Use of Chemometric Techniques to Design a Microbiological Method for Sulfonamide Detection in Milk

Orlando G. NAGEL[1], Maria PILAR MOLINA[2] and Rafael L. ALTHAUS[1]

[1]Department of Basic Science, Faculty of Veterinary Sciences, National University of Litoral, Esperanza, Argentina; [2]Department of Animal Science, Polytechnic University of Valencia, Valencia, Spain

Abstract

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We proposed an experimental design of a microbial bioassay of dichotomous response (positive or negative) using *Bacillus subtilis* BGA for the detection of sulfonamide residues. In the first stage, the bioassay response time was reduced to 6 h by increasing the spore concentration of *B. subtilis*. Then, the effects of spore, indicator, trimethoprim (TMP) concentration, and volume of the culture medium were examined with a Plackett Burman design (2^{4-1}). Finally, the effect of TMP concentration on the method detection capabilities and specificity was analysed using a logistic model with interaction. The detection capabilities of sulfonamides in milk are close to the MRLs when using 500 µg/l of TMP in the culture medium of the bioassay. It is concluded that the experimental design techniques and a logistic regression model can be used to design successfully a dichotomous response bioassay.

Keywords: *B. subtilis*; microbial inhibition method; bioassay; experimental design; logistic regression model

Sulfonamides (SAs) are used to treat a wide variety of bacterial and protozoal infections in animals (Lindsay & Blagburn 1995). The presence of these antimicrobials can produce a risk for consumers' health (Haagsma *et al.* 1989) and technological problems for the dairy industry (Berruga *et al.* 2007).

To prevent these problems and to control the presence of sulfonamide residues in foods, European Union authorities have established a Maximum Residue Limit (MRL) of 100 µg/l for sulfonamide residues in milk (Regulation UE 2010).

For the purpose of monitoring the presence of these residues, microbiological methods employing *G. stearothermophilus* are used (Pikkemaat 2009). Several authors (Nouws *et al*. 1999; Althaus *et al.* 2009) have proposed the use of a Microbiologic Multiplate System (MMPS) with Petri dishes to identify correctly the antimicrobial families in milk. *Bacillus subtilis* is used as the test strain for the detection of sulphonamides as being sensitive enough (Haagsma *et al.* 1989). However, the analysis done in Petri dishes involve long incubation times to identify SAs (18–24 h) and require personnel trained to work in microbiology.

For these reasons, Nagel *et al.* (2009a,b, 2011) proposed the use of chemometric techniques for the design of bioassays in microtiter plates to replace Petri dishes in microbial systems. These bioassays are easy to implement in laboratories for the analysis of large amounts of samples because they show a dichotomous response of simple interpretation (positive or negative) in a shorter response time.

Therefore, the objective of this study was to apply experimental design techniques and a lo-

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gistic regression model to optimise the bioassay in microtiter plates using *B. subtilis* which detects sulfonamide residues at levels similar to MRLs, and which replaces the Petri dishes used in MMPS.

MATERIAL AND METHODS

Relation between spore level and response time. Müeller Hinton culture medium (38 g/l, Merck®, Ref.105437) fortified with glucose (10 g/l; Sigma Aldrich®, Ref. G7528) and a mixture of indicators 2,3,5-trifenyltetrazolium chloride (150 mg/l; Sigma Aldrich®, Ref. T8877), toluidine blue (15 mg/l; Sigma Aldrich®, Ref. 198161), and spores suspension of *B. subtilis* BGA (Merck®, Ref. 1.10649) were employed.

Twelve microtiter plates were prepared randomly to evaluate the duplicates of six concentrations of *B. subtilis* (1.6×10^3, 8.0×10^3, 4.0×10^4, 2.0×10^5, 1.0×10^6, and 5.0×10^6 spores per ml).

To analyse the dose-response curve of the bioassays, 16 replicates of 12 sulfathiazole (Sigma Aldrich®, Ref. S0127) concentrations (STZ: 0, 1, 2, 4, 6, 8, 10, 12, 14, 16, 18, and 20 mg/l) were tested (Codex Alimentarius 2010). To each individual well, 50 µl of milk sample fortified with sulfathiazole were added. The microtiter plates were incubated in a water bath at 37 ± 1°C until the colour change of the indicators.

Visual interpretation was performed independently by three trained persons and was assessed visually as "negative" (pink) or "positive" (blue); "doubtful" (predominantly blue) qualifications were interpreted as positive.

To analyse the results, the logistic regression of SAS® (SAS 2001) was used according to the following statistical model:

$$L_{ijkl} = \text{logit}\, P_{ijkl} = \beta_0 + \beta_1 \text{STZ}_j + \beta_2 \text{Log}\, S_i + \beta_3 \text{Log}\, S_k^2 + \varepsilon_{ijkl} \quad \text{(I)}$$

where:

L_{ijkl} – linear logistic model

P_{ijkl} = logit $[Pp/(1 - Pp)]$; probability of "positive" response/probability of "negative" response)

β_0 – intercept

β_1, β_2, β_3 – estimated parameters for the model

STZ_i – sulfathiazole concentration (i = 12)

$\text{Log}\, S_j$ – logarithmic transformation of the spores concentration

$\text{Log}\, S_k^2$ – square of the logarithmic transformation of the spores concentration

ε_{ijkl} – residual error

The detection capability (CC_β) was calculated as the concentration at which the analyte can be quantified with an error probability of β = 0.05 (Codex Alimentarius 2010).

Exploratory study of factors by fractional factorial design. The effects of log spores concentration (Log S = 6–6.7), Triphenyltetrazolium/Toluidine blue indicator (I = 150/15–180/18 mg/l), Trimethoprim (TMP = 0–100 µg/l), and the volume of the culture medium (V = 100–120 µl) were analysed using a Plackett Burman design (2^{4-1}) with replication. To this end, duplicates of eight plates were prepared according to the fractional factorial design.

Then, 16 replicates of the 12 sulfathiazole concentrations (STZ = 0, 0.25, 0.5, 1, 2, 4, 6, 8, 10, 12, 14, and 16 mg/l) were analysed. The bioassays were incubated in a water bath at 37 ± 1°C until a change in the negative control.

The statistical analysis of the effects of each factor on the dose-response curve of sulfathiazole was performed using the following linear regression model:

$$L_{ijk} = \text{logit}\, P_{ijk} = \beta_0 + \beta_1 \text{STZ}_i + \beta_2 I_j + \beta_4 \text{Log}\, S_j + \beta_5 \text{TMPj} + \beta_6 V_j + \varepsilon_{ijk} \quad \text{(II)}$$

where:

I_j – indicator concentration

STZ_i – sulfathiazole concentration (i = 12)

TMP_j – trimethoprim level

V_j – volume of medium culture

ε_{ijk} – residual error

Effect of trimethoprim concentration on the specificity and CC_β of sulfonamides in milk. A Müeller Hinton (38 g/l) culture medium with glucose (10 g/l) and a combination of indicators (150 mg/l of triphenyltetrazolium and 15 mg/l of toluidine blue) was inoculated with 10^7 spores/ml of *B. subtilis* BGA. The culture medium was divided into aliquots which were fortified with TMP solution to obtain the following concentrations:

Specificity: TMP = 0, 200, 300, 400, 500, and 600 µg/l. There were four plates for each level of TMP (24 microplates).

Detection capabilities: TMP = 0, 200, 300, 400, and 500 µg/l. There were twelve plates for each level of TMP (60 microplates).

Specificity study: For this study, 192 antimicrobial-free milk samples were analysed in duplicate using the bioassays previously prepared with each TMP level, in accordance with the Codex Alimentarius (2010). For this purpose, 50 µl of

milk samples were added into each individual microplate well. Each microplate was floated in a water bath and then incubated at 37 ± 1°C for 6 hours. The samples were also analysed simultaneously by the Delvotest® method (DSM, Delft, the Netherlands), which is considered a reference method (AOAC 2000).

For each bioassay, specificity was calculated as:

$$\text{Specificity (\%)} = \left(1 - \frac{\text{positive samples}}{\text{total samples}}\right) \times 100 \quad \text{(III)}$$

A screening method has an adequate specificity if the alpha error is less than or equal to 5%.

Detection capabilities (CC_β) study. Milk samples were fortified with different sulfamides (Sigma Aldrich®, St. Louis, USA): sulfadiazine (Ref. S-8626), sulfadimethoxine (Ref. S-7385), sulfamerazine (Ref. S-8876), sulfamethoxazole (Ref. S-7507), sulfathiazole at the following concentrations: 0, 25, 50, 75, 100, 150, 200, 250, 300, 350, 400, and 500 µg/l, and sulfamethazine (Ref. S-5637) to levels 0, 50, 100, 150, 200, 250, 300, 350, 400, 500, 600, and 800 µg/l. For this study, 16 replicates of 12 concentrations of SAs in milk were tested for each TMP level. The interpretations of the bioassay results were performed in accordance with previous studies.

For the statistical analysis of the data, the stepwise option of the logistic procedure of SAS® (SAS 2011) was applied to the following model:

$$L_{ijk} = \text{logit}\, P_{ijk} = \beta_0 + \beta_1[\text{SAs}]_i + \beta_2[\text{TMP}]_j + \beta_3[\text{SAs}]_i \times [\text{TMP}]_j + \varepsilon_{ijk} \quad \text{(IV)}$$

where:

L_{ijk} – response variable of the linear logistic model

P_{ijk} – $[Pp/(1 - Pp)]$ or the ratio of the probability of a "positive" response/the probability of a "negative" response

$[\text{SAs}]_i$ – effect of sulfonamide concentration (i = 1, 2, ..., 12 levels, Table 3)

$[\text{TMP}]_j$ – effect of TMP concentrations (j = 0, 200, 300, 400, 500, or 600 µg/l)

$[\text{SAs}] \times [\text{TMP}]_{ij}$ – effect of the interaction between sulfonamide and TMP concentrations

$\beta_0, \beta_1, \beta_2, \beta_{12}$ – coefficients estimated in terms of their intercept, sulfonamide, TMP and interaction between sulfonamide and TMP

ε_{ijk} – residual error

RESULTS AND DISCUSSION

Effect of spore level on bioassay response time

The stepwise option of the logistic regression model indicated significant differences ($P < 0.05$) for STZ concentration ($\chi^2 = 502.061$) and $(\text{Log}\,[S])^2$ ($\chi^2 = 207.610$). The following equation shows the effect of spore concentration on the dose-response curve of STZ:

$$\text{Logit}\, P = 2.3619 + 0.00089[\text{STZ}] - 0.2659(\text{Log}\,[\text{S}])^2$$
(C = 86.6%)

The effect of spore levels on the response time and $CC_{\beta, STZ}$ in a bioassay using *B. subtilis* is shown in Table 1. An increase in the spore concentration reduced the response time due to the changes in the redox potential of the culture medium with *B. subtilis*. This time decreased from 11 h (Log S = 3.6) to 5.2 h (Log S = 7.1). In contrast, the $CC_{\beta STZ}$ increased from 4.7 mg/l (Log S = 3.60) to 10.7 mg/l (Log S = 7.10) because an increase in the spore concentration requires a higher STZ concentration to inhibit the bacteria test growth.

The decrease in bioassay response time was also pointed out by Nagel *et al.* (2011a), who indicated a reduced response time (9–5 h) when the concentration of *B. cereus* ATCC 11778 spores (Log S = 3.90–6.70) increased.

Exploratory study of factors by fractional factorial design

Only the TMP concentration significantly affected ($\chi^2 = 266.603$) the dose-response curve of the bioassay. In contrast, other factors (Log S, in-

Table 1. Effect of spore concentration logarithm (Log S) on the bioassay response times and detection capability

Log S	Time (h)	CC_β (mg/l)
3.60	11.0	4.7
4.30	8.5	5.4
5.00	7.0	6.2
5.70	6.0	7.3
6.40	5.5	8.7
7.10	5.2	10.7

Log S – spore concentration logarithm; CC_β – detection capability

Table 2. Effects of trimethoprim (TMP) on bioassay rate of non-compliant results (α) using *B. subtilis* BGA (*n* =192)

TMP (mg/l)	Positive	α
0	2	0.011
200	4	0.020
300	4	0.020
400	5	0.021
500	4	0.016
600	8	0.042
Delvotest® MCS	7	0.036

α – rate of non-compliant results (α = positives/total samples)

dicator concentration, volume of culture medium) did not significantly affect ($P > 0.05$) the method.

High levels of TMP led to an increase in the positive bioassay results, along with a decrease in the detection capability of STZ. In fact, the addition of TMP (100 µg/l) in the culture medium improved the sensitivity of the method for STZ detection in milk as it increased the frequency of the bioassay positive results.

Effect of trimethoprim concentration on the specificity and the CC_β of sulfonamides in milk

Specificity study: Table 2 summarises the results obtained after analysing 192 antibiotic-free milk samples by the bioassays containing different levels of TMP and by the Delvotest®. The specificity of the method with 500 µg/l of TMP (1.6%) was similar to that obtained for the bioassays fortified with 200, 300, and 400 µg/l of TMP (about 2.0%), and was higher than those obtained with 600 µg/l of TMP (4.2%) and by the Delvotest® MCS method (3.6%). Therefore, the CC_β study was carried out with the additions of 0, 200, 300, 400, and 500 µg/l of TMP, because they exhibit a (α) error less than 5%.

The bioassay with 500 µg/l of TMP (Table 2) presented similar values as calculated for other microbiological methods. Thus, it should be noted that the TMP levels used for the bioassay preparation were higher than 200 and 60 µg/l used by Koenen-Dierick and De Beer (1998) and Myllyniemi *et al.* (2001), respectively, for the diffusion tests in Petri dishes with *B. subtilis* BGA.

Detection capabilities study: Table 3 shows the logistic equations obtained in the study of the TMP effect on the bioassay response.

The β_1 coefficient was positive for all the SAs tested, although it was higher for SMX ($\beta_{1, SMX}$ = 0.0321) and STZ ($\beta_{1, STZ}$ = 0.0249) than for SDZ ($\beta_{1, SDZ}$ = 0.0091), SDM ($\beta_{1, SDM}$ = 0.0095), and SMZ ($\beta_{1, SMZ}$ = 0.0014). The high β_1 coefficient values of SMX and STZ indicate an increase in the slope of the dose response curves and an improvement in *B. subtilis* sensitivity for these SAs.

The addition of TMP in the medium increases the bioassay sensitivity for the detection of SDA ($\beta_{2, SDA}$= 0.0191), SDM ($\beta_{2, SDM}$ = 0.0240), SMX ($\beta_{2, SMX}$ = 0.0522), and STZ ($\beta_{2, STZ}$ = 0.0632) residues because the β_2 coefficients were positive. Moreover, the interaction between the SAs and TMP was significant for SDA ($\beta_{12, SDA}$ = 0.00011), SDM ($\beta_{12, SDM}$ = 0.00011), and SMZ ($\beta_{12, SMX}$ = 0.000089). These positive β_{12} coefficients values indicate a synergistic effect between the two antimicrobials.

Figure 1 depicts the TMP effect on the dose response curves of the six sulfonamides. The increase in TMP produces a shift of the logistic curves to lower SAs concentrations. The SME and SMT dose response curves show a parallelism in their slopes because the SAs-TMP interaction was not significant. On the other hand, the

Table 3. Logistic equations showing the effect of trimethoprim (TMP) levels on the dose-response curve of sulfamides in milk

SAs	Logit [*P*] = $\beta_0 + \beta_1$ [SAs] + β_2 [TMP] + β_{12} [SAs] ×[TMP]	C
SDA	Logit [*P*] = –7.8286 + 0.0047 [SDA] + 0.0038 [TMP] + 0.00011 [SDA] × [TMP]	88.3
SDM	Logit [*P*] = –8.4647 + 0.0210 [SDM] + 0.0079 [TMP] + 0.00007 [SDM] × [TMP]	87.3
SME	Logit [*P*] = –8.5767 + 0.0325 [SME] + 0.0128 [TMP]	84.3
SMT	Logit [*P*] = – 11.4453 + 0.0112 [SMT] + 0.0160 [TMP] + 0.00004 [SMT] × [TMP]	88.0
SMX	Logit [*P*] = –23.1012 + 0.0743 [SMX] + 0.0339 [TMP]	91.8
STZ	Logit [*P*] = – 8.7725 + 0.02780[STZ] + 0.0097 [TMP] + 0.00005 [STZ] × [TMP]	87.2

SAs – sulfamides; TMP – trimethoprim; SDA – sulfadiazine; SDM – sulfadimethoxine; SME – sulfamerazine; SMT – sulfamethazine; SMX – sulfamethoxazole; STZ – sulfathiazole; C – percentual concordance coefficient

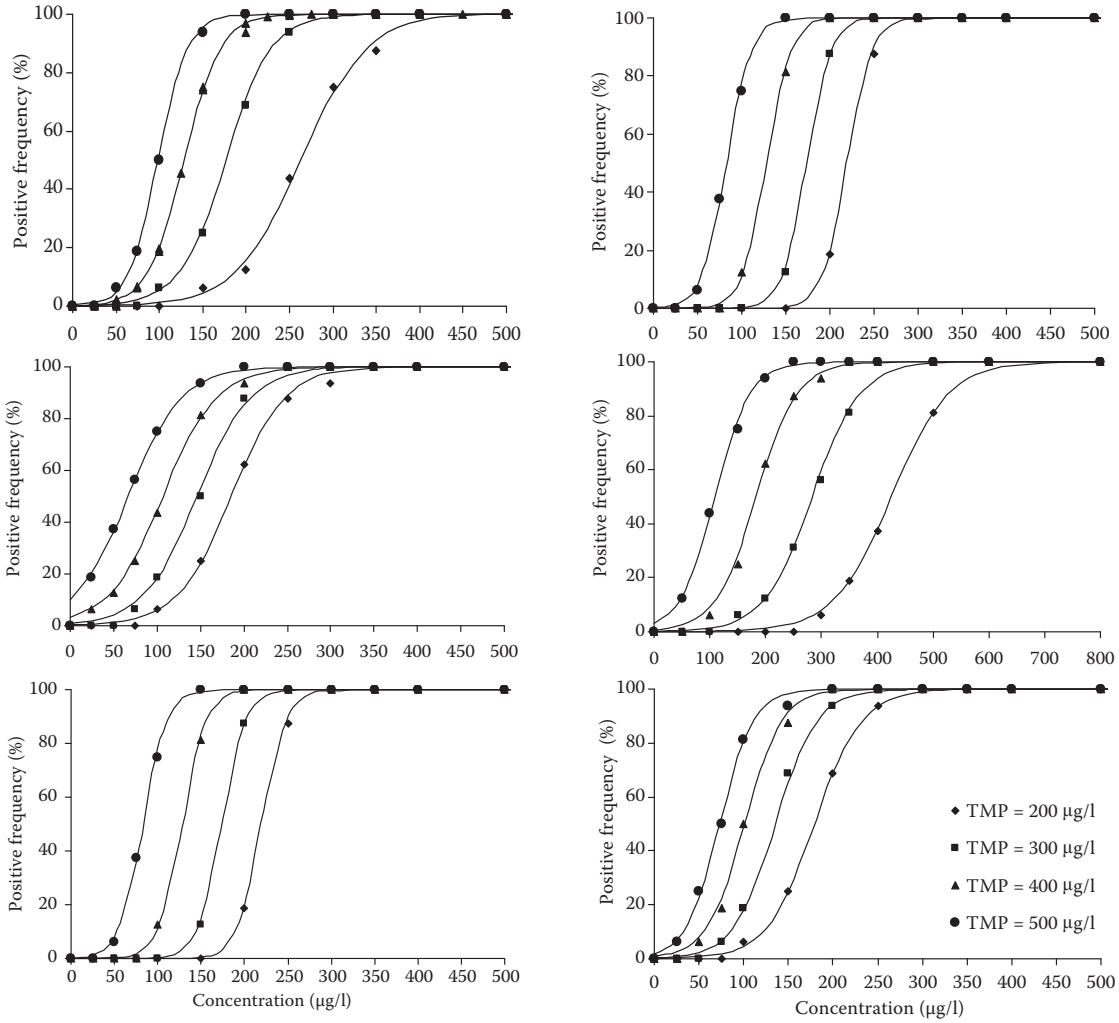

Figure 1. Effect of trimethoprim (TMP) on the dose-response curves of sulfonamides (SAs) in milk: (a) sulfadiazine, (b) sulfadimethoxine, (c) sulfamerazine, (d) sulfametazine, (e) sulfamethoxazole, and (f) sulfathiazole

synergistic effect of TMP with SDA, SDM, SMX, and STZ was visualised since increased levels of TMP raised the slopes of the dose-response curves of these SAs.

The CC_β bioassays for the different TMP concentrations were calculated using the logistic equations of Table 4. An increase in the TMP level added to the culture medium improved the detection capabilities. When bioassays were prepared with 500 µg/l of TMP, the detection capabilities of SDA, SDM, SMX, and STZ resembled to those established by the legislation (Regulation EU 2010).

Table 4. Effects of trimethoprim (TMP) on the detection capabilities (CC_β) of sulfamides in milk

SAs	TMP				MRL
	200	300	400	500	
SDA	144	120	101	86	100
SDM	283	217	170	135	100
SME	276	237	197	158	100
SMT	577	408	290	202	100
SMX	259	214	168	122	100
STZ	256	203	162	128	100

SAs – sulfamides; TMP – trimethoprim (µg/l); SDA – sulfadiazine; SDM – sulfadimethoxine; SME – sulfamerazine; SMT – sulfamethazine; SMX – sulfamethoxazole; STZ – sulfathiazole; MRL – Maximum Reside Limits (µg/l)

As regards the MMPS, Nouws *et al*. (1999) obtained low CC_β for SDA (30 µg/l), SDM (20 µg/l), and SMT (40 µg/l) when using 10^4 spores per ml of *B. subtilis* (fortified with 45 µg/l of TMP). The use of Petri dishes implies long incubation times (18–24 h) if compared with the bioassay developed in this work (6 h).

For other MMPS with Petri dishes, Tsai and Kondo (2001) calculated higher CC_β values of SAs (780 µg/l SDM, 3120 µg/l SDA, 1560 µg/l SMX, and 12 500 µg/l SMT) compared with those obtained in this work (500 µg/l of TMP; Table 4) using a diffusion assay with 10^5 spores per ml of *B. subtilis* containing 50 µg/l of TMP (incubation time 18–24 h).

CONCLUSION

The successive implementation of chemometric techniques and logistic regression models has allowed us to design a dichotomous response bioassay for the detection of sulfonamide residues in milk at levels allowed by legislation. This bioassay offers a response time of 6 h and could replace the Petri dish system using *B. subtilis* in current MMPSs.

These chemometric techniques can be used to design and optimise other dichotomous response bioassays, such as rapid methods using molecular receptors, enzymes or antibodies.

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Corresponding author:

Rafael Lisandro Althaus, PhD, Universidad Nacional del Litoral, Facultad de Ciencias Veterinarias, R.P.L. Kreder 2805, (3080) Esperanza - Santa Fe, Argentina; E-mail: ralthaus@fcv.unl.edu.ar