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Benefits of derivatization in GC–MS-based identification of new psychoactive substances

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HIGHLIGHTS

- Propionic anhydride derivatization increases peak shape and alters GC-selectivity.
- Derivatized NPS have shelf lives of over 6 years.
- Reproducible differences in mass spectra are obtained from derivatized ring-isomers.
- PCA-LDA identifies the isomeric form for mass spectra analyzed retrospectively.
- A cost-effective derivatization step eliminates the need for additional techniques.

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ABSTRACT

Drug isomer identification is a significant problem in forensic laboratories due to the rise of New Psychoactive Substances (NPS). Correct identification of the precise isomeric form is of crucial importance as legal controls can vary among individual isomers. Currently, GC–MS is the method of choice in routine drug identification. This technique, however, falls short for isomeric differentiations due to *i*) very similar chromatographic behavior, *ii*) almost identical mass spectra, and *iii*) limited availability of reference standards. Unambiguous NPS identification often requires additional analysis on advanced analytical instrumentation unavailable in small-scale routine laboratories. This work demonstrates the advantages of an easy and robust derivatization step for GC–MS-based NPS identification. Derivatized extracts yield shelf lives of multiple years, eliminating the need to frequently re-prepare reference standards. After derivatization, chromatographic selectivity and peak shapes are improved enabling identification on retention time. For fluoroamphetamine ring-isomers the mass spectra of their derivates differ more significantly allowing robust assignment through a simple ion abundance-ratio check. Mass spectra of the derivatized mephedrone ring-isomers were visually still very similar. However, isomers could be identified by using Linear Discriminant Analysis (LDA). In a retrospective analysis of 132 mass spectra recorded between 2015 and 2020, a 100% correct classification was achieved using external training data from 2020. Finally, derivatization aids structural elucidation of NPS by the inability to form derivates for tertiary amine isomers as shown for a dimethylated cathinone. This study shows that in specific cases NPS can robustly be identified using conventional GC–MS instrumentation in combination with active ingredient derivatization.

1. Introduction

The emergence of New Psychoactive Substances (NPS) presents a major analytical challenge for routine forensic drug-analysis laboratories. Between 2009 and 2020 more than 950 different substances have been reported to the United Nations Office on Drugs and Crime (UNODC) [1,2]. Among these NPS many closely related and isomeric substances are observed. The global workhorse technique for drug identification in high-volume forensic laboratories is single quadrupole GC–MS [3]. Reasons for this are its ability to process mixtures, its sufficient selectivity for traditional narcotics, the fact that most drugs-of-abuse substances show good gas-chromatographic behavior and the

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availability of affordable and robust routine instrumentation and electronic databases. However, a notable limitation of GC–MS is associated with the differentiation of isomeric, especially ring-isomeric, compounds. Due to their structural similarities these compounds yield almost identical retention times and electron ionization mass spectra. Since the emergence of NPS, encountering a substance that has several ring-positional isomers is becoming more routine. Another complicating factor is that legal control of NPS differs among countries and individual isomeric forms can be either controlled or uncontrolled [2]. For example, 4-MethylMethCathinone (4-MMC, mephedrone) is a controlled substance in The Netherlands whereas its 3-MethylMethCathinone (3-MMC) and 2-MethylMethCathinone (2-MMC) isomers are uncontrolled [4]. This has driven the development of various analytical approaches for unambiguous isomeric identification. Successful approaches using spectroscopic techniques coupled to GC include InfraRed detection (GC-IR) [5–7] and Vacuum UltraViolet detection (GC-VUV) [8–11]. Other analytical techniques capable of ring-isomeric identification are UPLC [12], ion mobility spectroscopy [13] and infrared ion spectroscopy [14]. In addition to these, GC–MS based approaches were developed using more advanced or modified MS-instrumentation including product ion spectroscopy [15,16], cold Electron Ionization (EI) [9,17], chemical ionization [18] and low energy EI [19].

Since single quadrupole GC–MS currently is the routine instrumentation available in the forensic laboratory, strategies for isomer identification on this platform provide a significant benefit as no instrumental investments and implementations are required. For some ring-isomers adequate chromatographic separation and retention time-based identification was achieved for the isomeric form, such as *ortho*-, *meta*- and *para*-methoxylated MethyleneDioxy-PyroValerone (MDPV) [20], methylenedioxy-cathinone isomers [21] and MMC-isomers [10,22] whereas other separations remain more challenging such as for the FluoroAmphetamine (FA) isomers [10]. A drawback of retention time-based approaches is the need of reference standards in sufficient amount to prepare standard solutions for each sequence. This can be challenging as reference materials are only available in small quantities or might even be unavailable due to the novelty of the NPS. In addition, import procedures can be expensive and time-consuming due to varying legislation procedures among countries. Therefore, approaches based on robust differences in mass spectra are preferable as they do not consume reference material on a daily basis. EI-mass spectra of most amphetamine-type drugs are uninformative as only one abundant ion is visible being the low *m/z*-ion resulting from the *alpha*-cleavage of the amine-group. However, other low abundant ions are present that could be exploited. Bonetti was the first to demonstrate the potential of applying multivariate statistics on mass spectra for NPS isomer differentiation [23]. Davidson and Jackson [24] showed an example of its applicability for NBOMe ring-positional isomers and Setzer and Waddell Smith compared various methods of variable selection for LDA on mass spectra of drug isomers [25]. In previous work our group recently showed that specific pre-processing and feature selection could further improve the selectivity of this ring-isomeric discrimination [19]. All previous work was performed on mass spectra of native (underivatized) substances.

For decades, derivatization had been a well-known strategy in analytical sciences to improve chromatographic behavior or detection of analytes. Before the routine availability of LC, derivatization was the method of choice to transform polar, non-volatile amines and hydroxides into volatile, relatively non-polar derivatives suitable for GC. Also, specific derivatizing reagents can remarkably increase detection sensitivity such as derivatization with fluorinated or chlorinated reagents for the halogen specific detection techniques Negative Chemical Ionization (NCI)-MS and Electron Capture Detection (ECD) [26,27]. As many synthetic drugs contain an amine-moiety and thus can yield challenging GC behavior (*e.g.* peak broadening by nonspecific column adsorption), derivatization is suggested as an optional technique by the UNODC [3]. One such derivatization strategy applied in GC drug analysis is acylation with acid-anhydrides where the active hydrogen on the amine group is converted into an amide [28]. Other derivatization techniques are silylation and alkylation, such as demonstrated by Ash *et al.* [29] for several illicit-drug substances. In this study dimethylformamide-dimethyl acetal (DMF-DMA) was -amongst others- used for derivatization of primary amines. As derivatization is not essential for identification of conventional synthetic drugs, most laboratories analyze the samples in their original form to maximize sample throughput and minimize the cost of analysis. However, the rise of NPS and the accompanying analytical challenges might justify derivatization especially when it eliminates the use of additional analytical methodology for robust chemical identification.

Clark and colleagues reported extensive studies on GC–MS analysis of acylated NPS. They compared various acylation reagents applied to MDMA and reported modified EI-fragmentation due to reduced nitrogen basicity and a decreased role of the *alpha*-cleavage reaction after acylation. As a result, other fragmentation pathways play a more prominent role resulting in more characteristic ions in the mass spectrum [30]. In this way, they were able to distinguish positional isomers of MDMA [31] and MDMA related compounds [32,33] based on the mass spectrum of their perfluorinated alkylamides. No differences in the mass spectra for the ring-isomers were reported, however ring isomer identification was obtained through the retention time of their reference standards as sufficient chromatographic separation of the *ortho*-, *meta*- and *para*-isomers was obtained [31,33,34]. In line with these MDMA-type drugs, Alsenedi and Morrison compared six different acylation reagents on cathinone NPS and also reported more informative mass spectra [35]. Derivatization experiments on the ring-isomers of fluoroamphetamine were performed by Nakazono [36] and Rösner [37], both reporting a better chromatographic behavior. Although no specific focus was set on the differentiation by mass spectra, both groups noticed a higher abundant *m/z* 136 ion for 4-FA compared to 2-FA and 3-FA after trifluoroacetylation [36] and acetylation [37] indicating that this ion could be an indicator of the isomeric form.

In this study we demonstrate the specific advantages of derivatization for single quadrupole GC–MS based NPS isomer analysis. Limitations in chromatographic behavior and robustness of conventional methods are presented and compared to alternative approaches using derivatization. Strategies for robust ring-isomer differentiation were developed based on increased chromatographic performance and more substantial differences in mass spectra. In this way, ion abundance-ratio checks could easily distinguish all FA-ring isomers after acylation. For the first time, we demonstrate the differentiation of MMC-ring isomers after PCA-LDA on the mass spectra of its propionyl-derivates. Finally, we show how derivatization could help in the identification process of unknown NPS as demonstrated for several actual case samples.

2. Materials and methods

2.1. Chemicals

2-FluoroAmphetamine (2-FA); 3-FluoroAmphetamine (3-FA); 3-FluoroMethAmphetamine (3-FMA); 4-FluoroMethAmphetamine (4-FMA); 2,3-ethylone; 2,3-methylone were obtained from Cayman Chemical Company (Ann Arbor, USA). 4-FluoroAmphetamine (4-FA); 2-FluoroMethAmphetamine (2-FMA); 3,4-methylenedioxy-N-ethylcathinone (ethylone); 3,4-methylenedioxy-N-methylcathinone (methylone); 3,4-methylenedioxy-N-dimethylcathinone (dimethylone), 2-MethylMethCathinone (2-MMC), 3-MetylMethCathinone (3-MMC), 4-MethylMethCathinone (4-MMC, mephedrone) and 2,5-dimethoxy-4-bromophenethylamine (2C-B) were pure seized case samples whose identities were established by prior FTIR and GC–MS analysis. These, and a case sample known for containing 4,5-dimethoxy-2-bromophenethylamine (2-Br-4,5-DMPEA) were provided by the Police

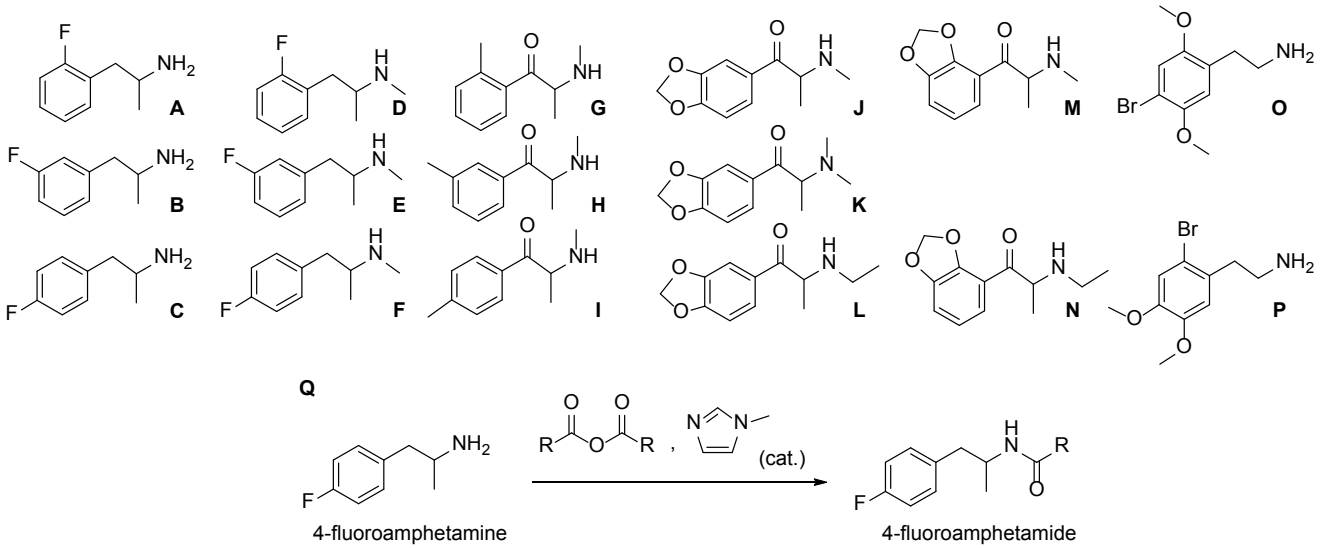

Fig. 1. Molecular structures of 2-FA (A), 3-FA (B), 4-FA (C), 2-FMA (D), 3-FMA (E), 4-FMA (F), 2-MMC (G), 3-MMC (H), 4-MMC (I), methylone (J), dimethylone (K), ethylone (L), 2,3-methylone (M), 2,3-ethylone (N), 2C-B (O), 2-Br-4,5-DMPEA (P) and an example of the derivatization for 4-FA (Q).

Laboratory. Molecular structures of all NPS used in this study are shown in Fig. 1. Case samples used in this study were seized by the Dutch Police between 2015 and 2020. Acetic Anhydride (AA) and Tri-FluoroAcetic Anhydride (TFAA) were obtained from Sigma Aldrich (St. Louis, USA), Propionic Anhydride (PA) and Butyric Anhydride (BA), N-methyl-imidazole, dichloromethane (for analysis), methanol (for analysis), sodium bicarbonate monohydrate (for analysis), Celite Hyflo Supercel, sodium hydroxide (for analysis) and hydrochloric acid (32%, for analysis) were obtained from Merck (Darmstadt, Germany).

2.2. Sample preparation and derivatization

Several sample preparation or derivatization methods were compared in this study. The method applied is indicated for each experiment and described in more detail below. Methanolic extraction (A): 10 mg of sample was dissolved in 5 mL of methanol; solutions were ultrasonicated for 10 min. Dichloromethane extraction (B): 10 mg of sample was dissolved in 5 mL of dichloromethane; solutions were ultrasonicated for 10 min. Neutralization (C): an aliquot from the methanolic solution of method A was transferred to a 2 mL vial containing 10 mg of sodium bicarbonate; the vial was capped, shaken for 20 min and the insoluble sodium bicarbonate residue was allowed to settle for several minutes; the supernatant was filtered over glass wool and transferred into a GC vial. Acid-base extraction (D): 10 mg of sample was dissolved in 1 mL of 1 M HCl and ultrasonicated for 10 min, 1 mL of 2 M NaOH was added directly followed by 5 mL of dichloromethane. The mixture was shaken for 10 min and the organic (lower) layer was filtered over glass wool and Hyflo Supercel and transferred to a GC vial. Derivatization (E): 25 µL of derivatization reagent and 25 µL of N-methyl-imidazole were added to a test tube containing 10 mg of reference material *or* 20 mg of sample. The test tube was ultrasonicated for 10 min at ambient temperature allowing the reaction to complete. 5 mL of dichloromethane was added and the mixture was again ultrasonicated for 10 min. An aliquot of the dichloromethane was filtered over glass wool and transferred into a GC vial. For samples and reference materials available at limited quantities; reduced amounts (down to 1 mg) were used by correcting all volumes equivalently.

The derivatization method (E) applied in this study is an N-methyl-imidazole catalyzed acylation with acid-anhydrides [27]. In this reaction N-methylimidazole-amides are *in situ* formed and react with the amine-group in the NPS to form a stable NPS-amide while N-methylimidazole is regenerated. In this way a high reaction rate is achieved allowing for a complete reaction in several minutes at ambient temperature thus eliminating the need for longer incubation times at elevated temperatures. An example of the derivatization reaction is shown in Fig. 1-Q for 4-FA. Fig. S1 gives the reaction mechanism of the N-methylimidazole catalyzed acylation with acid anhydride and Fig. S2 shows the molecular structures for the substances used in this study after derivatization (method E) with propionic anhydride. All derivatization experiments in this study were performed with propionic anhydride because a validated method using this reagent for synthetic drug identification has been used in the laboratory for many years and proved to yield stable and reproducible results; for experiments described at Section 3.5.1 the other acid anhydrides mentioned in Section 2.1 were also applied using the generic protocol described earlier.

2.3. GC–MS analysis

GC–MS experiments were performed on two identical systems consisting of a 7890B gas chromatograph and 5977B single quadrupole mass spectrometer from Agilent Technologies (Santa Clara, USA). For each extract a 1 µL volume was injected using a Combi PAL autosampler from CTC Analytics (Zwingen, Switzerland). The injector was operated in split mode at 300 °C with a split flow of 75 mL/min and column flow of 1.1 mL/min helium (split 1:68). An ultra-inert deactivated liner with glass wool was used. Chromatographic separation was achieved on a 15 m 5%-diphenyl-95%-dimethylpolysiloxane stationary phase DB-5MS column with 0.25 mm internal diameter and 0.25 µm film thickness. Degradation experiments on MMC were performed with a 30 m GC column from the same type. The oven program was in all cases as follows: start temperature at 100 °C with a hold time of 1.5 min, then ramping at 30 °C/min to 280 °C. The following MS parameters were applied: MS transfer line temperature 280 °C, 70 eV EI ionization, 230 °C source temperature and 150 °C quadrupole temperature. Full scan MS data was acquired from m/z 41 to m/z 462. A 1.5 min solvent delay time (3 min for the 30 m column) was applied to prevent the MS from detecting both the solvent peak and the excess of the derivatization reagents. Masshunter GCMS Data Acquisition B.07 and Masshunter Qualitative Analysis 10.0 were used for data acquisition and processing. The system's built-in autotune functionality was executed weekly on a routine basis.

2.4. Chemometrics

Principal Component Analysis (PCA) and Linear Discriminant Analysis (LDA) on mass spectral data were performed in Unscrambler 11 (Camo Analytics, Oslo, Norway). Mass spectral data was manually exported from MassHunter, data was zero-filled and ion intensities were rounded and binned at 1 Da intervals to compensate for minor mass calibration fluctuations. Unless specifically specified, the *m/z* 60–240 part of the mass spectrum was selected to exclude influences from the non-diagnostic base-peak ions. Ion intensities were area-normalized as pre-processing. PCA was applied for data reduction and LDA was performed on the first 3 principal components (PCs).

3. Results and discussion

3.1. Stability of underivatized extracts

Common GC-suitable solvents for drugs-of-abuse screening include dichloromethane and chloroform as both the neutral base from and hydrochloride salt form of traditional drugs (*e.g.* cocaine, heroin) are sufficiently soluble in these solvents. Contrary to this, sulphate or hydrochloride salts of many synthetic NPS drugs are only weakly soluble in these solvents. Therefore, more polar solvents such as methanol are routinely used for GC-analysis of NPS [8–10,17–19,21–23]. The use of these more polar solvents for the analysis of the amine-containing NPS drugs of the phenethylamine and cathinone class results in several chromatographic challenges. A well-known limitation in the gas chromatographic analysis of amines are peak distortions and peak broadening due to adsorption effects on active sites in the inlet liner or column. Although this effect could be minimized by frequent system cleaning, liner replacement and the use of dedicated deactivated consumables, this compromises overall robustness and routine stability. Additionally, peak distortion can be partly managed by peak focusing in the capillary column through film thickness selection and the temperature program settings. However, GC–MS methods applied should cover a broad range of compounds in terms of volatility and short analysis times are preferred to maintain a high throughput. In our study, the adsorption effects were most severe for the cathinone type NPS resulting in significant peak broadening on a 1-week old liner, while these effects disappeared for the same extract after liner replacement.

Another limitation of methanolic NPS extracts is reduced extract stability causing peak broadening in extracts that were not freshly prepared. This effect was described before [8,10] and the proposed explanation is an equilibrium of both the protonated amine and the neutral base form in the polar solution, resulting in slightly different evaporation characteristics during injection causing peak broadening. This effect was visible for all NPS included in this study but appeared more severe for the volatile (relatively unretained) phenethylamine-type NPS such as the fluoroamphetamines. Methanolic FA and FMA solutions yielded heavily distorted peaks as shown in Fig. S3 for a 4-FA sample and Fig. S4 for a 4-FMA sample. Fresh methanolic solutions of cathinone-type NPS that were ultrasonicated for several minutes and injected on a clean GC–MS system often yielded reasonable peak shapes, however severe peak distortions were sometimes observed (Fig. S5-A) and were attributed to the variable acid-base equilibrium in the methanolic extracts. A remedy for the peak distortions caused by this phenomenon was given by Skultety *et al.* [8] who described and explained a neutralization strategy using sodium bicarbonate. By applying this neutralization step (2.2, method C) symmetric chromatographic peaks could be obtained as visible in Figs. S3-C, S4-C and S5-C. For all compounds included in this study, peak broadening was observed in older extracts, even after prior neutralization. This suggests that the neutralization only had a temporary effect due to changing equilibria. The stability of the methanolic extracts varied per component and, although not further investigated in this study, sample concentration, sample volume and GC vial type seem to affect the sample stability. Fig. S6 shows the 6-fold replicate injection of the same methanolic MMC-solution over 17 h clearly showing severe peak broadening (double peak width and half peak height) over time. The peak shape could be restored by re-applying the neutralization procedure directly before re-analysis. However, this rapid decrease of peak shape clearly limits the possibilities to use methanolic extracts for routine GC–MS analysis in batch sequences using an autosampler.

The NPS compounds, often present in their salt form in case samples, are poorly soluble in dichloromethane. This leads to small peaks as visible in Figs. S3-B and S4-B. A known strategy to overcome this is to dissolve the salts in an aqueous acidic solution, change the pH to strongly alkaline conditions to produce the water insoluble free base form and extract these free bases with an organic solvent [3]. In this way, symmetric chromatographic peaks comparable with the peaks after $NaHCO_3$ neutralization were obtained for all compounds in this study (see plots D in Figs. S3 and S4 as an example). In similar fashion as the methanolic extracts, peak broadening was visible in older extracts.

3.2. Degradation of cathinones in methanolic extracts

While the adsorption and extract stability effects could be overcome by the described strategies, a more severe issue for NPS analysis is compound degradation. As described by Kerrigan *et al.* [38] cathinone type drugs are prone to thermal degradation due to enamine formation. In this way, a degradation product was formed that could be clearly observed in GC–MS as a chromatographic peak with a −2 Da mass shift of the base peak ion due to the formation of a double bound. (*e.g.* ion *m/z* 56 for the degradation product of MMCs that itself yields *m/z* 58 as most abundant ion by *alpha*-cleavage of the amine group). Kerrigan *et al.* [38] attributed this breakdown to thermal degradation in the injection system and described a reduction by using lower injection temperatures and working on clean systems. However, we observed this same degradation effect over time in methanolic extracts stored in the fridge. This effect was most severe for the *ortho*-positional isomers (*e.g.* 2-MMC, 2-MEC). A possible explanation for this is the presence of a hydrogen-bond between the *ortho*-methyl-substituent and the carbonyl-group that enhances the dehydrogenation. Another factor contributing to this degradation is the presence of alkaline conditions; increased degradation rates were observed for extracts after $NaHCO_3$ neutralization and acid-base extraction. Fig. 2 shows the degradation of a 2-MMC methanolic extract. The degradation products B and C could be attributed to 2-MMC enamines based on their *m/z* 56 base peak. These were already visible as minor contributions in the freshly prepared methanolic extracts and the 1-day old extract after neutralization (to remove the peak broadening effect as described in Section 3.1). The degradation accelerated after neutralization showing a degradation peak at 1/3th of the peak height of the main peak after 6 h. Although most severe for the 2-positional isomers, some degree of degradation was observed for all cathinone-type compounds included in this study, clearly limiting the shelf life of methanolic solutions.

3.3. Stability of derivatized extracts

Contrary to the extracts of the underivatized NPS, extracts of acylated NPS show excellent stability and reproducibility. No degradation or peak-broadening was visible in up to 3 years old dichloromethane extracts containing propionyl-derivates of MMCs stored in the fridge as shown in Fig. S7. This is in line with similar experiences with derivatized standards of amphetamine, MDMA and other phenethylamines. In applying a routine GC–MS identification method for amphetamine-type drugs by the Amsterdam Police Laboratory for many years, derivatized reference standards are routinely examined for shelf life stability and found to be extremely stable when properly stored. Fig. S8 gives an example chromatogram of an over 6 years old reference solution stored

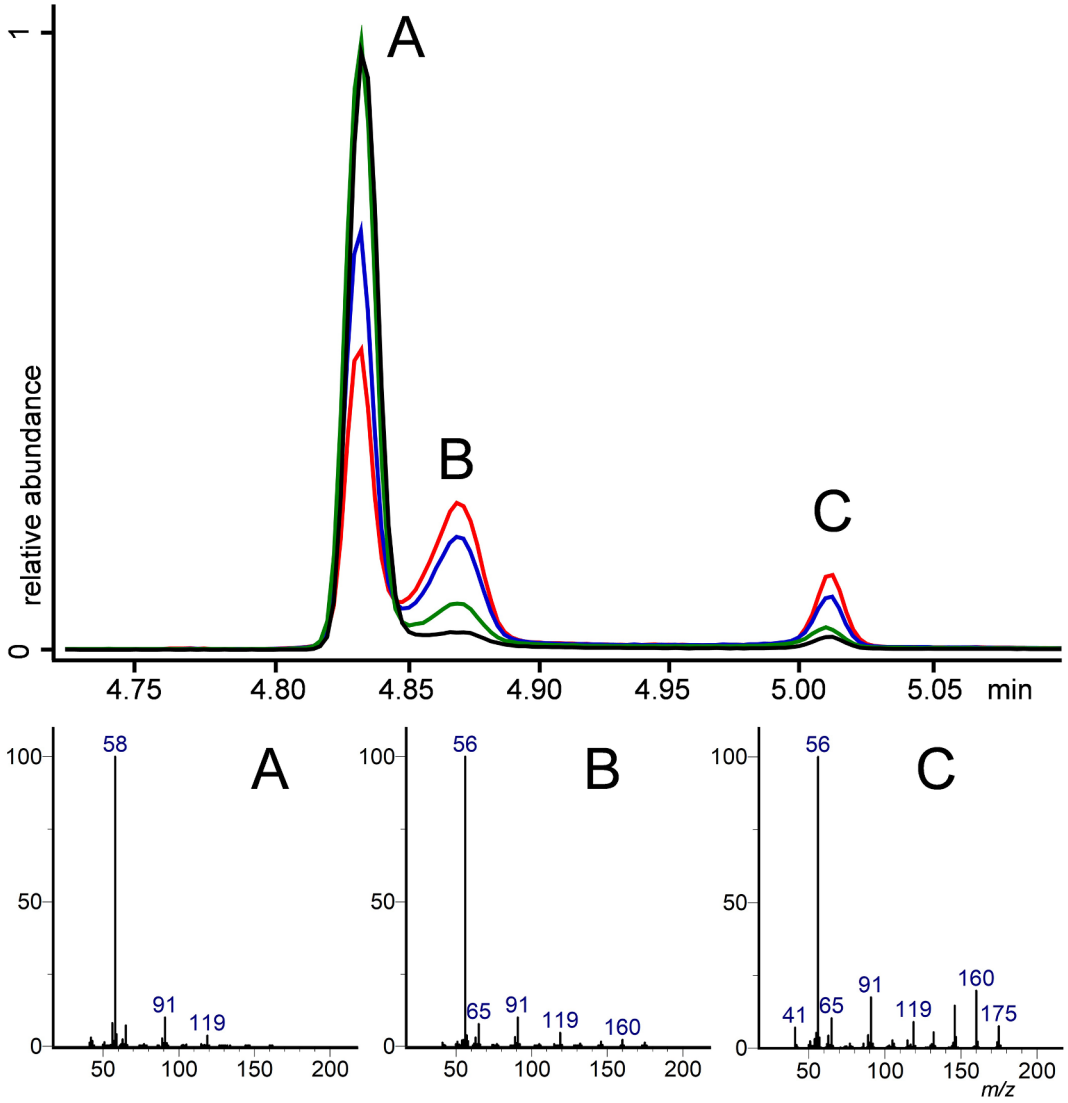

Fig. 2. GC–MS total ion chromatogram of a methanolic 2-MMC solution, freshly prepared (black), 1 day old extract after $NaHCO_3$ treatment (green), $NaHCO_3$ treated extract after 3 h (blue) and 6 h (red); with mass spectra of 2-MMC (A) and two degradation products (B,C). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

in a closed amber glass bottle at ambient temperature. For both solutions shown in Figs. S7 and S8 excellent peak shapes, correct mass spectra and no degradation product peaks were observed. Fig. 3 compares the chromatography of underivatized (A) and derivatized (B) extracts of 2-, 3-, and 4-MMC. The underivatized MMC only yielded adequate chromatographic peaks for the freshly prepared extracts analyzed on a clean GC–MS system (A, black trace). As older extracts gave peak broadening already after several hours as explained in Section 3.1, this chromatogram can be considered the best-case scenario for sample analysis without derivatization. In the fresh methanolic extract an impurity from the 2-MMC degradation product is already visible at the base of the 2-MMC peak, indicated with an asterisk. As the derivatization makes the molecule less volatile and reduces polarity, the derivates exhibit more GC retention resulting in longer retention times. This is beneficial in generic drugs-of-abuse screening methods using fast and uniform GC-methods since underivatized amphetamines as well as their related NPS (*e.g.* FAs, FMAs) are relatively volatile compared to the common drugs of abuse such as cocaine, heroin and THC. Analysis of underivatized amphetamines within the same method requires reduced temperature ramps with lower starting temperatures, resulting in longer analysis times.

3.4. Routine identification based on retention time of derivatized NPS

Since legal control of NPS can vary for individual ring-positional isomers, unambiguous identification of the correct isomeric form is of utmost importance for forensic laboratories. As both retention times and mass spectra for ring-isomeric NPS can be very similar [10,19,23] additional confirmation by retention time is typically enforced when confirmation by other techniques is not possible [20,22]. This requires the use of reference standards which in case of many NPS are only available in limited quantities at relatively high cost. Cumbersome import procedures due to varying international legislation may also lead to prolonged delivery times. As shown in Sections 3.1 and 3.2 the limited stability of methanolic extracts demands the frequent preparation of fresh standards and thus requires the availability of substantial amounts of material. Derivatized extracts show excellent stability and long shelf lives and are thus of far more practical use for retention time confirmation. As can be seen in Fig. 3 the elution order for both the underivatized and derivatized extracts was 2 < 3 < 4, or *ortho* for the first peak, then *meta*, followed by the *para*-isomer as the last eluting peak. Selectivity wise, for the underivatized MMCs the 2-isomer is more separated from the 3- and 4-isomers (0.19 min difference between 2-

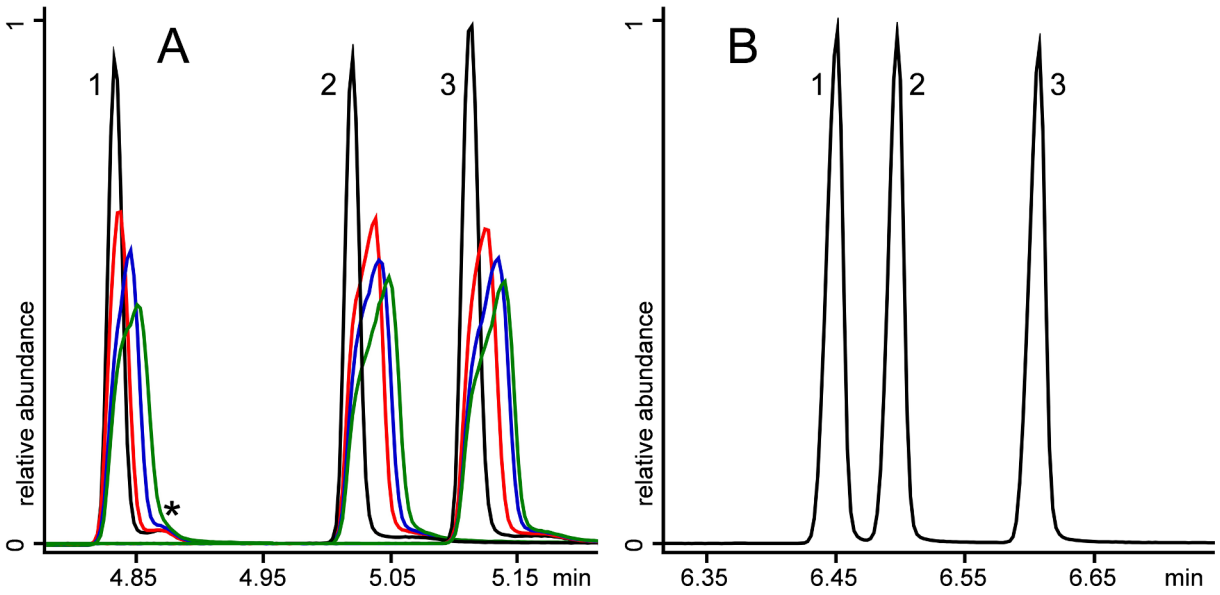

Fig. 3. GC–MS total ion chromatograms of underivatized (A) and PA-derivatized (B) 2-MMC (1), 3-MMC (2) and 4-MMC (3) solutions; for (A) replicate injections are shown of the freshly prepared methanolic extract (black), after 3.5 h (red), 7 h (blue) and 17 h (green). For (B) a 2-month-old derivatized solution was used. * indicates the 2-MMC degradation product already visible in the fresh extract. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

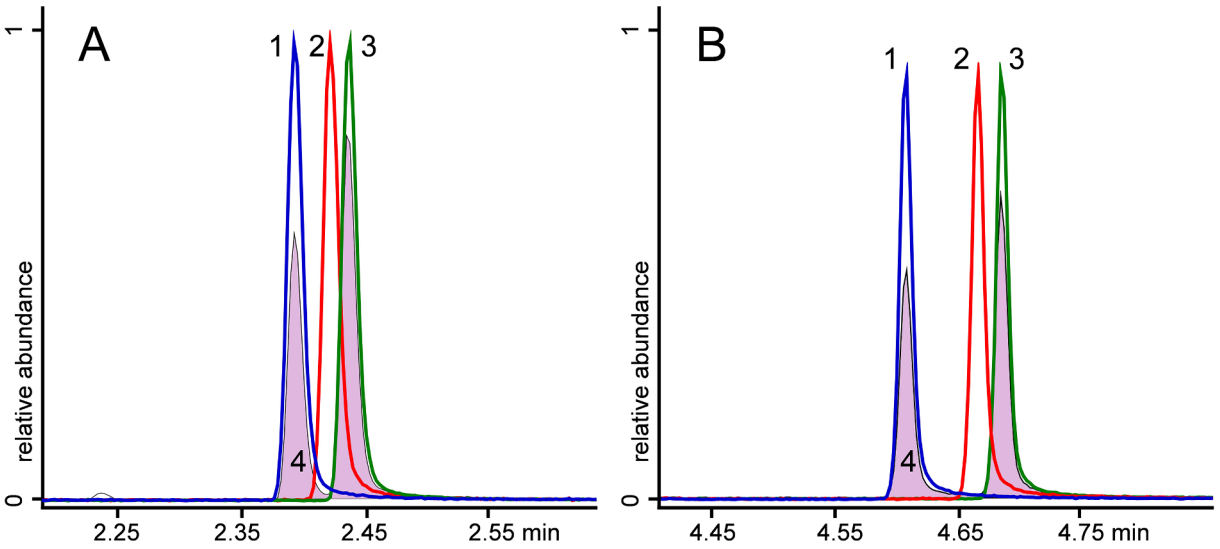

Fig. 4. GC–MS total ion chromatograms of (A) underivatized methanolic extracts after neutralization and (B) propionyl-derivates of 2-FMA (1), 3-FMA (2) and 4-FMA (3). For both plots reference standards were overlaid with the chromatogram of an unknown case sample containing two FMA isomers (4, purple shade). Retention times: A1: 2.390 min; A2: 2.418 min; A3: 2.433 min; B1: 4.606 min; B2 4.663 min; B3: 4.682 min. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

MMC and 3-MMC *vs.* 0.09 min difference between 3-MMC and 4-MMC, both for the freshly prepared solutions) whereas in the derivatized components, the 4-isomer is better resolved from the 2- and 3-isomers (0.05 min between 2-MMC and 3-MMC; 0.11 min between 3-MMC and 4-MMC). This was not found to be a general trend for all isomeric classes, as can be seen in Fig. 4 for a set of FMA-isomers where the 2-FMA was more separated (0.03 min *vs.* 0.06 min retention time difference) from its 3-, and 4-isomers after derivatization. In general, for most isomeric groups a slight shift in selectivity was observed after derivatization. Fig. 4 demonstrates the retention time-based identification of a case sample containing 2 different forms of FMA. The chromatographic separation of the underivatized compounds (A) improved after derivatization with PA (B). For this isomeric group the case sample could be identified as containing 2-FMA and 4-FMA for both methods. However, it must be noted that the underivatized chromatogram represents the ideal situation of freshly prepared and neutralized extracts directly injected into a clean GC-system with a new deactivated liner. This approach is thus only applicable in routine sequences after derivatization. Chromatographic peaks of derivatized NPS showed excellent reproducibility with a typical < 0.005 min deviation in retention time for as long as no maintenance on the GC was performed. Thus, for isomeric groups where the underivatized compounds co-elute, derivatization can help to provide additional chromatographic selectivity or even achieve baseline separation. One example is presented with a case sample containing a mixture of two different FluoroEthylAmpetamine (FEA) isomers shown in Fig. S9. The direct analysis shows co-elution for the two components whereas their respective propionyl-derivates were fully baseline separated using the same

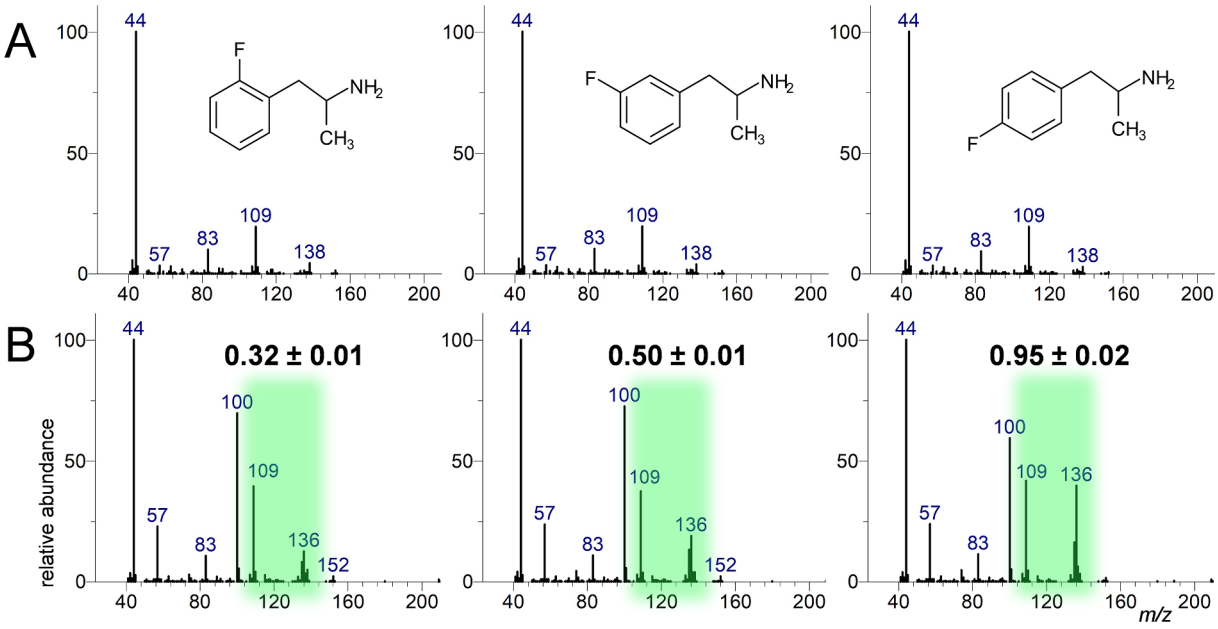

Fig. 5. Mass spectra of the FA-isomers (A) and propionyl-derivatives of the FA-isomers (B) with the abundance ratio of diagnostic ions *m/z* 136: *m/z* 109 shown in the green shade.

GC–MS method. Another example of increased chromatographic selectivity is provided by the 2C-B isomers described in Section 3.6. The combination of stable extracts, excellent peak shapes and adequate separation of the isomeric forms allows for a simple retention time check when an NPS known to contain isomeric forms is encountered in casework without the need to prepare fresh calibration samples each batch and thus significantly decreasing reference material consumption. Such a retention time check using a single solution containing the derivatized MMC isomers has successfully been implemented in the routine analysis protocol of the Amsterdam Police Laboratory for several years.

3.5. Identification of NPS isomers through MS spectra of their derivates

For all cathinone and amphetamine-type NPS in this study the EI-mass spectrum only shows one abundant yet uninformative low-*m/z* fragment. This is generally known by the experts and originates from the *alpha*-cleavage reaction of the amine [7,10,19,20]. The fragmentation mechanism of the amides formed after acylation of the amine-group is altered significantly leading to a broader range of abundant ions in the mass spectrum [30,31]. Fig. S10 shows the mass spectra for all NPS compounds studied in this work, while Fig. S11 shows the spectra of their respective propionyl-derivates. In line with earlier reports on mass spectra of NPS derivatized with other reagents, more fragments are also visible in the mass spectrum of the propionyl-derivates. Despite the additional fragments, no directly discernable differences in fragmentation were observed for ring-isomers. This finding is also in line with work from other teams listing characteristic ions for positional but not for ring-isomers (*i.e. ortho*, *meta* and *para*-isomers) [30–35]. An exception to these observations is the derivatization of fluoroamphetamine ring-isomers as highlighted in Fig. 5. Although their fragmentation pattern seems identical 'at first sight', notable intensity differences can be observed for the *m/z* 136 fragment, being much more abundant in 4-FA-propionyl. This effect was explained by Rösner *et al.* [37] for analogous acetyl- and trifluoroacetyl-derivates. They attributed this effect to a more favorable inductive route of the McLafferty rearrangement. Nakazono [36] also noticed this effect for other perfluorinated derivatization reagents and indicated its usefulness for mass spectral identification. However, both studies did not further discuss the added identification potential.

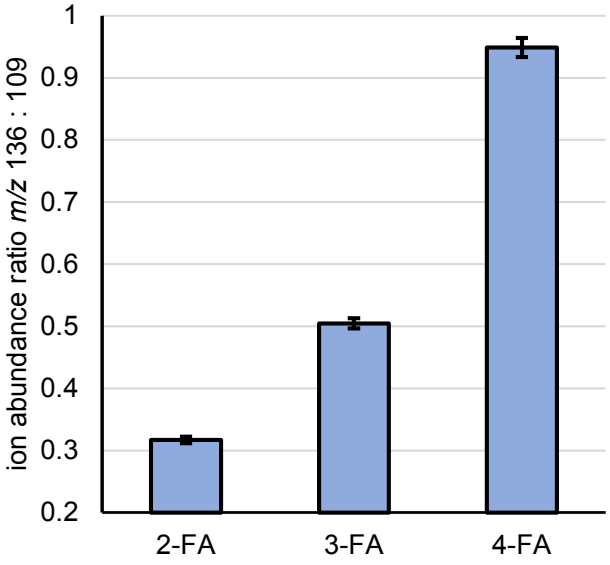

Fig. 6. The *m/z* 136:109 ion abundance ratio in 43 mass spectra of 2-FA-propionyl, 3-FA-propionyl and 4-FA-propionyl.

3.5.1. Identification by ion-ratio

The ion-ratio of the diagnostic *m/z* 136 ion to the *m/z* 109 ion was found to be a stable and reproducible indicator for isomeric differentiation at different reagents. The *m/z* 109 ion, being the fluorinated analogue of the *m/z* 91 tropylium-ion appeared relatively stable for various acylation reagents as visible in Fig. S12. The diagnostic *m/z* 136 ion showed major intensity differences, being the most abundant in all *para*-positional FA-derivates. The shortest chain reagents AA and TFAA tend to yield the highest *m/z* 136 abundance for 4-FA, although increase of abundance for 3-FA does not lead to a convincing preference for these reagents. In this study PA was selected based on its good derivatization performance and high stability of the resulting extracts. The reproducibility and selectivity of the *m/z* 136:109 ion abundance ratio was investigated on a set of 43 replicate injections acquired on two different GC–MS systems in a 2-month period. Fig. 6 shows a

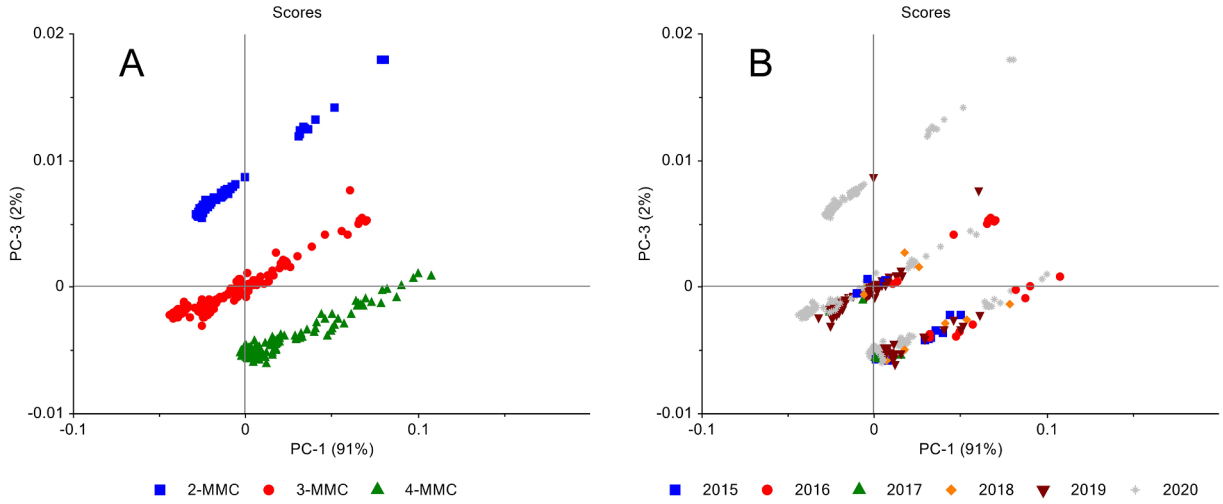

Fig. 7. PCA-plots of PC1 *vs.* PC3 following *m/z* 100–240 data selection and area normalization. Data grouped by known identity (A) and year of analysis (B), sample set: 261 reference standards (2020), 82 QC-samples (2015–2020) and 50 case samples (2015–2020).

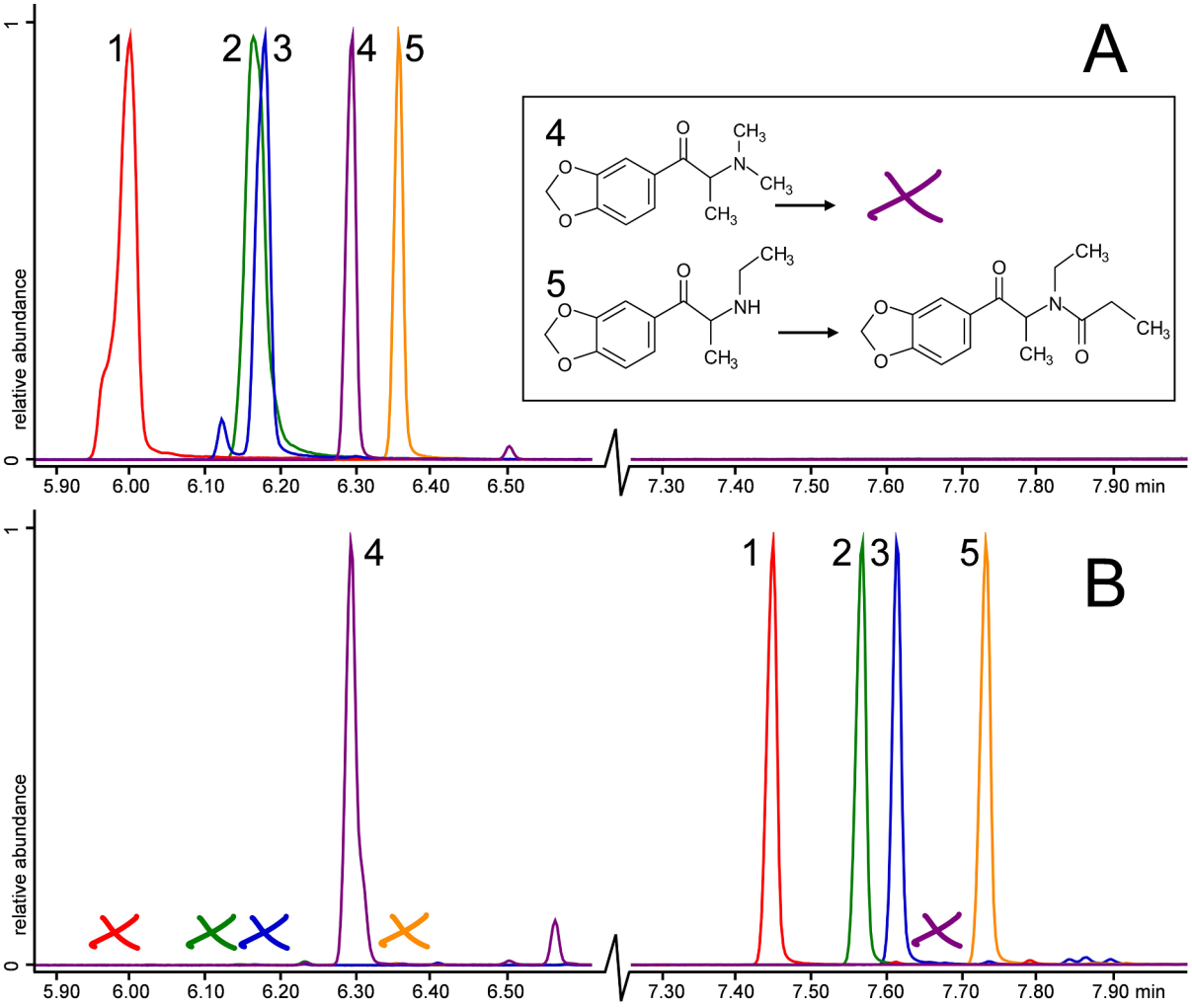

Fig. 8. Total ion chromatograms of methanolic extracts (A) and extracts after propionic anhydride derivatization (B) of 2,3-methylone (1); methylone (2); 2,3-ethylone (3); dimethylone (4) and ethylone (5). Left segment (5.90–6.50 min) shows the underivatized peaks, right segment (7.30–7.90 min) shows the derivatized peaks. Inset explains how the propionyl-derivate of ethylone (4) cannot be formed for the tertiary amine dimethylone (5).

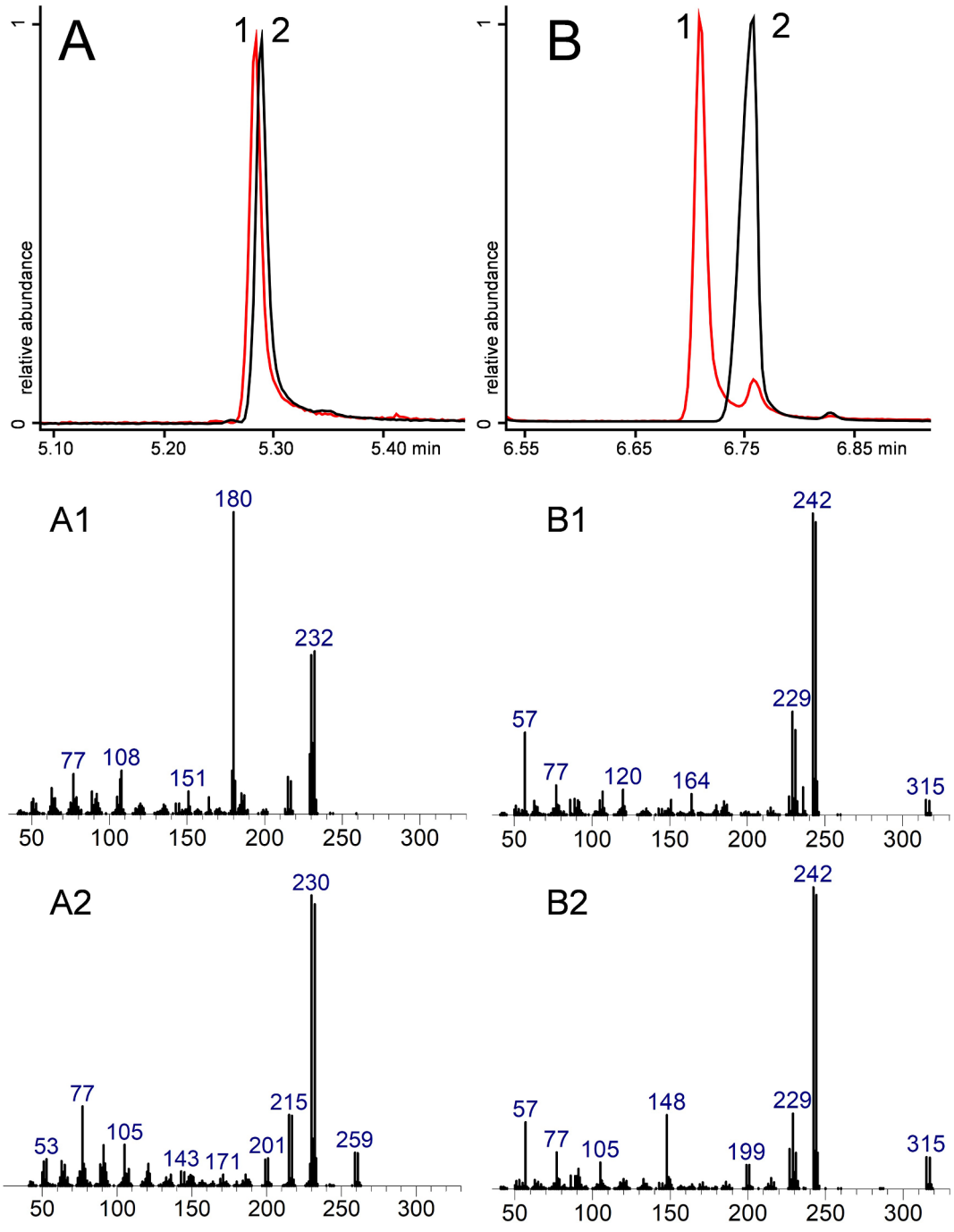

Fig. 9. Comparison of underivatized (A) and PA-derivatized (B) GC–MS selectivity of 2-Br-4,5-DMPEA (1) and 2C-B (2).

boxplot of the observed ion abundance ratios within these replicates demonstrating a clear separation in ranges. Average ratios with standard deviations were 0.95 ± 0.02 for 4-FA-propionyl, 0.50 ± 0.01 for 3-FA-propionyl and 0.32 ± 0.01 for 2-FA-propionyl. The observed minimal ratio for a 4-FA sample was 0.89 whereas the maximum ratio for a 3-FA sample was 0.52. This clearly demonstrates the applicability of this method to distinguish 3-FA from 4-FA. This is especially beneficial since these two FA-isomers are to most difficult to separate in gas chromatography [10]. A comparison of the ion abundance ratios of *m/z* 83, *m/z* 136 and *m/z* 138 towards *m/z* 109 for both native and derivatized FA-isomers is shown in Fig. S13. From this data it is obvious that the *m/z* 136:109 ratio of the derivatized isomers yields the best discrimination with smallest deviations. To a certain degree, all ratios involving *m/z* 136 or *m/z* 138 show ion-specific discrimination for both the native and derivatized forms. However, the *m/z* 138-ion in both forms and the *m/z* 136-ion for native FA has very low relative abundancy of ~4% and ~1% respectively. This can limit the usefulness of this approach at lower concentrations and can lead to increased deviations as is already visible in S13-B. The diagnostic nature of these low abundant ions for native FA is in line with the reported PCA loadings in earlier work [19] where the *m/z* 136 ion was found indicative for 3-FA and *m/z* 138 for 2-FA. Overall, these results demonstrate the potential for an ion-ratio based identification approach. Such ion-ratio based strategies have successfully been applied before for ring-isomeric differentiation [19,39], however, this is a first example focusing on mass spectral differences induced by derivatization. It must be emphasized that although weekly autotunes of the mass spectrometer were applied, no system maintenance was performed and the

vacuum system was not interrupted. Exploratory experiments after a system maintenance where the filament in the ion was replaced showed a ~10% decrease for all three ratios that could be attributed to a more intense *m/z* 109 peak. This demonstrates the need for periodic re-calibration of ion abundance ratios for isomer identification based on the relative intensity for only two ions in the mass spectrum. Strategies to overcome this could include chemometric approaches that consider the overall variation in mass spectra within an isomer class.

3.5.2. Retrospective identification by linear discriminant analysis of mass spectra

Contrary to the FAs, most mass spectra of ring-isomeric NPS did not show major visible differences among individual isomers after derivatization. One such example is given by the MMC-isomers (Fig. S11-DEF) yielding visibly similar PA-derivate spectra. A strategy to exhibit additional selectivity is by applying supervised discriminant analysis, such as LDA on mass spectral data. In this way, increased emphasis is put on minor but consistent differences between mass spectra of different NPS isomers [19,23–25]. The PCA-LDA approach developed in earlier work [19] was applied on mass spectra of the derivatized MMC compounds. A calibration set of 3 × 87 mass spectra of the individual isomers analyzed on 2 different GC–MS systems in a 2-month period was created. PCA and PCA-LDA analysis was performed on both the normalized full spectrum, the *m/z* 60–240 and *m/z* 100–240 part of the spectrum. This small modification to the original approach of *m/z* 100–200 was applied as the molecular weight of the MMC-propionyl molecule is 233 Da and several possibly diagnostic ions are visible in the *m/z* 60–100 region of the mass spectrum while still excluding the most abundant and potential saturated *m/z* 58 base peak. Exploratory PCA analysis revealed substantial separation in 3 classes corresponding to the individual isomers visible in the first 3 PC's that explained over 99% of the variance. Subsequent LDA showed 100% accurate classification with typical log LRs exceeding 100 for the hypothesis describing the correct classification against false-positive classification of the nearest other isomer (assuming equal prior probabilities).

As a retention time-based identification approach on propionyl-derivates of MMC-isomers was routinely put in place in the laboratory since 2014, a substantial amount of historic GC–MS sequences contained a quality control (QC) standard containing 3-MMC and 4-MMC. In addition to this, (inconclusive) mass spectra from propionyl-derivates of MMC-containing case samples and their identity based on the retention time check or FTIR analysis could be retrieved by a database search. This resulted in a set of mass spectra from 50 case samples and 82 QC-standards analyzed between 2015 and 2020 by the Amsterdam Police laboratory. These spectra were projected on and classified by the PCA-LDA model of the 2020 calibration set. Excitingly, this resulted in 100% correct classification even for all 5-year-old mass spectra. The LDA posterior probabilities, the predicted class and log LRs for all case samples and QC samples are shown in Table S1. For most samples, a log LR well above 100 was obtained, but observed minimum log LRs of around 60 still enable very convincing isomer attribution. A notable observation is that no long-term trend could be observed in the LRs over the 5-year period, although several maintenance procedures had taken place, such as filament replacement, ion source cleaning, vacuum system replacement and new column installations. This is also reflected in the lower PC scores following a PCA on all data as visible in the example for PC1 *vs*. PC3 in Fig. 7. Despite sample pre-processing including normalization and exclusion of the most abundant low mass fragments, a linear trend is reflected in the PCA-plots. As discussed in earlier work, a possible explanation are saturation effects in the ion source for the most abundant ions [19], also the linear trend is possibly exacerbated by the small portion of variation described by PCs 2 and 3. When evaluating the PCA loadings, it was evident that the abundant ions *m/z* 91 and *m/z* 114 were major contributors to the group selectivity and thus cannot be excluded. Other low abundant ions that were found to contribute to the differentiation are *m/z* 119 and *m/z* 148. Fig. S14 shows additional PCA-plots grouped by other variables such as instrument and sample type. No specific trends were observed for these features showing the robustness of the EI-MS data. Fig. S15 gives the corresponding loading plots of the PCA-plots shown in Fig. 7.

3.6. Derivatization as aid for structure elucidation

In addition to the above-mentioned strategies for ring-isomer differentiation, the fact that a specific derivative is formed can itself also provide a crucial clue for the identity of an NPS. The imidazole catalyzed acylation reaction is a well-known and reliable route for amine substitution showing a high rate at ambient temperatures. However, the reaction requires an active hydrogen and hence only works for primary and secondary amines. As such, secondary amines (*e.g.* ethylcathinones, ethylamphetamines) can be easily differentiated from their tertiary amine analogues (*e.g.* dimethylcathinones, dimethylamphetamines) by the presence of a derivate peak in the chromatogram. The tertiary amines simply cannot form the derivatization product as the amine-group is fully occupied and lacks an active hydrogen. This information can be especially beneficial to differentiate dimethylated from ethylated NPS isomers that yield identical mass fragments (*i.e. m/z* 72) as major -and often only significant- ion resulting from *alpha*-cleavage. This approach is demonstrated in Fig. 8 where dimethylone is differentiated from its isomers ethylone and 2,3-ethylone by both a resulting underivatized peak and a lacking peak from its PA-derivate. In addition to this effect, the increased peak shape and peak stability after derivatization (as described in Section 3.1) and increased chromatographic selectivity for methylone (peak 2) *vs.* 2,3-ethylone (peak 3) as described in 3.4 are visible. In similar fashion, the presence of FluorodiMethAmphetamine (FdMA) is excluded for the FEA ring-isomers containing case sample shown in Fig. S9 by the simple fact that both chromatographic peaks yield a derivatized equivalent.

In 2019 authorities in The Netherlands reported for the first time the presence of 2-Br-4,5-DMPEA in tablets, an isomer of 2C-B currently uncontrolled by the Dutch narcotic legislation [4]. As 2C-B itself is a controlled substance, additional efforts were required for forensic laboratories to prevent false-positive identifications. Both isomers co-eluted in their underivatized form for routine GC–MS conditions. (Fig. 9-A) Fortunately, a notable difference was visible in the mass spectra as *m/z* 180 was only present in the 2-Br-4,5-DMPEA mass spectrum (Fig. 9-A1). This is in line with an early report on 2C-B isomers attributing this specific fragment to 2C-B positional isomers with the bromine on the *ortho*-position [39]. Derivatization with propionic anhydride provided additional selectivity for this differentiation as the retention times for both derivatized compounds differed sufficiently to yield baseline separation and allowing identification on retention time. (Fig. 9-B) Surprisingly, the diagnostic *m/z* 180 ion completely disappeared by the modified fragmentation mechanism of the PA-derivate demonstrating that derivatization can also limit selectivity and thus must be applied with caution. Despite the lacking diagnostic *m/z* 180 ion, other differences in mass spectrum were introduced after derivatization: the derivatized 2C-B yielded ions *m/z* 148 and *m/z* 199, not visible in derivatized 2-Br-4,5-DMPEA (Fig. 9-B2).

4. Conclusions

Propionyl-derivatized NPS yield substantially improved sample solution stability (over 6 years shelf life) and more robust GC characteristics with increased peak shapes, plate numbers and chromatographic retention. In a routine setting, this enables retention time-based identification of NPS positional isomers with minimal consumption of reference standards. For NPS that are frequently encountered in a forensic setting and where reference materials of all isomeric forms are available this will eliminate the need for more advanced confirmatory techniques. After derivatization, most ring-isomeric NPS still show comparable chromatographic behavior and mass spectra. However, minor

selectivity changes may arise that allow for chromatographic differentiation not possible without derivatization. GC–MS peaks of underivatized 2C-B *vs.* 2-Br-4,5-DMPEA, methylone *vs.* 2,3-ethylone and 3-FMA *vs.* 4-FMA show severe co-elution whereas their PA-derivates are well resolved. In addition, specific differences in mass spectral fragmentation or fragment intensity may be present due to the lower basicity and altered fragmentation routes of the acyl-amides. Mass spectra of 2-, 3- and 4-FA-propionyl derivatives show major intensity differences for their *m/z* 136 fragment and a straight-forward ion abundance ratio check was adequate for mass spectral identification. For the visibly similar mass spectra of 2-, 3- and 4-MMC propionyl derivatives a PCA-LDA model sufficed for unambiguous mass spectral identification with 100% correct classification. The robust and stable mass spectra of the derivates enable retrospective identification of mass spectra several years after actual analysis. Correct isomeric forms were predicted for a set of 132 mass spectra from case material analyzed between 2015 and 2020. Typical log LR-values of around 100 illustrate tremendous discriminative power and long-term robustness. Besides additional chromatographic and mass spectral selectivity, the occurrence of the derivatization-reaction itself also provides helpful information. Differentiation of dimethylated and ethylated isomers, almost indistinguishable by mass spectrum, is obvious as the dimethylated species as tertiary amines are unable to form a derivate due to the absence of an active hydrogen. For case samples containing a mixture of FEA-isomers this aided the structural elucidation by excluding FdMA as potential candidates. In general, this work demonstrates that a fast and straightforward derivatization step in routine GC–MS analysis can eliminate the need for additional confirmatory experiments in NPS isomer identification. This is especially beneficial for routine laboratories equipped only with GC–MS instruments. For such laboratories, the relatively small investment in sample preparation time and cost of derivatization reagents to conduct chemical derivatization prior to GC–MS illicit drug screening could yield a high return on investment.

CRediT authorship contribution statement

Ruben F. Kranenburg: Conceptualization, Methodology, Investigation, Formal analysis, Data curation, Writing - original draft. **Joshka Verduin:** Investigation, Writing - review & editing. **Laura I. Stuyver:** Investigation, Writing - review & editing. **Renee Ridder:** Investigation. **Annique Beek:** Investigation. **Erik Colmsee:** Investigation. **Arian C. Asten:** Supervision, Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.forc.2020.100273.

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