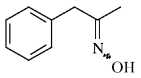
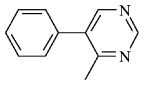
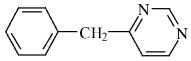
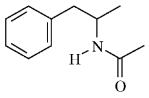
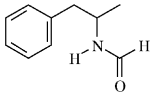
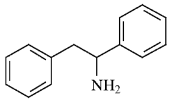
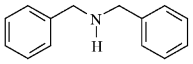
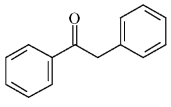
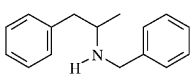
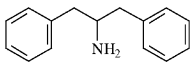
t_R (min)	Compound	Target ion (*m/z*)	Qualifier ions (*m/z*)
7.05	2-Methyl-3-phenylaziridine[a] 	132	117, 133
8.95 and 9.03	Phenyl-2-propanoneoxime (two isomers)[a] 	149 and 131	131, 132 and 116, 130
11.96	4-Methyl-5-phenylpyrimidine[a] 	170	102, 169
12.57	Unknown 1[b]	105	77, 163
12.63	4-Benzylpyrimidine[a] 	169	115, 170
12.97	*N*-acetylamphetamine[a] 	118	86, 117
13.23	*N*-formylamphetamine[a] 	118	72, 117
15.40	1,2-Diphenylethylamine[c] 	106	79, 107
15.70	*N,N*-dibenzylamine[c] 	106	196, 197
16.40	1,2-Diphenylethanone[c] 	105	77, 196
16.70	1,2-Dibenzylamphetamine[a] 	134	91, 135
16.95	1,3-Diphenyl-2-propylamine[a] 	120	103, 121

Table 3 (*Continued*)

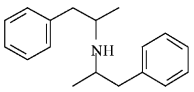
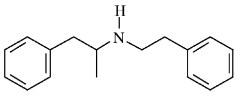
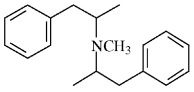
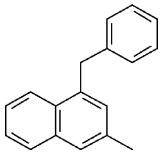
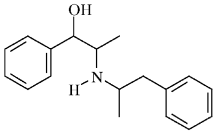
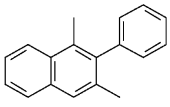
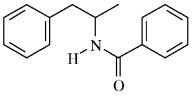
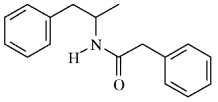
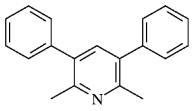
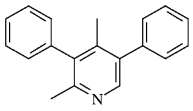
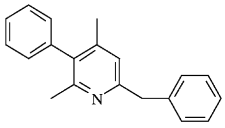
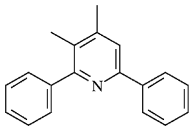
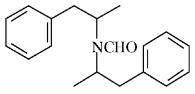
t_R (min)	Compound	Target ion (*m/z*)	Qualifier ions (*m/z*)
	N,N-di(β-phenylisopropyl)amine (two isomers)[a]		
17.41 and 17.49		162	119, 163
	α-Methyldiphenethylamine[d]		
17.74		148	105, 119
	N,N-di(β-phenylisopropyl)methylamine (two isomers)[a]	176	119, 177
19.07 and 19.15			
19.98	Unknown A2[b]	160	128, 143
	1-Benzyl-3-methylnaphthalene[e]		
20.16		232	215, 217
20.60	Unknown A3[b]	143	128, 160
	1-Hydroxy-*N,N*-di(β-phenylisopropyl)-amine[a]		
20.64		162	163, 178
	1,3-Dimethyl-2-phenylnaphthalene[e]		
20.80		232	215, 217
20.87	Unknown A4[b]	143	128, 160
	N-benzoylamphetamine[a]		
20.93		105	148, 149
21.19	Unknown B2[b]	120	143, 160
	2-Oxo-1-phenyl-(β-phenylisopropylamino)ethane[a]		
21.30		162	118, 163

Table 3 (*Continued*)

t_R (min)	Compound	Target ion (*m/z*)	Qualifier ions (*m/z*)
21.56	2,6-Dimethyl-3,5-diphenylpyridine[f] 	259	258, 260
21.64	2,4-Dimethyl-3,5-diphenylpyridine[a] 	259	258, 260
22.19	Pyridine 7 and 14[g]	258	244, 259
22.36	2,4-Dimethyl-3-phenyl-6-(phenylmethyl)-pyridine[h] [23] 	272	258, 273
23.11	2,6-Diphenyl-3,4-dimethylpyridine[f] 	258	244, 259
23.30 and 23.65	*N,N*-di(β-phenylisopropyl)formamide (two isomers)[a] 	190	119, 191

[a] Substance synthesised within the SMT project.
[b] Substance that could not be identified.
[c] Reference standards were available for this substance.
[d] Substance identified matching with a commercial mass spectra library.
[e] Reference standards for this substance were provided by the United Nations Office on Drugs and Crimes (UNODC).
[f] Reference standards for this substance were provided by the Netherlands Forensic Institute (NFI).
[g] Identified as a pyridine based on typical mass spectra of pyridines.
[h] Substance identified from mass spectra in a scientific publication.

$$\text{Similarity index (SI)} = \frac{100 \times 50}{n} \times \sum_{i=1}^{n} \left(\left(\frac{x_{kj}}{x_{lj}} - 0.25 \right)^{6} - 1 + 50 \right)^{-1},$$

$$\text{If } x_{kj} > x_{lj} \text{ then } \frac{x_{kj}}{x_{lj}} \text{ and if } x_{lj} > x_{kj} \text{ then } \frac{x_{lj}}{x_{kj}} \quad (4)$$

$$\text{Pearson correlation } (r_{kl}) = \frac{\sum_{j=1}^{n} (x_{kj} - \bar{x}_k)(x_{lj} - \bar{x}_l)}{\sqrt{\sum_{j=1}^{n} (x_{kj} - \bar{x}_k)^2 \sum_{j=1}^{n} (x_{lj} - \bar{x}_l)^2}} \quad (5)$$

$$\text{Squared sine } (\sin \alpha)^2 = 1 - \left(\frac{\sum_{j=1}^{n} x_{kj} x_{lj}}{\left[\sum_{j=1}^{n} x_{kj}^2 \sum_{j=1}^{n} x_{lj}^2 \right]^{1/2}} \right)^2 \quad (6)$$

$$\text{Quotient method } (q_j) = \frac{x_{kj}}{x_{lj}} \quad (7)$$

$$r_{kl} = \left| \frac{(q_k - q_l)}{(q_k + q_l)} \right| \quad (8)$$

$$r_{kl} < r_{\max} \quad (9)$$

Two of the distance methods, namely the Canberra distance and the similarity index, could not handle the presence of zeroes in the data matrix, i.e., when target compounds occur in the samples at concentrations that are below the detection limit of the GC method. Therefore, two different approaches for handling zeroes were evaluated. With the first method (hereafter refered to as zero-handling method 1), all impurities with a peak area of zero were excluded during numerical comparison of two profiles. With the second procedure (hereafter refered to as zero-handling method 2), the zeroes were replaced with a value corresponding to approximately half the detection limit of the GC method (i.e., 200). These two methods of handling zeros were used for all distance methods except the quotient technique. In the GC analysis, the target compounds often co-eluted with other substances present in the amphetamine extract. When co-eluting substances made substantial contributions to the peak areas of the target compounds, the target compounds in question were registered as missing (*m*); that is, the ''affected'' peak areas were replaced with an ''*m*'', and they were excluded when undertaking numerical comparisons including such samples. However, this was normally not a problem with GC–MS as coelution between a target compound and an unidentified compound with similar mass spectra was rarely observed.

Table 4
Steps involved in amphetamine profiling performed with the SMT method and the reference method

Step	Developed method		Reference method	
Sample preparation	- 200 ± 5 mg amphetamine dissolved in 4 ml TRIS buffer (1.0 M, pH 8.10) - No pH adjustment - Extracted with 200 μl toluene		- 200 ± 2 mg amphetamine dissolved in 2 ml phosphatebuffer (0.063 M, pH 7.00) - pH adjusted to 7.00 ± 0.05 - Extracted with 200 μl n-octane	
GC analysis	- Column: DB-35MS (30 m, 0.25 mm, 0.25 μm) - Temp. program: 90 °C (1 min), 8 °C/min, 300 °C (10 min) - Injection: 1–4 μl, splitless - Detection: MS		- Column: HP Ultra 2 (25 m, 0.2 mm, 0.33 μm) - Temp. program: 100 °C (1 min), 12 °C/min, 300 °C (10 min) - Injection: 2 μl, splitless - Detection: FID	
Data processing and evaluation	No. of impurities: Distance method: Pretreatment distance calculation:	26 Pearson Zeros replaced by 200, normalised + 4th root	No. Of impurities: Distance method: Pretreatment distance calculation:	23 Quotient method Raw data

When applying the quotient method to compare profiles, zeroes and missing values were treated in a somewhat different manner than was done in the other distance calculations. The quotient technique compares two profiles by dividing the value for each impurity in the first profile by the corresponding value for the same impurity in the second profile [24]. Cases in which a quotient had two zeroes or two missing values were regarded as a match, whereas all other combinations of zeroes and missing values were considered not to be a match. All distance calculations were automated, either with a program written in Q-basic from Microsoft [24] or one written in Visual Basic for Excel® [10].

2.8. Comparing the developed profiling method with a reference procedure

The method developed in the present study (herein refered to as the SMT method) and a profiling technique used at some forensic laboratories in Europe [24] were compared regarding their ability to distinguish between different batches of amphetamine. These two methods are briefly described in Table 4.

3. Results

3.1. Pretreatment of data

Forty-four well-defined samples were used in the study, and they were chosen to be representative of a total of 768 samples that were available. The selected samples were synthesised by the Leuckart reaction (25 samples), reductive amination of benzyl methyl ketone (12 samples), and the nitrostyrene route (seven samples). The 44 samples and the degree of similarity they exhibited are summarised in Table 5.

The following combinations of pretreatment methods were applied to the GC–MS data before comparing the samples:

- Normalisation (N)
- Normalisation followed by weighting (N + W)
- Weighting followed by normalisation (W + N)
- Normalisation followed by application of a logarithm (N + log)
- Normalisation followed by taking fourth root (N + 4th root)
- Weighting followed by normalisation and application of a logarithm (W + N + log)
- Weighting followed by normalisation and taking fourth root (W + N + 4th root)

The effect of pretreating the data was evaluated in two ways. First, PLS-DA [25] models were calculated to monitor the effect of pretreatment on the ability to discriminate between samples synthesised by different routes. Three ''dummy'' variables [25] were used in these calculations, one for each synthetic route (i.e., the Leuckart, the reductive amination, and the nitrostyrene methods). Leave-one-out cross-validation [26] was employed to validate the models that were obtained and to determine the number of significant principal components. The PLS-DA models were calculated using *The Unscrambler* version 7.6 (CAMO Process AS, Norway), and they were evaluated by contrasting predicted and measured values and by visual inspection of score plots.

The second type of assessment of the impact of data pretreatment focused on the ability of different methods to discriminate between linked and unlinked distances. In this study, a linked distance was defined as a distance acquired when comparing samples from the same batch or samples from

Table 5
Samples used in the evaluation of data pretreatment methods

Type of samples	Concentration of amphetamine (%)	No. of samples according to method of synthesis			
		Leuckart	Reductive amination	Nitrostyrene	All
From same batch	100 and 5	12	6	4	22
Synthesised using same recipe	100	2	0	0	2
Synthesised using same recipe	100–20	4	0	0	2
Synthesised using different recipes	100–20	3	0	3	9
Street samples	Unknown	4	6	0	9
Total no. of samples		25	12	7	44

different batches synthesised in a highly reproducible manner by the same recipe. Accordingly, an unlinked distance was defined as a distance obtained when comparing samples from batches synthesised using different conditions or routes. GC data of the 44 samples (Table 5) were pretreated with the investigated methods, and different numerical methods were employed to calculate correlations between the samples. The ratio between a value representing the mean minus the standard deviation (S.D.) of the obtained unlinked distances and a value representing the mean plus the S.D. of the linked distances (Eq. (10)) was calculated and used as a measure of the capacity of different pretreatment techniques to separate unlinked and linked distances; the higher the quotient, the larger the differences in the distances between unlinked and linked samples. Accordingly, the pretreatment method that resulted in the highest quotient was considered superior.

$$\text{Estimated discrimination} = \frac{\text{Mean (unlinked)} - \text{STD (unlinked)}}{\text{Mean (linked)} + \text{STD (linked)}} \quad (10)$$

The quotient method was used as it was first described by Jonson [24], which means that the numerical calculations were performed on raw data.

3.1.1. PLS-DA

Visual inspection of the score plots showed that samples from different synthetic routes were differentiated most successfully when GC data were pretreated in one of the following manners: N + 4th root (Fig. 1); W + N + 4th root; N + Log; W + N + Log.

When N were applied to the data set, two samples synthesised by the reductive amination route appeared in the Leuckart group (Fig. 2). A plausible explanation for this observation is the so-called closure effect [26], which might occur when applying N on a sample that contains one dominant variable. Application of N + Log or N + 4th root decreased the relative difference between the peak areas of the target compounds. That effect reduced the influence of large peaks

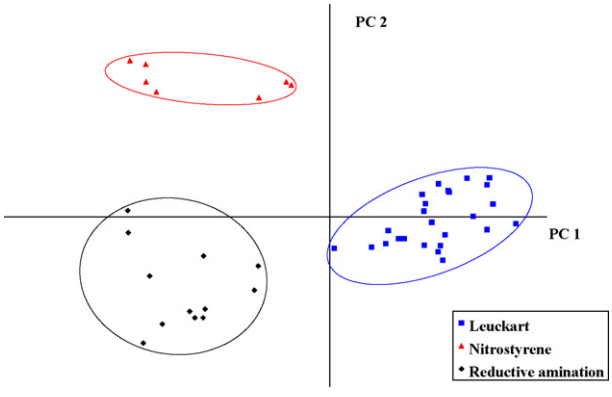


Fig. 1. Score plot (PC1 vs. PC2) for 44 amphetamine samples modelled by PLS-DA. The three groups respectively represent samples of amphetamine synthesised by the Leuckart reaction, the reductive amination route, and the nitrostyrene method. PLS-DA was performed on peak areas for 33 target compounds, after pretreating the GC–MS data with N + 4th root.

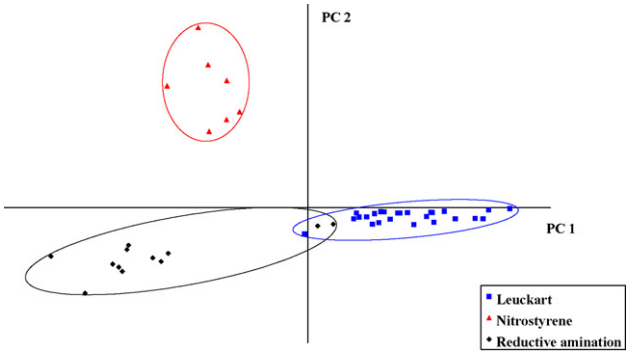


Fig. 2. Score plot (PC1 vs. PC2) for 44 amphetamine samples modelled by PLS-DA. The three groups, respectively, represent samples of amphetamine synthesised by the Leuckart reaction, the reductive amination route, and the nitrostyrene method. PLS-DA was performed on peak areas for 33 target compounds, after pretreating the GC–MS data with N + W.

and resulted in three different groups of samples, each representing one synthetic route. When W + N and N + W was applied to the data, one Leuckart sample and two reductive amination samples appeared in groups comprising samples synthesised by one of the other routes. More precisely, the Leuckart sample was visualised in the reductive amination group, and the two reductive amination samples were in the Leuckart group. There is no obvious explanation for this observation but pretreating the W + N data with 4th root or log resulted in well-defined groupings of samples.

The findings of the visual inspection of score plots were confirmed using plots of predicted versus pre-defined values of the "dummy" variables (i.e., so called "predicted versus measured" plots). This approach is useful to check the quality of the regression model fitted to the data, which in this case gave a direct measure of how well the three synthetic routes could be discriminated when applying the different pretreatment methods. Table 6 summarises calculated regression coefficients (R and Q) of these plots for each of the tested pretreatment alternatives. The regression coefficient Q is the more important value since the predicted values used in the calculation of this coefficient were generated by applying leave-one-out

Table 6
Regression coefficients (R, for calibration; Q, for validation) for the models, and the numbers of significant principal components (PCs) when calculating PLS-DA models based on GC–MS data of 33 target compounds pretreated in different ways

Type of pretreatment	Leuckart (n = 25)		Reductive amination (n = 12)		Nitrostyrene (n = 7)		No. of PCs
	R	Q	R	Q	R	Q	
N	0.963	0.934	0.957	0.889	0.980	0.938	8
N + W	0.950	0.911	0.942	0.890	0.979	0.962	4
W + N	0.972	0.909	0.964	0.883	0.980	0.955	7
N + Log	0.967	0.943	0.977	0.943	0.961	0.940	4
N + 4th root	0.975	0.964	0.968	0.941	0.976	0.960	4
W + N + Log	0.967	0.945	0.954	0.916	0.946	0.939	3
W + N + 4th root	0.974	0.964	0.965	0.942	0.961	0.941	3

Table 7
Ratios calculated between the mean minus standard deviation (S.D.) of the obtained unlinked distances ($n = 635$) and the mean plus S.D. of the linked distances ($n = 18$) according to Eq. (10)

Zero-handling method 1 (zeros omitted) Pretreatment method	Manhattan distance	Canberra Distance	Euclidean distance	Pearson correlation	Similarity index	Squared sine
Quotients						
N	3.01	2.17	2.76	14.19	2.27	9.17
N + W	2.22	2.17	2.08	9.69	2.27	5.83
W + N	1.77	1.89	1.56	1.98	1.99	5.37
N + log	1.72	3.83	2.17	0.83	12.53	5.79
N + 4th root	2.62	2.94	3.04	9.96	8.39	7.22
W + N + log	1.28	2.66	1.70	0.97	5.97	4.87
W + N + 4th root	1.57	2.37	2.01	1.86	5.76	5.33
Zero-handling method 2 (zeros replaced with 200) Pretreatment method	**Manhattan distance**	**Canberra distance**	**Euclidean distance**	**Pearson correlation**	**Similarity index**	**Squared sine**
Quotients						
N	4.57	1.27	3.63	14.21	1.23	11.19
N + W	3.73	1.27	2.81	9.78	1.23	6.23
W + N	1.98	1.21	1.27	2.01	1.21	1.60
N + log	1.77	2.55	1.68	3.09	9.51	3.25
N + 4th root	3.42	1.63	3.56	12.06	1.88	10.18
W + N + log	1.62	1.97	1.61	2.37	4.11	2.44
W + N + 4th root	2.20	1.50	1.79	2.44	1.50	2.88

cross-validation. Hence, the Q values describe the quality of the classifications; the higher the correlation coefficient, the better the pretreatment method. As can be seen in Table 6, the best overall discrimination between synthetic routes was achieved when GC data were pretreated with N + 4th root.

3.1.2. Distance calculations

The samples used for distance calculations in this study are summarised in Table 5. Distances were calculated between samples of the same batch (11 distances) and between samples of batches synthesised with the same recipe (seven distances), which gave a total of 18 linked distances. Distances were also computed between samples in Table 5 that had no common origin, resulting in a total of 635 unlinked distances. The values in Table 7 were calculated as the ratio between the mean minus the standard deviation (S.D.) of the unlinked distances and the mean plus the S.D. of the linked distances (Eq. (10)); the linked and unlinked distances were acquired by applying the tested numerical methods to GC–MS data that had been transformed with the evaluated pretreatment techniques. In the numerical calculations, zero-handling approaches one and two were applied to the zeros in the dataset.

The ratio between unlinked and linked distances according to Eq. (10) (Table 7) was similar for the tested distance methods regardless of whether zero handling approach one or two was applied to zeros in the GC–MS data. The best pretreatments for each of the distance methods were N + 4th root (Euclidean distance, Manhattan distances, Pearson correlation, and squared sine) and N + Log (Canberra distance and similarity index). Separation of linked and unlinked samples was better with N than with N + 4th root when using Manhattan distance, Pearson correlation, and squared sine. Nonetheless, N + 4th root was chosen for pretreatment of the data, because it entailed a smaller risk of closure effects.

3.2. Reduction of variables

The samples used in this study are summarised in Table 5. The aim of these experiments was to investigate to what extent the number of target compounds used in the evaluation of the profiles could be reduced without losing relevant information. The underlying cause for this approach was a goal to end up with a faster evaluation of chromatograms in order to make the method more adapted for routine purposes. The number of target compounds in the dataset were reduced as indicated in Table 8. The effects of such reduction on the separation of samples synthesised by the three different routes were determined by PLS-DA employing GC data pretreated with N + 4th root in the calculations, and the impact on separation of samples from different batches was monitored by calculating Pearson distances for data pretreated with N + 4th root and using zero-handling approach 2. The substances that were removed from the dataset were those that were either most difficult to identify in the chromatograms or were known to evaporate from amphetamine stored at room temperature. The regression coefficients (R, for calibration; Q, for validation) obtained by PLS-DA modelling using the reduced data sets are summarised in Table 9, and the values calculated as the ratio between the mean minus the standard deviation (S.D.) of the unlinked distances and the mean plus the S.D. of the linked distances (Eq. (10)) are shown in Table 10.

The visual classification of samples obtained in the PLS-DA score plots was approximately the same when using 26 target compounds as compared to 33 compounds, although the

Table 8
Different combinations of target compounds that were removed from the original dataset

No. of compounds in analysed data set	2,4-Dimethyl-3-phenyl-6-(phenylmethyl)pyridine	1-Hydroxy-*N*,*N*-di(beta-phenylisopropyl)amine	Unknown A4	4-Methyl-5-phenylpyrimidine	4-Benzylpyrimidine	2-Methyl-3-phenylaziridine	Unknown 1	Benzylmethylketoxime	Benzylmethylketoximes integrated as one substance
33									
29	X	X	X					X	
28	X	X	X			X		X	
27	X	X	X	X	X			X	
26	X	X	X	X	X	X		X	
25 (A)	X	X	X	X	X	X	X	X	
25 (B)	X	X	X	X	X	X		X	
25 (C)	X	X	X	X	X		X	X	
24	X	X	X	X	X	X	X	X	

Table 9
Regression coefficients (Q, for validation; R, for calibration), and the significant number of principal components when applying PLS-DA to the reduced data sets described in Table 8

No. of target compounds[a]	Leuckart		Reductive amination		Nitrostyrene		No. of PCs
	R	Q[b]	R	Q	R	Q	
33	0.975	0.964	0.968	0.941	0.976	0.960	4
29	0.976	0.965	0.968	0.941	0.979	0.963	4
28	0.976	0.965	0.967	0.939	0.976	0.958	4
27	0.963	0.946	0.957	0.922	0.981	0.969	4
26	0.962	0.946	0.957	0.921	0.980	0.965	4
25 (A)	0.967	0.946	0.970	0.939	0.979	0.964	5
25 (B)	0.961	0.943	0.956	0.916	0.968	0.943	4
25 (C)	0.967	0.946	0.968	0.927	0.966	0.939	5
24	0.972	0.946	0.978	0.931	0.962	0.909	7

[a] The target compounds in the different datasets are given in Table 8.
[b] The regression coefficients Q describe how well the models are fitted to data and give therefore a measure of how the different data sets perform in discriminating the three synthetic routes.

correlation values (Q) were somewhat better in the latter case (Table 9). The ratios between unlinked and linked distances (Table 10) showed that application of 26 compounds gave very good separation of unlinked and linked distances, and only removal of unknown 1 (leaving 25 compounds in the data set) resulted in greater improvement of the discrimination. Further reduction of the number of substances seemed to decrease the ability to discriminate between unlinked and linked distances. Moreover, since better PLS-DA models were attained with 26 compounds than with 25 (i.e., when unknown 1 was removed), it was elected to use 26 compounds for the developed amphetamine profiling method. The pretreatment experiments were subsequently repeated using these 26 target compounds, and the findings confirmed the results obtained with 33 compounds.

3.3. Evaluation of numerical methods

Numerical methods were investigated regarding their ability to discriminate between distances observed when comparing profiles of amphetamine originating from the same

Table 10
Ratios calculated between a value representing the mean minus standard deviation (S.D.) of the obtained unlinked distances and a value representing the mean plus S.D. of the linked distances according to Eq. (10)

Number of substances[a]	Quotient (of unlinked and linked distances)
33	11.2
29	12.6
28	12.7
27	18.6
26	19.0
25 (A)	19.7
25 (B)	18.5
25 (C)	18.9
24	18.5

[a] The target compounds in the datasets are summarised in Table 8.

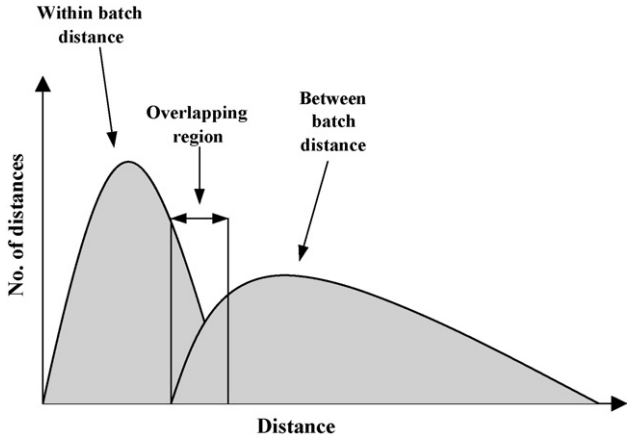


Fig. 3. Visualisation of overlapping samples. The samples in the area shared by the curves for within-batch and between-batch distances are referred to as overlapping samples.

(linked distances) and different batches (unlinked distances). This was done by calculating the number of distances in the overlapping region as is illustrated in Fig. 3.

Synthesised amphetamine samples was used to calculate distances between samples from the same batch (linked distances). Table 11 describes these samples and also indicates the number of distances calculated between them. Where applicable, different concentrations of amphetamine (5, 40, and 100%) were included in the calculations, and each sample was analysed in triplicate at three different laboratories. This generated 3901 linked distances. A total of 7800 unlinked distances were calculated between 20 synthesised samples (as indicated in Table 11) and 390 street samples seized in three different countries. The distance calculations were performed on the peak areas of 26 target compounds, using the best pretreatment method for each distance method. Both techniques for handling zeros (zero-handling methods 1 and 2) were tested. The number of overlapping distances for the different approaches are given in Table 12.

Table 11
Amphetamine samples (concentrations within parentheses) used to calculate linked distances

Samples (amphetamine conc. in %)	No. of distances
Reductive amination 1 (5, 40, and 100%)[a]	351
Reductive amination 2 (5, 40, and 100%)[a]	351
Reductive amination 3 (5, 40, and 100%)[a]	351
Leuckart 1 (5, 40, and 100%)[a]	351
Leuckart 2 (5, 40, and 100%)[a]	276
Leuckart 3 (5, 40, and 100%)[a]	276
Leuckart 4 (5, 40, and 100%)[a]	351
Leuckart 5 (5, 40, and 100%)[a]	351
Leuckart 6 (5, 40, and 100%)[a]	351
Nitrostyrene 1 (20, and 100%)[a]	153
Nitrostyrene 2 (20, and 100%)[a]	153
Leuckart (100%)[a,b]	3
Leuckart (50%)[a,b]	3
Leuckart (20%)[a,b]	3
Leuckart (10%)[a,b]	3
Leuckart (10%)[b,a]	3
Leuckart (15%)[a,b]	3
Nitrostyrene (6%)[a,b]	3
Nitrostyrene (6%)[a,b]	3
Nitrostyrene (6%)[c]	1
Leuckart (100%)[a,d]	561
Total	3901

[a] Samples that were compared with 390 samples from street seizures to generate 7800 unlinked distances. Only undiluted synthesised samples (100%) were used.
[b] The sample was analysed once at three different laboratories.
[c] The sample was analysed once at two different laboratories.
[d] The sample was analysed 11 times at each of three different laboratories (33 times in all).

Considering all the distance calculations that were tested, the best separation of linked and unlinked distances was achieved when applying squared sine and Pearson correlation (Fig. 4) in combination with the zero-handling method 2. The zero-handling method 1 was not as successful, because it entailed exclusion of target compounds with peak areas of zero, which meant that many of the differences in peak areas between different samples were not taken into account when comparing the samples. In other words, this way of handling zeros reduced the discriminatory power of the various numerical methods.

The sensitivity of the numerical methods to errors in the GC data was also examined. The results indicated that Pearson correlation was less sensitive than squared sine, hence Pearson correlation was considered to be the best alternative for separation of linked and unlinked distances. Zero-handling method 2 resulted in the smallest number of overlapping samples when employing Pearson correlation, and therefore this way of handling zeros was retained. However, it should be kept in mind that only a limited number of samples (11,701 distances) were used in this study. If a larger database had been used, it is possible that the outcome would have been slightly different.

3.4. Comparison of the SMT method with an existing profiling method

The samples used to calculate linked distances in this study originated from 17 different batches of amphetamine synthesised by the Leuckart reaction, one step reductive amination of benzyl methyl ketone, and the nitrostyrene route (Table 13). The concentrations of the samples varied between 5% and 100%. All samples were analysed in triplicate by both investigated methods at one of the participating laboratories. Unlinked distances were calculated by comparing 12 of the synthesised samples (as indicated in Table 13) with 131 samples of street amphetamine. The pairwise comparisons were conducted using GC–MS data provided by the developed SMT method and GC-FID data for the reference method. Inasmuch as the reference method employs the quotient procedure to find linked samples in a database, both the quotient technique and Pearson correlation were used to calculate distances. The number of distances in the overlapping area (Table 14) was regarded as a measure of the separation power of the profiling methods. In all, 351 linked distances and 1572 unlinked distances were calculated.

Table 12
Number of distances in the overlapping region obtained with each of the numerical methods, using both procedures for handling zeros in the data matrix

Distance method	Pretreatment of peak areas	Zero-handling method	No. of overlapping samples
Pearson	N + 4th root	1	4643
Pearson	N + 4th root	2	67
Squared sine	N + 4th root	1	456
Squared sine	N + 4th root	2	41
Euclidean	N + 4th root	1	855
Euclidean	N + 4th root	2	218
Manhattan	N + 4th root	1	2056
Manhattan	N + 4th root	2	241
Similarity index	N + log	1	2159
Similarity Index	N + log	2	356
Canberra	N + log	1	334
Canberra	N + log	2	452
Quotient (r_{max} 0.20)	No pretreatment	1	268
Quotient (r_{max} 0.15)	No pretreatment	1	461
Quotient (r_{max} 0.10)	No pretreatment	1	1212
Quotient (r_{max} 0.05)	No pretreatment	1	2905

The results of the comparison clearly indicated that the SMT profiling method was superior with respect to separating linked and unlinked distances. Indeed, no overlapping distances were obtained with the SMT approach (Fig. 5), whereas the reference method led to over 400 overlapping distances when using the quotient technique. The reference method performed better with Pearson correlation, although there were still over 300 overlapping distances (Fig. 5). Consequently, it was apparent that the profiling procedure developed in the present study surpassed the reference method with regard to the capacity to separate distances between linked and unlinked samples.

3.5. Application of the SMT profiling method

The SMT profiling method was evaluated regarding its ability to find amphetamine samples with the same impurity profile in an established database. Thirty blind samples were analysed, and their profiles were compared with 568 profiles in a database which included the profiles of the 30 blind samples. In order to make the test more authentic, the blind sample profiles stored in the database had been recorded by a different laboratory than the one attempting to retrieve these samples.

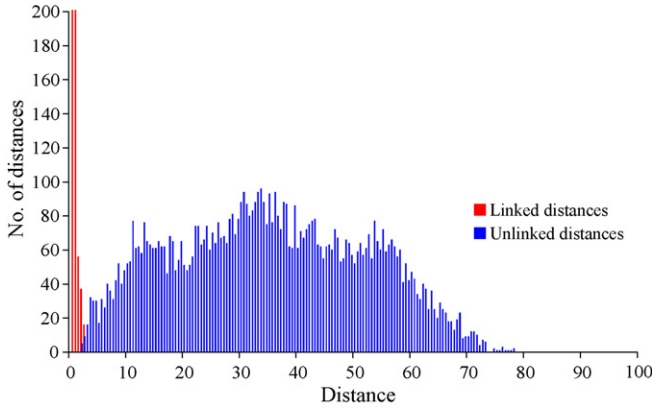


Fig. 4. Histogram of linked and unlinked distances obtained using Pearson correlation with the second method of handling zeros and pretreatment of data with N + 4th root.

Data were transformed according to the optimised pretreatment procedure (i.e., zeroes replaced with 200, followed by N + 4th root) and thereafter Pearson correlation distances were calculated. All 30 samples were correctly identified among the samples in the database. The correlation distances between the 30 samples and their references in the database ranged from 0.03 to 2.2 (mean 0.38, on a scale of 0–100). Those results can be considered in relation to the distances noted when comparing samples synthesised by different recipes (19 combinations) and routes (36 combinations), which had correlation values respectively ranging from 3.8 to 37.8 and from 14 to 69.4.

Table 13
Amphetamine samples used to calculate linked distances, and the number of distances between samples from each batch

Sample (amphetamine conc. in %)	No. of distances
Reductive amination 1 (100, 40, and 5%)[a]	36
Reductive amination 2 (100, 40, and 5%)[a]	36
Reductive amination 3 (100, 40, and 5%)[a]	36
Leuckart 1 (100, 40, and 5%)[a]	36
Leuckart 2 (100, 40, and 5%)[a]	15
Leuckart 3 (100, 40, and 5%)[a]	36
Leuckart 4 (100, 40, and 5%)[a]	36
Leuckart 5 (100, 40, and 5%)[a]	36
Leuckart 6 (100, 40, and 5%)[a]	36
Nitrostyrene 1 (100, and 20%)[a]	15
Nitrostyrene 2 (100, and 20%)[a]	15
Leuckart 7 (100%)	3
Leuckart 8 (100%)	3
Leuckart 9 (100%)	3
Leuckart 10 (100%)	3
Leuckart 11 (100%)	3
Leuckart 12 (100%)[a]	3
Total:	351

[a] Samples that were compared with 131 samples of steet amphetamine to generate 1572 unlinked distances. Only undiluted synthesised samples (100%) were used.

Table 14
Number of distances in the overlapping region (see Fig. 4) when calculating distances between linked and unlinked samples

Profiling method	Distance method	Pretreatment	No. of impurities	No. of overlaps
SMT	Pearson	N + 4th root	26	0
Reference	Pearson	N + 4th root	21	347
Reference	Quotient (r_{max} 0.2)	Raw data	23	432

Fig. 6 shows the profiles of two samples taken from the same batch of amphetamine and analysed at two different laboratories. Fig. 7 illustrates the profiles of two samples synthesised by the same route (Leuckart) but using different recipes, and Fig. 8 shows the profiles of two samples produced by different routes.

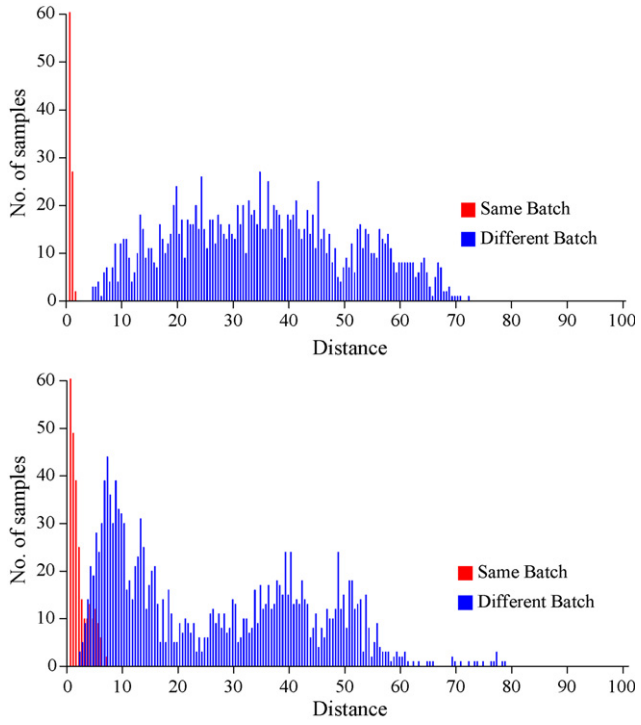


Fig. 5. Pearson distances between linked and unlinked samples. The illustrated histograms were obtained with the SMT method (above) and with the reference method (below).

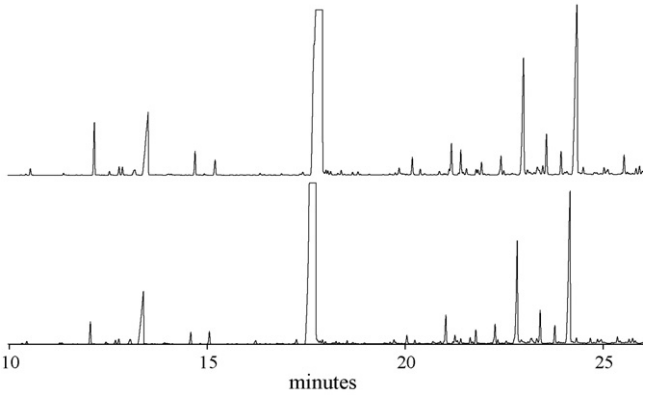


Fig. 6. Parts of impurity profiles of two samples from the same batch of Leuckart amphetamine analysed at different laboratories. The Pearson distance was 0.09.

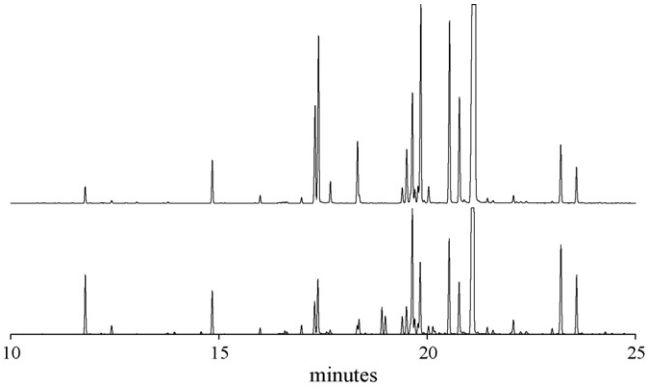


Fig. 7. Parts of impurity profiles of two amphetamine samples synthesised using different Leuckart recipes. The Pearson distance between the samples was 4.5.

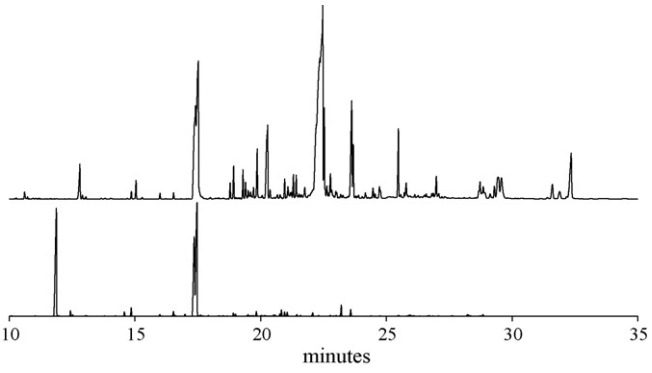


Fig. 8. Parts of impurity profiles of two amphetamine samples, respectively, synthesised by the Leuckart route and one-step reductive amination. The Pearson distance between the samples was 14.0.

4. Conclusion

In this study, Pearson correlation was the numerical method that most efficiently distinguished between linked and unlinked distances. Replacement of zeroes with 200, and pretreatment by normalisation to the peak area sum followed by the fourth root was shown to be the most efficient pretreatment alternative. Furthermore, the number of target compounds used in the comparison of amphetamine samples could be reduced to 26 without significant loss of information.

The profiling procedure that was developed (designated SMT) proved to be superior to an existing profiling method now in use at some forensic laboratories in Europe.

The SMT profiling method includes the following steps:

- Preparation of samples by an optimised liquid–liquid extraction procedure [18]
- Analysis of samples using an optimised GC–MS method [19];
- Integration of 26 target compounds in chromatograms;
- Replace zeros in data with 200;
- Pretreatment of GC-MS data using normalisation to the peak area sum followed by application of the fourth root;
- Numerical comparison of amphetamine impurity profiles by calculating Pearson correlation distances.

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