PAPER

CRIMINALISTICS

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Comparative Analysis of the Chemical Profiles of 3,4-Methylenedioxymethamphetamine Based on Comprehensive Two-Dimensional Gas Chromatography–Time-of-Flight Mass Spectrometry (GC × GC-TOFMS)*

ABSTRACT: The chemical profiling of illicit drugs is an important analytical tool to support the work of investigating and law enforcement authorities. In our work, comprehensive two-dimensional gas chromatography–time-of-flight mass spectrometry (GC × GC-TOFMS) combined with nontargeted, pixel-based data analysis was adapted for the chemical profiling of 3,4-methylenedioxymethamphetamine (MDMA). The validity and benefit of this approach was evaluated by analyzing a well-investigated set of MDMA samples. Samples were prepared according to a harmonized extraction protocol to ensure the comparability of the chemical signatures. The nontargeted approach comprises preprocessing followed by analysis of variances as a fast filter algorithm for selection of a variable subset followed by partial least squares discriminant analysis for reduction to promising marker compounds for discrimination of the samples according to their chemical profile. Forty-seven potential marker compounds were determined, covering most of the target impurities known from the harmonized one-dimensional profiling as well as other compounds not previously elucidated.

KEYWORDS: forensic science, Ecstasy, 3,4-methylenedioxymethamphetamine, synthetic drugs, chemical profiling, impurities, comprehensive two-dimensional gas chromatography–time-of-flight mass spectrometry, nontargeted chemometric analysis

Apart from the main active substance(s), various compounds resulting from the precursor and/or manufacturing process can be found in illicit drug samples. The presence and relative concentrations of these impurities depend on the type and quality of the precursor, the synthesis route, the parameters used for the synthesis process, environmental conditions in the clandestine laboratory, the purification step at the end of synthesis, packaging and storage of the samples, and aging. These compounds lead to a characteristic signature of every batch, the impurity profile, which can be related to the synthesis process of the drug (1). In contrast to these signature compounds, cutting agents (e.g., adulterants, diluents), which are frequently found in illicit drugs, can be added at any point during manufacture and distribution, and therefore provide differing levels of information. Using comparative analysis of samples based on their impurity profile, conclusions about linkage between

different samples originating from the same source (e.g., origin, manufacturing batch) can be drawn. Therefore, confirmation of linkage among different samples or different seizures via chemical analysis is an important support for investigating and law enforcement authorities.

3,4-Methylenedioxymethamphetamine (MDMA) is a structural derivative of beta-phenylethylamine and belongs to the group of amphetamines. The popularity of MDMA as a drug of abuse increased in the 1990s, and consequently, its illicit production also increased. Currently, MDMA is one of the most popular drugs of abuse. It is often known as Ecstasy, although the use of this term does not premise the presence of MDMA, as many other amphetamine-type stimulants (ATS) in tablet form can be referred to as Ecstasy.

Many different analytical methods are applied to obtain access to the chemical profiles of illicit drugs. Besides chromatographic methods, isotope ratio mass spectrometry (2–6) and nuclear magnetic resonance spectroscopy (3,6) are frequently described methods in the literature for the chemical profiling of MDMA. In forensic toxicology, many routine methods are based on gas chromatography (GC) among others for the identification of drug-relevant substances and their quantitative determination in seized material as well as in tissue or body fluids for confirmation of drug abuse. Chromatographic methods are also applied to study the chemical profiles of drug precursors. For the profiling of MDMA precursors, Huhn et al. (7,8) developed a micellar electrokinetic chromatographic method using ultraviolet and laser induced fluorescence

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detection. GC methods are also commonly applied for impurity profiling of MDMA (9–17). For the establishment of a database-assisted drug profiling program for the surveillance of seized drug samples over an extended period of time, it is of utmost importance to combine highly standardized sample preparation procedures and analysis techniques with excellent separation and identification power. Because of the variable sample matrix of illicit drugs, liquid–liquid extraction is primarily used for sample preparation, often followed by GC separation and mass selective detection. Illicit drug samples are generally dominated by the active ingredient(s) and in some cases by high-abundant additives as well. To obtain an efficient separation of main, minor, and trace compounds and a preferably comprehensive profile, a high separation power of the employed (chromatographic) system is required. As mostly minor and trace compounds are of interest besides the high separation power, a high sensitivity of the analytical system is preferred.

Comprehensive two-dimensional gas chromatography (GC × GC) provides such a comprehensive analysis combined with the desired increase in sensitivity achieved by the modulation process (18,19). Recently, GC × GC was successfully applied to different issues in the field of forensic science, for example, for the analysis of fire debris (20), chemical weapon precursors (21), and toxic waste (22). Several publications about drug analysis based on GC × GC and application of GC × GC in forensic toxicology have been published. One of the first reports refers to the application of GC × GC to drug analysis in doping control and was published in 2003 (23). Most of the following publications focused on screening of drug and doping substances and their corresponding metabolites in different matrices (23–26). Some of these application used heart-cut chromatography rather than a comprehensive approach by GC × GC (24,26). In contrast to drug screening in different matrices, forensic impurity profiling requires a comparative analysis of the (drug) material based on selected attributes or features, for example, the chemical impurity profile. An initial report of batch-to-batch comparison of drug seizures based on GC × GC has been published recently (27).

Higher dimensional techniques such as GC × GC–time-of flight mass spectrometry (GC × GC-TOFMS) lead to higher dimensional data offering novel opportunities for adaption of data analysis techniques known from other scientific fields. The two-dimensional output (chromatogram) can be handled as a picture in which the quantitative information is given within discrete values (pixels) of the raw signal, and the data analysis techniques from image processing can be applied. An increasing number of articles reporting this type of data analysis have been published recently, demonstrating their potential with respect to comparative data analysis (27–33). In the pixel-based data approach, data are represented as a matrix whose dimensions are given by the 1st retention time, the second retention time, and the mass axis, which is in the case of comparative analysis replaced by one specific mass (or optional the sum of selected ions or the total ion current [TIC]) extracted from all samples. An appropriate data pretreatment is required prior to further data analysis. Chemometric or statistical analysis is carried out on preprocessed data. The variables or so-called "features" are in this case the individual pixels of the two-dimensional chromatogram. The compounds corresponding to significant features extracted by the statistical analysis are identified at the end of the data analysis. Many forensic routine investigations of drug samples require a comparative analysis based on predefined marker compounds. Therefore, the identification of discriminating marker compounds is of major significance in the field of forensic science.

The results of a previously published proof-of-concept study about batch-to-batch comparison of real seizures of heroin and cannabis based on the obtained two-dimensional chemical profiles had been promising, and we were able to demonstrate the adaptability of GC × GC for forensic profiling (27). This work on MDMA, which was carried out in cooperation with the German Federal Criminal Police Office (BKA), focused on the comparative analysis of real samples, the identification of discriminative compounds, and the verification of the results by comparison with the results of standardized profiling routines. A set of MDMA samples from real seizures, which had been well-investigated during the pan-European project (CHAMP [Collaborative Harmonisation of Methods for Profiling of Amphetamine Type Stimulants]), was prepared according to the harmonized extraction procedure (9) to ensure the comparability of the chemical signatures and analyzed by GC × GC-TOFMS. For the verification of the GC × GC-TOF-MS analysis, the reproducibility of target compounds (peak areas) known from the standardized and harmonized one-dimensional profiling method was determined, and hierarchical cluster analysis (HCA) was applied based on normalized peak areas of those target compounds for comparison of classification results. Potential marker compounds for discrimination of the present samples were identified by a comprehensive comparative analysis of the chemical signatures. This part of the data analysis was accomplished using the nontargeted and pixel-based approach rather than the conventional peak-based approach, which requires deconvolution and peak integration *a priori*. Analysis of variance (ANOVA) and partial least squares discriminant analysis (PLS-DA) as part of the analysis routine were thereby applied on preprocessed raw data. Verification of the results could be achieved by taking the forensic background into consideration. Mass spectra allowed in most instances the assignment of the corresponding compound identification.

Materials and Methods

Materials and Reagents

Eighteen MDMA samples from real seizures were provided by the BKA. Because of the investigative background and results of the well-established and harmonized profiling analysis, six samples of this collection were known to be related to each other (MT17-19, MT26-27, MC4). Sample MC4 was extracted and analyzed in triplicate. The remaining samples, MC1, MX1-4, and MT20-25 had no known linkage either between them or to the related samples. Samples were available either in powdered or in tablet form. Each sample was homogenized using a mortar. One sample (MC3) was extracted seven times to determine the reproducibility of the developed method.

Toluene and potassium dihydrogen phosphate were purchased from Acros Organics (Geel, Belgium), and eicosane (>99%) and disodium hydrogen phosphate dihydrate were purchased from Fluka (Buchs, Germany). Ultrapure water was obtained from a Millipore water system (Bedford, MA).

Sample Preparation

Extracts of MDMA samples were prepared at the BKA laboratory according to a standardized protocol that was developed and optimized at the Netherlands Forensic Institute (9) and harmonized during the largest profiling study carried out on ATS, involving MDMA, in which several countries have taken part (CHAMP) (34). The well-established extraction process was optimized, that is, to achieve an efficient extraction of impurities while at the same time limiting the extraction of MDMA. Fatty acids that are often added as lubricants or binder during tableting process are

eliminated during extraction to prevent interferences in the chromatographic analysis.

A mass of 200 mg of homogenized MDMA powder was weighed and was dissolved in 4 mL phosphate buffer solution (pH 7 with a concentration of 0.33 M). The solution was vortexed, sonicated for 10 min, and then centrifuged at $3200 \times g$ for 8 min successively. Next, it was filtered (0.45-μm pore size; Schleicher & Schuell, Dassel, Germany) and extracted with 400 μL of toluene including eicosane as internal standard (ISTD) (20 mg/L). After rotational shaking for 20 min and centrifugation at $1900 \times g$ for 3 min, the organic layer was transferred into a GC vial, and samples were stored at −20°C until GC × GC analysis.

Comprehensive Two-Dimensional Gas Chromatography–Mass Spectrometry

For GC × GC-TOFMS analysis, an Agilent 6890N gas chromatograph (Agilent Technologies, Palo Alto, CA) equipped with a LECO GC × GC system (Leco Corp., St. Joseph, MI) was used. The system consists of a dual-stage, four-jet cryogenic (N_2) modulator. The GC system was coupled to a Pegasus III Time-of-Flight Mass Spectrometer (Leco Corp.). Injections were performed by a Combi PAL autosampler (CTC Analytics, Zwingen, Switzerland).

In the first dimension, an 1.5 m × 0.25 mm deactivated fused silica tubing as precolumn combined with a 25 m × 0.22 mm I.D. × 0.25 μm d_f (film thickness) capillary column (5% phenyl–polysilphenylene–siloxane, SGE) was used, and a 1.1 m × 0.1 mm I.D. × 0.1 μm d_f (film thickness) capillary column (50% phenyl–polysilphenylene–siloxane, SGE) in the second dimension, respectively. The initial temperature of the GC oven was set to 90°C for 2 min, followed by 10°C/min to 340°C. A temperature offset of 100°C was set on the modulator. Modulation time was adjusted to 2 sec. One-microliter injections were performed at 300°C in splitless mode with helium as carrier gas and with a head pressure of 434 kPa, which was reduced to 303 kPa after transfer time (120 sec). The pressure was then ramped parallel to the oven temperature program up to 434 kPa. The transfer line and ion source temperatures were set to 300 and 250°C, respectively. The mass range was adjusted to 40–500 m/z at an acquisition rate of 100 Hz. Detailed parameters about the harmonized, one-dimensional gas chromatography–quadrupole mass spectrometry (GC-qMS) method can be found elsewhere (9).

Software

ChromaTOF® software (2.0; Leco Corp.) was used for data acquisition and peak-based target analysis (version 3.25). The raw data were imported into Matlab® (R2008b; MathWorks Inc., Natick, MA) via network common data format. Integrated peak data were imported via character-separated values format. Further data processing and statistics were carried out in Matlab. For statistical operations, we used the PLS_Toolbox® (Eigenvector Research Inc. Wenatchee, WA), which was implemented in Matlab.

Peak-Based Target Analysis

All the samples were analyzed at the BKA by the harmonized GC-qMS profiling method (9) to provide a reference analysis. This one-dimensional GC method is based on relative quantification of 44 target impurities, using a characteristic m/z value of each compound. (Note that not all of the 44 target compounds are present in each sample.) According to the one-dimensional data processing procedure, absolute peak areas of target compounds are normalized

to their sum and prior to statistical analysis scaled via fourth root to decrease the influence of high-abundant compounds. Relative values compared with the %ISTD are stored in a database and can be used for monitoring of the illicit drugs market and its distribution networks. In a preliminary evaluation, the GC × GC-TOFMS method was verified by application of the one-dimensional data-processing procedures to GC × GC data and comparison with results of the harmonized GC-qMS method. For the peak-based target analysis, data were processed using the ChromaTOF® software. Target impurities could be identified by their mass spectra, and their characteristic ions were integrated. Peak areas of all known target impurities were loaded into Matlab, normalized to the sum of their areas, and scaled using their fourth root. Peak-based classification of samples was determined based on hierarchical clustering and compared with the known background of these samples.

Data Preprocessing and Pixel-Based Chemometric Data Analysis

Up to several hundred compounds could be detected in the two-dimensional chromatograms that had peaks with signal to noise (S/N) above 20. The targeted approach based on 44 preselected compounds, therefore, only uses a small fraction of all accessible data. In contrast, our work focused on a comprehensive analysis of the entire profile accessible by comprehensive GC × GC. Therefore, a different strategy for data analysis had to be applied. A comprehensive pixel-based approach for analysis of GC × GC data is described in some recent publications using commercial GC × GC software (33), laboratory customized software (27–31), or the adaption of an image-processing approach, which originated from the analysis of two-dimensional gels in the field of proteomics (32). An overview about recent developments regarding the application of chemometrics to data obtained by comprehensive two-dimensional separations is given in the literature (35).

Preprocessing of data is of crucial importance for the comparative pixel-based data analysis, as data comparison is based on comparison of single data points. A routine for preprocessing of data was generated. At first, sample-unspecific variation within the data set was removed. Column bleeding or solvent tailing was excluded by exclusion of the dominant mass traces (e.g., 91 m/z for toluene). Hence, data analysis was carried out on the TIC minus a few selected ions. For baseline correction, the data matrix was converted into a one-dimensional string along the second dimension, and the resulting baseline was fitted based on a weighted least square optimization algorithm using a third order polynomial. Subtraction of the found polynomial from the one-dimensional data string leads to the adjusted baseline, and the data string can be folded back into the two-dimensional chromatogram. Signal smoothing was achieved using a nonparametric algorithm based on a Gaussian Kernel function. For comparative purposes, an accurate match of data is essential, and therefore, data have to be aligned for compensation of small shifts in retention time, which might be caused during data acquisition. Alignment of both dimensions was made by implementation of the correlation optimized warping algorithm (36,37). This algorithm, which was originally developed for one-dimensional problems, could be satisfactorily applied to the two-dimensional shifting problem of this work. Normalization of data was made according to the summed detector signal to equalize the total response. MDMA as the major compound, is present in the samples in a comparatively immense amount with not evaluable peak shape, and as it is not of interest for data analysis, the total response of MDMA was excluded. Prior to statistical analysis, mean centering was applied (column-wise) on data for centering of

each variable. Scaling by the fourth root as was used for the target peak-based analysis is less practical in a pixel-based approach, because it leads to amplification of noise which is not desired. For uni-/multivariate data analysis, corresponding two-dimensional chromatograms of all samples were arrayed to a three-dimensional matrix with equivalent data points, or variables in terms of statistics, on top of each other.

Supervised discrimination techniques were applied based on the comprehensive two-dimensional profiles of the MDMA samples to identify potential discriminating marker compounds and therefore to verify whether the additional data yields additional valuable information. For identifying discriminating compounds in complex samples, ANOVA combined with PLS-DA has already been successfully applied to the analysis of tobacco smoke particulate matter (31). ANOVA handles multiclass problems and can be seen as an extension of a simple two-sample t-test. The output of ANOVA highlights the differences among the predefined groups according to a defined significance level. Here, ANOVA was used as filter algorithm for selection of a subset of significant variables out of the complete data set and was applied to the preprocessed data matrix. ANOVA was calculated for each variable, which means in this case for each pixel. The following step included the generation of a PLS-DA model based on the reduced data matrix to discover the directions in data space, which allow a direct discrimination of the predefined classes. Variable Importance in Projection (VIP) scores were calculated subsequently. Their values describe the significance of each variable for the prediction model and can be used for variable selection. Variables possessing VIP scores >1 are considered as significant (38). Those variables can be rearranged and illustrated as a chromatogram in which they indicate the position of potential marker compounds. Back in the original chromatograms, the related peaks or compounds, respectively, can be identified. In

Fig. 1, the data work flow is illustrated. The scoring plot of the PLS-DA as well as HCA based on those calculated markers can be used to verify whether the expected classification could be achieved.

Results and Discussion

Peak-Based Analysis

The reproducibility of the developed GC × GC-TOFMS method was determined by relative standard deviations (RSDs) of normalized peak areas of all target impurities found in the sevenfold extracted sample MC3. As mentioned before, not all of the originally 44 target compounds, which are used for profiling, are present in each sample. Thirty target compounds known from the CHAMP profiling method could be detected. Their identities and RSDs are given in Table 1. The values for the RSD ranged from 1.2 to 13.5%. Three compounds (Safrole, 3,4-Methylenedioxydimethylamphetamine and N-[2-(3,4-methylenedioxyphenyl)-1-methylvinyl]-N,N-dimethyl-amine) had high RSD values (>11%). The same observation could be found in the publication of van Deursen et al. (9) who developed the extraction method. The higher RSD values might be because of the relatively low extraction efficiency of these compounds.

In the following, HCA was applied on normalized and scaled peak data of the GC × GC-TOFMS analysis (selection of target analytes and data pretreatment were carried out according to the CHAMP method). Standard Euclidean distance was calculated, and samples were linked via the "nearest neighbor" method. The resulting dendrogram is shown in Fig. 2. A well-defined formation of one cluster can be observed. The cluster is composed of the previously mentioned sample MT17, MT18, MT19, MT26, MT27, and
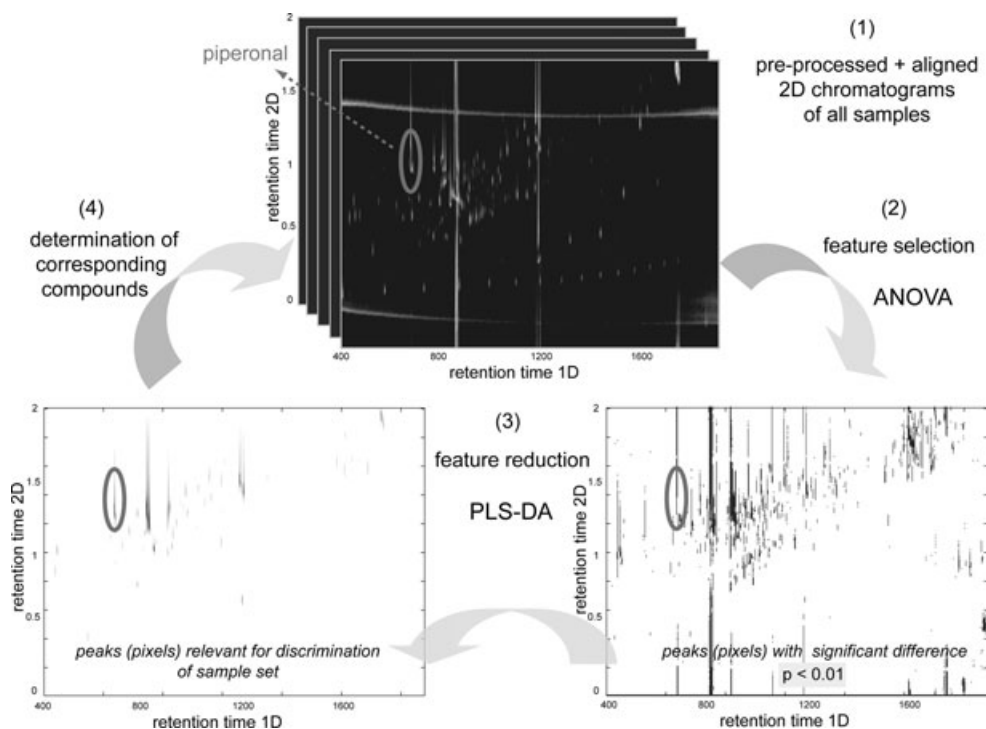

FIG. 1—*Nontargeted pixel-based data analysis for the identification of potential discriminative marker compounds that indicate variations in the impurity profile. Top: Preprocessed two-dimensional chromatograms of 3,4-methylenedioxymethamphetamine samples. Single data points are the features or variables, respectively, for the comparison of samples from different classes. Bottom right corner: ANOVA is used for variable selection. Bottom left corner: Using partial least squares discriminant analysis, discriminative features regarding the predefined classification are calculated. Back in the chromatogram, respective peaks and corresponding compounds are identified.*

TABLE 1—*RSD of target compounds.*

No	Compound	RSD %*
1	Safrole	13.1
2	3,4-Methylenedioxyphenylpropane	5.2
3	Piperonal	2.4
4	Piperonylmethylether	5.6
6	Isosafrole-trans	3.9
7	*N*-Methyl-3,4-(methylenedioxy)benzylamine[†]	
8	3,4-Methylenedioxyacetophenone	4.4
10	Unknown-176	5.8
11	3,4-Methylenedioxyphenyl-2-propanone	3.6
12	3,4-Methylenedioxyamphetamine	7.0
13	3,4-Methylenedioxyphenyl-2-propanol	3.5
16	3-(3,4-Methylenedioxyphenyl)-3-buten-2-one	5.9
17	3,4-Methylenedioxy-N-ethylamphetamine[†]	
18	Trimethyl-3,4-methylenedioxychromane	6.2
19	3,4-Methylenedioxydimethylamphetamine	11.3
20	2-(3,4-Methylenedioxyphenyl)-1-methylethyl acetate	4.4
24	3,4-(Methylenedioxy)benzylmethylketoxime	4.1
25	5-Methylenedioxyphenyl-4-methylpent-4-en-2-one	5.1
26	*N*-[2-(3,4-methylenedioxyphenyl)-1-methylvinyl]-*N,N*-dimethylamine	13.5
28	4-(3,4-Methylenedioxy)but-3-en-2-one	7.5
30	*N*-Methyl-*N*-formyl-methylenedioxybenzylamine	3.6
33	*N*-Methyl-*N*-acetyl-methylenedioxybenzylamine	4.3
34	*N*-Formyl-methylenedioxyamphetamine	4.4
35	*N*-Acetyl-methylenedioxyamphetamine	3.1
36	*N*-Formyl-methylenedioxymethamphetamine	1.2
37	*N*-Acetyl-methylenedioxymethamphetamine	2.9
38	*N*-(3,4-Methylenedioxyphenylmethyl)-*N*-[2-(3,4-methylenedioxyphenyl)-1-methylethyl]-*N*-methylamine	6.6
39	di-[1-(3,4-Methylenedioxyphenyl)-2-propyl]amine (1)	6.2
40	di-[1-(3,4-Methylenedioxyphenyl)-2-propyl]amine (2)	7.7
41	Unknown-192(b)	6.4
42	di-[1-(3,4-Methylenedioxyphenyl)-2-propyl]methylamine(1 + 2)	6.2
43	Unknown-218	8.3

RSD, relative standard deviation.

*Peak areas were standardized according to their sum.

[†]Compound was not be detected in sample MC3.

Discriminating compounds determined by analysis routine are underlined.

FIG. 2—*Batch-to-batch comparison—classification result from hierarchical cluster analysis based on target compounds known from the CHAMP project.*

MC4a–c. As mentioned before, sample MC4a–c is one and the same sample, but extracted and analyzed in triplicate (a–c). Beneath the last fusion level of the three samples (fusion between MC4a and the already linked samples MC4b and MC4c), samples cannot be discriminated as this distance is already necessary to link single samples from the very same sample material. The distance indicates, in this case, the inner-group variance between the same material induced by sample preparation and GC × GC analysis. As the classification is based on target analytes that are resultant from the synthesis process of illegal drug manufacturing, the classification result implicates the assumption that those clustered samples (MT17-19, MT26-27, and MC4a–c) are out of the same material. Between the remaining samples (linked above the dashed line), no relation among each other or to the clustered samples is known at all, and this case is reflected likewise in the resulting classification of cluster analysis.

Comprehensive Nontargeted and Pixel-Based Data Analysis

Samples were grouped according to their forensic background as it was described before. All samples were analyzed in duplicate (except for MC4, which was re-extracted in triplicate). Because of limitations in sample material, multiple extractions were not feasible. The "linked" samples (MT17-19, 26, and 27 with their respective replicate analysis and MC4a–c [triplicate extraction]) were grouped together. In case of the unlinked samples, individual "groups" were generated by double determination. Preprocessing was applied on raw data as described in a previous section. A selection of a subset of significant variables or features, respectively, was achieved using ANOVA as a filter algorithm, using a significance level of $p < 0.01$ as the decision level. Only those pixels that met the significance criteria were used as input for subsequent PLS discriminant analysis. The same group membership as described previously for the ANOVA was used for subsequent discriminant analysis. A PLS-DA model consisting of four latent variables (LVs) was generated. The model permits good separation of the linked samples from the unlinked ones. LV1 that explains approximately 30% of the variation already separates the known group from most of the other samples. Subsequently, VIP coefficients were calculated from the regression model. The threshold of "1" was used for determination of significance of the respective variable (38). The group-specific information that is provided by PLS-DA was summarized to a general result by summation of the VIP values (past application of the threshold). The result is illustrated in Fig. 3a. The picture displays those parts of the chromatogram that possess a high potential in discriminating the given set of samples according to the PLS-DA model.

For verification of the result, all features obtained by PLS-DA were used as data input for hierarchical clustering, using Euclidean distances combined with the "nearest neighbor" linkage method. Again, the formation of one cluster, consisting of samples MT17-19, 26, 27, and MC4a–c, can be observed (Fig. 3b). The obtained classification corresponds to the one obtained by the targeted peak-based approach (Fig. 2) and the known forensic background of the samples.

The indicated positions in the VIP plot (Fig. 3a) were then used to determine the corresponding compounds in the original chromatograms by back calculation of x- and y-values to the respective first and second retention times. More than half of all the potential marker compounds could be identified as target impurities used in the reference profiling method. These compounds had been selected as target compounds for MDMA profiling, and as they are derived from the synthesis process, they possess discriminative qualities
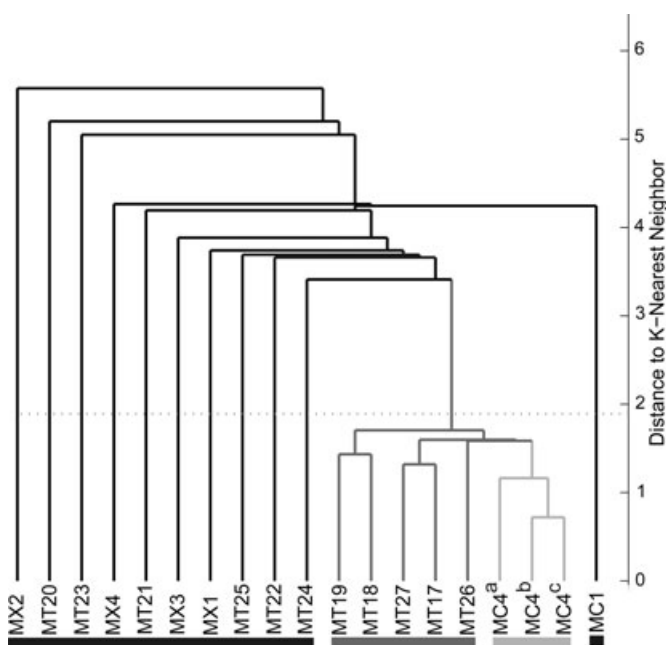
TABLE 2—*Potential marker compounds (nontarget compounds).*

No	Mass Peaks/Intensities					Compound	$n^{\ddagger}$
a	135	136	51	77	78	3,4-Methylenedioxytoluene*	17
	100	79	46	45	43		
b	43	91	65	92	134	Phenylacetone*	17
	100	47	21	18	11		
c	59	55	114	111	101	Dimethyladipate*	1
	100	69	55	43	37		
d	162	104	103	131	77	Isosafrole,* -cis	17
	100	78	63	61	60		
e	170	102	169	115	51	4-Methyl-5-phenylpyrimidine	16
	100	91	64	46	32		
f	151	95	123	77	180	Additive†	17
	100	69	59	54	8		
g	121	147	65	138	194	Additive†	17
	100	48	33	29	26		
h	57	165	137	180	91	Additive†	17
	100	66	33	33	26		
i	71	43	41	56	159	Additive†	17
	100	45	13	7	3		
j	135	58	136	164	165	Additive†	4
	100	96	81	74	55		
k	135	77	51	190	79		9
	100	54	42	23	20		
l	149	65	121	64	194		17
	100	33	30	28	6		
m	145	89	63	117	190		16
	100	95	74	56	48		
n	91	65	44	155	90		6
	100	39	26	25	23		
o	91	155	65	184	199		4
	100	58	42	26	8		
p	77	141	170	51	78	Additive†	15
	100	66	49	37	10		
q	194	109	67	55	82	Caffeine* (additive)	14
	100	96	92	87	60		
r	149	57	41	104	223	Dibutylphthalate* (lubricant)	17
	100	24	21	12	5		
s	44	43	190	86	147		2
	100	35	21	17	16		
t	176	77	149	51	91	N-[β-3,4-(methylenedioxy)-phenyl-	17
	100	33	25	23	20	isopropyl]-3,4-(methylenedioxy)-	
u	209	43	267	310	151	benzaldimine	14
	100	96	92	74	61		
v	58	270	152	151	59	Additive†	17
	100	3	3	3	3		
w	149	65	162	121	206		17
	100	21	18	18	11		
z	135	77	43	51	178		1
	100	35	24	22	13		

*Identified by library search (NIST) with probability >95%.
†See Fig. 4.
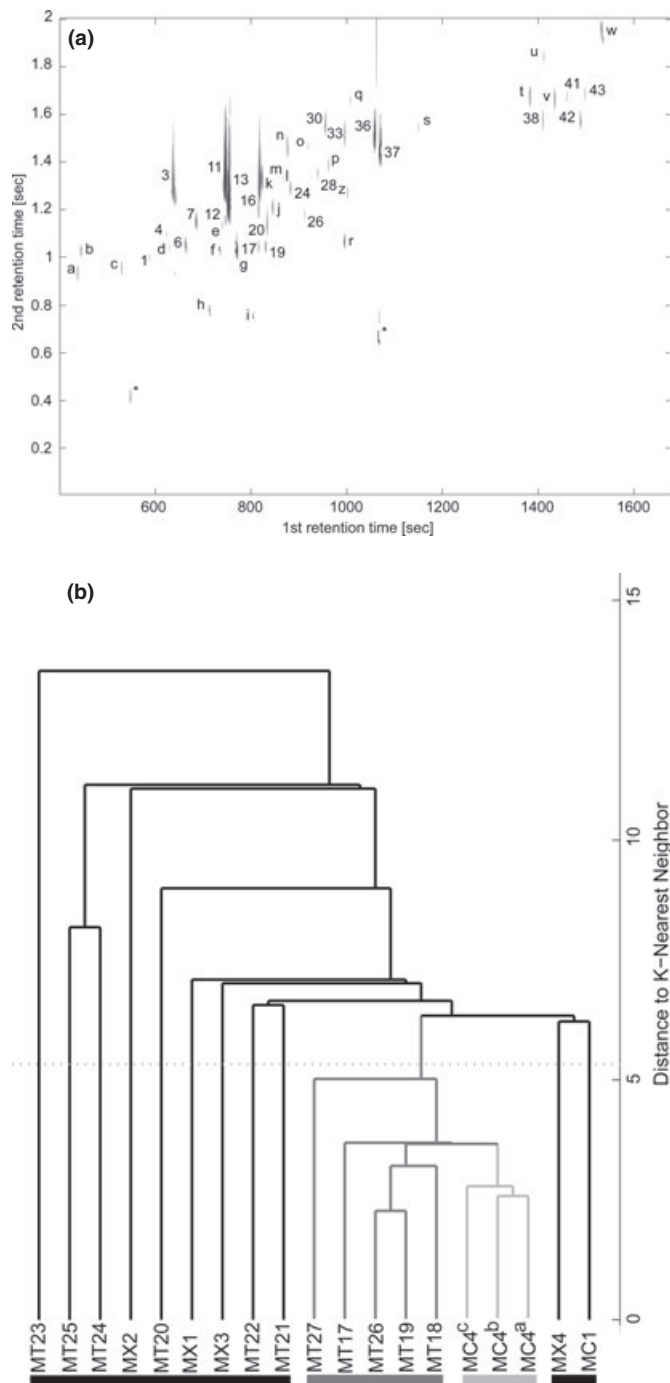‡Number of samples in which that compound was found.

FIG. 3—*(a) The result of the demonstrated data analysis (see Fig. 1) provides statistically significant peaks for discrimination of the given sample set. Identified markers labeled by numbers are target compounds known from one-dimensional standard profiling method (see Table 1). Main mass spectral features and possible identification of other potential marker compounds (a–z) are listed in Table 2. (b) Batch-to-batch comparison—classification result of hierarchical cluster analysis based on selected features of two-dimensional picture data.*

and are known to be stable. Therefore, those compounds have already been established as marker compounds, and their identification from Fig. 3a consequently confirms the validity of our data analysis approach. The identification of those marker compounds labeled by the respective peak number in Fig. 3a is given in Table 1 (underlined). Other potential discriminative compounds

identified from Fig. 3a, which do not appear in the target list of the one-dimensional profiling routine, are labeled alphabetically (a–z), and their main mass peaks (with relative intensities) are given in Table 2, together with the frequency of occurrence within this set of samples and possible identification (based on NIST spectral match). As we were dealing with real (seized) samples, additives such as diluents, adulterants, or excipients for tableting can be present in the samples and might contribute to the result from the comprehensive and nonrestrictive approach. Caffeine (q) (1,3,7-trimethylpurine-2,6-dione) is frequently found in MDMA samples as an organic adulterant. It was found in 15 samples, but often just in trace amounts, therefore it might be a contact impurity or contamination. Dibutylphthalate (r) was found in every sample. As this compound is present as a plasticizer in many polymer components, it can be frequently found as contaminant in every chromatogram irrespective of the sample. Dibutylphthalate has also been used as a

lubricant for tableting of MDMA (14). As the amount was quite high and with large variation across the samples, it might have been used indeed as an additive. One sample showed a high amount of dimethyladipate (c). The second isomer of isosafrole (d) was present in every sample as well as 3,4-methylenedioxytoluene (a) and phenylacetone (b), the latter being known, that is, as a precursor in the synthesis of amphetamines. Both 3,4-methylenedioxytoluene and 4-methyl-5-phenylpyrimidine (e) have been reported as impurities in MDMA samples (14). The spectra of *N*-[β-3,4-(methylenedioxy)-phenyl-isopropyl]-3,4-(methylenedioxy)-benzaldimine (t) can be found in the publication of van Deursen et al. (9).

Figure 4 shows the peak areas (for compounds a–z) of the linked samples MT17-19, 26, 27, and MC4a–c. Peak areas were normalized to the sum of target impurities. As those samples originate from the same batch, unknown peaks (a–z) that are related to synthesis process should result in similar quantitative measurement.

Based on the triplicate analysis of sample MC4a–c, RSDs were calculated for these peaks. The average RSD amounts to 8.9%. Peak shape and therefore also integration of the compound labeled (w) was very poor; therefore, it is neither included in the calculation nor in the diagram. Compounds a, b, d, l, m, and t showed similar responses and may, therefore, be considered as synthesis-related compounds. As noted earlier in this section, the presence of compounds a, b, d, and t in MDMA samples and their relation to synthesis process have been reported previously. Compounds d and t showed very high RSD values, which in the case of isosafrole (d) has also been reported in the literature (9). Compound (j) was only detected in sample MC4 and is not shown in Fig. 4. Compound (u) showed low responses in case of the linked samples; for samples MT17-19, 26, and 27, the values were below the applied S/N, and for compound MC4, the value was above the S/N threshold (not shown in Fig. 4). Therefore, the compound can be also considered as a compound that is related to the synthesis. For the other
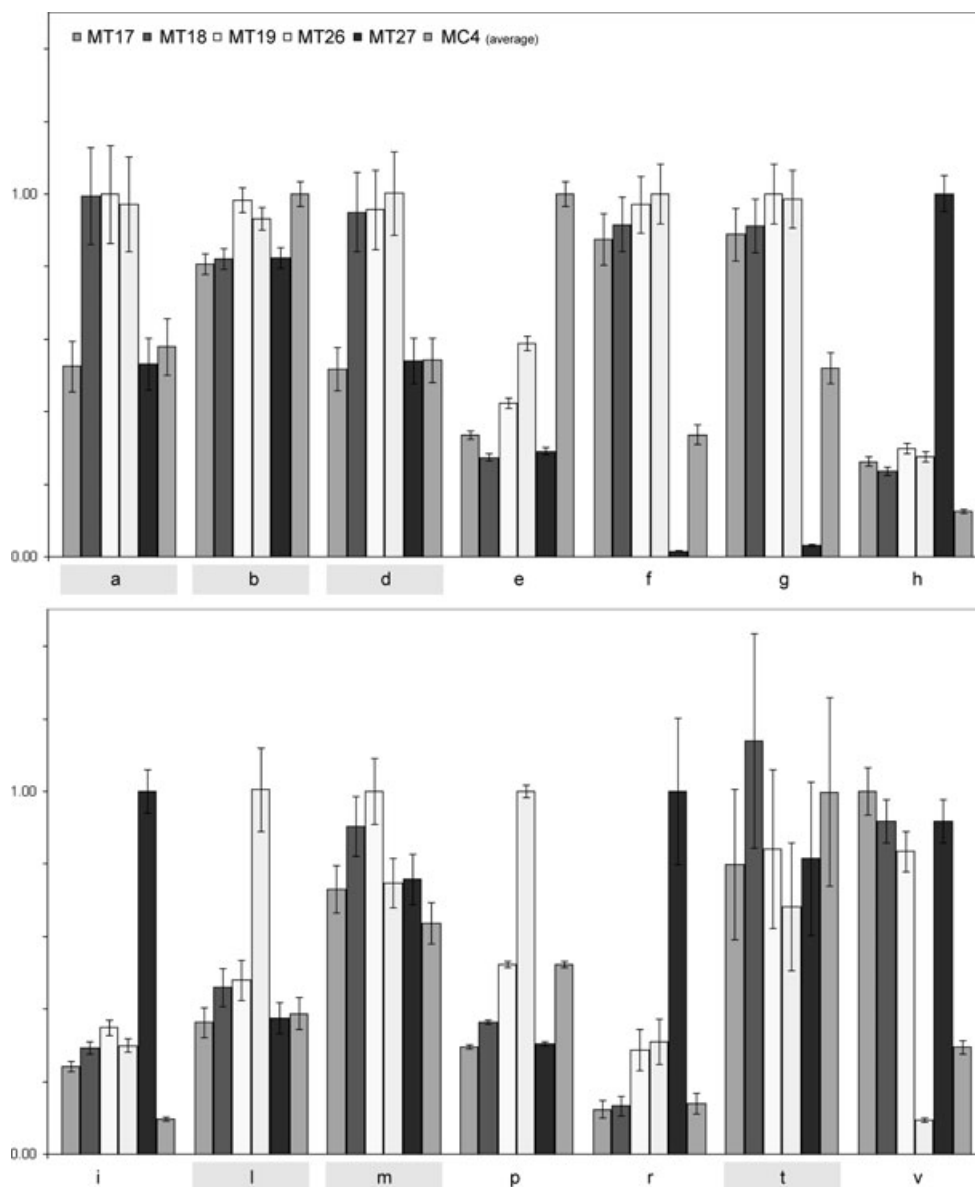

FIG. 4—*Normalized peak areas of unknown compounds of samples MT17-19, 26, 27, and MC4 (avg.). As these samples originated from the same basic material, compounds (a, b, d, l, m, t) directly related to manufacturing are supposed to appear in a similar quantity in all samples. Data values range from 0 to 1 for better graphical representation.*

compounds shown in the diagram (Fig. 4), higher variations were found among the clustered samples. Those compounds might be added during tableting or distribution process (e.g., lubricants for tableting process) or result from different storage conditions (e.g., plasticizers). For compounds k, n, o, s, and z, no conclusion can be drawn, as these compounds are not present in the clustered samples.

Conclusions

This work focuses on the demonstration of the nontargeted pixel-based routine used for comparative profiling analysis of MDMA extracts based on comprehensive GC × GC-TOFMS. The performance of the method combined with the nontargeted and pixel-based analysis routine could be verified by application to a well-investigated set of real samples from police seizures. The results of a classification analysis based on target impurities coincided with the results of the well-established and harmonized one-dimensional profiling method and the forensic background. Comprehensive pixel-based and nontargeted analysis was shown to enable a comprehensive exploration of the chemical profile of seized MDMA samples. The application of chemometric data analysis allowed the identification of potential marker compounds useful for discrimination of the set of samples. Most of the target impurities known from the well-established and harmonized 1D GC-qMS profiling routine could be identified by the nontargeted discrimination approach. Besides known target analytes, additional compounds could be determined based on the comprehensive two-dimensional analysis. The verified results obtained by this approach makes it a promising tool for the identification of discriminative compounds especially in unknown matrices or in cases in which no standard profiling routine with known targets exists. In a real comprehensive analysis approach, all peaks are treated as potential marker compounds, and individual compounds associated with the found features are identified at the end of the statistical analysis. This implies that there is no initial differentiation between impurity compounds, which are related to the synthesis process, and other organic compounds, which might be added during tableting process or distribution chain. If the identity of additives is initially known, they can be excluded from data analysis as was carried out for heroin samples in our previous work on illicit drugs (27). For identification of potential discriminating features or potential target compounds, respectively, in complex samples, a nontargeted comprehensive approach is of great benefit. As the chemical profiles obtained by analysis of drugs of abuse can become highly complex, comprehensive GC × GC is a promising alternative in this field.

Acknowledgments

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