

1 Chromatographic retention behaviour, modelling and optimization of a UHPLC-UV
2 separation of the regioisomers of the Novel Psychoactive Substance (NPS) methoxphenidine
3 (MXP)

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22

23 Abstract

24 A detailed investigation into the chromatographic retention behaviour and separation of the
25 three regioisomers of the Novel Psychoactive Substance (NPS) methoxphenidine (i.e. 2-, 3-
26 and 4-MXP isomers) has revealed the ionization state of the analyte and stationary phase, to
27 be the controlling factor in dictating which retention mechanism is in operation. At low pH,
28 poor separation and retention was observed. In contrast, at intermediate pH, enhanced
29 retention and separation of the three MXP isomers was obtained; it appeared that there
30 was a synergistic effect between the electrostatic and hydrophobic mechanisms. At high
31 pH, the MXP isomers were retained by hydrophobic retention. Accurate retention time
32 predictions (<0.5%) were achievable using non-linear retention models (3 x 3). This allowed
33 the optimization of the gradient separation of the MXP isomers using a two-dimensional
34 gradient and temperature design space. Prediction errors for peak width and resolution
35 were, in most cases, lower than 5%. The use of linear models (2 x 2) still afforded retention
36 time and resolution accuracies of < 2.3 and 11% respectively. A rapid and highly sensitive LC-
37 MS friendly method (i.e. $R_{s \text{ min}} > 3$ within 2.5 minutes) was predicted and verified. The

38 developed methodology should be highly suitable for the rapid, specific and sensitive
39 detection and control of MXP regioisomers.

40

41 Keywords

- 42 • Reversed phase HPLC
- 43 • Two-dimensional retention modelling
- 44 • Regioisomeric methoxyphenidines
- 45 • Novel Psychoactive Substance
- 46 • Chromatographic optimization
- 47 • Retention mechanisms

48

49 1 Introduction

50 Designer drugs are analogues of controlled substances that are designed to produce effects
51 similar to the controlled substances they mimic² [1]. The rate at which such substances are
52 appearing poses significant issues for forensic laboratories with respect to identification and
53 quantification, as validated analytical methods and reference standards are not usually
54 available [4].

55 Dissociative diarylethylamine anaesthetics (Figure 1) such as diphenidine (1) [5] and 2-
56 methoxyphenidine (2-MXP, 2) [6] are substances that distort perceptions, produce feelings of
57 detachment and induce a state of anaesthesia by antagonising ionotropic *N*-methyl-*D*-
58 aspartate receptors (NMDAR) in the central nervous system [7]. Though both the supply and
59 production of diphenidine and 2-methoxyphenidine is now controlled in the United Kingdom
60 by the Psychoactive Substances Act (2016) [8], the global prevalence of novel
61 diarylethylamine derivatives still raises considerable legal and analytical challenges in the
62 forensic identification of these materials. 2-MXP has been implicated in a number of
63 fatalities in Europe [9, 10] and is encountered in both tablet and powder forms. Recently,
64 the reversed-phase liquid chromatographic (RP-LC) separation of the regioisomers of
65 methoxyphenidine (2-MXP, 2; 3-MXP, 3 and 4-MXP, 4, see Figure 1) has been reported using
66 a superficially porous phenyl hexyl material (i.e. 2.6 µm Kinetex) coupled with a shallow
67 MeCN / formic acid gradient at 30 °C (i.e. 0.25% MeCN/min). While the 2-isomer was well
68 resolved from the other two isomers, only partial separation of the 3- and 4-isomers was
69 observed (the elution order was reported to be 3-MXP, 4-MXP, 2-MXP isomer). However,
70 the paper [6] did not prove evidence of any systematic investigation into the retention
71 behaviour. Analytical differentiation of regioisomers is a significant issue in forensic drug
72 analysis, because, in most cases, legal controls are placed on only one or two of the
73 conceivable isomers and require a forensic scientist to show unequivocally that a sample
74 submitted is in fact a controlled drug and not one of the non-controlled regioisomers. This
75 can be readily achieved using Nuclear Magnetic Resonance (NMR) spectroscopy, however,
76 few forensic laboratories have such instruments and the discrimination of regioisomers
77 using the technique is both cost and labour intensive. Geyer *et al.* has recently published a

78 validated GC-(EI)-MS protocol for the qualitative and quantitative analysis of thirteen
79 diarylethylamine derivatives (including 2-MXP and its isomers) in seized powder samples –
80 however, the published method has significant limitations in terms of overall analysis time
81 (*circa.* 45 mins) [11]. This HPLC method provides, for the first time, both a general screening
82 method and quantification of the active components for seized solid samples of
83 methoxphenidine, which is significantly superior to the previously reported GC-MS [11] and
84 HPLC [6, 10] methods in terms of overall run time (7 mins) and resolution of the
85 regioisomers.

86 In contrast, this current paper reports the retention behaviour and separation of the three
87 regioisomeric methoxphenidines as a function of pH, temperature, proportion of organic
88 modifier and buffer concentration on a variety of RP columns of widely differing
89 chromatographic selectivity. Six new generation RP silica phases were selected from the
90 same manufacturer in order to minimize any problems associated with differing base silica
91 acidities [12]. Three totally porous particles (TPP) (i.e. C18-AR, C18 and C18-PFP) were
92 selected as previously these stationary phases have demonstrated complementary
93 chromatographic selectivity to each other [12]. In addition, three high pH stable phases
94 (which have been shown to possess similar selectivity to their non-high pH stable TPP
95 counterparts [i.e. TPP C18 versus the TPP and superficially porous particles (SPP) SuperC18
96 materials plus the TPP C18-AR and SPP Super Phenyl hexyl phases] were additionally
97 selected in order to allow the basic MXP regioisomers to be chromatographed, at high pH, in
98 their ion-suppressed form. The three-high pH stable phases have been reported to show
99 good stability up to pH 11 [13].

100 A detailed investigation into the retention mechanism of these regioisomeric substances
101 was performed as a function of stationary phase chemistry, mobile phase pH, proportion of
102 organic modifier and buffer concentration. The most promising chromatographic conditions
103 were then subjected to retention modelling and optimization in order to develop a rapid,
104 highly selective and robust UHPLC-UV separation of the 2-, 3- and 4-MXP isomers, within
105 bulk forensic samples, using LC-MS friendly conditions.

106

107 2 Materials and methods

108 2.1 Chemicals and Reagents

109 All water and solvents used were HPLC grade, test analytes and mobile phase chemicals
110 were supplied by Sigma-Aldrich (Poole, UK) and Fisher Scientific (Loughborough, UK).
111 Samples of the three methoxphenidine isomers (2 – 4) were prepared, under UK [Home
112 Office] Drug Licence (No. 337201), as their corresponding hydrochloride salts at Manchester
113 Metropolitan University. The synthesis of the racemic target compounds was achieved using
114 the previously reported method [11] in 52 – 77% overall yield. The hydrochloride salts were
115 obtained as stable, colourless to off-white powders (Figure 1) and determined to be soluble
116 (10 mg mL^{-1}) in deionised water, methanol, dichloromethane and dimethylsulfoxide. To
117 ensure the authenticity of the materials utilized in this study the three synthesized samples

118 were fully structurally characterized by ¹H-NMR, ¹³C-NMR, GC-MS and ATR-FTIR and the
119 purity of all samples confirmed by elemental analysis (>99.5% in all cases) [11].

120

121 2.1.1 Methoxphenidine (MXP) isomers

122 Stock solutions of the individual isomers of methoxphenidine were made up in MeCN/water
123 (1:1 v/v) at a concentration of 1 mg mL⁻¹. A mixture of the isomers was prepared and then
124 diluted to 100 µg mL⁻¹ (of each isomer) with MeCN/water (1:1 v/v) for the chromatographic
125 studies.

126

127 2.2 Software

128 LogD and *pK_a* values were predicted (ACD/Percepta, Toronto, Canada, version 2016.1.1) and
129 retention modelling and optimization (ACD/LC Simulator, version 2016.1.1) were performed
130 using software from ACD/Labs (Advanced Chemistry Development Inc., Toronto, Canada).
131 Buffers of a desired pH and buffer concentration were determined by the Buffer Maker
132 software (ChemBuddy, Marki, Poland, version 1.0.1.55).

133

134 2.3 Instrumentation

135 2.3.1 UHPLC instrumentation

136 UHPLC was performed on the following instrumentation: Agilent 1290 Infinity UHPLC
137 systems (Agilent Technologies, Waldbronn, Germany) equipped with either binary (model
138 G4220A) or quaternary (model G4204A) pumps used in conjunction with an integrated
139 degasser (model G4220A), autosampler (model G4226A), column oven model (G1316C),
140 photodiode array detector (model G4212A) equipped with a 1 µL / 10 mm pathlength flow
141 cell, 380 µL Jet Weaver mixer and a 12 position / 13 port solvent selection valve (model
142 G1160A), was used to allow the automated selection of up to 12 different eluents from
143 mobile phase line C of the Agilent 1290 Infinity quaternary UHPLC, the system(s) was
144 controlled and data collected by means of ChemStation (Agilent Technologies, Waldbronn,
145 Germany, version B.04.03). Shimadzu Nexera X2 UHPLC (Shimadzu UK Ltd, Milton Keynes,
146 UK) equipped with LC-30AD pumps, DGU-20A5R degassers, SIL-30AC autosampler, CTO-
147 20AC column oven, SPD-M30A photodiode array detector equipped with a 10 µL / 10 mm
148 pathlength flow cell, 180 µL mixer, the system was controlled and data collected by means
149 of LabSolutions software (Shimadzu UK Ltd, Milton Keynes, UK, version 5.86).

150

151 2.4 Liquid Chromatography

152 pH measurements were recorded in the aqueous fraction of the mobile phase and quoted
153 as ^wpH. At least 20 column volumes of the appropriate mobile phase were flushed
154 through the columns prior to commencing the testing or on changing the mobile phase

155 conditions. The totally porous ACE C18, C18-PFP, C18-AR (5 μm , 100 \AA , 150 x 4.6 mm I.D.
156 format), C18-AR, SuperC18 (3 μm , 100 \AA , 50 x 4.6 mm I.D. format), ACE UltraCore
157 superficially porous SuperC18 and SuperPhenylhexyl (2.5 μm , 100 \AA , 50 x 4.6 mm I.D.
158 format) columns were as supplied by Advanced Chromatography Technologies (Aberdeen,
159 Scotland, UK). The integrity of all the columns was confirmed periodically throughout the
160 experiments by injecting a suitable non-polar test mixture (i.e. uracil, toluene, biphenyl,
161 dimethyl phthalate and phenanthrene) before and after the experiments. All columns gave
162 retention times, efficiency and peak symmetry levels >95% of their initial value. The mobile
163 phase was degassed and mixed on-line for the aqueous / organic mixtures.

164 The first baseline disturbance for a water injection was used as the dead time (t_M) marker.
165 A flow rate of 1.0 mL min^{-1} and a 2 μL injection was used in all experiments and a column
166 temperature was maintained between 20 – 70 $^\circ\text{C}$. The diode array detector was set to
167 monitor a wavelength of 278 nm with a reference at 360 nm. The data sampling rate was
168 set at 40 Hz. Peak width and symmetry was determined at half height as reported by the
169 ChemStation software or LabSolutions software. For the retention modelling the peak
170 width at base was calculated by multiplying the peak width at half height by 1.699 [to
171 generate the 4σ , United States Pharmacopeia (USP) peak width values]. Chromatographic
172 values reported are the average of duplicate injections. Retention factors (k) were
173 calculated for isocratic conditions using the following equation; $k = (t_R - t_M) / t_M$. Where t_R =
174 retention time of the isomer and t_M = void time of an unretained analyte.

175

176 2.4.1 Effect of ammonium acetate concentration on the retention of the MXP isomers (see
177 section 3.3)

178 Evaluation of the effect of ammonium acetate (pH 6.8) concentration (1 – 14 mM) on the
179 retention of the methoxphenidine isomers was performed on an ACE C18-AR 3 μm 50 x 4.6
180 mm column at 54 % MeCN concentration, 30 $^\circ\text{C}$, 1 mL min^{-1} using the Agilent 1290 Infinity
181 Quaternary UHPLC. Mobile phase A) 100 mM ammonium acetate (pH 6.8 unadjusted), B)
182 MeCN, C) water. The appropriate buffer concentrates were mixed on-line, for example 10
183 mM buffer in MeCN/water was prepared by mixing A:B:C in the ratio 10:54:36 v/v/v.

184

185 2.4.2 Effect of the proportion of acetonitrile (MeCN) on the retention of the MXP isomers
186 (see section 3.4)

187 Evaluation of the effect of the proportion of MeCN (18 – 63 % v/v) on the retention of the
188 methoxphenidine isomers was performed on an ACE C18-AR and ACE SuperC18, 3 μm , 50 x
189 4.6 mm column, 1 mL min^{-1} , 60 $^\circ\text{C}$, mobile phase A) 10 mM ammonium acetate (pH 6.8
190 unadjusted), 10 mM ammonium formate (pH 3) or 18.6 mM ammonia (pH 10.7) in water, B)
191 the appropriate buffer in MeCN/water (9:1 v/v) using the Agilent 1290 Infinity Binary
192 UHPLC.

193

194 2.4.3 Effect of temperature on the retention of the MXP isomers (see section 3.5)

195 Evaluation of the effect of temperature (20 -70 °C) on the retention of the methoxphenidine
196 isomers was performed on an ACE C18-AR, 3 µm, 50 x 4.6 mm column using 60 %B (i.e. 54 %
197 v/v MeCN), 1 mL min⁻¹, mobile phase A) 10 mM ammonium acetate (pH 6.8 unadjusted) in
198 water, B) 10 mM ammonium acetate (pH of 6.8 unadjusted) in MeCN/water (9:1 v/v) using
199 the Shimadzu Nexera X2 UHPLC.

200

201 2.4.4 Effect of pH on the retention of the MXP isomers (see section 3.2)

202 Evaluation of the effect of pH on the retention of the methoxphenidine isomers was
203 performed on ACE UltraCore SuperC18 and C18-AR columns, 2.5 and 3 µm respectively, 50 x
204 4.6 mm column at 60 %B (i.e. 54 % v/v MeCN), 50 °C, 1 mL min⁻¹, mobile phase A) 10 mM
205 ammonium formate pH 3, B) 10 mM ammonium acetate (unadjusted pH of 6.8) and c) 18
206 mM ammonia (unadjusted pH of 10.7) using the Agilent 1290 Infinity Quaternary UHPLC.

207

208 2.4.5 Effect of pH over the range pH 8 -10.7 on the retention of the MXP isomers (see
209 section 3.2.3)

210 Evaluation of the effect of high pH (pH 8, 9, 9.25, 9.5, 9.75, 10 and 10.7) on the retention of
211 the methoxphenidine isomers was performed on an ACE Ultracore SuperC18, 2.5 µm, 50 x
212 4.6 mm column using 10 mM ammonia / acetic acid buffers (ammonia concentration kept
213 constant) in MeCN/water (54:46 v/v), 50 °C, 1 mL min⁻¹ using the Agilent 1290 Infinity
214 Quaternary UHPLC. Stock pH buffers were prepared as described by the Buffer Maker
215 Software.

216

217 2.5 Retention modelling

218

219 2.5.1 Two-dimensional retention modelling and optimization: Gradient time *versus*
220 temperature on the C18-AR at pH 6.8 (see section 3.7.2)

221 An ACE C18-AR column (3 µm, 50 x 4.6 mm) was used at a flow rate of 1 mL min⁻¹ using the
222 Shimadzu Nexera X2 UHPLC. Sixteen input runs and six validation runs were performed
223 (see section 3.7.2, Figure 6). Mobile phase A consisted of 10 mM ammonium acetate
224 (unadjusted pH 6.8) and mobile phase B of 10 mM ammonium acetate (unadjusted pH 6.8)
225 in MeCN/water (9:1 v/v). A temperature range of 30 to 70 °C was investigated (see Figure
226 6). The %B gradient range was run between 40 and 70 %B. After the selected gradient run
227 time (t_G) was reached, a 5-minute hold time at 70%B, 1-minute ramp down to 40%B, and a
228 5-minute post time at 40%B were employed.

229

230

231 3 Results and Discussion

232

233 3.1 Chromatographic separation of the methoxphenidine (MXP) regioisomers as a
234 function of stationary phase chemistry

235 The TPP ACE C18, C18-AR and C18-PFP and the high pH stable SPP SuperC18 and
236 SuperPhenylhexyl phases, which possess differing bonded ligands on the silica, have
237 recently been showed to exhibit differing chromatographic selectivities (see Supplementary
238 electronic information Table SEI 1) due to the ligands' differing propensity to participate in
239 hydrophobic, aromatic (i.e. π acid and π base interactions), dipole – dipole interactions,
240 hydrogen bonding and electrostatic interaction with various analytes under a range of
241 chromatographic conditions [13]. Hence, it was somewhat surprising that these phases
242 failed to exhibit any major selectivity differences irrespective of mobile phase pH suggesting
243 that the MXP interactions with the differing stationary phase ligands was not the controlling
244 retention mechanism.

245

246 3.2 Chromatographic separation of the methoxphenidine (MXP) regioisomers as a
247 function of pH

248 The regioisomers of methoxphenidine are hydrophobic compounds with tertiary amine
249 functionality, with calculated pK_a values of 8.7, 9.1 and 9.4 for the 2-, 3- and 4-MXP isomers
250 respectively. Hence, the effect of pH was investigated in order to assess the influence of
251 hydrophobic and electrostatic interactions on their chromatographic retention.

252

253 3.2.1 Chromatographic separation of the methoxphenidine (MXP) regioisomers at low pH

254 Chromatography of the regioisomeric analytes (Figure 1, 2 – 4) on the TPP ACE C18, C18-AR
255 and C18-PFP, at low pH, resulted in low retention and only partial separation of the isomers
256 (data not shown). The low retention and the elution order observed on the three TPP
257 phases, at low pH with 10 mM ammonium formate pH 3 mirrored that was previously
258 reported by McLaughlin *et al* [6] using another phenylhexyl phase (i.e. the 2-isomer (2)
259 eluted after the partial separation of the 3- and 4- isomers). Separation selectivity was not
260 improved even when lower %MeCN containing mobile phases were employed in order to
261 improve retention (see Figure 4a). The low retention (see Figure 2a for a typical
262 chromatogram on the SPP SuperC18 column) may be attributed to the mutual repulsion of
263 the adsorbed protonated MXP isomers and the low acidity of the new generation silica
264 columns used in this study.

265

266

267 3.2.2 Chromatographic separation of the methoxphenidine (MXP) regioisomers at
268 intermediate pH

269 Chromatography at pH 6.8 (i.e. 10 mM ammonia acetate) using the C18-AR, SuperC18 and
270 SuperPhenylHexyl phases resulted in enhanced retention and excellent separation of the
271 regioisomers (the C18 and C18-PFP phases were not evaluated). Figure 2b is typical of the
272 separation that could be achieved on these phases at intermediate pH using the SPP
273 SuperC18. Once again, the same elution order (i.e. 2-MXP, 4-MXP, 3-MXP) was obtained on
274 each phase, which was surprising, given the large chromatographic selectivity differences
275 that exists between the C18 and phenyl phases (see Supplementary electronic information
276 Table SEI 1). The elution order at low and intermediate pH (i.e. 2-MXP, 4-MXP, 3-MXP) was
277 different to that observed at high pH (i.e. 4-MXP, 3-MXP, 2-MXP see Figures 2a -c).

278

279 3.2.3 Chromatographic separation of the methoxphenidine (MXP) regioisomers at high pH

280 Chromatography on the high pH stable SPP & TPP phases (i.e. SuperC18 and
281 SuperPhenylHexyl) at pH 10.7 (i.e. 18 mM ammonia) exhibited enhanced retention and
282 good resolution of all of the isomers with the same elution order (i.e. 4-MXP, 3-MXP, 2-
283 MXP) irrespective of the phase chemistry. Figure 2c highlights a typical separation at high
284 pH conditions using the SPP SuperC18 phase. Interestingly, the elution order of the isomers
285 at high pH was different to that observed using intermediate pH conditions (i.e. 2-MXP, 4-
286 MXP, 3-MXP). It is presumed that the high pH of the mobile phase renders the MXP
287 molecules uncharged hence eliminating the possibility of ion exchange interactions and
288 increasing the hydrophobic and π - π interaction of the neutral MXP analytes with the
289 stationary phase. As only small differences in selectivity were observed between the C18
290 and phenyl phases, we must conclude that there is minimal π - π interaction of the analytes
291 with the phenyl phase, this may be attributed to the fact that MeCN was used as the organic
292 modifier [14,15].

293 The retention of each of the isomers was in line with their estimated logD values in that
294 greater retention was observed at pH 10.7 when the MXP isomers were in their unionized
295 forms. (e.g. the 4-MXP's LogD values were estimated at pH 3, 6.8 and 10.7 to be 1.76, 2.41
296 and 4.84 respectively).

297 In order to gain a better understanding of the retention behaviour of the MXP isomers at pH
298 conditions spanning their estimated pK_a values [i.e. ACD Percepta estimates of 9.4 (4-MXP),
299 9.1 (3-MXP), and 8.7 (2-MXP)] their retention over the pH range of 8 – 11 was investigated
300 on the high pH stable SPP SuperC18 at constant ammonia concentration (see
301 Supplementary electronic information Figure SEI 1). Up to a w_w pH of 9.5, the elution order
302 remained the same as that at pH 6.8; the retention of all the isomers becoming
303 progressively longer presumably due to a greater influence from hydrophobic retention
304 mechanisms as the mobile phases becomes progressively more alkaline and the MXP
305 isomers less protonated. Between w_w pH 9.75 and 11 (the latter is the maximum operating
306 pH for this phase) a switch in the elution order was observed. The 2-MXP which between w_w
307 pH 6.8 – 9.5 eluted before the 4-MXP and 3-MXP isomers respectively, at w_w pH 11 eluted
308 after the 4-MXP and 3-MXP isomers respectively. The same observations were seen on

309 another high pH stable phase (i.e. the bridged ethyl hybrid - XBridge C18 phase – data not
310 shown).

311 Addition of sodium chloride into the high pH mobile phase with the TPP SuperC18 phase
312 (see Supplementary electronic information Figure SEI 2) failed to affect the retention time of
313 the MXP regioisomers due to the fact that they were chromatographed in their ion-
314 suppressed form at pH 10.7 (i.e. as the free bases). In comparison, the addition of sodium
315 chloride to the intermediate pH mobile decreased the retention of the methoxphenidine
316 isomer as expected due to competition of the positively charged sodium and MXP ions for
317 the negatively charged silanol groups on the surface of the stationary phase.

318 Due to the enhanced separation (i.e. resolution and speed) of the isomers at intermediate
319 pH, a more detailed study into the chromatographic parameters which control their
320 retention was performed at intermediate pH using the ACE C18-AR and SuperC18 phases as
321 phase chemistry did not appear to be a major factor in determining chromatographic
322 selectivity.

323

324 3.3 Effect of buffer concentration at intermediate pH

325 The effect of ammonium acetate concentration was investigated at 30 °C with a w_w pH 6.8
326 mobile phase on the C18-AR phase (see Figure 3). According to ion exchange theory [16-18]
327 retention has been proposed to be related to buffer concentration as expressed in Equation
328 1.

329

$$330 \log k = a + b \log x \quad \text{Equation 1}$$

331

332 where k = retention factor, a , b and c are coefficients and x = chromatographic variable (i.e.
333 proportion of organic or buffer concentration)

334

335 Equation 1 did not provide a good fit for the data shown in Figure 3 so a more complex
336 model, as described by Equation 2, was employed.

337

$$338 \log k = a + b \log x + c (\log x)^2 \quad \text{Equation 2}$$

339

340 The observation that increased buffer concentrations generated reduced retention of the
341 MXP isomers highlighted that there is an ion exchange mechanism contributing to retention
342 at intermediate pH.

343

344

345 3.4 Effect of the proportion of MeCN at intermediate pH

346 In contrast to the expected linear relationship (see Equation 3) between the $\log k$ of the
347 MXP isomers and the proportion of MeCN in the mobile phase [19, 20], a curved
348 relationship (see Equation 4) was observed between the retention of the MXP isomers and
349 the proportion of MeCN in the mobile phase at pH 6.8 (see Figure 4a for a typical example
350 on the SuperC18 phase). The use of the standard second order polynomial model (see
351 Equation 4) used in the retention modelling software was found to generate highly accurate
352 retention predictions (see retention modelling sections 3.7.1 and 3.7.2).

353

354 $\log k = a + b x$ Equation 3

355

356

357 $\log k = a + b x + c x^2$ Equation 4

358

359 The curved relationship suggested that, at intermediate pH, a mixed mode retention
360 mechanism was in operation. The negatively charged silanol groups on the phase may
361 attract the positively charged analytes, via an electrostatic attraction, into the hydrophobic
362 phase where it can interact with the bonded ligands. A curved relationship (i.e. second
363 order polynomial model) was also observed at low pH possibly due to a secondary ionic
364 repulsive interaction (see Figure 4b). In comparison the relationship at pH 10.7 was
365 observed to be much more linear (see Figure 4c) due to the fact that the MXP isomers were
366 chromatographed in their ion suppressed form and hence a simple hydrophobic retention
367 mechanism dominated.

368

369

370

371 3.5 Effect of temperature at intermediate pH

372 If a simple hydrophobic retention mechanism was in operation at pH 6.8, then as the
373 temperature was increased the retention time should decrease (i.e. van't Hoff relationship)
374 as shown in Equation 5.

375

$$376 \log k = a + \frac{b}{T} \quad \text{Equation 5}$$

377 Where T = temperature

378

379 However, if the retention is dependent on multiple interactions, then non-linear responses
380 may be generated and Equation 6 should be more appropriate [18, 21, 22].

381

$$382 \log k = a + \frac{b}{T} + \frac{c}{T^2} \quad \text{Equation 6}$$

383

384 As can be seen in Figure 5, the retention of each MXP isomer on the ACE C18-AR phase
385 behaved differently as a function of temperature in 10 mM ammonium acetate (pH 6.8)
386 MeCN/water (54:46 v/v). The 2-MXP isomer exhibited the expected reduction in retention
387 as temperature increased whereas temperature had little effect on the retention of the 3-
388 MXP and 4-MXP isomers. These observations may reflect differential changes in the pK_a of
389 the MXP isomers and the silanol groups on the stationary phase surface and the pH of the
390 organic / aqueous mobile phase as temperature is changed and hence the degree of
391 electrostatic interaction of the regioisomers with the ionized silanol groups. Therefore, it
392 was inferred that the mechanism controlling the retention and separation of the MXP
393 regioisomers at pH 6.8 was attributed to an electrostatic interaction which facilitated
394 hydrophobic interactions.

395

396

397 3.6 Retention behaviour conclusions

398 Stationary phase chemistry appears to have minimal influence on the chromatographic
399 selectivity of the three MXP regioisomers at low, intermediate or high pH mobile phase
400 conditions. At low pH mobile phase conditions, the analytes exhibited minimal retention as
401 a result of mutual repulsion of the adsorbed positively charged analyte on the low acidity
402 stationary phases. In comparison, at intermediate pH enhanced retention and separation of
403 the regioisomers was observed. This was attributed to a synergistic effect of the
404 electrostatic attraction between the ionized analyte and the silanol groups which attracts
405 the charged analyte into the lipophilic stationary phase where hydrophobic interactions
406 could take place. In comparison, at high pH the MXP analytes are chromatographed on the
407 SPP and TPP SuperC18 or phenyl hexyl phases in their neutral form and hydrophobic
408 interactions were the major retention mechanism.

409

410 3.7 Two-dimensional retention modelling and optimization

411 The chromatographic separation of the three isomers was greater at pH 6.8 than at either
412 pH 3 or 10.7 (see Figures 2a -c). This was further confirmed in preliminary two-dimensional
413 (gradient time versus temperature) retention modelling studies using the SPP Super
414 phenylhexyl and C18 phases, as a function of gradient time (i.e. 5 and 15 minutes) and
415 temperature (i.e. 30 to 65°C) at pH 3 (gradient range 4.5 - 45% MeCN), 6.8 (36 - 90% MeCN)
416 and 10.7 (36 - 90% MeCN). Four experimental input runs were used to construct the 2 x 2
417 models using Equations 3 and 5 in the commercial retention modelling software (see
418 Supplementary electronic information Figures SEI 3 and 4).

419

420 3.7.1 Selection of the most appropriate retention models

421 From the preliminary two-dimensional retention modelling the following operating
422 parameters were chosen to perform more detailed one-dimensional modelling studies using
423 the ACE C18-AR, which was observed to generate sharper MXP peaks, to confirm which
424 equations would generate the most accurate predictions. A temperature range 30 – 75 °C,
425 and a gradient time range 3 – 12 minutes were evaluated using an initial to final %MeCN of
426 36 – 63% MeCN. It was found that there was no need to re-define the dwell volume (V_D)
427 using an iterative process as excellent results were obtained with the calculated value of
428 517 μL using a slightly modified USP methodology for determining V_D [23].

429 Table 1 highlighted that the non-standard Equation 4 which described a curved relationship
430 between $\log k$ and % organic generated more accurate retention time predictions (Δt_R
431 $<0.11\%$) than ~~that of~~ the standard Equation 3 ($\Delta t_R <0.45\%$) for gradient time modelling.

432 In a similar manner, Table 2 highlighted that the non-standard Equation 6 which described a
433 curved relationship between \log retention factor (k) and $1/\text{temperature}$ generated more
434 accurate retention time predictions ($\Delta t_R <0.23\%$) than ~~that of~~ the standard Equations 5
435 ($\Delta t_R <2.19\%$) for temperature modelling.

436 The LC simulator software utilizes empirical models to calculate peak widths (w_{base}) as
437 shown in Equations 7, 8 and 9. Where α and β terms are fitted to minimize the residual for
438 the retention time of the front ($t_{R \text{ front}}$) and tail ($t_{R \text{ tail}}$) of the peak.

439

$$440 \quad t_{R \text{ tail}} = (1 - \alpha)t_R \quad \text{Equation 7}$$

$$441 \quad t_{R \text{ front}} = (1 + \beta)t_R \quad \text{Equation 8}$$

$$442 \quad w_{\text{base}} = t_{R \text{ front}} - t_{R \text{ tail}} \quad \text{Equation 9}$$

443

444 It should be noted that Equations 1-9 describe isocratic separations, however, by employing
445 numerical calculations where the gradients are divided into a large number of isocratic
446 segments, these equations can be equally applied to gradient separations as described here.

447 From Tables 1 and 2 it can be seen that the commercially employed equations are able to
448 model and predict the peak width to an acceptable degree with errors of <3% being
449 observed with the models associated with Equations 4 and 6. As a result of the excellent
450 retention time and acceptable peak width predictions excellent resolution predictions of
451 <2% were obtainable when Equations 4 and 6 were employed, see Tables 1 and 2.

452

453

454 3.7.2 Gradient time *versus* temperature on the C18-AR at pH 6.8

455 As a result of the one-dimensional investigation (see section 3.7.1) the more complex
456 Equations 6 and 4 were employed in the two-dimensional temperature and gradient time
457 modelling. In order to model the non-linear relationships of temperature and gradient time
458 on retention, described in Equations 6 and 4, sixteen input runs (i.e. 4 x 4) were used in
459 order to generate high quality data.

460 From the two-dimensional model (see Figure 6), it is possible to iteratively change the V_D in
461 order to minimize the predicted versus actual retention time errors for an experimental
462 condition (gradient = 4.5 minutes and temperature = 30°C, often classed as a calibration
463 run). However, the model using the determined V_D of 517 μL was shown to generate
464 <0.08% error for retention time and was hence not changed.

465 The accuracy of the non-linear 4 x 4 retention model (total of 16 input experiments) was
466 observed to be excellent. The prediction errors for t_R , peak width and resolution were <0.5
467 and <13.7% (most were below 5%), <7.8% respectively (see Table 3 and Figure 6) which is
468 very good compared to the accepted accuracies of 2, 20 and 20% for t_R , peak width and
469 resolution respectively [24, 25].

470 The resolution plot of gradient time versus temperature demonstrated that the
471 methodology was robust (i.e. $R_s > 2$) within the ranges of gradient time (3 to 12 minutes) and
472 temperature (30 to 75°C), see Figure 6a.

473 A simplified 3 x 3 retention model (i.e. gradient times of 3, 6 and 9 minutes and
474 temperatures of 30, 45 and 60°C, total of nine input experiments) which is sufficient to
475 generate second order polynomial relationships generated results very similar to that seen
476 in the more complex 4 x 4 model see Table 4.

477 It is interesting to note that if one employed the simple linear 2 x 2 retention modelling
478 using the linear Equations 3 and 5 in a cut down four input data experiment (i.e. gradient
479 times of 3 and 12 minutes and temperatures of 30 and 75°C), the retention time, peak
480 width and resolution were <2.3 and <16.4%, <10.7% respectively which is still impressive
481 given the substantially smaller number of experimental input runs that are required.

482 Conclusion

483 A detailed investigation into the retention behaviour and separation of the regioisomers of
484 ~~the~~ methoxphenidine (i.e. 2-MXP, 3-MXP and 4-MXP isomers) has shown that, for this
485 particular separation, the stationary phase chemistry is not a major selectivity parameter.
486 At low pH, poor separation and retention of the MXP isomers was observed presumably due
487 to mutual electrostatic repulsion of the adsorbed protonated analytes. In contrast, at
488 intermediate pH, enhanced retention and separation of all MXP isomers was obtained, it
489 appeared that there was a synergistic effect between the electrostatic and partitioning
490 mechanisms. At high pH, the MXP isomers were retained by a predominantly hydrophobic
491 mechanism due to their unionized form. It was observed that more complicated models
492 were necessary to fully describe the retention of the MXP isomers due to the fact that
493 multiple retention mechanisms were in operation. Using these non-linear models with 4 x 4
494 or 3 x 3 input runs, it was possible to predict with a high degree of certainty (<0.5%) the
495 retention behaviour of the MXP isomers and then to optimize the gradient separation of the
496 MXP isomers using a gradient and temperature design space. Prediction errors for peak
497 width and resolution were in most cases lower than 5%. If one wishes to slightly sacrifice
498 the prediction accuracy in favour of using a reduced number of experimental input runs,
499 the linear models using a 2 x 2 model still generated retention time accuracy <2.3% yielding
500 resolution accuracies of <11%.

501 Subsequently, from the 4 x 4 retention model, a rapid and highly sensitive LC-MS friendly
502 method (i.e. $R_{s \text{ min}} > 3$ within 2.5 minutes) was predicted and verified. The developed
503 methodology should be highly suitable for the rapid, specific and sensitive detection and
504 control of these novel illicit drugs within bulk forensic samples.

505

506 Conflict of interest

507 The authors have declared no conflict of interest.

508

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513 linear work respectively; Advanced Chemistry Development for providing the LC simulator
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515 for providing the columns used in this work.

516

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518 References

- 519 [1] L. A. King, Legal Classification of Novel Psychoactive Substances: An International
520 Comparison, In Novel Psychoactive Substances, edited by P.I. Dargan, D.M. Wood,
521 Academic Press, Boston, (2013), Chapter 1, 3-27, ISBN 9780124158160,
522 <http://dx.doi.org/10.1016/B978-0-12-415816-0.00001-8>.
- 523 [2] S.W. Smith, F.M. Garlich, Availability and Supply of Novel Psychoactive Substances, In
524 Novel Psychoactive Substances, edited by P.I. Dargan, D.M. Wood, Academic Press,
525 Boston, (2013), Chapter 3, 55-77, ISBN 9780124158160,
526 <http://dx.doi.org/10.1016/B978-0-12-415816-0.00003-1>.
- 527 [3] M.C. Van Hout, E.Hearne, "Word of Mouse": Indigenous Harm Reduction and Online
528 Consumerism of the Synthetic Compound Methoxphenidine, J. Psychoactive Drugs,
529 47 (2015) 30-41.
- 530 [4] L.A. King, A.T. Kicman, A brief history of 'new psychoactive substances', Drug Test.
531 Anal., 3 (2011) 401–403.
- 532 [5] J. Wallach, P.V. Kavanagh, G. McLaughlin, N. Morris, J.D. Power, S.P. Elliot, M.S.
533 Mercier, D. Lodge, H. Morris, N.M. Dempster, S.D. Brandt, Preparation and
534 characterisation of the "research chemical" diphenidine, its pyrrolidine analogue,
535 and their 2,2-diphenylethyl isomers, Drug Test Anal. 7(5) (2015) 358-267.
- 536 [6] G. McLaughlin, N. Morris, P.V. Kavanagh, J.D. Power, J. O'Brien, B. Talbot, S.P. Elliott,
537 J. Wallach, K. Hoang, H. Morris, S.D. Brandt, Test purchase, synthesis, and
538 characterization of 2-methoxydiphenidine (MXP) and differentiation from its meta -
539 and para -substituted isomers. Drug Test Anal., 8 (2016) 98-109.
540 <http://doi.org/10.1002/dta.1800>
- 541 [7] H. Morris, J. Wallach, From PCP to MXE: a comprehensive review of the non-medical
542 use of dissociative drugs, Drug Test Anal. 6(7-8) (2016) 614-632.
- 543 [8] P. Reuter, B. Pardo, Can new psychoactive substances be regulated effectively? An
544 assessment of the British Psychoactive Substances Bill, Addiction 112 (1) (2017) 25-
545 31.
- 546 [9] A. Helander, O. Beck, M. Baeckberg, Intoxications by the dissociative new
547 psychoactive substance diphenidine and methoxphenidine, Clin Toxicol 53(5) (2015)
548 446-453.
- 549 [10] S.P. Elliot, S.D. Brandt, J. Wallach, H. Morris, P.V. Kavanagh, First reported fatalities
550 associated with the "research chemical" 2-methoxydiphenidine, J Anal Toxicol 39(4)
551 (2015) 287-293.

552

553

- 554 [11] P.M. Geyer, M.C. Hulme, J.P.B. Irving, P.D. Thompson, R.N. Ashton, R.J. Lee, L.
555 Johnson, J. Marron, C.E. Banks and O.B. Sutcliffe, Guilty by Dissociation –
556 Development of Gas Chromatography-Mass Spectrometry (GC-MS) and other rapid
557 screening methods for the analysis of 13 diphenidine- derived New Psychoactive
558 Substances (NPSs), *Anal Bioanal Chem*, 408 (29) (2016) 8467-8481.
- 559 [12] M.R. Euerby, M. Fever, J. Hulse, M. James, P. Petersson, C. Pipe, Maximization of
560 Selectivity in Reverse Phase Liquid Chromatographic Method Development
561 Strategies, *LC•GC Europe*, 29 (2016) 8 – 21.
- 562 [13] <http://www.ace-hplc.com/technical-area/literature.aspx#2733>. Accessed 3rd May
563 2016.
- 564 [14] M.R. Euerby, P. Petersson, Chromatographic classification and comparison of
565 commercially available reversed-phase liquid chromatographic columns using
566 principal component analysis, *J. Chromatogr. A* 994 (2003) 13-36.
- 567 [15] M.R. Euerby, P. Petersson, W. Campbell, W. Roe, Chromatographic classification and
568 comparison of commercially available reversed-phase liquid chromatographic
569 columns containing phenyl moieties using principal component analysis, *J.*
570 *Chromatogr. A* 1154 (2007) 138-151.
- 571 [16] P. Jandera, M. Janderov, J. Churcek, Gradient elution in liquid chromatography: VIII.
572 Selection of the optimal composition of the mobile phase in liquid chromatography
573 under isocratic conditions, *J Chromatogr. A* 148 (1978) 79-97.
- 574 [17] Y. Baba, G. Kura, Computer-assisted retention prediction system for inorganic cyclic
575 polyphosphates and its application to optimization of gradients in anion-exchange
576 chromatography, *J Chromatogr. A* 550 (1991) 5-14.
- 577 [18] M.R. Euerby, J. Hulse, P. Petersson, A. Vazhentsev, K. Kassam, Retention Modelling in
578 Hydrophilic Interaction Chromatography, *Anal Bioanal Chem* 40 (2015) 9135-9152.
- 579 [19] L.R. Snyder, J.W. Dolan, J.R. Gant, Gradient elution in high-performance liquid
580 chromatography : I. Theoretical basis for reversed-phase systems, *J Chromatogr. A*
581 165 (1979) 3-30.
- 582 [20] P. Jandera, J. Churcek, L. Svoboda, Gradient elution in liquid chromatography: X.
583 Retention characteristics in reversed-phase gradient elution chromatography, *J*
584 *Chromatogr. A* 174 (1979) 35-50.
- 585 [21] P. Petersson, J. Munch, M.R. Euerby, A. Vazhentsev, M. McBrien, S.K. Bhal, K.
586 Kassam, Adaption of Retention Models to Allow Optimisation of Peptide and Protein
587 Separations. *Chromatography Today* 7 (2007) 15-18.
- 588 [22] T. Galaon, V. David, Deviation from van't Hoff dependence in RP-LC induced by
589 tautomeric interconversion observed for four compounds, *J Sep Sci* 34 (2011) 1423-
590 1428.

591 [23] Chromatographic system suitability tests signal to noise USP 37 <621>
592 <https://hmc.usp.org/sites/default/files/documents/HMC/GCs-Pdfs/c621.pdf>.
593 Accessed 19th December 2016

594 [24] R.G. Wolcott, J.W. Dolan, L.R. Snyder, Computer simulation for the convenient
595 optimization of isocratic reversed-phase liquid chromatographic separations by
596 varying temperature and mobile phase strength, *J Chromatogr. A* 869 (2000) 3-25,

597 [25] L.R. Snyder, J.W. Dolan, *High-Performance Gradient Elution: The Practical Application*
598 *of the Linear-Solvent-Strength Model*, Hoboken, NJ: Wiley, (2007) pp. 399.

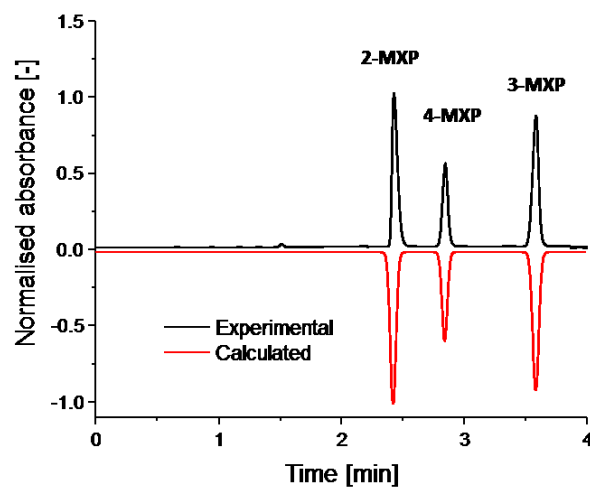
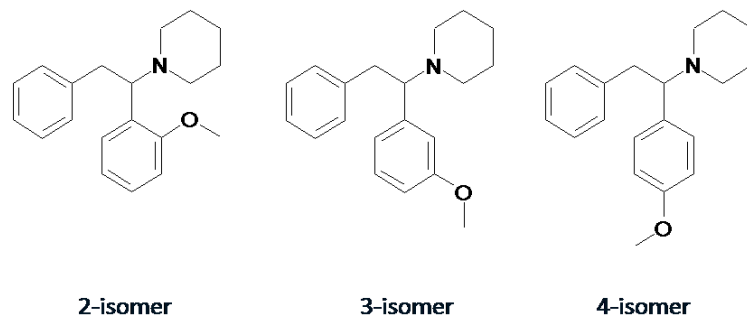
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603 Highlights

- 604 • Retention / separation of MXP regioisomers is controlled by electrostatic /
- 605 hydrophobic mechanisms
- 606 • Non-linear models were generated to describe the effect of % organic and
- 607 temperature on retention
- 608 • Two-dimensional (gradient time versus temperature) modelling was highly accurate
- 609 • Rapid separation of MXP regioisomers was achieved by retention modelling and
- 610 optimization
- 611 • A rapid / highly sensitive LC-MS method ($R_{s \text{ min}} > 3$ within 2.5 minutes) was predicted
- 612 and verified

613

614 Graphical highlight



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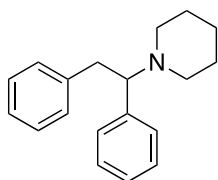
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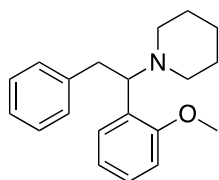
618 Figure 1. Structure of the diphenidine (1) and methoxydiphenidine regioisomers (2, 2-
619 MXP; 3, 3-MXP and 4, 4-MXP).

620

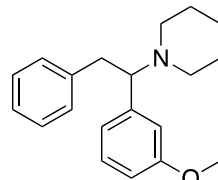
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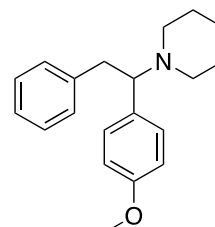
Diphenidine (1)



2-Methoxyphenidine
(2, 2-MXP)



3-Methoxyphenidine
(3, 3-MXP)



4-Methoxyphenidine
(4, 4-MXP)

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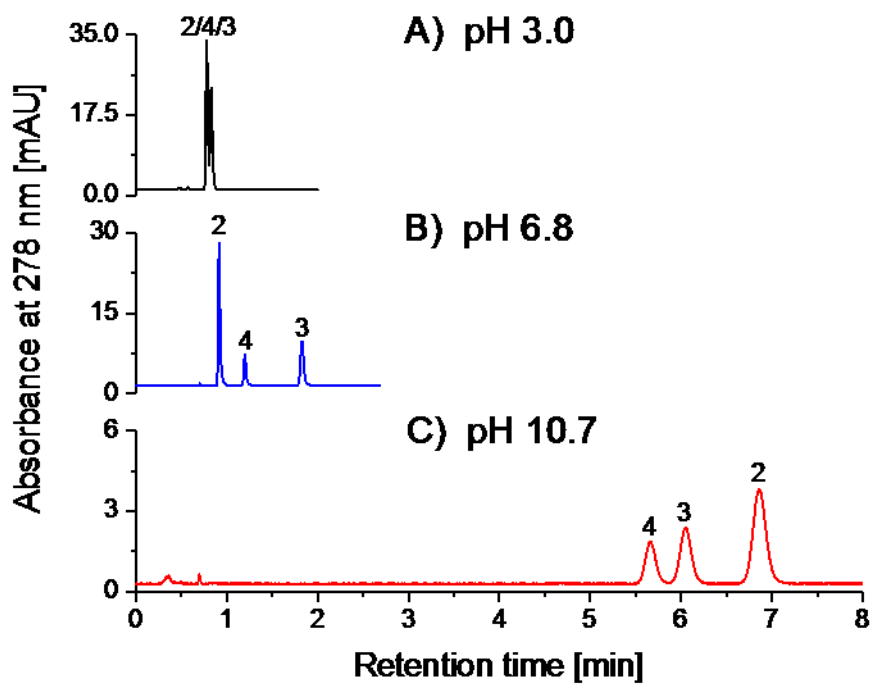
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626 Figure 2

627 Separation of the MXP isomers (2-, 3- and 4-isomers) on an ACE UltraCore
628 SuperC18 2.5 μm 50 x 4.6 mm column, 50 $^{\circ}\text{C}$, 1 mL min^{-1} , Agilent 1290 Infinity
629 Quaternary UHPLC, mobile phase of MeCN : water (54:46 v/v) containing a)
630 10 mM ammonium formate pH 3, b) 10 mM ammonium acetate (unadjusted
631 pH of 6.8) and c) 18 mM ammonia (unadjusted pH of 10.7). MXP isomer
632 assignment as shown in the chromatograms.



633

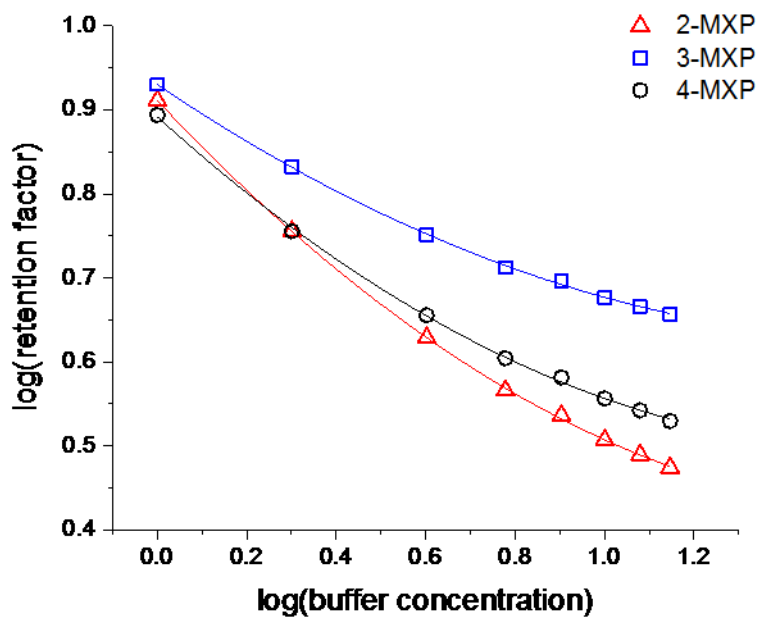
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636

637 Figure 3. Effect of buffer concentration on the retention on the regioisomers at pH 6.8
638 using an ACE C18-AR, 3 μm , 50 x 4.6 mm column, ammonium acetate (pH 6.8)
639 in MeCN/water (54:46 v/v), 30 $^{\circ}\text{C}$, 1 mL min^{-1} , Agilent 1290 Infinity
640 quaternary UHPLC.

641



642

643

644 Figure 4. The effect of the proportion of MeCN, on the retention of the MXP
645 isomers performed on an ACE SuperC18 3 μm 50 x 4.6 mm column, 1
646 mL min^{-1} , 60 $^{\circ}\text{C}$, Agilent 1290 Infinity binary UHPLC. Mobile phase A
647 buffer in water, mobile phase B buffer in MeCN/water (9:1 v/v).

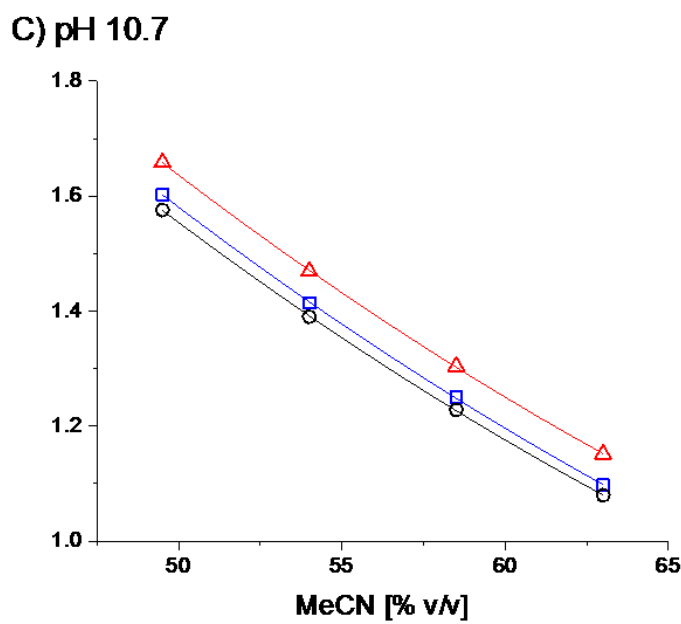
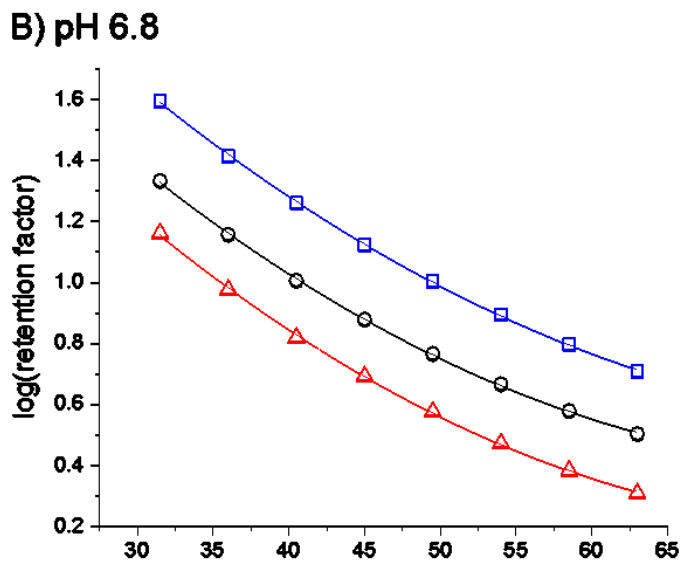
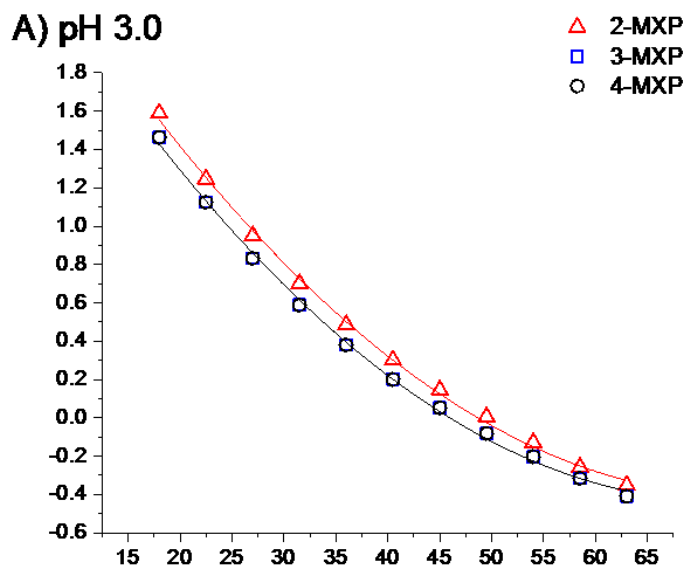
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649 4a) buffer 10 mM ammonium formate (pH 3.0).

650 4b) buffer 10 mM ammonium acetate (pH 6.8 unadjusted).

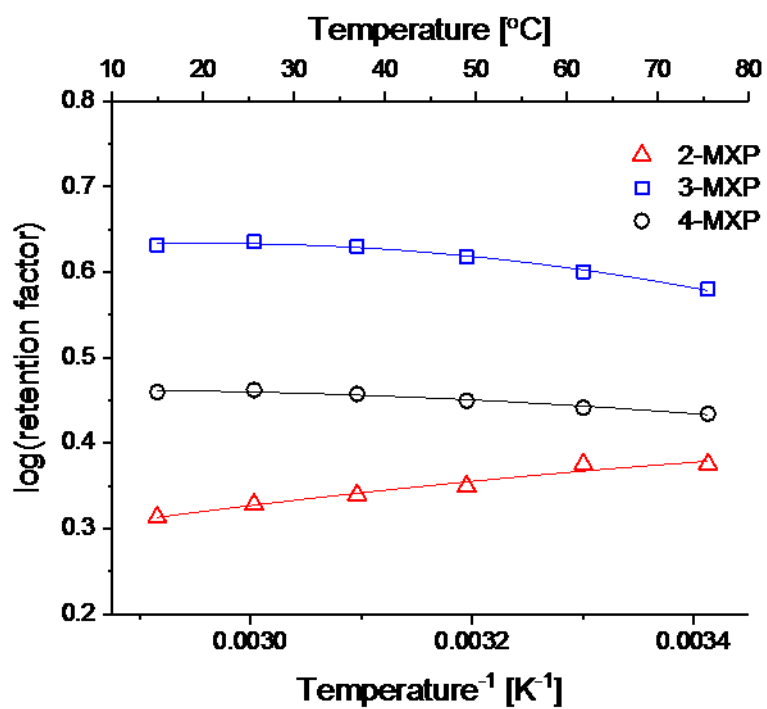
651 4c) buffer 18.6 mM ammonia (pH 10.7).

652



655 Figure 5. The effect of 1/temperature (°K) on the log of the retention factor of the MXP
656 isomers performed on an ACE C18-AR 3 μm 50 x 4.6 mm column using 10 mM
657 ammonium acetate (pH 6.8 unadjusted) in MeCN/water 54:46 v/v, 1 mL min⁻¹
658 using the Shimadzu Nexera X2 UHPLC.

659



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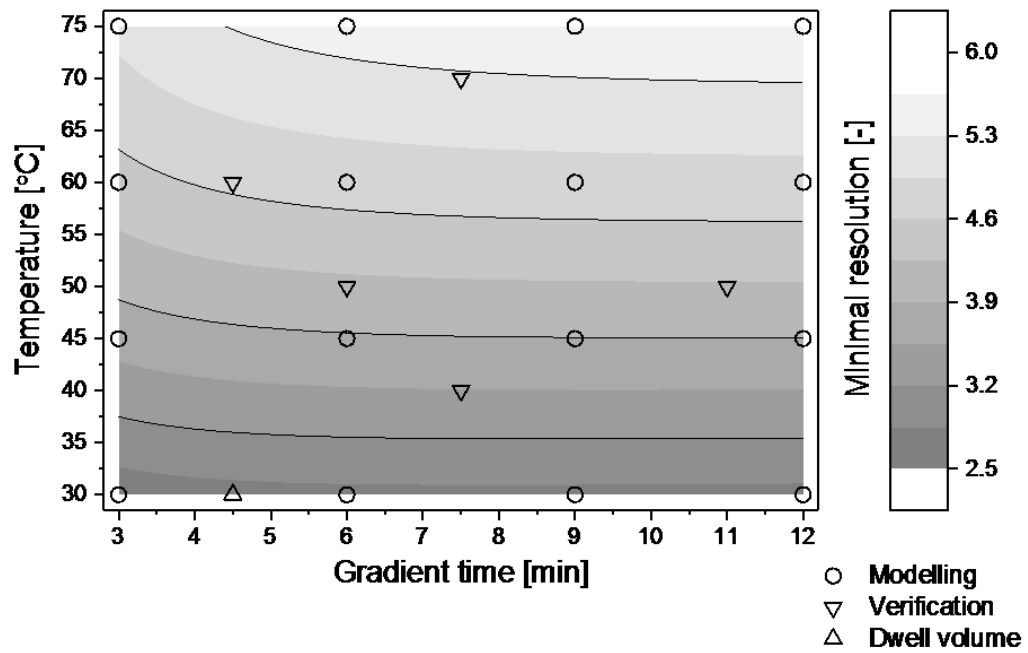
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662 Figure 6. a) Two-dimensional retention model (gradient time versus temperature) for
 663 the ACE C18-AR, 3 μm , 50 x 4.6 mm column, 1 mL min⁻¹, mobile phase A) 10
 664 mM ammonium acetate (pH 6.8 unadjusted) in water and B) 10 mM
 665 ammonium acetate (pH 6.8 unadjusted) in MeCN/water (9:1 v/v), gradient 40
 666 to 70%B, Nexera X2 UHPLC with a V_D and V_m of 517 and 458 μL respectively.
 667 b) Experimental and predicted chromatograms performed with a gradient
 668 and temperature of 4.5 min and 60 °C.

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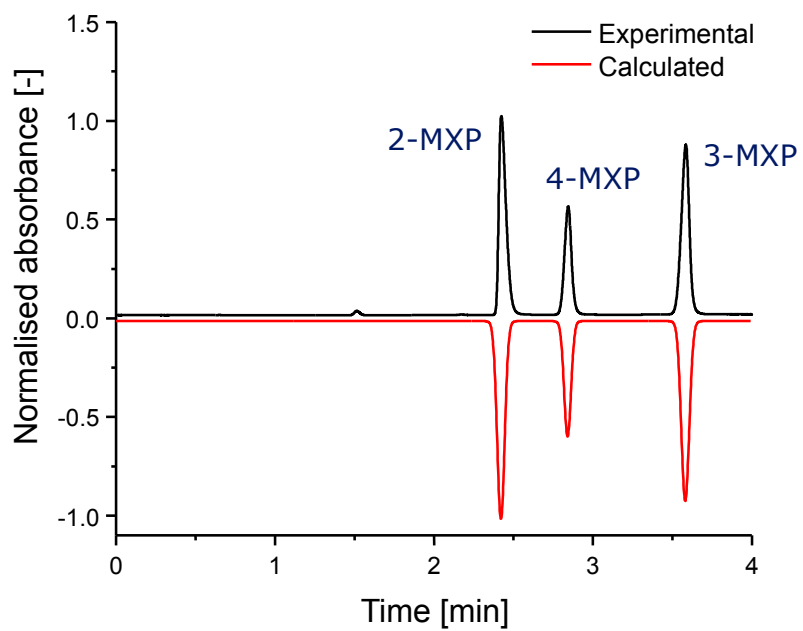
Figure 6a



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678 Table 1. Prediction errors for gradient time models using Equations 3 (gradient inputs of 3
 679 and 12 min) and 4 (gradient inputs of 3, 6, 9 and 12 min) as assessed by an interpolation of
 680 the retention at a gradient time of 4.5 minutes using a temperature of 30°C, where % Δ
 681 retention time (t_R) = (predicted t_R – actual t_R)/ actual t_R , % Δ peak width at 4 x standard
 682 deviation (4σ) = (predicted peak width at 4σ – actual peak width at 4σ)/ actual peak width at
 683 4σ , % ΔR_s at 4σ = (predicted resolution (R_s) at 4σ – actual R_s at 4σ)/ actual R_s at 4σ . V_D and the
 684 column void volume (V_m) = 517 and 458 μ L respectively.

685

Equation 3		Predicted			Actual			Δt_R (min)		Δ width (min)		ΔR_s (USP)		Model used
Peak Name	t_R (min)	Width (min)	R_s (USP)	t_R (min)	Width (min)	R_s (USP)	Δt_R (min)	% Δt_R	Δ width (min)	% Δ width	ΔR_s (USP)	% ΔR_s (USP)		
2-MXP	2.486	0.108		2.477	0.109		0.009	0.36	-0.001	-0.65			a = 4.9666, b = -7.8663e-2, c = 0.0000	
4-MXP	2.729	0.088	2.48	2.72	0.087	2.49	0.009	0.33	0.001	1.59	-0.01	-0.34	a = 4.8601, b = -7.2282e-2, c = 0.0000	
3-MXP	3.378	0.096	7.05	3.363	0.099	6.95	0.015	0.45	-0.003	-2.55	0.11	1.56	a = 5.0037, b = -6.6316e-2, c = 0.0000	

686

Equation 4		Predicted			Actual			Δt_R (min)		Δ width (min)		ΔR_s (USP)		Model used
Peak Name	t_R (min)	Width (min)	R_s (USP)	t_R (min)	Width (min)	R_s (USP)	Δt_R (min)	% Δt_R	Δ width (min)	% Δ width	ΔR_s (USP)	% ΔR_s (USP)		
2-MXP	2.475	0.110		2.477	0.109		-0.002	-0.08	0.001	1.19			a = 7.7101, b = -1.9844e-1, c = 1.2937e-3	
4-MXP	2.717	0.088	2.44	2.720	0.087	2.49	-0.003	-0.11	0.001	1.59	-0.04	-1.75	a = 7.0448, b = -1.6585e-1, c = 9.8928e-4	
3-MXP	3.361	0.100	6.85	3.363	0.099	6.95	-0.002	-0.06	0.001	1.51	-0.10	-1.37	a = 6.7427, b = -1.3715e-1, c = 7.0815e-4	

687

688

689 Table 2. Accuracy of the temperature models using Equations 5 (temperature inputs of 30
 690 and 70°C) and 6 (temperature inputs of 30, 45, 60 and 70°C) as assessed by an interpolation
 691 of the retention at 50°C using a gradient time of 6 minutes where % Δt_R = (predicted t_R –
 692 actual t_R)/ actual t_R , % Δ peak width at 4σ = (predicted peak width at 4σ – actual peak width
 693 at 4σ)/ actual peak width at 4σ , % ΔR_s at 4σ = (predicted R_s at 4σ – actual R_s at 4σ)/ actual R_s
 694 at 4σ . V_D and V_m = 517 and 458 μ L respectively.

695

Equation 5		Predicted			Actual			Δt_R (min)		Δ width (min)		ΔR_s (USP)		Model
Peak	t_R (min)	Width (min)	R_s (USP)	t_R (min)	Width (min)	R_s (USP)	Δt_R (min)	% Δt_R	Δ width (min)	% Δ width	ΔR_s (USP)	% ΔR_s (USP)		
2-MXP	2.547	0.106		2.574	0.100		-0.027	-1.05	0.006	5.78			a = 1.1179, b = 1.2912e+2	
4-MXP	2.947	0.097	3.94	2.988	0.093	4.28	-0.041	-1.37	0.004	3.83	-0.34	-7.84	a = 2.1439, b = -1.4570e+2	
3-MXP	3.747	0.112	7.66	3.831	0.109	8.34	-0.084	-2.19	0.003	3.03	-0.69	-8.22	a = 2.5724, b = -1.9410e+2	

696

Equation 6		Predicted			Actual			Δt_R (min)		Δ width (min)		ΔR_s (USP)		Model
Peak	t_R (min)	Width (min)	R_s (USP)	t_R (min)	Width (min)	R_s (USP)	Δt_R (min)	% Δt_R	Δ width (min)	% Δ width	ΔR_s (USP)	% ΔR_s (USP)		
2-MXP	2.568	0.103		2.574	0.100		-0.006	-0.23	0.003	2.78			a = -9.8483e-1, b = 1.4981e+3, c = -2.2174e+5	
4-MXP	2.985	0.095	4.21	2.988	0.093	4.28	-0.003	-0.10	0.002	1.69	-0.06	-1.50	a = -1.0249, b = 1.9168e+3, c = -3.3403e+5	
3-MXP	3.829	0.111	8.19	3.831	0.109	8.34	-0.002	-0.05	0.002	2.11	-0.15	-1.77	a = -2.5816, b = 3.1609e+3, c = -5.4338e+5	

697

698 Table 3. Predicted, actual and accuracy of retention time, peak width and resolution from
 699 the two-dimensional models (see Figure 6) using equation 4 (t_G inputs of 3, 6, 9 and 12 min)
 700 and equation 6 (temperature inputs of 30, 45, 60 and 70°C) as assessed by five interpolation
 701 conditions within the design space, where $\% \Delta t_R = (\text{predicted } t_R - \text{actual } t_R) / \text{actual } t_R$, $\% \Delta$
 702 peak width at $4\sigma = (\text{predicted peak width at } 4\sigma - \text{actual peak width at } 4\sigma) / \text{actual peak}$
 703 width at 4σ , $\% \Delta R_s \text{ at } 4\sigma = (\text{predicted } R_s \text{ at } 4\sigma - \text{actual } R_s \text{ at } 4\sigma) / \text{actual } R_s \text{ at } 4\sigma$. V_D and $V_m =$
 704 517 and 458 μL respectively.

705

Peak	Temperature (°C) t_G (min)		Predicted			Actual								
			t_R (min)	Width (min)	R_s (USP)	t_R (min)	Width (min)	R_s (USP)	Δt_R (min)	$\% \Delta t_R$	Δ width (min)	$\% \Delta$ width	ΔR_s (USP)	$\% \Delta R_s$ (USP)
2-MXP	70	7.5	2.585	0.111		2.576	0.098		0.010	0.37	0.013	13.27		
4-MXP			3.151	0.105	5.24	3.145	0.103	5.67	0.006	0.19	0.002	1.94	-0.43	-7.52
3-MXP			4.121	0.126	8.40	4.117	0.124	8.56	0.005	0.11	0.002	1.61	-0.16	-1.88
Peak	50	6	t_R (min)	Width (min)	R_s (USP)	t_R (min)	Width (min)	R_s (USP)	Δt_R (min)	$\% \Delta t_R$	Δ width (min)	$\% \Delta$ width	ΔR_s (USP)	$\% \Delta R_s$ (USP)
2-MXP			2.566	0.104		2.574	0.100		-0.008	-0.31	0.004	4.00		
4-MXP			2.982	0.095	4.18	2.988	0.093	4.30	-0.006	-0.20	0.002	2.15	-0.12	-2.77
3-MXP	3.827	0.113	8.13	3.831	0.109	8.30	-0.004	-0.10	0.004	3.67	-0.18	-2.11		
Peak	50	11	Predicted			Actual								
			t_R (min)	Width (min)	R_s (USP)	t_R (min)	Width (min)	R_s (USP)	Δt_R (min)	$\% \Delta t_R$	Δ width (min)	$\% \Delta$ width	ΔR_s (USP)	$\% \Delta R_s$ (USP)
2-MXP			2.793	0.124		2.792	0.123		0.001	0.04	0.002	1.22		
4-MXP	3.303	0.117	4.23	3.302	0.115	4.29	0.002	0.05	0.002	1.74	-0.06	-1.36		
3-MXP	4.415	0.146	8.46	4.411	0.144	8.58	0.004	0.09	0.003	1.74	-0.13	-1.49		
Peak	60	4.5	Predicted			Actual								
			t_R (min)	Width (min)	R_s (USP)	t_R (min)	Width (min)	R_s (USP)	Δt_R (min)	$\% \Delta t_R$	Δ width (min)	$\% \Delta$ width	ΔR_s (USP)	$\% \Delta R_s$ (USP)
2-MXP			2.420	0.094		2.423	0.085		-0.003	-0.12	0.009	10.59		
4-MXP	2.840	0.086	4.67	2.843	0.083	5.00	-0.003	-0.11	0.003	3.61	-0.33	-6.67		
3-MXP	3.580	0.099	8.00	3.584	0.095	8.32	-0.003	-0.10	0.004	4.21	-0.32	-3.85		
Peak	40	7.5	Predicted			Actual								
			t_R (min)	Width (min)	R_s (USP)	t_R (min)	Width (min)	R_s (USP)	Δt_R (min)	$\% \Delta t_R$	Δ width (min)	$\% \Delta$ width	ΔR_s (USP)	$\% \Delta R_s$ (USP)
2-MXP			2.680	0.112		2.672	0.117		0.009	0.32	-0.005	-4.27		
4-MXP	3.059	0.102	3.54	3.047	0.102	3.43	0.013	0.41	0.000	0.49	0.11	3.19		
3-MXP	3.955	0.122	8.00	3.940	0.122	7.99	0.016	0.39	0.000	0.00	0.01	0.11		

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707

708 Table 4. Predicted, actual and accuracy of retention time, peak width and resolution from
 709 the two-dimensional models using equation 4 (t_G inputs of 3, 6 and 9 min) and equation 6
 710 (temperature inputs of 30, 45 and 60°C) as assessed by three interpolation conditions within
 711 the design space, where $\% \Delta t_R = (\text{predicted } t_R - \text{actual } t_R) / \text{actual } t_R$, $\% \Delta \text{ peak width at } 4\sigma =$
 712 $(\text{predicted peak width at } 4\sigma - \text{actual peak width at } 4\sigma) / \text{actual peak width at } 4\sigma$, $\% \Delta R_s \text{ at } 4\sigma =$
 713 $(\text{predicted } R_s \text{ at } 4\sigma - \text{actual } R_s \text{ at } 4\sigma) / \text{actual } R_s \text{ at } 4\sigma$. V_D and $V_m = 517$ and $458 \mu\text{L}$
 714 respectively.

715

Peak	Temperature (°C) t_G (min)		Predicted			Actual			Δt_R (min) $\% \Delta t_R$		$\Delta \text{ width (min) } \% \Delta \text{ width}$		ΔR_s (USP) $\% \Delta R_s$ (USP)	
	50	6	t_R (min)	Width (min)	R_s (USP)	t_R (min)	Width (min)	R_s (USP)	Δt_R (min)	$\% \Delta t_R$	$\Delta \text{ width (min)}$	$\% \Delta \text{ width}$	ΔR_s (USP)	$\% \Delta R_s$ (USP)
2-MXP	50	6	2.567	0.107		2.574	0.100		-0.008	-0.31	0.004	4.00		
4-MXP			2.984	0.096	4.11	2.988	0.093	4.30	-0.006	-0.20	0.002	2.15	-0.19	-4.46
3-MXP			3.828	0.113	8.08	3.831	0.109	8.30	-0.004	-0.10	0.004	3.67	-0.22	-2.69
	60	4.5	Predicted			Actual			Δt_R (min) $\% \Delta t_R$		$\Delta \text{ width (min) } \% \Delta \text{ width}$		ΔR_s (USP) $\% \Delta R_s$ (USP)	
Peak			t_R (min)	Width (min)	R_s (USP)	t_R (min)	Width (min)	R_s (USP)	Δt_R (min)	$\% \Delta t_R$	$\Delta \text{ width (min)}$	$\% \Delta \text{ width}$	ΔR_s (USP)	$\% \Delta R_s$ (USP)
2-MXP			2.422	0.096		2.423	0.085		-0.003	-0.12	0.009	10.59		
4-MXP	2.841	0.086	4.60	2.843	0.083	5.00	-0.003	-0.11	0.003	3.61	-0.40	-7.91		
3-MXP	3.581	0.099	8.00	3.584	0.095	8.32	-0.003	-0.10	0.004	4.21	-0.32	-3.85		
	40	7.5	Predicted			Actual			Δt_R (min) $\% \Delta t_R$		$\Delta \text{ width (min) } \% \Delta \text{ width}$		ΔR_s (USP) $\% \Delta R_s$ (USP)	
Peak			t_R (min)	Width (min)	R_s (USP)	t_R (min)	Width (min)	R_s (USP)	Δt_R (min)	$\% \Delta t_R$	$\Delta \text{ width (min)}$	$\% \Delta \text{ width}$	ΔR_s (USP)	$\% \Delta R_s$ (USP)
2-MXP			2.679	0.115		2.672	0.117		0.009	0.32	-0.005	-4.27		
4-MXP	3.059	0.102	3.50	3.047	0.102	3.43	0.013	0.41	0.000	0.49	0.07	2.03		
3-MXP	3.955	0.123	7.96	3.940	0.122	7.99	0.016	0.39	0.000	0.00	-0.03	-0.33		

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