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Development and optimisation of a new derivatisation procedure for gas chromatographic–mass spectrometric analysis of dihydrostreptomycin
Comparison of multivariate and step-by-step optimisation procedures

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Abstract

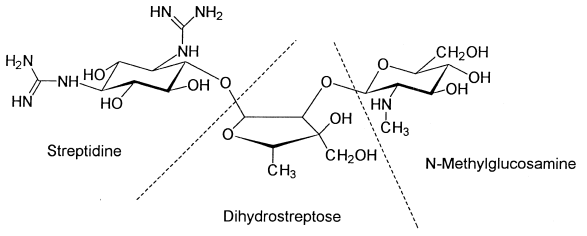
A sensitive capillary GC–MS method for the analysis of dihydrostreptomycin has been developed. This method involves a new derivatisation procedure for dihydrostreptomycin with silylation of the hydroxyl groups with trimethylsilylimidazole and cyclisation of the guanidino groups with hexafluoroacetylacetone. The mass spectrum (electron impact ionisation) of the resulting derivative is given and interpreted. The derivatisation procedure has been optimised using multivariate and step-by-step optimisation procedures. Both approaches are explained, the respective results and their interpretation are discussed in detail. Comparison of both methods reveals, that the multivariate optimisation method is the more efficient and more reliable procedure.

Keywords: Optimisation; Derivatisation, GC; Chemometrics; Experimental design; Multivariate methods; Dihydrostreptomycin; Antibiotics; Aminoglycosides; Streptomycin

1. Introduction

Dihydrostreptomycin is a semi-synthetic aminoglycoside antibiotic (Fig. 1) which is commonly used in food-animal production. Parenteral administration of dihydrostreptomycin can result in high, persistent residues in food of animal origin [1].

Chromatographic analysis of dihydrostreptomycin usually requires pre- or post-column derivatisation, as the compound itself lacks chromophores or fluoro-



Dihydrostreptomycin

Fig. 1. Structure of dihydrostreptomycin.

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phores and is not sufficiently volatile for gas chromatographic analysis. To our knowledge only one gas chromatographic method for the determination of dihydrostreptomycin has been published so far [2]. This method applies persilylation using a reagent mixture consisting of *N*,*O*-bis(trimethylsilyl)acetamide (BSA), trimethylsilylimidazole (TMSI) and trimethylchlorosilane (TMCS) in pyridine. However, the resulting *N*-trimethylsilyl (TMS) derivatives are rather susceptible to hydrolysis, so that removal of excess silylation agents or extraction of the derivatives into an organic solvent proved impossible according to our own experiments. Direct injection of excess silylation agent, however, is not compatible with some gas chromatography (GC) detection systems, for instance, with nitrogen–phosphorus detection. It may also adversely affect the performance of other selective and sensitive detection methods, like mass-selective detection by causing rapid deterioration of detector performance due to silicium dioxide deposits in the ion source of the mass spectrometer. Two-step derivatisation procedures combining silylation of hydroxyl groups and acylation of the guanidino groups failed to deliver a defined single product, so that we decided to derivatise the guanidino group of dihydrostreptomycin using a cyclisation reaction. The cyclisation of guanidino groups with acetylacetone to dimethylpyrimidine derivatives as reported by Beyermann and Wisser in 1969 [3] has been used to derivatise various guanidino compounds prior to gas chromatographic analysis [4–6]. Derivatisation of dihydrostreptomycin with acetylacetone and subsequent silylation of hydroxyl groups gave a single product, however, according to our own experience the reaction was rather irreproducible. Substituting hexafluoroacetylacetone (HFAA) for acetylacetone [7,8] finally led to a reproducible derivatisation procedure.

Our aim was to optimise this new derivatisation procedure for dihydrostreptomycin to achieve the best possible yield and to check the suitability of the optimised derivatisation procedure for residue analysis in conjunction with GC–mass spectrometry (MS). For the optimisation of the derivatisation parameters we compared the efficiency of the two most common optimisation methods. The classical approach of optimising one parameter after the other in a step-by-step procedure, while keeping all other potentially influential parameters at a constant level, is often addressed as the OVAT (one-variable-at-a-time) method. This method clearly has its drawbacks, as it fails to take interactions between two or more parameters into account, and only examines a very narrow range within all possible combinations of values (Fig. 2). As a consequence, the detected optimum is in many cases not identical with the global optimum. If interactions between two or more parameters occur, the global optimum can only be detected by varying several parameters simultaneously, i.e., by using multivariate methods [9–12].

Multivariate methods are based on the design of an experimental plan, i.e., a series of experiments in each of which the values for several parameters are changed at the same time. The results of these experiments are then evaluated using simple statistical methods like analysis of variance (ANOVA) and regression analysis. Two major groups of experimental designs are important: screening designs and optimisation designs. Screening designs are used to determine (i) which parameters have an effect on, for instance, the yield of a derivatisation reaction, (ii) which parameters have an interaction effect, (iii) whether these effects are positive or negative and (iv) whether they are significant. Classical screening designs such as fractional and full factorial designs use two levels for each parameter. Determining the

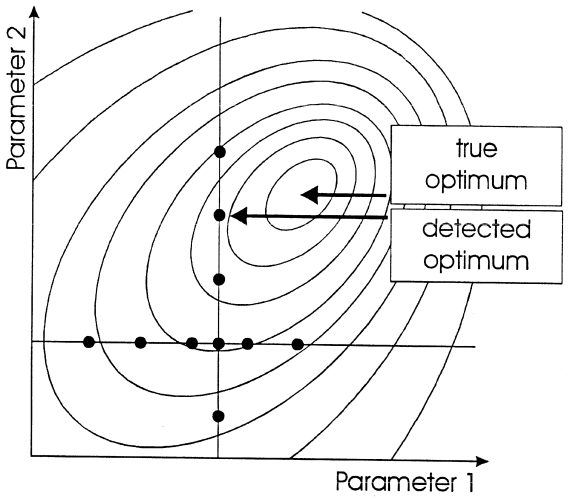

Fig. 2. Illustration of the step-by-step approach (OVAT method).

optimum, however, requires optimisation designs with at least three levels for each parameter. These designs are used to compute a non-linear correlation between parameters and response, the so-called response surface model. This non-linear model can be used to determine optima (maxima or minima), steepest ascents, etc. Classical optimisation designs are Box–Behnken designs or central composite designs. Further details about multivariate optimisation methods in general and the mentioned design-types in particular can be found in Refs. [9–12] or in a recent publication in this journal [13] which focuses on the use of multivariate methods for the optimisation of a derivatisation reaction for kanamycin and gentamicin for GC–MS analysis.

2. Experimental

2.1. Materials

All solvents used were of analytical-reagent grade. Water was deionised, distilled twice and finally distilled for a third time over sodium permanganate to remove traces of organic compounds. Dihydrostreptomycin sulphate was supplied by Serva (Heidelberg, Germany). The derivatisation agents TMSI and HFAA and sodium carbonate were obtained from Fluka (Buchs, Switzerland), as was the internal standard hexatriacontane ($C_{36}H_{74}$).

2.2. Derivatisation procedure

The developed derivatisation procedure consists of simultaneous silylation of hydroxyl groups with TMSI and cyclisation of guanidino groups with HFAA to give di(trifluoromethyl)pyrimidine derivatives (Fig. 3). To achieve faster reaction and better yield for the cyclisation reaction, dried and powdered sodium carbonate (Na_2CO_3) was added [3,4]. After the derivatisation, excessive silylation reagent was removed by the addition of water.

For the optimisation, the amounts of TMSI, HFAA and Na_2CO_3, as well as the reaction temperature and reaction time were varied, while all other parameters were kept constant. Derivatisation was carried out in 4-ml round-bottomed reaction vials (WGA, Düsseldorf, Germany) fitted with screw-caps. Fifty μl of an aqueous stock solution containing 50 μg of dihydrostreptomycin were evaporated to dryness under nitrogen at 60°C in a heating block. To the dry residue 500 μl of the internal standard solution (20 μg/ml hexatriacontane in heptane) were added. After the addition of Na_2CO_3, TMSI and HFAA (varying amounts for each optimisation experiment), the vial was sealed and the reaction mixture was ultrasonicated for 5 min. The vial was then transferred to a heating block, heating temperature and heating time again depending on the optimisation experiment. After removing the vial from the heating block, the mixture was allowed to cool down to ambient temperature for 7 min. Finally, 1000 μl of water were added, and, after vortex-mixing and centrifuga-

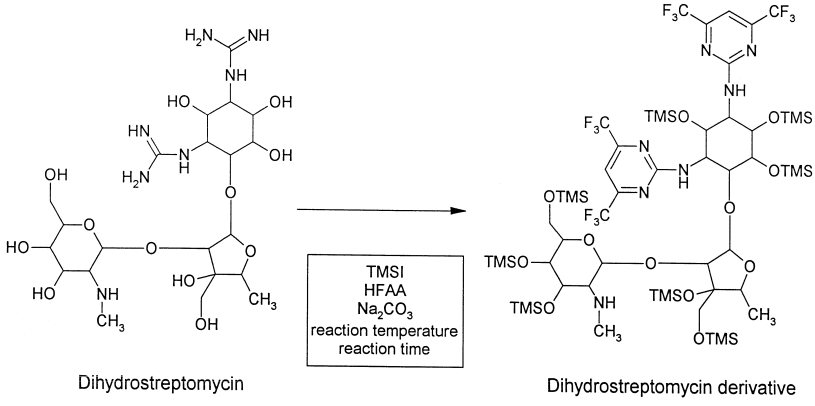

Fig. 3. Formation of the dihydrostreptomycin derivative.

tion, the upper heptane-layer containing the derivative was transferred into a 1-ml septum-capped vial and stored at −20°C. One μl of the heptane phase was injected into the GC system.

2.3. GC conditions

The GC system consisted of a Hewlett-Packard (Waldbronn, Germany) HP-5890 Series II gas chromatograph fitted with a Hewlett-Packard on-column injector for stainless steel syringe needles and a Hewlett-Packard 5971A MS system working in electron impact ionisation (EI) mode with 70 eV. A DB 1 fused-silica cross-linked methyl silicone gum capillary column (J&W Scientific, Cologne, Germany), 30 m×0.25 mm I.D. with 0.1 μm film thickness was used for chromatographic separation. The transfer line temperature was 290°C and the oven temperature programme was 107°C for 5 min, ramping at 58°C/min to 220°C and subsequently at 10°C/min to the final temperature of 290°C, which was held for 2 min. Helium (>99.999% purity) was used as carrier gas with an initial column head pressure of 0.76 bar at 107°C resulting in a flow of 1.0 ml/min equalling 37.2 cm/s linear velocity. The electronic pressure control was set to constant flow mode with vacuum compensation. Samples were injected on-column into a methyl-deactivated 3 m× 0.53 mm I.D. fused-silica retention gap with the injector set to oven-track mode, so that the injector temperature automatically remained several degrees above the oven temperature throughout the run.

2.4. Interpretation of mass spectra and selection of fragment ions for quantification

Identifying the most appropriate fragment ions for quantification by selected ion monitoring (SIM) required interpretation of the mass spectrum of the new dihydrostreptomycin derivative. Due to the limited mass range of the benchtop MS system used (50–650 u), we could not detect the molecular ion of the derivative. However the fragment ions from the derivatised streptidine ring as well as from the derivatised dihydrostreptose and *N*-methylglucosamine moieties in conjunction with the mass spectrum of the structurally related dimethylpyrimidine derivative, which was obtained by reaction with acetylacetone instead of hexafluoroacetylacetone, provided sufficient evidence for the compound to be identified as the expected di(trifluoromethyl)pyrimidyl-*O*-trimethylsilyl derivative. The fragmentation pathways are dominated by glycosidic cleavage and subsequent losses of silylated hydroxyl groups (Fig. 4).

On this basis we were able to select three ions for the SIM method which were either of high abundance (m/z 145 and m/z 392, both from the *N*-methylglucosamine moiety) or very characteristic for a specific part of the formed derivative (m/z 625 corresponding to the derivatised streptidine moiety).

3. Optimisation

3.1. Multivariate optimisation

3.1.1. Screening design

The multivariate optimisation of the derivatisation procedure was carried out in two steps. For all statistical calculations involved in this optimisation process we used the software THE UNSCRAMBLER, version 6.11 (CAMO ASA, Trondheim, Norway).

The initial screening design served to detect those variables having the highest influence on the yield of the derivatisation reaction. In addition it enabled us to monitor interactions between those variables. Five variables were included in the screening design: the amounts of Na_2CO_3, HFAA and TMSI and the reaction temperature and reaction time. In a heterogeneous reaction the surface area of Na_2CO_3 will also have an important impact on the reaction. However, as it is difficult to prepare different defined surface areas, we used a single batch of dried and powdered Na_2CO_3. That way the amount of Na_2CO_3 added to the reaction mixture was directly proportional to its surface area. The response was calculated by measuring the peak area of the dihydrostreptomycin derivative and normalising it using the peak area of the internal standard hexatriacontane. By this way, variations in injection volume and in the volume of the organic phase after extraction due to varying amounts of reagents could be corrected.

The screening design used was a two-level full factorial design, requiring 36 experiments (32 experimental points and four additional centre points),

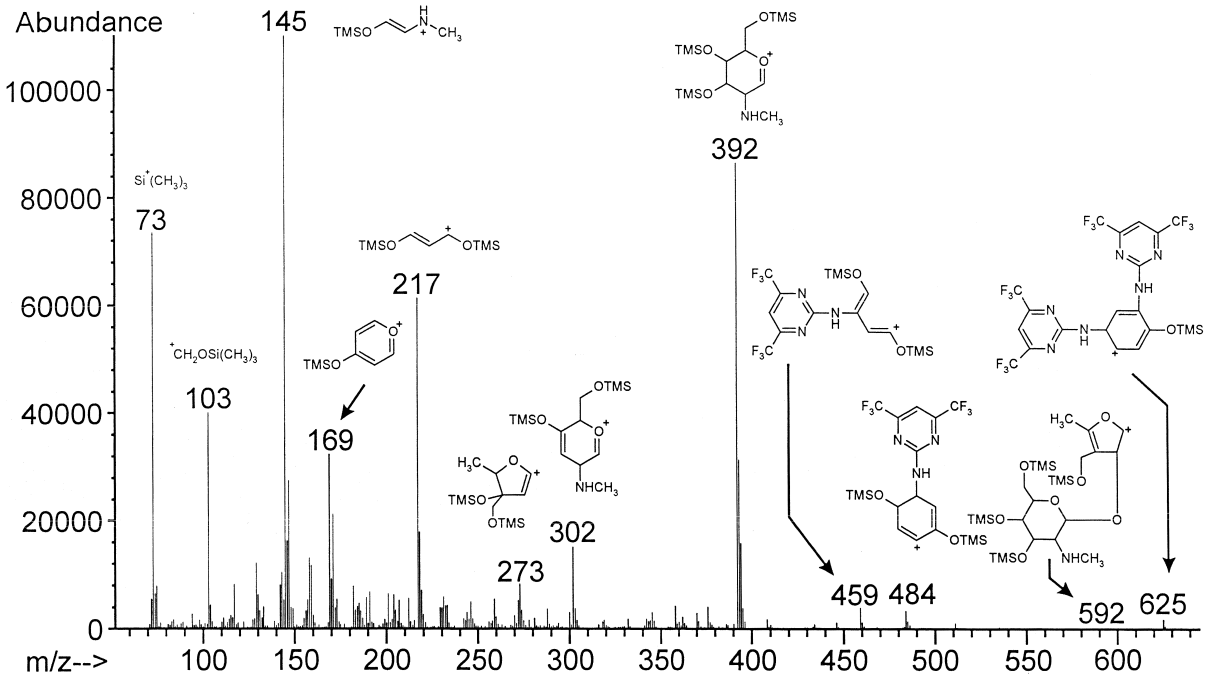

Fig. 4. Mass spectrum of the dihydrostreptomycin derivative including the structures of some important fragments. Fragments 145, 392 (corresponding to the derivatised *N*-methylglucosamine moiety) and 625 (derivatised streptidine moiety) were selected for SIM mode.

which were conducted in randomised order to avoid interferences from systematic errors.

Fig. 5 shows the results of the screening design in summarised form. The parameter effects related to the cyclisation step can be interpreted as follows: both, Na_2CO_3 (A) and HFAA (B), show negative main effects. This indicates, that both amounts should be kept rather low. However, the interaction Na_2CO_3–HFAA (AB) shows a significant positive effect. This interaction of both reagents should be expected considering the reaction mechanism: Na_2CO_3 serves to deprotonate the guanidino groups prior to their cyclisation reaction with HFAA. A positive interaction of both reagents indicates, that not both amounts should be set to low values at the same time. If, for instance, a low amount of Na_2CO_3 is added, a higher amount of HFAA is necessary to achieve good yield.

Regarding the silylation, TMSI (C) shows a rather significant positive effect, i.e., higher amounts of TMSI have a positive effect on the yield of the reaction. Even more significant is the positive interaction TMSI–HFAA (BC), and this is indeed an interesting phenomenon. This interaction indicates, that there is a connection between the amounts of TMSI and HFAA, although each of those reagents should react with different functional groups of the analyte. The interaction of both reagents actually reflects a side-reaction: the *O*-silylation of the enol form of HFAA to give *O*-trimethylsilylhexafluoroacetylacetone as described by Refs. [14–16]. The very significant positive interaction effect of HFAA and TMSI in conjunction with the positive main effect of TMSI and the negative main effect of HFAA can be interpreted in the following way: (i) setting both amounts to low values at the same time is not advisable as this ignores the positive main effect of TMSI and the positive interaction effect of both reagents; (ii) setting both amounts to high values fails to take into account the negative main effect of HFAA; (iii) setting the amount of TMSI to low values and the amount of HFAA to high values ignores the main effects of both reagents; (iv) setting the amount of HFAA to rather low values while

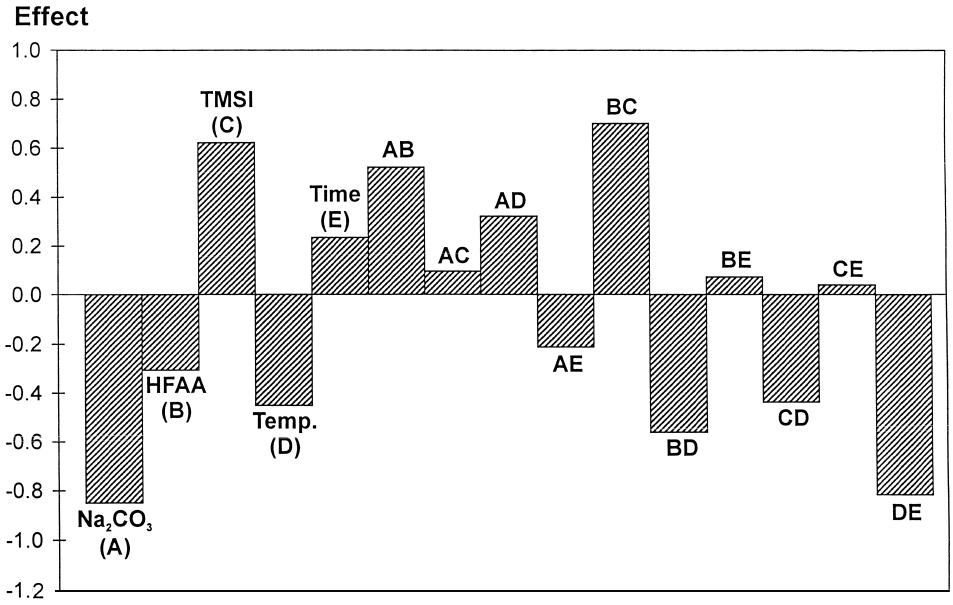

Fig. 5. Results of the screening design.

setting the amount of TMSI to higher values is in accordance with both main effects and the interaction effect.

The reaction temperature and reaction time affect both reactions, the silylation and the cyclisation process. Considering the reaction time, it becomes apparent, that this parameter does not have a significant main effect and shows only one significant interaction with the reaction temperature (DE). This interaction signifies, that, if the reaction temperature is set to a high value, a short reaction time is sufficient to achieve good yield and vice versa. The reaction temperature itself has a negative main effect (D) and negative interactions with HFAA (BD), TMSI (CD) and the reaction time (DE), all indicating a tendency towards a lower reaction temperature.

3.1.2. Optimisation design

The parameters for the optimisation could then be selected based on the results of the screening (Table 1). As it is advisable to keep the number of parameters for an optimisation design low to avoid a high amount of variability, and as only significant parameters should be included in an optimisation design, the reaction time was set to a constant value of 120 min for the subsequent optimisation experiments. For the remaining four parameters the ranges for the optimisation could be narrowed down in accordance with the results of the screening. The ranges for Na_2CO_3, HFAA and the reaction temperature were set to lower values, while the range for TMSI was set to higher values, mainly because of its positive main effect and its positive interaction with HFAA. Using THE UNSCRAMBLER, the optimal conditions within these ranges were determined via a Box–Behnken design, which required 28 experiments (24 experimental points and four centre points). These experiments were again conducted in randomised order.

Using response surface analysis based on multiple regression and analysis of variance, we computed a quadratic response surface model for the yield of

Table 1
Parameters and ranges selected for screening and optimisation using multivariate methods

	Parameter	Screening	Optimisation
A	Amount of Na_2CO_3	5–15 mg	3–10 mg
B	Amount of HFAA	50–250 μl	50–100 μl
C	Amount of TMSI	50–250 μl	100–250 μl
D	Reaction temperature	70–130°C	60–100°C
E	Reaction time	30–120 min	120 min

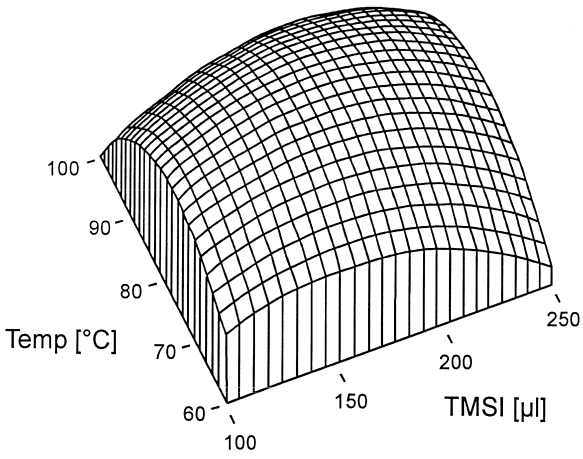

Fig. 6. Response surface plot TMSI vs. temperature.

derivatised dihydrostreptomycin. Fig. 6 shows the response surface plot for the amount of TMSI and the reaction temperature. This plot shows a clear optimum for both parameters at approximately 170 µl of TMSI and 78°C reaction temperature. Fig. 7 shows the response surface plot for the amount of TMSI and the amount of HFAA. This plot illustrates the interaction of both reagents which had also been detected in the preliminary screening design and is due to the side reaction leading to silylated HFAA. These response surface plots were used to determine the following optimal combination of all parameter settings which produces the highest possible yield of derivatised dihydrostreptomycin: 3 mg Na_2CO_3, 67 µl HFAA, 169 µl TMSI and 78°C reaction temperature.

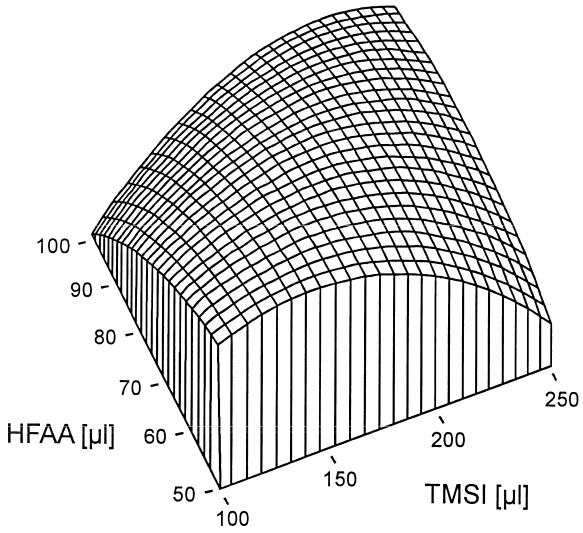

Fig. 7. Response surface plot TMSI vs. HFAA.

3.2. Step-by-step optimisation (OVAT method)

We also optimised the derivatisation parameters using the classical step-by-step method. We conducted two series of experiments, i.e., two different step-by-step optimisations – OVAT 1 and OVAT 2 – which differed in the initial parameter settings and in the sequence in which the optimisation of the parameters was carried out.

OVAT 1 started with initial values of 10 mg Na_2CO_3, 100 µl HFAA, 100 µl TMSI, a reaction temperature of 100°C and a reaction time of 60 min. The parameters were optimised in the following sequence: Na_2CO_3, HFAA, TMSI, reaction temperature and reaction time. The respective steps of this optimisation and their results are illustrated in Fig. 8. The detected optimal conditions are: 6 mg Na_2CO_3, 50 µl HFAA, 175 µl TMSI, 90°C reaction temperature and 80 min reaction time.

The second series of experiments, OVAT 2, started with 10 mg Na_2CO_3, 20 µl HFAA, 50 µl TMSI, a reaction temperature of 100°C and a reaction time of 30 min. The parameters this time were optimised in the order: HFAA, TMSI, Na_2CO_3, reaction temperature and reaction time. Fig. 9 illustrates the different steps of the optimisation OVAT 2 and their results. The optimum in this case was detected at 10 mg Na_2CO_3, 5 µl HFAA, 50 µl TMSI, 93°C and 80 min reaction time.

4. Discussion

In general, good consistency between the results of the step-by-step optimisations OVAT 1 and OVAT 2 and the model computed from the multivariate experiments can be observed: most effects apparent in the OVAT models can be explained by the results of the screening design and the response surface model. It was even possible to predict the results of each optimisation step of OVAT 1 using the response surface plots of the multivariate optimisation, as both optimisations covered similar ranges.

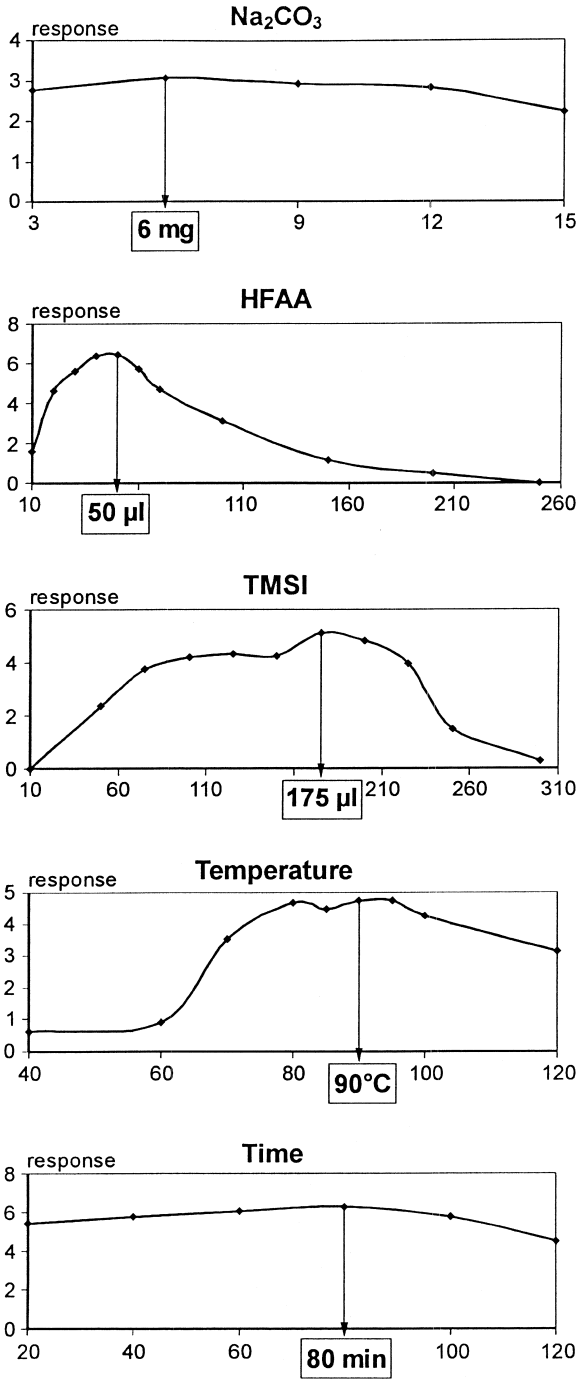

Fig. 8. Step-by-step optimisation OVAT 1.

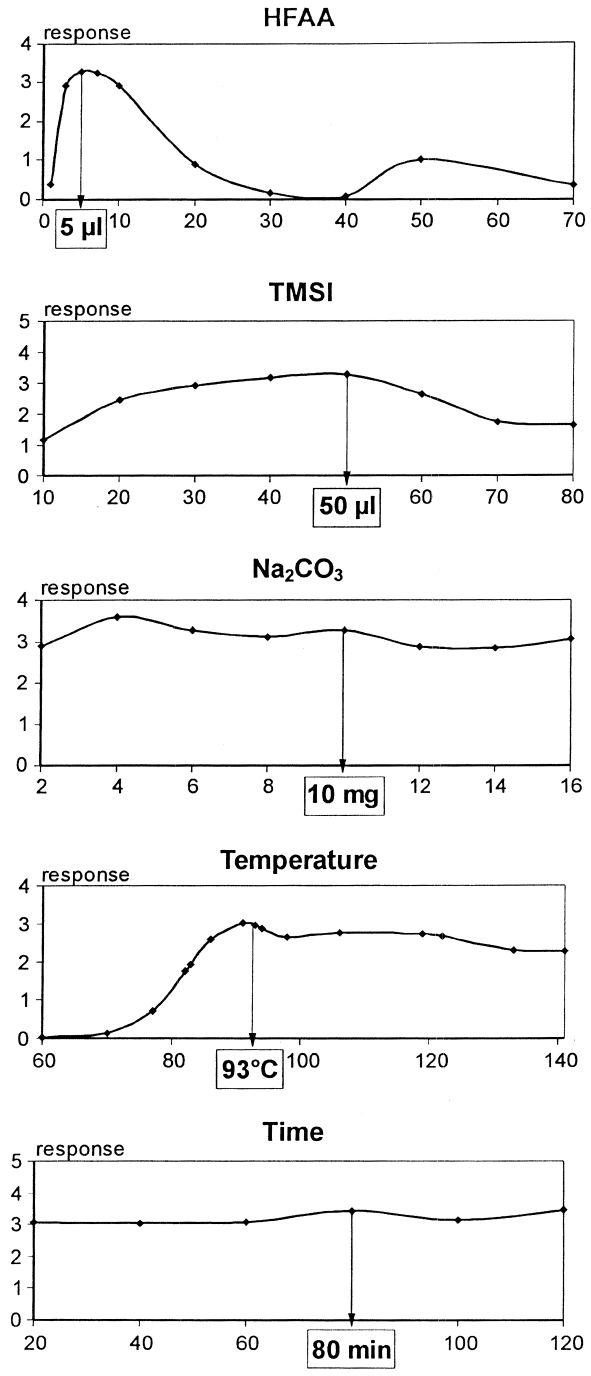

Fig. 9. Step-by-step optimisation OVAT 2.

Even contradictory results can be explained by having a closer look at the response surface plots. For example, both OVAT optimisations showed no significant influence of the amount of Na_2CO_3 on the yield of the derivatisation, while the screening design predicted a rather significant main effect for Na_2CO_3. The response surface plot of Na_2CO_3 vs. HFAA (Fig. 10) helps to explain these contradictory results. It becomes obvious, that the amount of Na_2CO_3 indeed does not have an effect when higher amounts of HFAA (e.g., 100 μl) are present. However, if only low amounts of HFAA are added (e.g., 50 μl), Na_2CO_3 does have a strong negative effect. This effect did not become apparent in the sequential optimisation OVAT 1, as the amount of HFAA was kept at a high level of 100 μl throughout the optimisation of the amount of Na_2CO_3. As the response surface model and OVAT 2 do not cover the same ranges, it is unfortunately impossible to use the response surface plots to explain why neither OVAT 2 detected a negative effect of Na_2CO_3.

The disadvantages of the step-by-step approach become apparent when comparing the results of the two step-by-step optimisations. Though the results of OVAT 1 and OVAT 2 seem to be consistent if considered separately, they deliver two very different sets of optimal conditions. This discrepancy can be explained by having a closer look at the interaction

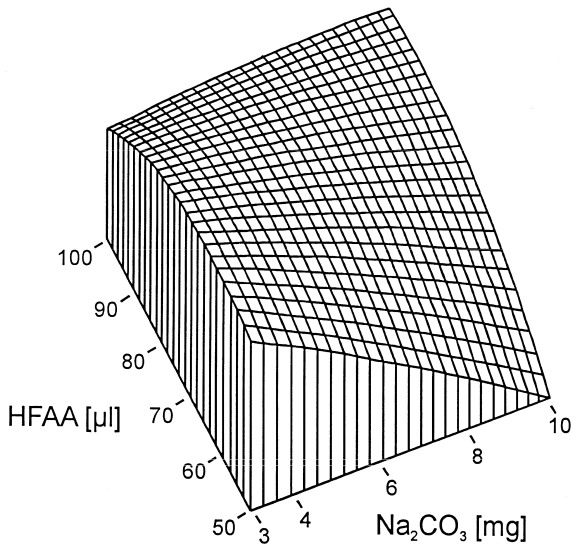

Fig. 10. Response surface plot Na_2CO_3 vs. HFAA.

effects. Both step-by-step methods cannot detect interaction effects, as for the optimisation of one parameter all other parameters are kept at a constant level. As a consequence, both models fail to take the interaction of HFAA and TMSI into account. This interaction was detected in the multivariate screening, however, and could be tracked down to the reaction of both reagents with each other. Bearing this in mind, the positive effect of this interaction could be interpreted as follows: if high amounts of HFAA are present, even higher amounts of TMSI are necessary to achieve silylation of the hydroxyl groups of dihydrostreptomycin. On the other hand, if low amounts of TMSI are present, only low amounts of HFAA may be added, as otherwise there are insufficient amounts of TMSI for the silylation of dihydrostreptomycin. In other words, a certain excess of TMSI is necessary. For OVAT 2 a rather low amount of 50 μl TMSI was added when optimising the amount of HFAA, so that, due to the interaction of both reagents, the optimum yield was achieved when very low amounts of HFAA were added.

Comparison of the optima detected with the different optimisation methods (Table 2) shows, that OVAT 1 delivered optimal conditions that are very similar to those obtained with the multivariate method. The yield of both optima is therefore comparable. However, OVAT 2 fails to detect this global optimum but delivers other optimal conditions which feature considerably lower yield. The main differences to the other two optima can be found in the amounts of Na_2CO_3, HFAA and TMSI. The failure to detect the global optimum is in this case clearly a consequence of the inability to detect the very important interactions of those parameters.

Another aspect worth mentioning when comparing multivariate and step-by-step methods is, that, although the multivariate method required a higher number of experiments than either of the OVAT procedures (see Table 2), it was still less time-consuming, as the experiments could be carried out simultaneously instead of sequentially.

The optimised derivatisation method (optimum obtained by multivariate optimisation) was tested for linearity between 0.1 and 100 μg of dihydrostreptomycin. Good linearity could be observed between 0.3 and 80 μg dihydrostreptomycin (slope 0.1334 ± 0.0010, intercept -0.0612 ± 0.0248, stan-

Table 2
Comparison of multivariate optimisation and step-by-step optimisations (OVAT 1 and 2)

	Multivariate method	OVAT 1	OVAT 2
No. of experiments	64 (26+28)	42	47
Optimum			
Na_2CO_3	3 mg	6 mg	10 mg
HFAA	67 μl	50 μl	5 μl
TMSI	169 μl	175 μl	50 μl
Temperature	78°C	90°C	93°C
Time	120 min	80 min	80 min
Response (normalised)	100%	88%	39%
Time	4 days	5 days	6 days

dard error 0.1323, correlation coefficient 0.9977, 15 data points, three replicates each). The coefficients of variation for the whole procedure (including derivatisation, extraction and GC analysis) were below 6% at all levels within this linear range. It turned out that the suggested GC–MS method for dihydrostreptomycin is very sensitive, the lower limit of the linear range equals approximately 600 pg of dihydrostreptomycin injected into the GC system. Considering the provisional MRL limits for dihydrostreptomycin of 1000 μg/kg kidney, 500 μg/kg muscle, liver or adipose tissue and 200 μg/kg milk [17], the method could even be suitable for use in residue analysis.

Finally, the developed derivatisation procedure was tested for the derivatisation of the aminoglycoside antibiotic streptomycin which could also be transferred to the corresponding derivative. Fig. 11 shows the chromatogram of a derivatised mixture of streptomycin and dihydrostreptomycin.

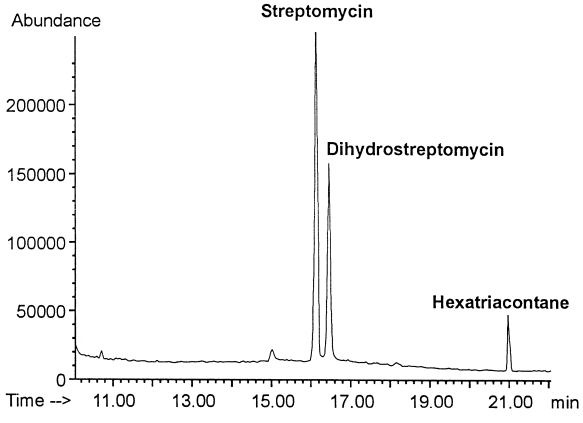

Fig. 11. Chromatogram of derivatised streptomycin and dihydrostreptomycin; GC conditions see Section 2.3, scan mode (50–650 u), the peaks correspond to 400 ng streptomycin and 100 ng dihydrostreptomycin.

5. Conclusion

A GC–MS method for the analysis of dihydrostreptomycin has been developed. The sensitivity of this method implies its suitability for residue analysis. The comparison of step-by-step and multivariate optimisation procedures made evident, that the latter are more powerful, more efficient and produce more reliable results. Requiring slightly more experiments but considerably less time, they provide a lot of additional information and perform a more thorough investigation of the influential parameters and their interactions. As a consequence, multivariate methods are more likely to detect the global optimum of a derivatisation reaction.

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