Supplementary Data

Analytical quality by design in the development of a paper-based microfluidic device for iron (II) determination as an impurity in iron (III) pharmaceuticals

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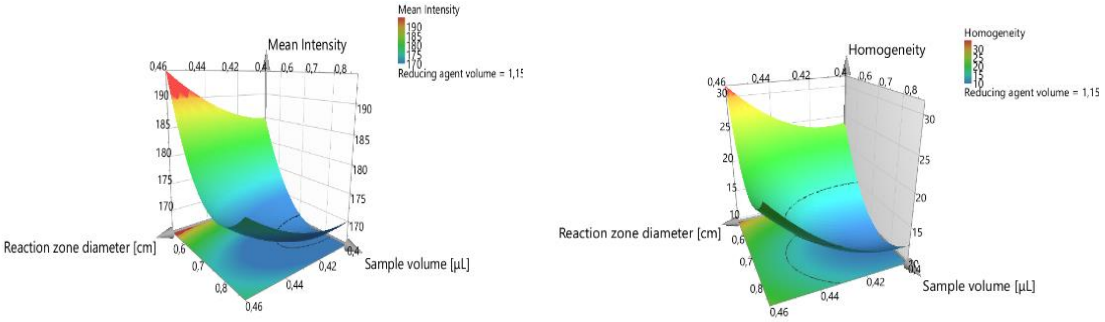

Figure S1. Graphic representation of response Mean Intensity and Homogeneity models.

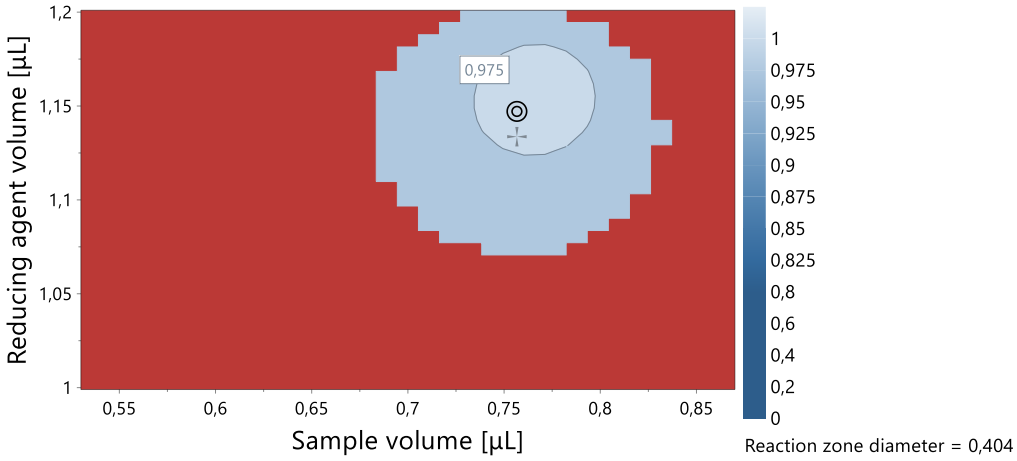

Figure S2. Desirability plot calculated within the MODR.

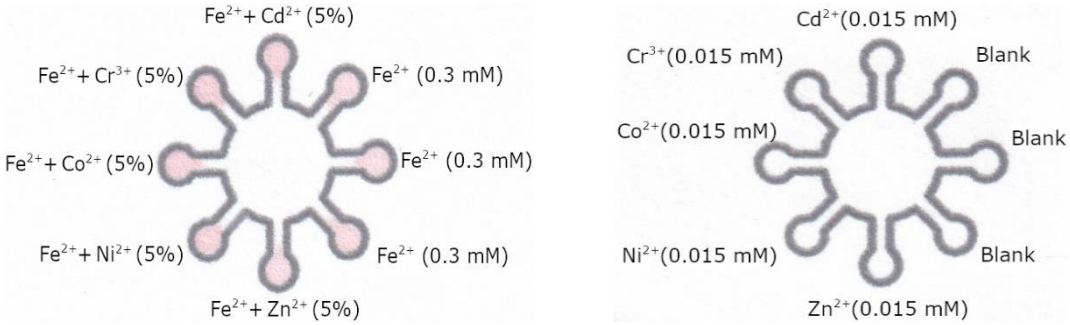

Figure S3. Selectivity of the µPAD method. **Left panel**: determination of Fe^{2+} (0.300 mM) in the absence and in the presence of potentially interfering transition metals spiked at 5% concentration relative to the analyte. For a statistical comparison of the obtained responses, refer to the main body of the article and Table S7. **Right panel**: analysis of individual transition metals standards at the spiking concentration (0.015 mM) to evaluate interferences.

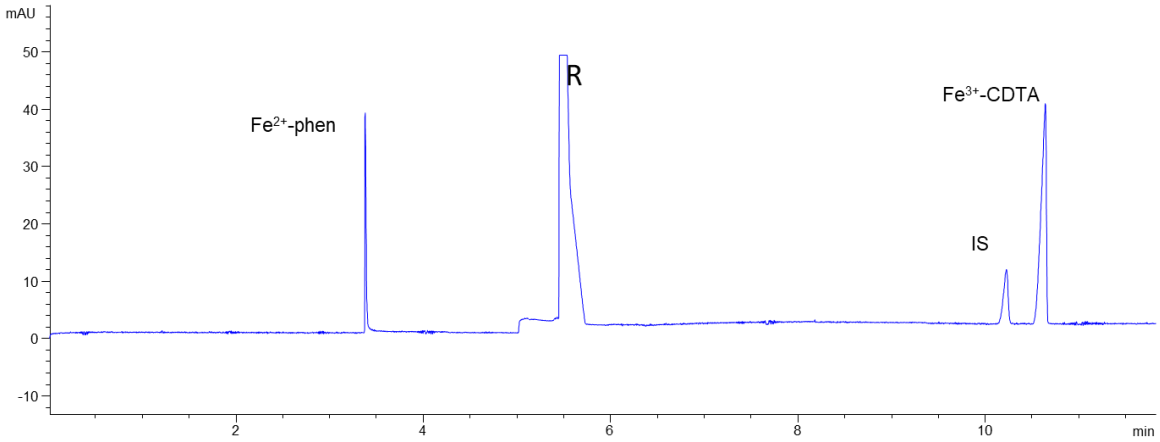

Figure S4. Electropherogram of a real sample of iron sucrose pharmaceuticals. Conditions: fused-silica capillaries (50 µm id, 64.5 cm total length, 56 cm length to the detector); running buffer: 60 mM sodium tetraborate; constant voltage of 25 kV; cartridge temperature was 25°C; hydrodynamic injection at 50 mbar x 10 s; wavelength 265 nm. Symbols: Fe^{2+}-phen and Fe^{3+}-CDTA are the complexes of Fe^{2+} and Fe^{3+} with 1,10-phenanthroline and 1,2-diaminocyclohexanetetra-acetic acid, respectively; R is the reagent mixture excess; IS is the internal standard (suprofen).

The sample (200 µL of the solution prepared as for µPAD analysis) was complexed with 200 µL of Phen solution 20 mM (water) and 200 µL of CDTA 20 mM (in NaOH 0.05 M). Suprofen was used as the internal standard (final concentration of 0.1 mg/mL) [33].

Table S1. D-Optimal design for the risk evaluation step

N°Exp	Reagent volume[(1)]	Sample volume[(1)]	Reducing agent volume[(1)]	Reaction zone diameter[(2)]	Channel length[(2)]	Channel width[(2)]	Mean Intensity[(3)]	SD of mean intensity	Homogeneity[(4)]
1	21	0.50	0.80	0.45	0.30	0.20	190.49	1.70	26.29
2	21	0.30	1.00	0.45	0.30	0.20	208.22	2.41	30.64
3	25	0.30	0.80	0.50	0.30	0.20	226.34	3.47	27.85
4	25	0.50	1.00	0.50	0.30	0.20	201.69	1.92	30.00
5	21	0.30	0.80	0.45	0.40	0.20	213.05	1.03	28.44
6	21	0.50	1.00	0.45	0.40	0.20	190.15	1.66	24.69
7	25	0.30	0.80	0.50	0.40	0.20	224.07	3.32	28.86
8	25	0.50	0.80	0.50	0.40	0.20	196.27	1.63	28.30
9	21	0.30	1.00	0.50	0.40	0.20	214.23	1.70	30.94
10	25	0.50	1.00	0.50	0.40	0.20	199.05	3.40	28.02
11	25	0.30	0.80	0.45	0.30	0.25	210.73	0.89	29.37
12	25	0.50	1.00	0.45	0.30	0.25	190.40	2.81	25.95
13	21	0.30	0.80	0.50	0.30	0.25	220.79	1.83	29.66
14	21	0.50	0.80	0.50	0.30	0.25	198.95	3.21	28.91
15	25	0.30	1.00	0.50	0.30	0.25	220.39	2.20	28.22
16	21	0.50	1.00	0.50	0.30	0.25	194.82	2.04	27.14
17	21	0.30	0.80	0.45	0.40	0.25	212.05	1.84	28.56
18	25	0.50	0.80	0.45	0.40	0.25	192.35	2.90	26.80
19	25	0.30	1.00	0.45	0.40	0.25	206.86	5.64	29.53
20	21	0.50	0.80	0.50	0.40	0.25	211.51	3.42	30.09
21	21	0.30	1.00	0.50	0.40	0.25	222.48	1.99	28.08

(1) µL
(2) cm
(3) mean grayscale intensity in the define detection zone
(4) standard deviation of the pixel intensity within the defined detection zone

Table S2. CCD experimental plan and measured responses values for the optimization step

N°Exp	Sample volume[(1)]	Reducing agent volume[(1)]	Reaction zone diameter[(2)]	Mean Intensity[(3)]	SD of mean intensity	Homogeneity[(4)]
1	0.60	1.04	0.412	179.83	2.37	19.05
2	0.80	1.04	0.412	168.45	1.86	13.05
3	0.60	1.16	0.412	177.35	1.42	17.63
4	0.80	1.16	0.412	167.19	2.12	9.694
5	0.60	1.04	0.448	187.41	3.61	24.31
6	0.80	1.04	0.448	174.53	1.87	18.01
7	0.60	1.16	0.448	182.82	1.46	22.85
8	0.80	1.16	0.448	172.31	1.08	17.47
9	0.53	1.10	0.430	185.79	2.20	23.69
10	0.87	1.10	0.430	170.88	1.64	15.58
11	0.70	0.999	0.430	173.79	1.78	18.53
12	0.70	1.20	0.430	168.30	1.99	11.93
13	0.70	1.10	0.400	165.58	1.19	11.96
14	0.70	1.10	0.460	174.45	1.69	18.33
15	0.70	1.10	0.430	169.48	1.15	12.61
16	0.70	1.10	0.430	169.98	1.39	11.70
17	0.70	1.10	0.430	169.10	1.63	13.66
18	0.70	1.10	0.430	169.94	1.31	13.04

(1) µL
(2) cm
(3) mean grayscale intensity in the define detection zone
(4) standard deviation of the pixel intensity within the defined detection zone

Table S3. Experimental plan for robustness testing

N°Exp	Rand	Sample volume[(1)]	Reducing agent volume[(1)]	Reaction zone diameter[(2)]	Mean intensity[(3)]	SD of mean intensity	Homogeneity[(4)]
1	2	0.77	1.23	0.406	164.29	1.24	8.56
2	3	0.77	1.23	0.406	165.01	2.07	8.03
3	7	0.75	1.23	0.426	165.42	1.57	8.45
4	8	0.75	1.23	0.426	166.06	0.98	10.71
5	5	0.75	1.03	0.426	165.55	0.57	9.38
6	4	0.75	1.03	0.426	165.71	0.72	8.28
7	6	0.75	1.03	0.406	165.35	0.85	8.51
8	1	0.75	1.03	0.406	165.30	0.86	8.42

(1) µL
(2) cm
(3) mean grayscale intensity in the define detection zone
(4) standard deviation of the pixel intensity within the defined detection zone

Table S4. ANOVA for the response Mean Intensity

Source of variation	Sum of squares	Degrees of freedom	Mean squares	Ratio	Significance
Regression	1.4409	3	0.4803	4.0656	10.4
Residual	0.4725	4	0.1181		
Total	1.9134	7			

Table S5. ANOVA for the response SD of Mean Intensity

Source of variation	Sum of squares	Degrees of freedom	Mean squares	Ratio	Significance
Regression	1.2085	3	0.4028	3.0156	15.7
Residual	0.5343	4	0.1336		
Total	1.7428	7			

Table S6. ANOVA for the response Homogeneity

Source of variation	Sum of squares	Degrees of freedom	Mean squares	Ratio	Significance
Regression	1.9580	3	0.6527	0.7908	55.9
Residual	3.3014	4	0.8253		
Total	5.2594	7			

Table S7. Results of the selectivity study in Fe^{2+} determination (mM, n = 3) in the presence of transition metals as potential interferences

Analyte[(1)]	Found Fe^{2+} ± SD	p-value[(2)]
Fe^{2+}	0.296 ± 0.005	n/a
Fe^{2+} + Zn^{2+}	0.301 ± 0.010	0.48
Fe^{2+} + Ni^{2+}	0.309 ± 0.010	0.11
Fe^{2+} + Co^{2+}	0.292 ± 0.002	0.26
Fe^{2+} + Cr^{3+}	0.318 ± 0.020	0.13
Fe^{2+} + Cd^{2+}	0.301 ± 0.002	0.18

(1) Spike of Zn^{2+}, Ni^{2+}, Co^{2+}, Cr^{3+}, Cd^{2+} at 5% each with respect to the Fe^{2+} content (nominal concentration was 0.300 mM)
(2) p-values were calculated using a student's t-test; the results are reported at a significance level of $\alpha = 0.05$ (corresponding to a 95 % confidence level).

Table S8. Analytical characteristics of µPAD devices used for iron quantitation

Sample/Analyte	Dimensions			Fabrication	Detection[4]	DL or QL (µM)	Range (µM)	Rec%	Ref
	Ø[1]	Ch-l[2]	Ch-w[3]						
Water (tap/river/sea) Fe^{3+}	3	n/r[5]	n/r	Test-spot type lamination	Naph-3,4-HPO[6]	DL 1.25	4.5 - 35.8	100.3%	28
Serum Fe^{2+}/Fe^{3+}	10	10	5	marker pen	Ferrozine Ferrozine + immunopurification	DL 53.7 DL 5.37	90 - 717	<34 % 96 %	29
Urine Total Fe	5	n/r	n/r	Test-spot type lamination	Bathophenanthroline	DL 0.36	1.25 – 21.5	n/r	30
Wine Fe^{2+}/Fe^{3+}	5	n/r	n/r	Test-spot type lamination	Bathophenanthroline	DL 1.1	4.5 – 89.6	n/r	31
Water/plasma (surface/ground) Fe^{3+}	10	n/r	n/r	circular disks hydrophobic paper	CTAB-AgNPs[7]	DL 0.36	0.9 – 16.1	91.3-95%	32
Welding fumes Fe^{3+}	6	3	2	screen printing	Phe	DL 82.4	1.8 – 896.0	95-99%	38
Water (drinking/river) Fe^{3+}	7	16	2.5	wax	Bathophenanthroline	DL 8.96	65.8 – 268.8	62-90 %	42
Water Hot spring Fe^{3+}	n/r	n/r	n/r	wax	Phe	QL 717	717 - 6272	n/r	43
Water (river and tap) Fe^{2+}/Fe^{3+}	n/r	n/r	n/r	Photolithography lamination	Phe	DL 3.96	8.9 - 89	~100 %	44
Pharmaceutical injections Fe^{2+}, total Fe	4	3.5	2	wax	Phe	DL 30	100 - 500 Fe^{2+}	87-105 %	This study

(1) diameter of detection zone (mm)
(2) channel length (mm)
(3) channel width (mm)
(4) colorimetric detection/derivatization reagents
(5) not reported
(6) naphthalene-3-hydroxy-4-pyridione
(7) cetyltrimethylammonium bromide-modified silver nanoparticles

Table S9. Trueness and precision data (mM, n = 3) for Fe^{2+} and total iron

Level	Fe^{2+}	Total Iron
Low	0.10	2.00
Found%	93.3%	89.6%
RSD%	6.00%	2.51%
Medium	0.30	5.00
Found%	105.4%	104.4%
RSD%	3.75%	1.0%
High	0.50	8.00
Found%	98.6%	98.1%
RSD%	2.34%	0.50%

Table S10. Comparison of Iron release efficiency between HCl and phosphoric acid

	Acid	Mean[(1)] ± SD	t-test p value [(2)]
Fe^{2+}	HCl	0.124 ± 0.008	p = 0.8
	Phosphoric acid	0.123 ± 0.007	
Total Iron	HCl	3.59 ± 0.01	p = 0.4
	Phosphoric acid	3.60 ± 0.02	

1) mM, (n = 3)
2) p-values were calculated using a student's t-test; the results are reported at a significance level of $\alpha = 0.05$ (corresponding to a 95 % confidence level).

Table S11. Fe^{2+} and Total iron concentration (mM) in the drug preparation and expired drug samples as analysed by CE and µPAD

Sample type	Iron species	Platform	Replicate 1	Replicate 2	Replicate 3	Mean ± SD	t-test, p value(1)
Viable drug	Fe^{2+}	CE	0.197	0.199	0.197	0.197 ± 0.001	p = 0.5
	Fe^{2+}	µPAD	0.188	0.197	0.200	0.195 ± 0.006	
	Total Iron	CE	3.62	3.80	3.92	3.78 ± 0.15	p = 0.3
	Total Iron	µPAD	3.54	3.69	3.72	3.65 ± 0.1	
Expired drug	Fe^{2+}	CE	0.334	0.329	0.319	0.327 ± 0.01	p = 0.7
	Fe^{2+}	µPAD	0.331	0.344	0.318	0.331 ± 0.01	
	Total Iron	CE	3.76	3.65	3.31	3.57 ± 0.2	p = 0.8
	Total Iron	µPAD	3.76	3.67	3.41	3.61 ± 0.2	

1) p-values were calculated using a student's t-test; the results are reported at a significance level of $\alpha = 0.05$ (corresponding to a 95 % confidence level).