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RESEARCH ARTICLE

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Sensitive screening of synthetic cannabinoids using liquid chromatography quadrupole time-of-flight mass spectrometry after solid phase extraction

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Abstract

Analysis of synthetic cannabinoids still poses a challenge for many institutions due to the number of available substances and the constantly changing drug market. Both new and well-known substances keep appearing and disappearing on the market, making it hard to adapt analytical methods in a timely manner.

In this study, we developed a qualitative screening approach for synthetic cannabinoids and their metabolites by means of liquid chromatography quadrupole time-of-flight mass spectrometry (LC-QTOF-MS). Samples were measured in data-dependent auto-MS/MS mode and identified by fragment spectra, retention time and accurate mass.

Two established solid phase extractions were compared using fortified serum and urine samples. Mixes of 199 synthetic cannabinoids and 110 metabolites were used in 1- and 10-ng/ml concentrations. Up to 93% of synthetic cannabinoids and 74% of metabolites were detected in fortified 1-ng/ml samples.

From February 2018 to October 2020, we analyzed 1492 cases, of which 73 cases were positive for synthetic cannabinoids or metabolites. 5F-MDMB-PICA, 4F-MDMB-BINACA, MDMB-4en-PINACA, and 4F-MDMB-BICA were most frequently detected. Hydrolysis metabolites were detected in many blood samples, providing a longer detection window. Quantification was conducted via liquid chromatography triple quadrupole mass spectrometry after liquid–liquid extraction. Concentrations were mostly close to 1 ng/ml in blood samples. LC-QTOF-MS was able to detect substances above trace quantities (< 0.1 ng/ml) in most cases, therefore fulfilling its purpose as a sensitive general screening approach. Expansion of the screening library was uncomplicated and enables future additions for up to thousands of targets.

KEYWORDS
forensic blood analysis, high-resolution mass spectrometry, screening, solid phase extraction, synthetic cannabinoids

1 | INTRODUCTION

The number of new psychoactive substances (NPS) reported by the European Monitoring Centre for Drugs and Drug Addiction (EMCDDA) has reached a total of 790 at the end of 2019, with about 50 new substances appearing annually since 2017.[1] Synthetic cannabinoids (SCs) are, besides cathinones, the largest subclass of NPS.[1] Almost 200 SCs are currently known to the EMCDDA.[2] Fortunately, the amount of new SCs appearing per year has dropped from 20–30 (2011–2015) to approximately 10 per year (2016–2019),[1] presumably due to introduction of new national laws such as the New Psychoactive Substances Law (Neue Psychoaktive Stoffe Gesetz, NpSG) in Germany in 2016.[3] Nevertheless, numbers of SCs in circulation remain high: since 2015, about 100 different SCs were detected in forensic cases each year in Europe.[1] In the ever-changing market, substances appear and disappear regularly, making it hard to adapt methods to focus on currently relevant SCs. Low prevalence of SC consumption (0.3% to 0.6%)[1] and consequent low case numbers can result in poor cost–benefit ratios of dedicated analysis methods. Nonetheless, forensic institutions cannot simply neglect rare analytes and must solve this problem one way or another.

The key to successful chromatographic mass spectrometric identification of SCs is knowledge about their mass, retention time and fragmentation pattern. Getting these data can be challenging, as purchasable reference standards are often not available in the first period after the discovery of a new substance. Early data about metabolites can be gathered by various metabolism experiments[4–6] or by chemical hydrolysis of parent compounds,[7] if their structure has the necessary moiety.[8] However, having access to chemically pure substances usually yields the best results in terms of clean spectra acquisition, reliable retention times and basic validation data.

Usually, SCs are detected by means of liquid chromatography (LC) coupled with tandem mass spectrometry. Various methods with different setups have been published, like multiple reaction monitoring (MRM) approaches via triple-quadrupole mass spectrometry (QqQ-MS),[9–11] detection by means of ion trap mass spectrometry[9] or quadrupole time-of-flight mass spectrometry (QTOF-MS).[10,11] Each approach has advantages, such as high sensitivity and throughput for MRM methods (QqQ-MS) or comprehensive screening and retrospective analysis for QTOF-MS. Also, numerous extraction procedures are described for blood and urine samples, primarily liquid–liquid extraction (LLE)[12–14] and solid phase extraction (SPE).[13,15,16]

The aim of this study was to develop a qualitative LC-QTOF-MS screening method to cover SCs and their metabolites. With the use of fortified serum and urine samples, two routinely used solid phase extractions were evaluated in terms of suitability and resulting limits of identification (LOIs). Furthermore, we discussed chromatographic and mass spectrometric results as well as options to add new spectrum library entries, including a crowd-sourced consensus library. The results of 73 authentic samples gathered in Rhineland-Palatinate (Germany) were evaluated with regard to consumed SCs, temporal occurrence and blood concentration levels. LC-QTOF-MS analysis results were compared with those of a validated quantitative LC-QqQ-MS MRM method to verify the results. In particular, the differences between the two methods in terms of SC findings were discussed.

2 | EXPERIMENTAL

2.1 | Materials and reagents

Internal standards (ISTD) utilized were protriptyline (LGC Standards, Wesel, Germany), fludiazepam and JWH-018-D11 (Lipomed, Arlesheim, Switzerland). Synthetic cannabinoid reference substances used for retention time (RT) determination and spectra acquisition were kindly provided by the Institute of Forensic Medicine, University Medical Center in Freiburg. Approximately 80% of all substances were obtained from Cayman Chemical (Ann Arbor, MI, USA) as reference standards, while others were bought as research chemicals or have been provided by other forensic laboratories. Substances without analysis certificates had their purity determined by gas chromatography mass spectrometry (GC–MS), LC-QTOF-MS and nuclear magnetic resonance (NMR) spectroscopy. β-glucuronidase/arylsulfatase from *Helix pomatia* (Roche, Mannheim, Germany) was used for glucuronide and sulfate cleavage in urine samples. SPE was conducted with either Bond Elut Certify (C8 + SCX [strong cation exchanger], 300 mg; Agilent, Waldbronn, Germany) or BAKERBOND spe™ C18 (500 mg, 3 ml, polypropylene; VWR International GmbH, Darmstadt, Germany).

2.2 | Methods

Sixty-five blood and two urine samples were collected and sent by the police of Rhineland-Palatinate (Germany) to be tested for drugs or medication. Blood samples were stored at 4°C until sera were obtained by centrifugation. Sera were then spiked to 5 g/L sodium fluoride (NaF) and stored at −20°C. Blood samples collected in NaF collection tubes were mostly hemolyzed by the time they arrived in our institute. After centrifugation, the resulting supernatants were stored at −20°C. Urine samples were stored at −20°C with no addition of preservatives. When no frozen serum was left at the time of this study, blood samples stored at 4°C were used instead, despite their advanced hemolysis (further referred to as *whole blood [hemolyzed]*).

Bodily fluids of seven in-house autopsy cases (seven femoral blood samples; six urine samples) were also subject to testing. Investigated urine and femoral blood samples were stored at −20°C with no addition of preservatives.

Samples were subject to a general screening by LC-QTOF-MS or specific SC analysis by LC-QqQ-MS. Findings of either method were then checked against the other method.

2.2.1 | Qualitative screening via LC-QTOF-MS

Sample preparation

One-millimeter sample volume was used for SPE. Urine samples were incubated with 50 µl β-glucuronidase/arylsulfatase for 2 hr at 37°C. Afterward, all samples were fortified with a mixture of ISTD (50 µl, 0.1 µg/ml each; fludiazepam and protriptyline for extraction; JWH-018-D11 for retention time offset) and vortexed briefly.

Extraction

Two different solid phase extractions were used, furthermore specified as *SPE A* and *SPE B*. Details are displayed in Table 1.

Prepared samples were diluted accordingly, vortexed briefly and centrifuged for 10 min at 3000 rpm. After conditioning the SPE columns, the supernatant was added. Columns were washed with the specified washing mixtures and centrifuged for 10 min at 3000 rpm to leave the columns dry. Elution occurred in two subsequent steps. Both eluates were collected separately and combined afterward. The resulting combined extracts were then evaporated until dryness, transferred to microliter vials using 300-µl methanol and evaporated again. The samples were then reconstituted in 50 µl of methanol/acetonitrile/water (3:3:2) and vortexed briefly.

Chromatography

Chromatographic separation was achieved via liquid chromatography (Agilent 1260 Infinity) using an EC-C18 Poroshell (2.1 mm × 100 mm, particle size 2.7 µm; Agilent, Waldbronn, Germany) column with an equivalent guard column (2.1 mm × 5 mm, particle size 2.7 µm). Eluents used were water with 2mM ammonium acetate (Solvent A) and methanol (Solvent B). Total runtime was 24 min using the following gradient: 0 min 10% B, 8 min 50% B, 20 min 100% B, 24 min 100% B, re-equilibration for 6 min with 10% B. Column temperature was 50°C; flow rate 0.4 ml/min; injection volume 5.0 µl.

High-resolution mass spectrometry

Ion source. The extracts were analyzed with a coupled accurate-mass quadrupole time-of-flight mass spectrometer (6530 QTOF-MS Agilent Technologies) using electrospray ionization (ESI) in positive mode. The source conditions were as follows: gas temperature 320°C; nebulizer pressure 35 psi; drying gas flow 8 L/min; sheath gas temperature 380°C; sheath gas flow 11 L/min; VCAP 3000 V.

Acquisition of spectra and retention times. Most spectra and retention times were acquired by measuring methanolic reference standards at concentrations of 1 µg/ml. Spectra were recorded at five collision energies ranging from 10 to 40 eV (target 50,000 counts/spectrum). A suitable collision energy (CE) was chosen for each compound to allow unambiguous distinction from other analytes. The use of multiple CEs reduced dwell time to the disadvantage of spectra quality and was therefore rejected.

SCs which degrade to their carboxylic acid metabolites in vivo[8] could be produced by in vitro alkaline hydrolysis of the parent compound, for example, 5F-MDMB-PICA or 4F-MDMB-BINACA.[7] Accordingly, 30 µL reference standard (20 µg/ml) were incubated for 5 min at room temperature using 30 µL of 1 M potassium hydroxide solution. After neutralization with 30 µl of 1-M hydrochloric acid, the samples were analyzed as described above.

In cases that involved novel SCs, spectra and RTs were gained from authentic samples to bridge the time until commercial reference standards were available. This could only be done if the presence of any yet unknown SC was already confirmed otherwise: through statements of the accused, substance seizures by the police or analysis by our assisting laboratory (see Section 2.2.3). This procedure was used for 4F-MDMB-BICA, 4F-MDMB BICA (hydr. metab.), 5F-AB-P7AICA M13 (hydr. metab.), Cumyl-CBMICA and MDMB-4en-PINACA (hydr. metab.).

TABLE 1 Comparison of applied SPE methods

	SPE A Bond Elut certify, C8 + SCX	SPE B Bakerbond, C18
Sample dilution	5-ml phosphate buffer (pH 6)	4-ml water
Conditioning	2 × 3-ml methanol 2 × 2-ml water	1 × 3-ml methanol/diethylamine (994:6) 1 × 3-ml 0.1 M $KHCO_3$ in water/acetonitrile (90:10)
	Sample addition	Sample addition
Washing	2 × 2-ml water 2 × 2-ml water/methanol (80:20) 1 × 1-ml 0.1 M acetic acid in water	3 × 1-ml water/acetonitrile (80:20)
Centrifugation	10 min at 3000 rpm	10 min at 3000 rpm
Elution	3 × 1-ml dichlormethane/acetone (1:1) 3 × 1-ml dichlormethane/2-propanol/ammonia water 32% (40:10:2)	2 × 1.2-ml acetonitrile/methanol/water (3:3:2) 2 × 1.2-ml (methanol/diethylamine (994:6)
Vaporization	40 °C and nitrogen	95 °C and nitrogen
Reconstitution	50 µl acetonitrile/methanol/water (3:3:2)	50 µL acetonitrile/methanol/water (3:3:2)

Abbreviation: SPE, solid phase extraction.

Currently, the method includes 199 synthetic cannabinoids and 130 metabolites of 56 different SCs. Tables S7 and S8 contain all substances with their corresponding m/z values, RTs, CEs, and LOIs.

Acquisition method. QTOF-MS analysis was operated in auto-MS/MS mode, a data-dependent acquisition mode switching between MS1 (3 spectra/s, 100–1300 Da) and MS2 (5 spectra/s, 40–1300 Da) cycles. After each MS1 cycle, the quadrupole selects precursor ions of high abundance for fragmentation (*isolation width* ± 2 Da). A *preferred list* was used to focus the precursor selection algorithm on desired masses prior to ions of high abundance. All investigated SCs and metabolites were added to this list with the following conditions: maximum mass error of ± 20 ppm within ± 0.5 min of their expected RT and fragmentation at their individually specified CEs (Tables S7 and S8). Total cycle time was 1.033 s, with a maximum of three selected precursors per cycle (target 10000 counts/spectrum; active exclusion after 1 spectrum for 0.09 min, precursor threshold 1000 counts, precursor exclusion range 100–125 and 600–1300 m/z).

Data analysis. Raw data was processed with *Agilent Qualitative Analysis B.10.0* to extract compounds using the *find by molecular feature* compound mining algorithm. All compounds were then identified by library spectra comparison via *Agilent Personal Compound Database and Libraries* (*PCDL*) and given a score from 0 to 100. Only *reverse score* was used for identification, which means when all fragments of a given library compound entry were present in the acquired spectrum with correct abundance ratios, the score would be 100. Additionally, mass error (Δ ppm) and retention time (Δ min) were used to rate all findings. When offered multiple matches by the software, each finding was evaluated manually to approve their correctness by verifying all three parameters. As algorithms calculated the scores, no absolute cut-offs were used to prevent false negative results.

2.2.2 | Extraction suitability and LOI estimation

Blank serum or urine samples were fortified to 10 and 1 ng/ml, respectively, with 195 SCs or 110 metabolites to estimate approximate LOIs and extraction suitability. SCs were divided into seven mixes and metabolites into five mixes to avoid interference of substances with similar RTs (±0.5 min) and masses (±2 Da). As the quadrupole isolation width was set to ±2 Da, co-eluting substances inside this range would result in mixed fragment ion spectra.

Extraction was assessed as suitable by evaluation of the 10-ng/ml mixtures with the following criteria: signal-noise-ratio > 3; mass error < 20 ppm; RT offset < 0.2 min. Additionally, signal heights were compared to methanolic reference standards and post-extraction-spiked samples to differ between poor ionization and insufficient extraction. Signal heights across all three samples had to be roughly in the same scale (decimal power).

LOIs were estimated by evaluating findings of both 10- and 1-ng/ml mixtures. The acquisition of at least one MS2 spectrum for each compound in each run was mandatory to fulfill the identification criteria. Additional criteria were as stated above: signal-noise-ratio > 3; mass error < 20 ppm; RT offset < 0.2 min. Later, more accurate approximations of LOIs were made by evaluating authentic samples with known blood concentrations.

2.2.3 | Quantitative analysis

Quantitative analysis was conducted by the Institute of Forensic Medicine, University Medical Center in Freiburg by means of liquid chromatography tandem mass spectrometry (LC–MS/MS) after liquid–liquid extraction. Kneisel et al.[17] described the basics of the serum analysis in 2012. Mogler[18] described the metabolite analysis in urine in 2019. The portfolio of targets is constantly adapted for current SCs and their metabolites. Most synthetic cannabinoids were calibrated down to 0.1 ng/ml, which also represented the limit of quantification (LOQ).

3 | RESULTS AND DISCUSSION

3.1 | Method evaluation

Fortified samples were extracted once and measured twice. Extraction was successful when both fludiazepam (first eluate; acidic) and protriptyline (second eluate; basic) were detected. Maximum allowed relative retention time deviation was ± 0.2 min. Library scores were averaged for each substance and concentration. Results are summarized in Table 2.

3.1.1 | Extraction

For synthetic cannabinoids, SPE B delivered the best results in terms of number of identified compounds in 1-ng/ml samples. Average library scores were 6% lower in 1-ng/ml samples. Across both extraction groups, average mass error was 1.3 ppm (SD = 2.9 ppm; $n = 752$) in 10-ng/ml samples and 4.3 ppm (SD = 7.0 ppm; $n = 690$) in 1-ng/ml samples.

For SC metabolites, SPE A surpassed SPE B in total number of identified compounds at both concentrations (92% vs. 73% and 74% vs. 66%). Average library score was 2% lower in 1-ng/ml samples. Across all three groups, average mass error was 1.7 ppm (SD = 5.9 ppm; $n = 362$) in 10-ng/ml samples and 5.2 ppm (SD = 7.1 ppm; $n = 308$) in 1-ng/ml samples. The difference between both extractions could be explained by analyzing the washing solution of SPE B (3 × 1-ml water/acetonitrile [80:20]). Analysis showed that 24 of 30 missing metabolites were detected in the washing solution. Most missing metabolites were carboxylic acids and could not be retained sufficiently on the SPE columns due to their higher polarity. It can be concluded that SPE A performed better than SPE B for metabolites and was able to extract the more polar, acidic compounds.

TABLE 2 Analysis results of fortified serum and urine samples

		10-ng/ml samples		1-ng/ml samples	
		Identified	ø library score	**Identified**	ø library score
Synthetic cannabinoids	SPE A	98% (190/194)	94% (62%–99%)	85% (164/194)	88% (54%–99%)
	SPE B	96% (186/194)	95% (51%–99%)	93% (181/194)	89% (47%–99%)
SC metabolites	SPE A	92% (101/110)	96% (52%–99%)	74% (81/110)	94% (74%–99%)
	SPE B	73% (80/110)	96% (55%–100%)	66% (73/110)	94% (71%–99%)

Abbreviations: SC, synthetic cannabinoid; SPE, solid phase extraction.

The overall extraction results showed that SPE is a suitable extraction method besides LLE, though the extraction procedure should be optimized if only SCs and SC metabolites are to be investigated to prevent unnecessary substance loss. Of our routine solid phase extractions, SPE B should be used for parent compounds and SPE A for SC metabolites. For detailed information on undetected substances, see Tables S7 and S8.

3.1.2 | Chromatography

The described chromatography using an EC-C18 Poroshell column was able to cover all investigated SCs and SC metabolites in the given run time of 24 min. Elution of parent compounds ranged from 12.5 to 23.2 min and metabolites eluted from 4.1 to 19.9 min. A better distribution of parent compounds over the entire run time was achieved using a phenyl-hexyl Poroshell column (2.1 mm × 150 mm, particle size 2.7 μm; Agilent, Waldbronn, Germany) with elution ranging from 4.5 to 21.2 min. However, as this screening is to be expanded for other substance classes, we stuck to a broad chromatography using C18 columns.

Isomeric substances with close retention time and identical fragmentation patterns cannot be distinguished with the current chromatography. This mainly affects substances that differ only in their differently positioned side chain on aromatic structures, like AB-FUBINACA and its 2-fluorbenzyl and 3-fluorbenzyl isomerics. Other examples are JWH-210 (RT 20.12) and JWH-234 (RT 20.04), whose RTs are so close that differentiation is only possible with RT comparison of control samples. This issue could be solved by chromatographic changes: alteration of run time, gradients, solvents or chromatographic columns (e.g., phenyl-hexyl columns).

3.1.3 | Data acquisition and analysis

Auto-MS/MS mode is an untargeted, data-dependent acquisition mode, which primarily selects precursor ions for fragmentation based on abundance. The use of a *preferred list* is useful when confronting compounds of low abundance, which is typical for synthetic cannabinoids due to low blood concentrations around 1 ng/ml. As a result, those analytes can be detected more reliably, while other analytes can still be detected. The use of a *preferred list* does not impede its general operation principle as long as retention time and mass error windows are kept narrow.

Another advantage of untargeted screening is that it is unnecessary to reduce the total amount of targets. As auto-MS/MS mode only selects and fragments present precursors, there is no loss of sensitivity by expanding the spectral library or *preferred list* per se. Therefore, all parent compounds and metabolites plus any other desired targets can be included in one acquisition method.

Retrospective analysis in data-dependent auto-MS/MS mode can be used to identify substances that were not included in the spectral library at the initial time of investigation. This is restricted by the efficiency of the precursor selection algorithm, as substances cannot be identified reliably without fragmentation.[10] Data-independent acquisition (DIA) allows for more reliable retrospective analysis, but our approaches suffered from insufficient fragment ion abundances and strong noise from matrix at low concentrated spiked samples. We therefore prioritized low LOIs and clean spectra above comprehensive retrospective investigations.

Average spectra quality was well above 80%. Library matches should be evaluated manually, though scores over 80% are very reliable if mass error and retention time are within tolerated ranges. Average mass error was below 5 ppm in all clusters, whereas individual outliers could exceed the soft limit of 20 ppm (stated in *preferred list*). This might be attributed to co-eluting substances within the quadrupole resolution of ±2 Da, resulting in good RT and library score, but higher mass error.

Isomeric substances and compounds of similar mass (±2 Da quadrupole isolation width) cannot be detected simultaneously, if peaks are not properly separated. This can result in mixed fragment spectra and consequently lower library scores. By evaluating possible matches, this can be noticed when comparing the acquired fragment spectra with those of the suggested matches. This knowledge is relevant, as in 73% of all cases multiple SCs have been detected simultaneously. However, due to the amount of available SCs on the market, this has not occurred in any authentic cases yet.

3.1.4 | Sensitivity and limits of identification

LC-QTOF-MS can be run in various acquisition modes, each with its advantages and disadvantages regarding sensitivity and specificity. Manufacturers offer individual targeted/untargeted and data-

dependent/data-independent acquisition modes, complicating head-to-head sensitivity comparisons. Gundersen et al.[10] evaluated LOIs for six SCs, ranging from 2–20 ng/ml, though LLE was conducted with less sample volume (200 µl) and injection volume (2 µl). In another approach, LOIs between 0.04 and 17.5 ng/ml were determined for 35 SC metabolites in urine, extracted by SPE in targeted MS/MS mode.[19] Mollerup et al.[20] pursued a combined targeted and untargeted approach, with LOIs from 1–100 (targeted) and 20–100 ng/ml (untargeted). However, both approaches follow a different workflow for identification. Sundström et al.[21] developed a method that could be compared to ours, with SC metabolite LOIs from 0.2–60 ng/ml, with half of them below 1 ng/ml. A mixed-mode SPE (C4, cation exchange) was used for extraction, with similar sensitivity results, depending on the individual analyte. Other methods run in DIA mode either provided no LOI information or found their LOIs well above typical SC blood concentration levels, making it hard to compare sensitivity.[22–24] Our screening was developed to be as sensitive as possible, and exceeded the mentioned methods in terms of sensitivity for most substances while also covering much more compounds. Further narrowing down the LOI below 1 ng/ml can be useful, but not yet considered necessary for our purposes.

3.1.5 | Library expansion

As described in Section 2.2.1, most spectra and RTs were obtained using commercial reference standards. Some hydrolysis metabolites were obtained by alkaline hydrolysis of parent compounds, and few were extracted from authentic cases. For the latter, which were measured at only one CE, the resulting spectrum had to be verified with current literature to eliminate false fragments. Therefore, this procedure has strong limitations in its application, though repeated findings decrease spectrum and RT deviations.

Fragment ions can be predicted a lot easier once the molecular structure is known, but RT prediction can be difficult if the applied chromatography utilizes different columns and solvents. However, as many SCs are structurally related to each other, retention time can often be estimated roughly. For example, 4F-MDMB-BINACA (RT 16.08 min) and 4F-MDMB-BICA (RT 15.66 min) only have one structural difference in their core structure, resulting in similar RTs. Until further verification, the RT detection window in the *preferred list* could be widened or removed entirely.

Further expansion of the spectra library is generally uncomplicated, as the untargeted auto-MS/MS acquisition mode is independent of the amount of substances present in the library. As the used acquisition mode is data-dependent (DDA), retrospective analysis is—as stated above—not possible in every case. This makes updating the library with spectra of new substances important and necessary.

HighResNPS consensus library

Other ways to expand a spectrum library would be crowd-sourced projects, such as www.highresnps.com, which offer information on fragment ions and retention times of established methods. Additionally, new prediction models for both RTs and NPS fragment spectra are being developed. Once refined, such sources can ease the entrance into NPS analytics by saving both time and money. Davidsen, Mardal et al.[25] demonstrated how the HighResNPS consensus library can be used for high-resolution mass spectrometry screening approaches.

After importing our library to HighResNPS, a library was created, containing predicted retention times and simplified fragment spectra (arbitrary abundance ratios and reduced number of fragments) for each compound. We then compared the HighResNPS library (January 2021 [Mainz]) with our own created library by using the exact same data analysis method (see Section 2.2.1) on the raw data of fortified synthetic cannabinoid samples extracted with SPE B. The results are summarized in Table 3. The HighResNPS library missed three substances in 10-ng/ml samples and nine substances in 1-ng/ml samples which were detected by our own library. Average library scores were 13% lower in 10-ng/ml samples and 6% lower in 1-ng/ml samples, which can be primarily attributed to the arbitrary abundance ratios of consensus spectra (example in Figure 1). Predicted retention times were very accurate, deviating on average by only −0.004 min (−0.166 to 0.093 min) in 10-ng/ml samples and −0.012 min (−0.072 to 0.127 min) in 1-ng/ml samples. Identification without retention times might not be possible for many compounds with similar fragment spectra due to the reduced amount of fragments and missing abundance ratios. However, these already good results can surely be improved in the future by enabling fragment spectra prediction for specific collision energies.

3.2 | Authentic case results

From February 2018 to October 2020, 20,850 cases were subject to forensic toxicological analysis. Thereof, 1492 cases (7.16%) were demanded to be analyzed for SCs, of which 73 were positive. SC analysis were ordered by police without further hints of consumption. Therefore, the total number of cases is rather arbitrary and the increasing percentage of positive cases from 2018 to 2020 could be coincidental. Investigated SC cases have increased since this study began and the case numbers of 2020 are expected to exceed those of the previous year again. The percentage of positive cases has increased from 3.55% in 2018 to currently 6.46% in 2020. Table 4 summarizes all case numbers.

No cases were excluded to provide the complete set of results. The LC-QTOF-MS screening library was updated with all targets in May 2019 (from case 22). Samples of previous cases were then extracted and measured. Afterwards, routine LC-QTOF-MS screening was able to detect SCs and samples were measured in a timely manner. This partly explains the percentage increase of positive cases, as SCs were now also detected in cases where SC analysis was not ordered explicitly. In 13 of 73 positive cases (18%) SCs were only detected by accident during routine screening.

Initially, SPE A was used for authentic samples analogously to our routine cannabinoid extraction. However, fortified sample analysis

TABLE 3 Comparison of HighResNPS library with own created spectral library

Synthetic cannabinoids (SPE B)	10-ng/ml samples		1-ng/ml samples	
	Identified	ø library score	Identified	ø library score
Own created library	96% (186/194)	95% (51%–99%)	93% (181/194)	89% (47%–99%)
HighResNPS library	94% (183/194)	82% (46%–100%)	89% (172/194)	83% (52%–100%)

Abbreviation: SPE, solid phase extraction.

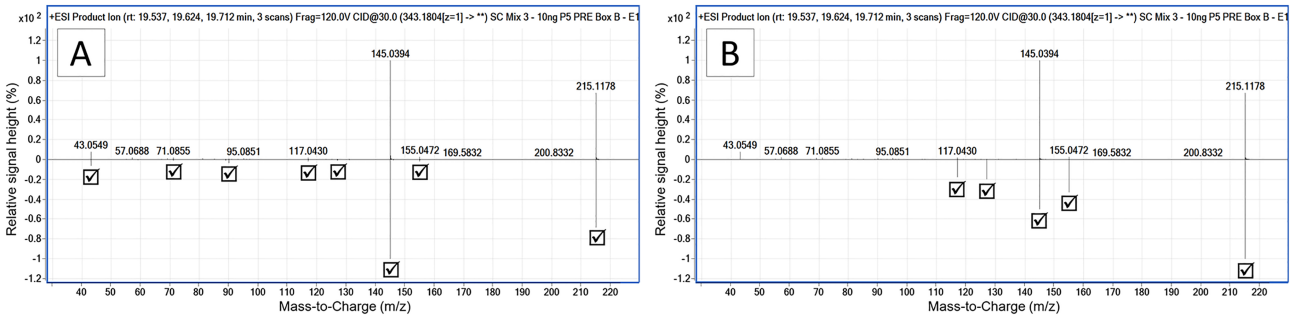

FIGURE 1 Comparison of the acquired MS2 spectrum of THJ-018 (A/B top)with self-made reference standard library (A bottom) and HighResNPS consensus library (B, bottom) at 30 eV CE. Matching fragments are marked for better visibility. The nearly ideal abundance ratios of fragment ions in (A) result in a score of 96.74. In (B) all library fragments are present in the acquired spectrum, but abundance ratios are far off, resulting in a score of 67.91 [Colour figure can be viewed at wileyonlinelibrary.com]

TABLE 4 Case numbers and positive SC cases per year during the time of investigation (02/2018 to 10/2020)

	Total cases	SC cases	SC cases/total cases	Positive cases	Pos. Cases/SC cases	Pos. Cases/total cases
2018	6650	422	6.35%	15	3.55%	0.23%
2019	7640	559	7.32%	25	4.47%	0.33%
2020	6560	511	7.79%	33	6.46%	0.50%
Total	20850	1492	7.16%	73	4.89%	0.35%

Abbreviation: SC, synthetic cannabinoid.

revealed SPE B to be superior for identification in lower concentration levels (see Section 3.1). As SC concentrations in authentic samples were usually close to 1 ng/ml (Tables 5 and 6), SPE B was used from then on for blood samples.

Quantification was conducted for parent compounds in blood samples when commercial reference standards were available. In urine samples, metabolites were neither quantified nor specified, though at least two metabolites of a specific SC had to be present for proof. Hydrolysis metabolites (hydr. metab.) were distinguished from other metabolites for comparison purposes. Details of all examined cases are presented in Table 6. Results of 12 cases (11, 12, 16, 21–23, 25, 26, and 31–34) have been previously published in a case report on 5F-MDMB-PICA.[7]

3.2.1 | Synthetic cannabinoid findings

All synthetic cannabinoids detected in authentic cases were evaluated regarding their times of occurrence, total qualitative and quantitative findings, concentration ranges and median values as well as the lowest authentic concentration of each substance detected by LC-QTOF-MS after SPE (Table 5). Median concentrations were calculated with all data, including trace quantities (counted as 0.1 ng/ml) and outliers.

In 2018, there were 15 cases with 21 individual SC findings, dominated by 5F-ADB (five cases) and 5F-Cumyl-PEGACLONE (3 cases). In 2019, there were 25 cases with 44 individual SC findings, led by 4F-MDMB-BINACA (13 cases) and 5F-MDMB-PICA (11 cases). In 2020, up to and including October, there were 33 cases with 74 individual SC findings: 5F-MDMB-PICA (18 cases) and MDMB-4en-PINACA (17 cases) were detected most frequently, followed by 4F-MDMB-BICA (11 cases) and 4F-MDMB-BINACA (nine cases). The increase in SC cases in the second and third quarter of 2020 might be explained by effects of the current corona virus pandemic. Cannabis resin and herbal cannabis retail prices increased, while supply chains and logistics of drug trafficking in Europe were restricted because of social distancing measures and border controls.[26] Additionally, online drug distribution increased,[26] where easy access to NPS comes into play.

TABLE 5 Overview of 73 authentic SC findings: Occurrence per quarter, total findings, concentration ranges, concentration median and lowest concentration detected by LC-QTOF-MS after SPE

	2018				2019				2020								
Substance	Q1[c]	Q2	Q3	Q4	Q1	Q2	Q3	Q4	Q1	Q2	Q3	Q4[c]	Total findings$_a$ [n]	Quantifications$_b$ [n]	Range [ng/ml]	Median [ng/ml]	Lowest conc. Detected by LC-QTOF [ng/ml]
5F-MDMB-PICA				2	2	6	2	1	1	5	11	1	31	18	< 0.1–52	0.45	< 0.1
4F-MDMB-BINACA					2	7	2	2	2	3	4		22	17	< 0.1–42	0.41	0.19
MDMB-4en-PINACA							1			5	8	4	18	12	< 0.1–1.0	0.21	< 0.1
4F-MDMB-BICA										5	4	2	11	–	–	–	–
5F-ADB		2	1	2				3					8	7	< 0.1–0.3	< 0.1	0.15
JWH-122			1		1			1		1	1		5	5	< 0.1–0.3	< 0.1	0.2
JWH-210					1					1	1	1	4	4	< 0.1–0.98	0.23	< 0.1
Cumyl-4CN-BINACA		1							2		1		4	4	< 0.1–56	3.7	0.29
5F-Cumyl-PEGACLONE			2	1	1								4	4	< 0.1–0.21	0.13	–
5F-MDMB-P7AICA					2			1					3	3	< 0.1–7.5	2.32	2.32
5F-AB-P7AICA								1		1			2	–	–	–	–
Cumyl-CBMICA							1			1			2	1	12	–	12
Cumyl-CBMEGALONE										2			2	–	–	–	–
5F-Cumyl-P7AICA				1				1					2	2	< 0.1–3.4	1.75	3.4
Cumyl-PEGACLONE		1						1					2	1	0.26	–	–
MDMB-FUBINACA	2												2	2	< 0.1–2.4	1.25	2.4
AB-FUB7AICA										1			1	1	0.23	–	–
ADB-BINACA										1			1	–	–	–	–
Cumyl-CBMINACA										1			1	–	–	–	–
FUB-144										1			1	–	–	–	–
JWH-018										1			1	1	0.14	–	0.14
JWH-073										1			1	1	< 0.1	–	–
JWH-081										1			1	1	< 0.1	–	–
5F-ADB-PINACA								1					1	–	–	–	–
5F-AMB								1					1	–	–	–	–

TABLE 5 (Continued)

Substance	2018				2019				2020				Total findings$_a$ [n]	Quantifications$_b$ [n]	Range [ng/ml]	Median [ng/ml]	Lowest conc. Detected by LC-QTOF [ng/ml]
	Q1[c]	Q2	Q3	Q4	Q1	Q2	Q3	Q4	Q1	Q2	Q3	Q4[c]					
AMB-CHMICA								1					1	1	0.12	–	–
FUB-AMB/EMB-FUBINACA								1					1	–	–	–	–
5F-Cumyl-PINACA					1								1	1	0.29	–	–
5F-Cumyl-PICA				1									1	1	< 0.1	–	–
NE-CHMIMO			1										1	1	< 0.1	–	–
ADB-FUBINACA		1											1	1	0.51	–	–
AB-FUBINACA	1												1	1	< 0.1	–	–
FUB-AMB	1												1	1	< 0.1	–	–
Findings/quarter[a] [n]	4	5	5	7	10	13	6	15	5	31	30	8	139	91			
Pos. cases/quarter [n]	2	4	4	5	6	11	4	4	4	10	15	4					

Note: Sorted by total findings and time of occurrence.

Abbreviations: LC-QTOF-MS, liquid chromatography quadrupole time-of-flight mass spectrometry; SPE, solid phase extraction.

[a]Parent substance or metabolite detected.

[b]Only parent substances in blood samples were quantified.

[c]Time of investigation: February 2018–October 2020.

TABLE 6 Overview of authentic cases analyzed by both LC-QTOF-MS and LC-QqQ-MS

Case no. /age/sex	Time of event	Sample type[a]	Sample volume [µL][a]	LC-QTOF-MS findings	LC-QqQ-MS findings[b]	Concentration [ng/ml][a]	Extraction[a]
1/19 y/male	02/2018	Whole blood (hemolyzed)	500	FUB-AMB hydr. metab.	FUB-AMB MDMB-FUBINACA	<0.1 <0.1	SPE A
2/29 y/male	03/2018	NaF blood	1000	MDMB-FUBINACA (+ hydr. metab.) AB-FUBINACA hydr. metab.	MDMB-FUBINACA AB-FUBINACA	2.4 <0.1	SPE A
3/35 y/male	04/2018	Whole blood (hem.)	700	–	ADB-FUBINACA Cumyl-PEGACLONE	0.51 0.26	SPE A
4/23 y/male	04/2018	Serum	700	–	Cumyl-4CN-BINACA	<0.1	SPE A
5/27 y/male	05/2018	NaF blood	500	–	5F-ADB	<0.1	SPE A
6/35 y/male	06/2018	Whole blood (hem.)	150	–	5F-ADB	<0.1	SPE A
7/32 y/male	08/2018	Whole blood (hem.)	1000	–	JWH-122	<0.1	SPE A
8/25 y/male	08/2018	Serum	700	–	5F-Cumyl- PEGACLONE NE-CHMIMO	<0.1 <0.1	SPE A
9/53 y/male	08/2018	Serum	650	–	5F-ADB	<0.1	SPE A
10/34 y/female	09/2018	Whole blood (hem.)	40	–	5F-Cumyl- PEGACLONE	0.21	SPE A
11/29 y/male	11/2018	Whole blood (hem.)	400	5F-MDMB-PICA (+ hydr. metab.)	5F-MDMB-PICA	approx. 16	SPE A
12/33 y/female	12/2018	Femoral blood	1000	5F-MDMB-PICA (+ hydr. metab.)	5F-MDMB-PICA 5F-Cumyl-P7AICA 5F-Cumyl-PICA	1.7 <0.1 <0.1	SPE A
		Urine	1000	5F-MDMB-PICA hydr. metab.	No analysis		SPE A
13/32 y/female	12/2018	NaF blood	1000	–	5F-Cumyl- PEGACLONE	<0.1	SPE A
14/18 y/male	12/2018	Serum	1000	5F-ADB hydr. metab.	5F-ADB	0.3	SPE A
15/29 y/male	12/2018	Serum	1000	–	5F-ADB	<0.1	SPE A
16/38 y/male	01/2019	Serum	950	5F-MDMB-PICA (+ hydr. metab.) 4F-MDMB-BINACA	5F-MDMB-PICA	0.89	SPE A
17/29 y/male	01/2019	Serum	1000	5F-MDMB-P7AICA (+ hydr. metab.)	5F-MDMB-P7AICA	2.32	SPE A
18/35 y/male	01/2019	Serum	1000	–	JWH-210 JWH-122	0.35 <0.1	SPE B
19/26 y/male	02/2019	Serum	1000	–	5F-Cumyl-PINACA	0.29	SPE A
20/31 y/male	03/2019	Serum	900	–	5F-Cumyl- PEGACLONE	0.16	SPE A
21/22 y/male	03/2019	NaF blood	800	5F-MDMB-PICA (+ hydr. metab.) 4F-MDMB-BINACA	5F-MDMB-PICA 4F-MDMB-BINACA 5F-MDMB-P7AICA	0.26 0.25 <0.1	SPE A
22/26 y/male	05/2019	NaF blood	1000	5F-MDMB-PICA (+ hydr. metab.)	5F-MDMB-PICA	0.54	SPE A
23/22 y/male	05/2019	Serum	1000	5F-MDMB-PICA	5F-MDMB-PICA	2.39	SPE A
24/30 y/male	05/2019	Serum	1000	–	4F-MDMB-BINACA	0.41	SPE A
25/50 y/male	05/2019	Serum	1000	5F-MDMB-PICA 4F-MDMB-BINACA	5F-MDMB-PICA 4F-MDMB-BINACA	approx. 6.95 approx. 6.55	SPE B

TABLE 6 (Continued)

Case no. /age/sex	Time of event	Sample type[a]	Sample volume [µL][a]	LC-QTOF-MS findings	LC-QqQ-MS findings[b]	Concentration [ng/ml][a]	Extraction[a]
26/29 y/male	05/2019	Serum	600	5F-MDMB-PICA (+ hydr. metab.) 4F-MDMB-BINACA hydr. metab.	5F-MDMB-PICA	2.5	SPE A
27/25 y/male	05/2019	Femoral blood	1000	4F-MDMB-BINACA	4F-MDMB-BINACA	approx. 42	SPE B
28/26 y/male	05/2019	Whole blood (hem.)	850	–	4F-MDMB-BINACA	<0.1	SPE A
29/23 y/male	06/2019	Serum	1000	–	4F-MDMB-BINACA	<0.1	SPE A
30/20 y/male	06/2019	Serum	900	–	4F-MDMB-BINACA	<0.1	SPE A
31/20/male	06/2019	Whole blood (hem.)	800	–	5F-MDMB-PICA	0.11	SPE A
32/ unknown/ male	06/2019	Serum	1000	5F-MDMB-PICA	5F-MDMB-PICA	<0.1	SPE A
33/65 y/male	07/2019	Femoral blood	1000	4F-MDMB-BINACA	4F-MDMB-BINACA 5F-MDMB-PICA	0.48 0.14	SPE A
		Urine	1000	–	4F-MDMB-BINACA metabolites 5F-MDMB-PICA metabolites	(+) (+)	SPE A
34/19 y/male	07/2019	Whole blood (hem.)	700	4F-MDMB-BINACA	4F-MDMB-BINACA 5F-MDMB-PICA	1.62 0.14	SPE A
35/35 y/female	09/2019	Serum	1000	Cumyl-CBMICA	Cumyl-CBMICA	(+)	SPE B
36/37 y/male	09/2019	Serum	1000	–	MDMB-4en-PINACA	(+)	SPE B
37/30 y/male	10/2019	Serum	500	–	5F-ADB	<0.1	SPE B
38/28 y/male	10/2019	Serum	1000	5F-Cumyl-P7AICA 5F-MDMB-PICA 4F-MDMB-BINACA 5F-ADB Cumyl-PEGACLONE	5F-Cumyl-P7AICA 5F-MDMB-PICA (+ hydr. metab.) 4F-MDMB-BINACA (+ hydr. metab.) JWH-122 5F-ADB (+ hydr. metab.) AMB-CHMICA (+ hydr. metab.)	3.4 0.35/(+) 0.30/(+) 0.20 0.15/(+) 0.12/(+)	SPE B
39/42 y/male	11/2019	Femoral blood	800	5F-MDMB-P7AICA (+ hydr. metab.) 5F-AB-P7AICA hydr. metab.	5F-MDMB-P7AICA (+ hydr. metab.)	7.5/(+)	SPE B
		Urine	1000	5F-AB-P7AICA hydr. metab. 5F-ADB hydr. metab. 5F-AMB 5F-MDMB-P7AICA hydr. metab. FUB-AMB hydr. metab. /EMB-FUBINACA hydr. metab.	5F-AB-P7AICA hydr. metab. 5F-ADB hydr. metab. 5F-ADB-PINACA metabolites 5F-AMB metabolites 5F-MDMB-P7AICA hydr. metab. FUB-AMB hydr. metab. /EMB-FUBINACA hydr. metab	(+) (+) (+) (+) (+) (+)	SPE A
40/25 y/male	12/2019	Serum	1000	4F-MDMB-BINACA	4F-MDMB-BINACA (+ hydr. metab.)	0.75/(+)	SPE B

(Continues)

TABLE 6 (Continued)

Case no. /age/sex	Time of event	Sample type[a]	Sample volume [µL][a]	LC-QTOF-MS findings	LC-QqQ-MS findings[b]	Concentration [ng/ml][a]	Extraction[a]
41/22 y/male	02/2020	Femoral blood	0	No analysis (lack of sample)	5F-MDMB-PICA (+ hydr. metab.)	0.18/(+)	SPE B
		Urine	1000	5F-MDMB-PICA hydr. metab.	No analysis	–	SPE A
42/19 y/male	03/2020	Serum	1000	4F-MDMB-BINACA (+ hydr. metab.)	4F-MDMB-BINACA (+ hydr. metab.)	9.2/(+)	SPE B
43/21 y/male	03/2020	Serum	1000	4F-MDMB-BINACA Cumyl-4CN-BINACA	4F-MDMB-BINACA (+ hydr. metab.) Cumyl-4CN-BINACA	1.11/(+) 0.29	SPE B
44/39 y/male	03/2020	NaF blood	180	Cumyl-4CN-BINACA	Cumyl-4CN-BINACA	approx. 56	SPE B
45/50 y/male	05/2020	Femoral blood	1000	Cumyl-CBMICA	Cumyl-CBMICA	approx. 12	SPE B
		Urine	1000	-	Cumyl-CBMEGACLONE metabolites Cumyl-CBMICA metabolites Cumyl-CBMINACA metabolites	(+) (+) (+)	SPE A
46/19 y/male	05/2020	Serum	700	–	AB-FUB7AICA	0.23	SPE B
47/48 y/male	06/2020	Serum	1000	JWH-210 JWH-122 JWH-018 JWH-073 3-OH-butyl metabolite	JWH-210 JWH-122 JWH-018 JWH-073 JWH-081	0.98 0.30 0.14 <0.1 <0.1	SPE B
48/ 35 y/ male	06/2020	NaF blood	900	4F-MDMB-BINACA	4F-MDMB-BINACA (+ hydr. metab.) 5F-MDMB-PICA hydr. metab.	0.45/(+) (+)	SPE B
49/19 y/male	06/2020	Serum	1000	–	5F-MDMB-PICA hydr. metab.	(+)	SPE B
50/14 y/male	06/2020	Serum	1000	MDMB-4en-PINACJA 4F-MDMB-BICA	MDMB-4en-PINACA (+ hydr. metab.) 4F-MDMB-BICA (+ hydr. metab.)	0.19/(+) (+)/(+)	SPE B
51/16 y/male	06/2020	Serum	1000	MDMB-4en-PINACA 4F-MDMB-BINACA 4F-MDMB-BICA	MDMB-4en-PINACA (+ hydr. metab.) 4F-MDMB-BINACA (+ hydr. metab.) 4F-MDMB-BICA (+ hydr. metab.) 5F-MDMB-PICA hydr. metab.	0.43/(+) 0.19/(+) (+)/(+) (+)	SPE B
52/19 y/male	06/2020	Serum	1000	MDMB-4en-PINACA (+ hydr. metab.) 4F-MDMB-BICA (+ hydr. metab.)	MDMB-4en-PINACA (+ hydr. metab.) 4F-MDMB-BICA (+ hydr. metab.)	0.21/(+) (+)/(+)	SPE B
53/19 y/male	06/2020	Serum	1000	MDMB-4en-PINACA (+ hydr. metab.) 4F-MDMB-BICA (+ hydr. metab.)	MDMB-4en-PINACA (+ hydr. metab.) 4F-MDMB-BICA (+ hydr. metab.) 5F-MDMB-PICA hydr. metab.	0.50/(+) (+)/(+) (+)	SPE B

TABLE 6 (Continued)

Case no. /age/sex	Time of event	Sample type[a]	Sample volume [µL][a]	LC-QTOF-MS findings	LC-QqQ-MS findings[b]	Concentration [ng/ml][a]	Extraction[a]
54/51 y/male	06/2020	Femoral Blood	1000	4F-MDMB-BICA (+ hydr. metab.)	MDMB-4en-PINACA (+ hydr. metab.) 4F-MDMB-BICA (+ hydr. metab.) 5F-MDMB-PICA hydr. metab.	0.2/(+) (+)/(+) (+)	SPE B
		Urine	1000	4F-MDMB-BICA (+ hydr. metab.) 5F-MDMB-PICA hydr. metab.	4F-MDMB-BICA metabolites 4F-MDMB-BINACA metabolites 5F-AB-P7AICA metabolites 5F-MDMB-PICA metabolites ADB-BINACA metabolites Cumyl-CBMEGACLONE metabolites FUB-144 metabolites MDMB-4en-PINACA metabolites	(+) (+) (+) (+) (+) (+) (+) (+)	SPE A
55/39/ female	07/2020	Serum	1000	5F-MDMB-PICA (+ hydr. metab.)	5F-MDMB-PICA (+ hydr. metab.)	approx. 52/(+)	SPE B
56/37 y/male	07/2020	Serum	1000	5F-MDMB-PICA	5F-MDMB-PICA hydr. metab.	(+)	SPE B
57/21 y/male	07/2020	Urine	1000	5F-MDMB-PICA (+ hydr. metab.) 4F-MDMB-BINACA hydr. metab.	No analysis	–	SPE A
58/18 y/male	08/2020	Serum	1000	4F-MDMB-BICA 5F-MDMB-PICA MDMB-4en-PINACA	4F-MDMB-BICA (+ hydr. metab.) 5F-MDMB-PICA hydr. metab. MDMB-4en-PINACA (+ hydr. metab.)	(+)/(+) (+) (+)/(+)	SPE B
59/22 y/male	08/2020	Serum	950	5F-MDMB-PICA MDMB-4en-PINACA 4F-MDMB-BICA	5F-MDMB-PICA (+ hydr. metab.) MDMB-4en-PINACA (+ hydr. metab.) 4F-MDMB-BICA (+ hydr. metab.)	0.34/(+) <0.1/(+) (+)/(+)	SPE B
60/30 y/male	08/2020	Serum	1000	Cumyl-4CN-BINACA MDMB-4en-PINACA (+ hydr. metab.)	Cumyl-4CN-BINACA MDMB-4en-PINACA (+ hydr. metab.)	7.1 0.69/(+)	SPE B
61/17 y/female	08/2020	Serum	200	-	4F-MDMB-BINACA hydr. metab. 5F-MDMB-PICA hydr. metab.	(+) (+)	SPE B
62/14 y/male	08/2020	Serum	1000	–	4F-MDMB-BINACA (+ hydr. metab.)	<0.1/(+)	SPE B
		Urine	1000	4F-MDMB-BINACA hydr. metab.	No analysis		SPE A
63/17 y/female	09/2020	NaF blood	900	4F-MDMB-BICA	4F-MDMB-BICA (+ hydr. metab.)	(+)/(+)	SPE B

(Continues)

TABLE 6 (Continued)

Case no. /age/sex	Time of event	Sample type[a]	Sample volume [µL][a]	LC-QTOF-MS findings	LC-QqQ-MS findings[b]	Concentration [ng/ml][a]	Extraction[a]
64/19 y/male	09/2020	Serum	400	MDMB-4en-PINACA (+ hydr. metab.) 4F-MDMB-BINACA	MDMB-4en-PINACA (+ hydr. metab.) 4F-MDMB-BINACA (+ hydr. metab.) 5F-MDMB-PICA hydr. metab.	1.0/(+) <0.1/(+) (+)	SPE B
65/32 y/female	09/2020	Serum	400	5F-MDMB-PICA 4F-MDMB-BICA (+ hydr. metab.)	5F-MDMB-PICA (+ hydr. metab.) 4F-MDMB-BICA (+ hydr. metab.)	<0.1/(+) (+)/(+)	SPE B
66/32 y/male	09/2020	Serum	1000	JWH-210 5F-MDMB-PICA (+ hydr. metab.)	JWH-122 JWH-210 5F-MDMB-PICA hydr. metab. MDMB-4en-PINACA hydr. metab.	<0.1 <0.1 (+) (+)	SPE B
67/34 y/male	09/2020	Serum	200	5F-MDMB-PICA hydr. metab.	5F-MDMB-PICA hydr. metab. MDMB-4en-PINACA hydr. metab.	(+) (+)	SPE B
68/22 y/male	09/2020	Serum	200	MDMB-4en-PINACA (+ hydr. metab.)	MDMB-4en-PINACA (+ hydr. metab.) 5F-MDMB-PICA hydr. metab.	<0.1/(+) (+)	SPE A
69/15 y/male	09/2020	Serum	1000	–	MDMB-4en-PINACA hydr. metab.	(+)	SPE B
70/23 y/male	10/2020	Serum	1000	4F-MDMB-BICA (+ hydr. metab.) 5F-MDMB-PICA (+ hydr. metab.) MDMB-4en-PINACA JWH-210	4F-MDMB-BICA (+ hydr. metab.) 5F-MDMB-PICA (+ hydr. metab.) MDMB-4en-PINACA (+ hydr. metab.) JWH-210	(+)/(+) 2.4/(+) 0.18/(+) 0.11	SPE B
71/29 y/male	10/2020	Serum	1000	–	MDMB-4en-PINACA hydr. metab.	(+)	SPE B
72/22 y/male	10/2020	Serum	300	–	MDMB-4en-PINACA (+ hydr. metab.)	<0.1/(+)	SPE B
73/25 y/male	10/2020	Serum	1000	4F-MDMB-BICA (+ hydr. metab.)	4F-MDMB-BICA (+ hydr. metab.) MDMB-4en-PINACA (+ hydr. metab.)	(+)/(+) 0.35/(+)	SPE B

Note: Sorted by time of event. Concentrations below the limit of quantification (LOQ) are trace quantities (e.g., < 0.1 ng/ml). Concentrations above the calibration range are specified as approximately (approx.). Qualitative results are labeled as (+).

[a]Liquid chromatography-triple-quadrupole mass spectrometry (LC-QqQ-MS) quantitative analysis was always conducted with 1000-µl serum using LLE (postmortem: femoral blood, urine).

[b]Sample type, sample volume and extraction method relate to LC-QTOF-MS analysis.

The most frequently detected SCs were 5F-MDMB-PICA (31 findings, from 11/2018), 4F-MDMB-BINACA (22 findings, from 01/2019), MDMB-4en-PINACA (18 findings, from 09/2019) and 4F-MDMB-BICA (11 findings, from 06/2020). All four were still detected at the end of this study. 5F-ADB (eight cases, 05/2018–11/2019) was only detected until the end of 2019. Other frequent SCs were JWH-122 (five cases), JWH-210 (four cases), CUMYL-4CN-BINACA (four cases), and 5F-Cumyl-PEGACLONE (four cases). The latter was only detected in a short period from Aug. 2018 to Mar. 2019, possibly because in July 2019 5F-Cumyl-Pegaclone was added to the Narcotics Law (Betäubungsmittelgesetz, BtMG) and its gamma-carboline core structure was added to the NpSG.[27] However, since most detected substances are subject to the NpSG, other reasons such as pharmacological and toxicological drug effects might have played a

role. Of the substances detected only once, all but AB-FUB7AICA and 5F-Cumyl-PINACA were detected alongside other SCs. Herbal mixtures can contain multiple SCs at once, but an offset in time of consumption would also be possible.

In 35 of 73 cases (48%), consumption of multiple synthetic cannabinoids was proven (blood and urine samples combined). Two SCs were found in 21 cases (29%); three SCs were found in seven cases (10%); four SCs were found in three cases (4%); five, six, seven and eight SCs were found once.

3.2.2 | Authentic case findings: LC-QTOF-MS vs. LC-QqQ-MS

In LC-QTOF-MS analysis, 68 SCs were found in blood samples of 47 cases. LC-QqQ-MS analysis detected 103 SCs in blood samples of 73 cases. This results in 55% more cases and 51% more findings for LC-QqQ-MS analysis. Thirty-one SCs were quantified in trace concentrations (<0.1 ng/ml), of which LC-QTOF-MS analysis detected six parent compounds (cases 32, 59, 64–66, 68) and three metabolites (cases 1, 2, 47). Fourteen SC findings were not quantified due to lack of reference standard (4F-MDMB-BICA, MDMB-4en-PINACA and Cumyl-CBMICA), of which 13 were detected in LC-QTOF-MS analysis.

At least one hydrolysis metabolite was detected in blood samples of 41 cases: by LC-QTOF-MS analysis in 24 cases (cases 1, 2, 11, 12, 14, 16, 17, 21, 22, 26, 39, 42, 52–55, 60, 64–68, 70, 73) and by LC-QqQ-MS analysis in 31 cases (cases 38–43, 48–56, 58–73). Considering only cases after case 38 (when hydrolysis metabolites were introduced in the LC-QqQ-MS method), LC-QqQ-MS analysis found 322% more hydrolysis metabolites (58 vs. 18 findings) in 29% more cases (31 vs. 24 cases). Regarding individual findings of all cases, LC-QTOF-MS analysis found 30 hydrolysis metabolites in blood, 24 of them alongside their parent compound. LC-QqQ-MS analysis found 58 hydrolysis metabolites in blood, 41 of them alongside their parent compounds. Thus, over 70% of all detected hydrolysis metabolites in blood samples were found alongside their parent compound.

In 26 cases, LC-QTOF-MS failed to detect any SCs or SC metabolites (cases 3–10, 13, 15, 18–20, 24, 28–31, 36, 37, 46, 49, 61, 69, 71, 72). Fourteen of those cases contained parent compounds only at trace quantities (cases 4–9, 13, 15, 28–30, 37, 62, 72), thereof mostly 5F-ADB (five cases) and 4F-MDMB-BINACA (four cases). Four cases contained only hydrolysis metabolites of unknown concentrations (cases 49, 61, 69, 71) and in four cases analysis lacked proper sample volume and/or quality (cases 5, 6, 10, 37).

Due to other investigations, LC-QTOF-MS analysis sometimes had to use suboptimal sample volumes or sample types. Especially older cases (cases 1–11) with many preceding investigations had no serum left, but only hemolyzed whole blood (refrigerated) or NaF blood (frozen). A minimum sample volume of 700 μL was determined rather arbitrarily to be sufficient without losing too much sensitivity. Adequate sample volume (>700 μL) was unavailable in 16 cases (cases 1, 5, 6, 9–11, 26, 34, 37, 44, 46, 64, 65, 67, 68, 72), from which SCs have been detected completely in three cases (cases 11, 26, 44) and partially in four cases (cases 1, 34, 67, 68). Blood samples other than serum were used in 23 cases: whole blood in nine cases (cases 1, 3, 6, 7, 10, 11, 28, 31, 34), NaF blood in eight cases (cases 2, 5, 13, 21, 22, 44, 48, 63) and femoral blood in seven cases (cases 12, 27, 33, 39, 45, 54). SC findings were complete in six cases (cases 11, 22, 27, 39, 44, 45) and partially complete in nine cases (cases 1, 2, 12, 21, 33, 34, 48, 54, 63). No blood analysis was conducted in case 41 due to lack of sample volume.

In three cases, LC-QTOF-MS analysis was able to detect substances when QqQ-MS did not: 4F-MDMB-BINACA was found retrospectively in case 16 and its hydrolysis metabolite was found in case 26. Both analytes were not included in the QqQ-MS method at the time. Cumyl-PEGACLONE was found only by LC-QTOF-MS in case 38, which was verified by the laboratory in Freiburg upon request, but not quantified due to improper ratios of both present mass transitions. The resulting hints for Cumyl-PEGACLONE were not reported.

As QqQ-MS instruments are often built for trace analytics and benefit from specialized ion funneling techniques, they can exceed other tandem mass spectrometry instruments like QTOF-MS in terms of sensitivity. LC-QTOF-MS was unable to detect SCs in trace quantities (<0.1 ng/ml) reliably. The impact of low sample volume and suboptimal sample quality could not be compared thoroughly, as QqQ-MS analysis was always performed with 1000-μl serum.

Another aspect relating to differing SC findings are substance specific stability issues. Krotulski et al.[8] found three prevalent SCs (FUB-AMB, 5F-ADB and 5F-MDMB-PICA) to be unstable especially at room temperature and refrigerated. Rapid degradation into their hydrolysis metabolites would impede the detection of their parent compounds. This might especially explain the discrepancies in cases 1, 2, 14, 56, 58 and 66 (parent compounds vs. hydrolysis metabolite), as samples were shipped by mail uncooled. For these cases it was crucial to include hydrolysis metabolites in blood analysis. Blood samples sent to our lab by the police were not stored frozen until serum was separated by centrifugation, which could take several days from blood sampling to arrival. The resulting loss of substance could be crucial for detection of trace quantities, especially by less sensitive instruments. Hess et al.[28] investigated stability of 84 SCs at various conditions, and recommended continuous storage at −20°C. However, stability data of both studies are not completely consistent, for example for 5F-ADB. Summarizing, comprehensive and reliable data on stability are hard to come by, especially for recently emerged substances. Thus, samples should be stored and transported frozen whenever possible.

Hydrolysis metabolites were included in our LC-QTOF-MS blood sample screening method from the beginning and subsequently added to the QqQ-MS method in October 2019 (after case 38). For LC-QTOF-MS analysis, this has proven to be useful when parent compounds were not detected (cases 1, 2, 14). Finding hydrolysis metabolites alongside their parent compounds can be considered an additional contribution of confirmation. They also serve as biomarkers to prolong the detection window after SC consumption.[8,29] Some hydrolysis metabolites retain cannabinoid receptor 1 (CB_1) activity at

varying extents, potentially contributing to the overall pharmacological effect in vivo.[30] Also, not restricting serum screening to parent compounds and urine screening to metabolites can be useful sometimes: urine containing 5F-AMB (case 39), 4F-MDMB-BICA (case 54) or 5F-MDMB-PICA (case 57); serum containing JWH-073-3-OH-butyl (case 47) as well as 41 cases of detected hydrolysis metabolites in blood samples.

Urine analysis, although usually only conducted in post mortem cases in our institution, was restricted due to missing metabolite entries in the spectra library, except for hydrolysis metabolites. This affected especially more recent synthetic cannabinoids.

4 | CONCLUSION

The present study demonstrates that LC-QTOF-MS after SPE is an excellent screening procedure for sensitive detection of most synthetic cannabinoids. Usual blood concentrations around 1 ng/ml as well as metabolites in urine samples were detected reliably. At least one synthetic cannabinoid or metabolite was detected in most cases, leading to further analysis and quantification by a more sensitive LC-QqQ-MS MRM method. This way, serving as a general screening for potentially thousands of pharmacological and toxicological compounds, LC-QTOF-MS reliably fulfilled its purpose to indicate SC consumption.

Results of fortified serum samples were in line with findings in authentic cases. All substances above 1 ng/ml were detected in authentic samples, while many could be detected at even lower levels, down to 0.1 ng/ml. Only trace amounts below 0.1 ng/ml were not detected reliably. Hydrolysis metabolites of synthetic cannabinoids were found frequently in blood samples, confirming findings of their respective parent compounds and potentially prolonging the detection window of unstable SCs. 5F-MDMB-PICA, 4F-MDMB-BINACA, MDMB-4en-PINACA and 4F-MDMB-BICA were detected most frequently in authentic samples.

The creation of a spectrum library containing 199 synthetic cannabinoids and 130 metabolites was uncomplicated and enables further expansion in the future. The data-dependent auto-MS/MS acquisition mode makes it unnecessary to adjust targets due to changing SC markets. Sources other than reference standards for spectra and retention time acquisition were used successfully to bridge the time until standards were commercially available. Additionally retrospective analysis could be conducted to reinvestigate past cases.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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