Simplex-optimized and Flow Injection Spectrophotometric Assay of Tetracycline Antibiotics in Drug Formulations

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The modified simplex method was applied to the selection of the proper experimental conditions for the flow injection spectrophotometric determination of tetracycline, chlortetracycline, oxytetracycline and demeclocycline. In the method a 157 mm^3 sample volume was injected, for all the compounds, into the carrier stream of iron(III) of concentrations 554, 626, 701 and 447 ppm flowing at rates of 3.72, 4.37, 3.72 and 3.72 cm^3 min^{-1}, thus passing through a reaction coil of length 55, 85, 45 and 45 cm for the respective compounds, all in 0.001 mol dm^{-3} sulfuric acid as an over-all reaction medium. A high sampling frequency of the order of at least 170 h^{-1} was attained for all the compounds. A high precision with a relative standard deviation of less than 0.9% (n = 5) was also obtained. The accuracy was found to be high as the Student t-values were calculated to be less than the theoretical values when the results were compared with those obtained by the conventional spectrophotometric method. There were no interferences from excipients in dosage forms when the method was applied to pharmaceutical preparations.

Keywords: *Simplex method; flow injection; spectrophotometry; tetracycline antibiotics; drug formulations*

Tetracyclines and derivatives are extensively used in current therapy owing to their broad-spectrum antibacterial activity. Tetracycline hydrochloride, oxytetracycline, chlortetracycline and demeclocycline hyclate have recently been assayed spectrophotometrically[1] through complexation with iron(III) in sulfuric acid media and subsequent measurement of the soluble brown complex formed at the corresponding wavelength. The introduction of flow injection (FI) and the trend to utilize this technique for routine and batch analyses led us to apply it to drug formulations to maximize the sensitivity and response and to optimize the throughput and economy of the drugs and other reagents. However, the failure of the iterative univariate procedure to achieve the highest throughput with the lowest concentrations of reagents in a recent FI method for the determination of oxytetracycline,[2] probably owing to relatively high interactions of FI and chemical variables, necessitated the use of a modified computerized simplex method[3-5] with a program designed for the optimization of the FI system and chemical variables.

Few FI methods for the assay of tetracyclines have been reported; one requires a special and complicated amperometric detector[6] and another a chemiluminescence detector suitable for the determination of tetracycline only at relatively high concentrations with poor detection limits.[7] Both methods require the use of a difficult buffering procedure and suffer low throughput. The official British Pharmacopoeial (BP) method is tedious and time consuming, requiring prolonged incubation.[8] Other methods for the assay of tetracycline and its derivatives using different techniques such as chromatography, electrochemistry and spectrophotometry have been reviewed elsewhere,[1,2,9] and each has advantages and disadvantages.

Experimental

Apparatus

Apparatus obtained from the Alitea USA/FIA Laboratory (Sweden) was used for all experimental work. The apparatus, which has been described previously,[10] consists of a four-channel peristaltic pump, a Rheodyne Model 5041 injector, a reactor module, a Spectronic Mini 20 spectrophotometer as a detector with a 20 mm^3 ultra-micro flow-through cell having a pathlength of 1.0 mm and a Cole Parmer Model 0555 single-channel strip-chart recorder.

A Perkin-Elmer Lambda 5 ultraviolet/visible spectrophotometer with 10.00 mm cells was used for measuring the absorbances of samples prepared for analysis by the original spectrophotometric method[1] for comparison purposes.

Reagents

Tetracycline standard solution

This was freshly prepared from the pure compounds supplied by Pfizer (Belgium) by directly dissolving the powder in 0.001 mol dm^{-3} sulfuric acid to give a 1000 ppm stock solution, from which other concentrations were prepared by appropriate dilution. Oxytetracycline required warming at 60 °C for 20 min, whereas the others dissolved at room temperature.

Tetracycline proprietary drugs

Capsules. The contents of ten capsules were carefully transferred into a beaker and accurately weighed. An amount of powder equivalent to 250 mg of the drug was dissolved in 150 cm^3 of 0.001 mol dm^{-3} sulfuric acid, warmed at 60 °C for 20 min and transferred into a 250 cm^3 calibrated flask through a filter-paper, cooled to room temperature and diluted to the mark with the same sulfuric acid.

Syrups. A volume of syrup containing a certain mass of the tetracycline was pipetted into a suitable calibrated flask and diluted to the mark with the required sulfuric acid solution to give a final solution of the drug in 0.001 mol dm^{-3} acid.

Iron(III)

A 5.6 mg cm^{-3} iron(III) solution was prepared by dissolving exactly 24.2000 g of dried ammonium iron(III) sulfate [$NH_4Fe(SO_4)_2 \cdot 12H_2O$] in 400 cm^3 of 0.001 mol dm^{-3} sulfuric acid and diluting to 500 cm^3 with the same acid in a calibrated flask.

Manifold

The single-configuration FI manifold is shown in Fig. 1. Poly(vinyl chloride) (PVC) tubing of 1.3 mm i.d. was used as pump tubing for the carrier stream. A Rheodyne Model 5041 four-way poly(tetrafluoroethylene) (PTFE) rotary valve with a 157 mm^3 sample loop was used for injecting the sample into the carrier stream. Poly(tetrafluoroethylene) tubing of 0.5 mm i.d. and different lengths (55 cm for tetracycline, 45 cm for oxytetracycline and demeclocycline and 85 cm for chlortetracycline) was used as reactor coils.

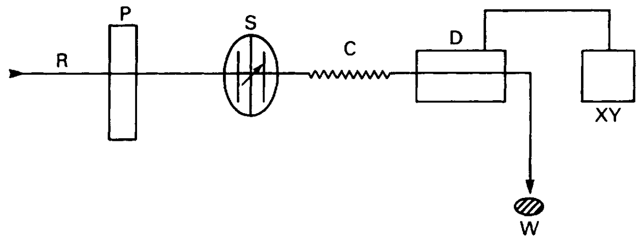


Fig. 1 Schematic diagram of the FI manifold used: R, reagent carrier of iron(III) in sulfuric acid; P, peristaltic pump; S, sample injector of 157 mm³ loop size; C, reaction coil; D, Spectronic Mini 20 spectrophotometer; XY, recorder; and W, waste

Optimization and Procedure

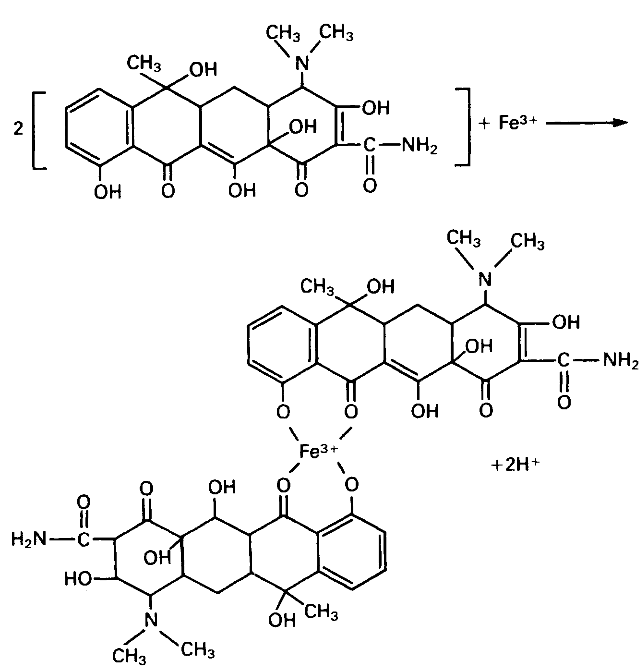
The parameters of the program and the upper and lower boundaries for each variable were fed into the computer. Five experiments were designed according to the procedure suggested by Betteridge and co-workers[11,12] as the initial simplex, as the simplex is a geometric figure whose vertices are $n + 1$, where n is the number of variables. Solutions were prepared accordingly, the carrier stream was allowed to flow for a few minutes at a certain flow rate and the baseline absorbance was adjusted to zero for both the spectrophotometer and recorder. The drug sample was injected into the flowing stream and the absorbance of the coloured complex was recorded at the corresponding maximum wavelength. The absorbance values of each of the five initial experiments were entered into the computer and the program calculated the next single set of conditions to be prepared and tried. The process continued in this way until satisfactory results were obtained.

Samples of unknown concentrations were then analysed and the results were calculated by comparing the absorbance given by the sample with the calibration equation for the generic drug under investigation.

Results and Discussion

Chemical System

As reported earlier,[1] tetracycline, oxytetracycline, chlortetracycline and demeclocycline react with iron(III) in sulfuric acid instantly forming soluble, stable, brown complexes with absorption maxima at 423, 435, 435 and 435 nm, respectively, and according to the reaction scheme shown below.



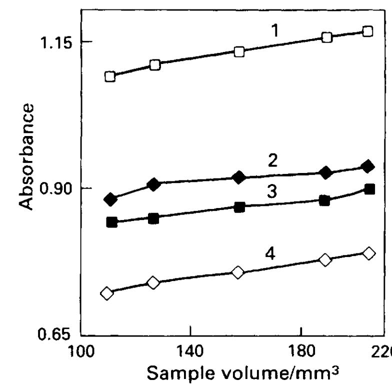


Fig. 2 Effect of variation of the sample loop size on absorbance for: 1, 680 ppm demeclocycline; 2, 600 ppm oxytetracycline; 3, 460 ppm tetracycline hydrochloride; and 4, 450 ppm chlortetracycline

Table 1 Simplex optimization of chemical and FI variables for tetracycline hydrochloride

Experiment No.*	Coil length/cm	Flow rate/ $cm^3 min^{-1}$	$[H_2SO_4]$/ mol dm^{-3}	[Iron(III)] (ppm)	Peak absorbance
1	100	4.78	0.050	100	0.198
2	300	4.78	0.050	100	0.192
3	45	5.31	0.050	100	0.162
4	45	3.90	0.010	300	0.516
5	45	3.90	0.100	600	0.372
6R	200	3.72	0.055	450	0.408
7C	84	4.12	0.030	290	0.381
8R	136	3.72	0.046	544	0.462
9C	81	4.04	0.029	311	0.402
10R	45	3.72	0.035	649	0.528
11E	45	3.72	0.028	924	0.528
12R	82	3.99	0.001	311	0.738
13E	101	4.03	0.001	167	0.720
14R	56	3.84	0.006	528	0.708
15C	85	3.99	0.009	293	0.588
16R	55	3.72	0.001	554	0.840
17E	45	3.72	0.001	688	0.828
18R	93	3.83	0.009	736	0.648
19C	57	3.79	0.005	602	0.690
20	99	4.05	0.001	50	0.558

* R = reflection; C = contraction; E = expansion.

The univariate method of optimization was used to study the effect of changing the sample loop size as it was expected to have little influence on absorbance, and this was confirmed as shown in Fig. 2. The effect of change of loop size between 110 and 210 mm³ was studied under various conditions, with Fig. 2 showing a typical cycle in which the flow rate, reaction coil length, iron(III) concentration and sulfuric acid concentration, respectively, were as follows for the four compounds: 4.10 $cm^3 min^{-1}$, 85 cm, 600 ppm and 0.001 mol dm^{-3} for tetracycline; 3.80 $cm^3 min^{-1}$, 100 cm, 600 ppm and 0.001 mol dm^{-3} for oxytetracycline; 3.81 $cm^3 min^{-1}$, 140 cm, 600 ppm and 0.001 mol dm^{-3} for chlortetracycline; and 3.77 $cm^3 min^{-1}$, 100 cm, 600 ppm and 0.001 mol dm^{-3} for demeclocycline. The optimum volume found and used in all other experiments was 157 mm³ for all the compounds.

Having fixed the sample loop size, the flow rate, reaction coil length, iron(III) concentration and acid concentration variables were optimized by the modified simplex procedure for each compound independently as follows.

Tetracycline

Table 1 gives the result of the four-variable optimization. Points 1–5 represent the first cycle and cover an optimization area of 20–60%. The best point attained was point 4 with a peak absorbance of 0.516 and the worst was point 3 with a peak absorbance of 0.162. Therefore, point 3 was reflected through the centroid of other points to obtain point 6. An experiment was then performed utilizing the variable setting as the reflected point and an absorbance of 0.408 was obtained. As this value was better than that at point 3, the latter was rejected and replaced with point 6. A contraction was then performed, as this point was not better than the best point, point 4. Then, by using the experimental setting of variables generated by contraction, a peak absorbance of 0.384 was obtained, which was not better than the reflection point; hence this completes the cycle. The reflection point 10 gave an absorbance better than the best one leading to an expansion point, the absorbance of which was the same as that at the reflection point, and therefore the reflection point replaced the worst point of the previous simplex. Point 16 represents the highest response obtained. However, attempts were made to make further improvements towards maximization, but this was found to necessitate additional experiments and was deemed not to be worthwhile, so the procedure was halted.

The optimum conditions for the assay of tetracycline were: sample volume 157 mm^3, flow rate 3.72 cm^3 min^{-1}, reaction coil length 55 cm, iron(III) 554 ppm and sulfuric acid 0.001 mol dm^{-3}.

Chlortetracycline

Table 2 summarizes the four-variable optimization, which clearly indicates that point 17 offers the highest absorbance, and at this point it became obvious that further improvement was not possible and further continuation to point 21 resulted in no significant improvement. Therefore, the optimum conditions for the assay of chlortetracycline were: sample volume 157 mm^3, flow rate 4.37 cm^3 min^{-1}, reaction coil length 85 cm, iron(III) 626 ppm and sulfuric acid 0.001 mol dm^{-3}.

Oxytetracycline

In Table 3, it is clear that a total of 19 experiments were performed and it was then found convenient to halt further experimentation. Point 15 gave the highest absorbance and the optimum conditions were: sample volume 157 mm^3, flow rate 3.72 cm^3 min^{-1}, reaction coil length 45 cm, iron(III) 701 ppm and sulfuric acid 0.001 mol dm^{-3}.

Demeclocycline

As indicated in Table 4, a total of 26 experiments were required to decide the optimum conditions and the system did not merit further experimentation to improve the response. Point 20 gave the best response and the highest absorbance value of 1.098. The optimum conditions were: sample volume 157 mm^3, flow rate 3.72 cm^3 min^{-1}, reaction coil length 45 cm, iron(III) 447 ppm and sulfuric acid 0.001 mol dm^{-3}.

For all the compounds investigated, 0.001 mol dm^{-3} sulfuric acid was found to be the optimum concentration for the determination, which is in agreement with the conventional spectrophotometric method.[1] Poorer responses were always obtained at higher acid concentrations, indicating dissociation of the complex at higher acidities.

The relatively high flow rate and short reaction coil length for all the compounds confirms that the reaction kinetics and

Table 2 Simplex optimization of chemical and FI variables for chlortetracycline

Experiment No.*	Coil length/cm	Flow rate/ cm^3 min^{-1}	[H_2SO_4]/ mol dm^{-3}	[Iron(III)] (ppm)	Peak absorbance
1	100	4.87	0.0500	100	0.204
2	400	4.87	0.0500	100	0.168
3	45	5.31	0.0500	100	0.186
4	45	3.90	0.1000	300	0.27
5	45	3.90	0.0100	600	0.576
6R	45	4.61	0.0750	200	0.258
7C	96	4.32	0.0360	375	0.423
8R	98	3.89	0.0680	337	0.324
9C	58	4.25	0.0350	409	0.426
10R	50	3.80	0.0600	492	0.378
11C	60	4.11	0.0330	448	0.474
12R	85	4.40	0.0017	466	0.756
13E	104	4.65	0.0010	549	0.786
14R	65	4.08	0.0049	552	0.744
15C	83	4.40	0.0100	515	0.606
16R	76	4.29	0.0010	566	0.792
17E	85	4.37	0.0010	626	0.810
18R	91	4.46	0.0010	534	0.780
19C	86	4.40	0.0023	575	0.768
20R	91	4.47	0.0013	612	0.792
21C	86	4.40	0.0012	583	0.780

* R = reflection; C = contraction; E = expansion.

Table 3 Simplex optimization of chemical and FI variables for oxytetracycline

Experiment No.*	Coil length/cm	Flow rate/ cm^3 min^{-1}	[H_2SO_4]/ mol dm^{-3}	[Iron(III)] (ppm)	Peak absorbance
1	100	4.78	0.050	100	0.198
2	300	4.78	0.050	100	0.186
3	45	5.31	0.050	100	0.168
4	45	3.90	0.010	300	0.462
5	45	3.90	0.100	600	0.36
6R	200	3.72	0.055	450	0.288
7C	84	4.12	0.031	288	0.378
8R	45	3.72	0.046	544	0.402
9C	56	4.04	0.029	311	0.384
10R	45	3.72	0.035	649	0.564
11E	45	3.72	0.028	924	0.564
12R	70	4.00	0.001	311	0.702
13E	82	4.03	0.001	167	0.642
14R	45	3.73	0.002	563	0.72
15E	45	3.72	0.001	701	0.786
16R	52	3.72	0.001	734	0.768
17C	49	3.78	0.005	612	0.696
18R	66	3.73	0.008	902	0.660
19C	50	3.77	0.005	651	0.702

* R = reflection; C = contraction; E = expansion.

Table 4 Simplex optimization of chemical and FI variables for demeclocycline

Experiment No.*	Coil length/cm	Flow rate/ cm^3 min^{-1}	[H_2SO_4]/ mol dm^{-3}	[Iron(III)] (ppm)	Peak absorbance
1	100	4.78	0.0500	100	0.312
2	400	4.78	0.0500	100	0.324
3	45	5.31	0.0500	100	0.252
4	45	3.90	0.0100	300	0.780
5	45	3.90	0.1000	600	0.570
6R	250	3.72	0.0550	450	0.546
7C	96	4.12	0.0310	288	0.570
8R	193	3.72	0.0460	544	0.714
9C	96	4.04	0.0290	311	0.654
10R	45	3.72	0.0350	649	0.774
11C	58	3.94	0.0270	337	0.720
12R	45	3.77	0.0520	487	0.660
13C	53	3.92	0.0260	345	0.738
14R	80	4.00	0.0010	50	0.264
15 Red	45	3.90	0.0550	450	0.546
16R	80	4.00	0.0010	196	0.63
17C	54	3.93	0.0160	312	0.72
18R	45	3.81	0.0100	335	0.87
20E	45	3.72	0.0010	447	1.098
21R	45	3.79	0.0010	314	1.080
22C	47	3.79	0.0070	336	0.954
23R	45	3.74	0.0060	352	0.984
24C	46	3.78	0.0060	340	0.972
25R	45	3.72	0.0010	318	1.080
26C	45	3.76	0.0040	339	0.954

* R = reflection; C = contraction; E = expansion; Red = reduction.

rate of formation of the complex are fast. The lower response values obtained when using longer reaction coil lengths could be attributed to dispersion.[13]

Analytical Appraisals

The FI method was run for each compound using series of drug standards. Typical FI results are shown in Fig. 3 for chlortetracycline in the range 40–300 ppm, indicating excellent reproducibility. Beer's law for the four compounds was found to be valid in the ranges 20–200, 40–300, 40–260 and 20–220 ppm for tetracycline, chlortetracycline, oxytetracycline and demeclocycline, with the calibration equations $A = -0.0249 + 0.0026c$ ($r = 0.999$), $A = -0.0160 + 0.0023c$ ($r = 0.999$), $A = 0.00245 + 0.0019c$ ($r = 1.00$) and $A = 0.00870 + 0.0027c$ ($r = 0.998$), respectively, where A is absorbance, c is the concentration of the tetracycline (mol dm^{-3}) and r is the correlation coefficient.

The peak width at 60% of the peak height was calculated as a measure of the total sample dispersion and was found to be 3, 2.4, 2.4 and 2.8 s for the above four compounds, respectively, thus indicating poor dispersion. In all instances the relative standard deviation was less than 0.9% ($n = 5$) and a sample throughput of at least 170 h^{-1} was attained, which is ten times the throughput attained by the previous method for oxytetracycline.[2]

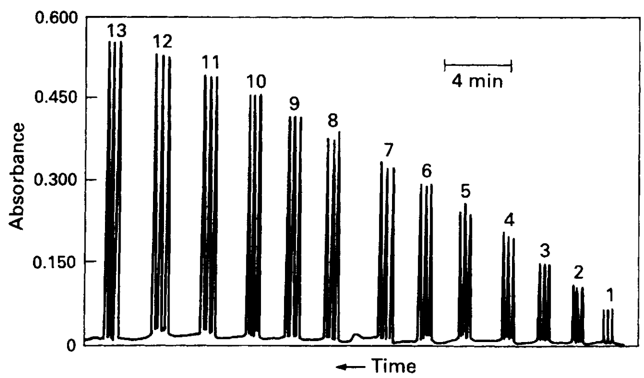


Fig. 3 Typical FI calibration graph for a series of standard chlortetracycline solutions of: 1, 40; 2, 60; 3, 80; 4, 100; 5, 120; 6, 140; 7, 160; 8, 180; 9, 200; 10, 220; 11, 260; 12, 280; and 13, 300 ppm

Application and Statistical Evaluation

The method was applied to the determination of tetracycline and its derivatives in proprietary drugs in both capsules and injection formulations (Table 5). The capsules are for human treatment and the injection form is for camels. The recorder tracings in Fig. 4 show good reproducibility with relative standard deviations within the standard calibration range.

The results, when compared with the spectrophotometric method,[1] indicated no interferences either from excipients added to capsules or from those added to syrups such as starch, glucose and sugar. Moreover, the presence of glucosamine hydrochloride in the proprietary drug Latycin and sodium metaphosphate in Hostacycline P capsules also showed no interference in the proposed method, whereas the presence of phosphate in the latter drug caused large interferences in the spectrophotometric method, which could be attributed to the fact that phosphate equilibrates in acidic media and buffers the solution, raising its pH and leading to higher absorbances and high results. This effect was minimal in the FI method as only micro-amounts are used. Finally, the Student's t-test values indicated high precision.

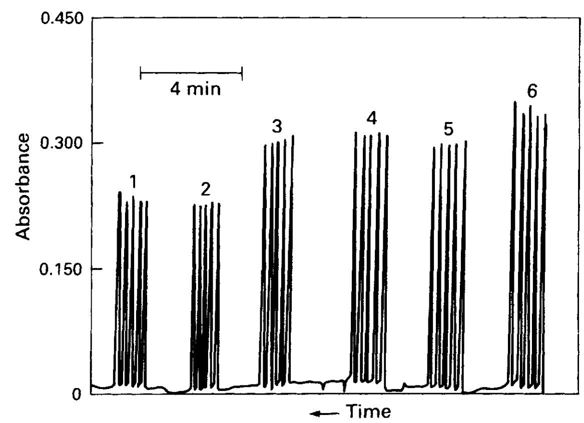


Fig. 4 Typical FI recorder tracings for: 1, Oxytetracycline syrup, 200 mg cm^{-3}; 2, Terramycin capsules, 250 mg; 3, Balkacycline capsules, 250 mg; 4, Latycin G capsules, 250 mg; 5, Hostacycline capsules, 500 mg; and 6, Dumocycline capsules, 250 mg

Table 5 Results of the analysis of pharmaceutical products containing tetracycline hydrochloride and oxytetracycline by the proposed method and the conventional spectrophotometric method

			Mean recovery ± SD (%)*		
Drug	Supplier	Stated contents	FI method	Conventional spectrophotometric method†	t‡
Dumocycline capsules	Dumex, Denmark	Tetracycline HCl 250 mg	99.72 ± 0.42	101.88 ± 0.46	1.15
Hostacycline P capsules	Hoechst, Germany	Tetracycline HCl 500 mg, sodium hexametaphosphate 220 mg	102.31 ± 0.65	122.88 ± 0.55	—
Latycin capsules	Biochemie, Austria	Tetracycline HCl 250 mg, glucosamine HCl 250 mg	104.23 ± 0.46	98.78 ± 0.73	1.71
Balkacycline capsules	Arab Pharmaceutical Manufacturing, Jordan	Tetracycline HCl 250 mg	101.79 ± 0.58	103.19 ± 0.46	1.63
Terramycin capsules	Pfizer, Belgium	Oxytetracycline 250 mg	99.62 ± 0.87	103.72 ± 0.62	1.99
Oxytetracycline 20% L.A. injection	Dopharma, The Netherlands	Oxytetracycline 200 mg cm^{-3}	99.21 ± 0.63	102.19 ± 0.56	1.23

* $n = 5$.
† Ref. 1.
‡ t theoretical = 2.31, $n = 5$.

Conclusion

The proposed method is as accurate as other reported methods but is more suitable for the assay of tetracyclines in drug formulations as it does not suffer interferences from excipients and other common additives in such compounds. Being an FI method, it has the distinct advantage of providing high sample throughputs and shorter times are required for analysis.

The chemical system permitted the successful application of the modified simplex method of optimization, the maximum response with optimum operating conditions being reached faster and with a smaller number of experiments than with the iterative univariate method.

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