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Use of experimental design to optimise a flow injection analysis assay for L-*N*-monomethylarginine

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Abstract

A flow injection analysis (FIA) method to determine L-*N*-monomethylarginine, based on the reaction with *ortho*-phthalaldehyde in the presence of a suitable thiol-group, was optimised using experimental design. Two different approaches were followed wherein, (i) critical factors were identified in a screening design, and (ii) the simplex algorithm was used for further optimisation. In the first approach, the chemical reaction was optimised off-line and the optimal chemical conditions were transferred to the FIA-system. In the second approach the reaction and the FIA-system parameters were optimised together. The on-line approach is preferred.

Keywords: Flow injection analysis; Fractional factorial design; L-*N*-monomethylarginine

1. Introduction

L-*N*-monomethylarginine is a modified amino-acid which is being developed for the treatment of septic shock. The drug is a competitive inhibitor of nitric oxide synthase isoenzymes which catalyse the formation of nitric oxide from L-arginine. High concentrations of nitric oxide, found in patients with septic shock, seem to be responsible for vasodilatation, organ dysfunction and eventually death [1]. Inhibiting the formation of nitric oxide by administering L-*N*-monomethylarginine, can prevent the vasodilatation and tissue damage. L-*N*-monomethylarginine is currently administered as a solution containing no other excipients. To determine the content of the drug in a pharmaceutical quality assurance laboratory, an analytical technique is required which is rapid, sensitive, reproducible and which can be automated for routine analysis. Flow injection analysis (FIA) fulfils these requirements [2,3]. In this FIA application a small volume of sample is injected into a flowing stream, which contains reagent. Reaction between the sample and the reagent takes place and the reaction product is measured downstream. In fact two processes occur simultaneously, namely the chemical reaction, and the dispersion of both the injected substance and the

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reaction product along the flowing stream [3]. It is necessary to find a compromise between both processes so that on the one hand there is time for a sufficient formation of the reaction product, while on the other excessive dispersion of the reaction product, leading to smaller peaks, is avoided. In other words, a balance between sensitivity and rapidity has to be established.

An univariate strategy, in which one factor at a time is optimised, is most frequently applied to find the optimal operational conditions for which the responses of the method are appropriate. The peak height, which is proportional to the sample concentration, should be maximal while the residence time, the time from injection to detection, is considered appropriate within a given interval selected based on a compromise between reaction and dispersion. Often the chemical reaction is not completed at the moment the reaction product is measured. The univariate strategy is time consuming because of the large number of experiments to be performed and does not take into account interaction effects that can occur [4,5]. To counteract these disadvantages, an experimental design

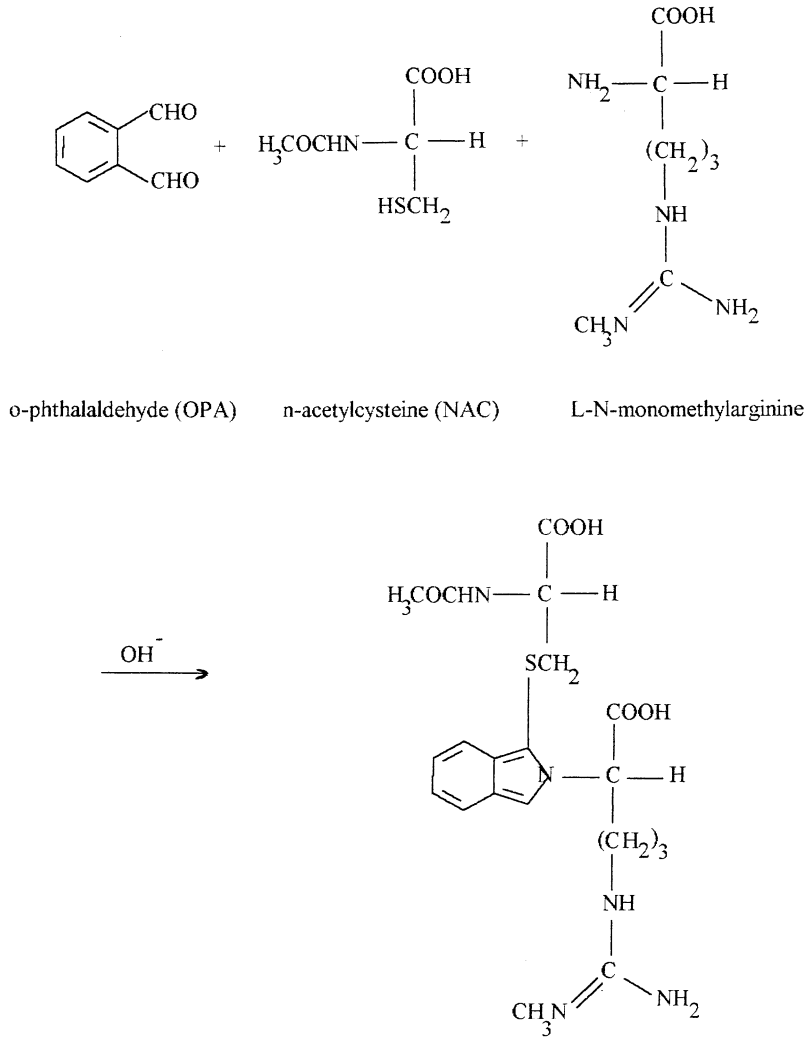

Fig. 1. Chemical reaction performed in the assay of L-*N*-monomethylarginine.

Table 1
2^{5-1} design for the optimisation off-line[a]

Experiment	Factors					Response absorbance
	Concentration OPA (= A)	Concentration NAC (= B)	pH buffer (= C)	Ionic strength (= D)	Time (= E)	
1	−1	−1	−1	−1	−1	0.262
2	1	−1	−1	1	−1	0.343
3	−1	1	−1	1	−1	0.206
4	1	1	−1	−1	−1	0.353
5	−1	−1	1	1	−1	0.111
6	1	−1	1	−1	−1	0.319
7	−1	1	1	−1	−1	0.201
8	1	1	1	1	−1	0.343
9	−1	−1	−1	−1	1	0.305
10	1	−1	−1	1	1	0.343
11	−1	1	−1	1	1	0.252
12	1	1	−1	−1	1	0.353
13	−1	−1	1	1	1	0.158
14	1	−1	1	−1	1	0.334
15	−1	1	1	−1	1	0.246
16	1	1	1	1	1	0.343

[a] Generator D = ABC.

approach can be used. Only a few examples of the use of experimental design in FIA have been published. In these cases the simplex algorithm [4] is mostly used [6–8].

Factorial designs, where several variables (factors) are varied at the same time according to systematic multivariate optimisation schemes, can also be applied. Duarte et al. [9] used a factorial design for the optimisation of an FIA-configuration. Janse et al. [10] employed factorial designs for the optimisation of the FIA determination of phosphate. Previously we used factorial designs to screen variables in the optimisation of a glycine assay [11].

The aim of the present study was to optimise an FIA-method for L-*N*-monomethylarginine, using a factorial design to screen possible variables, and simplex to optimise the variables found to be important in the screening. Two different strategies were examined: an optimisation of the chemical reaction off-line and transferring the optimal chemical conditions to the FIA-system, and an optimisation of the reaction and the FIA-system parameters at the same time (on-line). We wanted to compare the two approaches to decide which one is more convenient.

The chemical reaction to determine L-*N*-monomethylarginine was based on the reaction of its primary amine function with *ortho*-phthalaldehyde (OPA) and a suitable thiol-group at alkaline pH [12]. Mercaptoethanol is often used as provider of the thiol-group in reactions with

Table 2
Levels of the factors examined in the screening designs

Factors	(−1) level	(+1) level
Chemical reaction		
Concentration OPA (= A)	15 mg%	200 mg%
Concentration NAC (= B)	15 mg%	150 mg%
pH buffer solution (= C)	9.3	11.7
Ionic strength buffer (= D)	0.05	0.15
Time (= E)	15 s	23 s
FIA-system		
Flow rate (= F)	0.65 ml/min	1.20 ml/min

Table 3
Effects of the factors on the absorbance in the off-line optimisation using a 2^{5-1} design

Factors	Effects	Normalised effects (%)
Concentration OPA (A)+BCD	0.124	44.25
Concentration NAC (B)+ACD	0.015	5.47
pH buffer (C)+ABD	−0.045	−16.20
Ionic strength buffer (D)+ABC	−0.034	−12.27
Time (E)	0.024	8.76
Interactions		
AB+CD	−0.002	−0.71
AC+BD	0.032	11.45
AD+BC	0.038	13.41
AE	−0.021	−7.44
BE	−0.002	−0.61
CE	0.002	0.82
DE	−0.001	−0.43
Lenth		
ME	0.035	12.27
SME	0.070	24.54

OPA. However since mercaptoethanol is smelly and toxic, and the method has to be applied in routine analysis, mercaptoethanol was replaced by *N*-acetylcysteine (NAC). The reaction product is measured spectrophotometrically.

2. Experimental

2.1. Reagents and solutions

L-*N*-monomethylarginine was obtained from Glaxo–Wellcome (Dartford, UK). Sample solutions with a concentration of 100 μg/ml were prepared from a stock solution of 1 mg/ml L-*N*-monomethylarginine in MilliQ water (MilliQ water purification system, Millipore, Bedford, MA). The sample solution was injected in the FIA manifold. For the off-line reaction optimisation, 400 μl sample solution was added to 3 ml reagent solution in a quartz cell with a path length of 1 cm, which was stirred for 5 s.

The FIA carrier stream consisted of *N*-acetylcysteine (Sigma, Steinheim, Germany) which was dissolved in alkaline buffer. The alkaline buffer was prepared with boric acid (Merck, Darmstadt, Germany) dissolved in water and adjusted to pH with a 2 M NaOH solution. The NaOH pellets used to prepare the 2 M solution were obtained from Merck. *ortho*-Phthalaldehyde 97% (Sigma, Steinheim, Germany), dissolved in 25 ml of methanol (BDH, Poole, England), was added to the NAC-buffer solution and the volume was adjusted to 500 ml with the alkaline buffer. The carrier solution was filtered through a membrane with a pore size of 0.2 μm and was sonicated to release possible air bubbles. The pH and the ionic strength of the buffer, the concentrations of NAC and of OPA, used in the different experiments, varied according to the experimental design requirements.

2.2. Equipment

The flow injection analysis was performed on a Burkard (Burkard Scientific, Uxbridge, UK) FIA-flo flow injection system equipped with PTFE six-port valves. PTFE tubing (0.5 mm ID) was used for all connections. The injection volume was 15 μl.

A Merck–Hitachi L-4200 variable wavelength UV–Vis Detector, equipped with a 5-mm flow-cell, was applied to monitor the reaction product. The detection wavelength was 336 nm. Peak heights were measured with a Merck–Hitachi D-7500 integrator.

For the off-line reaction optimisation, the absorption of the derivative was monitored using a Perkin Elmer Lambda 20UV/Vis Spectrophotometer (Norwalk, CT, USA).

2.3. Theory

The effects of the factors, examined in fractional factorial designs, on the response (peak height) were calculated as $E_x = (\Sigma Y(+))/n - (\Sigma Y(-))/n$ where $\Sigma Y(+)$ and $\Sigma Y(-)$ are the

sums of the responses where factor x is at its high ($+1$) and at its low (-1) level respectively and n is the number of times each factor is at the ($+1$) or (-1) level [13]. Normalised effects were calculated as $\%E_x = (E_x/\bar{Y})100$ where $\bar{Y}$ is the average response of the design experiments [14]. Normalised effects from both strategies can be compared since they are dimensionless and indicate the percent change a factor has on the response. Statistical significance of the effects was checked by applying both a t-test [14], and a non parametric method, published by Lenth [15], where a margin of error (ME) and simultaneous margin of error (SME)-value are calculated. Normal probability plots [14] were also drawn to study graphically the significance of effects.

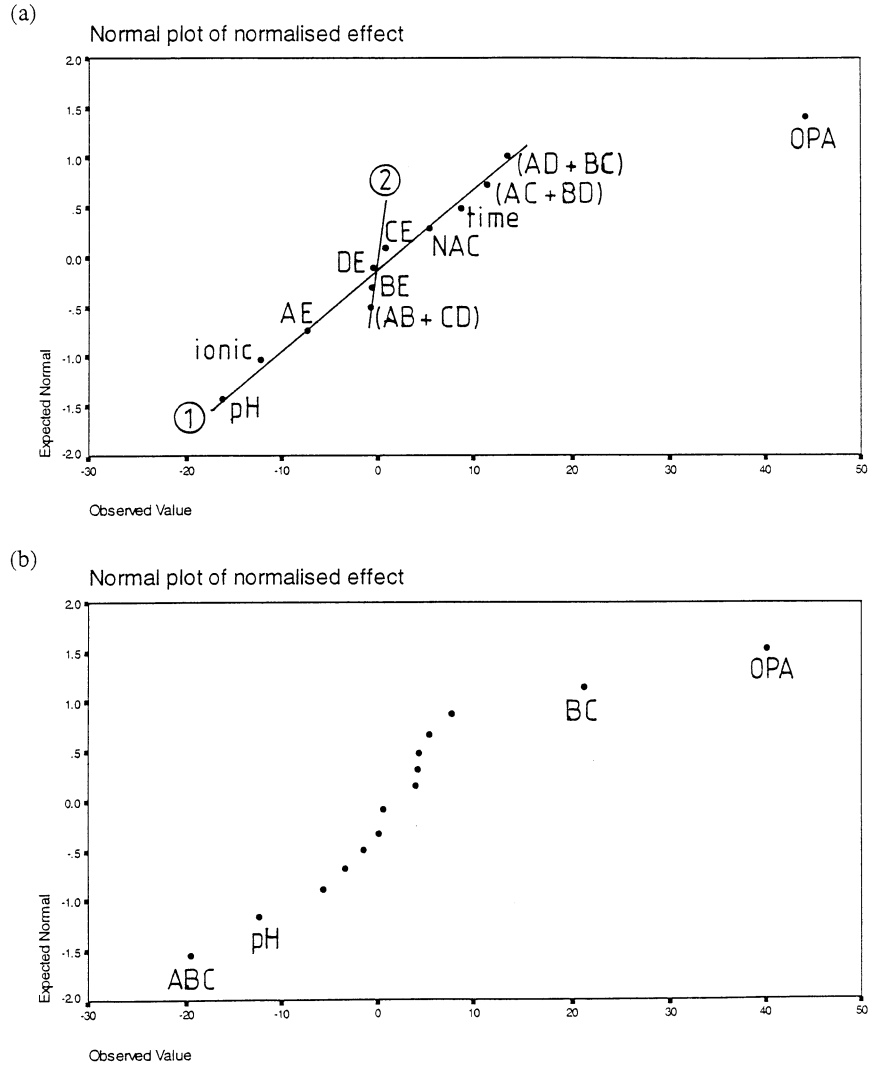

Fig. 2. Normal probability plot of the effects on the absorbance in the off-line optimisation using (a) a 2^{5-1} design and (b) a 2^4 design.

Table 4
2^4 full factorial design for the optimisation off-line

Experiment	Factors				Absorbance 20 s
	Concentration OPA (= A)	Concentration NAC (= B)	pH buffer (= C)	Ionic strength (= D)	
1	−1	−1	−1	−1	0.302
2	1	−1	−1	−1	0.348
3	−1	1	−1	−1	0.186
4	1	1	−1	−1	0.359
5	−1	−1	1	−1	0.142
6	1	−1	1	−1	0.335
7	−1	1	1	−1	0.228
8	1	1	1	−1	0.341
9	−1	−1	−1	1	0.319
10	1	−1	−1	1	0.343
11	−1	1	−1	1	0.223
12	1	1	−1	1	0.351
13	−1	−1	1	1	0.131
14	1	−1	1	1	0.320
15	−1	1	1	1	0.299
16	1	1	1	1	0.353

3. Results and discussion

3.1. Optimisation off-line

In a first approach the chemical reaction (Fig. 1) was optimised off-line. The response considered was the optical absorbance of the reaction product at 336 nm. A half-fraction factorial design for five factors at two levels (2^{5-1}-resolution IV) was executed to identify important factors (Table 1). The factors examined were the concentrations of OPA (A) and NAC (B) in the reagent, the pH (C) and the ionic strength (D) of the buffer. Reaction time was included as a fifth factor (E) in the design to study the time dependence of the reaction. For this reason the absorbance was measured at 15 and 23 s, which is the time interval considered acceptable in the on-line application. For each factor an upper (+1) and a lower (−1) level were selected based on literature data [16] (Table 2). Effects on the absorbance were calculated for each factor (Table 3). It was observed that the concentration of OPA (+ interaction NAC–pH–ionic strength), the pH of the buffer (+ interaction OPA–NAC–ionic strength), the ionic strength (+ interaction OPA–NAC–pH), the interaction terms OPA–pH + NAC–ionic strength (AC + BD) and OPA–ionic strength + NAC–pH (AD + BC) have larger values than the rest of the estimated effects. Statistical significance was determined by calculating a critical effect from the t-test in which the two-factor interactions are used to estimate the experimental error. It should be noted that the critical effect from this t-test could depend on occasional important two-factor interactions. Only the two-factor interactions, which on the basis of the normal probability plot are not considered as potentially significant, were used in the estimation of the experimental error. Plotting the effects in a normal probability plot is also used to allow visual confirmation of the statistical test results. Calculating the critical effect, based on the four effects (CE, DE, BE and AB + CD) which form a straight line through zero in Fig. 2a, line 2, results in significance of all the other effects, even the concentration of NAC. The critical effect is probably underestimated in this way. When doing the same but including now also the interaction AE (Fig. 2a, line 1) in the calculation of the critical effect, the concentration of OPA and the pH were found to be significant at $\alpha = 0.01$ and

the ionic strength and the interactions (AC + BD) and (AD + BC) at $\alpha = 0.05$. Excluding none of the two-factor interactions in the calculation of the critical effect indicates the concentration of OPA to have a significant effect. Lenth's method [15] also indicates the concentration of OPA to be important. The effects of the pH and of the confounded two-factor interaction terms OPA–ionic strength + NAC–pH (AD + BC) are situated between (ME) and (SME) which means that it should be verified whether the effects are 'active' or whether they are a consequence of inactive effects [15]. It should be noted that the ME and SME from Lenth's method depend very much on the value of the median as was shown in [11]. Therefore, interpretation of significance should be done with care because depending on the method to determine the significance levels used, the experimental error will be over- or underestimated and effects will be indicated sometimes as significant and sometimes as not. Several methods for determining the significance should be compared before conclusions are drawn.

Table 5
Effects of the factors on the absorbance in the off-line optimisation using a 2^4 design

Factors	Effects	Normalised effects (%)
Concentration OPA (A)	0.115	40.14
Concentration NAC (B)	0.012	4.32
pH buffer (C)	−0.035	−12.36
Ionic strength (D)	0.012	4.24
Interactions		
OPA–NAC (= AB)	0.002	0.65
OPA–pH (= AC)	0.022	7.73
OPA–ionic strength (= AD)	−0.016	−5.72
NAC–pH (= BC)	0.060	21.18
NAC–ionic strength (= BD)	0.015	5.46
pH–ionic strength (= CD)	0.002	0.65
OPA–NAC–pH (= ABC)	−0.056	−19.52
OPA–NAC–ionic strength (= ABD)	−0.001	−3.45
NAC–pH–ionic strength (= BCD)	0.000	0.13
OPA–pH–ionic strength (= ACD)	0.011	3.97
OPA–NAC–pH–ionic strength (= ABCD)	−0.004	−1.44
Critical effect $\alpha = 0.05$	0.026	9.32
Critical effect $\alpha = 0.01$	0.038	13.39

The time-interval (factor E) is found not to have an important effect on the response.

Looking more carefully at Table 1, one can notice that the absorbencies measured for experiments 5 and 13, which have the same experimental conditions, but measured at different time-intervals since all the factors are at the same levels except the factor time, are very low compared to those of all other experiments. These low absorbencies can be due to a specific factor combination, which has a negative influence on the reaction. To draw straightforward conclusions about this, the half-fraction factorial design (resolution IV) does not deliver enough information about these observations because of the confounding pattern (main effects with three-factor interactions and two factor interactions with other two-factor interactions). To check which factors or interactions indeed have an effect and if the design of Table 1 was appropriate to decide on significancies, a full factorial design for four factors at two levels was executed (Table 4). The factor reaction time was not further considered since it was never important, neither as a main effect nor in the interaction effects. The effects on the absorbance, measured at 20 s, i.e. with a constant reaction time, were calculated (Table 5). After drawing the normal probability plot, the two-factor interaction NAC–pH (BC) and the three-factor interaction OPA–NAC–pH (ABC) were excluded in the calculation of the critical effect because they deviate from the normal distribution (Fig. 2b). The concentration of OPA, the pH, the interactions NAC–pH and OPA–NAC–pH can be selected as important from Table 5. The low responses found for experiments 5 and 13 in Table 1 become also clearer now. These experiments were executed with the concentrations of OPA and NAC at their low levels and the pH at high level. Table 5 indicates the effect of the concentration of OPA as positive, which means that the response is lower when the concentration is low. The same reasoning can be followed for the concentration of NAC and for the pH. In

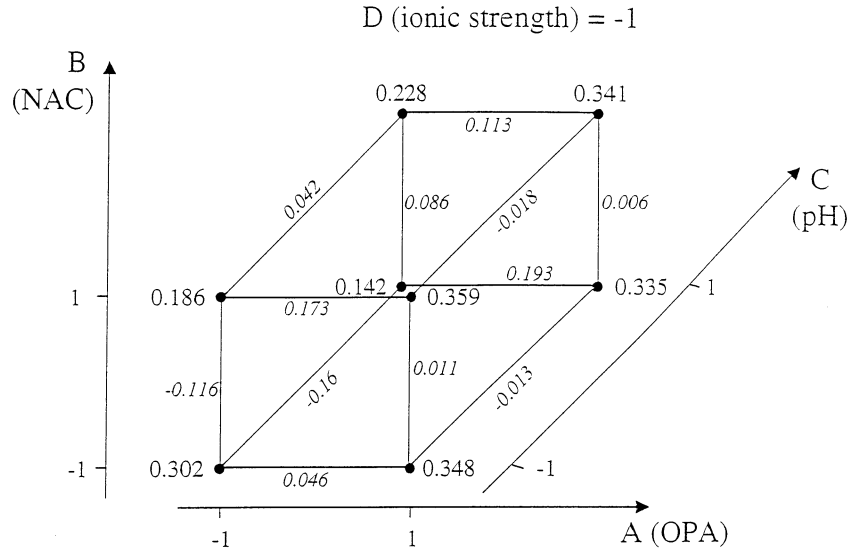

Fig. 3. Visual plot of responses and effects of the 2^4 full factorial design (off-line) with the ionic strength at low level.

Table 6
Optimisation of OPA concentration off-line and on-line using the uniplex approach [11,17]

Experiment number	Simplex	Concentration OPA (mg%)	Off-line absorbance	On-line peak height
1		150	0.352	227839
2		250	0.345	221528
3	Reflection	50	0.339	194102
4	Contraction	200	0.350	224494
5	Reflection	100	0.348	223123

Table 7
2^4 design with OPA at optimal concentrations

Experiment	Factors				Absorbance
	Concentration NAC	pH of the buffer	Ionic strength	Time	
1	−1	−1	−1	−1	0.350
2	1	−1	−1	−1	0.353
3	−1	1	−1	−1	0.305
4	1	1	−1	−1	0.350
5	−1	−1	1	−1	0.349
6	1	−1	1	−1	0.348
7	−1	1	1	−1	0.296
8	1	1	1	−1	0.349
9	−1	−1	−1	1	0.350
10	1	−1	−1	1	0.353
11	−1	1	−1	1	0.335
12	1	1	−1	1	0.351
13	−1	−1	1	1	0.349
14	1	−1	1	1	0.350
15	−1	1	1	1	0.330
16	1	1	1	1	0.349

experiments 5 and 13 there is such a combination of the factor levels that can explain the low responses found. When comparing Tables 3 and 5, the effects of the factors found in Table 3 are confirmed by those of Table 5. The effect of the concentration of OPA is comparable in both tables. The same can be observed for the concentration of NAC and for the pH of the buffer. However, the conclusion for the ionic strength differs: from the half-fraction factorial design it would be assumed important while the full factorial design indicates that not the ionic strength, but the three-factor interaction OPA–NAC–pH is. The effect of OPA–pH + NAC–ionic strength (AC + BD) in Table 3 was due to the combination of two intermediate effects (Table 5), while the effect of OPA–ionic strength + NAC–pH (AD + BC) (Table 3) is due to NAC–pH (BC) (Table 5). The interpretation of a half-fraction factorial design in optimisation should thus be done with care.

Table 8
Effects of the factors on the absorbance when OPA is at optimal concentrations

Factors	Effects	Normalised effects (%)
Concentration NAC	0.017	5.08
pH buffer	−0.017	−5.01
Ionic strength	−0.003	−0.99
Time	0.008	2.45
Interactions		
NAC–pH	0.016	4.64
NAC–ionic strength	0.006	0.18
NAC–time	−0.008	−2.23
pH–ionic strength	−0.001	−0.25
pH–time	0.008	2.30
Ionic strength–time	0.001	0.18
NAC–pH–ionic strength	0.002	0.62
NAC–pH–time	−0.008	−2.38
NAC–ionic strength–time	−0.000	−0.11
pH–ionic strength–time	0.000	0.04
NAC–pH–ionic strength–time	−0.009	−0.25

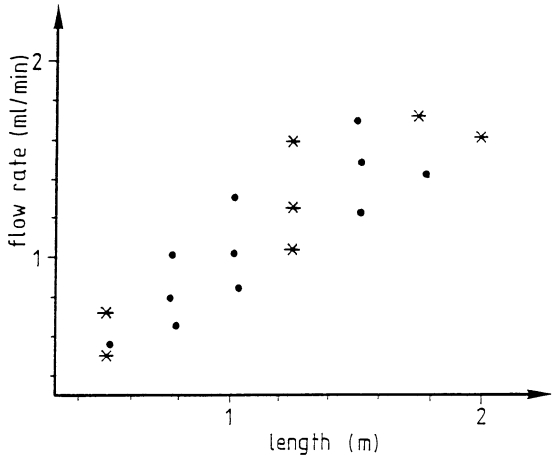

Fig. 4. Region where the residence time is appropriate, selected using Eq. (1). Experiments indicated with (*) were selected with D-optimality.

A chemical explanation for the two-factor interaction NAC–pH could be the following. The reaction of OPA with a thiol and a primary amine forms an isoindol. A possible reaction mechanism is shown in ref. [17]. First the thiol group of NAC binds to OPA, followed by the primary amine and finally, a second ring in the structure is formed. The degree of ionisation of NAC and of L-*N*-monomethylarginine depends on the pH and so is the speed of formation of the isoindol five ring. Moreover the amount of NAC used can influence the reaction rate since the more thiol is available, the quicker the reaction performs. There is an interaction between NAC and the pH. The three-factor interaction OPA–NAC–pH indicates that the amount of ionised NAC that reacts with OPA depends on the amount of the latter. This can be visualised by looking at Fig. 3. At low OPA concentrations, the effect of changing the concentration of NAC from high to low level with the pH at low level is −0.116 while with the pH at high level 0.086. There is an interaction between the concentration of NAC and the pH. Moreover, at high OPA concentrations, a different pattern is observed: the effect of changing the concentration of NAC from high to low level with the pH at low level is 0.011 while with the pH at high level the effect is 0.006. A clear two-factor interaction NAC–pH is found when OPA was at (−1) level while this was not the case for OPA at (+1) level: there is a three-factor interaction.

Since the concentration of OPA is found to be the dominant factor, it was further optimised with

Table 9
Experiments performed to model the peak height in the selected region

Length reaction coil (m)	Flow rate (ml/min)	Peak height	Residence time (s)
2	1.61	115000	22
0.5	0.5	232887	23
1.25	1.61	183395	14
1.25	1	167872	21
0.5	0.75	290951	16
1.25	1.25	171195	19
1.75	1.72	146329	18

the uniplex method [18], which was also used in the optimisation of an FIA-method for glycine [11]. The optimisation of the OPA concentration was carried out at the following levels for the other factors: 150 mg% NAC, pH of the buffer solution 9.3 and ionic strength 0.05. The screening showed that the high level of the concentration of OPA leads to a higher absorbance. The starting points for the uniplex were selected as 150 and 250 mg%. The absorbance is measured after 20 s. Table 6 shows the path that was followed. The optimisation was stopped after five experiments and the optimal concentration OPA was found to be 150 mg% (highest absorbance). Moreover it is situated in a rather rugged region because the response remains constant for deviating OPA concentrations (100–200 mg%). With OPA at optimal concentrations, it was then checked if the absorbance is robust when changing the concentration of NAC (B) in the reagent, the pH (C) and the ionic strength (D) of the buffer and the time (E), from low level to high level as shown in Table 2. This can be considered as a ruggedness test with extreme broad factor-level intervals. A 2^4 design was performed (Table 7) and the effects were calculated (Table 8). The effects of the concentration of NAC, the pH of the buffer and the interaction NAC–pH are of the same magnitude. Calculation of the effects on the absorbance from the full factorial design (2^3), created from Table 4 with the experiments where OPA is at its high level, confirms the results of Table 8. When selecting from Table 7 experiments 3 and 7 (at 15 s) and 11 and 15 (at 23 s), which have the worst factor combination (as was explained earlier), the absorbances now obtained are still lower but are more comparable to these of the other design experiments, which confirms the limited influence of the concentration of NAC, the pH and the ionic strength of the buffer on the response. In practice one will work with the optimal OPA concentration and for the other factors, the levels combination which resulted in the highest response measured, being the concentration of NAC at high level and the pH and the ionic strength of the buffer at low level. This combination performs well and even if the levels are changed to the factor levels combination giving the lowest response for an optimal OPA concentration, the measured response is affected only relatively slightly. The concentration of NAC, the pH and the ionic strength of the buffer were therefore not optimised further.

After the optimal off-line reaction conditions were established, the FIA-system is considered. A

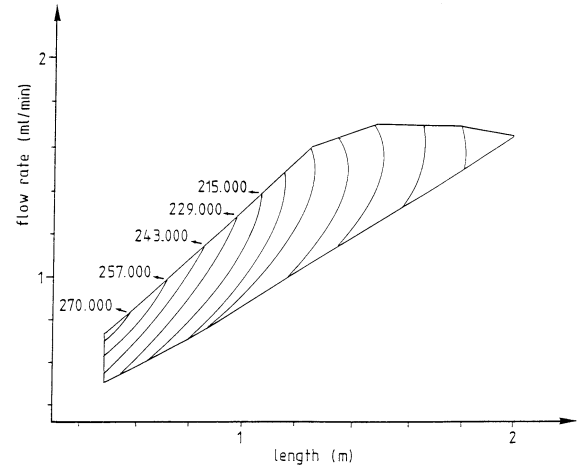

Fig. 5. Contour plot for the peak height in the selected region.

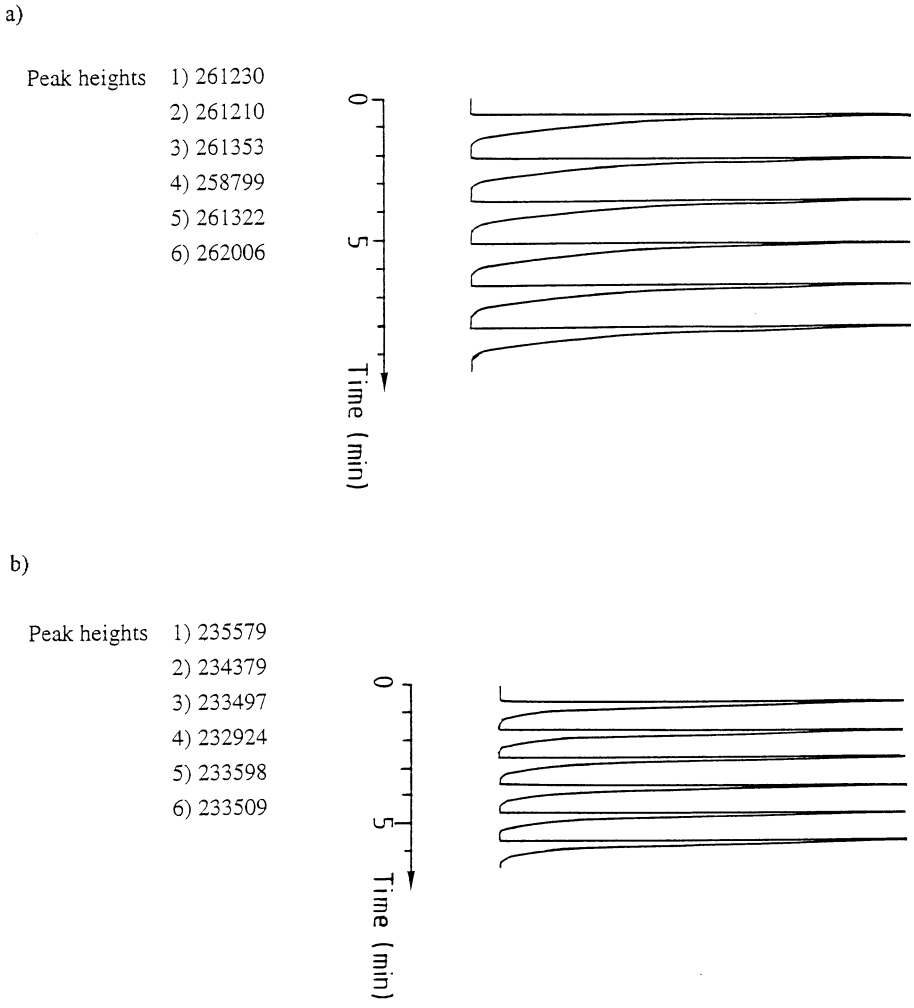

Fig. 6. FIA-traces found with: (a) 150 mg% OPA, 150 mg% NAC, a buffer solution pH 9.3 with ionic strength 0.05, a reaction coil 0.5 m, a flow rate 0.6 ml/min and (b) the optimal conditions being the concentration OPA 150 mg%, concentration NAC 150 mg%, pH buffer solution 9.3, ionic strength buffer 0.05, flow rate 0.9 ml/min, length reaction coil 0.75 m. 15 μl of a 100 μg/ml solution of L-*N*-monomethylarginine was injected.

one-stream FIA-configuration was used. Parameters to be adjusted are the length and the internal diameter of the reaction coil, the flow rate of the reagent stream and the injection volume of the sample. An injection volume of 15 μl was selected. Two internal diameters were tested, namely 0.5 and 0.8 mm. The latter gives, as expected, lower but broader peaks. An internal diameter of 0.5 mm was therefore chosen for further experiments. The length of the reaction coil and the flow rate could be varied between 0.5 and 2 m, and between 0.5 and 2 ml/min respectively. An equation to predict the residence time, as a function of the length of the tubing and the flow rate, was empirically derived based on the knowledge that the residence time is proportional to the length of the reaction coil and inversely proportional to the flow rate ($Tq/l = \text{const}$) [2]. This means that

$$T_2 = \frac{T_1 q_1 (l_2 + 0.1)}{(l_1 + 0.1) q_2} \quad (1)$$

with l_1 being a length of tubing (m), q_1 a flow rate

(ml/min), which produce a measured residence time T_1, and with l_2 a new length of tubing, q_2 a new flow rate, which are responsible for a new residence time T_2. In our system we also had to take into account the dimensions of the inlet tubing of the detector, which are constant in all experiments, namely a length of 40 cm and an internal diameter of 0.25 mm. This is equivalent to an additional 0.1 m length of 0.5 mm ID tubing.

Since we are only interested in a residence time between 15 and 23 s, equation (1) is used to select the appropriate region (Fig. 4). The upper limit of the flow rate (2 ml/min) could not be used in practice due to back-pressure at the detector. A flow rate of 1.7 ml/min was the maximal value that could be applied without problems. Experiments can now be performed in the selected area to evaluate how the peak height changes as a function of the length and of the flow rate. To minimise the number of experiments, the D-optimal algorithm [19] was used to select seven experiments in this irregular domain (Fig. 4, Table 9). A multiple linear regression model for the peak height (y) was built:

$$y = 264770 - 366820x_1 + 324040x_2 + 118400x_1x_2 + 27180x_1^2 - 163100x_2^2$$

with x_1 the length of the reaction coil and x_2 the flow rate. The contour plot (Fig. 5) shows that the maximal peak height is to be expected with a short reaction coil and a relative high flow rate. A length of tubing of 0.5 m was however not selected because although a high peak is obtained, the peak width at the baseline was too broad due to the low flow rate (0.6 ml/min) needed to have a residence time of 20 s (Fig. 6a). A length of 0.75 m and a flow rate of 0.9 ml/min gave a residence time within 20 s, although the peak height then is slightly smaller (Fig. 6b).

Optimal conditions from the off-line optimisation were thus found as a concentration of 150 mg% OPA, 150 mg% NAC, a buffer of pH 9.3 with an ionic strength of 0.05, a reaction coil of 0.75 m and a flow rate of 0.9 ml/min.

Table 10
2^{5-1} (V) design for the on-line optimisation[a]

Experiments	Factors					Response Peak height
	Concentration OPA (=A)	Concentration NAC (=B)	pH buffer (=C)	Ionic strength buffer (=D)	Flow rate (=F)	
1	−1	−1	−1	1	−1	131082
2	1	−1	−1	−1	−1	199563
3	−1	1	−1	−1	−1	87177
4	1	1	−1	1	−1	154339
5	−1	−1	1	−1	−1	58559
6	1	−1	1	1	−1	133911
7	−1	1	1	1	−1	119453
8	1	1	1	−1	−1	207518
9	−1	−1	−1	−1	1	122522
10	1	−1	−1	1	1	194449
11	−1	1	−1	1	1	85455
12	1	1	−1	−1	1	233579
13	−1	−1	1	1	1	43423
14	1	−1	1	−1	1	175431
15	−1	1	1	−1	1	90577
16	1	1	1	1	1	204102

[a] Generator: D = ABCF.

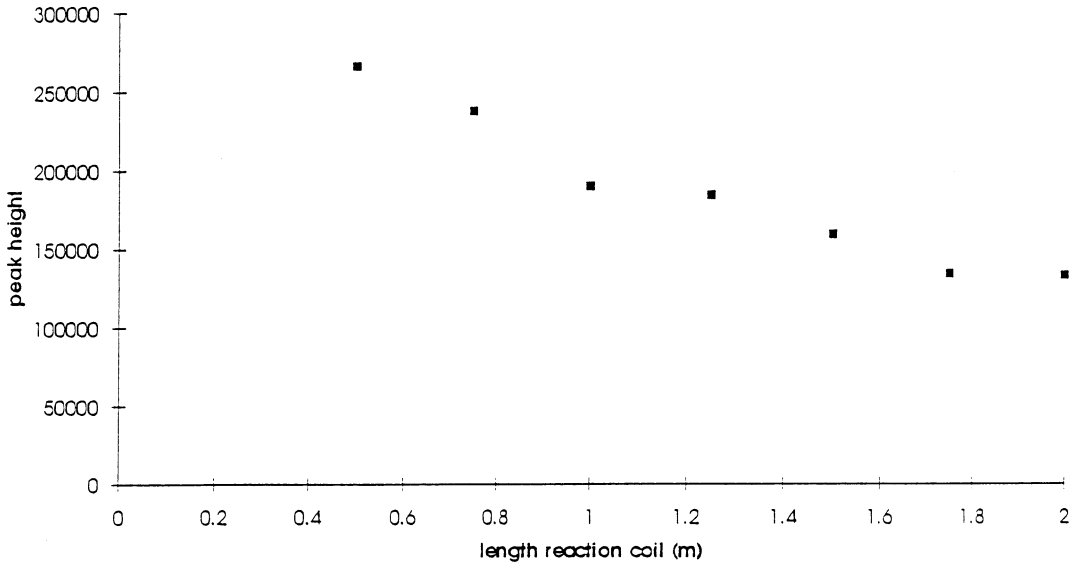

Fig. 7. Influence of the length of the reaction coil on the peak height. Flow rate, 1.25 ml/min. Carrier stream: OPA 107.5 mg/v%, NAC 82.5 mg/v%, pH buffer 10.5, ionic strength buffer 0.1.

3.2. Optimisation on line

In a second approach the factors of the chemical reaction and those of the FIA-instrument were optimised simultaneously. The results were compared with those obtained off-line. Again, first a screening is applied, followed by an optimisation of the critical factors. The response to be optimised now is the peak height.

The factors considered for screening were the chemical reaction factors, namely the concentrations of OPA (A) and of NAC (B) in the reagent, the pH (C) and the ionic strength (D) of the buffer solution, and the system factors, flow rate and length of the reaction coil. However, since both the flow rate and the length of the reaction coil determine the residence time [2] as shown in Eq. (1), it is unwise to define them both as factors in the same screening design because if both are varied at the same time, a residence time will often be obtained which is not situated between 15 and 23 s. By keeping one of both factors constant and varying the other one, an appropriate residence time can be obtained. Therefore, the length of the reaction coil was held constant in the screening while the flow rate was included as a factor (F) (Table 10). To determine which length of reaction coil was to be used in the screening experiments, the length was varied between the limits 0.5 and 2 m in a preliminary experiment. A carrier stream consisting of 1.075 g/l OPA, 0.825 g/l NAC, ionic strength 0.1 and pH 10.5 (which were the original nominal levels of the factors from which the extreme levels in the screening were derived, Table 2), was pumped through the system with a flow rate of 1.25 ml/min. The peak height and the residence time were measured for lengths of reaction coils between the above mentioned limits. These experiments showed that a shorter reaction coil gives higher peaks (Fig. 7). A length of reaction coil of 0.75 m was chosen in further experiments because the repeatability for this length of tubing was better than for a length of 0.5 m.

A 2^{5-1} (resolution V) design was selected to execute the screening (Table 10). The same levels for the factors were used as in the optimisation of the chemical reaction off-line (Table 2). For the

flow rate, the levels were chosen based on the length of reaction coil (0.75 m) in order to give a residence time of 15 and 23 s.

Effects of the factors on the peak height were calculated (Table 11). The concentration of OPA is significant and the interaction term NAC–pH probably is too, which is confirmed by the normal probability plot (Fig. 8). The flow rate had been expected to be more important than it was found to be.

The normalised effects (Table 11) were compared with the ones calculated for the chemical reaction off-line (Table 3). The effect of the pH and the ionic strength of the buffer are similar in both designs. The effect of the flow rate on-line is comparable to the effect of the time factor off-line. The concentration of OPA is in both designs by far the most important factor.

Table 11
Effects of the factors on the peak height in the on-line optimisation

Factors	Effects	Normalised effects (%)
Concentration OPA (A)+BCDF	95580	68.24
Concentration NAC (B)+ACDF	15410	11.00
pH buffer (C)+ABDF	−21900	−15.63
Ionic strength buffer (D)+ABCF	−13590	−9.70
Flow rate (F)+ABCD	7242	5.17
Interactions		
OPA–NAC+CDF	8639	6.17
OPA–pH+BDF	6657	4.75
OPA–ionic strength+BCF	−18730	−13.37
OPA–flow rate+BCD	20820	14.86
NAC–pH+ADF	37170	26.53
NAC–ionic strength+ACF	−286	−0.20
NAC–flow rate+ACD	4065	2.90
pH–ionic strength+ABF	5790	4.13
pH–flow rate+ABD	−8719	−6.22
Ionic strength–flow rate+ABC	−10080	−7.20
Critical effect $\alpha = 0.05$	35320	25.21
Critical effect $\alpha = 0.01$	50230	35.86
Length		
ME	24156	17.25
SME	49065	35.03

Although the interaction term NAC–pH is on the limit of significance, in analogy with the off-line optimisation, the factors NAC and pH were not included in the further optimisation procedure, because their effect is much smaller than that of the concentration of OPA. The concentration of OPA was again optimised using the uniplex procedure, but now on-line (Table 6). The optimisation was executed at a concentration of 150 mg% NAC, a buffer pH 9.3 with ionic strength 0.05, a length of tubing of 0.75 m and a flow rate of 0.9 ml/min. A concentration of 150 mg% OPA gave the highest peaks. The sample was injected six times under these conditions to determine the repeatability. A % RSD of 0.36% was found which is far below the 1% limit. At the optimal OPA concentrations, a design could again be executed similar to the one of Table 7. This was however not done, because the effects calculated from Table 7 were less important compared to the ones of Table 5. Moreover, in both the screening and in the optimisation with uniplex, the same results were found off-line and on-line.

4. Conclusions

Using an experimental design approach made it possible to optimise the determination of L-*N*-monomethylarginine. The two strategies that were executed, optimisation on-line and off-line, lead to the same results. Although it is not always clear why some factors have only little influence on-line (e.g. the flow rate), an explanation for this was found off-line. The best approach seems to be to optimise on-line, using a screening design first and optimisation of the important factors afterwards. This approach takes less time to perform all the experiments and the system can be automated. Using a fractional factorial design for screening should however be done with care because of the confounding of factors, since it was observed that two- and even three-factor interactions can be important.

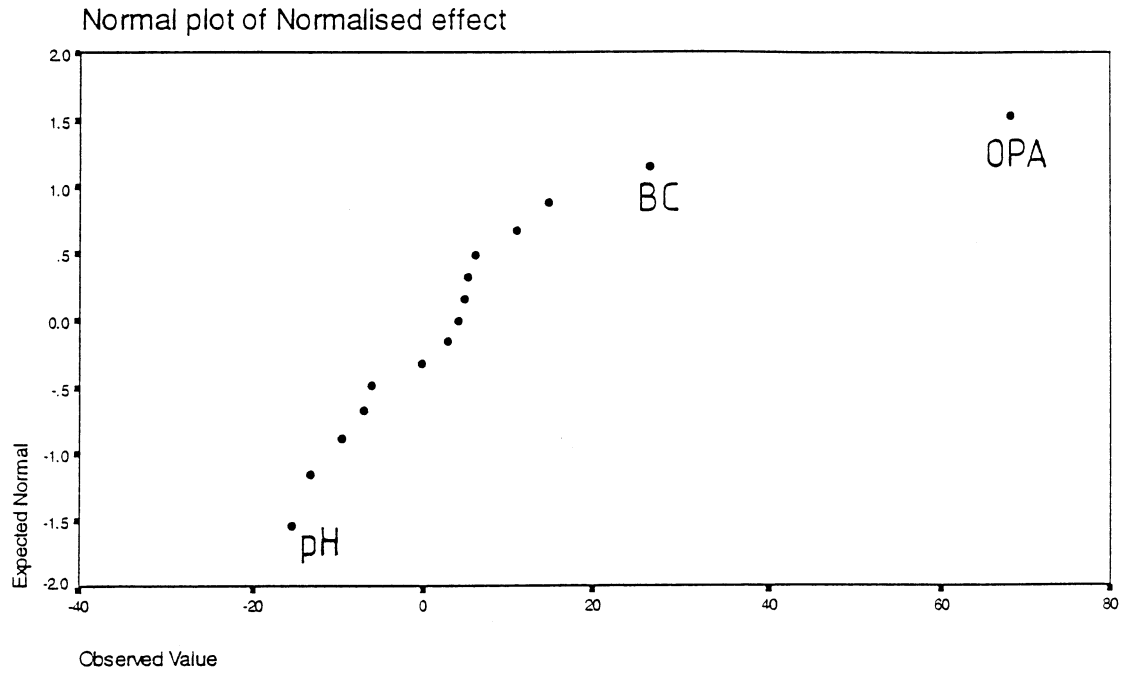

Fig. 8. Normal probability plot of the effects in the screening on-line.

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