

1 **In field implementation of NIR quantitative methods for medicines analysis**
2 **in tropical environments.**

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27 **ABSTRACT**

28 NIR spectroscopy is actually a well-established technique that demonstrated its performances
29 in the frame of detection of poor-quality medicines. The use of low-cost handheld NIR
30 spectrophotometers in low-resource contexts can allow an inexpensive and more rapid detection
31 compared to laboratory methods. Considering these points, it has been decided to develop,
32 validate and transfer methods for the quantification of ciprofloxacin and metronidazole tablets
33 samples, using a NIR handheld spectrophotometer in transmission mode (NIR-M-T1) coupled
34 to chemometrics such as Partial Least Square Regression (PLS-R) algorithm. All the models
35 were validated with the total error approach using accuracy profile as decision tool, with $\pm 10\%$
36 specifications and a risk α set at 5%. Quantitative PLS-R models were firstly validated in
37 Belgium, a temperate oceanic climate zone. Secondly, they were transferred in Cameroon, a
38 tropical climate zone, where issues regarding the prediction of new validation series with the
39 initial models were highlighted. Two augmentation strategies were then envisaged to make the
40 predictive models robust to environmental conditions, incorporating the potential variability
41 linked to environmental effects in the initial calibration sets. The resulting models were then
42 used for in field analysis of ciprofloxacin and metronidazole tablet samples collected in three
43 cities in Cameroon. The contents results obtained for each sample with the two strategies were
44 close and not statistically different. Nevertheless, the first one is more easy to implement and
45 the second is the best regarding model diagnostic measures and accuracy profiles. Two samples
46 were found non-compliant in terms of content, and these results were confirmed by HPLC taken
47 as reference method.

48 **Keywords:** NIR handheld device; Transmission; PLS-R; Model transfer; Environmental effect;
49 in field analysis; Substandard and Falsified Medicines; Screening devices.

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52 **Introduction**

53 According to the World Health Organization (WHO), 10.5 % of medicines circulating in limited
54 resource settings are substandard or falsified (SF).¹⁻³ Those proportions may be higher in some
55 regions than in others, sometimes reaching up to 50 % prevalence rates. A recent study
56 highlighted the fact that Africa held the highest prevalence of SF medicines with an average
57 proportion of 18.9%.⁴

58 These SF medicines are not harmless from a public health perspective, with negative
59 repercussions like: treatment efficacy compromised, intoxications, antimicrobial resistance
60 (AMR), increased mortality, and prolonged illness. This scourge is also impacting the socio-
61 economic aspect of many countries.^{2,5-8}

62 Detection is an essential cornerstone for the identification of SF medicines and therefore for
63 their quarantine and withdrawal from the supply chain. This detection has traditionally been
64 possible thanks to pharmacopeial methods. Unfortunately, their implementation remains
65 problematic, especially in low-resource settings where national pharmaceutical regulatory
66 authorities have a low level of maturity with weak laboratory capacities unable to reach
67 international standards. These laboratories are challenged by many issues like weak analytical
68 infrastructures and distribution logistics, lack of qualified staff and supply strategies for
69 consumables and chemicals, and unpredictable power shortages. A recent field survey
70 highlighted such problems in Cameroon.⁹ These difficulties generally lead to long results-
71 reporting deadlines or no report at all. This fact prevents SF medicines from being detected and
72 removed from the pharmaceutical market.^{10,11}

73 Considering the above, some authors advocate a two-stage detection approach in the post-
74 marketing surveillance strategy, namely, a screening phase and a confirmatory one.

75 The last four decades have been marked by a special focus on near-infrared (NIR) spectroscopy
76 as screening technique.¹²⁻¹⁴ Such interest is mainly related to some merits of NIR among which:

77 its ease of use, short analysis time, little or no sample preparation. The recent progresses in
78 optical spectroscopy technology enabled the development of inexpensive NIR handheld
79 devices.¹⁵⁻¹⁸

80 On the one hand, these handheld devices can be used on-site during routine inspection for
81 qualitative purpose e.g., at customs and frontier posts thanks to their non-destructive character,
82 the goal being either to confirm the presence of the expected active pharmaceutical ingredient
83 (API) and/or the identification of a specific formulation.^{19,20} Some studies have shown the
84 usefulness of NIR devices for qualitative inspection of medicines.^{21,22} However, their
85 implementation comes with some constraints related to the physical aspect of the samples, the
86 multiplicity of pharmaceutical forms (capsules, tablets, ...) and the need for representative data
87 sets for the calibration of the models.^{23,24} On the other hand, quantitative approach with NIR
88 handheld devices can be useful for near-site analysis of a large number of samples. This
89 screening step enables a wiser allocation of the resources. Handheld transmission NIR
90 spectroscopy based quantitative methods have been successfully developed and validated for
91 the assay of medicines in various pharmaceutical formulations.^{24,25} The quantitative partial
92 least squares regression (PLS-R) models may also be deployed on different devices using
93 various calibration transfer strategies to manage spectral variations between instruments.²⁵

94 Nevertheless, these quantitative methods are not free from drawbacks. Indeed, the regression
95 models are developed in laboratories with environmental conditions different from the field.
96 Another limitation concerns the maintenance plan of the models that implies a control strategy
97 of the model's performances and possibly requires model adjustments. In addition to that, since
98 NIR spectroscopy is not a sensitive technique, the analysis of low-dosed formulations is
99 problematic.²⁶

100 Another enduring challenge concerning NIR-based methods is their sensitivity to various
101 factors that can lead to spectral variability and therefore to poor prediction. According to the

102 European Pharmacopoeia,²⁷ the different factors affecting the spectral response are (i) the
103 environment's temperature and humidity, (ii) the sample presentation area, (iii) the sample
104 temperature, (iv) the moisture and solvent residues, (v) the sample thickness, (vi) the sample
105 optical properties, (vii) the solid state forms and (viii) the sample's shelf life.²⁸⁻³² When
106 working in transmission mode on solubilized samples, only the factors (i) and (iii) remain.²⁴
107 While these factors may be well controlled in laboratory during the development of the
108 regression model, one must keep in mind that the models will be used in various locations with
109 possibly different environmental conditions (e.g., tropical conditions) that may affect the
110 spectral response. Temperature of both detector and samples are known to influence NIR
111 spectra.^{31,33}

112 In the present study, we were interested to investigate the factors that may influence the transfer
113 of a regression model for quantification purpose from a controlled environment to tropical
114 conditions. Several updating approaches can be implemented to correct the environmental
115 influence while maintaining the predictive abilities of the models. One can mention among
116 others: slope and bias adjustments, automatic model updating, robust variable selection,
117 external parameter orthogonalization of PLS (EPO-PLS), complete recalibration and the
118 augmentation strategy.^{32,34,35} The latter which consists in adding samples to the calibration set
119 was the one envisaged. Therefore, two model augmentation strategies were applied to reduce
120 the environmental effects on regression models. The first one considered only temperature as
121 potential influencing factor by expanding the calibration model with heated sample solutions.
122 This strategy only corrects the disturbing factor (iii). The second aims at correcting factors (i)
123 and (iii), augmenting the calibration set with supplementary validation series on the transfer
124 site. The main advantage of the first strategy being that it may be realized when calibrating the
125 model in the laboratory while the second strategy requires a model augmentation in the
126 receiving country.

127 This study has been designed for the assay of two different APIs: ciprofloxacin hydrochloride
128 and metronidazole. These substances are antibiotics that are used alone or in combination in the
129 treatment of infections caused by aerobic and anaerobic germs.³⁶⁻³⁹ Such infections are very
130 common in low- and middle-income countries among which Cameroon. Firstly, the models
131 were developed and validated in temperate oceanic climatic conditions (Liège, Belgium).
132 Secondly, these models were applied under tropical conditions in three Cameroonian cities
133 namely Yaoundé, Douala and Garoua, and updated regarding prediction bias obtained with the
134 initial models. These different study sites were chosen regarding their climatic variabilities. The
135 updated models were then used for in field analysis of samples gathered from the Cameroonian
136 pharmaceutical market. The predicted contents were compared to the results of High-
137 Performance Liquid Chromatography (HPLC) considered as a gold standard method. In
138 addition, as it is important to monitor the analytical performances of the models during their
139 routine use, quality control (QC) samples were analyzed along with the samples. These QC
140 samples could be either validation samples or samples tested with confirmatory techniques. In
141 this case, the second option was preferred.

142 **Material and methods**

143 *Chemicals and reagents*

144 Hydrochloric acid (HCl) 37%, orthophosphoric acid 85% of analytical grade and triethylamine
145 of HPLC grade were purchased from VWR (Leuven, Belgium). Ciprofloxacin hydrochloride
146 monohydrate and metronidazole reference standards were purchased from TCI Europe N.V.
147 (Zwijndrecht, Belgium). Ultrapure water was obtained with a MilliQ Plus 185 system
148 (Millipore, Molsheim, France). Bottled mineral water with low total dissolved solids amount
149 (TDS = 38mg/mL) was bought in Belgium (Spa[®] Reine, Spadel, Spa, Belgium).

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152 *NIR calibration and validation solution preparation*

153 Calibration and validation individual solutions were prepared according to Table S1, dissolving
154 weighted mixture of powdered reference standards and excipients in a diluent. The calibration
155 levels were: 1.0, 1.5, 2.0 and 2.5 % (w/v) for both ciprofloxacin and metronidazole. Two
156 additional validation concentration levels were included around the target (2%) for both
157 ciprofloxacin and metronidazole at 1.75 and 2.25 % (w/v), respectively. Gravimetric data were
158 taken as reference for the building of PLS models.

159 The excipients mixture was composed of starch (28.10%), croscarmellose (18.20 %), povidone
160 (14.80 %), hypromellose (14.15 %), polyethylene glycol 20000 (7.70 %), lactose (8.85 %), and
161 sodium starch glycolate (8.20 %). These excipients were chosen to mimic the composition of
162 the real ciprofloxacin and metronidazole samples available on the Cameroonian market. The
163 average target ratio of 2/3 of API and 1/3 of excipients was used regarding the average ratio in
164 medicines available on the pharmaceutical market.

165 Three calibration and three validation series were prepared for metronidazole and ciprofloxacin
166 in Liège. A fourth validation series was prepared with bottled mineral water with a composition
167 similar to that commonly found in Cameroon for both ciprofloxacin and metronidazole. This
168 was done to prevent possible shortage of milliQ water in the field. All the solutions were
169 independently prepared in triplicates.

170 The diluent for ciprofloxacin calibration and validation samples was water and for
171 metronidazole ones, we used HCl 1M. This choice of diluent is based on preliminary study on
172 the solubility of these APIs. Before being analyzed, the powders were diluted and hand shaken
173 for a maximum of 5 minutes, then left to stand for 10 minutes. The supernatant liquid was then
174 filtered through a 0.45 µm PTFE syringe filter system and brought to NIR system. For each
175 solution, 3 measurements were made.

176

177 *HPLC standard solution preparation*

178 Ciprofloxacin hydrochloride and metronidazole reference standard powders were weighted and
179 diluted with 0.025M orthophosphoric acid buffer adjusted to pH 2.0 with
180 triethylamine/methanol in 75/25 (v/v) in order to obtain a target concentration of 16 µg/mL for
181 each substance. ⁹

182 *Instrumentation*

183 A handheld dispersive NIR spectrometer in transmission mode (NIR-M-T1, Innospectra Corp.,
184 Taiwan) was used. Each spectrum corresponds to the average of 32 scans, programmable gain
185 amplifier (PGA) of 16 in the range of 900 to 1700 nm. The lamp was turned ON for 1 hour
186 before starting the analyses to reach a stable detector's temperature. Then, a new background
187 was measured before each sample scan and the lamp remained lit up during the analysis of the
188 whole series. Solutions were directly scanned in Hellma UV quartz cell, 2 mm light path, with
189 a 700 µL filling.

190 A handheld dispersive NIR spectrometer in reflection mode (NIR-S-G1, Innospectra Corp.,
191 Taiwan) was used to analyze ciprofloxacin and metronidazole chemical reference substances
192 with the same above acquisition parameters as the transmission measurements.

193 The confirmatory samples analyses were performed with an HPLC system consisting of a
194 Waters 2695 separation module coupled to a Waters selector valve 7678 and a Waters 996
195 Photodiode array (PDA) detector (Waters, Milford, MA, USA). A X-Bridge BEH C18 column
196 (100 x 2.1 mm, 3.5 µm particle size) was purchased from Waters.

197 *Samples description*

198 A total of 15 ciprofloxacin and 15 metronidazole samples of different brands were collected in
199 Yaoundé, Douala and Garoua (5 samples of each API per city).

200 These samples were all tablet dosage forms with a claimed content of 500 mg per tablet. The
201 samples were collected from both formal (private and public) and informal sectors, using a

202 mystery shopper strategy. An average of 20 tablets were collected per sample. The samples
 203 description as stated on the packaging is shown in Table 1.

204 **Table 1:** Description of samples. The samples in bold have been used as QC samples (See QC
 205 samples section). All information presented in this table comes from the sample packaging.

City sampled	Sample number	Product name	Country of origin	Sampling Sector	Manufacturing date	Expiry date
Yaoundé	1	Boncipro 500	India	Formal	03/2021	02/2024
	2	Cipro Denk 500	Germany	Formal	10/2020	10/2023
	3	AD Ciprolif 500	India	Formal	10/2020	09/2023
	4	Cipflacin 500	India	Informal	07/2021	06/2024
	5	Ciprovif 500	India	Informal	04/2021	03/2024
	6	Metronidazole BP 500	Cameroon	Formal	04/2021	03/2024
	7	Flagyl 500	Senegal	Formal	07/2021	06/2024
	8	Metzole 500	India	Formal	05/2021	04/2024
	9	Metronidazole 500mg tablets	China	Informal	Not indicated	09-2024
	10	Metronidazole BP 500mg	India	Informal	10/2021	09/2024
Garoua	11	Ciprofloxacin tablets USP 500mg	Cameroon	Formal	01/2022	12/2024
	12	Fluoxine 500	India	Formal	03/2021	02/2024
	13	Ciprofloxacin Tablets USP 500mg	India	Formal	02/2021	01/2024
	14	Cipromax Fort 500	China	Informal	05/2021	04/2024
	15	Tekacipro Ciprofloxacin tablets BP	India	Informal	09/2020	09/2023
	16	Metronidazole BP 500mg	Cameroon	Formal	Not indicated	09/2024
	17	Metronidazole 500mg Comprimés	France	Formal	Not indicated	02/2023
	18	Metronidazole 500mg	France	Formal	Not indicated	03/2023
	19	Metronidazole 500mg tablets	China	Informal	Not indicated	09/2024
	20	Metronidazole 500mg tablets	China	Informal	Not indicated	09/2024
Douala	21	Serviflox 500	Germany	Formal	04/2019	04/2023
	22	Ciprofloxacin 500mg Tablets USP	China	Formal	01/2020	01/2023
	23	Ciprofloxacin tablets USP	Cameroon	Informal	09/2020	08/2023
	24	Ciprofloxacin 500 mg tablets USP	India	Informal	05/2021	04/2024

25	Ciprofloxacin tablets 500 mg USP	India	Informal	08/2021	07/2024
26	Supplin 500	Austria	Formal	10/2019	10/2024
27	Flagyl 500	Senegal	Formal	07/2021	06/2024
28	Metronidazole BP 500mg	Cameroon	Formal	Not indicated	09/2024
29	Metronidazole 500mg	-	Informal	Not indicated	11/2023
30	Metronidazole BP 500mg	U.K.	Informal	Not indicated	06/2024

206

207 *Sample preparation and analysis*

208 *NIR spectroscopy*

209 *In field analysis*

210 The 30 samples collected in Cameroon were analyzed in triplicates, i.e. three tablets per sample.

211 Each tablet was introduced into a pre-filled tube with a sufficient volume of solvent allowing

212 to reach the target concentration (20 mg/mL). The claimed content of each sample (as written

213 on the packaging) was taken as reference. The vial was shaken until the tablet was completely

214 disintegrated and then left to stand for 10 minutes. The supernatant was then collected and

215 filtered using the 0.45 μm PTFE syringe filter system before being brought to the handheld NIR

216 spectrophotometer for measurement. Three spectra were collected per solution. The samples

217 were analyzed indoor in their respective collection cities (see Figure S1). The analysis room

218 did not have any laboratory facility nor controlled environment.

219 *Quality control (QC) samples*

220 Three ciprofloxacin samples and three metronidazole samples (N° 1, 3, 5, 6, 8, 10 according to

221 Table 1) collected and analyzed in the first sampling city (Yaoundé) were selected to constitute

222 QC samples. The average weights of these samples were determined, then the samples were

223 crushed and an amount of powdered sample corresponding to 40 mg of API was placed in

224 sealed tubes. These aliquots were diluted on-site with enough solvent allowing to reach the

225 target concentration (20 mg/mL). They were filtered and analyzed in each city at the same time

226 as the other samples. These samples were used to evaluate the reproducibility of the method
227 and the impact of the different environmental conditions on the prediction results of real
228 samples.

229 *Environmental parameters of study sites*

230 According to the Köppen classification, three climatic regions were involved in the present
231 study: Marine West Coast (Belgium), Tropical Savanna (Garoua and Yaoundé, Cameroon) and
232 Tropical monsoon (Douala, Cameroon).⁴⁰ According to the data of the Belgian Royal
233 Meteorological Institute,⁴¹ the observed annual average temperatures in the study locations are
234 11.2, 23.8, 26.7 and 28.3°C for Liège (Belgium), Yaoundé, Douala and Garoua respectively
235 with a total annual precipitation of 821, 3854, 1628 and 853mm for Liège (Belgium), Yaoundé,
236 Douala and Garoua, respectively.

237 *HPLC*

238 The method used for identification and assay of both ciprofloxacin and metronidazole is a
239 validated method described in Table S2.⁹ 10 tablets per sample were crushed using mortar and
240 pestle. A quantity of powder corresponding to 40 mg of ciprofloxacin or metronidazole was
241 weighed per sample before being analyzed. The final solutions were filtered through a 0.45 µm
242 PTFE syringe-filter system and then brought to HPLC for analysis. The samples were analyzed
243 in triplicates.

244 *Data analysis*

245 *Software*

246 The software used to acquire the spectra was ISC_NIRScan_GUI (version 3.5.7, Innospectra
247 Corp., Taiwan). Predictive models were developed using MATLAB version R2018b
248 (MathWorks Inc., Natick, MA, USA) and the PLS_Toolbox[®] version 8.6.2 (Eigenvector
249 Research Inc., Wenatchee, WA, USA). Empower (Waters) was used to monitor the HPLC

250 analyses. Statistical analysis for the comparison between cities was performed using JMP® Pro
251 Version 15.2.1. (SAS Institute Inc., Cary, NC, USA).

252 *Spectral preprocessing*

253 The selected spectral range was 1549-1682nm for ciprofloxacin, and 1546-1665nm for
254 metronidazole (See Figures S2 a and b). The applied pretreatments were Savitzky-Golay 1st
255 Derivative (order: 2, window: 9pt) and Mean Center for ciprofloxacin and Savitzky-Golay 2nd
256 Derivative (order: 2, window: 11pt) and Mean Center for metronidazole. The cross-validation
257 method was venetian blinds w/10 splits and blind thickness of 3 (See Table 2).

258 **Table 2** : PLS regression models parameters

	Ciprofloxacin model	Metronidazole model
Spectral range (nm)	1549 - 1682	1546 - 1665
Preprocessing	Savitzky-Golay 1 st Derivative (Polynomial order: 2, window: 7 pt) Mean Center	Savitzky-Golay 2 nd Derivative (Polynomial order: 2, window: 11 pt) Mean Center
Number of LV	5	5

259

260 *PLS-regression analysis*

261 Several PLS models were built, applying different pretreatments, selection of spectral ranges
262 and number of latent variables (LV) using the model optimizer of the PLS_Toolbox®. Few
263 models were chosen using root mean square error of prediction (RMSEP) as quality criterion.
264 The latter were compared based on their respective accuracy profiles and the best ones were
265 selected.

266 *Validation*

267 The obtained PLS models were validated using the total error approach with the accuracy
268 profile as decision tool. Acceptance limits were set at $\pm 10\%$ and α risk at 5%. All validation
269 calculations were carried out with E-noval 4.0b (Pharmalex, Mont-saint-Guibert, Belgium).

270 *Comparison between cities*

271 The comparison of the assay results obtained in Douala, Garoua and Yaoundé has been
272 performed by an equivalence test. First, the highest intermediate precision standard deviation
273 (IP) obtained during validation has been used to compute the equivalence margins ($2 \times \text{IP}$).
274 Then the obtained concentration in each city for each sample has been fitted by a linear mixed
275 model with cities as fixed effect and samples as random effect. A pairwise comparison with the
276 Tukey HSD correction has been performed for multiplicity testing. To declare equivalence, the
277 upper and lower 90% confidence intervals must fall within the obtained equivalence margins.

278 **Results and discussion**

279 *PLS calibration and validation models*

280 The initial regression models were built using PLS algorithm based on the spectral data of
281 ciprofloxacin and metronidazole calibration samples prepared in Belgium under controlled
282 environmental conditions. The optimized parameters of each PLS regression model are shown
283 in Table 2.

284 The specificity of the different models was demonstrated as recommended by the Food and
285 Drug Administration (FDA) guidelines,⁴² and the bands of the regression vectors were
286 superimposable to those of the pretreated pure spectra of ciprofloxacin or metronidazole in
287 solution (at the target concentration) from which the spectrum of the solvent used for dilution
288 was subtracted (See Figures S3a and S3b).

289 Validation series measured in Liège were predicted using the developed models and validated
290 using the total error approach as suggested by De Bleye *et al.*⁴³ One can observe that, for both
291 ciprofloxacin and metronidazole, the validation samples were within the $\pm 10\%$ total error
292 acceptance limits (See Figures 1a and 1b). The RMSEP values for ciprofloxacin and
293 metronidazole equal to 0.44 and 0.24mg/mL respectively, were higher than root mean square

294 error of calibration (RMSEC) ones of 0.34 and 0.19 mg/mL, but close to the root mean square
 295 error of cross-validation (RMSECV) ones of 0.42 and 0.22 mg/mL (See Table 3).

296 **Table 3.** Figures of merit of the different PLS-R models

Model parameters	Initial model		Initial model predicting Cameroon and Belgium validation samples		1 st strategy of model augmentation		2 nd strategy of model augmentation	
	CIP	MTZ	CIP	MTZ	CIP	MTZ	CIP	MTZ
RMSEC (mg/mL)	0.34	0.19	0.34	0.19	0.36	0.21	0.37	0.20
RMSECV (mg/mL)	0.42	0.22	0.42	0.22	0.41	0.23	0.42	0.23
RMSEP (mg/mL)	0.44	0.24	0.59	0.25	0.46	0.24	0.44	0.22
Q ² (R ² of prediction)	0.993	0.998	0.990	0.998	0.991	0.998	0.993	0.998

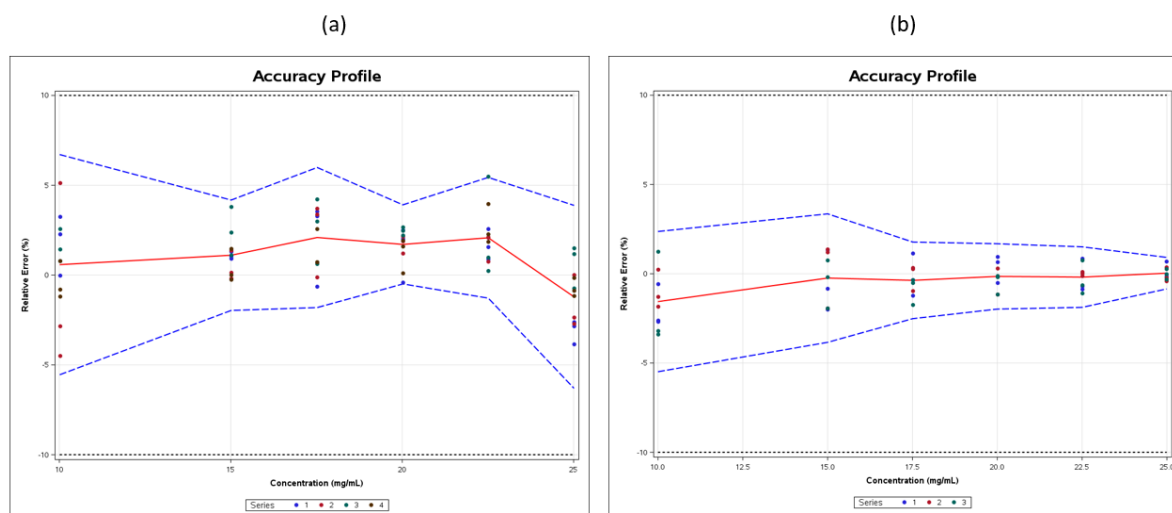
297 Legend: CIP=Ciprofloxacin, MET=Metronidazole

298 The accuracy profiles obtained showed that the ICHQ2(R1) criteria were met in terms of
 299 linearity, trueness, accuracy and dosing range.⁴⁴ The ciprofloxacin method was considered as
 300 valid between 10.01 and 25.01 mg/mL, and the metronidazole one between 10.00 and 24.99
 301 mg/mL (see Figures 1a and 1b, Tables S3 and S4). The concentration ranges chosen for the
 302 development and validation of the models were based on the solubility of APIs and quantifiable
 303 concentration with NIR spectroscopy.

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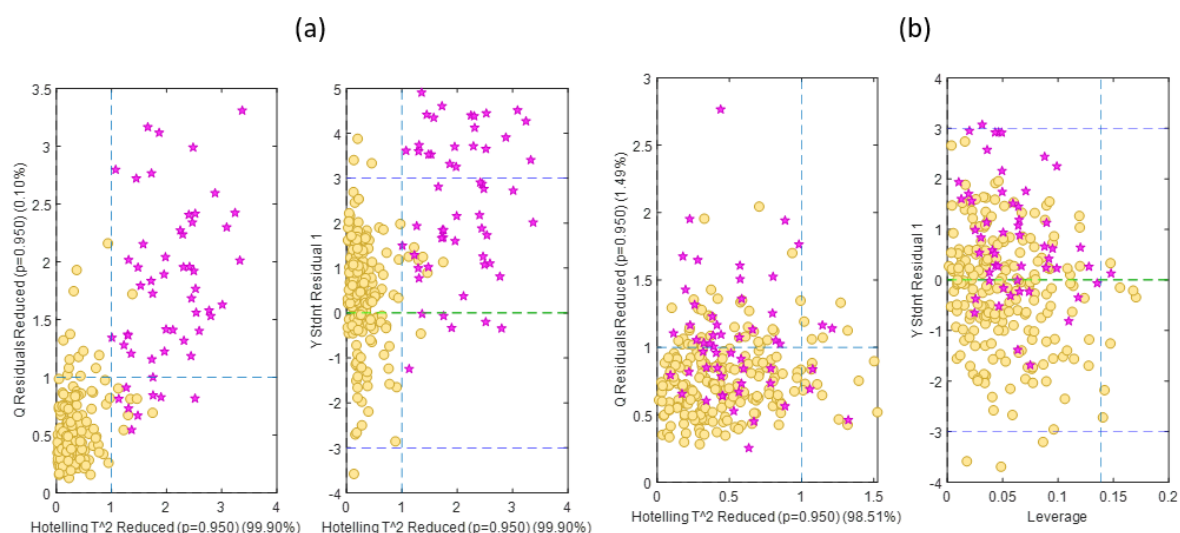
307
 308 **Figure 1:** Accuracy profiles resulting from the prediction of 4 validation series measured in
 309 Belgium obtained with the initial calibration models: (a) ciprofloxacin and (b) metronidazole
 310 accuracy profiles. The plain red line is the relative bias, the dashed blue lines are the 95 % β -
 311 expectation tolerance intervals, and the dashed black lines represent the acceptance limits of \pm
 312 10 %. The dots represent the relative error of the results and are plotted with respect to their
 313 targeted concentration.

314 Regarding the ciprofloxacin validation results (Figure 1a and Table S3), a negative bias is
 315 observed at the highest concentration level. This bias could be related to the proximity of this
 316 concentration to the solubility limit of ciprofloxacin in water. Nevertheless, this concentration
 317 level was selected to potentially detect over-dosed samples despite the fact that under-dosed
 318 samples are the most encountered in practice.⁴⁴ About the relatively important dispersion of
 319 predicted concentrations at the lowest concentration level for both ciprofloxacin and
 320 metronidazole accuracy profiles, it could be related to the important proportion of excipients at
 321 this level compared to others. This situation could also explain the negative bias observed at
 322 the lowest concentration level of metronidazole accuracy profile.

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325 *Models evaluation with Cameroon external validation samples*

326 Once in Cameroon, an external validation series was prepared per API and predicted by the
327 models previously validated in Liège. This stage was performed only in the city of Yaoundé
328 with access to laboratory facilities such as analytical balances. The RMSEP values of the
329 augmented validation sets were slightly higher than those of the original ones: 0.59 and 0.25
330 mg/mL for ciprofloxacin and metronidazole respectively (See Table 3). For ciprofloxacin, the
331 RMSEP value was the double of the RMSEC one. An inspection of the distances to the model
332 indicates that the new ciprofloxacin validation series measured in Cameroon are not well
333 modelled and should be considered as outliers (Figure 2a). This phenomenon is less marked for
334 metronidazole (Figure 2b).



335
336 **Figure 2:** Prediction results of spectra solutions measured in Belgium and Cameroon. (a)
337 ciprofloxacin and (b) metronidazole plots representing reduced Q residuals versus reduced
338 Hotelling's T^2 distances (left) and predicted residuals versus leverage (right). Pink stars:
339 Cameroon validation samples, yellow circles: Belgium validation samples.

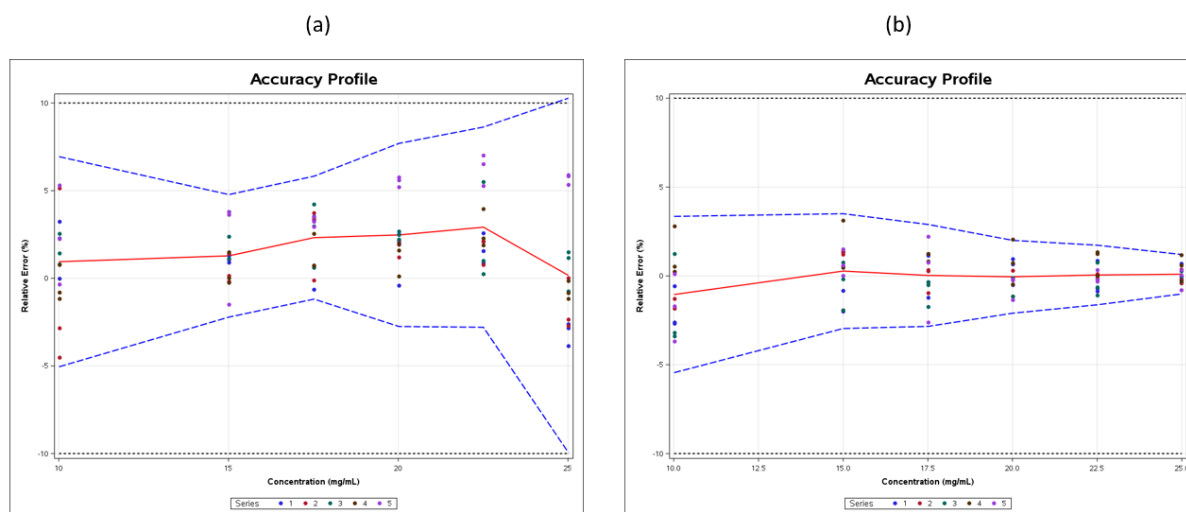
340 When plotting the average spectra measured in Liège with the one of the samples measured in
341 Cameroon, a spectral blueshift is observed (See Figures S4 a and b). This blueshift is mainly
342 observable at the maximum absorption of the water band which moves from 1459 to 1425 nm.

343 As one can see, this shift is less pronounced with metronidazole solutions which were diluted
344 in HCl 1M compared to ciprofloxacin ones diluted in water. A principal component analysis
345 (PCA) of the spectra (Figures S5 and S6) and the correspondent loadings of the spectra collected
346 in Belgium and Cameroon confirms that there is a difference between the two sets. The
347 inspection of the PC1 loading confirms that the blueshift is responsible for this difference
348 (derivative-like pattern). A possible explanation of the blueshift is a difference in the sample's
349 solution temperature. This hypothesis is reinforced by the temperature data recorded in the NIR
350 device during the collection of spectra (Figure S7), the minimum temperature was obtained in
351 Liège (38.9°C) and the maximum in Douala (51.3°C). Regarding the relative humidity data, the
352 minimum and maximum were obtained in Garoua (7.2%) and Douala (47.2%) respectively (see
353 Table S5).

354 Despite this blueshift, the accuracy profiles obtained with the original models developed in
355 Liège on the augmented validation sets remain valid with β -expectation tolerance intervals
356 within the ± 10 % total error acceptance limits for both ciprofloxacin and metronidazole (See
357 Figures 3a and 3b). Nevertheless, it is not good practice to predict samples that are outside the
358 modeled variability. Therefore, two model updating strategies were investigated to manage this
359 blueshift and incorporate this variability in the regression model.

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361



362
 363 **Figure 3:** Accuracy profiles resulting from the prediction of 4 validation series measured in
 364 Belgium and one external validation series measured in Cameroon with the initial calibration
 365 models. (a) ciprofloxacin and (b) metronidazole accuracy profiles. The plain red line is the
 366 relative bias, the dashed blue lines are the 95 % β -expectation tolerance intervals, and the
 367 dashed black lines represent the acceptance limits of ± 10 %. The dots represent the relative
 368 error of the results and are plotted with respect to their targeted concentration.

369 *Models updating*

370 *First strategy: before the field*

371 The first strategy is envisaged to compensate the sample solution temperature and has the
 372 advantage that it can be realized in the laboratory during the model development.

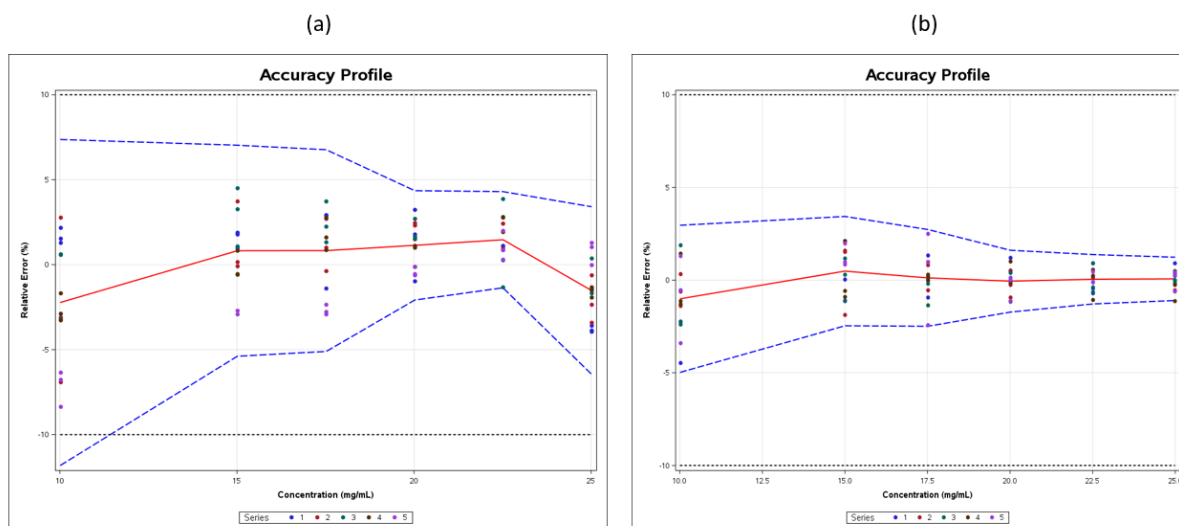
373 It consisted in heating a solution composed of a mixture of API and excipients at the target
 374 concentration of 2 % (w/v) in a water bath at different temperatures which were increased by
 375 5°C steps from 25 to 60°C. These temperatures were in line with the ones observed during the
 376 Belgian and the Cameroonian measurements. After each 5°C temperature increase step, the
 377 solutions were measured immediately after their removal from the water bath in order to avoid
 378 any temperature variation with the NIR-M-T1 device. For each temperature, 5 replicates were
 379 performed, in order to assign high weights to the new spectra as recommended by Stork *et al.*⁴⁵

380 The resulting 40 new spectra were added to those of the initial calibration samples in order to

381 obtain more robust models for ciprofloxacin and metronidazole. The same spectral band
382 selection, latent variables and preprocessing as for initial models were applied.

383 The visual inspection of the sample solution spectra measured at the different temperatures
384 confirms the presence of a blueshift as the water bath temperature rises. According to some
385 authors, an increase of temperature leads to intensity increase of pure water spectrum, peak shift
386 towards lower wavelengths and band narrowing.^{35,46} From a vibrational perspective, these
387 changes are attributed to the fact that the strength of the hydrogen bonds of the water O-H group
388 decreases in accordance with temperature. This, in turn, induces an increase of the O-H bond
389 energy, and therefore an increase in the frequency of vibration.⁴⁷

390 Regarding predictions, the new RMSEPs have improved and were close to those of the initial
391 models but lower than those obtained with new external validation samples on initial models:
392 0.43 and 0.24 mg/mL for ciprofloxacin and metronidazole respectively (See Table 3). In
393 addition, the specificity of the new models was verified (Figures S8 a and b). One can see a
394 clear improvement of the accuracy profiles (Figures 4a and 4b) compared to those obtained
395 with the prediction of the new validation samples with initial models. Compared to the initial
396 models, the upper and lower limits of quantification remained the same for metronidazole while
397 for ciprofloxacin, the lower one has increased from 10.01 to 11.43 mg/mL (See Tables S3 and
398 S4) with this first augmented strategy. The highest robustness of the model is at the expense of
399 a lowest validated concentration range. This concentration range still remains in line with the
400 final purpose of the method.



401
 402 **Figure 4:** Accuracy profiles resulting from the prediction of 4 validation series measured in
 403 Belgium and one external validation series measured in Cameroon with the first strategy of
 404 model augmentation. (a) ciprofloxacin and (b) metronidazole accuracy profiles. The plain red
 405 line is the relative bias, the dashed blue lines are the 95 % β -expectation tolerance intervals, and
 406 the dashed black lines represent the acceptance limits of ± 10 %. The dots represent the relative
 407 error of the results and are plotted with respect to their targeted concentration.

408 In light of the above, one can say that the temperature factor which was influencing the
 409 prediction with the initial models was therefore taken into account with the new ones. As shown
 410 by their respective accuracy profiles, the metronidazole updated model was less influenced by
 411 thermal effect than the ciprofloxacin one. This could be related to the nature of the diluent. In
 412 addition, metronidazole absorbs more than ciprofloxacin in the selected spectral band, from
 413 1540 to 1680 nm (See Figure S2). This might decrease the relative influence of the blueshift
 414 related to the thermal effect on the water spectrum.

415 *Second strategy: in the field*

416 The second strategy consisted in preparing a calibration series per API in Cameroon in order to
 417 augment the previous calibration sets prepared in Belgium and build new models. This strategy
 418 is based on the hypothesis that the spectral differences are not completely explained by the

419 difference in samples solution temperature. However, it requires that the presence of laboratory
420 equipment and furniture at the local site which, in our case, was only possible in Yaoundé.
421 Therefore, augmented models were built by the introduction of new calibration series prepared
422 in Yaoundé to the initial ciprofloxacin and metronidazole calibration sets. The same
423 preprocessing, spectral band selection used in the initial model were applied.

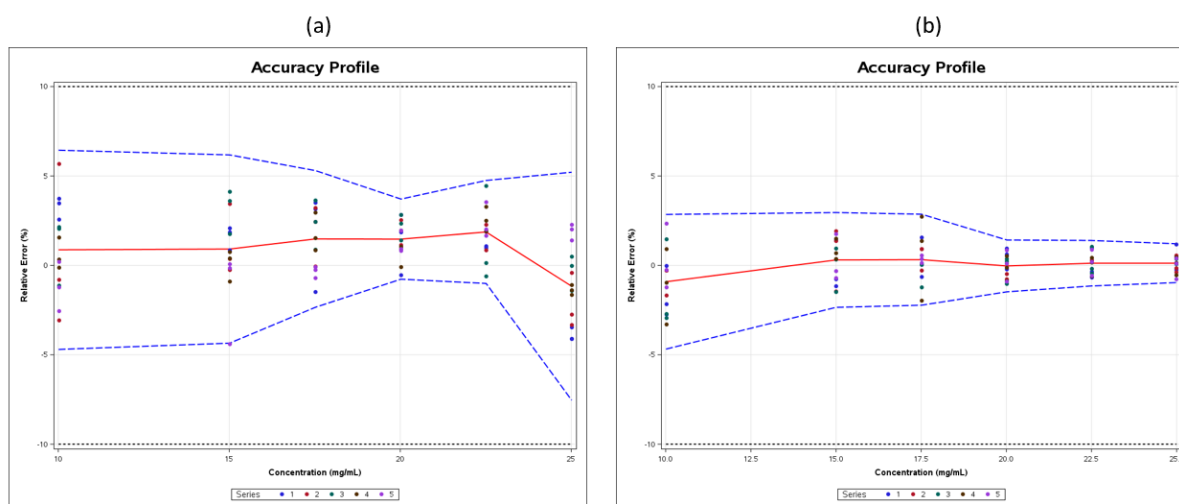
424 Regarding predictions, the new RMSEPs have improved and were better than those of the initial
425 models predicting Cameroon's validation samples and the ones updated with the first strategy:
426 0.40 and 0.22 mg/mL for ciprofloxacin and metronidazole respectively (Table 3). The
427 specificity of the new models was verified (See Figures S9 a and b). Regarding the new
428 accuracy profiles obtained (See Figures 5a and 5b), one can see a clear improvement, especially
429 for the ciprofloxacin one, since the negative bias observed at the lowest concentration level in
430 the accuracy profile obtained with the first strategy was no longer present as for the initial
431 model. However, regarding the metronidazole new accuracy profile, it seems to show no
432 significant difference with the previous models (See Tables S3 and S4).

433 One can observe that the environmental factors, possibly more complex than just the influence
434 of temperature, were efficiently taken into account in the new models.

435

436

437



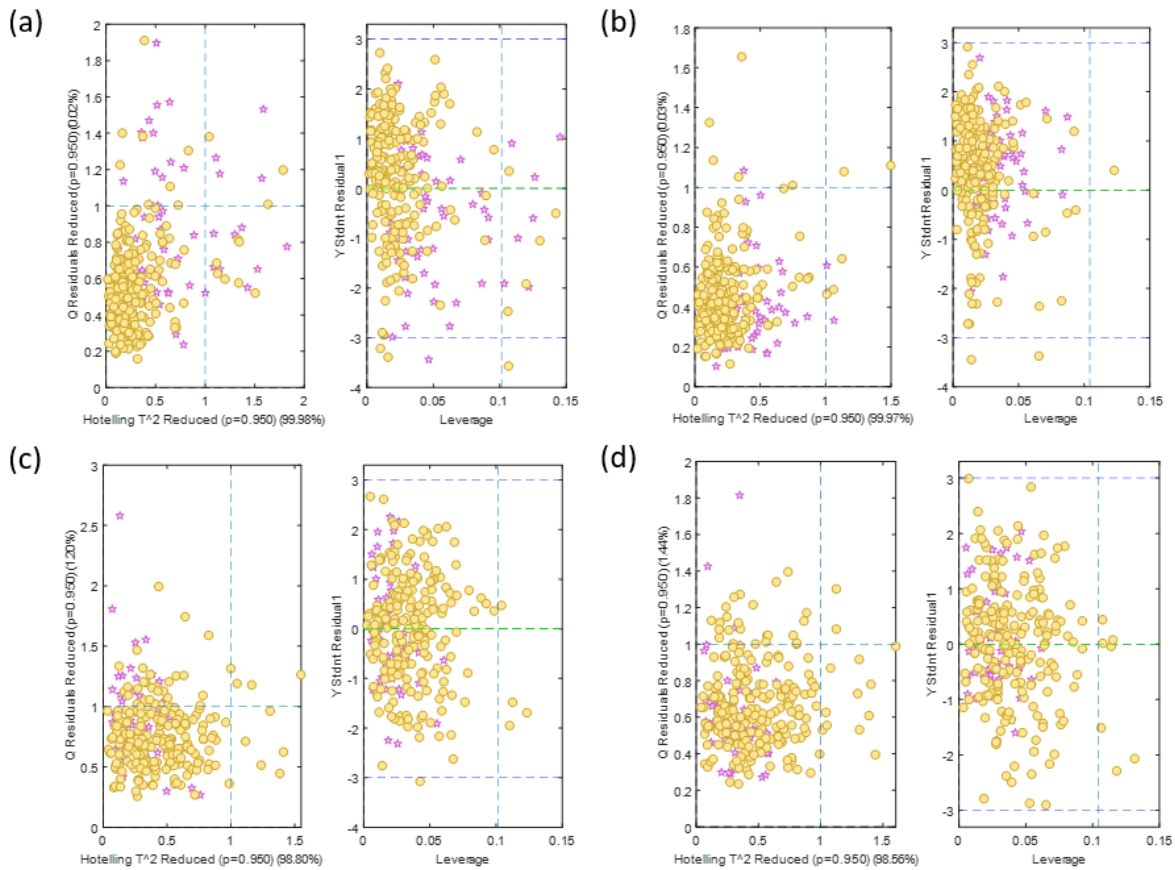
438
 439 **Figure 5:** Accuracy profiles resulting from the prediction of 4 validation series measured in
 440 Belgium and one external validation series measured in Cameroon with the second strategy of
 441 model augmentation. (a) ciprofloxacin and (b) metronidazole accuracy profiles. The plain red
 442 line is the relative bias, the dashed blue lines are the 95 % β -expectation tolerance intervals, and
 443 the dashed black lines represent the acceptance limits of ± 10 %. The dots represent the relative
 444 error of the results and are plotted with respect to their targeted concentration.

445 *Comparison of the augmented strategies*

446 The two strategies allowed to make the new models more robust (See Figures 6 a, b, c and d)
 447 to the influence of environmental factors as shown by the distances to the model of the
 448 validation samples previously observed (See Figures 2a and b). The first augmented strategy
 449 can be considered before moving to the field, where the method will be applied, taking into
 450 consideration the ambient temperature. It is a simple and economical strategy as recommended
 451 by Wise *et al.*³⁴ It can be applied to many APIs in order to include temperature variability as
 452 long as the stability of the latter allows it.

453 In addition, stability studies showed that ciprofloxacin remains stable below 100°C and
 454 metronidazole is resistant to thermal stress.^{48,49} Therefore, no API degradation was expected
 455 during the experiment which is confirmed by the good recoveries observed that were within
 456 97.8 and 101.5%.

457 The second augmenting strategy should be considered once in field setting. According to Figure
 458 6, It showed better validation results than the first strategy. However, it requires more material
 459 and time for the calibration set extension.



460

461 **Figure 6:** Prediction results of spectra solutions measured in Belgium and Cameroon with the
 462 first (a and b) and the second (c and d) strategies of model augmentation: (a and c) ciprofloxacin
 463 and (b and d) metronidazole plots representing the reduced Q residuals versus reduced
 464 Hotelling's T^2 (left) and predicted residuals versus leverage (right). a and b were obtained with
 465 the first strategy augmentation model; b and c were obtained with the second strategy
 466 augmentation model. Pink stars: Cameroon validation samples, yellow circles: Belgium
 467 validation samples.

468 Considering the results of the predicted contents of the validation samples using the initial and
 469 updated models, one can say that it was likely not just the temperature that influenced the

470 models. Therefore, the first augmentation strategy with water bath was not enough to include
471 environmental variations in the models. As one can see, it is more interesting to use the second
472 approach, which increases calibration samples with data taken in local conditions, building
473 models including the complex environmental variations.

474 Overall, the results obtained were similar to those of Thygesen *et al.* which used several
475 strategies to handle temperature influence in NIR measurement of moisture in wood and found
476 that adding spectra representing the relevant temperatures to the calibration set allowed better
477 prediction of unknown samples.⁵⁰

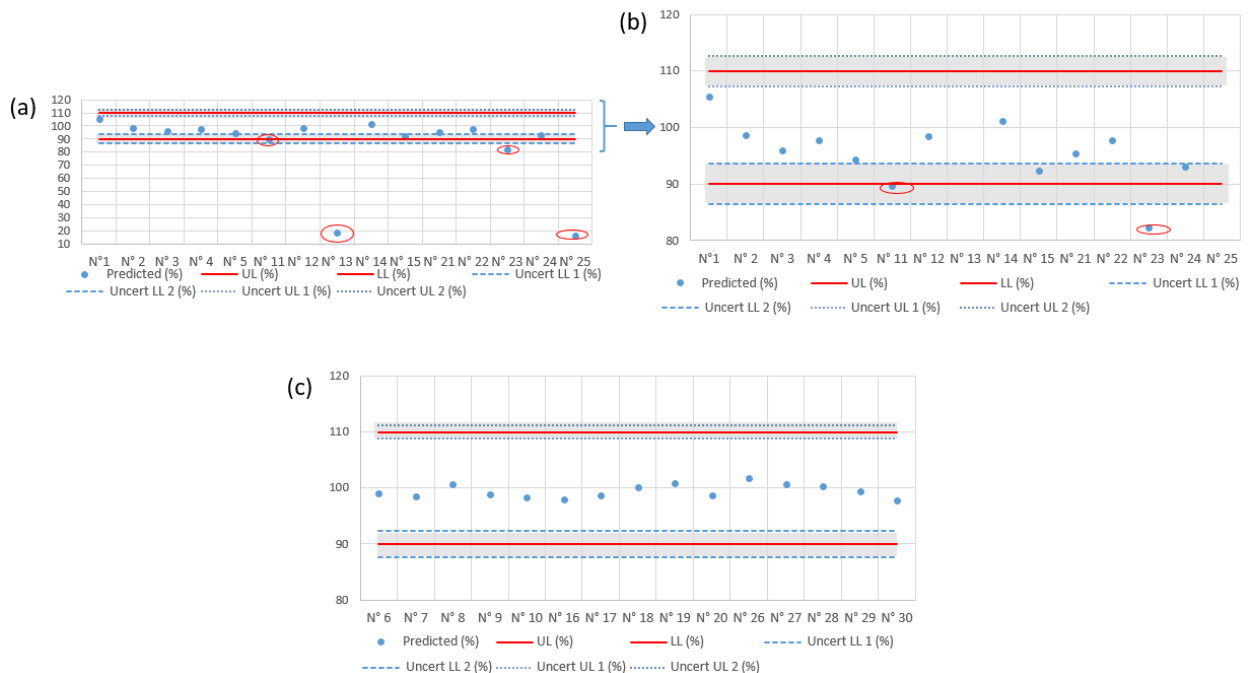
478 *In field methods application*

479 The analyses of all the samples were performed under real field conditions and the resulting
480 spectra predicted with models obtained with the second augmentation strategy. The contents
481 obtained for QC samples were very close for each sample across the cities. During the
482 equivalence study, the acceptance margins were of $\pm 1.1\%$ and $\pm 0.4\%$ for ciprofloxacin and
483 metronidazole respectively. All 90% confidence intervals of the cities pairwise comparisons
484 fell inside the defined acceptance margins. Therefore, the equivalence study between the three
485 cities shown that all cities provided equivalent results for each active ingredient (See Figure
486 S10).

487 Regarding ciprofloxacin samples, the NIR method allowed the detection of four suspicious
488 samples (Figure 7a and 7b): N° 11, 13, 23, and 25. These samples had out-of-specification
489 (OOS) predicted contents: 89.6, 18.2, 82.3, and 15.6% of the declared nominal content
490 respectively. Compared to HPLC results, these NIR contents were very close, (See Figure 8a)
491 except for the previous suspicious samples, N° 11, 13, 23 and 25 with HPLC contents of 96.2,
492 48.8, 92.7 and 42.6% of the declared nominal content respectively.

493 The important discrepancy between the contents obtained with NIR and HPLC methods for the
494 ciprofloxacin samples with weakest contents (N° 13 and 25) can be explained by the fact that

495 the real contents obtained with the reference method (i.e. 9.8 and 8.5 mg/mL respectively) were
 496 below the lowest calibration solution (10.01 mg/mL). Therefore, these results should be
 497 reported as “lower than the validated concentration range”. In addition, after complete
 498 disintegration, when the solutions of these two samples were left to stand for about 15 minutes,
 499 the solutions settled in mass. This is possibly the consequence of a specific excipient.



500

501 **Figure 7:** Assay results of collected samples obtained with the second augmentation strategy.

502 (a) Assay results of ciprofloxacin samples with y axis (representing the content of the samples)

503 set between 80 - 120%, (b) Assay results of ciprofloxacin samples with y axis zoomed between

504 80 - 120 % for better visualization of the uncertainty regions, and (c) Assay results of

505 metronidazole samples. The blue dots represent the predicted contents of the samples. Those

506 encircled with red circles represent suspect samples which need to be re-analyzed with

507 confirmatory method. The plain red lines are the acceptance limits (UL and LL), the dashed

508 blue lines are the lower and upper limits of the guard bands (Uncert LL1, Uncert LL2, Uncert

509 UL1, Uncert UL2) based on the relative expanded uncertainty values obtained during

510 validation.

511 Further investigations are ongoing since no information on the qualitative composition of the
512 tablets is provided. The very low contents (3.6 and 3.1mg/mL respectively) predicted by the
513 NIR method for these samples could be explained by this matrix effect. The sample N° 13 was
514 collected from the formal sector and the sample N° 25, from the informal sector. According to
515 some authors,^{4,51,52} since the contents of these samples were below 50 %, they should be
516 considered as falsified. These results were reported to the Cameroonian national regulatory
517 authority (NRA) and the WHO global surveillance and monitoring system. Concerning the
518 Cameroonian NRA, a circular letter was emitted in order to quarantine the medicines in case
519 they were found in the supply chain.

520 Regarding the other two ciprofloxacin samples (N° 11 and 23), they were considered as suspect
521 since their predicted contents were OOS (89.7 and 82.3 % respectively, see Figure 7a). One of
522 them (sample 11) was within the guard bands around the acceptance limits based on the
523 accuracy profile. These regions in grey (see Figures 7b and 7c) were determined considering
524 the relative expanded uncertainty values at 90 and 110% for both ciprofloxacin and
525 metronidazole. Since the objective of these methods was to be able to detect non-compliant
526 samples and remove them from the supply chain, one can say that the validated methods are fit-
527 for-purpose. According to the correspondents HPLC results (Figure 8a), these samples were
528 compliant as their contents were within the acceptance limits set at ± 10 % with contents of 96.2
529 and 92.7 % respectively. This underlines the fact that confirmatory methods are still needed to
530 confirm the results of the screening methods. Moreover, the results obtained highlight how a
531 fast and easy NIR screening method could detect SF medicines and drastically reduce the
532 number of samples to be analyzed by confirmatory methods. All the metronidazole samples
533 were compliant (Figure 7c), and these results were confirmed by HPLC results (Figure 8b).

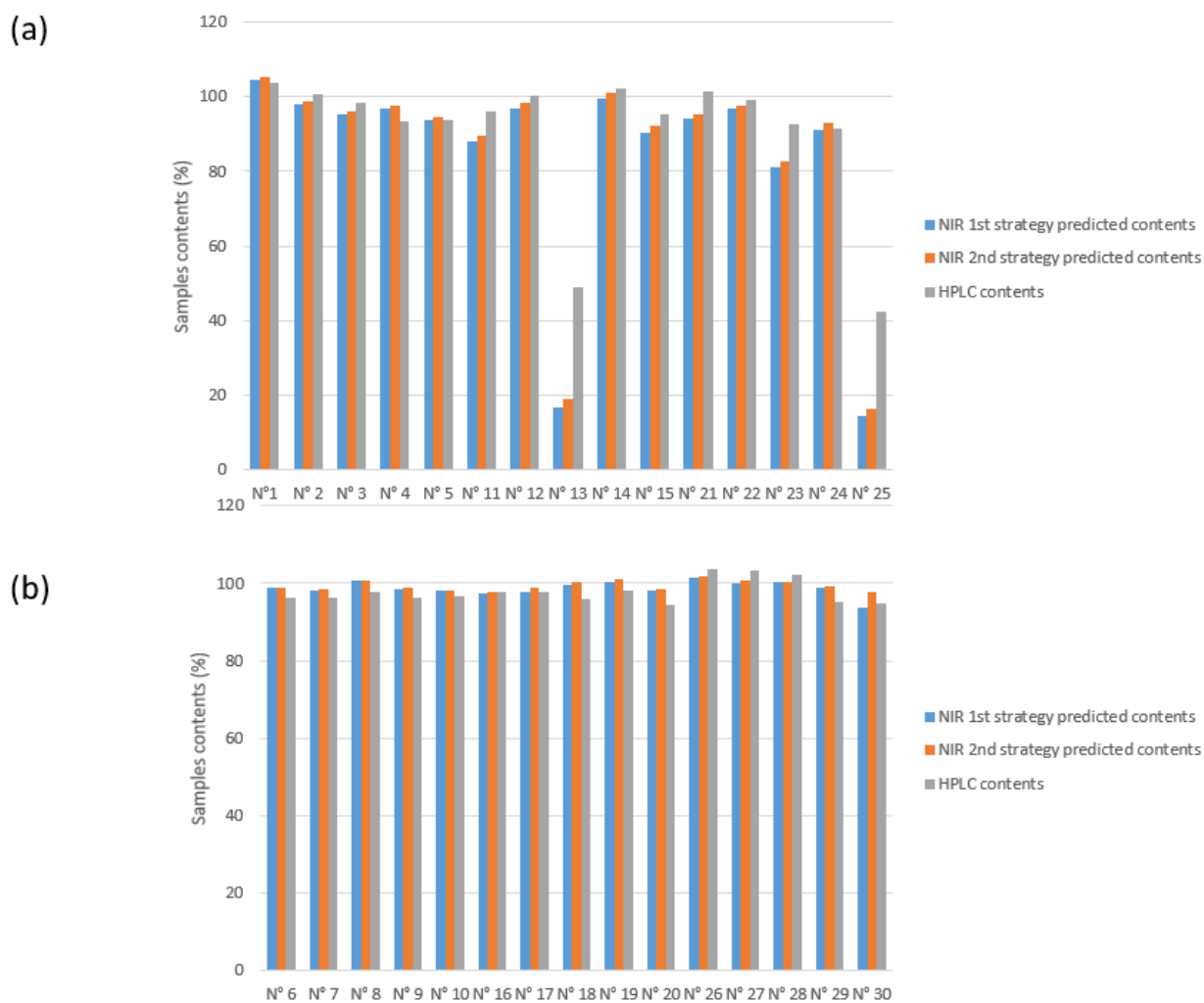
534 It is important to notice that the sample preparation is different between HPLC and NIR
535 spectroscopy. In the case of HPLC, 10 tablets are crushed and a quantity of powder

536 corresponding to the average weight of one tablet is taken for analysis, while 1 tablet was diluted
537 with NIR. In the two cases, the samples were analyzed in triplicates. Although the preparation
538 procedures were not the same for NIR and reference methods, the content results obtained were
539 very close, except for the four suspect ones.

540 These findings are close to those of Ciza *et al.* for which the contents obtained with the handheld
541 NIR devices were consistent to those obtained with a benchtop NIR spectrometer for quinine
542 samples and to those obtained with UV-spectroscopy reference method for metformin samples.
543 ^{24,25} Their analyses were realized in the laboratory (under controlled environmental conditions)
544 while those of the present work took place out of the laboratory in the different cities: Yaoundé,
545 Douala and Garoua respectively, near to the collection sites.

546 When predicting the samples contents with the first NIR augmentation strategy, the results were
547 very close for each sample to those of the second one (Figures 8a and 8b).

548



549

550 **Figure 8:** Assay results of collected samples obtained with the first and second augmented PLS-
 551 R models, and HPLC method. (a) Ciprofloxacin and (b) Metronidazole results

552 *Proposal about the practical implementation of NIR spectroscopy as in field screening tool*

553 In practice, all samples detected as OOS should be re-tested with confirmatory methods.

554 Furthermore, it could be suggested that those with contents within the uncertainty region around

555 the acceptance limits (see Figure 7b), should be re-tested as well. Nevertheless, this decision

556 would be left to the final user's appreciation if there is not decision rule.⁵³ In addition, randomly

557 selected compliant samples (e.g., 10%) should undergo confirmatory testing in order to monitor

558 the analytical performances of the regression models as part of its control strategy.⁵⁴

559 Considering the emergency nature of decision-making about the negative consequences of SF
560 medicines, NIR spectroscopy-based screening methods are suitable, especially in hard-to-reach
561 areas and resource-limited settings for medicines quality control since poor samples shipping
562 conditions could contribute to their degradation and therefore to biased results. The methods
563 validated in the present study did not require any reagents that are difficult to acquire (water
564 and HCl are cheap and readily accessible) and the sample preparation was quite simple with
565 only one dilution step. It has also been demonstrated that milli-Q water could be replaced by
566 bottled water. Moreover, the NIR device is easily transportable even if the spectra acquisition
567 requires a computer, a tablet, or a smartphone thanks to the Bluetooth® connection possibility.
568 It could also reduce the number of samples to be analyzed with confirmatory methods. Suspect
569 samples could thereby be quarantined and quickly removed from the supply chain, preventing
570 them from harming the population's health. Practically, in the present study, out of thirty
571 samples, only four were considered as suspicious.

572 Another advantage of the proposed NIR methodology is that, the sample preparation step could
573 be predictive of the disintegration test. This has been shown with ciprofloxacin samples
574 collected during a previous field study.⁹ 13 tablets were non-compliant to the disintegration
575 test. These non-compliant samples required in average 33.1 ± 1.03 minutes to dissolve
576 completely in the 25,0 mL of diluent for the NIR measurements. This was clearly different from
577 the compliant samples that required in average 5.4 ± 2.74 minutes to disintegrate. A maximum
578 disintegration time could be set and the samples having disintegration times exceeding the
579 defined cut-off value should also be considered as suspect and submitted to pharmacopeia
580 disintegration testing for confirmation purpose.

581 The sample preparation step may vary from 15 to 45 minutes per sample, depending on the
582 tablet's dissolution time. The spectra acquisition took about one minute per sample. Once the
583 model is validated, the prediction phase is not time-consuming.

584 It is important to think about waste management on-site, since the analyses generate liquid
585 waste, even if the quantities are not significant compared to laboratory methods. In our case,
586 after the analysis, wastes were transported to the national laboratory where there is a dedicated
587 waste management procedure.

588 **Conclusion**

589 Two strategies for calibration augmentation were considered for the application of NIR PLS-R
590 models for the quantification of ciprofloxacin and metronidazole in tropical conditions. The
591 challenge to overcome was the environmental effect on NIR spectra, as these models were
592 transferred from Belgium, a temperate oceanic climate zone, to Cameroon, a tropical climate
593 zone.

594 The different PLS-R models were used in field conditions to analyze some ciprofloxacin and
595 metronidazole tablet samples gathered on formal and informal Cameroonian markets. These
596 results suggested that, as the sample contents obtained with the two strategies were not
597 statistically different, the first one targeting only temperature as potential influencing factor can
598 be implemented considering its easier application. However, regarding the models' diagnostic
599 measures, the use of the second strategy which considered temperature and other complex
600 environmental factors in transfer site's conditions, is more ideally suited.

601 These simple NIR approaches allowed the detection of two SF medicines and these results were
602 confirmed by HPLC method taken as reference. They highlighted the fact that low-cost NIR
603 spectrophotometers despite their limited spectral window and resolution, can be used
604 successfully as screening tools for the detection of SF medicines in different environmental
605 conditions. The purpose of handheld devices according to Crocombe would be therefore
606 fulfilled, allowing the instrument to reach the sample, and moving the laboratory to the point
607 of use.¹⁷ It is therefore interesting for national medicines regulatory systems in resource-limited
608 settings to implement the use of this type of device that can be performed without constant

609 power supply and little training in order to strengthen the capacity of inspectors during their
610 pharmaceutical market surveillance operations, ensuring that SF medicines are detected as
611 quickly as possible and removed from the supply chain. Their use in the screening phase allows
612 saving costs.

613 The principal limitation of the present work was the low number of samples. Therefore, we
614 suggest that larger-scale studies should be carried out to confirm the results obtained and cost-
615 effectiveness studies of the validated methods for more efficient post-marketing surveillance.

616 In addition, another limitation concerns the inability to carry out additional validation runs in
617 Garoua and Douala cities. Nevertheless, falsified samples were detected in these two cities, and
618 the contents obtained for the samples were generally close to those of the HPLC (except the
619 two falsified samples). Furthermore, as demonstrated by the statistical tests carried out on the
620 QC samples, there was no significant difference between the levels obtained in the different
621 cities.

622 Since some methods have been developed yet for the quantification of monotherapies and APIs
623 soluble in aqueous phase, the development of methods for the analysis of combinations and
624 hydrophobic APIs would be considered, the goal being to extend these NIR methods to most
625 essential medicines and various pharmaceutical forms. Furthermore, model transfer to different
626 types of devices, different environmental conditions, and the use of low-cost portable systems
627 with wider spectral bands would also be potential research areas.

628

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635 **Supplemental Material**

636 All supplemental material mentioned in the text is available in the online version of the journal.

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815 **Figures captions**

816 **Figure 1:** Accuracy profiles resulting from the prediction of 4 validation series measured in
817 Belgium obtained with the initial calibration models. Legend: (a) ciprofloxacin and (b)
818 metronidazole accuracy profiles. The plain red line is the relative bias, the dashed blue lines are
819 the 95 % β -expectation tolerance intervals, and the dashed black lines represent the acceptance
820 limits of ± 10 %. The dots represent the relative error of the results and are plotted with respect
821 to their targeted concentration.

822 **Figure 2:** Prediction results of spectra solutions measured in Belgium and Cameroon. Legend:
823 (a) ciprofloxacin and (b) metronidazole plots representing reduced Q residuals versus reduced
824 Hotelling's T^2 distances (left) and predicted residuals versus leverage (right). Pink stars:
825 Cameroon validation samples, yellow circles: Belgium validation samples.

826 **Figure 3:** Accuracy profiles resulting from the prediction of 4 validation series measured in
827 Belgium and one external validation series measured in Cameroon with the initial calibration
828 models. Legend: (a) ciprofloxacin and (b) metronidazole accuracy profiles. The plain red line
829 is the relative bias, the dashed blue lines are the 95 % β -expectation tolerance intervals, and the
830 dashed black lines represent the acceptance limits of ± 10 %. The dots represent the relative
831 error of the results and are plotted with respect to their targeted concentration.

832 **Figure 4:** Accuracy profiles resulting from the prediction of 4 validation series measured in
833 Belgium and one external validation series measured in Cameroon with the first strategy of
834 model augmentation. Legend: (a) ciprofloxacin and (b) metronidazole accuracy profiles. The
835 plain red line is the relative bias, the dashed blue lines are the 95 % β -expectation tolerance
836 intervals, and the dashed black lines represent the acceptance limits of ± 10 %. The dots
837 represent the relative error of the results and are plotted with respect to their targeted
838 concentration.

839 **Figure 5:** Accuracy profiles resulting from the prediction of 4 validation series measured in
840 Belgium and one external validation series measured in Cameroon with the second strategy of
841 model augmentation. Legend: (a) ciprofloxacin and (b) metronidazole accuracy profiles. The
842 plain red line is the relative bias, the dashed blue lines are the 95 % β -expectation tolerance
843 intervals, and the dashed black lines represent the acceptance limits of ± 10 %. The dots
844 represent the relative error of the results and are plotted with respect to their targeted
845 concentration.

846 **Figure 6:** Prediction results of spectra solutions measured in Belgium and Cameroon with the
847 first (a and b) and the second (c and d) strategies of model augmentation: (a and c) ciprofloxacin
848 and (b and d) metronidazole plots representing the reduced Q residuals versus reduced
849 Hotelling's T^2 (left) and predicted residuals versus leverage (right). Legend: (a) and (b) were
850 obtained with the first strategy augmentation model; (b) and (c) were obtained with the second
851 strategy augmentation model. Pink stars: Cameroon validation samples, yellow circles:
852 Belgium validation samples.

853 **Figure 7:** Assay results of collected samples obtained with the second augmentation strategy.
854 Legend: (a) Assay results of ciprofloxacin samples with y axis (representing the content of the
855 samples) set between 80 - 120%, (b) Assay results of ciprofloxacin samples with y axis zoomed
856 between 80 - 120 % for better visualization of the uncertainty regions, and (c) Assay results of
857 metronidazole samples. The blue dots represent the predicted contents of the samples. Those
858 encircled with red circles represent suspect samples which need to be re-analyzed with
859 confirmatory method. The plain red lines are the acceptance limits (UL and LL), the dashed
860 blue lines are the lower and upper limits of the guard bands (Uncert LL1, Uncert LL2, Uncert
861 UL1, Uncert UL2) based on the relative expanded uncertainty values obtained during
862 validation.

863 **Figure 8:** Assay results of collected samples obtained with the first and second augmented PLS-
864 R models, and HPLC method. Legend: (a) Ciprofloxacin and (b) Metronidazole results

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