Liquid chromatography-tandem mass spectrometry determination of synthetic cathinones and phenethylamines in influent wastewater of eight European cities

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Abstract

The popularity of new psychoactive substances (NPS) has grown in recent years, with certain NPS commonly and preferentially consumed even following the introduction of preventative legislation. With the objective to improve the knowledge on the use of NPS, a rapid and very sensitive method was developed for the determination of ten priority NPS (N-ethylcathinone, methylenedioxypyrovalerone (MDPV), methylone, butylone, methedrone, mephedrone, naphyrone, 25-C-NBOMe, 25-I-NBOMe and 25-B-NBOMe) in influent wastewater. Sample clean-up and pre-concentration was made by off-line solid phase extraction (SPE) with Oasis MCX cartridges. Isotopically labelled internal standards were used to correct for matrix effects and potential SPE losses. Following chromatographic separation on a C_{18} column within 6 minutes, the compounds were measured by tandem mass spectrometry in positive ionisation mode. The method was optimised and validated for all compounds. Limits of quantification were evaluated by spiking influent wastewater samples at 1 or 5 ng/L. An investigation into the stability of these compounds in influent wastewater was also performed, showing that, following acidification at pH 2, all compounds were relatively stable for up to 7 days. The method was then applied to influent wastewater samples from eight European countries, in which mephedrone, methylone and MDPV were detected. This work reveals that although NPS use is not as extensive as for classic illicit drugs, the application of a highly sensitive analytical procedure makes their detection in wastewater possible. The developed analytical methodology forms the basis of a subsequent model-based back-calculation of abuse rate in urban areas (*i.e.* wastewater-based epidemiology).

Key Words: New psychoactive substances, Ultra high-performance liquid chromatography, Triple Quadrupole, Wastewater, Stability, Matrix Effects

Introduction

New psychoactive substances (NPS) are emerging narcotic or psychotropic drugs that are not controlled by legislation, but which may pose a public health threat. It must be noted that here, the term 'new' does not necessarily refer to new inventions but to substances that have recently become available (UNODC, 2014). The use of NPS has grown rapidly over the past decade and there have been increasing reports of the availability and manufacture of such substances, with the number of NPS reported globally more than doubling between 2009-2013 (UNODC 2014). In 2014 alone, 101 NPS were for the first time reported to the EU Early Warning System (EMCDDA, 2015a). Given the nature of the NPS market, with developers limited only by their imagination and ability to side-step legislation (Reid and Thomas, 2016) there is a sustained need to analyse the extent of the NPS problem.

The analysis of wastewater to estimate (illicit) drug consumption based on biomarkers, has traditionally focussed on the most common illicit drugs - cocaine, cannabis, amphetamine, methamphetamine and 3,4-methylenedioxymethamphetamine (ecstasy, MDMA) (Ort et al., 2014; Thomas et al., 2012), leaving a large information gap on other illicit drugs and NPS. Little research has been made on NPS and their suitable biomarkers, let alone their stability. In the few papers that have been published until now on the determination of NPS in wastewater, the target analytes included are commonly the synthetic cathinones mephedrone, methylone and MDPV (Baz-Lomba et al., 2016; Borova et al., 2015; Castrignanò et al., 2016; Chen et al., 2013; Kankaanpää et al., 2014; Kinyua et al., 2015a; Mwenesongole et al., 2013; Reid et al., 2014; Senta et al., 2015; van Nuijs et al., 2013). Within these studies, the most commonly detected NPS in wastewater are mephedrone and MDPV, generally found at the low ng/L range.

Liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) is the technique of choice for the quantitative determination of illicit drugs in wastewater, due to the low concentrations involved and the high sensitivity of the instrument. In addition to the required validation at realistic concentrations that can be found in the samples, relevant issues must be considered, such as the way to correct/minimize matrix effects, and the proper identification of the compound detected. The use of isotopically-labelled internal standards (ILIS) is one of the most efficient tools to correct for matrix effects as well as potential losses from solid phase extraction (SPE). When utilising LC-MS/MS instruments in selected reaction monitoring (SRM) mode, at least two transitions should be incorporated in the method (one for quantification and the other(s) for confirmation). However, the specificity of the transitions should be taken into account, as non-specific transitions (such as the loss of water) can lead to false negatives due to the non-compliance of ion ratios (Pozo et al., 2006).

The purpose of this study was to develop and validate a sensitive LC-MS/MS method for the quantitative determination of a number of NPS of the synthetic cathinone and phenethylamine families: butylone, ethylone, methylone, naphyrone, methedrone, methylenedioxypyrovalerone (MDPV), mephedrone, 25-I-NBOMe, 25-C-NBOMe and 25-B-NBOMe. These compounds were selected on the basis of their frequent detection in analytical, forensic and toxicological studies (Borova et al., 2015; Chen et al., 2013; Elliott and Evans, 2014; Ibáñez et al., 2014; Kankaanpää et al., 2014; Kinyua et al., 2015a; Mwenesongole et al., 2013; Reid et al., 2014; Senta et al., 2015; Uralets et al., 2014) as well as reports from the EMCDDA (EMCDDA, 2015b) and UNODC (UNODC, 2014). The developed method, using Oasis MCX SPE cartridges for sample pre-treatment, followed by UHPLC-MS/MS measurement, has been applied to influent wastewater samples from around Europe, with an additional study on stability being

made. Special emphasis is placed on the reliable confirmation of the NPS detected in water, with up to three SRM transitions being acquired, which, together with ion ratios, allowed simultaneous detection, quantification and confirmation of positive samples.

2. Experimental

2.1 Chemicals and Materials

See Supporting Information for this section as well as the structures of all compounds (Figure S1).

2.2 Samples

A number of different influent wastewater (IWW) samples were utilised in the development and validation of the present method: from Zurich, Switzerland; Copenhagen, Denmark and Castellon, Spain. The developed method was applied to IWW samples. The 24-h composite samples were taken in March 2015 from Zurich, Switzerland; Copenhagen, Denmark; Oslo, Norway; Castellon, Spain; Milan, Italy; Brussels, Belgium, Utrecht, The Netherlands and Bristol, United Kindom. All samples were collected in high density polyethylene bottles, transported to Castellon and stored in the dark at -20ºC until pre-treatment.

2.3 Instrumentation

A Waters Acquity UHPLC system (Milford, MA, USA) was interfaced to a triple quadrupole mass spectrometer (Xevo TQS, Waters Micromass, Manchester, UK) equipped with Z-Wave devices and an electrospray ionization interface (ESI) operated in positive-ion mode. The chromatographic separation was performed using an Acquity UPLC BEH C_{18} column, 1.7 μm, 50 mm × 2.1 mm (i.d.) (Waters) at a flow rate of 0.3 mL min^{-1}. The mobile phases used were water with 5 mM ammonium acetate and 0.01% formic acid (solvent A) and MeOH with 0.01% formic acid (solvent B). The percentage of B changed linearly as follows: 0 min, 10 %; 3 min, 90 %; 3.5 min, 90 %; 3.6 min, 10 %; 6 min 10 %, equilibration of the column. Cone gas as well as desolvation

gas was dry nitrogen, with flows set to 150 and 800 L h^{-1}, respectively. For operation in MS/MS mode, collision gas was argon 99.995 % (Praxair, Madrid, Spain) with a pressure of 4×10−3 mbar in the collision cell (0.15 mL min^{-1}). Further parameters optimized were: capillary voltage, 3.0 kV; source temperature, 150 °C and desolvation temperature, 650 °C. Dwell times of 0.01 s/transition were automatically selected.

All acquired data were processed using MassLynx v4.1 software (Waters, Manchester, UK).

2.4 Sample Pre-treatment

All water samples were acidified to pH 2 with formic acid (98%), then centrifuged for 5 minutes at 6000 rpm. SPE cartridges were conditioned with methanol (6mL), water (3mL) and acidified water (pH 2 with formic acid, 3mL). The IWW samples (100mL) were spiked with mixed surrogate ILIS to give a final in sample concentration of 20 ng/L, then percolated through the cartridges at ±5 mL/min. The cartridges were then washed with 5 mL acidified methanol (pH 2 with formic acid) and dried under vacuum for 10 minutes. The analytes were eluted with 5 mL MeOH (2% ammonia), evaporated to dryness at 35ºC under nitrogen and reconstituted to 1mL with 10:90 methanol:water.

Analyses were performed by injecting 3 µL of the final extract in the UHPLC-MS/MS system.

2.5 Quantification and Method Validation

SRM mode was used to acquire all data, with the precursor ion of each compound being the protonated molecule. In general, the most abundant product ion of each target analyte was used for quantification, with one or two additional product ions and their ion ratios used for confirmation. Furthermore, LC retention time was compared to that of reference standards (tolerance of ± 0.1 minutes). These steps are in line with the SANCO guidelines for analytical quality control and validation procedures (SANCO, 2013), which has been utilised previously by research groups for quantification of illicit drugs in wastewater (Bijlsma et al., 2009). Each compound was quantified using its ILIS as a surrogate internal standard, except N-ethylcathinone and methedrone, which were quantified using butylone-d_3 and methylone-d_3, respectively. These ILIS were chosen based on their ability to correct for matrix effects as well as having similar retention times and chemical structures closely related to the analyte of interest.

The performance of the method was evaluated in terms of linearity, limits of detection and quantification, accuracy and precision.

The linearity was studied by analysing standard solutions in solvent in triplicate at seven concentration levels ranging from 0.5-100 ng/L. Linearity was deemed satisfactory when the correlation coefficient (r) was >0.99, using weighted (1/X) least squares regression.

For limits of quantification and detection (LOQ and LOD), two different concentration levels (1 and 5 ng/L) were tested so as to provide a more accurate measure. The limit of quantification (LOQ) was established as the lowest level for which the method was fully validated using spiked IWW samples with satisfactory accuracy based on recovery experiments (between 70-120%) and precision (relative standard deviation (RSD)

176 ≤20%). Furthermore, a minimum of two MS/MS transitions were required at the LOQ
177 level for confirmation. All recovery experiments were performed in quintuplicate.

178 The limit of detection (LOD) was estimated using blank samples spiked at the lowest
179 analyte concentration tested, based on a signal-to-noise level of three from the
180 quantification SRM chromatogram.

181 *2.6 Stability experiments*

182 Stability experiments were performed under different conditions (IWW at natural pH
183 and acidified to pH 2, filtered influent wastewater at natural pH and acidified to pH 2
184 and MilliQ water) and at three different temperatures (20ºC, 4ºC and -20ºC) for 24
185 hours, 48 hours, 7 days, and 30 days (at -20ºC only). The samples were each initially
186 spiked at a concentration level of 100ng/L. One mL of each sample was collected at the
187 time intervals outlined above and spiked with ILIS (100 ng/L) just before analysis.

188

3. Results and Discussion

3.1 Selection of Compounds

Only parent compounds were selected for this study. This is based on the previous metabolism studies performed on the selected ten as well as related NPS in human and rat urine (Caspar et al., 2015; Ibáñez et al., 2016; Kamata et al., 2006; Mardal and Meyer, 2014; Meyer et al., 2010; Uralets et al., 2014), which showed that in spite of the number of metabolites identified, the parent compound could still be detected in urine. It has also been shown that the metabolic patterns of selected phenethylamine-based designer drugs show rather slow metabolism rates, with parent drugs being the primary biomarkers of consumption (Lai et al., 2015; Senta et al., 2015), somewhat expected due to their structural similarity to amphetamine and MDMA (Ort et al., 2014).

In addition, for the phenethylamine-based 25-X-NBOMe compounds, the parent compound is generally used in both qualitative and quantitative toxicological studies (Kinyua et al., 2015b; Poklis et al., 2014; Tang et al., 2014).

3.2 Instrument Optimisation

Initially, all cone voltages were optimised concurrently for all compounds, using a mixed standard solution (10 µg/L), with the cone voltage ranging from 10-50 V at 10 V intervals. For all compounds, the precursor ion was $[M+H]^+$. Collision energies were optimised for each compound independently using a collision energy ranging from 10-50eV at 10eV intervals. The most sensitive transition was typically selected for quantification (Q) while an additional one (or two) were selected for confirmation (q). Some compounds only had two product ions of significant intensity, meaning that only two transitions (i.e. Q and q1 only) could be monitored (**Table 1**). Non-specific transitions such as the loss of water were avoided as much as possible (except for

methylone, mephedrone and N-ethylcathinone where they were used as confirmation transitions) in order to reduce the likelihood of false positives (Bijlsma et al., 2009). For the three 25-X-NBOMe compounds, the same product ions were seen (*m/z* 121 and 91), corresponding to the cleavage of the methoxybenzoyl moiety (*m/z* 121) and the further loss of the methoxy group producing the tropylium ion (*m/z* 91). These transitions are not very specific, and can come from any compound with a methoxybenzoyl moiety, however as there were no other transitions available for these compounds, they had to be used as quantification and confirmation transitions. Regarding the ILIS, only a single transition was monitored.

The chromatographic separation was initially based on a mobile phase composed of water:MeOH, which resulted in very poor peak shapes. Different concentrations of ammonium acetate buffer and pH (formic acid) were investigated and the effects of pH and mobile phase ionic strength on the peak shapes, resolution and efficiencies were evaluated by varying the buffer concentration. An optimal mobile phase consisting of water with 5 mM ammonium acetate and 0.01% formic acid (solvent A) and MeOH with 0.01% formic acid (solvent B) was finally selected.

3.3 Optimisation of extraction procedure

An investigation into the filtration losses of these compounds was made in samples at both natural and pH 2, using a vacuum filter (Whatman, 0.45μm mixed cellulose ester membrane). It was found that for naphyrone and the 25-X-NBOMe compounds, less than 5% of the compounds were recovered following filtration, while for MDPV a recovery of 55-71% was found. (**Table S1**) With these results, filtration was not employed in this method, instead only centrifugation.

Two cartridges were evaluated in the optimisation of the SPE procedure: Oasis HLB and Oasis MCX, which are two of the most popular cartridges for the extraction of different types of illicit drugs in environmental waters (Baker and Kasprzyk-Hordern, 2011). The backbone of the Oasis sorbents consists of apolar moieties (benzyl groups, aliphatic chains) and polar groups (pyrrolidone). HLB is only synthesised from these two monomers and is neutral. MCX has extra sulfonic acid functional groups (1.01mmol/g) implemented at a fixed location and contain permanently charged groups at any relevant pH (Bäuerlein et al., 2012).For both cartridges, 100 mL of IWW was spiked with the compounds (100 ng/L) before and after extraction and the recovery was used to determine the better cartridge for further optimisation. No pH adjustment was made for the Oasis HLB cartridges, while for the MCX, samples were acidified (pH 2) in order to protonate the basic analytes, thereby enhancing their ionic interactions with the sorbent (Senta et al., 2015). This led to a reduction in matrix interferences, giving higher sensitivity and peak shapes for the majority of the compounds (**Figure S2**). The SPE procedure followed for the HLB cartridges was the same as in previously published studies. (Bade et al., 2015; Bijlsma et al., 2014)

Higher recoveries were obtained when using Oasis MCX cartridges (**Figure S3**) and therefore these were selected for further optimisation. For the washing step, water, pure MeOH and acidified MeOH (pH 2) were tested. Drying under vacuum (or not) was also tested, to determine the optimal washing/drying steps (**Figure S3**).

As seen in **Figure S3**, the optimal SPE procedure involved washing with 5mL acidified methanol and drying for 10 minutes, with recoveries ranging from 83%-93% (**Figure S3, MCX 3**).

In previous methods for NPS using MCX cartridges, different percentages of ammonia in methanol have been used for elution, ranging from 0.5% to 4% (Kinyua et al., 2015a; Reid et al., 2014; Senta et al., 2015; van Nuijs et al., 2013). A compromise of 2% was utilised in this work, and showed good recoveries for all compounds. An elution volume of 5mL of 2% ammonia in MeOH solution was finally chosen. This was based on the comparison of the recoveries of 5mL and 10mL elution volumes, with recoveries only 2% less for the 5mL than the 10mL.

3.4 Method Validation

Before applying the developed methodology to IWW samples, the method was fully validated for linearity, precision and accuracy (recovery), LOD, LOQ and q/Q ratio (**Tables 1** and **2**). A calibration curve of seven points was made in solvent from 0.5-100 ng/L (in triplicate). Correlation coefficients were greater than 0.99 for all compounds, except for naphyrone and the NBOMe compounds, which had values between 0.97-0.98. For precision and accuracy, blank IWW samples were spiked at four concentration levels (1 ng/L, 5 ng/L, 10 ng/L and 50 ng/L), and analysed in quintuplicate. These two sets correspond to the different LOQs of some of the compounds (either1 ng/Lor 5 ng/L, see **Table 1**). Accuracy was measured by means of recovery, with all compounds showing satisfactory recovery (between 70-120%), and precision (all within RSD 20%).

LOQs were estimated from a non-statistical point of view, utilising an LOQ objective: the lowest level in sample for which the method was fully validated in terms of accuracy and precision. This criterion has been utilised in the past in published results (Bijlsma et al., 2009) and is used in other fields, such as pesticide residue analysis (SANCO, 2013). This gives a more realistic LOQ value, albeit generally higher than those estimated from statistical criteria (i.e. signal-to-noise of 10 at the lowest validation

283 level tested); thus, comparison of LOQs reported in the literature for different analytical
284 methods is commonly difficult due to the distinct criteria applied by the authors.

285 Average intensity q/Q ratios were calculated from reference standards in solvent of
286 different concentrations (**Table 1**). With RSD% values ≤11% for all q/Q ratios, there
287 was high confidence that the ratios are not concentration dependent. These were
288 compared to those experimentally obtained from sample extracts spiked at the lowest
289 level validated. The aim of this was to test the robustness of these values and to check
290 for potential matrix interferences that could affect the q/Q ratios and consequently, the
291 confirmation process. As **Table 2** shows, average q/Q deviations were all below 30%
292 (SANCO, 2013), except for 25-I-NBOMe and 25-C-NBOMe at the lowest level of 1
293 ng/L. This finding returns to the previous discussion of the non-specificity of the
294 NBOMe transitions, whereby although these transitions could be seen at this level,
295 matrix interferences let the q/Q deviations far exceed the threshold of the SANCO
296 guidelines, which were being followed in this work. This led to the LOQ for these two
297 compounds being increased to 5 ng/L.

298 Matrix effects are a known drawback of LC-MS/MS, which can adversely affect the
299 compounds of interest, leading to signal enhancement or suppression, thereby affecting
300 the quantification process. These effects are most pronounced in more complex
301 matrices, such as IWW. In this work, matrix effects were calculated by comparing the
302 peak areas of Set 1 (SPE eluates (IWW previously extracted) spiked with the mixed
303 standard and ILIS solutions) with Set 2 (mixed standard solution in solvent, including
304 ILIS). All samples were performed in triplicate and averaged to perform the following
305 calculations (Matuszewski et al., 2003):

306 $$\text{Matrix effects (\%)} = \frac{\text{average peak area (Set 1)}}{\text{average peak area (Set 2)}} \times 100$$

These calculations showed the true impact of the matrix on the compounds, with all showing significant matrix enhancement (**Table 2**). To correct for these interferences, ILIS were included in the calculation:

$$\text{Matrix effects (\%)} = \frac{\text{average peak area (Set 1,ILIS) / average peak area (Set 2,ILIS)}}{\text{average peak area (Set 1) / average peak area (Set 2)}} \times 100$$

Including ILIS to the calculations led to corrected matrix effects under 10% for all compounds.

Additional data pertaining to the use of ILIS is shown in **Table 2**, for SPE recovery and matrix effects, showing how well the ILIS are able to correct for matrix interferences.

315 *3.5 In-sample Stability*

316 Stability of analytes is a matter of concern in any analysis, particularly when dealing
317 with very low analyte concentrations in complex matrices such as wastewater. It is
318 imperative that target analytes do not degrade during the 24-hour composite samples
319 collection or during their storage before extraction. Composite samples are normally
320 collected at 4°C and are stored either at that temperature, or at -20°C, depending on the
321 time lapsing between collection and extraction(Senta et al., 2015), while extraction is
322 usually carried out at room temperature. For these reasons, the stability of the target
323 analytes in this study was studied at 4°C, -20°C and 20°C. The study was carried out in
324 both raw and filtered wastewater (as some research groups filter their samples prior to
325 storage) as well as in Milli-Q water. Furthermore, the impact of acidification was also
326 examined, with the samples examined at both natural pH and at pH 2.

327 In this section, low (60-100% transformation), medium (20-60% transformation) and
328 high stability (0-20% transformation) is defined as by McCall *et al*(McCall et al., 2016),
329 with all results being normalised to t=0. It must be stated that there was little difference
330 between the filtered and unfiltered wastewater for most analytes, with the compounds
331 less stable at natural pH. At 20ºC, N-ethylcathinone and mephedrone, showed low
332 stability after 24 hours. However, at pH 2, all compounds exhibited high stability at all
333 temperatures, for at least 7 days.

334 The results show that if the samples are not acidified they should be refrigerated (at
335 4°C), or frozen (at -20°C), and analysed within 7 days to ensure that no significant
336 degradation occur. Alternatively, if samples are acidified to pH 2, all investigated
337 compounds are more stable and can even be maintained at room temperature for up to 7
338 days. However, after 30 days, there were some distinct stability issues with some

compounds: mephedrone, methedrone and butylone all exhibiting low stability (up to 84% transformation); N-ethylcathinone, MDPV and methylone showing medium stability while the 25-X-NBOMe compounds and naphyrone still had high stability (less than 10% transformation) (**Figure 1**). All other results of the stability tests (i.e. in raw unacidified wastewater as well as both acidified and unacidified filtered wastewater and Milli-Q water) can be found in the supporting information (**Figures S4-S7**). These results also mirror those of the recent studies of Senta *et al*(Senta et al., 2015), and Reid *et al* (Reid et al., 2014), who investigated the stability of some of these compounds.

Although compounds were found to be stable at pH 2 and -20ºC for at least one week, storage at lower temperatures have advantages in terms of green and sustainability analytical chemistry such as energy and cost saving. Furthermore, storage at -20ºC is sometimes limited and not always feasible for wastewater treatment plants and/or laboratories. In this regard, storage at 4ºC is possible for at least one week as tested in the current study.

3.6 Application to Real Samples

A selection of samples from throughout Europe were investigated for the presence of NPS. In total, IWW from eight cities were investigated: Bristol, United Kingdom; Brussels, Belgium; Castellon, Spain; Oslo, Norway; Copenhagen, Denmark; Milan, Italy; Utrecht, The Netherlands and Zurich, Switzerland, with samples from weekends (Saturday and Sunday) and weekdays (Monday-Friday).

In every sequence of analysis, water samples were injected between two calibration curves. Two quality control samples (QCs), i.e. a blank water sample (previously analyzed) spiked at LOQ and 10×LOQ levels, were also analysed. QC recoveries were considered satisfactory when they were in the range of 70–120% for each analyte.

As shown in **Table 3**, mephedrone was the most commonly identified NPS, with concentrations ranging from <5 ng/L (Brussels, Oslo, Copenhagen and Utrecht) to 106 ng/L (Bristol). The high concentrations found in Bristol are similar to another study in the United Kingdom, which found concentrations up to 114 (± 15) ng/L. (Castrignanò et al., 2016) These data also follow that from the UNODC, whereit is stated that mephedrone has the highest prevalence rate of any NPS in the United Kingdom(UNODC, 2014). The only other compounds confirmed in our study were methylone and MDPV, with concentrations ranging from below LOQ to 12 ng/L (methylone) and 6 ng/L (MDPV), with these low concentrations in line with previous studies in Croatia and Switzerland (Kinyua et al., 2015a; Senta et al., 2015). These overall results are also comparable with EMCDDA seizure data, with mephedrone (20%), methylone (7%) and MDPV (9%) some of the most commonly seized synthetic cathinones. Furthermore, the United Kingdom had one of the most synthetic cathinone seizures in Europe, implying a high consumption of these NPS (EMCDDA, 2015b).

Figure 2 shows the confirmation of MDPV, mephedrone and methylonein three IWW samples. All show the compliance of all the necessary criteria: retention time compatibility with the standard (in the form of the QC), at least two transitions, and ion ratio within 30% for at least one confirmation transition. It must be noted that the q2/Q ratio for both MDPV and methylone are outside the accepted ±30% ion ratio window, but as the q1/Q ratio for both are within the window, both can be confirmed.

383 *3.6.1 Occurrence of false positives*

384 With quantification methods, it is easy to distinguish positive findings from negative,
385 using strict criteria such as ion ratio (±30%), retention time (± 0.1 min) and at least one
386 additional, confirmatory transition. However, there are some scenarios where the rulings
387 are not so obvious.

388 For some compounds, only one, the most sensitive, transition may be observed, but may
389 not be confirmed with a subsequent transition, suggesting that the compound could be
390 present, although only at very low concentrations. If criteria are not strictly adhered to,
391 this could lead to false positive findings. This was the case for MDPV in a sample from
392 Zurich (**Figure S8**). Within this sample, the Q transition was found at the correct
393 retention time, but the two q transitions were between 0.09-0.2 minutes away. This
394 suggests that two different compounds were present in the samples showing the above
395 transitions. Even though the shift in retention time itself is enough to reject the detection
396 of this compound, the ion ratios were still checked. The q1 transition was found within
397 the q/Q ratio threshold (30%). Although the q2 ratio was more than 100% lower than
398 expected, if the sample was not well checked, it could have been erroneously assigned
399 as a false positive on the basis of one q/Q ratio accomplishment.

400 **Figure S8** also shows the curious case of butylone, which seemed to have been detected
401 in an IWW sample from Bristol. As this figure shows, all three transitions can clearly be
402 seen, albeit at a retention time 0.17 minutes lower than in the quality control. Although
403 one q/Q ratio is within the ±30% threshold, the fact that the retention time is greater
404 than 0.10 minutes from the standard is enough evidence to say that this is not butylone.
405 However, with all the same transitions present, there is a possibility that this compound
406 could be related to butylone. Due to the retention time being less than the reference

standard, this compound could be a metabolite, which, due to its more polar characteristics would lead it to elute earlier. Alternatively, it could be a related cathinone-derivative, for example ethylone, which has the same fragmentation, and has been found in previous European samples (Kinyua et al., 2015a). However, more research is needed to confirm its identity.

Conclusions

A very sensitive analytical method based on UHPLC-MS/MS has been developed for the determination of ten NPS of the synthetic cathinone and phenethylamine classes in influent wastewater. The overall analytical procedure, based on an off-line SPE step using Oasis MCX cartridges prior to the determination by UHPLC–MS/MS using a triple quadrupole analyzer, has been fully validated, obtaining satisfactory accuracy and precision. Extra emphasis was made on the SPE cartridge, clean-up step and matrix effects. An additional study was also made into the stability of these compounds in five matrices at -20°C, 4°C and 20°C, with advice given on the optimal means of storage.

The method was tested on samples from throughout Europe, with mephedrone, methylone and MDPV able to be identified in various samples. These data support the applicability of the method to influent wastewaters, despite the very low concentrations observed, and also support the previous information available on NPS use in Europe, where these compounds are in agreement with prevalence and seizure data.

In the future, with information on flow data for the IWW plants and metabolic data to correct for excretion, this method could be used to obtain accurate analytical concentrations which subsequently could allow the estimation of NPS consumption within a population.

Contributions

Richard Bade (RB), Lubertus Bijlsma (LB), Juan V. Sancho (JVS) and Félix Hernández(FH) planned and designed the study. LB, Jose. A. Baz-Lomba, Sara Castiglioni, Erika Castrignanò, Ana Causanilles, Emma Gracia-Lor, Barbara Kasprzyk-Hordern, Juliet Kinyua, Ann-Kathrin McCall, Alexander L.N. van Nuijs, Christoph Ort, Benedek G. Plósz, Pedram Ramin, Nikolaos I. Rousis, Yeonsuk Ryu, Kevin V. Thomas,

Pim de Voogt and Ettore Zuccato organised the collection of the wastewater samples. RB and LB developed the method and RB performed the validation. RB analysed the samples and interpreted the results with contribution from all co-authors. RB, with significant contributions from LB, JVS and FH drafted the manuscript, which was critically revised by all co-authors. All authors are aware of the content, and accept responsibility, for the manuscript.

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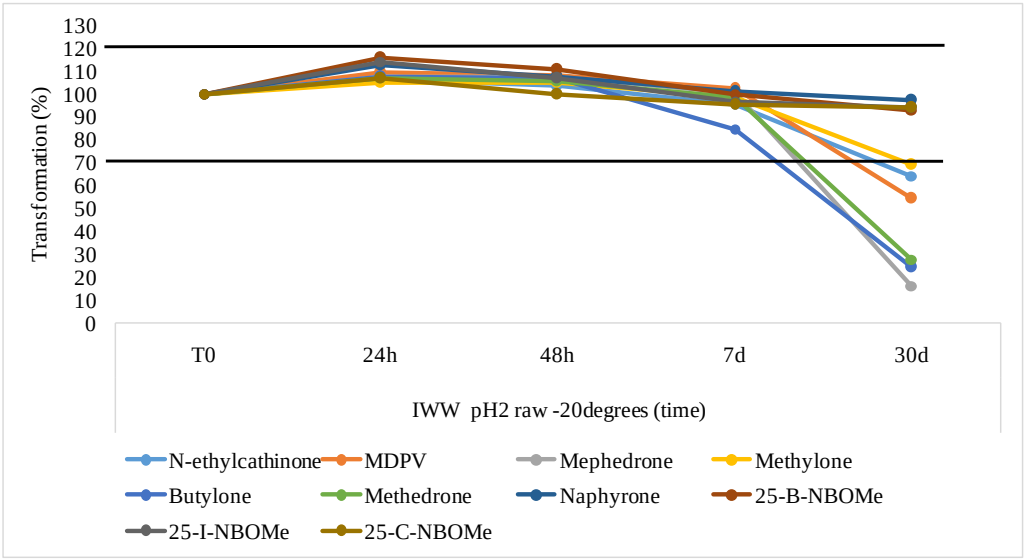
610

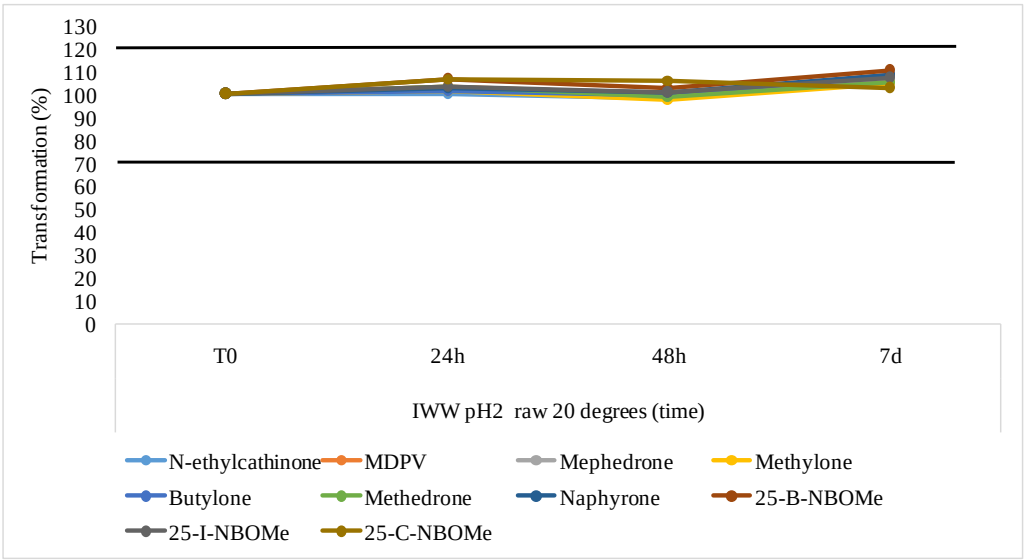
Figure Captions:

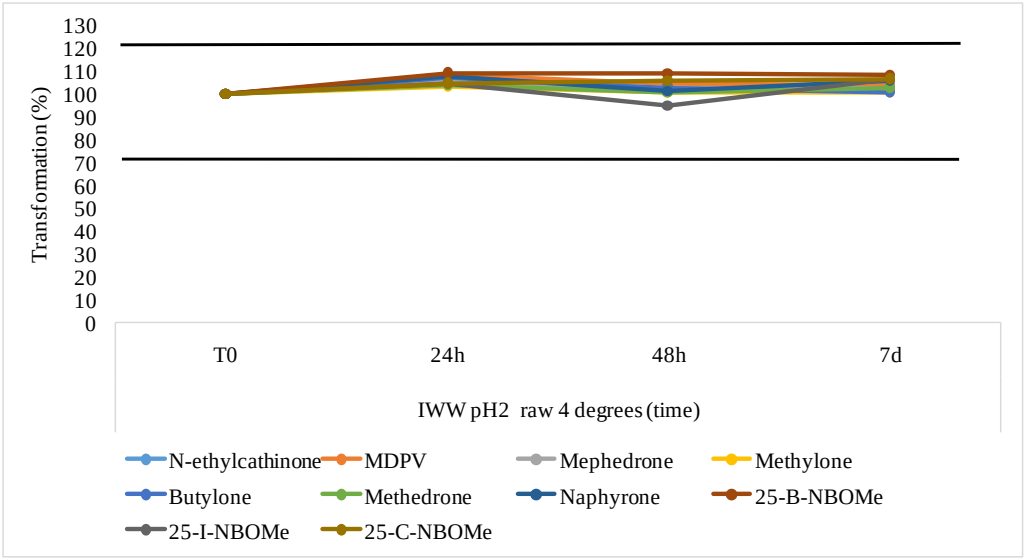
Figure 1: Stability of all compounds in raw IWW at (pH 2) at 20ºC (TOP), 4ºC (MIDDLE) and -20ºC (BOTTOM). Transformation (y axis) shows the value of the analyte remaining (i.e. a value of 20% means a transformation of 80%). The acceptable interval of 70-120% has also been included (black lines).

Figure 2: Positive findings (from left to right) of MDPV, mephedrone and methylone in IWW of Milan, Bristol and Copenhagen, respectively. The uppermost transition for each compound is the confirmation (Q) transition in the QC (low, 5 ng/L), while the lower three are all from the sample. All include three transitions, and the q/Q ratio, together with the deviation from the QC.

623 **Figure 1:**

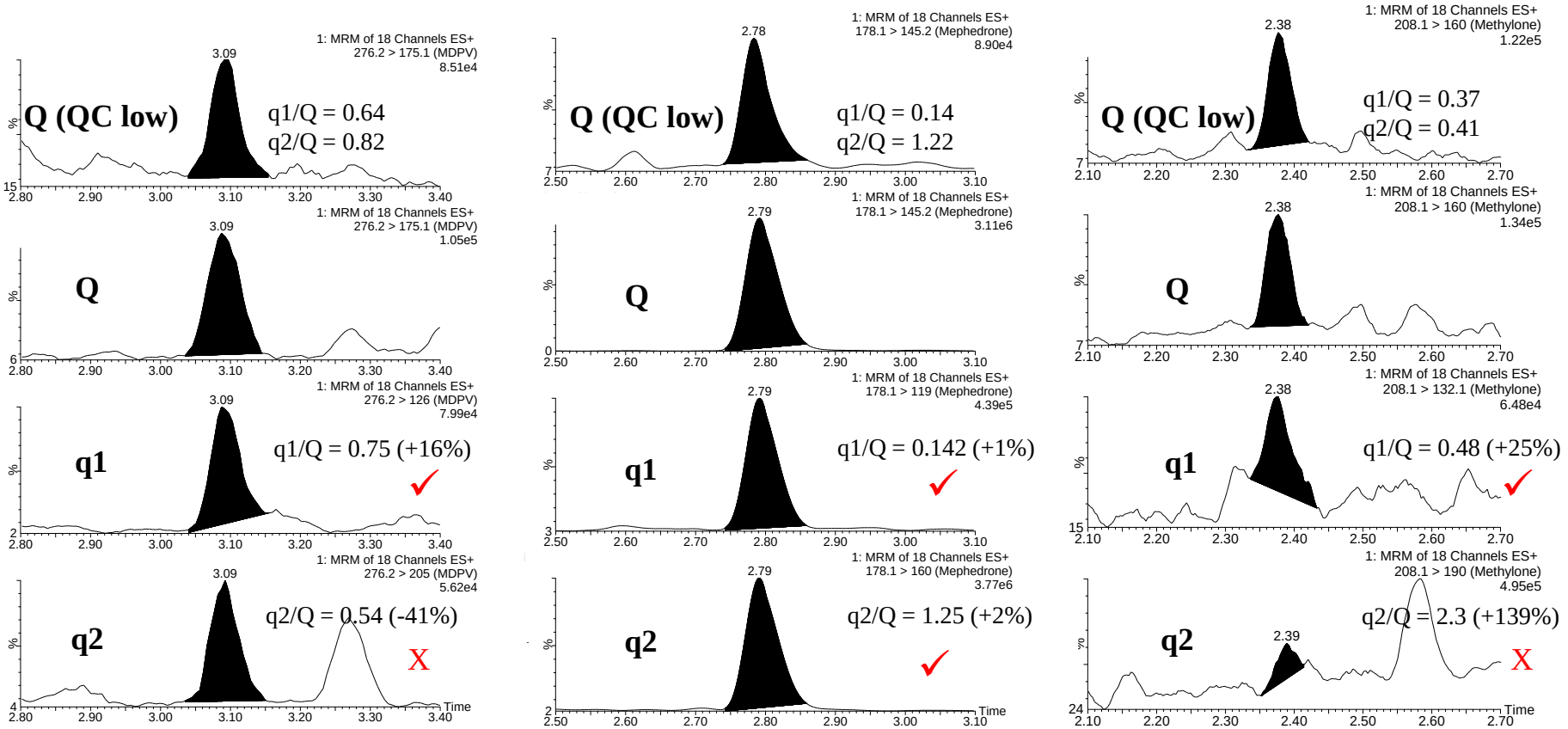






624

Figure 2:


1: MRM of 18 Channels ES+
276.2 > 175.1 (MDPV)
8.51e4
Q (QC low)
q1/Q = 0.64
q2/Q = 0.82
1: MRM of 18 Channels ES+
276.2 > 175.1 (MDPV)
1.05e5
Q
1: MRM of 18 Channels ES+
276.2 > 126 (MDPV)
7.99e4
q1
q1/Q = 0.75 (+16%)
1: MRM of 18 Channels ES+
276.2 > 205 (MDPV)
5.62e4
q2
q2/Q = 0.54 (-41%)
X
Time
1: MRM of 18 Channels ES+
178.1 > 145.2 (Mephedrone)
8.90e4
Q (QC low)
q1/Q = 0.14
q2/Q = 1.22
1: MRM of 18 Channels ES+
178.1 > 145.2 (Mephedrone)
3.11e6
Q
1: MRM of 18 Channels ES+
178.1 > 119 (Mephedrone)
4.39e5
q1
q1/Q = 0.142 (+1%)
1: MRM of 18 Channels ES+
178.1 > 160 (Mephedrone)
3.77e6
q2
q2/Q = 1.25 (+2%)
Time
1: MRM of 18 Channels ES+
208.1 > 160 (Methylone)
1.22e5
Q (QC low)
q1/Q = 0.37
q2/Q = 0.41
1: MRM of 18 Channels ES+
208.1 > 160 (Methylone)
1.34e5
Q
1: MRM of 18 Channels ES+
208.1 > 132.1 (Methylone)
6.48e4
q1
q1/Q = 0.48 (+25%)
1: MRM of 18 Channels ES+
208.1 > 190 (Methylone)
4.95e5
q2
q2/Q = 2.3 (+139%)
X
Time

627 **Table 1:** MS/MS optimised conditions for all studied compounds

Compound	Retention time (mins)	Precursor ion $[M+H]^+$ (m/z)	Cone Voltage (V)	Collision Energy (eV)	Product Ions (m/z)	LOQ[a] (ng/L)	LOD[b] (pg L^{-1})	q/Q ratio (RSD %)
25-B-NBOMe	3.76	380.1	40	20	121.0	1	100	
				30	91.0			0.4 (6)
25-C-NBOMe	3.70	336.1	20	20	121.1	5	70	
				40	91.0			0.6 (6)
25-I-NBOMe	3.86	428.1	20	20	121.0	5	100	
				50	91.0			0.5 (6)
Butylone	2.68	222.1	20	20	174.0	5	200	
				20	146.0			0.3 (6)
				30	131.2			0.3 (7)
Mephedrone	2.77	178.1	30	20	145.2	5	200	
				20	119.0			0.1 (11)
				10	160.0			1.3 (7)
Methedrone	2.57	194.1	20	20	161.1	5	200	
				20	146.0			0.4 (4)
MDPV	3.11	276.2	50	20	175.1	1	200	

				20	126.0			0.6 (5)
				20	205.0			0.8 (9)
Methylone	2.36	208.1	50	20	160.0	5	50	
				20	132.1			0.4 (6)
				10	190.0			0.5 (11)
Naphyrone	3.71	282.2	50	20	141.2	1	50	
				20	211.1			0.5 (6)
				40	127.4			0.2 (6)
N-ethylcathinone	2.42	178.1	20	10	130.0	5	200	
				20	117.1			1.1 (7)
				30	160.1			0.7 (10)
25-B-NBOMe-d_3	3.76	383.3	40	20	124.0			
25-C-NBOMe-d_3	3.70	339.1	20	20	124.0			
25-I-NBOMe-d_3	3.86	431.1	20	20	124.0			
Butylone-d_3	2.68	225.1	20	20	177.1			
Mephedrone-d_3	2.77	181.1	30	20	145.2			
MDPV-d_8	3.11	284.2	50	20	205.0			
Methylone-d_3	2.36	211.1	50	20	163.1			
Naphyrone-d_5	3.71	287.2	50	20	141.2			

[a]LOQ objective. Lowest concentration tested for the method being fully validated with satisfactory results.

[b]LOD estimated for a signal-to-noise level of three from the quantification SRM chromatogram of blank samples spiked at the lowest analyte concentration tested.

Table 2: Method validation for all compounds in IWW at 2 validation levels (all values are in %), together with recovery and matrix effects before and after correction with internal standard (n=5). Linearity is also shown.

Compound	Matrix effects (%)[a]		q1/Q ratio		q2/Q ratio		SPE Recovery (%)				Linearity
			Low[b]	High[c]	Low[b]	High[c]	Low[b]		High[c]		
		No ILIS						No ILIS		No ILIS	
25-B-NBOMe	-4	60	-11 (12)	-9 (4)	-	-	106 (7)	87 (13)	104 (4)	89 (16)	0.9722
25-C-NBOMe	-7	50	-7 (8)	-2 (0)	-	-	92 (9)	89 (14)	97 (7)	90 (15)	0.9737
25-I-NBOMe	-6	66	5 (8)	-13 (1)	-	-	98 (9)	85 (16)	103 (6)	86 (16)	0.9722
Butylone	-6	29	-13 (12)	-4 (6)	24 (26)	-11 (4)	100(10)	91 (13)	101 (4)	89 (8)	0.9923
MDPV	-7	48	6 (8)	10 (4)	17 (13)	9 (8)	98 (10)	94 (8)	98 (9)	91 (10)	0.9900
Mephedrone	-9	26	-6 (11)	12 (4)	0 (5)[d]	15 (4)[d]	94 (10)	90 (8)	97 (7)	85 (6)	0.9914
Methedrone	-10	31	-12 (4)	-7 (6)	-	-	103 (8)	83 (7)	104 (5)	87 (7)	0.9923
Methylone	-6	30	6 (7)	-1 (8)	[d]	[d]	101 (8)	93 (4)	101 (4)	84 (7)	0.9919
Naphyrone	-10	62	-5 (5)	-7 (9)	-5 (9)	-13 (3)	108 (10)	86 (10)	101 (8)	87 (15)	0.9883
N-ethylcathinone	3	27	-33 (21)	1 (7)	[d]	5 (6)[d]	96 (7)	74 (12)	98 (7)	83 (5)	0.9975

ILIS (Isotopically-labelled Internal Standard)

[a]: for matrix effects, a negative value (-) denotes matrix suppression, while positive values indicate matrix enhancement.

[b]: Low refers to the LOQ value of all compounds: 1 ng/L for MDPV, Naphyrone and 25-B-NBOMe and 5 ng/Lfor all other compounds

[c]: High refers to the 10xLOQ value of all compounds: 10 ng/L for MDPV, Naphyrone and 25-B-NBOMe and 50 ng/Lfor all other compounds

[d]: These transitions were for a loss of water. The non-specificity associated with these transitions combined with the complex IWW matrix led to some of these transitions being non-observed/interfered. However, as they were all q2 transitions, the method could still be validated based on q1.

638 **Table 3:** *Concentrations (in ng/L) of investigated NPS in IWW throughout Europe (weekday/weekend)*

Compound	Bristol	Brussels	Castellon	Copenhagen	Milan	Oslo	Utrecht	Zurich
N-ethylcathinone	-	-	-	-	-	-	-	-
MDPV	-	-	-	-	-/3	-/2	-	6/-
Mephedrone	61/106	d/-	-	5/5	-	d/d	-/d	-/d
Methylone	-/d	-	-	-/12	-	-	7/5	-
Butylone	-	-	-	-	-	-	-	-
Methedrone	-	-	-	-	-	-	-	-
Naphyrone	-	-	-	-	-	-	-	-
25-B-NBOMe	-	-	-	-	-	-	-	-
25-I-NBOMe	-	-	-	-	-	-	-	-
25-C-NBOMe	-	-	-	-	-	-	-	-

639 d: detected, at a concentration below LOQ; -: not detected (<LOD)

640

641