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

Richard Bade[a], Lubertus Bijlsma[a], Juan V. Sancho[a], Jose A. Baz-Lomba[b,c], Sara Castiglioni[d], Erika Castrignanò[e], Ana Causanilles[f], Emma Gracia-Lor[a,d], Barbara Kasprzyk-Hordern[e], Juliet Kinyua[h], Ann-Kathrin McCall[i], Alexander L.N. van Nuijs[h], Christoph Ort[i], Benedek G. Plósz[j], Pedram Ramin[j], Nikolaos I. Rousis[d], Yeonsuk Ryu[b,c], Kevin V. Thomas[b], Pim de Voogt[f,g], Ettore Zuccato[d] , Félix Hernández[a,*]

[a] Research Institute for Pesticides and Water, University Jaume I, Avda. Sos Baynat s/n, E-12071 Castellón, Spain.

[b] Norwegian Institute for Water Research (NIVA), Gaustadalléen 21, 0349 Oslo, Norway

[c] Faculty of Medicine, University of Oslo, PO box 1078 Blindern, 0316, Oslo, Norway

[d] IRCCS—Istituto di Ricerche Farmacologiche "Mario Negri", Department of Environmental Health Sciences, Via La Masa 19, 20156 Milan, Italy

[e] University of Bath, Department of Chemistry, Faculty of Science, Bath BA2 7AY, UK

[f] KWR Watercycle Research Institute, Chemical Water Quality and Health, P.O. Box 1072, 3430 BB, Nieuwegein, The Netherlands

[g] Institute for Biodiversity and Ecosystem Dynamics, University of Amsterdam, P.O. Box 94248, 1090 GE, Amsterdam, The Netherlands

[h] Toxicological Center, Department of Pharmaceutical Sciences, Campus Drie Eiken, University of Antwerp, Universiteitsplein 1, 2610 Antwerp, Belgium

[i] Eawag, Swiss Federal Institute of Aquatic Science and Technology, CH-8600, Dübendorf, Switzerland

[j] Department of Environmental Engineering, Technical University of Denmark, Miljøvej, Building 115, DK-2800 Kgs. Lyngby, Denmark

*Corresponding author: felix.hernandez@uji.es Tel.: +34 964 387366 Fax: +34 964 387368

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).

57 Key Words: New psychoactive substances, Ultra high-performance liquid

58 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

108   made. Special emphasis is placed on the reliable confirmation of the NPS detected in

109   water, with up to three SRM transitions being acquired, which, together with ion ratios,

110   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

134   gas was dry nitrogen, with flows set to 150 and 800 L h^{-1}, respectively. For operation in

135   MS/MS mode, collision gas was argon 99.995 % (Praxair, Madrid, Spain) with a

136   pressure of 4×10−3 mbar in the collision cell (0.15 mL min^{-1}). Further parameters

137   optimized were: capillary voltage, 3.0 kV; source temperature, 150 °C and desolvation

138   temperature, 650 °C. Dwell times of 0.01 s/transition were automatically selected.

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

140   UK).

141   *2.4 Sample Pre-treatment*

142   All water samples were acidified to pH 2 with formic acid (98%), then centrifuged for 5

143   minutes at 6000 rpm. SPE cartridges were conditioned with methanol (6mL), water

144   (3mL) and acidified water (pH 2 with formic acid, 3mL). The IWW samples (100mL)

145   were spiked with mixed surrogate ILIS to give a final in sample concentration of 20

146   ng/L, then percolated through the cartridges at ±5 mL/min. The cartridges were then

147   washed with 5 mL acidified methanol (pH 2 with formic acid) and dried under vacuum

148   for 10 minutes. The analytes were eluted with 5 mL MeOH (2% ammonia), evaporated

149   to dryness at 35ºC under nitrogen and reconstituted to 1mL with 10:90 methanol:water.

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

151   system.

2.5 Quantification and Method Validation

152

153 SRM mode was used to acquire all data, with the precursor ion of each compound being

154 the protonated molecule. In general, the most abundant product ion of each target

155 analyte was used for quantification, with one or two additional product ions and their

156 ion ratios used for confirmation. Furthermore, LC retention time was compared to that

157 of reference standards (tolerance of $\pm$ 0.1 minutes). These steps are in line with the

158 SANCO guidelines for analytical quality control and validation procedures (SANCO,

159 2013), which has been utilised previously by research groups for quantification of illicit

160 drugs in wastewater (Bijlsma et al., 2009). Each compound was quantified using its

161 ILIS as a surrogate internal standard, except N-ethylcathinone and methedrone, which

162 were quantified using butylone-d$_3$ and methylone-d$_3$, respectively. These ILIS were

163 chosen based on their ability to correct for matrix effects as well as having similar

164 retention times and chemical structures closely related to the analyte of interest.

165 The performance of the method was evaluated in terms of linearity, limits of detection

166 and quantification, accuracy and precision.

167 The linearity was studied by analysing standard solutions in solvent in triplicate at seven

168 concentration levels ranging from 0.5-100 ng/L. Linearity was deemed satisfactory

169 when the correlation coefficient (r) was >0.99, using weighted (1/X) least squares

170 regression.

171 For limits of quantification and detection (LOQ and LOD), two different concentration

172 levels (1 and 5 ng/L) were tested so as to provide a more accurate measure. The limit of

173 quantification (LOQ) was established as the lowest level for which the method was fully

174 validated using spiked IWW samples with satisfactory accuracy based on recovery

175 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

213 methylone, mephedrone and N-ethylcathinone where they were used as confirmation

214 transitions) in order to reduce the likelihood of false positives (Bijlsma et al., 2009). For

215 the three 25-X-NBOMe compounds, the same product ions were seen (m/z 121 and 91),

216 corresponding to the cleavage of the methoxybenzoyl moiety (m/z 121) and the further

217 loss of the methoxy group producing the tropylium ion (m/z 91). These transitions are

218 not very specific, and can come from any compound with a methoxybenzoyl moiety,

219 however as there were no other transitions available for these compounds, they had to

220 be used as quantification and confirmation transitions. Regarding the ILIS, only a single

221 transition was monitored.

222 The chromatographic separation was initially based on a mobile phase composed of

223 water:MeOH, which resulted in very poor peak shapes. Different concentrations of

224 ammonium acetate buffer and pH (formic acid) were investigated and the effects of pH

225 and mobile phase ionic strength on the peak shapes, resolution and efficiencies were

226 evaluated by varying the buffer concentration. An optimal mobile phase consisting of

227 water with 5 mM ammonium acetate and 0.01% formic acid (solvent A) and MeOH

228 with 0.01% formic acid (solvent B) was finally selected.

229 *3.3 Optimisation of extraction procedure*

230 An investigation into the filtration losses of these compounds was made in samples at

231 both natural and pH 2, using a vacuum filter (Whatman, 0.45μm mixed cellulose ester

232 membrane). It was found that for naphyrone and the 25-X-NBOMe compounds, less

233 than 5% of the compounds were recovered following filtration, while for MDPV a

234 recovery of 55-71% was found. (**Table S1**) With these results, filtration was not

235 employed in this method, instead only centrifugation.

236   Two cartridges were evaluated in the optimisation of the SPE procedure: Oasis HLB

237   and Oasis MCX, which are two of the most popular cartridges for the extraction of

238   different types of illicit drugs in environmental waters (Baker and Kasprzyk-Hordern,

239   2011). The backbone of the Oasis sorbents consists of apolar moieties (benzyl groups,

240   aliphatic chains) and polar groups (pyrrolidone). HLB is only synthesised from these

241   two monomers and is neutral. MCX has extra sulfonic acid functional groups

242   (1.01mmol/g) implemented at a fixed location and contain permanently charged groups

243   at any relevant pH (Bäuerlein et al., 2012).For both cartridges, 100 mL of IWW was

244   spiked with the compounds (100 ng/L) before and after extraction and the recovery was

245   used to determine the better cartridge for further optimisation. No pH adjustment was

246   made for the Oasis HLB cartridges, while for the MCX, samples were acidified (pH 2)

247   in order to protonate the basic analytes, thereby enhancing their ionic interactions with

248   the sorbent (Senta et al., 2015). This led to a reduction in matrix interferences, giving

249   higher sensitivity and peak shapes for the majority of the compounds (**Figure S2**). The

250   SPE procedure followed for the HLB cartridges was the same as in previously published

251   studies. (Bade et al., 2015; Bijlsma et al., 2014)

252   Higher recoveries were obtained when using Oasis MCX cartridges (**Figure S3**) and

253   therefore these were selected for further optimisation. For the washing step, water, pure

254   MeOH and acidified MeOH (pH 2) were tested. Drying under vacuum (or not) was also

255   tested, to determine the optimal washing/drying steps (**Figure S3**).

256   As seen in **Figure S3**, the optimal SPE procedure involved washing with 5mL acidified

257   methanol and drying for 10 minutes, with recoveries ranging from 83%-93% (**Figure

258   S3, MCX 3**).

259 In previous methods for NPS using MCX cartridges, different percentages of ammonia

260 in methanol have been used for elution, ranging from 0.5% to 4% (Kinyua et al., 2015a;

261 Reid et al., 2014; Senta et al., 2015; van Nuijs et al., 2013). A compromise of 2% was

262 utilised in this work, and showed good recoveries for all compounds. An elution volume

263 of 5mL of 2% ammonia in MeOH solution was finally chosen. This was based on the

264 comparison of the recoveries of 5mL and 10mL elution volumes, with recoveries only

265 2% less for the 5mL than the 10mL.

266 *3.4 Method Validation*

267 Before applying the developed methodology to IWW samples, the method was fully

268 validated for linearity, precision and accuracy (recovery), LOD, LOQ and q/Q ratio

269 (**Tables 1** and **2**). A calibration curve of seven points was made in solvent from 0.5-100

270 ng/L (in triplicate). Correlation coefficients were greater than 0.99 for all compounds,

271 except for naphyrone and the NBOMe compounds, which had values between 0.97-

272 0.98. For precision and accuracy, blank IWW samples were spiked at four concentration

273 levels (1 ng/L, 5 ng/L, 10 ng/L and 50 ng/L), and analysed in quintuplicate. These two

274 sets correspond to the different LOQs of some of the compounds (either1 ng/Lor 5 ng/L,

275 see **Table 1**). Accuracy was measured by means of recovery, with all compounds

276 showing satisfactory recovery (between 70-120%), and precision (all within RSD 20%).

277 LOQs were estimated from a non-statistical point of view, utilising an LOQ objective:

278 the lowest level in sample for which the method was fully validated in terms of

279 accuracy and precision. This criterion has been utilised in the past in published results

280 (Bijlsma et al., 2009) and is used in other fields, such as pesticide residue analysis

281 (SANCO, 2013). This gives a   more realistic LOQ value, albeit generally higher than

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

307   These calculations showed the true impact of the matrix on the compounds, with all

308   showing significant matrix enhancement (**Table 2**). To correct for these interferences,

309   ILIS were included in the calculation:

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

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

312   compounds.

313   Additional data pertaining to the use of ILIS is shown in **Table 2**, for SPE recovery and

314   matrix effects, showing how well the ILIS are able to correct for matrix interferences.

3.5 In-sample Stability

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

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

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

339   compounds: mephedrone, methedrone and butylone all exhibiting low stability (up to

340   84% transformation); N-ethylcathinone, MDPV and methylone showing medium

341   stability while the 25-X-NBOMe compounds and naphyrone still had high stability (less

342   than 10% transformation) (**Figure 1**). All other results of the stability tests (i.e. in raw

343   unacidified wastewater as well as both acidified and unacidified filtered wastewater and

344   Milli-Q water) can be found in the supporting information (**Figures S4-S7**). These

345   results also mirror those of the recent studies of Senta *et al*(Senta et al., 2015), and Reid

346   *et al* (Reid et al., 2014), who investigated the stability of some of these compounds.

347   Although compounds were found to be stable at pH 2 and -20ºC for at least one week,

348   storage at lower temperatures have advantages in terms of green and sustainability

349   analytical chemistry such as energy and cost saving. Furthermore, storage at -20ºC is

350   sometimes limited and not always feasible for wastewater treatment plants and/or

351   laboratories. In this regard, storage at 4ºC is possible for at least one week as tested in

352   the current study.

353   *3.6 Application to Real Samples*

354   A selection of samples from throughout Europe were investigated for the presence of

355   NPS. In total, IWW from eight cities were investigated: Bristol, United Kingdom;

356   Brussels, Belgium; Castellon, Spain; Oslo, Norway; Copenhagen, Denmark; Milan,

357   Italy; Utrecht, The Netherlands and Zurich, Switzerland, with samples from weekends

358   (Saturday and Sunday) and weekdays (Monday-Friday).

359   In every sequence of analysis, water samples were injected between two calibration

360   curves. Two quality control samples (QCs), i.e. a blank water sample (previously

361   analyzed) spiked at LOQ and 10×LOQ levels, were also analysed. QC recoveries were

362   considered satisfactory when they were in the range of 70–120% for each analyte.

363   As shown in **Table 3**, mephedrone was the most commonly identified NPS, with

364   concentrations ranging from <5 ng/L (Brussels, Oslo, Copenhagen and Utrecht) to 106

365   ng/L (Bristol). The high concentrations found in Bristol are similar to another study in

366   the United Kingdom, which found concentrations up to 114 (± 15) ng/L. (Castrignanò et

367   al., 2016) These data also follow that from the UNODC, whereit is stated that

368   mephedrone has the highest prevalence rate of any NPS in the United

369   Kingdom(UNODC, 2014). The only other compounds confirmed in our study were

370   methylone and MDPV, with concentrations ranging from below LOQ to 12 ng/L

371   (methylone) and 6 ng/L (MDPV), with these low concentrations in line with previous

372   studies in Croatia and Switzerland (Kinyua et al., 2015a; Senta et al., 2015). These

373   overall results are also comparable with EMCDDA seizure data, with mephedrone

374   (20%), methylone (7%) and MDPV (9%) some of the most commonly seized synthetic

375   cathinones. Furthermore, the United Kingdom had one of the most synthetic cathinone

376   seizures in Europe, implying a high consumption of these NPS (EMCDDA, 2015b).

377   **Figure 2** shows the confirmation of MDPV, mephedrone and methylonein three IWW

378   samples. All show the compliance of all the necessary criteria: retention time

379   compatibility with the standard (in the form of the QC), at least two transitions, and ion

380   ratio within 30% for at least one confirmation transition. It must be noted that the q2/Q

381   ratio for both MDPV and methylone are outside the accepted ±30% ion ratio window,

382   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

407   standard, this compound could be a metabolite, which, due to its more polar

408   characteristics would lead it to elute earlier. Alternatively, it could be a related

409   cathinone-derivative, for example ethylone, which has the same fragmentation, and has

410   been found in previous European samples (Kinyua et al., 2015a). However, more

411   research is needed to confirm its identity.

412

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,

437 Pim de Voogt and Ettore Zuccato organised the collection of the wastewater samples.

438 RB and LB developed the method and RB performed the validation. RB analysed the

439 samples and interpreted the results with contribution from all co-authors. RB, with

440 significant contributions from LB, JVS and FH drafted the manuscript, which was

441 critically revised by all co-authors. All authors are aware of the content, and accept

442 responsibility, for the manuscript.

References

Bade, R., Rousis, N.I., Bijlsma, L., Gracia-Lor, E., Castiglioni, S., Sancho, J. V., Hernandez, F., 2015. Screening of pharmaceuticals and illicit drugs in wastewater and surface waters of Spain and Italy by high resolution mass spectrometry using UHPLC-QTOF MS and LC-LTQ-Orbitrap MS. Anal. Bioanal. Chem. 407, 8979–8988. doi:10.1007/s00216-015-9063-x

Baker, D.R., Kasprzyk-Hordern, B., 2011. Critical evaluation of methodology commonly used in sample collection, storage and preparation for the analysis of pharmaceuticals and illicit drugs in surface water and wastewater by solid phase extraction and liquid chromatography–mass spectrometry. J. Chromatogr. A 1218, 8036–8059. doi:10.1016/j.chroma.2011.09.012

Bäuerlein, P.S., ter Laak, T.L., Hofman-Caris, R.C.H.M., de Voogt, P., Droge, S.T.J., 2012. Removal of charged micropollutants from water by ion-exchange polymers - Effects of competing electrolytes. Water Res. 46, 5009–5018. doi:10.1016/j.watres.2012.06.048

Baz-Lomba, J.A., Reid, M.J., Thomas, K. V., 2016. Target and suspect screening of psychoactive substances in sewage-based samples by UHPLC-QTOF. Anal. Chim. Acta 914, 81–90. doi:10.1016/j.aca.2016.01.056

Bijlsma, L., Beltrán, E., Boix, C., Sancho, J. V, Hernández, F., 2014. Improvements in analytical methodology for the determination of frequently consumed illicit drugs in urban wastewater. Anal. Bioanal. Chem. 406, 4261–4272. doi:10.1007/s00216-014-7818-4

Bijlsma, L., Sancho, J. V, Pitarch, E., Ibanez, M., Hernandez, F., 2009. Simultaneous ultra-high-pressure liquid chromatography-tandem mass spectrometry determination of amphetamine and amphetamine-like stimulants, cocaine and its metabolites, and a cannabis metabolite in surface water and urban wastewater. J. Chromatogr. a 1216, 3078–3089. doi:10.1016/j.chroma.2009.01.067

Borova, V.L., Gago-Ferrero, P., Pistos, C., Thomaidis, N.S., 2015. Multi-residue determination of 10 selected new psychoactive substances in wastewater samples by liquid chromatography–tandem mass spectrometry. Talanta 144, 592–603. doi:10.1016/j.talanta.2015.06.080

Caspar, A.T., Helfer, A.G., Michely, J.A., Auwarter, V., Brandt, S.D., Meyer, M.R., Maurer, H.H., 2015. Studies on the metabolism and toxicological detection of the new psychoactive designer drug 2-(4-iodo-2,5-dimethoxyphenyl)-N-[(2-methoxyphenyl)methyl]ethanamine (25I-NBOMe) in human and rat urine using GC-MS, LC-MS, and LC-HR-MS/MS. Anal Bioanal Chem 6697–6719. doi:10.1007/s00216-015-8828-6

Castrignanò, E., Lubben, A., Kasprzyk-Hordern, B., 2016. Enantiomeric profiling of chiral drug biomarkers in wastewater with the usage of chiral liquid chromatography coupled with tandem mass spectrometry. J. Chromatogr. A 1438, 84–99. doi:10.1016/j.chroma.2016.02.015

Chen, C., Kostakis, C., Irvine, R.J., White, J.M., 2013. Increases in use of novel synthetic stimulant are not directly linked to decreased use of 3,4-methylenedioxy-N-methylamphetamine (MDMA). Forensic Sci. Int. 231, 278–283.

507   doi:10.1016/j.forsciint.2013.06.007

508   Elliott, S., Evans, J., 2014. A 3-year review of new psychoactive substances in
509   casework. Forensic Sci. Int. 243, 55–60. doi:10.1016/j.forsciint.2014.04.017

510   EMCDDA, 2015a. European Drug Report: Trends and Developments.

511   EMCDDA, 2015b. New psychoactive substances in Europe. EU early Warn. Syst. 12.
512   doi:10.2810/372415

513   Ibáñez, M., Pozo, Ó.J., Sancho, J. V., Orengo, T., Haro, G., Hernández, F., 2016.
514   Analytical strategy to investigate 3,4-methylenedioxypyrovalerone (MDPV)
515   metabolites in consumers' urine by high-resolution mass spectrometry. Anal.
516   Bioanal. Chem. 408, 151–164. doi:10.1007/s00216-015-9088-1

517   Ibáñez, M., Sancho, J. V., Bijlsma, L., van Nuijs, A.L.N., Covaci, A., Hernández, F.,
518   2014. Comprehensive analytical strategies based on high-resolution time-of-flight
519   mass spectrometry to identify new psychoactive substances. TrAC Trends Anal.
520   Chem. 57, 107–117. doi:10.1016/j.trac.2014.02.009

521   Kamata, H.T., Shima, N., Zaitsu, K., Kamata, T., Miki, A., Nishikawa, M., Katagi, M.,
522   Tsuchihashi, H., 2006. Metabolism of the recently encountered designer drug,
523   methylone, in humans and rats. Xenobiotica 36, 709–723.
524   doi:10.1080/00498250600780191

525   Kankaanpää, A., Ariniemi, K., Heinonen, M., Kuoppasalmi, K., Gunnar, T., 2014. Use
526   of illicit stimulant drugs in Finland: A wastewater study in ten major cities. Sci.
527   Total Environ. 487, 696–702. doi:10.1016/j.scitotenv.2013.11.095

528   Kinyua, J., Covaci, A., Maho, W., McCall, A.-K., Neels, H., van Nuijs, A.L.N., 2015a.
529   Sewage-based epidemiology in monitoring the use of new psychoactive
530   substances: Validation and application of an analytical method using LC-MS/MS.
531   Drug Test. Anal. 7, 812–818. doi:10.1002/dta.1777

532   Kinyua, J., Negreira, N., Ibáñez, M., Bijlsma, L., Hernández, F., Covaci, A., van Nuijs,
533   A.L.N., 2015b. A data-independent acquisition workflow for qualitative screening
534   of new psychoactive substances in biological samples. Anal. Bioanal. Chem. 407,
535   8773–8785. doi:10.1007/s00216-015-9036-0

536   Lai, F.Y., Erratico, C., Kinyua, J., Mueller, J.F., Covaci, A., van Nuijs, A.L.N., 2015.
537   Liquid chromatography-quadrupole time-of-flight mass spectrometry for screening
538   in vitro drug metabolites in humans: investigation on seven phenethylamine-based
539   designer drugs. J. Pharm. Biomed. Anal. 114, 355–375.
540   doi:10.1016/j.jpba.2015.06.016

541   Mardal, M., Meyer, M.R., 2014. Studies on the microbial biotransformation of the novel
542   psychoactive substance methylenedioxypyrovalerone (MDPV) in wastewater by
543   means of liquid chromatography-high resolution mass spectrometry/mass
544   spectrometry. Sci. Total Environ. 493, 588–595.
545   doi:10.1016/j.scitotenv.2014.06.016

546   Matuszewski, B.K., Constanzer, M.L., Chavez-Eng, C.M., 2003. Strategies for the
547   assessment of matrix effect in quantitative bioanalytical methods based on HPLC-
548   MS/MS. Anal. Chem. 75, 3019–3030. doi:10.1021/ac020361s

549   McCall, A.-K., Bade, R., Kinyua, J., Lai, F.Y., Thai, P.K., Covaci, A., Bijlsma, L., van
550   Nuijs, A.L.N., Ort, C., 2016. Critical review on the stability of illicit drugs in
551   sewers and wastewater samples. Water Res. 88, 933–947.
552   doi:10.1016/j.watres.2015.10.040

553   Meyer, M.R., Du, P., Schuster, F., Maurer, H.H., 2010. Studies on the metabolism of
554   the α-pyrrolidinophenone designer drug methylenedioxy-pyrovalerone (MDPV) in
555   rat and human urine and human liver microsomes using GC-MS and LC-high-
556   resolution MS and its detectability in urine by GC-MS. J. Mass Spectrom. 45,
557   1426–1442. doi:10.1002/jms.1859

558   Mwenesongole, E.M., Gautam, L., Hall, S.W., Waterhouse, J.W., Cole, M.D., 2013.
559   Simultaneous detection of controlled substances in waste water. Anal. Methods 5,
560   3248–3254. doi:10.1039/c3ay40655e

561   Ort, C., van Nuijs, A.L.N., Berset, J.-D., Bijlsma, L., Castiglioni, S., Covaci, A., de
562   Voogt, P., Emke, E., Fatta-Kassinos, D., Griffiths, P., Hernández, F., González-
563   Mariño, I., Grabic, R., Kasprzyk-Hordern, B., Mastroianni, N., Meierjohann, A.,
564   Nefau, T., Östman, M., Pico, Y., Racamonde, I., Reid, M., Slobodnik, J., Terzic,
565   S., Thomaidis, N., Thomas, K. V, 2014. Spatial differences and temporal changes
566   in illicit drug use in Europe quantified by wastewater analysis. Addiction 109,
567   1338–1352. doi:10.1111/add.12570

568   Poklis, J.L., Nanco, C.R., Troendle, M.M., Wolf, C.E., Poklis, A., 2014. Determination
569   of 4-bromo-2,5-dimethoxy-N-[(2-methoxyphenyl)methyl]-benzeneethanamine
570   (25B-NBOMe) in serum and urine by high performance liquid chromatography
571   with tandem mass spectrometry in a case of severe intoxication. Drug Test. Anal.
572   6, 764–769. doi:10.1002/dta.1522

573   Pozo, Ó.J., Sancho, J. V., Ibáñez, M., Hernández, F., Niessen, W.M. a., 2006.
574   Confirmation of organic micropollutants detected in environmental samples by
575   liquid chromatography tandem mass spectrometry: Achievements and pitfalls.
576   TrAC Trends Anal. Chem. 25, 1030–1042. doi:10.1016/j.trac.2006.06.012

577   Reid, M., Thomas, K., 2016. New Psychoactive Substances: analysis and site-specific
578   testing, in: Castiglioni, S. (Ed.), Assessing Illicit Drugs in Wastewater: Advances
579   in Wastewater-Based Drug Epidemiology. pp. 57–65.

580   Reid, M.J., Derry, L., Thomas, K. V., 2014. Analysis of new classes of recreational
581   drugs in sewage: Synthetic cannabinoids and amphetamine-like substances. Drug
582   Test. Anal. 6, 72–79. doi:10.1002/dta.1461

583   SANCO, 2013. Guidance document on analytical quality control and validation
584   procedures for pesticides residues in food and feed [WWW Document]. SANCO
585   /12571/2013. URL
586   http://ec.europa.eu/food/plant/pesticides/guidance_documents/docs/qualcontrol_en.
587   pdf

588   Senta, I., Krizman, I., Ahel, M., Terzic, S., 2015. Multiresidual analysis of emerging
589   amphetamine-like psychoactive substances in wastewater and river water. J.
590   Chromatogr. A 1425, 204–212. doi:10.1016/j.chroma.2015.11.043

591   Tang, M.H., Ching, C.K., Tsui, M.S., Chu, F.K., Mak, T.W., 2014. Two cases of severe
592   intoxication associated with analytically confirmed use of the novel psychoactive

593   substances 25B-NBOMe and 25C-NBOMe. Clin Toxicol 52, 561–565.
594   doi:10.3109/15563650.2014.909932

595 Thomas, K. V, Bijlsma, L., Castiglioni, S., Covaci, A., Emke, E., Grabic, R.,
596   Hernández, F., Karolak, S., Kasprzyk-Hordern, B., Lindberg, R.H., Lopez de Alda,
597   M., Meierjohann, A., Ort, C., Pico, Y., Quintana, J.B., Reid, M., Rieckermann, J.,
598   Terzic, S., van Nuijs, A.L.N., de Voogt, P., 2012. Comparing illicit drug use in 19
599   European cities through sewage analysis. Sci. Total Environ. 432, 432–439.
600   doi:10.1016/j.scitotenv.2012.06.069

601 UNODC, 2014. Global Synthetic Drugs Assessment: Amphetamine-type stimulants and
602   new psychoactive substances.

603 Uralets, V., Rana, S., Morgan, S., Ross, W., 2014. Testing for designer stimulants:
604   Metabolic profiles of 16 synthetic cathinones excreted free in human urine. J. Anal.
605   Toxicol. 38, 233–241. doi:10.1093/jat/bku021

606 van Nuijs, A.L.N., Gheorghe, A., Jorens, P.G., Maudens, K., Neels, H., Covaci, A.,
607   2013. Optimization, validation, and the application of liquid chromatography-
608   tandem mass spectrometry for the analysis of new drugs of abuse in wastewater.
609   Drug Test. Anal. 861–867. doi:10.1002/dta.1460

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:**
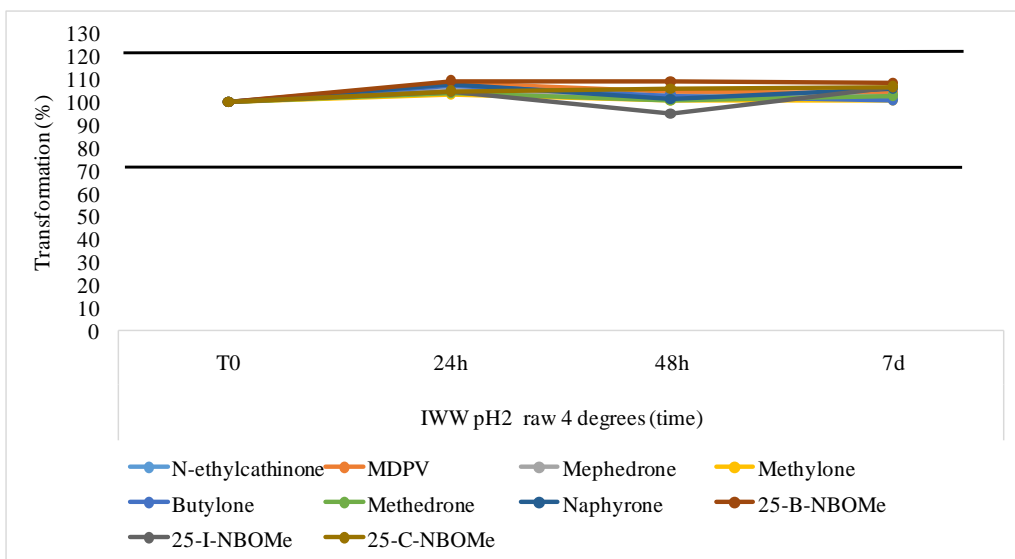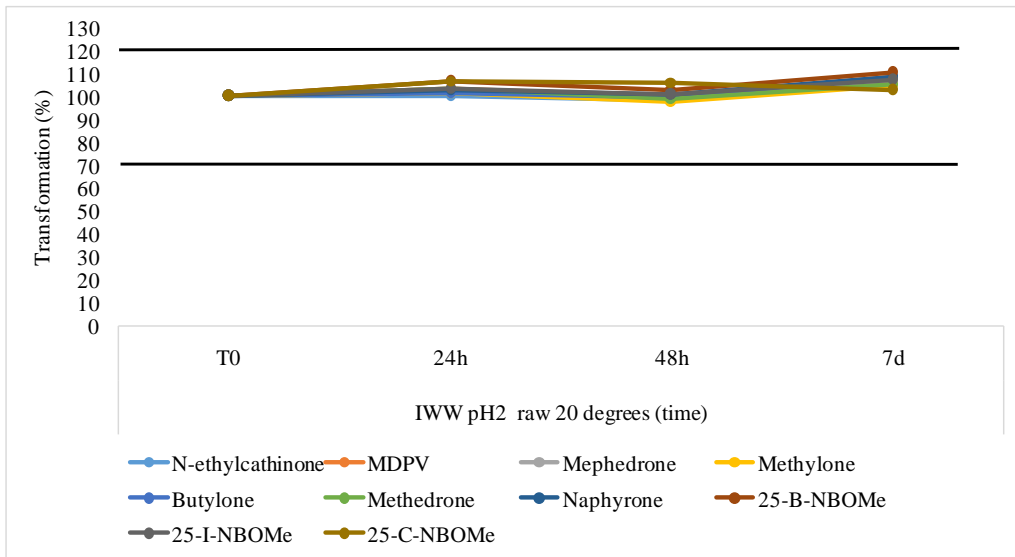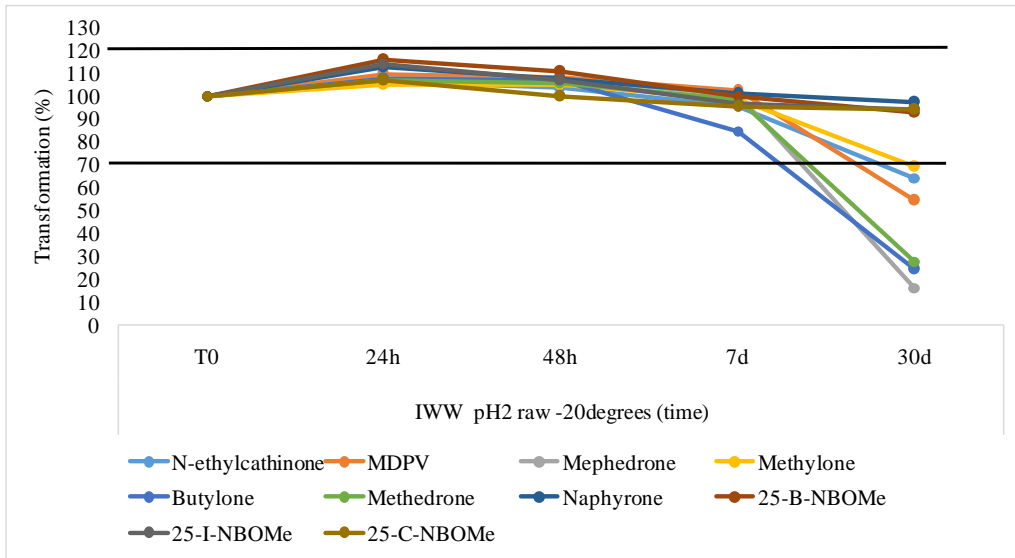
624

625 **Figure 2:**
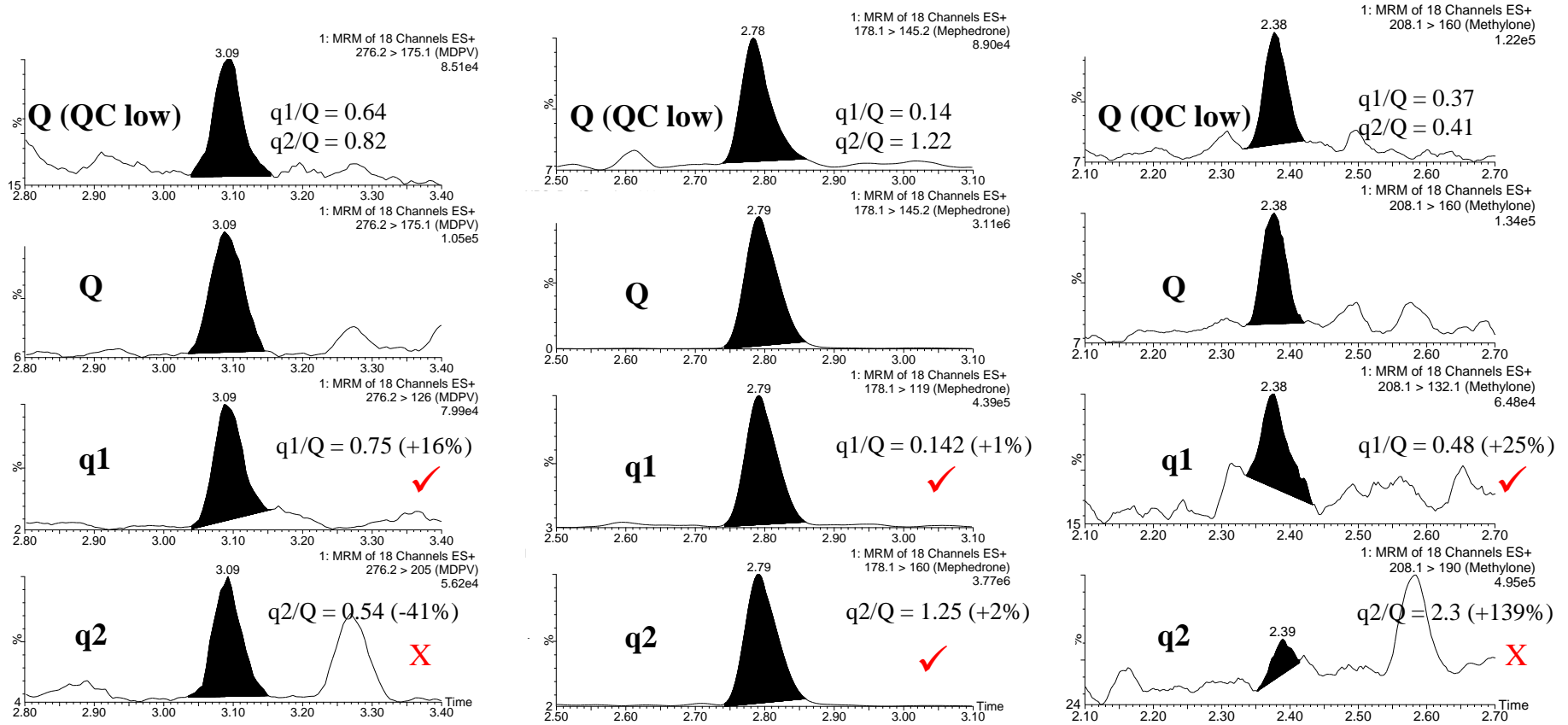
626

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			

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

629   [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