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

4  
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33

34 **Abstract**

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

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

59 **Introduction**

60 New psychoactive substances (NPS) are emerging narcotic or psychotropic drugs that  
61 are not controlled by legislation, but which may pose a public health threat. It must be  
62 noted that here, the term ‘new’ does not necessarily refer to new inventions but to  
63 substances that have recently become available (UNODC, 2014). The use of NPS has  
64 grown rapidly over the past decade and there have been increasing reports of the  
65 availability and manufacture of such substances, with the number of NPS reported  
66 globally more than doubling between 2009-2013 (UNODC 2014). In 2014 alone, 101  
67 NPS were for the first time reported to the EU Early Warning System (EMCDDA,  
68 2015a). Given the nature of the NPS market, with developers limited only by their  
69 imagination and ability to side-step legislation (Reid and Thomas, 2016) there is a  
70 sustained need to analyse the extent of the NPS problem.

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

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

96 The purpose of this study was to develop and validate a sensitive LC-MS/MS method  
97 for the quantitative determination of a number of NPS of the synthetic cathinone and  
98 phenethylamine families: butylone, ethylone, methylone, naphyrone, methedrone,  
99 methylenedioxypropylone (MDPV), mephedrone, 25-I-NBOMe, 25-C-NBOMe and  
100 25-B-NBOMe. These compounds were selected on the basis of their frequent detection  
101 in analytical, forensic and toxicological studies (Borova et al., 2015; Chen et al., 2013;  
102 Elliott and Evans, 2014; Ibáñez et al., 2014; Kankaanpää et al., 2014; Kinyua et al.,  
103 2015a; Mwenesongole et al., 2013; Reid et al., 2014; Senta et al., 2015; Uralets et al.,  
104 2014) as well as reports from the EMCDDA (EMCDDA, 2015b) and UNODC  
105 (UNODC, 2014). The developed method, using Oasis MCX SPE cartridges for sample  
106 pre-treatment, followed by UHPLC-MS/MS measurement, has been applied to influent  
107 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.

## 111 **2. Experimental**

### 112 *2.1 Chemicals and Materials*

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

### 115 *2.2 Samples*

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

### 124 *2.3 Instrumentation*

125 A Waters Acquity UHPLC system (Milford, MA, USA) was interfaced to a triple  
126 quadrupole mass spectrometer (Xevo TQS, Waters Micromass, Manchester, UK)  
127 equipped with Z-Wave devices and an electrospray ionization interface (ESI) operated  
128 in positive-ion mode. The chromatographic separation was performed using an Acquity  
129 UPLC BEH C<sub>18</sub> column, 1.7 µm, 50 mm × 2.1 mm (i.d.) (Waters) at a flow rate of 0.3  
130 mL min<sup>-1</sup>. The mobile phases used were water with 5 mM ammonium acetate and  
131 0.01% formic acid (solvent A) and MeOH with 0.01% formic acid (solvent B). The  
132 percentage of B changed linearly as follows: 0 min, 10 %; 3 min, 90 %; 3.5 min, 90 %;  
133 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<sup>-1</sup>, respectively. For operation in  
135 MS/MS mode, collision gas was argon 99.995 % (Praxair, Madrid, Spain) with a  
136 pressure of 4×10<sup>-3</sup> mbar in the collision cell (0.15 mL min<sup>-1</sup>). 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 35°C 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.

152 *2.5 Quantification and Method Validation*

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<sub>3</sub> and methylone-d<sub>3</sub>, 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  $\leq 20\%$ ). 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 (20°C, 4°C and -20°C) for 24  
185 hours, 48 hours, 7 days, and 30 days (at -20°C 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

### 189 3. Results and Discussion

#### 190 3.1 Selection of Compounds

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

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

#### 203 3.2 Instrument Optimisation

204 Initially, all cone voltages were optimised concurrently for all compounds, using a  
205 mixed standard solution (10 µg/L), with the cone voltage ranging from 10-50 V at 10 V  
206 intervals. For all compounds, the precursor ion was  $[M+H]^+$ . Collision energies were  
207 optimised for each compound independently using a collision energy ranging from 10-  
208 50eV at 10eV intervals. The most sensitive transition was typically selected for  
209 quantification (Q) while an additional one (or two) were selected for confirmation (q).  
210 Some compounds only had two product ions of significant intensity, meaning that only  
211 two transitions (i.e. Q and q1 only) could be monitored (**Table 1**). Non-specific  
212 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, 0.45 $\mu$ m 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 (Bauerlein 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 (either 1 ng/L or 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  $\leq 11\%$  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 
$$\text{Matrix effects (\%)} = \frac{\text{average peak area (Set 1,ILIS)} / \text{average peak area (Set 2,ILIS)}}{\text{average peak area (Set 1)} / \text{average peak area (Set 2)}} \times 100$$

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.

### 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 20°C, 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

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 -20°C 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 -20°C is  
350 sometimes limited and not always feasible for wastewater treatment plants and/or  
351 laboratories. In this regard, storage at 4°C 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 ( $\pm$  15) ng/L. (Castrignanò et  
367 al., 2016) These data also follow that from the UNODC, where it 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 methylone in 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  $q_2/Q$   
381 ratio for both MDPV and methylone are outside the accepted  $\pm 30\%$  ion ratio window,  
382 but as the  $q_1/Q$  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 ( $\pm 30\%$ ), retention time ( $\pm 0.1$  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  $\pm 30\%$  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

## 413 **Conclusions**

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

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

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

## 431 **Contributions**

432 Richard Bade (RB), Lubertus Bijlsma (LB), Juan V. Sancho (JVS) and Félix  
433 Hernández(FH) planned and designed the study. LB, Jose. A. Baz-Lomba, Sara  
434 Castiglioni, Erika Castrignanò, Ana Causanilles, Emma Gracia-Lor, Barbara Kasprzyk-  
435 Hordern, Juliet Kinyua, Ann-Kathrin McCall, Alexander L.N. van Nuijs, Christoph Ort,  
436 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.

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610

611 **Figure Captions:**

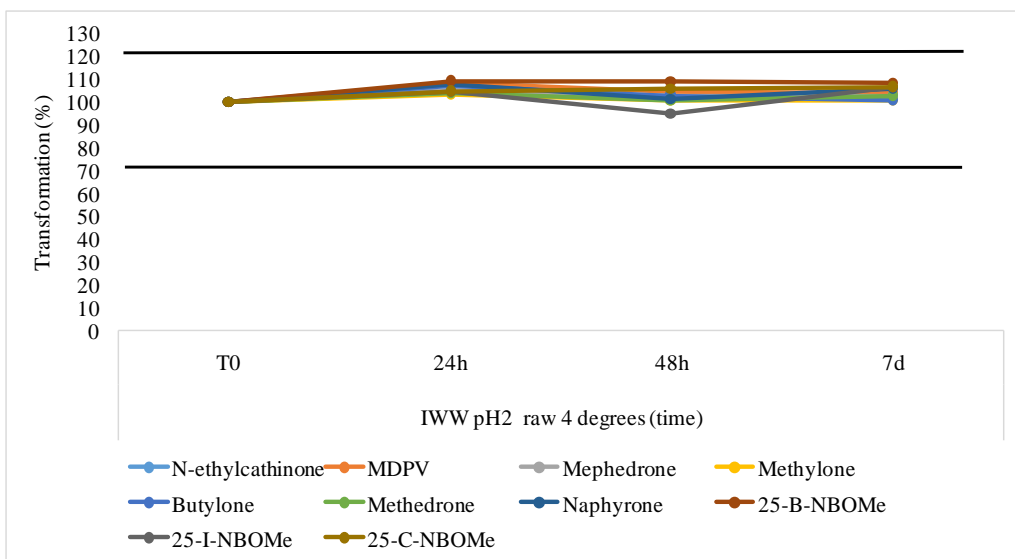
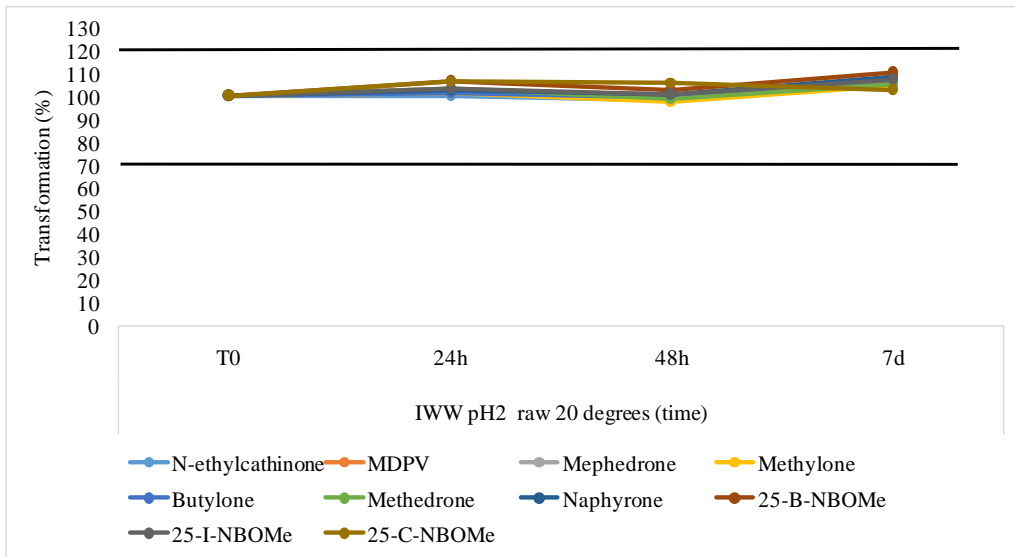
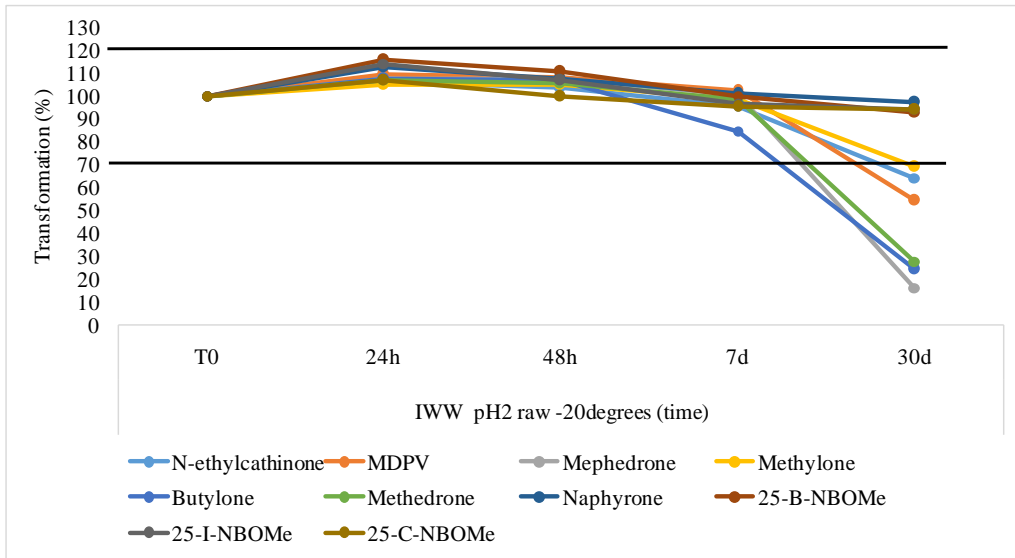
612

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

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

622

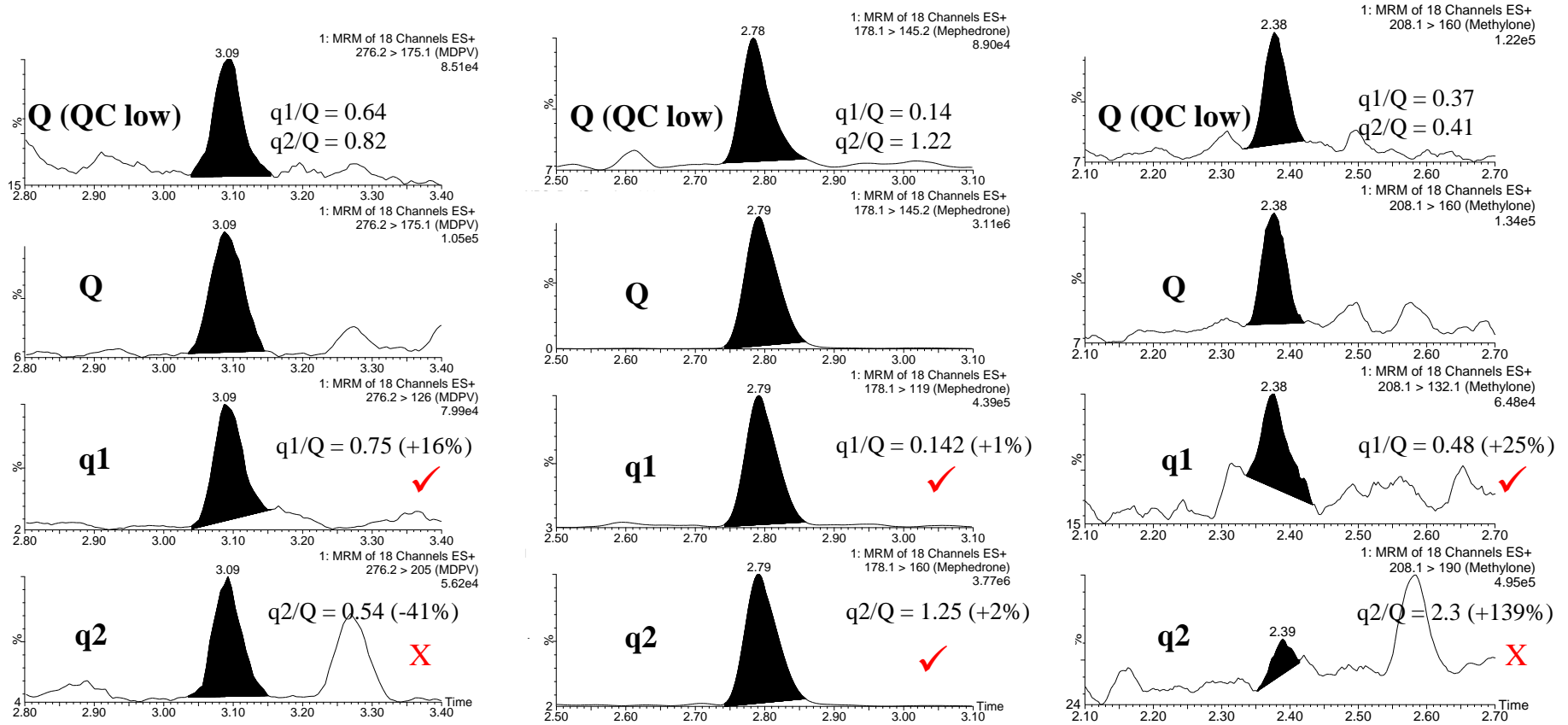
623 **Figure 1:**



624



625 **Figure 2:**



626

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

Compound	Retention time (mins)	Precursor ion [M+H] <sup>+</sup> (m/z)	Cone Voltage (V)	Collision Energy (eV)	Product Ions (m/z)	LOQ <sup>a</sup> (ng/L)	LOD <sup>b</sup> (pg L <sup>-1</sup> )	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 <sub>3</sub>	3.76	383.3	40	20	124.0			
25-C-NBOMe-d <sub>3</sub>	3.70	339.1	20	20	124.0			
25-I-NBOMe-d <sub>3</sub>	3.86	431.1	20	20	124.0			
Butylone-d <sub>3</sub>	2.68	225.1	20	20	177.1			
Mephedrone-d <sub>3</sub>	2.77	181.1	30	20	145.2			
MDPV-d <sub>8</sub>	3.11	284.2	50	20	205.0			
Methylone-d <sub>3</sub>	2.36	211.1	50	20	163.1			
Naphyrone-d <sub>5</sub>	3.71	287.2	50	20	141.2			

628 <sup>a</sup>LOQ objective. Lowest concentration tested for the method being fully validated with satisfactory results.

629 <sup>b</sup>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.

630 **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  
 631 internal standard (n=5). Linearity is also shown.

Compound	Matrix effects (%) <sup>a</sup>		q1/Q ratio		q2/Q ratio		SPE Recovery (%)				Linearity
			Low <sup>b</sup>	High <sup>c</sup>	Low <sup>b</sup>	High <sup>c</sup>	Low <sup>b</sup>		High <sup>c</sup>		
		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) <sup>d</sup>	15 (4) <sup>d</sup>	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)	<sup>d</sup>	<sup>d</sup>	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)	<sup>d</sup>	5 (6) <sup>d</sup>	96 (7)	74 (12)	98 (7)	83 (5)	0.9975

632 ILIS (Isotopically-labelled Internal Standard)

633 <sup>a</sup>: for matrix effects, a negative value (-) denotes matrix suppression, while positive values indicate matrix enhancement.

634 <sup>b</sup>: Low refers to the LOQ value of all compounds: 1 ng/L for MDPV, Naphyrone and 25-B-NBOMe and 5 ng/L for all other compounds

635 <sup>c</sup>: High refers to the 10xLOQ value of all compounds: 10 ng/L for MDPV, Naphyrone and 25-B-NBOMe and 50 ng/L for all other compounds

636 <sup>d</sup>: 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  
 637 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)

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