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Development of a gas chromatography–mass spectrometry method for the analysis of aminoglycoside antibiotics using experimental design for the optimisation of the derivatisation reactions

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Abstract

A packed column GC–electron-capture detection method for the analysis of the aminoglycoside antibiotics kanamycin and gentamicin was adapted to capillary GC–MS. The analytes were derivatised using a two-step procedure involving trimethylsilylation of the hydroxyl groups with trimethylsilylimidazole and acylation of the amino groups with heptafluorobutyrylimidazole. Electron impact mass spectra of the resulting derivatives of kanamycin A and gentamicins C_1, C_{1a} and C_2 are given and interpreted. The derivatisation procedure was optimised using experimental design. This chemometrical approach considers main effects as well as interactions of the influential parameters, thus conducting a more thorough investigation of the method than the common step-by-step approach. Optimisation using fractional factorial and Box Behnken Designs produced a derivatisation method featuring better yield than previously published methods while in many cases requiring less reagents and shorter reaction times.

Keywords: Chemometrics; Experimental design; Pharmaceutical analysis; Aminoglycosides; Antibiotics; Kanamycin; Gentamicin

1. Introduction

Kanamycin and gentamicin belong to the therapeutically important class of aminoglycosides, which are water-soluble broad spectrum antibiotics with a narrow therapeutic range. Kanamycin is composed of the main component kanamycin A and only small amounts of structurally related components like kanamycin B and C [1]. Gentamicin is a complex mixture consisting of three major components, C_1, C_{1a} and C_2, and several minor components [2] (Fig. 1).

The analysis of these aminoglycosides with chromatographic methods like high-performance liquid chromatography (HPLC) and gas chromatography (GC) is rather complicated due to their lack of chromophores and fluorophores and their insufficient volatility, so that usually pre- or post-column derivatisation is required.

Several gas chromatographic methods for the determination of aminoglycosides have been published, most of them employing trimethylsilylation to derivatise hydroxyl as well as amino groups [3–7]. This procedure, however, is not without drawbacks. Trimethylsilylation of primary amino groups often fails to produce one single product and is very

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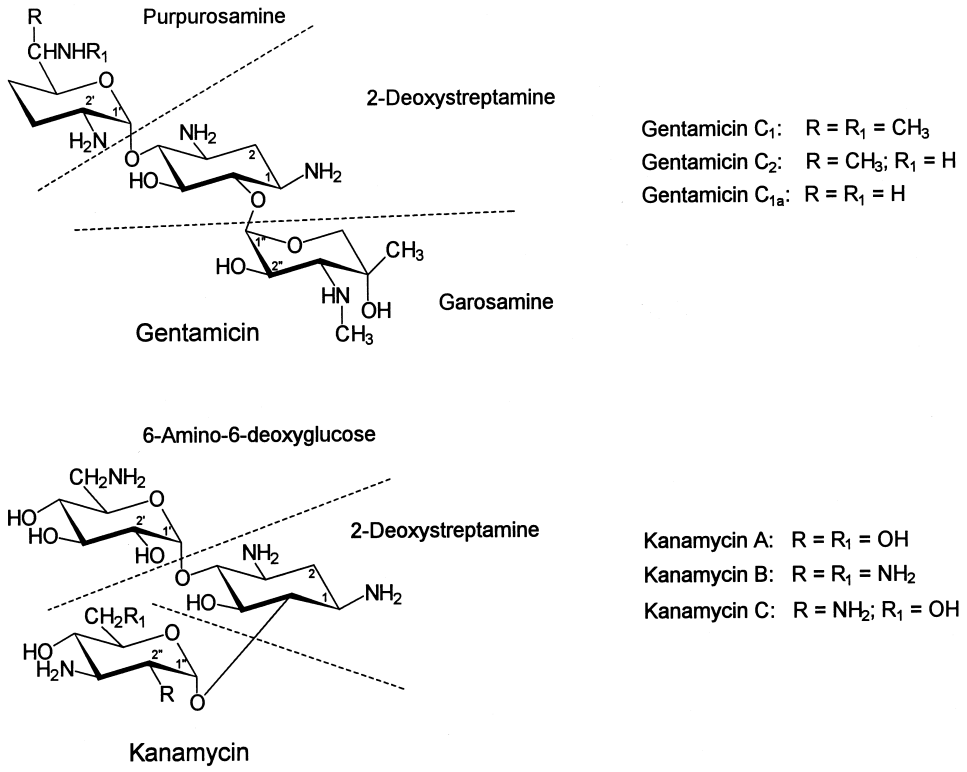

Fig. 1. Structures of the most important isomers of kanamycin and gentamicin.

sensitive even to minor changes in the derivatisation conditions. In addition, the resulting *N*-trimethylsilyl (TMS) derivatives are very susceptible to hydrolysis and, as a consequence, removal of excess silylation agent and extraction of the derivatives into an organic phase is often impossible. Direct injection of excess silylation agent, however, is not compatible with some GC detectors like, for instance, thermoionic specific detectors as the nitrogen–phosphorus detector. It may also adversely affect the performance of other detectors like the flame ionisation detector or the mass-selective detector by causing rapid deterioration of detector performance due to silicium dioxide deposits on the flame tip of a flame ionisation detector or in the ion source of a mass spectrometer.

Some authors also describe two-step derivatisation procedures involving silylation of the hydroxyl groups only and acylation of the amino groups. Whereas one of these procedures employing *S*-ethyl trifluorothioacetate proved to be rather time-consuming [4], a method first described by Mayhew and Gorbach [8] and later adopted by Nakaya et al. [9] using trimethylsilylimidazole (TMSI) for silylation of hydroxyl groups and heptafluorobutyrylimidazole (HFBI) for acylation of amino groups produced stable and sufficiently volatile derivatives in comparatively short time. Both use electron-capture detection (ECD), thus benefiting from the high number of halogen atoms being introduced into the analyte in the course of acylation. However, one usually tries to avoid using a highly selective detection method like ECD to detect functional groups that are not part of the analyte but of the derivatisation reagent used. This approach diminishes the potential selectivity of the ECD and frequently leads to methods that are very susceptible to interferences, e.g., from matrix components.

Our aim was therefore to adapt this ECD method to capillary GC–mass spectrometry (MS) to obtain a method which not only features good selectivity for the analytes but also provides additional information

in the form of mass spectral data useful in further confirming the identity of the analytes. Adapting the described method to GC–MS required adjustment of the derivatisation method to higher absolute amounts of analytes, for which both, the Mayhew and Gorbach as well as the Nakaya procedure delivered an unsatisfactory turnover. As in our own experiments Nakayas method, which requires extraction of the intermediate products between the two derivatisation steps, featured even worse results, we focused on Mayhew and Gorbachs method.

Consequently, we had to optimise the Mayhew and Gorbach method according to our requirements. For this purpose we did not rely on the step-by-step optimisation used for instance by Nakaya but employed a chemometrical experimental design. The approach of optimising a complex process by optimising one parameter after the other in a step-by-step procedure, while keeping all other potentially influential parameters at a constant level, is widely known as the OVAT-method (one-variable-at-a-time). Several aspects of this method must be considered critical: (i) interactions between variables cannot be detected and taken into account, (ii) only a very narrow range within all possible combinations of values is examined (Fig. 2), and (iii) the detected optimum is, as a consequence, in many cases not identical with the real optimum.

Using chemometrical experimental design on the other hand, each parameter can be examined and optimised in a predefined range by conducting a series of experiments in each of which the values for several parameters are changed at the same time [10–13].

Two major groups of experimental designs are important: screening designs and optimisation designs. Screening designs are used to determine which parameters have an effect on, for instance, the yield of a derivatisation reaction. This can be achieved by selecting a high and a low level for each parameter. Between those two levels the effects of the parameters can then be examined by conducting experiments with all combinations of high and low levels for all parameters. Fig. 3 shows an example of such a two-level full factorial design with three parameters. The results of the corresponding experiments are then statistically evaluated – usually by analysis of variance or by regression analysis. In that way the individual effect of each parameter and the interaction effect between any two or more parameters can be calculated and tested for significance (further details are given in Refs. [10–13]). Two-level full factorial designs require 2^k experiments if k parameters are to be studied. Consequently, the number of experiments increases rapidly with the number of parameters. However, the number of experiments can be kept low by employing fractional factorial designs. These are based on the assumption that interaction effects between three or more parameters are small compared to main effects and two-variable interaction effects. Thus it is possible to select a subset (or fraction) of the full factorial design and

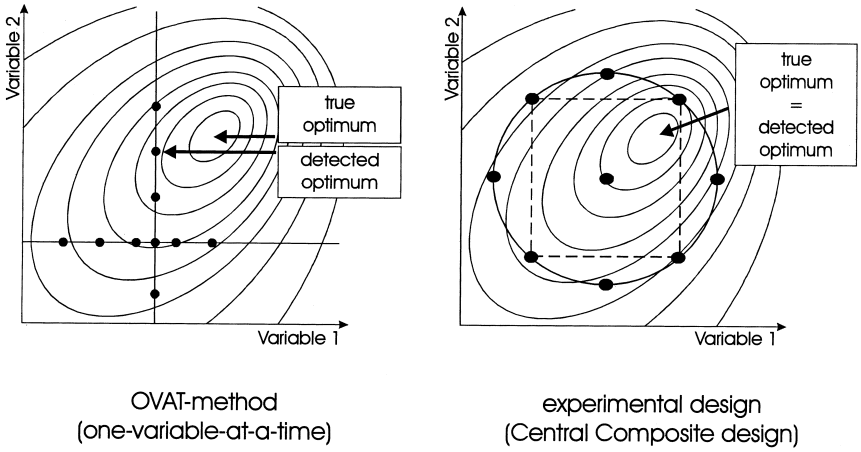

Fig. 2. Comparison of OVAT method and experimental design.

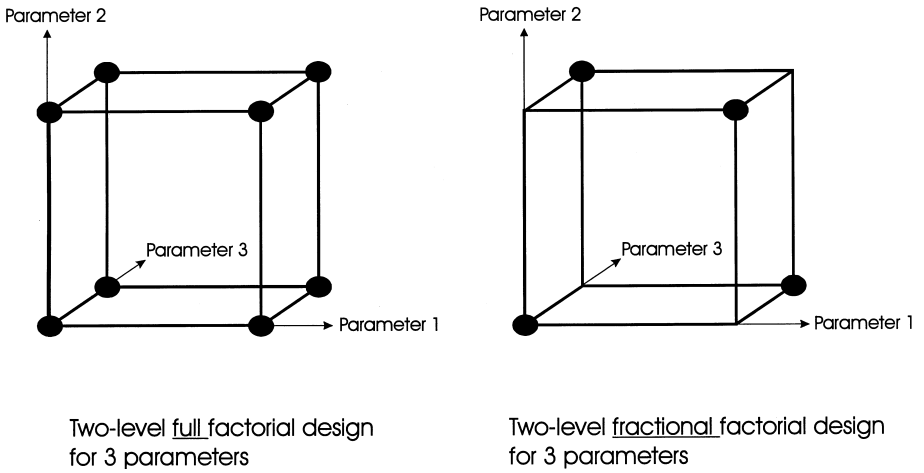

Fig. 3. Examples of screening designs: the black dots indicate the necessary experiments.

omit several combinations of parameters from the experimental plan (Fig. 3). The resulting fractional factorial design requires considerably fewer experiments at the expense of a certain loss of information – called confounding. Several effects are said to be confounded if the sum of these effects can be computed but it is not possible to determine mathematically the contribution of each effect to this sum. How much confounding a fractional factorial design involves is usually referred to as its resolution.

To determine the optimum conditions for a reaction on the other hand, one has to use optimisation designs which assume non-linear models. These so-called response surface models require at least three levels for each parameter. Fig. 4 shows two important types of optimisation designs. The central composite design (CCD) is based on a full factorial design which is augmented by centre points, i.e., experiments where all parameters are set to their mean values, and axial points. For a three parameter design as shown in Fig. 4 these axial points are located on a sphere surrounding the two-level fac-

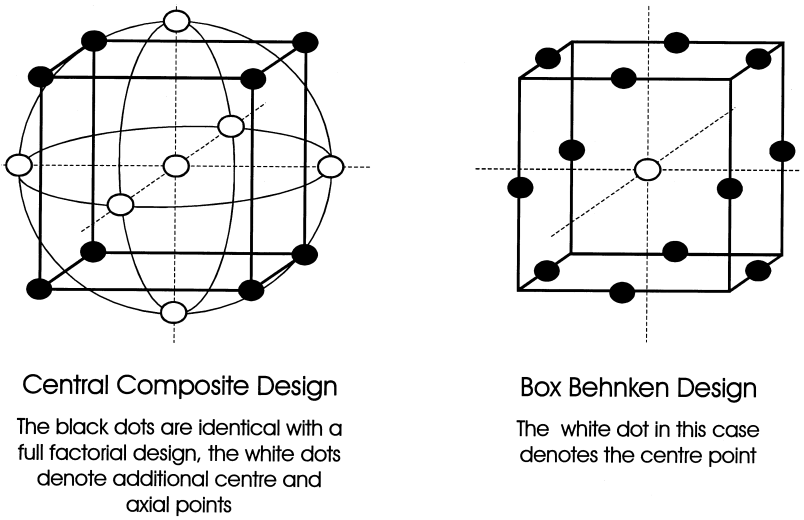

Fig. 4. Examples of optimisation designs.

torial design. A Box Behnken design is not directly based on a full factorial design as it uses middle points instead of corner points. The experimental plan is laid out according to the following pattern: when two (or three) parameters have a combination of their extreme levels, all others are set to their mean value. For a three-parameter Box Behnken design, for example, all experimental points are located on the edges of a cube around the centre points. Box Behnken designs require fewer experiments than CCDs but cover a slightly smaller experimental region. This can actually be an advantage if extreme situations have to be avoided. Based on the results of the experiments of the CCD or Box Behnken design experimental plan, a response surface model can then be computed, i.e., a polynomial regression equation which fits to the experimental data as well as possible. This response surface can be visualised as a three-dimensional response surface plot and can be used to determine the optimum conditions.

The number of experiments required for this kind of optimisation is not necessarily higher than for the common OVAT method and is usually substantially lower if several responses have to be optimised simultaneously. Planning and computing such experimental designs is facilitated by corresponding software programs.

2. Experimental

2.1. Materials

All solvents used were of analytical-reagent quality. Water was deionised, distilled twice and finally distilled for a third time over sodium permanganate to remove traces of organic compounds. Gentamicin sulfate and kanamycin (acid) sulfate were supplied by Serva (Heidelberg, Germany). The derivatisation agents TMSI and HFBI were obtained from Pierce (Rockford, IL, USA), as was silylation grade pyridine. The internal standard hexatriacontane ($C_{36}H_{74}$) was obtained from Fluka (Buchs, Switzerland). Thin-layer chromatography (TLC) detection reagent fluorescamine was obtained from Serva.

2.2. Derivatisation procedure

The derivatisation procedure described by Mayhew and Gorbach consists of a silylation of hydroxyl groups with TMSI in pyridine and a subsequent acylation of amino groups with HFBI. After the derivatisation, the derivatives are extracted into heptane which can be directly injected into the GC system.

In the course of our optimisation experiments we varied the amounts of pyridine, TMSI and HFBI as well as the respective reaction temperatures and reaction times, while keeping all other parameters constant. Derivatisation was carried out in 4-ml round-bottomed reaction vials (WGA, Düsseldorf, Germany) fitted with screw-caps. One hundred μl of an aqueous stock solution containing 200 μg of kanamycin and gentamicin each were evaporated to dryness under nitrogen at 60°C in a heating block. To the dry residue pyridine[1] and TMSI[1] were added and after 3 min ultrasonication the reaction vial was transferred to a heating block[1]. After removing the vials from the heating block, the mixture was allowed to cool down to ambient temperature for 5 min. Then HFBI[1] was added and after thorough shaking, the vial was transferred to the heating block[1] again. After 5 min cooling, 500 μl of the internal standard solution containing 0.21 mg/ml hexatriacontane in heptane and, after mixing by shaking, 1000 μl of water were added. Thus the derivatives could be extracted into the heptane phase while, at the same time, the added water reacted with excess reagent to water-soluble compounds which were removed with the aqueous phase. After vortex-mixing and centrifugation, the organic phase was transferred into a 1-ml septum-capped vial and 1 μl 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 mass-selective detec-

[1]Varying amounts, temperatures and times were used; see Table 1.

tor working in electron ionisation (EI) mode with 70 eV. The derivatised aminoglycosides were separated on a 0.1 μm HP Ultra-1 fused-silica cross-linked methylsilicone gum capillary column (Hewlett-Packard), 14 m×0.2 mm I.D. or 50 m×0.2 mm I.D. and a 0.2 μm OV-1 LV methylsilicone column from WGA with 50 m×0.25 mm I.D. The transfer line temperature was 310°C and the oven temperature programme was 108°C for 2 min, ramping at 50°C/min to 200°C, subsequently at 5°C/min to 240°C and finally at 2°C/min to 260°C. Helium (>99.999% purity) was used as carrier gas with an initial column head pressure of 0.88 bar at 108°C for the 14 m column resulting in a flow of 1.0 ml/min equalling 54.4 cm/s linear velocity and 0.90 bar for the 50 m column (0.7 ml/min; 24.1 cm/s). 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.

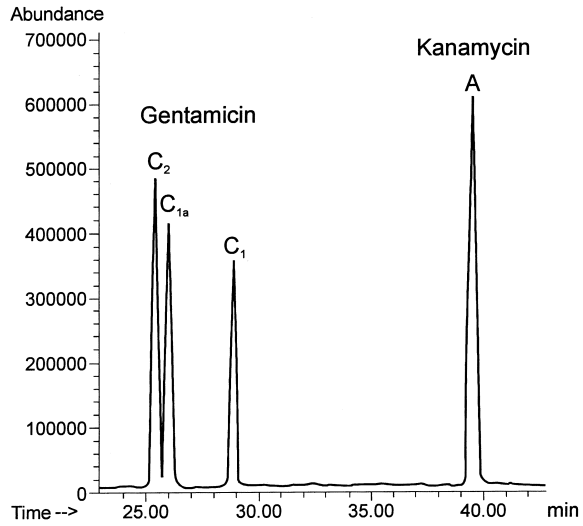

Fig. 5. Gas chromatogram of gentamicin and kanamycin derivatives (gentamicin signals are corresponding to approximately 100 ng each, kanamycin signal to 300 ng); column 50 m×0.25 mm I.D., d_f=0.25 μm, constant flow=0.7 ml/min, oven program 108°C for 2 min, 50°C/min to 200°C, 5°C/min to 240°C, 2°C/min to 260°C, hold 15 min.

2.4. Identification of the gentamicin isomers

Commercial samples of kanamycin are mainly composed of kanamycin A with only negligible amounts of the structurally related isomers kanamycin B and C present, usually amounting to less than 3% for the B isomer and less than 1% for the C isomer [1,14]. Gentamicin however consists of three major components, C_1, C_{1a} and C_2, which may be present in almost equal amounts. Commercial preparations contain 30–45% C_1, 12–30% C_{1a} and 19–43% C_2 [2,15]. Using the GC conditions described above and a 50 m methylsilicone capillary column, derivatised gentamicin complex gave three peaks (Fig. 5).

These peaks could be identified as the corresponding isomers by interpretation of their mass spectra which displayed characteristic mass fragments in the higher mass range of m/z 350 to 630 that could be assigned to the different purpurosamine structures of the isomers (Fig. 6).

These results could be confirmed by injecting the isolated isomers which were obtained by separating the isomers from the gentamicin complex using a TLC method described by Wilson et al. [16]. The zones of the silica gel TLC plate with the respective isomers were scraped off and the isomers eluted by adding a solvent system similar to the one used for separation (lower layer of a mixture of methanol, chloroform and ammonium hydroxide 25%, dried with anhydrous sodium carbonate) and stirring for 24 h. After filtration and evaporation of the solvent the isolated isomers were ready for confirmatory TLC and GC experiments. For the optimisation experiments we used a capillary column of only 14 m column length to render GC analysis less time-consuming, at the expense of coelution of the C_2 and C_{1a} isomers.

2.5. Interpretation of mass spectra and development of MS method

Developing a selected-ion monitoring (SIM) method for the mass-selective detector required interpretation of the mass spectra of the derivatised analytes. Although fragmentation pathways for underivatised kanamycin and gentamicin as well as some of their derivatives have been published [5,17–

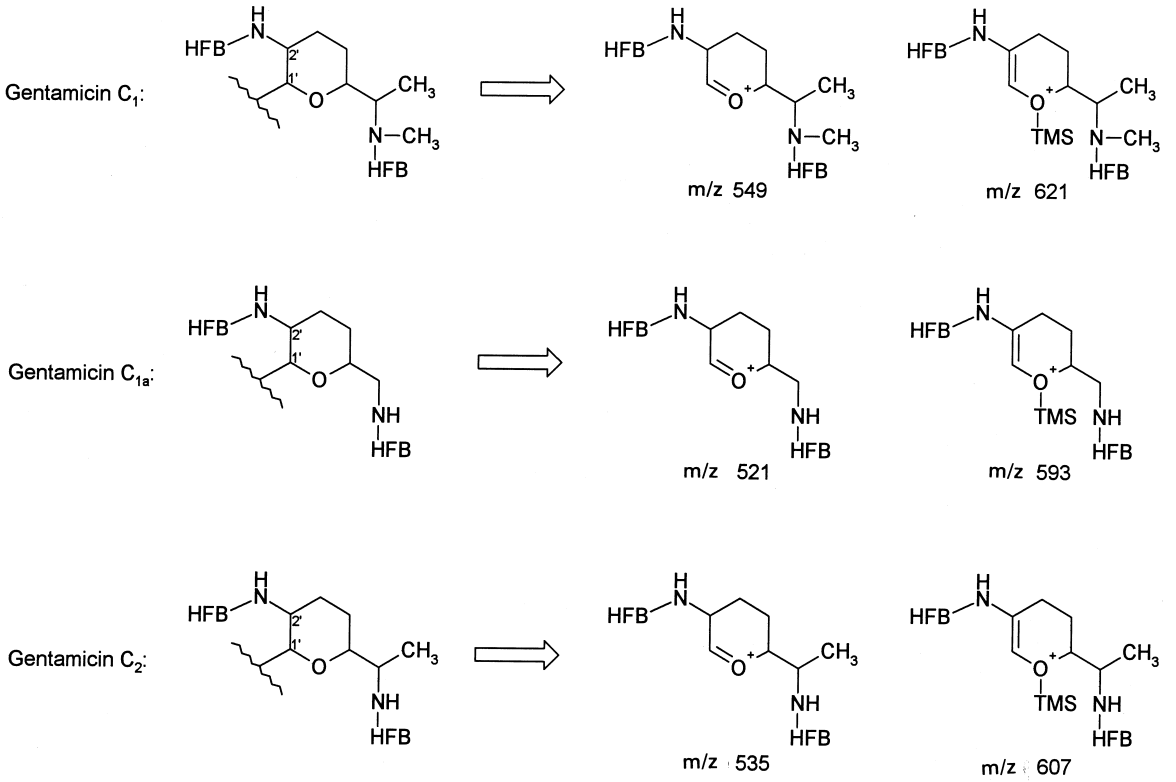

Fig. 6. Some characteristic fragment ions of the different gentamicin isomers.

20] the fragmentation of their *N*-heptafluorobutyryl-*O*-trimethylsilyl derivatives has to our knowledge not been published yet.

Due to the limited mass range of the benchtop MS system used, we could not detect the molecular ions. However the fragment ions from the 2-deoxystreptamine ring as well as from both respective sugar moieties provided sufficient evidence for the compounds to be identified as the expected *N*-heptafluorobutyryl-*O*-trimethylsilyl derivatives (Figs. 7 and 8). The fragmentation pathways are dominated by glycosidic cleavages and subsequent losses of acylated or silylated functional groups with occasional transfers of TMS groups.

On this basis we were able to select three ions of medium to high abundance per analyte, each of which was specific for a different sugar moiety (see Figs. 7 and 8), so that in the course of our optimisation experiments we could ensure, that the produced derivative was still the desired one. Regarding gentamicin, however, we avoided including fragments from the purpurosamine moiety, so that all three isomers could be covered by the same fragment ions.

3. Experimental design and optimisation

3.1. Screening design

The optimisation of the derivatisation procedure described by Mayhew and Gorbach was carried out in two steps using experimental design. 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. Seven variables were included in the screening design, notably the amounts of pyridine, TMSI and

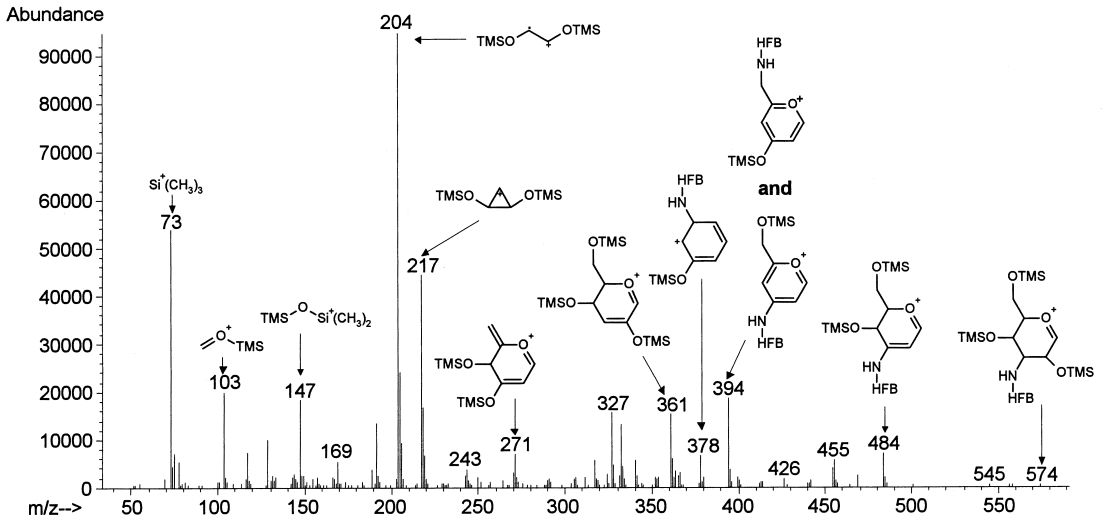

Fig. 7. Mass spectrum of N-HFB-O-TMS-kanamycin A derivative including the structures of some important mass fragments. Fragments 361 (corresponding to the 3-amino-3-deoxyglucose moiety), 378 (deoxystreptamine moiety) and 394 (6-amino-6-deoxyglucose and 3-amino-3-deoxyglucose moiety) were selected for SIM mode.

HFBI, the temperatures of the silylation and acylation steps and the reaction times allowed for those two steps. The variables and their respective ranges, which were defined basing on the conditions described by Mayhew and Gorbach are listed in Table 1. Responses were the peak areas of the kanamycin and gentamicin derivatives in the resulting gas chromatogram. These areas were normalised using the internal standard hexatriacontane to correct for variations in injection volume and, more important, variations in the resulting volume of the organic phase after extraction due to varying amounts of reagents added. The screening design used was a two-level fractional factorial design with resolution IV, i.e., main effects are confounded with three-variable interaction effects, the latter in most cases

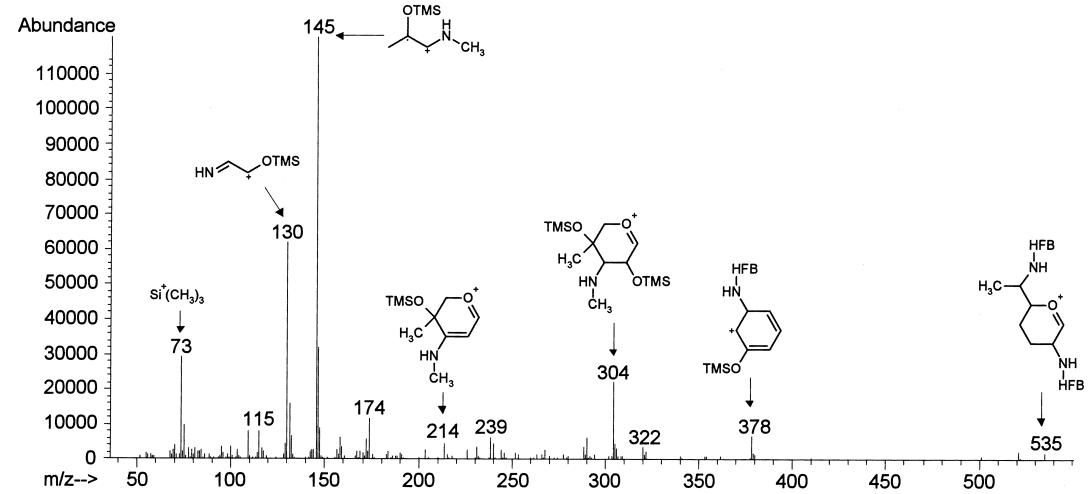

Fig. 8. Mass spectrum of N-HFB-O-TMS-gentamicin C_2 derivative including the structures of some important mass fragments. Fragments 145 (corresponding to the garosamine moiety), 304 (garosamine moiety) and 378 (2-deoxystreptamine moiety) were selected for SIM mode.

Table 1
Parameters and range selected for the screening design

	Variable	Mayhew and Gorbach method [8]	Screening
A	Amount of pyridine	100 μl	10–500 μl
B	Amount of TMSI	100 μl	10–250 μl
C	Silylation temperature	41–43°C	30–100°C
D	Silylation time	7–10 min	5–120 min
E	Amount of HFBI	50 μl	10–250 μl
F	Acylation temperature	41–43°C	30–100°C
G	Acylation time	7–10 min	5–120 min

being negligible compared to the main effects, and the two-variable interaction effects are confounded with each other three by three [10]. Thus the number of experiments for the screening design was reduced to 34 experiments (16 analyses, two replicates each and two additional centre points).

As can be seen in Fig. 9, all variables of the silylation, i.e., the amount of TMSI, the silylation temperature and the respective reaction time, had a significant positive main effect on the yield of both, derivatised kanamycin and derivatised gentamicin. This indicates that their values should be set to a high level to achieve good yield. Regarding the acylation step, all variables displayed significant negative main effects for both analytes, so that their values should rather be set to low levels. Only the amount of pyridine appeared to have different effects on kanamycin, where its influence was not significant, and gentamicin, where it showed a positive influence.

The interactions of these variables are summarised in Fig. 10. As three interactions are confounded, the observed effects are the sums of those three single interactions. However, in many cases the contribution of one interaction dominates, while the other two are negligible. The dominating interaction can in some cases be identified with the help of analytical background knowledge.

For instance for kanamycin the interaction AF (amount of pyridine and acylation temperature) is confounded with BE (amount of TMSI and amount of HFBI) and CD (silylation temperature and silylation time). Considering the strong negative main effect of the acylation temperature (F) and the non-significant main effect of the amount of pyridine (A), a very significant negative interaction AF of these

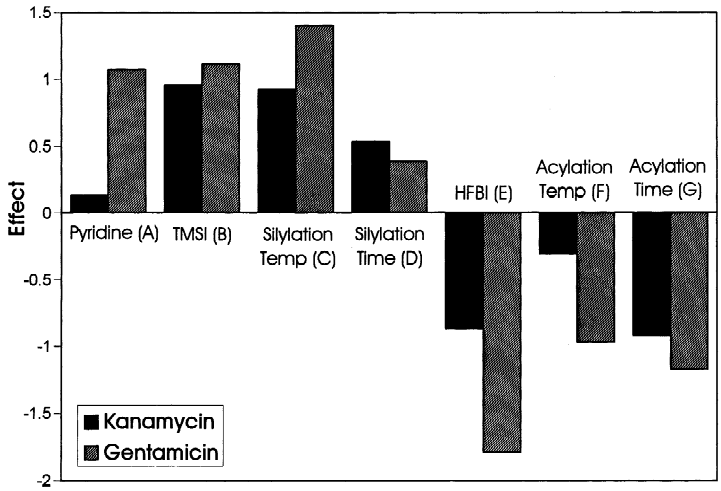

Fig. 9. Results of the screening design: main effects of the seven screened variables on kanamycin and gentamicin (effect: average change in the response value – in this case the area response from the GC–MS – when the design variable goes from its low to its high level).

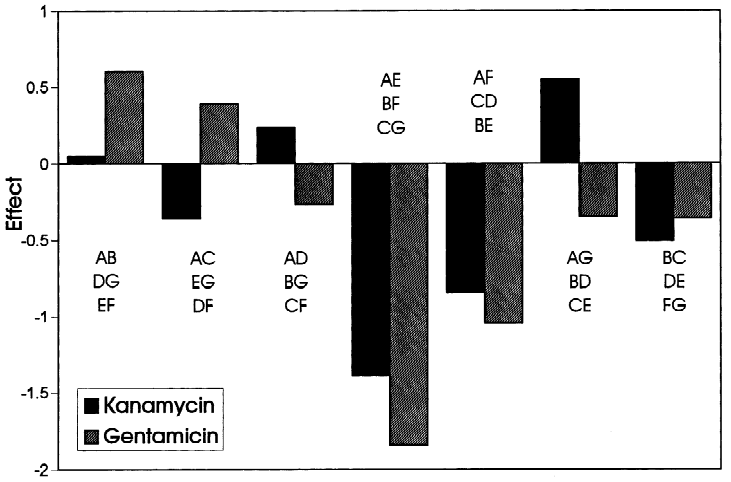

Fig. 10. Results of the screening design: interaction effects of the seven screened variables (effect: average change in the response value – in this case the area response from the GC–MS – when the design variable goes from its low to its high level).

two variables seems highly unlikely. Regarding the mechanism of the derivatisation reaction, an interaction between the amount of TMSI (B) and the amount of HFBI (E) can also be considered as rather insignificant. Thus the dominating influence in this case in all probability derives from the interaction of the silylation temperature (C) and the silylation time (D). The observed negative effect of this interaction indicates that, although considered separately, the silylation time and the silylation temperature have positive main effects and should consequently be set to high values, they should not both be set to high values at the same time. In other words, if a high silylation temperature is chosen, a short reaction time is sufficient to produce high yield, and vice versa.

The results of the screening design can be summarised in the following way:

(i) In most cases the influence on kanamycin and gentamicin was similar.

(ii) All variables of the silylation had positive main effects, i.e., their values should be high to achieve good yield.

(iii) Some variables of the silylation, however, had negative interactions, i.e., both variables should not be set to high values at the same time.

(iv) All variables of the acylation had negative main effects, i.e., they should be set to low values.

(v) Most variables of the acylation also had negative interactions, i.e., they can both be set to low values at the same time.

(vi) The interaction of the amount of HFBI (E) and the acylation temperature (F) however had a positive effect for gentamicin, i.e., if the amount of HFBI is set to a low value the reaction temperature should not be set too low.

3.2. Optimisation

The parameters for the optimisation could then be selected based on the results of the screening. It is advisable to keep the number of parameters for an optimisation design low to avoid very complex response models with a high amount of variability. Keeping this in mind we set the amounts of pyridine, TMSI and HFBI to fixed values in accordance with their main effects and interactions. Pyridine did not display a significant main effect for kanamycin and had a positive effect on gentamicin, but almost all of the confounded two-variable interactions involving pyridine showed negative effects, so we decided to set it at a medium level of 200 μl. TMSI had strong positive main effects but in most cases negative interactions with silylation time and silylation temperature. This indicates that if a larger amount of TMSI is selected (we chose 150 μl) the temperature and time required for the silylation can be kept low.

HFBI featured strong negative main effects for both analytes, while its interactions were mostly positive and less significant, so we fixed it at a rather low level of 10 μl. For the remaining four variables the ranges for the optimisation could be narrowed down. The range for the silylation temperature was set at higher values from 60 to 120°C, so that the range for the corresponding reaction time could be set at lower values from 1 to 10 min, taking into account the negative interaction of those two variables. In a similar way the range for the acylation temperature was set to low values of 20 to 60°C, while the range for the corresponding reaction time was set at higher values of 30 to 120 min. To avoid non-linearities due to the relatively wide ranges of variation of some variables, we transformed the values for the reaction times using the square root when we devised the experimental plan. Using The Unscrambler, the optimal conditions within these ranges were determined via a Box Behnken design, which required 81 experiments (28 analyses, three replicates each, and nine additional centre points). Table 2 shows an excerpt from the corresponding experimental plan; the required experiments were conducted in randomised order to avoid systematic errors.

Using response surface analysis based on multiple regression and analysis of variance, we first computed two separate models for kanamycin and gentamicin. The resulting models describe the experimental data quite well considering the complexity of the whole design. Using the respective response surfaces for the silylation and acylation steps, the conditions delivering the highest yield could then be determined for kanamycin and gentamicin separately. Fig. 11 shows the response surface plot for the acylation of kanamycin as an example. The resulting optimal conditions were as follows: kanamycin: silylation at 65°C for 10 min, acylation at 38°C for 57 min; gentamicin: silylation at 73°C for 10 min, acylation at 47°C for 30 min.

By combining the response surfaces for kanamycin and gentamicin it was finally possible to determine the optimal conditions delivering the highest possible yields for kanamycin as well as for gentamicin in a simultaneous analysis. The common optimum was detected at 70°C and 10 min for the silylation and 43°C and 36 min for the acylation.

4. Results and discussion

The final model was checked by several confirmatory experiments. The results showed good consistency with the predictions of the model as can be seen in Table 3.

Comparing the yield of the optimised method to

Table 2
Excerpt from the experimental plan of the selected Box Behnken design

Experiment No.	Silylation temperature (°C)	Silylation time ($\sqrt{}$min)	Acylation temperature (°C)	Acylation time ($\sqrt{}$min)
001	60	1	40	8.215
002	120	1	40	8.215
003	60	3.16	40	8.215
004	120	3.16	40	8.215
005	60	2.08	20	8.215
006	120	2.08	20	8.215
007	60	2.08	60	8.215
008	120	2.08	60	8.215
.	.	.	.	.
.	.	.	.	.
.	.	.	.	.
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023	90	2.08	20	10.95
024	90	2.08	60	10.95
Centre point	90	2.08	40	8.215

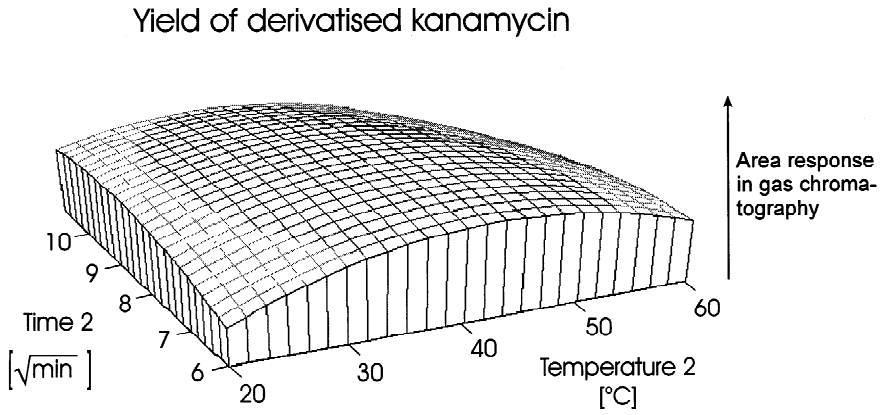

Fig. 11. Response surface plot for the acylation of kanamycin.

the one we achieved with the Mayhew and Gorbach method, we observed a significant increase in yield with the optimised method for higher amounts up to 200 μg of analyte by a factor of 3 for kanamycin and by a factor of 4 for gentamicin.

We also performed quantitative TLC experiments based on the method used for the isolation of the isomers to check the aqueous phase of the reaction mix for residues of non-derivatised analytes. For these experiments we used HPTLC silica gel 60 plates (Merck, Darmstadt, Germany) and fluorescamine, which is a more sensitive detection reagent for kanamycin and gentamicin than ninhydrin. Detection was carried out by dipping the plate for 10 s into a solution of 10 mg fluorescamine in 100 ml acetone and then allowing it to dry at ambient temperature. Using this procedure we determined a limit of detection of approximately 5 ng for both non-derivatised analytes. We examined three different concentrations which were subjected to derivatisation, corresponding to absolute amounts of 2 μg, 20 μg and 200 μg of kanamycin and gentamicin each. We could not detect non-derivatised gentamicin or kanamycin in the aqueous phase (1 μl and 10 μl spotted) obtained after derivatisation with the optimised method at any level examined. Using the Mayhew and Gorbach method we could detect between 5 and 7.5% non-derivatised analytes. With respect to the 3- to 4-fold increase in yield of the derivatised analytes, this leads to the conclusion, that the major part of the analytes was only partially derivatised when we applied the Mayhew and Gorbach method. These postulated incompletely derivatised analytes could not be detected with the TLC and GC methods used.

Finally, we even ended up with an optimised method that showed better turnover than both, the method described by Mayhew and Gorbach and the

Table 3
Results of the confirmatory experiments: comparison of measured area responses in GC–MS with responses predicted by the computed response surface models

	Kanamycin		Gentamicin	
	Predicted	Measured	Predicted	Measured
Common optimum 70°C/10 min – 43°C/36 min	4.20	3.95	7.14	6.88
Optimum kanamycin 65°C/10 min – 38°C/57 min	4.25	4.02	–	–
Optimum gentamicin 73°C/10 min – 47°C/30 min	–	–	7.27	6.98

Table 4
Comparison of the previously published methods and the optimised method

	Mayhew and Gorbach	Nakaya et al.	Optimised method
Pyridine	100 μl	400 μl	200 μl
TMSI	100 μl	200 μl	150 μl
Silylation temperature	41–43°C	80°C	70°C
Silylation time	7–10 min	30 min	10 min
HFBI	50 μl	200 μl	10 μl
Acylation temperature	41–43°C	80°C	43°C
Acylation time	7–10 min	30 min	36 min

Nakaya method. The main feature of the optimised method is that a higher reaction temperature saves reaction time and considerable amounts of expensive reagent (Table 4).

Finally, the optimised method was tested for linearity between 2 and 200 μg of kanamycin and gentamicin, respectively. Good linearity could be observed for kanamycin between 10 μg and 200 μg (slope 0.0438 ± 0.0007, intercept -0.4646 ± 0.0627, standard error 0.1298, correlation coefficient 0.9981, 10 data points), and for gentamicin between 30 and 200 μg (slope 0.0398 ± 0.0009, intercept -1.2027 ± 0.1000, standard error 0.1401, correlation coefficient 0.9974, 7 data points). The coefficients of variation for the whole method (including derivatisation, extraction and GC analysis) were below 7% at all levels within those linear ranges.

5. Conclusions

The GLC method described by Mayhew and Gorbach could easily be adapted to capillary GC with on-column injection. Replacing ECD by MS, however, resulted in an unusual loss of sensitivity due to the high molecular masses of the derivatised analytes of more than 1200 and the limited mass range of most benchtop mass-selective detectors, usually featuring upper limits around 800–1000. In addition the mass spectra obtained with EI at 70 eV featured a multitude of fragment ions of rather low abundance so that in this case even in SIM mode, MS detection could not compete with ECD in matters of sensitivity. However, using MS offers the advantage of delivering additional spectroscopic data thus allowing the identification of the analyte.

Experimental design proved to be a very powerful and efficient tool for optimising derivatisation reactions. It usually requires hardly more experiments (in this case 115 experiments for screening and optimisation compared to a minimum of 105 experiments which would be necessary for step-by-step optimisation of seven variables at five levels each, including three replicates of each experiment) but provides a lot of additional information. The most important advantage certainly is that experimental design performs a more thorough investigation of the influential parameters and their interactions than the step-by-step approach, thus being more likely to produce the real optimum. Furthermore experimental design combined with response surface analysis is the only way to ensure that a clear picture can be obtained from confusing individual experimental results with high variability.

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