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Modelling the effect of combined antimicrobials: a base model for multiple-hurdles

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Abstract

Combining antimicrobials to reduce microbial growth and to combat the potential impact of antimicrobial resistance is an important subject both in foods and in pharmaceutics. Modelling of combined treatments designed to reduce or eliminate microbial contamination in foods (microbiological predictive modelling) has become commonplace. Two main reference models are used to analyse mixtures: the Bliss Independence and the Loewe reference models (LRM).

By using optical density to analyse the growth of *Aeromonas hydrophila, Cronobacter sakazakii* and *Escherichia coli*, in combined NaCl/NaCl (a mock combination experiment) and combined NaCl/KCl experiments, previous models for combined antimicrobials in foods, based on the Bliss approach, were shown to be inconsistent and that models based on the LRM more applicable.

The LRM was shown, however, to be valid only in the specific cases where the concentration exponents of all components in a mixture were identical. This is assured for a mock combination experiment but not for a true mixture. This, essentially, invalidates the LRM as a general reference model. A new model, based on the LRM but allowing for mixed exponents, was used to analyse the combined inhibition data, and concluded that the NaCl/KCl system gave the additive effect expected from literature studies. This study suggests the need to revise current models used to analyse combined effects.

1 Introduction

Combining appropriate antimicrobials whether in foods or in pharmaceutics is a strategy to reduce the total loading of the combined preservatives or drugs, potentially reduce drug toxicity, increase the spectral range of the mixture beyond that of any one adjunct, and of increasing importance - to help combat the emergence of antimicrobial resistance (CDC 2013, Krueger et al., 2014). In foods the combination of several preservation methods can be used to reduce organoleptically deleterious effects of using a single or a few factors to preserve food products. This approach, known as combined hurdle technology, although distinct from combined antimicrobials in pharmaceutics has the same goal – to reduce a negative effect through combination (Leistner and Gorris 1995).

Much effort has gone into developing and advancing mathematical models for the prediction of growth of food borne pathogens in foods preserved by combinations of hurdles such as thermal processing, holding temperature, acidity, water activity, multiple preservatives, initial inoculum size, the shelf-life and the impact of transportation. These models have become an integral part of modern-day food microbiology, e.g. in HACCP and microbiological risk analysis (Dominguez and Schaffner 2009; Membré and Lambert 2008; Nychas et al., 2008).

One particular approach to modelling microbial growth in foods is the Gamma approach in which individual effects are combined multiplicatively and is based on Leistner's Hurdle idea (Zwietering et al., 1992). For each inhibitory effect a growth factor is calculated based on the ratio of the applied level to the optimum level for microbial growth. Multiplication of these gamma factors (γ) gives the overall growth factor which alters, for example, the growth rate from its optimum value.

$$\gamma_{total} = \frac{\mu}{\mu_{opt}} = \gamma(T).\gamma(pH).\gamma(Aw).\gamma(Pres)$$

Eqn. 1. The Gamma model combining the gamma factors (γ) for temperature (T), pH, water activity (Aw) and applied preservatives (Pres) to predict the microbial growth rate (μ), relative to the optimal growth rate(μ_{opt}).

As presented the Gamma hypothesis collates the applied factors as independent entities. This is an oversimplification, and Eqn.1 can only be considered a first approximation. The reason being that temperature affects pH, water activity and also the efficacy of preservatives – especially those that have partition abilities and furthermore weak acid preservatives are affected by temperature, pH and water activity. Some of these effects can be incorporated into a modelling scheme (e.g. pH and weak acids through the use of the pKa), whilst others have to be modelled on a case-by case basis (e.g., Arroyo-Lopez et al., 2012; Coroller et al., 2012; Lambert and Bidlas 2007). Combinations of hurdles which appear to give a greater effect than that described by the Gamma model may claim to show synergy: the magnitude of the synergy is claimed relative to the expected effect (Eqn. 1) (Augustin and Carlier 2000a, 2000b).

Previously, the effect of individual preservatives against spoilage and pathogenic bacteria had been successfully modelled using a monotonic exponential decay function (Lambert and Pearson 2000). Later studies of inhibition using multiple inhibitory factors assumed that the gamma factor for an individual preservative could be expanded for combinations, giving a model, based upon the Gamma hypothesis, which simply combined the contribution from each component (Eqn 2).

$$\gamma(Pres)_{total} = \gamma(Pres_1).\gamma(Pres_2).\gamma(Pres_3)\ldots$$

Eqn. 2.

For example the combined effect of pH, acetic and propionic acids against *Aeromonas hydrophila* was given as

$$\gamma(Pres) = exp\left\{-\left[\left(\frac{10^{-pH}}{P_1}\right)^{m_1} + \left(\frac{Acetic}{P_2}\right)^{m_2} + \left(\frac{Propionic}{P_3}\right)^{m_3}\right]\right\}$$

Eqn. 3. A Gamma model used for the prediction of the effect of combined acetic and propionic acids at a given pH. P_i are concentration parameters and m_i are the concentration exponents.

This model gave a very good fit to the observed data and gave us confidence in describing the combination as additive (in the sense of independent action (Lambert and Bidlas 2007)).

Within pharmaceutics the basis of much of the literature on drug combinations is based on one of two reference models, the Bliss independence model, of which the Gamma model (Eqn.1) is an example, and the Loewe reference model (LRM, Eqn.4) (Chou 2006; Greco et al., 1995).

$$\sum_{i=1}^{n} \frac{x_i}{X_i} = 1$$

Eqn. 4 The Loewe Reference Model (LRM): An n-component mixture has a given effect, which is elicited individually at concentrations Xi; in the mixture the fractional amount of each component, x_i/X_i, sums to give the same effect.

Equation 4 is the equation of a (n-1)-dimensional hyperplane and it defines the expected additive behaviour of a mixture and "deviation from expectation unequivocally indicates an interaction and its type" (Berenbaum 1985). A mixture, which satisfies the LRM, is labelled as Loewe additive; if the combination achieved the effect, but with a value less than 1 then the mixture is labelled as synergistic, and antagonistic if it is greater than 1. For binary combinations a linear line (an isobole) joining X_1 and X_2 indicates additive behaviour,

a concave line describes the presence of synergy and a convex one the presence of antagonism (Berenbaum 1978).

One of the most used methods for analysing synergy in pharmaceutical combinations is that of Chou and Talalay (CT), (Chou 2006). This uses the Hill model to describe the action of individual drugs (Goutelle et al., 2008). The CT method, however, does not model an overall effect, but calculates a measure of the interaction - the Combination Index (CI) for each observed combination of drugs, based on the LRM. The CI is therefore identical to the sum of the fractional inhibitory concentrations (ΣFIC) much used in the analysis of antimicrobial combinations (Hall et al., 1983).

Herein we present a more general model for combined antimicrobials, through a revision of the LRM, which gives a more consistent framework for producing more complex models – both in foods and with pharmaceutics. To achieve this we have examined the effect of NaCl and/or KCl on the growth of 3 organisms: *Aeromonas hydrophila, Cronobacter sakazakii and Escherichia coli*.

2 Methods

2.1 MICROBES AND EXPERIMENTAL SET UP

Cronobacter sakazakii (FSM263, isolated from a factory producing infant formula), *Aeromonas hydrophila* (ATCC 7966) or *Escherichia coli* (ATCC 11229) were grown overnight in a flask containing 80 ml tryptone soya broth (TSB; Oxoid CM 129) shaking at 30^{o}C. The cells were harvested, centrifuged to a pellet, washed and re-suspended in peptone water. A standard inoculum was produced by diluting the culture to an optical density (OD) of 0.5 at 600nm. This standardized culture was then further diluted to produce the starting inoculum of approximately $1x10^{5}$ cfu ml^{-1}.

All analyses were performed in Bioscreen Microbiological Analysers (Bioscreens), Labsystems Helsinki, Finland.

The analysis of NaCl or KCl on the organisms used twenty linear dilutions of a stock solution (10% (wt/vol) to 0.5% in 0.5% intervals) of sodium chloride or potassium chloride (Sigma Aldrich, UK) prepared in TSB. Each dilution (200μl) was placed in a column of the Bioscreen plate, giving 10 replicates per concentration (2 plates per experiment). For each protocol diluted standard inoculum was added (50μl) to all wells except the negative control wells (+50 μl of TSB). Plates were incubated for 7 days at 30^{o}C taking OD measurements automatically every ten minutes at 600nm.

For combined NaCl/NaCl and NaCl/KCl experiments a 20 x 20 grid over 4 Bioscreen plates was used. Linear dilutions of each test antimicrobial were made (10% (wt/vol) to 0.5% in 0.5% intervals) and each dilution (100μl) placed in either a column or a row of the Bioscreen plates. Standard inoculum (100μl) was then added to each well. Plates were incubated in

two Bioscreens for 7 days at 30°C taking OD measurements automatically every ten minutes at 600nm.

The time to detection (TTD) was defined as the time to produce an OD = 0.2, the time to detection was obtained through polynomial interpolation and has an accuracy of ± 1min.

2.2 THEORY AND MODEL DEVELOPMENT

For a single bioactive, with a monotonic response to concentration and which follows the Lambert-Pearson model (Lambert and Pearson 2000, LPM), two parameters are required to describe its action (Eqn. 5). If a system of combined hurdles is purely additive, then observations should be predictable using the parameters derived from the fitting of the LPM to each of the individual bioactives used.

$$eff = \exp\left[-\left(\frac{X}{P}\right)^{m}\right]$$

Eqn. 5. Where eff is the effect measured, P is the concentration at the inflexion point and m *is the concentration exponent and X is the concentration of the bioactive substance.*

2.2.1 Mock experiment

A standard method used in the development of combination models is the combination of self with self, known as the mock experiment; this cannot be synergistic only additive. Consider an antimicrobial compound *a*, and another compound *b*, which are given to the experimenter each of which follows the LPM. Unknown to the experimenter, compound *b* is in fact compound *a* but deviously labelled as *b*. Analysis of each reveals identical P and m parameters; and for any given effect *a*/2 +*b*/2 gives the effect of *a* by itself (or *b*) (labelled as

A or B). For any given effect if *a*/A is plotted against *b*/B then a linear line connects the points – a linear isobole – since the ratios of the fractional effects must sum to 1. Therefore since in this (mock) experiment there can be no synergy a linear isobole is assumed to be equivalent to an additive effect between the components in a mixture.

$$eff = \exp\left[-\left(\frac{a}{P}\right)^m\right] = \exp\left[-\left(\frac{b}{P}\right)^m\right] = \exp\left[-\left(\frac{a/2}{P} + \frac{b/2}{P}\right)^m\right]$$

Eqn. 6. In the mock experiment a = b

2.2.2 Identical Exponents

Consider two distinct antimicrobials x_1 and x_2, both of which can be modelled by the LPM, and in which the exponents, *m*, are equivalent, then a model describing the combined effect is given by

$$eff = \exp\left[-\left(\frac{x_1}{P_1} + \frac{x_2}{P_2}\right)^m\right]$$

Eqn. 7

The combined model cannot be

$$eff = \exp\left[-\left(\left(\frac{x_1}{P_1}\right)^m + \left(\frac{x_2}{P_2}\right)^m\right)\right]$$

Eqn. 8

as this violates the requirement of the mock experiment unless m = 1.

2.2.3 Extended LPM Model and an adaptation of the LRM

Consider again two bioactives x_1 and x_2, both of which can be modelled by the LPM, and in which their exponents are *not* equivalent. Eqn. (7) is no longer applicable as the equation cannot produce the individual exponents. The format of Eqn. (7) does however provide a

clue as to how to proceed along a different line of investigation. The expansion of the values within the bracket follows a standard binomial expansion when *m* is an integer and the non-integral (Newtonian) expansion when *m* is real.

A particular solution to the problem of mixed exponents for a binary system is given by Eqn. 9.

$$eff = \exp\left[-\left(\left(\frac{x_1}{P_1}\right)^{\frac{m_1}{m_2}} + \frac{x_2}{P_2}\right)^{m_2}\right] where \qquad m_1 \le m_2$$

Eqn. 9

If $m_1 = m_2$ then the model reduces to Eqn.7; if x_2 tends to zero then the LPM for x_1 is obtained and vice-versa. For a system of *n* bioactives this model expands to give

$$eff = \exp\left[-EffC^{m_n}\right]$$

where

$$EffC = \left\{\left(\left(\ldots\left(\left(\left(x_1^{\frac{m_1}{m_2}} + x_2\right)^{\frac{m_2}{m_3}} + x_3\right)^{\frac{m_3}{m_4}} + x_4\right)^{\frac{m_4}{m_5}} + \cdots x_{n-1}\right)^{\frac{m_{n-1}}{m_n}} + x_n\right)\right\}$$

Eqn. 10

where $m_1 \le m_2 \le m_3 \le \ldots \le m_n$ and x_1, $x_{2,\ldots}x_n$ are the ratios of the amount of x_i in the mixture to the P_i value for that component, *EffC* is defined as the effective concentration, and we have termed Eqn.10 the Extended Lambert Pearson Model (ELPM). This model is a series of nested binomial expansions; if all the exponents are equivalent then this reduces to the simple additive model (Eqn.11).

$$eff = \exp\left[-\left(\sum_{i=1}^{n}\frac{x_i}{P_i}\right)^{m}\right]$$

Eqn. 11. The simple additive model (SAM), where all the exponents of the components in a mixture are equal.

Eqn. 11 can be rearranged to produce an expression known as the Sum of the Fractional Inhibitory Concentrations (*ΣFIC*, see Appendix), which is equivalent to the LRM (Eqn. 1). For a binary system, with different concentration exponents, Eqn. 9 can also be shown to produce a format akin to the LRM;

$$\left(\frac{x_1}{X_1}\right)^{\frac{m_1}{m_2}} + \frac{x_2}{X_2} = 1$$

Eqn. 12. The Extended Loewe Reference Model.

We have termed this format of the LRM, the Extended LRM, as it represents an extension to the current model.

2.2.4 Fitting procedures

The LPM is an exponential decay function, and as such only approaches the 'zero' value at large concentrations. Lambert (2010) produced an extension to the basic model which allowed it to cut the concentration axis at the minimum inhibitory concentration (MIC). The function given for the effective concentration (Eqn. 8 of that publication) is only valid in the special cases where the concentration exponents are approximately 1. To be able to use the new insights into combinations the following composite function was used;

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$$RTD = \begin{cases} \textit{if} \quad EffC = 0, P_0 \\ \textit{else if} \quad EffC < 1 \\ \textit{then} \quad P_0 \exp\left(- EffC^{\,m_n}\right) \\ \textit{else if} \quad EffC > \exp\left(\frac{1}{m_n}\right), \quad 0 \\ \textit{else} \quad \frac{P_0}{\exp(1)}\left(1 - m_n \ln[EffC]\right) \end{cases}$$

248 *Eqn. 13.The Extended Lambert-Pearson Model modified to allow the model to cross the concentration*
249 *axis. RTD is the reciprocal of the time to detection, P_0 is the RTD of the positive control.*

250 The MIC contour or surface is given by the expression

251
$$EffC = exp\left(\frac{1}{m_n}\right)$$

252 *Eqn. 14*

253 Model fitting was carried out using the non-linear fitting procedure of JMP (SAS Institute,
254 Cary NC USA), or by *Mathematica* 8 (Wolfram Ill).

3 Results

3.1 Effect of NaCl and KCl on time to detection

The optical density/time curves for each of the organisms examined show similar patterns; a shift to the right of the OD/time curve with increasing salt concentration and a decrease in the maximum OD attained (results not shown). The parameters obtained from the analyses of the time to detection data and the fitting of the LPM are given in Table 1. Comparisons of the NaCl and KCl experiments for each organism are shown in Figures 1 to 3 for *A. hydrophila, C. sakazakii* and *E.coli* respectively; from the calculated MIC, the ratio of NaCl/KCl were 0.76, 0.77, and 0.77 respectively. This is in line with the ratio of the molecular weights of NaCl and KCl (0.784). The concentration exponents were found to range from 1.51 to 2.72.

3.2 Mock Experiments

Mock combination experiments using a 20x20 well format were carried out using NaCl against *A. hydrophila* and *C. sakazakii*. The concentrations in the wells were added together and the TTD data analysed using the LPM (Eqn. 5). The fitted data resulted in a set of parameters similar to those previously found (compare parameters in Table 1 with Table 2). The data, as two independent inhibitors, were then analysed using the ELPM (Eqn. 10, n = 2). The fitting of the ELPM to the separate concentration data resulted in an almost identical fit as the LPM, with statistically equivalent concentration exponents (Table 2).

Figure 4 plots the calculated effective concentration (using the parameters from the ELPM) for the mock experiment with *C. sakazakii* against the observed RTD data, along with the data modelled using the simple additive model (Eqn. 11). There is no evidence that the exponents are statistically distinct – as required by the hypothesis of the mock experiment. However, the values for P_1 and P_2 were statistically distinct (the 95% confidence intervals did not overlap) suggesting that small errors in the dilution sequences or other experimental

errors may be present. Contour plots (isoboles) of the observed *C. sakazakii* data and the modelled data are linear (figures not shown).

3.3 COMBINED NACL AND KCL

The format of the mock experiments was repeated but using KCl as the second antimicrobial. TTD data were fitted using both the SAM and the ELPM. Table 3 gives the parameters obtained from the fittings of the ELPM. Parameters obtained were consistent with the individual parameters previously found (Table 1). For *A. hydrophila* and *E. coli*, the concentration exponents were statistically equivalent and hence the SAM and the ELPM fitted equally well, whereas for *C. sakazakii* the difference between the concentration exponents gave a slightly better fit with the ELPM. Figure 5 gives a stereo view of the observed and modelled data for the combined NaCl/KCl against *C. sakazakii*. Combining the total amount of moles of NaCl and KCl, a plot of the observed and fitted (ELPM) data is given in Figure 6. This essentially shows that the two humectants can be interchanged (compare Figure 6 with Figure 4) and that the effective concentration is an alternative scaling. The salt combinations used for *C. sakazakii* were not concentrated enough to give full inhibition, whereas for *E. coli* the MIC contour line can be seen in Figure 7, which gives a stereo view of the observed and fitted data; again plotting the isoboles gave linear lines (figure not shown).

4 Discussion

A previous modelling study of preservatives in foods, based on the Gamma hypothesis, produced a model with good fits to the observed data (Lambert and Bidlas 2007). By considering, however, a mock experiment with two components each with a concentration exponent of 2, it was shown that this published model was inconsistent, and incompatible with the observations of combined salts against the three organisms studied. A Gamma model which contained functions for NaCl, and KCl as in the Eqn. 8 would have resulted in a conclusion of synergy, which is contrary to the observation of additive effects (Boziaris *et al.* 2007). Hence for combined antimicrobials the Bliss model and therefore the Gamma concept as stated (Eqn. 1) are inappropriate in these cases.

The second of the two main combination paradigms is the Loewe reference model, from which the sum of the fractional inhibitory concentrations (ΣFIC) and the idea of the combination index flow (Chou 2006). The mock experiment with m = 2 is wholly compatible with the LRM, and therefore the LRM is a better basis for the construction of a model for combined antimicrobials than Bliss (which forms a subset of the LRM when all exponents are equal to 1). Our studies using NaCl in mock combination experiments are in agreement with the LRM; and the isobologram (not shown) described linear isoboles connecting equivalent levels of inhibition as expected.

The models used to analyse the effect of the antimicrobials (e.g. the Hill model or the LPM) are each monotonic with respect to concentration. If the dose response is not monotonic then these models are not valid in their current guise. When formulating a model to analyse combinations of inhibitors two pieces of information are required for each component – the concentration at the inflexion point of the dose response curve and a measure of the slope at that point. For the LPM these are the P and the m values; and for the Hill model the EC50 and h values. A previous study (Lambert and Lambert 2003) had suggested an empirical model for a binary system (with three fitted exponents) and had

stated that the exponents could not be predicted from the individual data; this model was used to study combined NaCl and KCl (Bidlas and Lambert 2008). Serendipitously, the model used, although empirical and over-parameterised gave good fits because the salts had almost identical dose responses, for a given organism, and so the resulting equation was essentially compatible with the LRM.

The mock experiments using NaCl and the combined NaCl and KCl experiments are particularly useful in the synergy modelling debate; both are known to have concentration exponents of approximately 2, and it is well known from the literature that NaCl and KCl act in a similar way and that one can be replaced partially by the other on a molar basis and achieve the same antimicrobial effect (Bidlas and Lambert 2008; Boziaris et al 2007; Cebrian et al 2014; Gimeno et al 1999).

The LRM is, however, only applicable if the components in the mixture have identical concentration exponents (see appendix for an explanation). This also leads to an interesting argument: linear isoboles are obtained from mock combination experiments therefore these must indicate additive behaviour since self cannot synergise with self, whereas curved isoboles do not occur with self against self therefore these isoboles cannot indicate additive behaviour. But the LRM is only applicable if the components in the mix have identical concentration exponents and in these cases can only give linear isoboles. Indeed, this is only guaranteed if the components in a mix are identical, and from Table 1 these values are themselves subject to a statistical range. Thus it can be argued that linear isoboles can only occur when components in a mix have the same concentration exponents and only then does Berenbaum's labelling of synergy, antagonism and additivity apply. If the components have (statistically) different concentration exponents then the LRM is not a valid reference model and Berenbaum's labels are void. Interestingly, Loewe (1953) stated that when compounds with different dose responses were mixed he did not believe that the LRM was applicable.

The ELPM can be shown to default to the LRM when all components have equivalent concentration exponents, and the LRM defaults to Bliss addition when these are equal to 1. Figures 4 to 7 show that the model and observed data agree and that NaCl and KCl are molar replacements for each other (Fig. 6). For a system to act additively (in the sense of acting independently) there can be no more than 2n parameters (where n = the number of components). We suggest that if the results of a mixed system can be evaluated or can be predicted on the basis of the individual parameters then that system cannot be synergistic.

For a binary system the ELPM can be shown to produce a format akin to the LRM, but one which preserves the concentration exponent information from each component. This equation has a significant prediction – that if components in the mix act independently and have different concentration exponents, then these will produce concave isoboles. A concave isobole is currently considered to be proof of a synergy between components in the mix. Synergy, however, is a phenomenon that gives more than the expected 'additive' effect. Any model of synergy would require additional parameters to describe the interaction between the actives - in addition to the activities of the components themselves. If all components in a mix have identical concentration exponents then any departure from a linear isobole or (n-1) hyperplane is indicative of either synergy or antagonism. If any of the components has a statistically different concentration exponent then a curved isobole, or hypersurface for a given effect, is expected; deviation from this indicates synergy or antagonism i.e. the ELPM will not fit the data or will give parameters far from the predicted values (those of the individual adducts). Essentially the ELPM has generalised the reference model previously used and suggests that curved isoboles may no longer indicate synergy.

This new insight has impacts both in predictive modelling in foods and also modelling combinations in pharmaceutics. Leistner (2000) had encouraged food microbiologists to study the pharmaceutical literature for combined systems, but this study shows that the LRM (Eqn.4) and the SAM (Eqn. 11) are rearrangements of each other; the Chou-Talalay CI method uses the LRM format but does not consider the effect of disparate concentration

exponents. The rearrangement of the LRM in such cases results in multiple solutions, which invalidates the CI methodology used in pharmaceutical drug discovery. The new insight does not invalidate the Gamma approach used in food microbiology, however, because it has simply shown an error in the assumed function for combined antimicrobials (Eqn.2). The ELPM can be used to give the overall Gamma factor for the contribution of all the antimicrobials – if they act independently. The Gamma hypothesis (e.g., Eqn.1) is, by its very nature, an approximation, and introducing the ELPM (or similar functions) will refine that approximation. The ELPM is also a proposed solution to mixed exponents, but further work is needed to validate or refute this model.

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Tables

Table 1. Lambert-Pearson Model: Fitted parameters

	A. hydrophila		*C. sakazakii*		*E. coli*	
Parameter	NaCl	KCl	NaCl	KCl	NaCl	KCl
MIC (%)	3.997 (3.843-4.162)	5.24 (5.111-5.377)	7.16 (7.199-7.126)	9.349 (9.140-9.565)	7.624 (7.482-7.772)	9.885 (9.679-10.100)
P_0(/h)	0.2 (0.196-0.204)	0.204 (0.202-0.207)	0.225 (0.223-0.227)	0.217 (0.214-0.218)	0.156 (0.154-0.157)	0.152 (0.150-0.153)
P(%)	2.698 (2.657-2.739)	3.496 (3.462-3.529)	3.691 (3.659-3.723)	5.171 (5.126-5.216)	5.281 (5.241-5.321)	6.26 (6.211-6.309)
m	2.545 (2.390-2.710)	2.47 (2.375-2.568)	1.509 (1.478-1.540)	1.688 (1.649-1.729)	2.723 (2.640-2.809)	2.189 (2.126-2.254)
RMSE/df	0.0072/86	0.0053/115	0.0032/147	0.0044/197	0.0040/187	0.0034/146

RMSE: root mean square error of fit; df: degrees of freedom; 95% Asymptotic confidence intervals given in brackets ;concentrations are %(wt/vol)

Table 2. Fitted parameters for the NaCl/NaCl mock experiments.

	A. hydrophila		*C. sakazakii*	
Parameter	NaCl (total)	NaCl (Mock)	NaCl (total)	NaCl(Mock)
MIC_1 (%)	3.717 (3.566-3.871)	3.547 (3.386-3.723)	6.872 (6.771-6.975)	6.786 (6.616-6.964)
MIC_2(%)	-	3.804 (3.583-4.050)	-	7.010 (6.850-7.176)
P_0 (/h)	0.263 (0.255-0.271)	0.262 (0.254-0.270)	0.223 (0.221-0.225)	0.223 (0.221-0.225)
P_1 (%)	2.565 (2.523-2.605)	2.474 (2.420-2.529)	3.868 (3.846-3.889)	3.806 (3.762-3.851)
P_2(%)	-	2.623 (2.534-2.716)	-	3.954 (3.907-4.003)
m_1	2.696 (2.526-2.889)	2.778 (2.587-2.980)	1.740 (1.712-1.758)	1.729 (1.688-1.772)
m_2	-	2.689 (2.502-2.889)	-	1.746 (1.713-1.781)
RMSE/df	0.0123/207	0.0117/205	0.0032/395	0.0031/393

NaCl (total) data fitted by the LPM; NaCl (Mock) data fitted by the ELPM. RMSE: root mean square error of fit; df: degrees of freedom; 95% Asymptotic confidence intervals given in brackets; concentrations are %(wt/vol)

Table 3. ELPM fitted parameters for the NaCl/KCl combined experiments.

Parameter	*A. hydrophila*	*C. sakazakii*	*E.coli*
MIC NaCl (%)	4.082(3.945-4.229)	7.381(7.206-7.565)	7.841(7.636-8.052)
MIC KCl(%)	5.363(5.135-5.611)	9.980(9.741-10.228)	9.600(9.359-9.845)
P_0 (/h)	0.191(0.188-0.194)	0.193(0.192-0.194)	0.166(0.164-0.1680)
P_1, NaCl (%)	2.784(2.741-2.827)	4.020(3.977-4.065)	5.096(5.028-5.164)
P_2, KCl(%)	3.569(3.484-3.659)	5.233(5.175-5.292)	6.388(6.316-6.460)
m_1, NaCl	2.612(2.483-2.747)	1.646(1.610-1.682)	2.321(2.251-2.393)
m_2, KCl	2.456(2.338-2.578)	1.549(1.518-1.581)	2.457(2.374-2.542)
RMSE/df	0.00648/273	0.00235/373	0.003245/195

RMSE:root mean square error of fit; df: degrees of freedom; 95% Asymptotic confidence intervals given in brackets; concentrations are %(wt/vol)

Legends to figures

Figure 1. *A. hydrophila*: effect of added salt (%wt/vol) on the fractional inhibition at 30°C in TSB. Observed data (NaCl, □; KCl ○) and the fitted LPM models (dashed and solid lines).

Figure 2. *C. sakazakii*: effect of added salt (%wt/vol) on the fractional inhibition at 30°C in TSB. Observed data (NaCl, □ ; KCl ○) and the fitted LPM models (dashed and solid lines).

Figure 3. *E. coli*: effect of added salt (%wt/vol) on the fractional inhibition at 30°C in TSB. Observed data (NaCl, □ ; KCl ○) and the fitted LPM models (dashed and solid lines).

Figure 4. *C. sakazakii*: NaCl/NaCl mock experiment; effective concentration (modelled by the ELPM) against the observed RTD (symbols, n = 391) and fitted model (Simple additive model, solid line).

Figure 5. Stereo view of the combined NaCl/KCl (%wt/vol) effect on *C. sakazakii*; observed data (symbols) and the modelled data (grid).

Figure 6. Effect of combined NaCl and KCl (as total mol/l) on *C. sakazakii* (n = 378). Observed –symbols and fitted model (ELPM) solid line.

Figure 7*: E. coli*; stereo view of the NaCl/KCl (%wt/vol) combinations on the observed (symbols) and modelled (grid) RTD.

Appendix 1

FAILURE OF LOEWE REFERENCE MODEL

The Lambert-Pearson inhibition model can be expressed as

$$eff = exp\left\{-\left(\frac{X}{P_1}\right)^{m_1}\right\}$$

Rearranging gives

$$P_1\left(ln\left(\frac{1}{eff}\right)\right)^{1/m_1} = X$$

For a given effect (*Eff*) this gives the concentration, X_i for the given parameters P_i, and m_i for each individual compound in the mixture.

For a two component mixture, the LRM is given as

$$\frac{x_1}{X_1} + \frac{x_2}{X_2} = 1$$

Substituting for X_i

$$\frac{x_1}{P_1\left(ln\left(\frac{1}{eff}\right)\right)^{1/m_1}} + \frac{x_2}{P_2\left(ln\left(\frac{1}{eff}\right)\right)^{1/m_2}} = 1$$

This is the general model used in the Chou-Talalay method to obtain the combination index values.

Case 1; $m_1 = m_2$

$$1 = \frac{x_1}{P_1\left(ln\left(\frac{1}{eff}\right)\right)^{1/m_1}} + \frac{x_2}{P_2\left(ln\left(\frac{1}{eff}\right)\right)^{1/m_1}}$$

554 $$\equiv \left(ln\left(\frac{1}{eff}\right)\right)^{1/m_1} = \frac{x_1}{P_1} + \frac{x_2}{P_2}$$

555

556 $$\equiv ln\left(\frac{1}{eff}\right) = \left(\frac{x_1}{P_1} + \frac{x_2}{P_2}\right)^{m_1}$$

557 $$\equiv \left(\frac{1}{eff}\right) = exp\left(\frac{x_1}{P_1} + \frac{x_2}{P_2}\right)^{m_1}$$

558

559 Hence, this leads to the simple additive model

560 $$eff = exp\left[-\left(\frac{x_1}{P_1} + \frac{x_2}{P_2}\right)^{m_1}\right]$$

561

562

563 Case 2; $m_1 \neq m_2$

564 $$1 = \frac{x_1}{P_1\left(ln\left(\frac{1}{eff}\right)\right)^{1/m_1}} + \frac{x_2}{P_2\left(ln\left(\frac{1}{eff}\right)\right)^{1/m_2}}$$

565 (i) Multiplying through with $\left(ln\left(\frac{1}{eff}\right)\right)^{1/m_1}$ gives

566 $$\left(ln\left(\frac{1}{eff}\right)\right)^{1/m_1} = \frac{x_1}{P_1} + \frac{x_2\left(ln\left(\frac{1}{eff}\right)\right)^{1/m_1}}{P_2\left(ln\left(\frac{1}{eff}\right)\right)^{1/m_2}}$$

567

568 $$\equiv \left(ln\left(\frac{1}{eff}\right)\right)^{1/m_1} = \frac{x_1}{P_1} + \frac{x_2}{P_2}\left(ln\left(\frac{1}{eff}\right)\right)^{1/m_1 - 1/m_2}$$

569 $$eff = exp\left[-\left(\frac{x_1}{P_1} + \frac{x_2}{P_2}\left(ln\left(\frac{1}{eff}\right)\right)^{1/m_1 - 1/m_2}\right)^{m_1}\right]$$

(ii) Multiplying through with $\left(ln\left(\frac{1}{eff}\right)\right)^{1/m_2}$ leads to

$$eff = exp\left[-\left(\frac{x_1}{P_1}\left(ln\left(\frac{1}{eff}\right)\right)^{1/m_2 - 1/m_1} + \frac{x_2}{P_2}\right)^{m_2}\right]$$

The expressions (i) and (ii) are only equivalent if $m_1 = m_2$. Consider the case where $P_1 = P_2$, but $m_1 = 1$ and $m_2 = 2$. This leads to a situation where there are two solutions to the LRM; hence the LRM is an invalid model in situations where the concentration exponents are not equivalent.



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Modelling the effect of combined antimicrobials: A base model for multiple-hurdles

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