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Development of a green HPLC method for the analysis of artesunate and amodiaquine impurities using Quality by Design

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Abstract

Greening analytical methods has become of great interest in the field of pharmaceutical analysis to protect both the operators' health and the environment. In this work, an innovative methodology combining Quality-by-Design (QbD) and Green Chemistry principles was followed to develop a single, green and robust RP-HPLC method for the quantitative analysis of impurities of both artesunate and amodiaquine drugs. Ethanol was selected as the best ecofriendly alternative solvent in substitution to the commonly used organic solvents such as acetonitrile and methanol. To achieve method objectives, resolutions between the 10 peaks were chosen as critical method attributes (CMAs) to be optimized through QbD approach. Based on a quality risk assessment, pH, temperature, and gradient slope were then selected as critical method parameters (CMPs) and a three level full factorial design was used to model the CMAs as function of the CMPs. Response surface methodology associated to Monte Carlo simulations allowed to determine the method operable domain region (MODR), i.e., the multidimensional combination of CMPs where CMAs simultaneously satisfied specifications ($R_s \geq 1.5$) with a probability at least equal to 95%. Inside the MODR, the working point was chosen based on green criteria, involving a mobile phase composed of ethanol and 10 mM acetic acid only as pH modifier. The method was successfully validated for all impurities using accuracy profile methodology, which was fully compliant with the ICH Q2(R1) requirements. Finally, the method was applied to the analysis of amodiaquine and artesunate impurities in raw materials and formulations.

Key words: Green chromatography, Quality by Design, design space, impurity analysis, amodiaquine, artesunate, method validation

39 1. Introduction

40 Green Analytical Chemistry (GAC) emerged from green chemistry and has gained increasing
41 interest and acceptance among researchers to reduce environmental impacts and improve the
42 health safety of analysts [1,2]. GAC consists in eliminating or reducing hazardous chemicals
43 from analytical processes to improve environmental and health friendliness, without
44 compromising method performance [3]. High-Performance Liquid Chromatography (HPLC)
45 in the reversed-phase mode is the most widely used analytical technique for pharmaceutical
46 quality control (QC) and is involved in the analysis of active pharmaceutical ingredients (API)
47 as well as their impurities [4]. However, RP-HPLC methods commonly use large amounts of
48 hazardous organic solvents such as acetonitrile and produce high quantities of waste to be
49 disposed, leading to important issues in terms of ecological impact and operator safety [5,6].
50 Developing green HPLC methods becomes therefore highly desirable [7].

51 Based on the 12 principles of green chemistry, some strategies are commonly implemented to
52 achieve greener liquid chromatography methods [2,8]. One strategy is to reduce solvent
53 consumption through a decrease of the length, internal diameter, and/or particle size of the
54 column. However, this approach often implies the use of expensive ultra-high-performance
55 liquid chromatography (UHPLC) instruments which limits its access in QC laboratories
56 especially in resource-limited countries. Another way to make liquid chromatography
57 ecofriendly is to substitute classically used organic solvents, mainly acetonitrile and methanol
58 by greener ones [9]. Ethanol is one of the best green alternative solvent because of its
59 sourcing and safety of handling by operators [10].

60 Unfortunately, the use of green analytical methods is still limited in the pharmaceutical field,
61 particularly in QC activities. This could be partly explained by analytical methods described
62 in Pharmacopeia monographs which are usually not ecofriendly [11]. Other reasons may be
63 the lack of knowledge or experience and the high cost of equipment renewal to reach higher
64 performances, e.g. the purchase of an UHPLC instrument. However, green methods can be
65 developed even with conventional apparatus without requiring expensive equipment or
66 technology, leading to cheaper methods.

67 Artesunate (AS) and amodiaquine (AQ) association is one of the WHO-recommended
68 Artemisinin-based combination therapies to treat uncomplicated *Plasmodium falciparum*
69 malaria. Both artesunate and amodiaquine are part of the WHO Model lists of Essential
70 Medicine [12] and their fixed dose combination tablets are available on the market. Besides
71 the quantitative determination of APIs, quality control also requires the determination of
72 impurities in the bulk drugs as well as in the finished products. In the International
73 Pharmacopeia [13], the related substances analysis of artesunate is done by RP-HPLC using
74 acetonitrile while amodiaquine impurities are analyzed by Thin Layer Chromatography using
75 chloroform which has toxicity concerns. In literature, many analytical methods are described
76 for the determination of related substances and degradation products for either artesunate
77 [14,15] or amodiaquine [16,17] but to the best of our knowledge, there is no published
78 method for the single analysis of artesunate and amodiaquine impurities. Also, green
79 chemistry principles were not applied during method developments.

80 In pharmaceutical QC, the simultaneous analysis of an API and its related impurities is most
81 often challenging because of the similarity of their chemical structures and the high difference
82 in quantities between API and impurities. To overcome these difficulties, a systematic
83 approach to method development using Quality by Design (QbD) is highly recommended
84 [18]. QbD is a concept of quality initially introduced by the Food and Drug Administration in
85 2004 to be applied in the pharmaceutical industry for manufacturing processes [19]. Since
86 2009, QbD methodology is supported by ICH through the ICH Q8 (R2) guideline [20]. When
87 applied to analytical methods, QbD is commonly called Analytical Quality by Design

88 (AQbD) and can be defined as a systematic approach to development that begins with
89 predefined objectives and emphasizes analytical method understanding and control, based on
90 sound science and quality risk management [21]. The main objective of QbD methodology is
91 the definition of the design space also called the method operable domain region (MODR)
92 corresponding to the multidimensional robustness space where any combination of variables
93 has demonstrated to provide assurance of method quality [22,23]. Dealing with
94 pharmaceutical analysis, QbD approach has been mainly applied to impurities or degradation
95 products analysis [18,19,21]. This concept has also been used for the screening of APIs but to
96 a lower extent [24,25]. However, examples combining QbD strategy with green chemistry
97 principles remain limited [11,25].

98 In this work, an AQbD approach was developed to achieve a single and green RP-HPLC
99 method for the analysis of artesunate and amodiaquine impurities. This development was
100 particularly challenging due to the complexity of the mixture to be separated composed of 9
101 molecules including acidic, basic and structurally related compounds (**Fig. 1**). Moreover, the
102 use of an elution gradient was required raising detection concerns due to ethanol wavelength
103 cut-off and the presence of compounds with poor detection in UV. The developed method was
104 validated for impurity analysis of AS and AQ raw materials.

105 **2. Materials and methods**

106 **2.1. Chemicals and reagents**

107 Amodiaquine hydrochloride (>99%) and artesunate (>99%) United States Pharmacopeia
108 (USP) reference Standards, 7-chloro-4-(4-hydroxyphenylamino)quinolone (Impurity 3), 4,7-
109 dichloroquinoline (Impurity 4, 99%), and artemisinin (Impurity A, 98%) were supplied from
110 Sigma Aldrich (St Louis, MO, USA). 4-acetamido-2-diethylaminomethylphenol (Impurity 1,
111 >95%) and anhydrodihydro-artemisinin (Impurity C, 98%) were purchased from Carbosynth
112 Ltd (Berkshire, UK). Paracetamol (Impurity 2, 99%), and dihydroartemisinin (Impurity B)
113 were respectively bought from Cooper (Melun, France) and Mangalam Drugs and organics
114 Ltd (India). Amodiaquine dihydrochloride dihydrate raw materials were obtained from Parke
115 Davis (Senegal) while artesunate raw materials were purchased from Knoll BASF Pharma
116 (Liestal, Switzerland). Ethanol (EtOH) 96% HPLC grade, acetic acid HPLC grade ($\geq 99.9\%$),
117 formic acid LC/MS grade (99%), and ammonium acetate (99%) were purchased from VWR
118 chemicals (VWR International, Fontenay-sous-Bois, France). Ammonium formate was
119 obtained from Sigma Aldrich (St Louis, MO, USA). HPLC grade water (minimum resistivity
120 of 18.2 M Ω) was produced in house by ELGA Millipore system (France).
121 Artesunate/amodiaquine 100mg/270mg fixed dose tablets were from Sanofi Winthrop
122 (Morocco).

123 **2.2. Instrumentation**

124 Chromatographic experiments were performed on an Accela UHPLC system (Thermo
125 Scientific, USA) equipped with a pumping device, an autosampler, a column oven, and a
126 diode array detector.

127 **2.3. Analytical conditions**

128 The chromatographic columns used in this study were Xbridge BEH C18 (150 mm x 3 mm, 5
129 μm) purchased from Waters (Milford, MA, USA). All analyzes were performed in gradient
130 mode.

131 UV spectra were systematically recorded from 200 to 400 nm for peak identification. To
132 ensure a satisfactory sensitivity, peak integrations were performed at 210 nm for AS and its

133 impurities, and 254 nm for AQ and its impurities. For all experiments, the sample injection
134 volume was 10 μ L. An experimental design was used to vary simultaneously gradient slope,
135 pH of mobile phase A, and column temperature.

136 Mobile phase A was an aqueous solution with pH modifiers to reach the desired pH. For
137 method development, pH 2.9 was obtained by mixing 10 mM formic acid with 10 mM
138 ammonium formate while pH 3.5 was fixed using a mixture of 10 mM acetic acid and 10 mM
139 ammonium acetate. A pH of 3.2 was obtained with a 20 mM acetic acid solution. At the
140 working point, a pH of 3.35 was fixed using 10 mM acetic acid. Mobile phase B was
141 composed of 96% EtOH containing the same proportion of pH modifiers as mobile phase A to
142 prevent ionic concentration change during the gradient. The gradient profile consisted in a
143 linear ramp from 5% to 80% of mobile phase B, which corresponded to a variation of 72% in
144 pure ethanol from 4.8% to 76.8%. Since an UHPLC system was used and for a suitable
145 transfer of the method to a classical HPLC apparatus, an isocratic plate of 3 min consisting in
146 5% of mobile phase B was added, at the beginning of the gradient for all analyses. Flow rate
147 was set 0.4 mL/min.

148 **2.4. Sample preparation**

149 **2.4.1. Sample preparation for method development**

150 Stock solutions of AQ and AS at 10 mg/mL and 40 mg/mL, respectively, were prepared in
151 96% EtOH.

152 For each impurity, a stock solution of 1 mg/mL were prepared in 96% EtOH.

153 Working concentration of AQ was set at 2 mg/mL while AS, which has low absorbance in
154 UV, was injected at 20 mg/mL.

155 Each AQ impurity was studied at 0.5 % (0.01 mg/mL) of AQ concentration. Working
156 concentration of AS impurities was set at 0.5% (0.1 mg/mL) for impurity A and 2.5 % (0.5
157 mg/mL) for Impurity B, relative to AS concentration. Concentration levels were determined
158 according to the International Pharmacopeia related substance specifications of these two
159 drugs [13].

160 For AQ and its impurities, solutions were prepared by diluting appropriate volumes of stock
161 solutions in water - 96% EtOH (95/5, v/v) while solutions of AS and its impurities were
162 obtained by diluting stock solutions using water - 96% EtOH (50/50, v/v). A higher
163 percentage of EtOH was required for dilution of AS and its relative impurities samples
164 because of solubility problems whereas at such high percentage of EtOH, the early eluted
165 compounds, AQ and the majority of its impurities, evidenced peak broadening. Two mixtures
166 were prepared for design space checking and working point analysis by considering critical
167 pairs of peaks. The first mixture was composed of AQ and its impurities at 1 mg/mL and
168 0.005 mg/mL, respectively in water - 96% EtOH (95/5, v/v). The second mixture was
169 prepared in water - 96% EtOH (50/50, v/v) by adding AS at a concentration of 20 mg/mL,
170 impurity A at 0.1 mg/mL, impurity B at 0.5 mg/mL, and impurity 4 at 0.01 mg/mL to check
171 its critical separation with the second peak of impurity B.

172 Impurity C was not added during the method development due to its high price and
173 preliminary studies which showed that it was the last eluted compound.

174 **2.4.2. Sample preparation for method validation**

175 **2.4.2.1. Amodiaquine impurities**

176 Each day, a stock solution of each impurity was prepared at 1 mg/mL in 96% EtOH. The
177 target concentration of each impurity was 0.005 mg/mL (corresponding to 0.5% of an AQ
178 concentration of 1 mg/mL).

179 For the calibration set, stock solutions of each impurity were diluted with water – 96% EtOH
180 (95/5 v/v) containing 10 mM acetic acid to obtain three different concentration levels: 0.0025
181 mg/mL, 0.005 mg/mL, and 0.0075 mg/mL. Calibration standards were prepared in duplicate
182 by repeating the dilution step.

183 For the validation set, five concentration levels were injected for each impurity: 0.0025
184 mg/mL, 0.004 mg/mL, 0.005 mg/mL, 0.006 mg/mL, and 0.0075 mg/mL. AQ was added to
185 samples by mixing appropriate volumes of stock solutions and diluting in water – 96% EtOH
186 (95/5 v/v) containing 10 mM acetic acid to reach the desired impurity concentration and a
187 concentration of 1 mg/mL for AQ. Validation standards were prepared in triplicate by
188 repeating the dilution step.

189 This procedure was repeated at three different days and each solution was analyzed under the
190 chromatographic conditions of the working point.

191 **2.4.2.2. Artesunate impurities**

192 Each day, a stock solution of each impurity was prepared at 1 mg/mL in 96% EtOH.

193 For the calibration set, stock solutions of each impurity were diluted with water - 96% EtOH
194 (50/50 v/v) containing 10 mM of acetic acid to obtain three different levels of concentrations:
195 0.05, 0.1, and 0.15 mg/mL for impurity A, 0.1, 0.3, and 0.6 mg/mL for impurity B, 0.02, 0.04,
196 and 0.072 mg/mL for impurity C. Calibration standards were prepared in duplicate by
197 repeating the dilution step.

198 For the validation set, five concentration levels were injected: 0.05, 0.08, 0.1, 0.12, and 0.15
199 mg/mL for impurity A, 0.1, 0.2, 0.3, 0.5, and 0.6 mg/mL for impurity B, and 0.02, 0.03, 0.04,
200 0.06, and 0.072 mg/mL for impurity C. AS was added to samples by mixing appropriate
201 volumes of stock solutions and diluting in water - 96% EtOH (50/50, v/v) containing 10 mM
202 of acetic acid to reach the desired impurity concentration and a concentration of 20 mg/mL for
203 AS. Validation standards were prepared in triplicate by repeating the dilution step.

204 This procedure was repeated at three different days and each solution was analyzed under the
205 chromatographic conditions of the working point.

206 Because impurity B (dihydroartemisinin) exists in two forms in equilibrium (α -
207 dihydroartemisinin and β -dihydroartemisinin), leading to two peaks in HPLC [26], its area
208 was calculated taking the sum of areas of the two peaks.

209 **2.4.3. Sample preparation for raw materials and tablets**

210 AQ raw materials were prepared at 1 mg/mL in water - 96% EtOH (95/5 v/v) containing 10
211 mM acetic acid while AS raw materials were prepared at 20 mg/mL in water - 96% EtOH
212 (50/50 v/v) containing 10 mM acetic acid.

213 For the analysis of fixed dose tablets, two preparations from the powdered tablets were done,
214 one allowing to analyze AQ at 1 mg/mL in water - 96% EtOH (95/5 v/v) containing 10 mM
215 acetic acid and the second allowing to analyze AS at 20 mg/mL in water - 96% EtOH (50/50
216 v/v) containing 10 mM acetic acid.

217 **2.5. Software**

218 JMP 11.0 software (package) (S.A.S Institute Inc, Cary, NC, USA) was used to build design
219 of experiments, to model responses and to compute the Design Space using Monte Carlo
220 Simulations. Iso-response surfaces were built using Design Expert 9.0.6 software (Stat-Ease
221 Inc, Minneapolis, MN, USA).

222 3. Results and discussion

223 3.1. Method development and optimization

224 The development of the method was conducted according to AQBd approach which started
225 with the definition of the analytical target profile (ATP) and the subsequent choice of critical
226 method attributes (CMAs). Critical method parameters (CMPs) were then selected through a
227 quality risk assessment and a design of experiment (DoE) was carried out to optimize method.
228 Finally, Monte Carlo simulations were used to define the MODR i.e. the multidimensional
229 combination of CMPs where CMAs simultaneously satisfied specifications with a defined
230 probability [21,22].

231 3.1.1. Analytical target profile

232 ATP is defined as the purpose and the scope of the analytical method [18,22]. In our study, it
233 consisted in developing a green and robust analytical method, able to implement in resource-
234 limited laboratories, for the quantitative analysis of impurities of both AS and AQ. According
235 to the International Pharmacopeia and literature, AS and AQ have three and four main
236 impurities, respectively [13,16,17]. The structures of both APIs: AS and AQ, and their related
237 compounds are reported in **Fig. 1**.

238 Based on the ATP, RP-HPLC was chosen as the separation technique to achieve a baseline
239 separation of AQ, AS and their impurities. In fact, HPLC instrument is a commonly used
240 analytical tool for pharmaceutical analysis [4]. It is available even in resource-limited QC
241 laboratories. In addition, the substitution of toxic solvents by more eco-friendly ones is a
242 possible approach to green RP-HPLC [9].

243 3.1.2. Critical method attributes

244 To reach ATP, CMAs were selected. They are measured responses that should be within an
245 appropriate limit or range to ensure the desired quality of the analytical method [22]. In this
246 study, resolutions (R_s) between all peaks were retained as CMAs. In that case, since the
247 elution order was kept the same on the whole experimental space investigated, R_s were
248 expected to be monotonic responses, and thus could be well modelled with multiple linear
249 regressions. As impurity C was not included during method development and two peaks were
250 detected for impurity B, 8 resolutions between adjacent peaks were considered as CMAs. To
251 achieve a good quantification, a separation between peaks with resolutions at least equal to
252 1.5 was targeted.

253 3.1.3. Critical method parameters

254 The next step was to define CMPs which are parameters whose variability has an impact on
255 one or more CMA(s) and therefore should be monitored or controlled to ensure that the
256 method meets the desired quality. As several factors can affect the performance of a
257 chromatographic method, an approach based on quality risk assessment was followed. An
258 Ishikawa diagram was used to identify parameters having the highest risk of affecting CMAs,
259 to be further studied (**Fig. 2**). This diagram gathers the different types of CMPs which could
260 have a potential effect on CMAs. Three different categories were distinguished by a color:
261 CMPs related to the HPLC equipment are in black, CMPs fixed from background knowledge
262 are justified in the text below and appear in blue, and CMPs in red were optimized through
263 DoE.

264 Two wavelengths were required to detect the two types of molecules. A low wavelength (i.e.
265 210 nm) was needed for AS and its impurities. However, this wavelength could not be used
266 for AQ and its relative substances because of too high UV response. Therefore, a second
267 wavelength was selected at 254 nm.

268 **3.1.3.1. Column selection**

269 The Xbridge BEH C18 column was selected because it offered, not only a high stability at
270 low and high pH values but also satisfactory peak shape for basic compounds such as AQ and
271 some of its impurities (Impurities 1 and 3). In fact, the presence of ethylene bridges in the
272 silica structure and the trifunctional bonding limits free residual silanols and their
273 accessibility. It is well known that free silanols, when ionized, can interact with positively
274 charged compounds (case of basic compounds at low pH), leading to peak tailing and peak
275 broadening. The column geometry (150 mm x 3 mm, 5 μ m) was selected in order to be able to
276 work with classical HPLC equipment. The internal diameter was selected as low as possible
277 to remain compatible with the apparatus and limit the flow rate in order to limit solvent
278 consumption.

279 **3.1.3.2. Green organic solvent and reagent selection for mobile phase and sample**
280 **preparation**

281 Mobile phases in RP-HPLC are generally composed of an aqueous part with reagents to
282 adjust pH and an organic solvent. Acetonitrile and methanol are the two common organic
283 solvents used in RP-HPLC mainly because of the low viscosity of their mixture with water
284 and their low UV cut-off wavelength [5]. However, they present environmental impact and
285 health safety issues. In fact, they have been ranked as hazardous solvents due to their inherent
286 toxicity and the great requirements to dispose their waste [6]. Greening RP-HPLC methods
287 can be done by replacing acetonitrile and methanol by greener ones [5,6,9]. Among
288 alternative solvents, ethanol has been selected due to its advantages: low toxicity, bio-
289 sourcing and biodegradability. Nevertheless, its main drawbacks are baseline drift during
290 gradient, especially at low wavelength, and a high viscosity of its mixture with water, leading
291 to high column backpressure [9]. Therefore, in this study, flow rate was fixed at 0.4 mL/min
292 to limit the backpressure at 200 bars and achieve a method compatible with a classical HPLC
293 apparatus.

294 pH modifiers as green as possible were also investigated, preferentially acetic acid or formic
295 acid alone or else in combination with their corresponding salt, ammonium acetate or
296 ammonium formate.

297 Dealing with sample preparation, solvents selected were similar to those used for the mobile
298 phase, thereby keeping the GAC approach also for this step of the analytical development.
299 However, solvent composition of samples was adapted to the solubility and eluotropic
300 strength requirement for the elution of each compound. Moreover, to limit the effect of
301 sample solvent on the elution of early eluting peak, a 10 μ L injection volume was selected.

302 **3.1.4. Response-surface DoE**

303 A three level full factorial design was performed to establish the polynomial mathematical
304 models linking the CMAs to the three CMPs (pH of the aqueous phase, gradient slope, and
305 column temperature). Five center points were added to estimate the experimental error
306 allowing model lack of fit assessment. A total of 32 experiments ($3^3 + 5$) were therefore
307 carried out by DoE. Experiments were performed in a random order to transform unknown
308 systematic errors into random ones except for center points that were regularly distributed to
309 be able to notice changes occurring in the responses during the whole DoE, and thus
310 repeatability problems. The experimental matrix with the measured responses is reported in
311 Supplementary data (**Table S1**).

312 Variation domains for the three CMPs were determined based on preliminary studies: pH of
313 the aqueous phase between 2.9 and 3.5; gradient slope between 1.6 and 2.8 %/min; and

314 column temperature between 25 and 35°C. The third level was the middle of the variation
315 range of each parameter (3.2 for pH, 2.2%/min for gradient slope and 30°C for column
316 temperature). pH range was restricted to allow good peak shape for basic compounds. In fact,
317 working at a low pH range reduced the number of negative charges at the surface of the silica
318 coming from free silanols and consequently, the interaction between positively charged basic
319 compounds and column surface was weaker, limiting peak tailing. Moreover, temperatures
320 above 35°C led to bad peak shape for impurity B peaks which were not baseline separated.
321 The gradient slope range was fixed, thanks to preliminary studies, with the objective to
322 minimize the risk of not finding any good separation within it.

323 During analysis, compounds were eluted in this order: impurity 1, impurity 2, AQ, impurity 3,
324 first peak of impurity B, impurity A, AS, second peak of impurity B and lastly Impurity 4.
325 Three responses were found more critical: Rs (impurity A; first peak of impurity B), Rs (AS;
326 impurity A), and Rs (second peak of impurity B; impurity 4).

327 A full quadratic model was selected and fitted to obtain a predictive model for each CMA.
328 The model is written as follows:

$$329 \quad y(X_{1 \leq i \leq 3}) = \beta_0 + \sum_{i=1}^3 \beta_i X_i + \sum_{i=1}^3 \beta_{ii} X_i^2 + \sum_{i=2}^3 \sum_{j=1}^{i-1} \beta_{ij} X_i X_j + \epsilon$$

330 Where $y(X_{1 \leq i \leq 3})$ represent the CMAs, X_i the CMPs, β_0 the intercept, β_i the linear
331 coefficients, β_{ii} the quadratic coefficients, β_{ij} the first-order interaction coefficients, and ϵ the
332 random error of the method.

333 The analysis of variance (ANOVA) was used to assess the significance of the models.
334 Obtained results (**Table 1**) showed that all models were significant (p-values <0.0001).
335 Calculated R^2 ranging from 0.80 to 0.97 and insignificant lack of fit (p-values > 0.05) were
336 obtained for all CMAs, indicating that a high proportion of variation in the responses could be
337 explained by the models, and therefore proving the good quality of the models. Iso-response
338 surfaces (contour plots) of the most critical resolutions are given in Supplementary data (**Fig.**
339 **S1**). Rs (impurity A; first peak of impurity B) and Rs (AS; impurity A) were mostly
340 influenced by the gradient slope while Rs (second peak of impurity B; impurity 4) was
341 particularly impacted by pH and temperature. The gradient slope had a negative effect on Rs
342 (impurity A; first peak of impurity B) and Rs (AS; impurity A). On the contrary, pH and
343 temperature had positive effects on Rs (second peak of impurity B; impurity 4).

344 In order to satisfy the ATP, the established mathematical models were used to determine the
345 MODR.

346 **3.1.5. Method operable design region**

347 The MODR is defined as a zone of robustness where the quality of the method is guaranteed
348 with a high level of assurance [21]. In practice, it corresponds to the variation range of CMPs
349 where CMAs simultaneously satisfy the specifications with a defined probability.

350 The MODR was established through Monte-Carlo simulations which take into account
351 parameter uncertainty and propagate it to the modelled responses to be able to establish
352 probability maps for the CMAs to meet the specifications, i.e. $R_s \geq 1.5$ [22].

353 Monte-Carlo simulation experiments were performed over the whole experimental space. The
354 3D experimental domain was divided in 1 million points and centered on each of them, 5000
355 random runs were simulated. From these simulations which take into account model error, a
356 distribution of each CMA corresponding to prediction uncertainty was achieved. At each
357 simulated point, an overall defect rate was then calculated, corresponding to the probability
358 that at least one CMA is out of specification, i.e. R_s lower than 1.5. The MODR was then

359 determined as the multidimensional combination of CMPs where the 8 predicted resolutions
360 were at least 1.5 with a probability higher or equal to 95%. **Fig. 3** represents the defect rate
361 map as function of pH and temperature for different gradient slope ranges. The MODR is
362 represented by the blue area for which the defect rate is inferior or equal to 5 %,
363 corresponding to the following ranges of CMPs: pH, 3.25-3.50; column temperature, 29.5-
364 34.0°C; and gradient slope, 1.60-2.08 %/min.

365 The edge of the MODR was experimentally checked using a two-level full factorial design.
366 Each CMP was varied between the limits of the MODR. For each tested point, a minimum
367 resolution of 1.7 was obtained for all CMAs, as shown in **Table 2**. The requirements inside
368 the MODR were therefore fulfilled.

369 **3.1.6. Working point**

370 Inside the MODR, the working point was selected based on green criteria. Therefore, a pH
371 corresponding to 10 mM acetic acid only (pH 3.35) was chosen. In addition, this pH value
372 was not at the edge of the MODR (pH range from 3.25 to 3.50) for a better robustness of the
373 working point. Retained values for temperature and gradient slope were 30°C and 1.9%/min,
374 respectively. The selected gradient slope was found as a good compromise between reducing
375 analysis time and obtaining a robust separation. **Fig. 4** presents the chromatograms obtained
376 under the experimental conditions of the working point. The impurity C of AS, which was not
377 included during method development because of its high price, was injected under the final
378 conditions. As expected, because of its high lipophilic and neutral nature, it was the last
379 eluting compound and was well separated from the other compounds. This impurity was
380 therefore considered during the validation step.

381 The greenness of the developed method was assessed using two different tools: the National
382 Environmental Methods Index (NEMI) [27] and the Analytical Eco-Scale [28]. NEMI
383 labelling results in an easy-to-read pictogram including four terms: PBT (persistent, bio
384 accumulative, and toxic), Hazardous, Corrosive, and Waste. Each term is colored green or
385 blank depending on whether this particular criterion is fitting or not. The Analytical Eco-scale
386 is a more quantitative approach, based on subtracting penalty points from a total of 100,
387 considering the amount and hazard of reagents, energy consumption, occupational hazards,
388 and amount of waste generated.

389 Results of the greenness evaluation with a NEMI pictogram colored in green for the four
390 terms (**Fig. S2**) and an Eco-scale score of 95 (**Table S2**), allowed to confirm the excellent
391 greenness of the developed method.

392 **3.2. Method validation**

393 The developed RP-HPLC method was validated in order to quantify impurities in AQ and AS
394 raw materials.

395 The validation step was performed using accuracy profile approach based on β -expectation
396 tolerance intervals [29] with acceptance limits set at ± 15 % for each impurity. Such accuracy
397 profile methodology is fully compliant to ICH Q2 requirements [30]. For each impurity,
398 method validation was carried out during 3 days at 5 concentration levels performed in
399 triplicates. The chosen calibration models were linear regression models through 0 for all
400 impurities. Using the calibration curves, concentrations were back calculated to assess the
401 linearity, the relative bias, the repeatability, the intermediate precision, and the β -expectation
402 tolerance intervals at 95% probability level.

403 Taking into account the International Pharmacopeia specifications for AS and AQ related
404 substance analysis, the concentration ranges were: 0.0025-0.0075 mg/mL for impurity 1, 2, 3

405 and 4, 0.05-0.15 mg/mL for impurity A, 0.1-0.6 mg/mL for impurity B, and 0.02-0.072
406 mg/mL for impurity C. The target concentrations were 0.1 mg/mL for impurity A, 0.3 mg/mL
407 for impurity B, and 0.04 mg/mL for impurity C, corresponding to 0.5 %, 1.5%, and 0.2 % of
408 AS concentration set at 20 mg/mL, respectively, based on International Pharmacopeia related
409 substance specifications for AS.

410 For AS impurities, the selected ranges were slightly larger than those required by the
411 specifications of related substance assay. The aim was to be able to use this method also for
412 finished product control. In fact, concentration ranges were chosen in order to cover both
413 finished product and raw material specifications.

414 Validation results are presented in **Table 3** for AS impurities and in **Table 4** for AQ
415 impurities.

416 **3.2.1. Selectivity**

417 Since the objective of this method was to quantify AQ and AS impurities in raw materials, the
418 selectivity was demonstrated by the fact that both APIs and their related impurities were
419 separated with a resolution at least equal to 1.5, as guaranteed by the MODR.

420 **3.2.2. Linearity**

421 To assess linearity criterion, linear regression models were fitted between the back calculated
422 concentrations and the introduced concentrations over the whole calibration range. Linearity
423 results are presented in **Tables 3 and 4** for AS and AQ impurities, respectively. For all
424 impurities, slopes were close to 1 with intercepts close to 0 and R² values were higher than
425 0.98, showing the good linearity of the developed method.

426 **3.2.3. Trueness and precision**

427 Trueness is defined as the mean bias observed between the concentrations back calculated
428 from the calibration curves the series of measurements and the introduced concentrations.
429 Trueness is expressed in terms of relative bias (%). As can be seen in **Tables 3 and 4** for AS
430 and AQ impurities, respectively, relative biases were found lower than 10%, and thus
431 acceptable given the scope of the method which was to quantify impurities at low
432 concentrations.

433 Precision was determined by calculating the relative standard deviation (RSD (%)) of
434 calculated concentrations for repeatability (intra-day) and intermediate precision (inter-day) at
435 each level of validation standards. Precision results are shown in **Tables 3 and 4** for AS and
436 AQ impurities, respectively. RSD values for repeatability and intermediate precision appeared
437 to be lower than 5% for all impurities, indicating a good precision of the method.

438 **3.2.4. Accuracy**

439 Accuracy refers to the closeness of agreement between the test result and the accepted
440 reference value, conventionally accepted as the true value. The accuracy takes into account
441 the total error (random and systematic errors) i.e. the trueness and the precision associated to
442 the analytical method. As shown in **Tables 3 and 4** for AS and AQ impurities, the β -
443 expectation tolerance limits were between $\pm 15\%$ at 95% probability level for all impurities,
444 proving that the method was able to provide accurate results over the whole concentration
445 range for each impurity. The lower and upper limits of quantification (LLOQ and ULOQ)
446 correspond to the dosing range for each impurity: 0.0025-0.0075 mg/mL for AQ impurities,
447 0.05-0.15 mg/mL for AS impurity A, 0.1-0.6 mg/mL for AS impurity B, and 0.02-0.072
448 mg/mL for AS impurity C.

449 3.2.5. Column inter-batch repeatability

450 To check the column inter-batch repeatability, AS, AQ and their impurities were analyzed on
451 3 different column batches. As shown in **Table 5**, whatever the column batch, compounds
452 were well separated ($R_s \geq 1.6$) with a good repeatability for most resolutions (RSDs < 6 %),
453 except for the resolution between impurities 1 and 2 which was always satisfactory ($R_s > 2$)
454 but varied greatly depending on the column batch (RSD = 48.5%). This could be explained by
455 the retention time variation observed for impurity 1 which was the first eluting compound.
456 However, good column inter-batch repeatability of peak areas was achieved for all impurities
457 (RSDs < 4 %) (**Table 6**).

458 3.3. Applications

459 The developed and validated method was applied to analyze two different batches of AS and
460 AQ raw materials (**Tables S3 and S4**). For AS raw materials, impurities B and C were found
461 below 0.5 % and 0.1 %, respectively. These values were largely under International
462 Pharmacopeia specifications which are 1% and 0.2 % for impurities B and C, respectively.
463 Impurity 3 and some unknown compounds were found in AQ raw materials at percentages
464 inferior to 0.1 %, meeting International Pharmacopeia specification ($\leq 0.5\%$).

465 Although the method was validated for related substance assay of raw materials, , an analysis
466 of excipient mixture and AS and AQ in fixed dose tablets, was attempted to evidence that this
467 method can provide an analytical response for quality control of AQ and AS combination
468 therapy. The excipient peaks were separated from the peaks of AQ, AS and their impurities
469 (**Fig. S3**). It highlighted an appropriate selectivity of the developed method for its potential
470 application to analyze impurities in combination therapy of AS and AQ.

471 In addition, this study showed the potential of this method for finished product analysis.
472 Impurity 4 of AQ was observed and separated during tablets analysis from AS and its
473 degradation products (DHA), thereby they can be correctly quantify.

475 4. Conclusion

476 In this work, a single, green and robust RP-HPLC method using an ethanol-based mobile
477 phase was developed according to Quality by Design approach for the quantitative analysis of
478 impurities of both artesunate and amodiaquine drugs. This method development was
479 particularly challenging since it targets single chromatographic condition for analyzing 9
480 molecules including acidic, basic, and structurally related compounds. A gradient elution
481 using ethanol was therefore needed, raising detection issues due to the high UV cut-off of
482 ethanol and the poor detection in UV of AS related compounds, requiring a detection at 210
483 nm. QbD strategy enabled to define the method operable domain region with a high assurance
484 level of 95%. Inside the robustness space, a mobile phase composed of ethanol and 10 mM
485 acetic acid was selected to achieve a green HPLC method as assessed by NEMI and Eco-scale
486 approaches. This method was validated through a total error approach using accuracy profiles
487 and successfully applied to the quality control of raw materials and artesunate/amodiaquine
488 fixed dose combination tablets. Moreover, the generated column backpressure under 200 bars
489 showed that this analysis can be performed using conventional HPLC equipment and
490 consequently could be implemented in resource-limited laboratories. Through the use of QbD
491 development approach and the integration of green chemistry principles, this work opens new
492 opportunities in greening HPLC methods in the framework of pharmaceutical quality control.

493 **Acknowledgments**

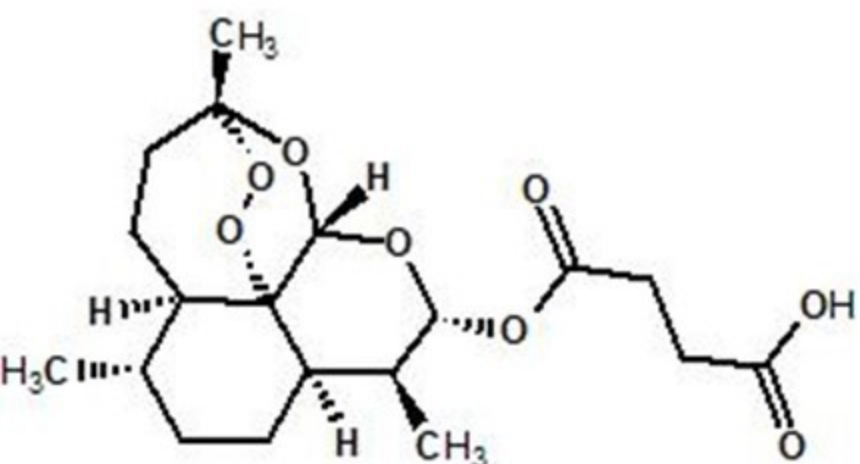
494 The Foundation Pierre Fabre is gratefully acknowledged for scholarship support to M. Yabré.

495 **References**

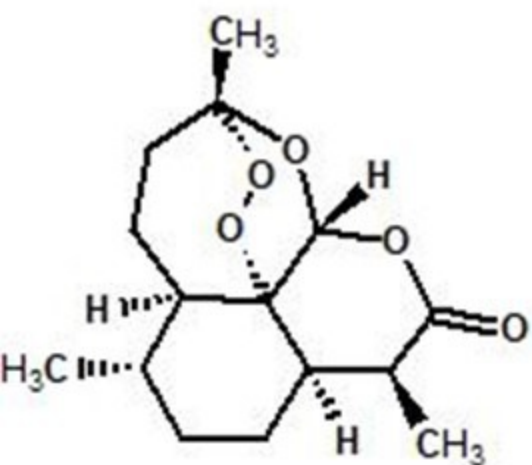
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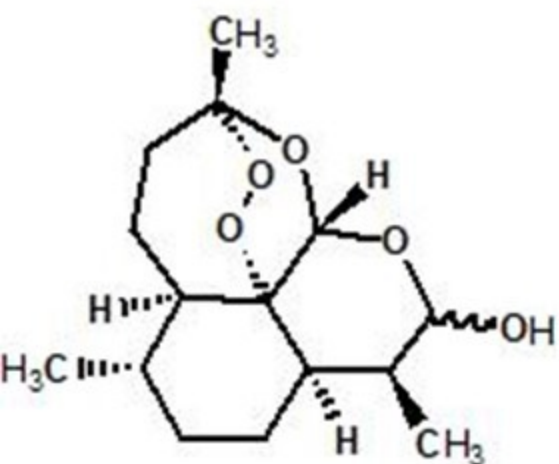
A



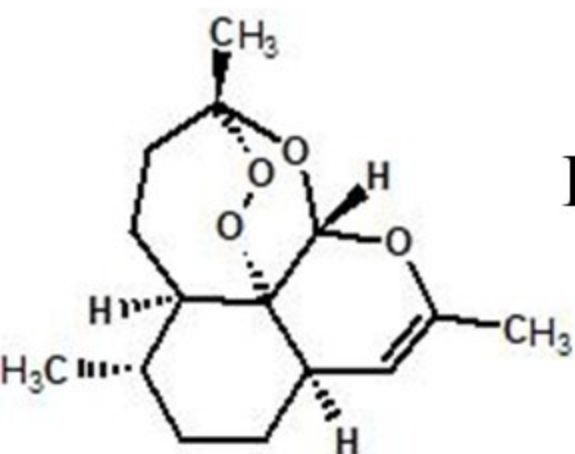
AS (pKa 4.35)



Impurity A

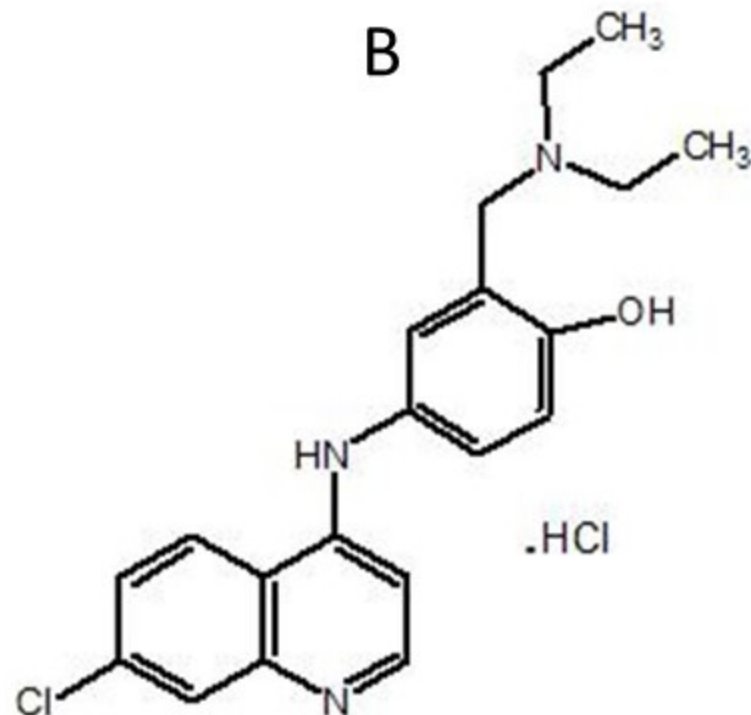


Impurity B

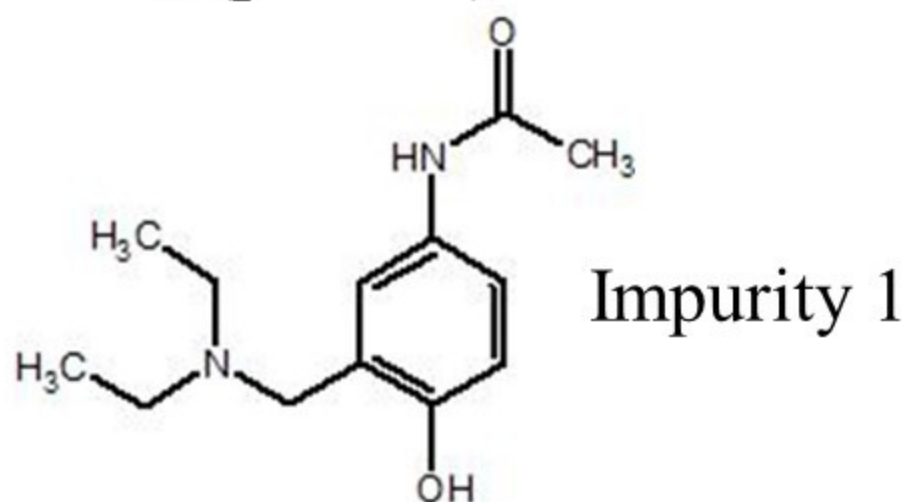


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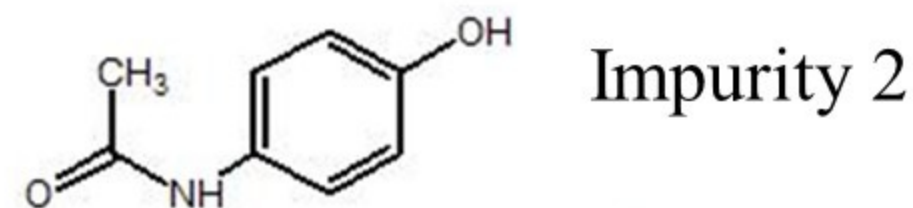
B



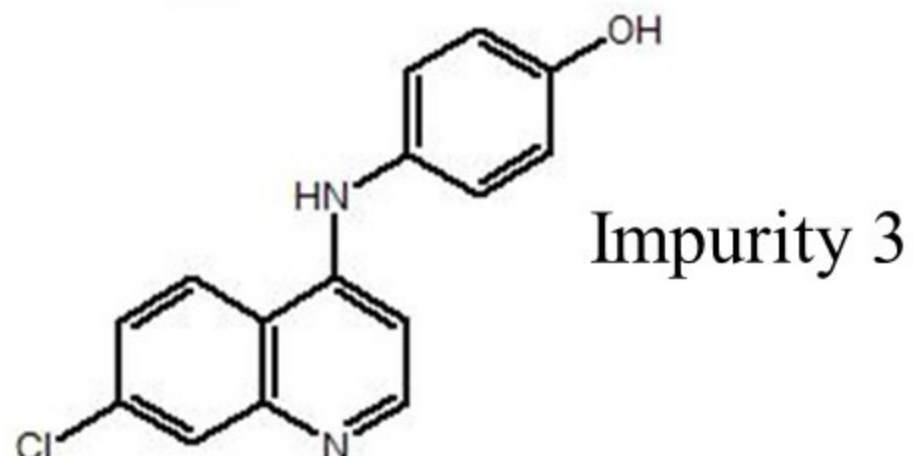
AQ (pKa 9.2)



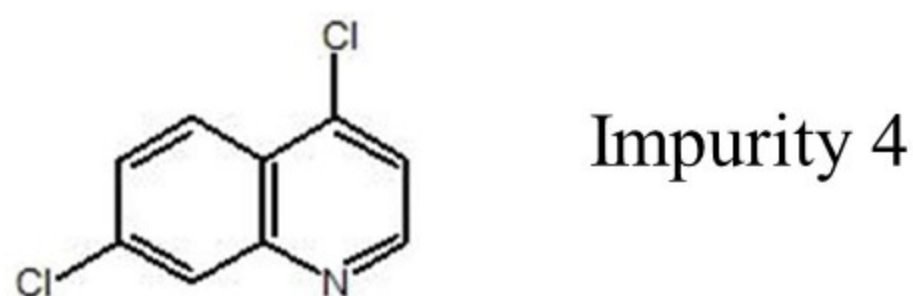
Impurity 1



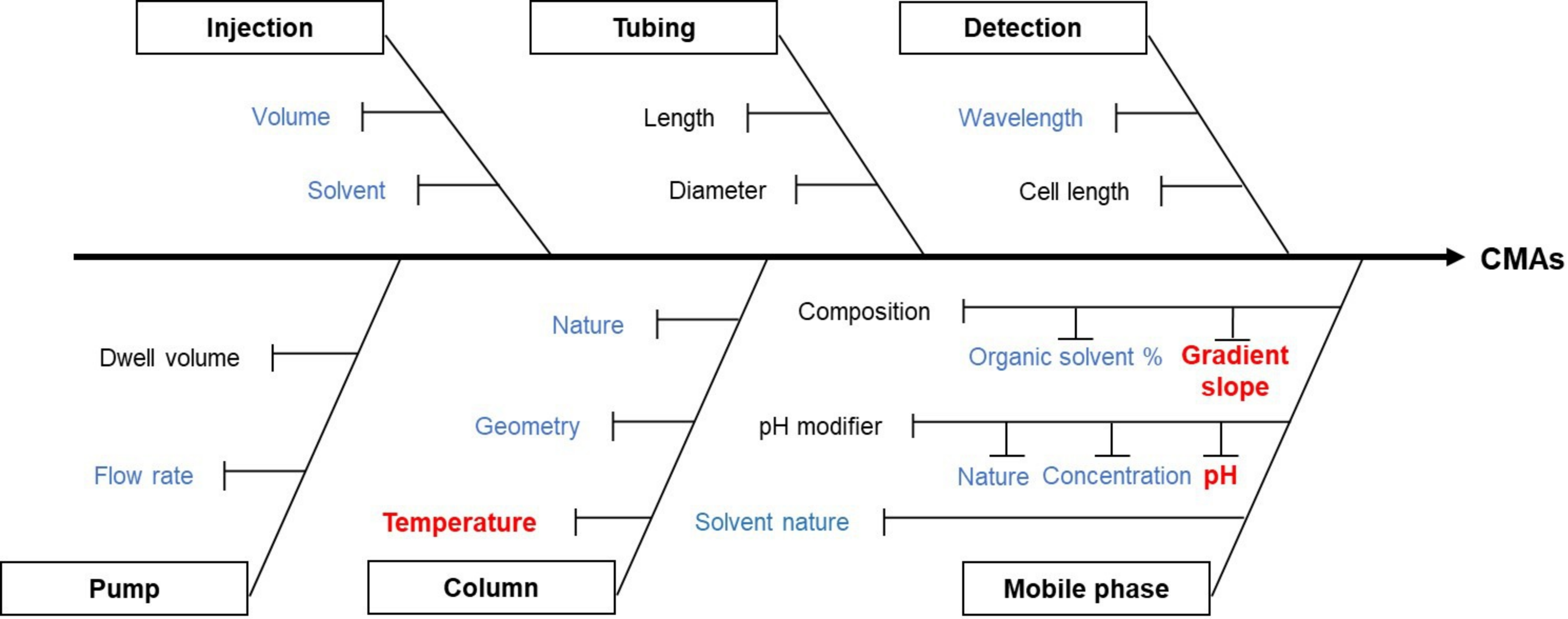
Impurity 2



Impurity 3



Impurity 4



Gradient slope

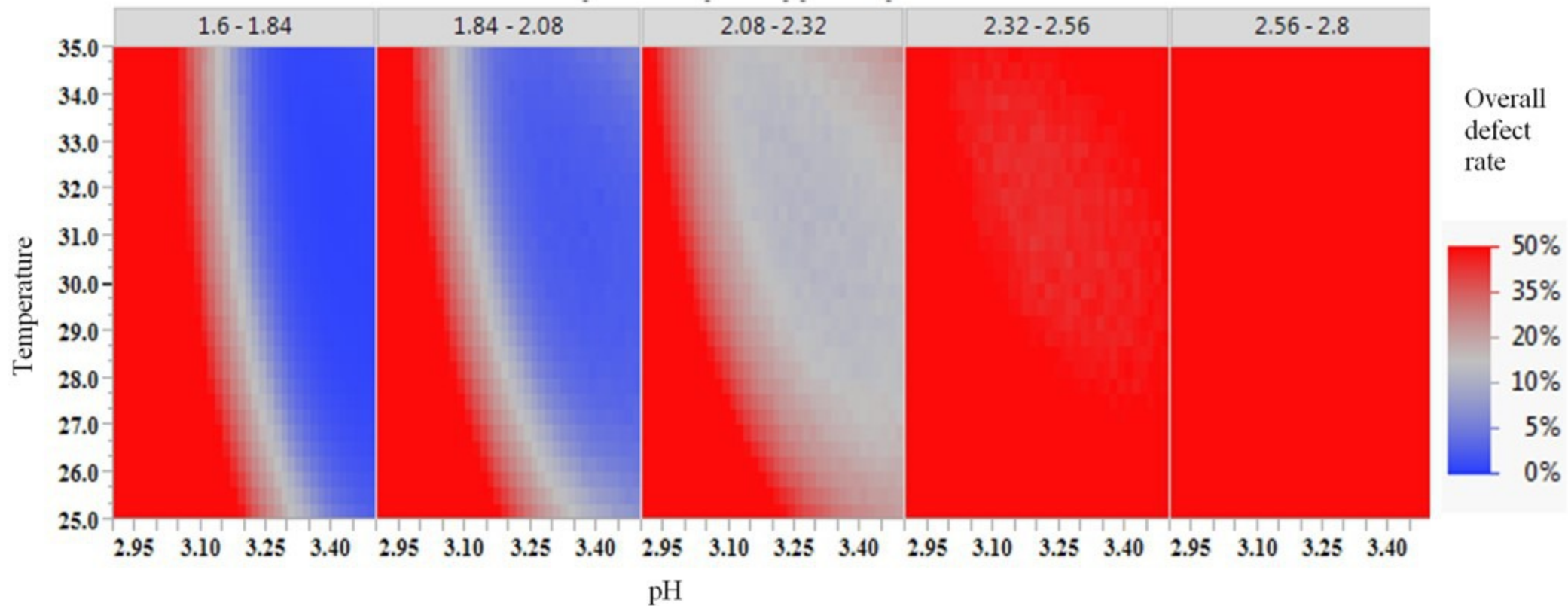


Table 1. Coefficients of determination (R^2), p-values to test model significance (ANOVA) and lack of fit of the multiple linear regressions for the 8 CMAs.

CMAs	p-values model	R^2	p-values Lack of fit
Rs 1	<0.0001	0.82	0.3713
Rs 2	<0.0001	0.96	0.5675
Rs 3	<0.0001	0.97	0.2889
Rs 4	<0.0001	0.97	0.5415
Rs 5	<0.0001	0.81	0.7760
Rs 6	<0.0001	0.82	0.3524
Rs 7	<0.0001	0.80	0.1120
Rs 8	<0.0001	0.90	0.2450

Rs 1 = Rs (impurity 2; impurity 1), Rs 2 = Rs (AQ; impurity 2), Rs 3 = Rs (impurity 3; AQ), Rs 4 = Rs (first peak of impurity B; impurity 3), Rs 5 = Rs (impurity A; first peak of impurity B), Rs 6 = Rs (AS; impurity A), Rs 7 = Rs (second peak of impurity B; AS), Rs 8 = Rs (impurity 4; second peak of impurity B).

Table 2. Experimental verification results at the border of the MODR

pH	Temperature (°C)	Gradient slope (%/min)	Rs 1	Rs 2	Rs 3	Rs 4	Rs 5	Rs 6	Rs 7	Rs 8
3.50	34.0	2.08	7.1	2.4	7.6	53.0	1.7	1.8	7.0	2.0
3.25	29.5	1.60	7.3	2.8	9.9	54.8	1.8	2.5	7.3	1.7
3.25	34.0	2.08	7.0	2.4	8.0	54.0	1.7	2.0	6.5	1.9
3.50	29.5	1.60	6.9	2.5	9.4	53.9	1.9	2.5	7.6	1.9
3.50	34.0	1.60	6.7	2.4	8.3	55.2	2.0	2.4	7.0	2.0
3.25	29.5	2.08	7.9	2.6	8.8	50.3	1.8	2.2	6.8	1.8
3.50	29.5	2.08	7.1	2.5	9.1	49.2	1.8	2.3	6.9	1.9
3.25	34.0	1.60	7.2	2.6	8.4	55.7	1.9	2.6	6.7	1.8

Rs 1 = Rs (impurity 2; impurity 1), Rs 2 = Rs (AQ; impurity 2), Rs 3 = Rs (impurity 3; AQ), Rs 4 = Rs (first peak of impurity B; impurity 3), Rs 5 = Rs (impurity A; first peak of impurity B), Rs 6 = Rs (AS; impurity A), Rs 7 = Rs (second peak of impurity B; AS), Rs 8 = Rs (impurity 4; second peak of impurity B).

Table 3. Validation results of AS impurities

Validation criteria		Impurity A		Impurity B		Impurity C	
Linearity	Range (mg/mL)	0.05-0.15		0.1-0.6		0.020-0.072	
	Slope	1.0922		1.0655		1.0665	
	Intercept	-0.0078		-0.0191		-0.0029	
	R ²	0.9952		0.9966		0.9975	
Trueness	Level	Conc. (mg/mL)	Relative bias (%)	Conc. (mg/mL)	Relative bias (%)	Conc. (mg/mL)	Relative bias (%)
	1	0.050	-5.35	0.100	-7.18	0.020	-5.76
	2	0.080	-1.04	0.200	-2.88	0.030	-3.88
	3	0.100	-0.67	0.300	-3.06	0.040	-1.24
	4	0.120	4.02	0.500	2.55	0.060	2.25
	5	0.150	4.02	0.600	4.14	0.072	2.66
Precision	Level	RSD% of Repeatability / Intermediate Precision		RSD% of Repeatability / Intermediate Precision		RSD% of Repeatability / Intermediate Precision	
	1	1.40/1.74		1.81/2.00		2.89/3.35	
	2	2.32/2.70		1.47/1.47		0.78/0.78	
	3	1.49/2.85		2.25/2.58		2.84/2.84	
	4	0.84/0.84		0.68/0.68		1.50/1.99	
	5	1.45/1.45		2.18/2.18		1.36/1.36	
Accuracy	Level	β -expectation tolerance limits (%)		β -expectation tolerance limits (%)		β -expectation tolerance limits (%)	
	1	[-9.43, -1.27]		[-11.72, -2.64]		[-13.48, 1.97]	
	2	[-7.27, 5.19]		[-6.17, 0.40]		[-5.61, -2.15]	
	3	[-8.59, 7.24]		[-9.01, 2.89]		[-7.57, 5.10]	
	4	[2.15, 5.88]		[1.02, 4.08]		[-2.63, 7.12]	
	5	[0.78, 7.26]		[-0.73, 9.00]		[-0.37, 5.69]	

Table 4. Validation results of AQ impurities

Validation criteria		Impurity 1		Impurity 2		Impurity 3		Impurity 4	
Linearity	Range (mg/mL)	0.0025-0.0075		0.0025-0.0075		0.0025-0.0075		0.0025-0.0075	
	Slope	1.0378		1.0348		1.0804		1.0118	
	Intercept	-0.0003		-0.0003		-0.0004		0.000006	
	R ²	0.9921		0.9905		0.9892		0.9919	
Trueness	Level	Conc. (mg/mL)	Relative bias (%)	Conc. (mg/mL)	Relative bias (%)	Conc. (mg/mL)	Relative bias (%)	Conc. (mg/mL)	Relative bias (%)
	1	0.0025	-9.43	0.0025	-3.17	0.0025	-5.76	0.0025	2.38
	2	0.0040	-3.30	0.0040	-4.04	0.0040	-2.42	0.0040	4.62
	3	0.0050	-3.15	0.0050	-4.84	0.0050	-5.02	0.0050	2.25
	4	0.0060	-0.50	0.0060	-0.71	0.0060	0.52	0.0060	0.25
	5	0.0075	-0.82	0.0075	1.18	0.0075	3.46	0.0075	2.78
Precision	Level	RSD% of Repeatability / Intermediate Precision		RSD% of Repeatability / Intermediate Precision		RSD% of Repeatability / Intermediate Precision		RSD% of Repeatability / Intermediate Precision	
	1	1.44/1.44		3.24/3.79		2.28/3.41		2.38/3.68	
	2	4.66/4.66		2.06/2.06		1.97/1.97		2.39/2.39	
	3	2.75/3.53		1.25/2.83		2.72/2.72		1.52/2.86	
	4	2.30/2.30		1.80/1.80		3.36/3.36		0.86/2.33	
	5	2.65/3.09		1.26/1.26		3.06/3.13		2.41/3.47	
Accuracy	Level	β -expectation tolerance limits (%)		β -expectation tolerance limits (%)		β -expectation tolerance limits (%)		β -expectation tolerance limits (%)	
	1	[-12.64, -6.21]		[-11.92, 5.58]		[-14.37, 2.85]		[-6.93, 11.70]	
	2	[-13.70, - 7.10]		[-8.63, 0.54]		[-6.80, 1.97]		[-0.70, 9.94]	
	3	[-11.60, - 5.29]		[-12.72, 3.04]		[-11.10, 1.06]		[-5.67, 10.16]	
	4	[-5.64, - 4.64]		[-4.72, 3.31]		[-6.98, 8.02]		[-6.78, 7.28]	
	5	[-7.97, - 6.33]		[-1.63, 3.98]		[-3.55, 10.47]		[-5.95, 11.52]	

Table 5. Column inter-batch repeatability of resolutions

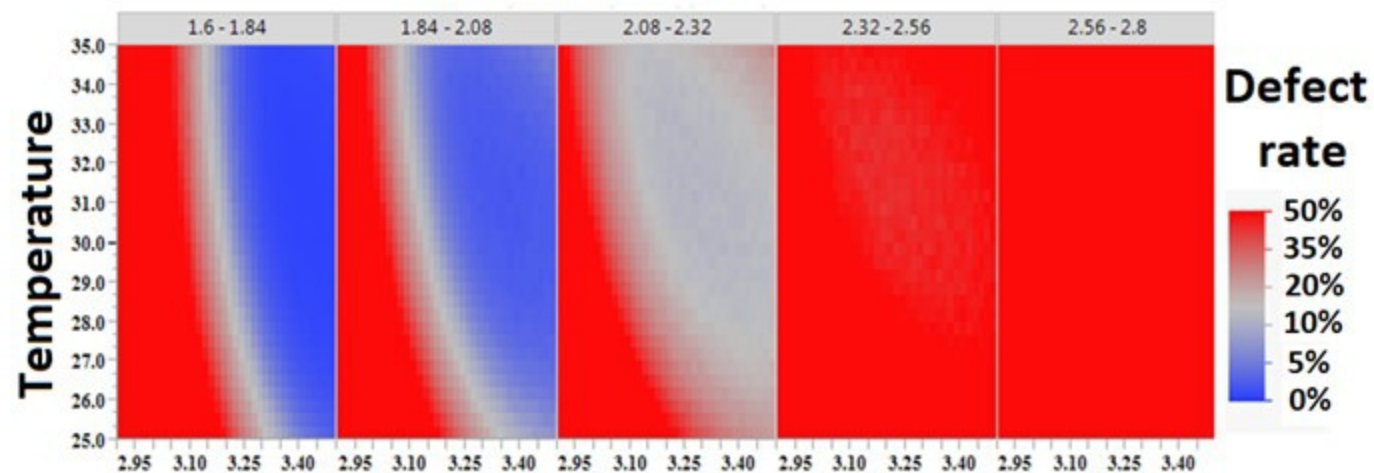
	Rs 1	Rs 2	Rs 3	Rs 4	Rs 5	Rs 6	Rs 7	Rs 8	Rs 9
Batch 1	3.0	2.4	8.8	44.8	1.7	2.0	5.0	2.1	6.6
Batch 2	2.1	2.2	8.5	42.9	1.6	2.0	4.9	1.9	7.1
Batch 3	5.4	2.3	9.0	42.4	1.7	2.1	5.4	2.1	7.4
Mean	3.5	2.3	8.8	43.4	1.7	2.0	5.1	2.1	7.0
RSD (%)	48.5	5.3	2.4	2.9	3.3	2.1	5.3	5.7	5.7

Rs 1 = Rs (impurity 2; impurity 1), Rs 2 = Rs (AQ; impurity 2), Rs 3 = Rs (impurity 3; AQ), Rs 4 = Rs (first peak of impurity B; impurity 3), Rs 5 = Rs (impurity A; first peak of impurity B), Rs 6 = Rs (AS; impurity A), Rs 7 = Rs (second peak of impurity B; AS), Rs 8 = Rs (impurity 4; second peak of impurity B), Rs 9 = Rs (Impurity C; impurity 4).

Table 6. Column inter-batch repeatability of peak areas

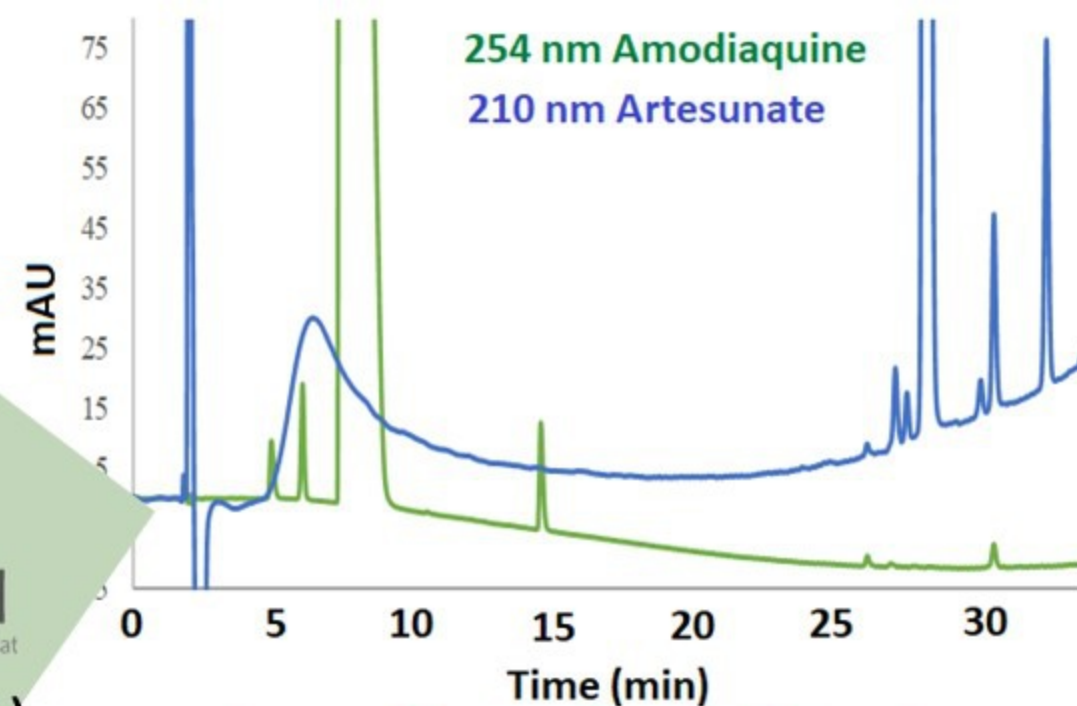
	Impurity 1	Impurity 2	Impurity 3	Impurity 4	Impurity A	Impurity B	Impurity C
Batch 1	1.55	3.16	3.43	0.78	2.17	6.54	11.16
Batch 2	1.54	3.18	3.65	0.77	2.20	6.61	11.25
Batch 3	1.57	3.18	3.43	0.77	2.13	6.72	11.32
Mean	1.55	3.17	3.50	0.77	2.16	6.62	11.24
RSD (%)	1.15	0.43	3.67	0.82	1.64	1.39	0.69

Gradient slope



pH Method Operable Design Region

Validation



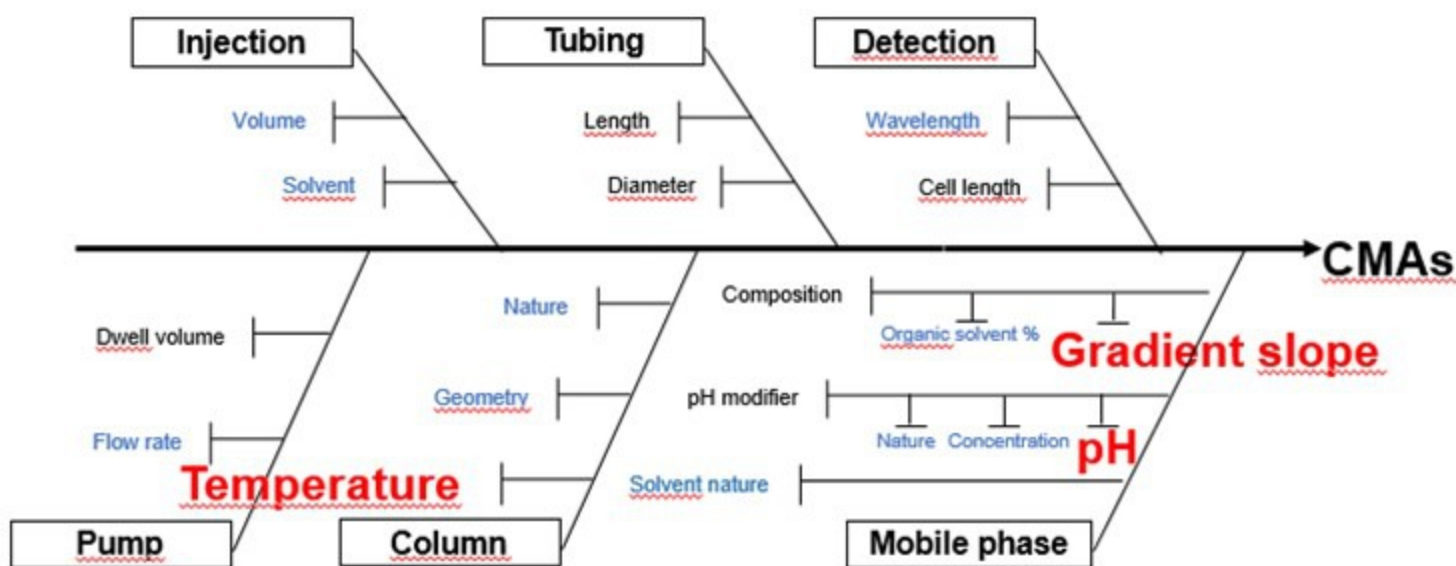
Ethanol-based mobile phase with acetic acid

Quality-by-Design



Critical Method Parameters

Critical Method Attributes
➤ 8 Resolutions



Analytical Target Profile

- Green RP-HPLC method
- Related substances analysis
- Antimalarial Combination therapy

