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An experimental design approach to the optimisation of a flow injection analysis method for glycine

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Abstract

A flow injection analysis method for the determination of glycine, based on the reaction with *ortho*-phtalaldehyde and *N*-acetylcysteine in a basic buffer, was optimised. In the first step screening of the variables, to select the most important ones, was performed using: (i) a half-fraction factorial design and (ii) a quarter-fraction factorial design, for five factors at two levels. The effects of the factors on the peak height were calculated from both screening designs and compared. For the half-fraction factorial design (resolution IV), the significance of the factor effects on the peak height was checked by: (i) comparing them with a critical effect, calculated from two-factor interactions and based on a *t*-test, (ii) using a non parametric approach and (iii) drawing a normal probability plot. For the quarter-fraction factorial design (resolution III) the significance of the effects of the factors on the peak height was checked using: (i) a randomization test method, (ii) the non parametric method and (iii) a normal probability plot. In the second step, the factor found to be of importance was optimised using the uniplex method.

Keywords: Flow injection analysis; Glycine; Fractional factorial design; Uniplex

1. Introduction

Flow injection analysis (FIA) has become a technique of increasing importance in pharmaceutical analysis because of its implicit simplicity, low cost and rapid approach [1,2]. FIA is a continuous flow method in which a small plug of sample is injected into a flowing reagent stream. Mixing occurs by diffusion and the product of the reaction is monitored downstream to give a transient peak signal. The response usually measured for quantitative FIA determinations is the peak height. The peak height not only depends on the parameters which describe the FIA system, such as the length and the diameter of the reaction tubing and the flow rate of the reagent, but also on the parameters of the chemical reaction involved, such as the concentration of the reagent(s), the pH of the reagent(s) and the ionic

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strength of the buffer [3]. To reach optimal conditions, i.e. where the peak height is maximal, the peak width minimal and the residence time (which is the time from injection to the maximum of the peak) appropriate, one can use the 'one-factor-at-a-time' strategy. In this strategy one variable is changed while the others are held constant and the response is measured. When the best response for a given variable value is found, this value is held constant and another variable is investigated. In the literature concerning optimisation of FIA methods, this univariate strategy is the one most frequently used [3,4]. However, this strategy is time-consuming because it requires many experiments and does not take into account the interaction effects that can occur. Another disadvantage is that, depending on the starting conditions, it is possible that one never finds the global optimum but will be trapped in a local optimum [5]. Another approach uses experimental designs. Optimisation of FIA methods is sometimes executed using the simplex design [6–8]. However one can apply other types of experimental designs. In this paper the optimisation of the determination of glycine by FIA was carried out using an experimental design approach based on using first screening designs.

Glycine is an amino-acid which is the critical component in several pharmaceutical buffer solutions needed to ensure the optimum stability of pH sensitive drugs. This is particularly true in lyophilised products where glycine is an essential component of the product formulation. To control the efficiency of the buffering solution it is necessary to routinely determine the amount of glycine in this solution. The derivatisation reaction of glycine with *ortho*-phtalaldehyde (OPA) in the presence of *N*-acetylcysteine (NAC) (which contains a thiol group) at alkaline pH was used (Fig. 1) [9]. A reagent consisting of OPA and NAC in an alkaline buffer is pumped through the FIA manifold. Solutions of glycine are injected in the flowing stream. The glycine reacts with the reagent stream yielding a derivative which can be measured spectrophotometrically.

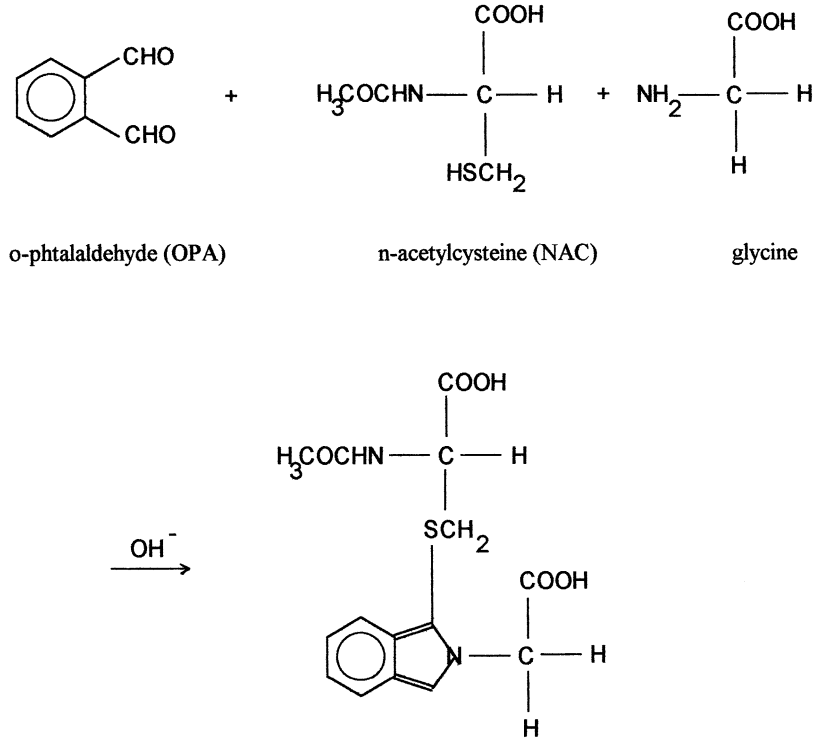

Fig. 1. Chemical reaction performed in the assay of glycine.

Table 1
Half-fraction factorial design 2^{5-1}_{IV}

Experiments	Factors					Peak height
	A	*B*	*C*	*D*	*E*	
1	−1	−1	−1	−1	1	373241
2	1	−1	−1	−1	−1	308001
3	−1	1	−1	−1	−1	306301
4	1	1	−1	−1	1	391880
5	−1	−1	1	−1	−1	322989
6	1	−1	1	−1	1	366471
7	−1	1	1	−1	1	389305
8	1	1	1	−1	−1	306285
9	−1	−1	−1	1	−1	298425
10	1	−1	−1	1	1	347450
11	−1	1	−1	1	1	344229
12	1	1	−1	1	−1	286799
13	−1	−1	1	1	1	306909
14	1	−1	1	1	−1	286329
15	−1	1	1	1	−1	306368
16	1	1	1	1	1	347100

Generator: $E = ABCD$.

2. Theory

To select the most important variables, a full factorial design can be used for screening [10,11]. However the number of experiments that has to be performed increases rapidly with the number of variables examined. In a fractional factorial design on the other hand only a fraction of the full factorial design is performed. For five factors at two levels for example, a full factorial design requires $2^5 = 32$ experiments, while a half-fraction factorial design only needs $2^{5-1} = 16$ experiments (Table 1). By creating a fractional design with resolution IV, confounding of the main effects with two-factor interaction effects is avoided. In the latter design it is still possible to estimate the effect of the main factors (variables) though they are confounded with three-factor interaction effects which are considered negligible. For economical reasons saturated fractional factorial designs are interesting. In these designs the smallest fraction, in which the main effects can still be estimated without confounding among each other, is executed. For five variables only 2^{5-2} (8) experiments have to be performed (Table 2). In the latter design the main effects are confounded with two-factor and higher order interactions (resolution III).

The effect of each variable on the response is calculated as the difference between the average results at the (+1) level and at the (−1) level of the variable:

$$E_x = \frac{\sum Y(+)}{n} - \frac{\sum Y(-)}{n}.$$

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. Normalised effects ($\%E_x$) can be calculated as $\%E_x = (E_x/\bar{Y})*100$ with $\bar{Y}$ being the average nominal peak height.

2.1. Determination of the significance of the factor effects

To determine whether an effect is statistically significant or not, different methods can be used.

The first method is to apply a *t*-test by comparing the effect of the factor with a critical effect [12]. If the absolute value of the effect is larger than this critical effect, the factor is statistically significant. The critical effect is calculated as $E_{critical} = t_{critical}*(S.E.)_e$ with $(S.E.)_e$ being the standard error of the effect, which is calculated as:

Table 2
Quarter-fraction factorial design 2^{5-2}_{III}

Experiments	Factors					Peak height
	A	*B*	*C*	*D*	*E*	
1	−1	−1	−1	1	1	354144
2	1	−1	−1	−1	−1	329069
3	−1	1	−1	−1	1	377220
4	1	1	−1	1	−1	303933
5	−1	−1	1	1	−1	274461
6	1	−1	1	−1	1	355407
7	−1	1	1	−1	−1	353600
8	1	1	1	1	1	349836

Generators: $D = AB$, $E = AC$.

$$(\text{S.E.})_e = \sqrt{\frac{\sum E^2_{X_i Y_j}}{n_{X_i Y_j}}}$$

with $E_{X_i Y_j}$ the effect of a two-factor interaction (which is not confounded with a main factor), $n_{X_i Y_j}$ the number of these effects used in the calculation of $(\text{S.E.})_e$ and t_{critical} being a tabulated t-value for the $n_{X_i Y_j}$ degrees of freedom. For saturated designs however it is not always possible to calculate a reliable critical effect since there are no or not enough interaction terms available and the t-critical values to be used are too high.

Secondly, randomization tests [13,14] can then be used as a statistical interpretation method. By using these tests, the significance is determined from the data as such, instead of using statistical tables. A P-value is given to the examined factor effect depending on the number of data permutations larger or equal to the factor effect examined. The effects are said to be significant if the P-value is smaller than 0.01 ($\alpha = 0.01$) or 0.05 ($\alpha = 0.05$).

A third method to identify significant effects or in other words, parameters that are 'active', in factorial and fractional factorial designs was published by Lenth [15]. In this method, also, a non parametric approach is used. In the first step, s_0 is calculated as $s_0 = 1.5 * \text{median} |E_x|$ with $|E_x|$ the absolute values of the factor effects. In the second step a pseudo standard error (P.S.E.) is calculated as

$$\text{P.S.E.} = 1.5 * \underset{|E_x| < 2.5 s_0}{\text{median}} |E_x|$$

where the effects of the factors with a value larger than $2.5 * s_0$ are excluded when selecting the median. This P.S.E. is used to calculate what the author calls a margin of error (M.E.) and a simultaneous margin of error (S.M.E.). The effects larger than S.M.E. are 'active', the effects smaller than M.E. are 'not active' and the effects situated between S.M.E. and M.E. should be looked at carefully.

The margin of error is calculated as $\text{M.E.} = t_{0.975;d} * \text{P.S.E.}$ and the simultaneous margin of error as $\text{S.M.E.} = t_{\gamma;d} * \text{P.S.E.}$ where d is the number of degrees of freedom ($d = m/3$ with m the number of effects that can be calculated from the experimental design performed) and $\gamma = (1 + 0.95^{1/m})/2$. For more theoretical background on the calculation of M.E. and S.M.E. we refer to Ref. [15]. The t-values used to calculate M.E. and S.M.E. can also be found in Ref. [15].

A fourth possibility to help to decide whether an effect is significant, is to draw a normal probability plot. The effects that deviate from the normal distribution around zero and thus from the straight line formed by the effects of no importance, are considered significant [11,12].

3. Experimental

3.1. Reagents

Glycine for the sample solutions was obtained from Merck (Darmstadt, Germany). Sample solutions were made by preparing a stock solution of 1 mg ml^{-1} glycine in Milli Q water (Milli Q water purification system, Millipore, Bedford, MA). The stock solution was diluted with Milli Q water to obtain a concentration of 50 μg ml^{-1} glycine. This solution was injected into the FIA manifold.

The carrier (reagent) stream consisted of NAC (Sigma, Germany) which was dissolved in an alkaline buffer solution. The alkaline buffer solution was made with boric acid (Merck, Darmstadt, Germany) dissolved in water and adjusted to the desired pH with a 2 M NaOH solution. The NaOH pellets were obtained from Merck. OPA (97% pure, Sigma, Steinheim, Germany), dissolved in 25 ml methanol (BDH, UK), was added to this solution and the volume was adjusted to 500 ml with alkaline buffer solution. The value of the pH of the buffer, the ionic strength of the buffer, the amount of NAC and the amount of OPA which was used for each experiment, varied according to the experimental design. The solution was sonicated to release possible air bubbles. The carrier solution was then pumped through the FIA manifold.

3.2. Apparatus

The apparatus consisted of a Burkard (Burkard Scientific, Uxbridge, UK) FIA-flo flow injection

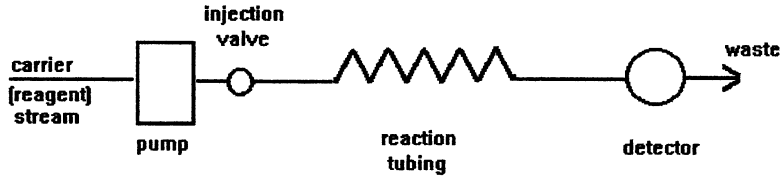

Fig. 2. Schematic representation of the apparatus for the determination of glycine by FIA.

Flow rate = 1.25 ml/min

Carrier stream consists of 0.15 g/v % OPA, 58 mg/100 ml NAC, pH buffer 10.4, ionic strength buffer 0.1.

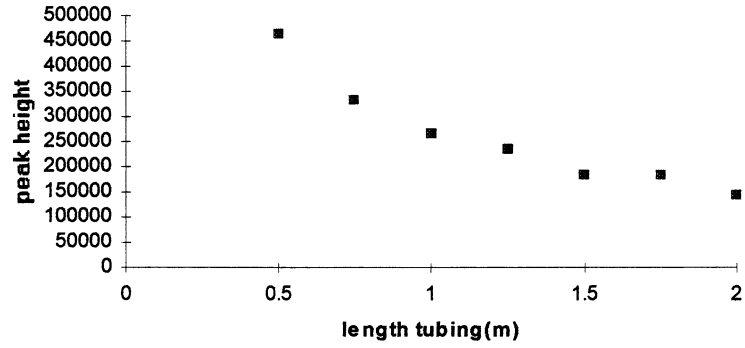

Fig. 3. Influence of the length of the tubing on the peak height.

system equipped with PTFE six-port valves (Fig. 2). PTFE tubing (0.5 mm i.d.) was used for all connections. The injection volume was 15 μl.

A Merck-Hitachi L-4200 variable wavelength UV–vis Detector was applied to monitor the reaction derivative. The detection wavelength was 336 nm.

The calculations for the randomization test and the correction factor were performed by a computer program written in Matlab and developed by Questier et al. [14].

4. Results and discussion

To ensure a rapid method, a residence time of approximately 20 s was desired. Since the residence time is proportional to the length of the tubing and inversely proportional to the flow rate [3], one can vary the residence time by holding for instance the length of the tubing constant and changing the flow rate, in such a way that the residence time remains between 15 and 25 s. The length of the tubing was first varied between the limits of 0.5 and 2 m. The flow rate was set at 1.25 ml min^{-1} and a carrier stream consisting of 0.15 g per volume percent OPA, 0.58 g l^{-1} NAC, ionic strength 0.1 and pH 10.4 was pumped through the system to determine the peak height and the residence time for lengths of tubing between the given limits. From these experiments it was seen that the shorter the length of the tubing, the higher the peak obtained (Fig. 3). This result is what one expects for fast chemical reactions, because the shorter the length, the smaller will be the dispersion. However the repeatability for a length of tubing of 0.5 m was not good. Therefore a length of tubing of 0.75 m was chosen to be used in further experiments.

In the first step a selection of the most important variables was performed using a screening design. After selecting the most important factors, they were further optimised in a second step.

Table 3
Levels for the factors examined in the screening designs

Factors	Levels	
	(−1)	(+1)
A (mg%)	30	200
B (mg%)	15	100
C	9.4	11.4
D	0.05	0.15
E (ml min^{-1})	0.6	1.2

Table 4
Effects of the factors on the peak height calculated from the half-fraction factorial design 2^{5-1}_{IV}

	Effect	Effect (%)
Factors		
A	−931	−0.28
B	8557	2.58
C	−3071	−0.92
D	−30110	−9.07
E	55640	16.76
Interactions		
AB	−2603	−0.78
AC	−3915	−1.18
AD	3868	1.16
AE	10740	3.23
BC	8034	2.42
BD	2789	0.84
BE	11055	3.33
CD	−4478	−1.35
CE	−8683	−2.62
DE	−13690	−4.13

Table 5
Effects of the factors on the peak height calculated from the quarter-fraction factorial design 2^{5-2}_{III}

	Effect	Effect (%)	*P*-value (randomisation)
Factors			
A (+*BD*+*CE*)	−5295	−1.53	0.914
B (+*AD*)	17880	5.18	0.600
C (+*AE*)	−7766	−2.25	0.686
D (+*AB*)	−33230	−9.62	0.057
E (+*AC*)	43890	12.71	0.057
Interactions			
BC+*DE*	18910	5.48	0.514
BE+*CD*	−9125	−2.64	0.629

4.1. Screening experiments

The factors investigated in the screening were the concentration of OPA (*A*) and of NAC (*B*) in the reagent, the pH (*C*) and the ionic strength (*D*) of the buffer and the flow rate (*E*).

For each factor an upper (+1) and a lower (−1) level were defined (Table 3). These levels were based on data from the literature [16], on stoichiometric calculations for the chemical reaction parameters and on the experiments for selecting the flow rate so that the residence time levels would be between 15 and 25 s. The flow rate was known to be important, but we included it as a factor in the design, to verify that the experimental design methodology performs correctly.

Two different two-level screening designs were executed for the five factors, namely a half-fraction factorial design (Table 1) and a quarter-fraction factorial design (Table 2).

For both designs, the effects of the factors on the peak height were calculated (Tables 4 and 5).

To identify the significant effects in both designs, different approaches were used.

For the half-fraction factorial design with 16 experiments a critical effect at significance levels $\alpha = 0.05$ and 0.01 was calculated using the two-factor interaction effects. The values obtained were 17680 (5.33%) and 25150 (7.58%), respectively. This means that the ionic strength and the flow rate are considered significant at $\alpha = 0.01$ (Table 4). The method of Lenth was also used to select the important parameters. First, s_0 was calculated using the median of all effects: $s_0 = 1.5*|8034| = 12051$. Then the identical calculation was performed excluding the effect that exceeds $2.5s_0 = 30127.5$ to obtain P.S.E. $= 1.5*|6256| = 9384$. The M.E. and the S.M.E. were calculated to be 24117 and 48984. Comparing the effects of the factors (Table 4) with these M.E. and S.M.E. values shows that the flow rate is (correctly) to be considered very important since its value is larger than the S.M.E. and the ionic strength is situated between the two values (Fig. 4). Drawing a normal probability plot confirms these results (Fig. 5).

For the quarter-fraction factorial design with eight experiments, a randomization test [14] was

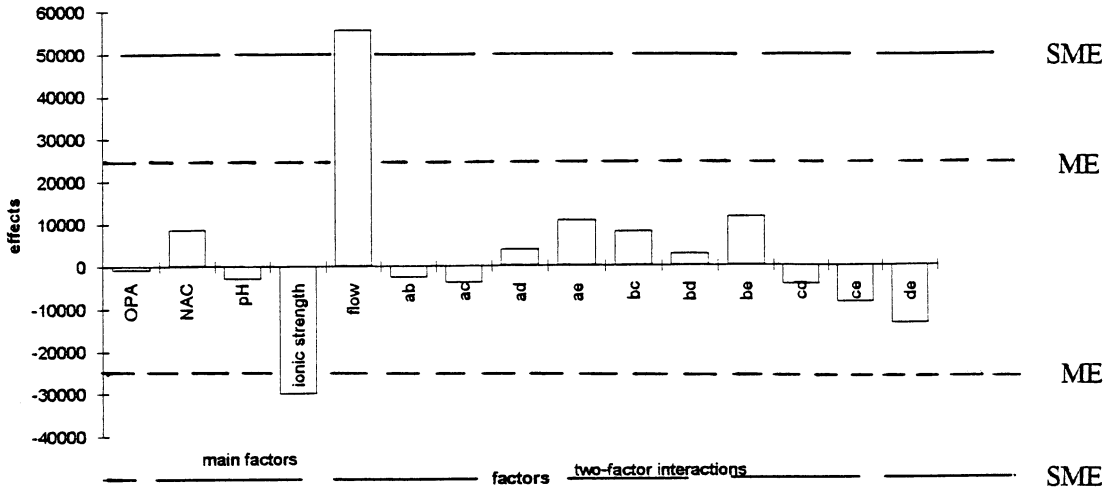

Fig. 4. Selection of 'active' effects using the method of Lenth in the half-fraction factorial design for five factors (2^{5-1}_{IV}).

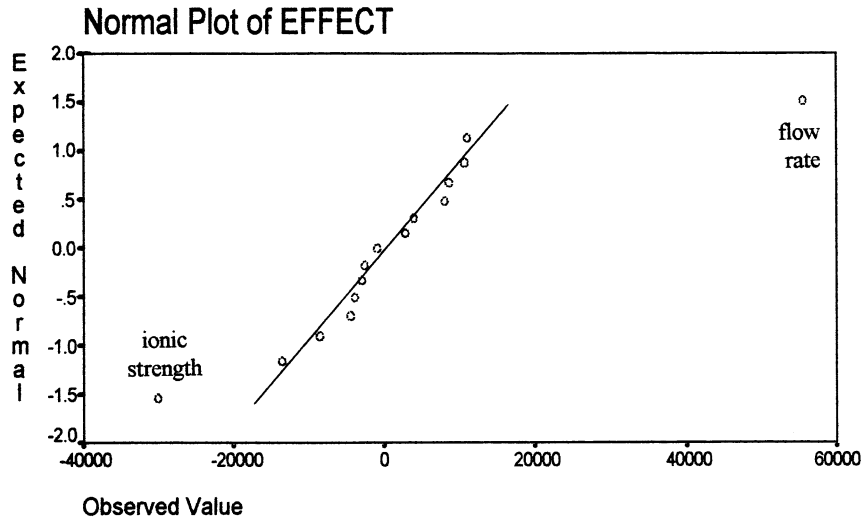

Fig. 5. Normal probability plot of the effects for the 2^{5-1}_{IV} design.

used to select the statistically significant effects. The P-values for the factors are shown in Table 5. From this table the flow rate and the ionic strength are considered significant at the 0.05 level since the value of $P = 2/35 = 0.057$. With this method the same factors are therefore found to be significant as in the half-fraction factorial design, which requires twice as many experiments. Again a normal probability plot confirms these results. The method of Lenth was also applied. Again s_0 was computed using the median of all effects: $s_0 = 1.5*|17880| = 26820$. The value of $2.5s_0 = 67050$ excludes no effects so that the P.S.E. is equal to s_0. The M.E. and S.M.E. gave 100843 and 241648, respectively. Since all the effects are smaller than the M.E. no factors were considered to be important by this method. This result is clearly not satisfactory.

An explanation of the failure of the method

of Lenth to identify the important effects in highly fractionated designs could be as follows. The first remark is that for the quarter-fraction factorial design, the number of degrees of freedom is lower than for the half-fraction factorial design, which results in higher t-values and thus in higher limits (M.E. and S.M.E. values). Another reason for the failing can be explained by what is called the influence curve of the median [17]. This can be intuitively explained as follows. The ranked absolute effects of Table 5 are 5295, 7766, 9125, 17880, 18910, 33230 and 43890. The median is 17880, but one observes that the ranking jumps from 9125 to 17880: the median depends very much on the value of a single result and this effect is all the greater when the number of data is low. When the median is relatively high (and thus s_0 and $2.5*s_0$), no effects will be excluded in the selection of the median to calculate P.S.E. This can be seen in the quarter-fraction factorial design where the flow rate is not excluded in the calculation of P.S.E. When an

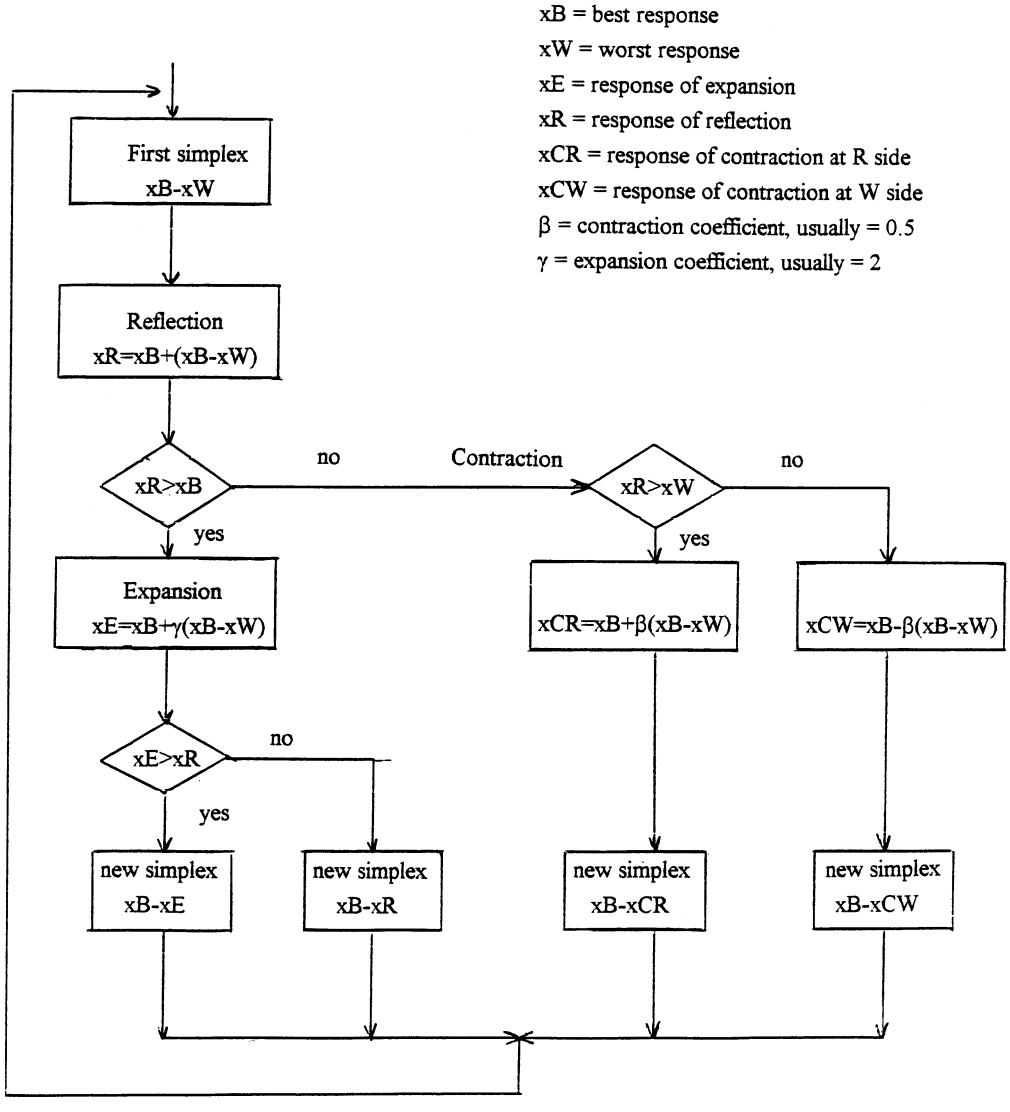

Fig. 6. Schematic representation of the uniplex procedure.

Table 6
Uniplex search for the optimisation of the ionic strength

Sequence number	New uniplex	Vertices of uniplex	Peak height
1	*xB–xW*	0.03–0.07	366242–356723
2	*xR*	−0.01	0
3	*xB–xCw*	0.03–0.05	366242–363913
4	*xR*	0.01	341865
5	*xB–xCw*	0.03–0.04	366242–363360
6	*xR*	0.02	370962
7	*xB–xCw*	0.02--0.025	370962–367485
8	*xR*	0.015	358151

effect is larger than $2.5*s_0$ and excluded in the selection of the median to calculate P.S.E., the new median will be lower and thus P.S.E., M.E. and S.M.E. will have smaller values than when the effect was not excluded, which was the case for the half-fraction factorial design.

4.2. Optimisation

From the screening it was seen that the flow rate (or residence time) and the ionic strength are important variables. As explained earlier, the flow rate was added as a factor only as a test to see whether the experimental design methodology works correctly. Therefore in further optimisation, the flow rate was set at 0.9 ml min^{-1} which corresponds to a residence time of approximately 20 s.

The optimisation was executed with the concentration of OPA at 30 mg%, the concentration of NAC at 100 mg%, pH 9.4 and, as said earlier, the length of the tubing was 0.75 m.

The ionic strength was further optimised using the uniplex method [18]. This method is a simpler version of the simplex optimisation, based on reflection, contraction and expansion rules, but with only one variable to be optimised. One starts with the selection of two points, making up the first uniplex. The rules to be followed in the uniplex procedure are explained schematically in Fig. 6.

From the screening design it was seen that the effect of the ionic strength on the peak height is negative. This means that the low level of the ionic strength (0.05) gave a higher peak than the high level (0.15). Therefore the starting points of the uniplex method were selected around the 0.05 value. In this way it is possible to see if the response obtained is better at a value lower or higher than 0.05. The starting points of the uniplex method were chosen as 0.03 and 0.07 (Table 6). It was seen that an ionic strength of 0.03 gave a better response so that the reflection of 0.07 selects an ionic strength of -0.01 as the new experimental conditions. Since this value can not be measured, a very bad response value (peak height = 0) was allocated to it. A new vertex 0.05 is obtained by contraction of the starting region. The new vertex is now 0.03–0.05. The peak height obtained with 0.05 was worse than with ionic strength 0.03 and the reflection leads to an ionic strength of 0.01. The response is again worse than 0.05 so a new contraction is performed to obtain a new experiment with ionic strength 0.04. The reflection of 0.04 over the best response 0.03 leads to a new experiment with ionic strength 0.02. The response obtained is better than with $\mu = 0.03$ so that an expansion is done, which leads again to an ionic strength of 0.01 which already gave a bad response. Therefore a new contraction is done yielding an experiment with ionic strength 0.025. The reflection of this vertex leads to a ionic strength of 0.015, which does not give a better result either. The procedure was stopped at this point because the difference in peak height was too small. The optimal ionic strength was defined as 0.025 because when looking at the responses for $\mu = 0.02$ and 0.03 the difference is small and the response reached a plateau there. The ionic strength 0.02 gave the highest response but was not selected as optimal because for smaller ionic strengths a fast decrease in peak height was observed. This means that $\mu = 0.025$ though giving a somewhat smaller peak height than $\mu = 0.02$, is situated in a more rugged region.

The repeatability of the conditions found to be optimal, was checked by injecting six replicates of the glycine sample. A percent R.S.D. of 0.13% was found (Fig. 7), which is significantly below the 1% limit required.

5. Conclusions

An experimental design approach allows us to find the optimal conditions for the determination of glycine with FIA in relatively few experiments. When one uses a screening design where the main factors are not confounded with the two-factor interactions, the interpretation of the significant (active) effects can be done by comparing the effects with a critical effect or with the method proposed by Lenth. However when using the smaller screening design, interpretation with the method proposed by Lenth is no longer possible.

If the number of experiments is not considered too high by the analyst, it is better to carry out a design where the main factors are not confounded with the two-factor interactions. In this way the selection of the significant effects is easier and more straightforward. However, when the number of factors is high, a design with resolution III can be applied instead and interpreted with a randomisation test.

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Optimal conditions :	concentration OPA	30 mg%
	concentration NAC	100 mg%
	pH buffer solution	9.4
	ionic strength buffer	0.025
	flow rate	0.9 ml/min
	length of tubing	0.75 m
	injection volume	15 µl

The concentration of glycine injected was 50 µg/ml.

peak number	peak height	
1	367001	
2	367612	average peak height = 367485
3	366937	% RSD = 0.13 %
4	368076	
5	367891	
6	367395	

Fig. 7. FIA traces obtained with the proposed optimal conditions.

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