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Title:

Comparing the antimicrobial effectiveness of NaCl and KCl with a view to salt/sodium replacement

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Running title: Salt replacement

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Abstract

A study using a small range of pathogenic bacterial species (*Aeromonas hydrophila*, *Enterobacter sakazakii*, *Shigella flexneri*, *Yersinia enterocolitica* and 3 strains of *Staphylococcus aureus*) has shown that potassium chloride has an equivalent antimicrobial effect on these organisms when calculated on a molar basis. Combined NaCl and KCl experiments were carried out and data analysed using a modification to the Lambert and Lambert (2003) model for combined inhibitors (J. Appl. Microbiol. 95, 734–743) and showed that in combination KCl is a direct 1:1 molar replacement for the antimicrobial effect of common salt. If this is a general finding then, where salt is used to help preserve a product, partial or complete replacement by KCl is possible.

Keywords: Predictive modelling, hurdles, salt, potassium chloride, NaCl, KCl

1. Introduction

Salt (NaCl) is generally added to foodstuffs to 1. improve taste and 2. as a preserving agent. Indeed, historically, salt was among the very few effective preserving methods known. With the advent of refrigeration, better processing, packaging, transport and storage, there is less need for high salt levels to maintain product integrity. Furthermore, consumers want products with reduced sodium levels (e.g. due to its relationship with hypertension), but where salt has been added as a preservation hurdle, removal or reduction of the salt will reduce shelf-life and could affect safety in more microbiologically fragile products.

The most obvious replacement for salt (NaCl) in food products is potassium chloride (KCl). Strong, Foster and Duncan (1970) reported that for the growth of *Clostridium perfringens*, solute identity had a bearing on the amount of growth for a given a_w, with KCl having a demonstrably greater effect than NaCl. Beuchat (1974), however, reported that at equivalent a_w NaCl and KCl had equivalent effects against *Vibrio parahaemolyticus*; it was reported that in fermented meat products, the replacement of NaCl with KCl did not affect the degree of inhibition and or inactivation, but did alter the taste of the foodstuffs (Gimeno, Astiasaran, and Bello 1999; Gimeno, Astiasaran, and Bello 2001). More recently, Boziaris, Skandamis, Anastasiadi, and Nychas (2007) have reported that equal-molar concentrations of NaCl or KCl exerted similar inhibitory effects against *Listeria monocytogenes* in terms of lag phase duration, growth or death rate and that NaCl can be replaced by KCl without risking the microbiological safety, with respect to *L. monocytogenes*, of the product. They also stated that in order to confirm this observation as general, a greater number of organisms needs to be studied.

62 In the work reported herein, we simply wanted to answer the following question: can KCl
63 be a direct or partial replacement for NaCl? Since the area of investigation is potentially
64 vast, we concentrated our initial efforts on a few species of pathogenic bacteria with which
65 we already had extensive modelling expertise on and which complimented other published
66 work.

2. Materials and methods

2.1. Culture Preparation

Aeromonas hydrophila (ATCC 7092), *Enterobacter sakazakii* (1387-2NL), *Staphylococcus aureus* (ATCC 6538, ATCC 25923 (labeled as ST121 in this report), ST55 (isolated from pasta)), *Yersinia enterocolitica* (ATCC 9610) or *Shigella flexneri* (ATCC 12022) was 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 (512g, 10 mins, 15^{o}C), washed and re-suspended in peptone water (0.1%). A standard inoculum was produced by diluting the culture to an OD of 0.5 at 600nm. This standardized culture was then further diluted in TSB to produce the starting inoculum (approx 1x10^{5} CFU ml^{-1} in the microtitre plate).

2.2. Experimental method

Experiments were carried out either using half-fold dilutions using the method of Lambert & Pearson (2000) or by using linear dilutions from stock solutions of sodium chloride or potassium chloride for the effect of individual inhibitors or the method of Lambert and Lambert (2003) for combined inhibitors.

2.3. Data analyses and model fitting

The data obtained from the Bioscreen are tables of optical density (OD) and time. The time to detection was defined as the time to produce an OD =0.2 at 600nm. The assumption being made was that at an OD =0.2, each well had identical numbers of microorganisms. Furthermore, microscopic checks were performed to see if cell elongation occurred at the highest salt levels used: no such elongation was observed. Data were transformed to the

reciprocal in order to stabilise data variance. Wells which showed no visible growth during the period of the experiment were removed from the analysis (censored data).

Previous publications (e.g. Lambert and Bidlas 2007) had used a general model for the fitting of time to detection data (TTD). A modified form of this model, which allows for a definitive MIC for individual inhibitors - the linear-exponential model (E-L), was used to analyse the data obtained, Eq.(1).

If [x] = 0 , RTD = P_0

Else if [x] < [P_1],

$$\text{then} \quad \text{RTD} = P_0 \exp\left(-\left(\frac{[x]}{P_1}\right)^{P_2}\right) \qquad (1)$$

$$\text{Else,} \quad \text{RTD} = \frac{P_0}{e}\left(1 - P_2\left(\log_e[x] - \log_e P_1\right)\right)$$

Where [x] is the concentration of the inhibitor, P_i are experimental parameters and e is the exponential, RTD is the reciprocal of the time to detection (min^{-1}).

The minimum inhibitory concentration was calculated from the parameter values obtained for each inhibitor using

$$MIC = P_1 \exp\left(\frac{1}{P_2}\right) \qquad (2)$$

For combined inhibitors (combinations of NaCl and KCl) the model of Lambert and Lambert (2003) was modified in a similar way to Eq.(1) allowing a definitive growth-no growth boundary to be constructed for combinations, Eq.(3)

113
$$\begin{cases} \textit{if} & \sum_{i=1}^{n}[x_i] = 0, RTD = P_0 \\ \textit{else if} & \\ & \ln[EffC] < 0 \\ \textit{then} & \\ & RTD = P_0 \exp\left(-[EffC]^{P_c}\right) \\ \textit{else} & \\ & RTD = \frac{P_0}{e}(1 - P_c \ln[EffC]) \end{cases} \quad (3)$$

114 Where $[EffC] = \sum_{i=1}^{n}\left(\frac{[x_i]}{P_{2i-1}}\right)^{P_{2i}}$ and P_c is the multinomial exponent for the combined system

115 (Lambert and Lambert 2003).

116 Data were fitted to the equation using non-linear regression using the minimised sum of

117 squares as the search criterion. Analyses were done using the JMP Statistical Software

118 (SAS Institute Cary, NC).

3. Results

3.1. Growth inhibition by sodium chloride and potassium chloride

Three species – the Gram negatives *E. sakazakii* and *A. hydrophila* and the Gram positive *S. aureus* are used below to highlight the results obtained. Three strains of the latter organism were used, due to the importance of humectant activity to control the growth of this organism. The results from these and the other two organisms examined in this study are summarised in Table 1.

3.2 Enterobacter sakazakii

Optical density/incubation time data were collected using half-folded dilutions of NaCl or KCl. When analysed using the modified RTD model there was an excellent fit; Figure 1 shows the results for the effect of KCl on the RTD. At KCl concentrations less than $1x10^4$ mg l^{-1} (1 %), there is little effect on the rate to detection, i.e. shows uninhibited growth. Above 1%, inhibition increases and above $1.2x10^5$ mg l^{-1} (12%) KCl no growth was observed.

Table 1 gives the experimental parameters found in percent and in mol l^{-1}. In terms of mol l^{-1} the MIC of NaCl and KCl are within the 95% confidence interval of the mean (calculated from the parameters P_1 and P_2) given for each humectant. Figure 2 compares the observed and fitted data for both the NaCl and KCl inhibition. From these results, when expressed in mol l^{-1}, there is no evidence that NaCl and KCl have different inhibitory effects against *E. sakazakii*. It was also found that in both cases there was evidence of a threshold concentration of approx. 0.1M added salt before any observation of growth inhibition.

144

145 *3.3. Staphylococcus aureus*

146 Comparison of NaCl and KCl were carried out on three strains of *S. aureus* (two standard
147 strains and a factory isolate). Figure 3 shows the results of both experiments in terms of
148 mol l^{-1} for strain ST121. The time to detection was transformed using the natural logarithm.
149 The curvature observed in Fig. 3 is typical for salt inhibition. Similar results were found
150 for the other two *S. aureus* strains examined.

151

152 It can also be observed from the figure that as conditions become more harsh, the
153 variability (or observed error) in the time to detection also increases, even although the
154 logarithm transformation has been used. There is a general observation throughout
155 predictive microbiology that the variance increases as conditions become harsher and
156 without variance stabilisation the accuracy of models is reduced the closer they approach
157 the MIC of the preservative. In this particular case the reciprocal transformation performs
158 better than the logarithmic.

159

160 *3.4. Combination NaCl and KCl experiments*

161 If the hypothesis that NaCl and KCl are mutually replaceable is correct, then the
162 antimicrobial effect of combinations of NaCl and KCl should be predictable. If the
163 calculation is performed using a combined humectant concentration in terms of mol^{-1} then
164 the modelling is facile. If, however, the separate identities are kept, then a complication
165 arises due to the non-unity dose response. Previous work done on combined hurdles has
166 used the following equation to describe the effect on RTD (Lambert and Bidlas 2007)

167 $$RTD = P_0 \exp\left(-\left(\sum_{i=1}^{n}\left(\frac{[x_i]}{P_{2i-1}}\right)^{P_{2i}}\right)\right) \quad (4)$$

For combined NaCl and KCl equation (4) will only give an approximation to the observed pattern of inhibition because the parameter P_{2i} for both humectants is approximately 2 and the above equation ignores the binomial expansion of the combined response. Equation (4), which takes this into account was used to examine the observed RTD data for *A. hydrophila* from a chequerboard of NaCl/KCl mixtures (observed data Figure 4, modelled data Figure 5). There is a very good fit of the model to the observed data. Figure 6 shows a plot of the observed and modelled RTD against the Effective Concentration; that the *EffC* is made up from any combination of NaCl and KCl and that the observations all lie on or close to the modelled line is a simple graphical representation to show that there is no synergy between the two inhibitors, since any 'true' synergy would cause a mismatch. Further, the predicted values for the combined experiment (as opposed to the direct modelling of the observed data) are given in Table 2 for both the *A. hydrophila* and the identical *E. sakazakii* experiments. The predicted parameters are in agreement with those modelled from the observed data.

4. Discussion

This work focuses on the ability of potassium chloride to replace salt as an antimicrobial humectant. From this study KCl is a direct 1:1 molar replacement for the antimicrobial effect of common salt. This study enhances the work done by Boziaris et al. (2007) and delivers the same conclusion for a larger spectrum of pathogens. There is still, however, clarification required for some of the other common pathogens as the paper by Strong et al. (1970) would suggest. There is also one point which needs to be made: there is no differentiation in this hypothesis of the equivalence of KCl and NaCl for the antimicrobial effect to be due to the chloride ion. The use of calcium chloride and other similar salts would immediately differentiate these two possibilities.

The model and the definition used for the effective concentration are direct applications of the Gamma hypothesis (Zwietering, Wijtzes, De Wit and Van't Riet 1992) - that individual inhibitors act independently against the growth of microorganisms. Recently, attempts had been made to expand the hypothesis to include the possibility of factor interaction, based on the observation of the shape of the Growth/No growth boundary for combined antimicrobials (Augustin and Carlier 2000; Le Marc, Huchet, Bourgeois, Guyonnet, Mafart and Thuault 2002). The model described here adequately describes the shape of these G/NG boundaries without recourse to altering the Zwietering hypothesis.

Figure 7 shows the calculated Growth/No Growth boundary for combinations of NaCl and KCl for the organisms studied. From equation (3) when the effective concentration is greater than 1, the linear function takes over the description of the level of inhibition. For a given RTD this describes a contour for a twin-mix of inhibitors, a surface for ternary-mixes and hypersurfaces for mixtures with greater numbers of components. The RTD = 0 contour, i.e. the absolute growth/no growth boundary is given by

$$RTD = 0 = \frac{P_0}{e}(1 - P_c \ln[EffC])$$

208 $$\therefore$$

$$EffC = Exp(1/P_c)$$

209 For any combination of inhibitors where the calculated effective concentration is equal to
210 $Exp(1/P_c)$, this combination will define a point on the MIC contour (surface, etc). Any
211 combination with $EffC > Exp(1/P_c)$ lies in a NG zone, and any with $EffC < Exp(1/P_c)$ lies
212 in a G zone. For a given organism, conditions beyond the calculated boundary line will
213 result in no growth. If total salt concentrations, for a given product, are within a boundary
214 line, then other preservative factors (e.g. temperature or weak acid preservatives) have to
215 be used to ensure no growth.
216
217 Most antimicrobial hurdles examined previously such as pH and weak acids give a linear
218 relationship between the log of the time to detection and the concentration, hence the dose
219 response parameter $P_2 \cong 1$ (Lambert and Bidlas 2007). Combinations of such hurdles will
220 also give $P_c \cong 1$. The humectants used in this study, NaCl and KCl are unusual in that the
221 dose response of each was approximately 2. Dose responses of other antimicrobials can
222 vary substantially e.g. phenolics have dose responses (or dilution coefficients) >6,
223 (Lambert and Johnston 2000; Lambert and Lambert 2003).
224
225 Modelling offers a cost-effective approach to understanding the microbial growth response
226 in foods. Indeed Zwietering (Zwietering et al.1996) has said that using a model to predict
227 the consequences of changing a formulation on microbial growth is a factor of 1000
228 quicker than attempting a large scale storage trial. Of course the formulator needs access to
229 the model in the first place and must also have an idea of its robustness. However, the use
230 of mathematical models can help to reduce the need for storage trials, challenge tests,
231 product reformulations and process modifications, which are labour intensive, time

consuming and expensive. The above example of NaCl vs. KCl is an example whereby a relatively rapid method of obtaining relevant information in conjunction with a robust model leads to a simple algorithm for NaCl replacement, allowing a product developer to gain insight perhaps 1000 times faster.

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289 # Tables

290 Table 1. Modelled parameters values for NaCl and KCl inhibition for Pathogens used in this
291 study

Organism	Humectant	P_1 (mg l^{-1}) (SErr)	P_1 (mol l^{-1}) (SErr)	P_2 (SErr)	MIC (mg l^{-1})	MIC (mol l^{-1}) (95% CI)
E. sakazakii	**NaCl**	**48180 (160)**	**0.824 (0.003)**	**1.609 (0.010)**	**89810**	**1.537 (1.516-1.561)**
	KCl	**62970 (390)**	**0.845 (0.005)**	**1.587 (0.0182)**	**118200**	**1.586 (1.545–1.630)**
***A. hydrophila* (ATCC 7966)**	**NaCl**	**32290 (330)**	**0.552 (0.006)**	**2.080 (0.050)**	**52230**	**0.893 (0.857–0.932)**
	KCl	**41870 (410)**	**0.562 (0.006)**	**1.910 (0.0395)**	**70670**	**0.948 (0.911-0.987)**
***Y. enterocolitica* (ATCC 9610)**	**NaCl**	**32700 (262)**	**0.560 (0.0045)**	**2.034 (0.061)**	**53460**	**0.915 (0.873–0.961)**
	KCl	**37930 (172)**	**0.509 (0.0023)**	**1.859 (0.030)**	**64950**	**0.871 (0.848–0.895)**
***Sh. flexneri* (ATCC 12022)**	**NaCl**	**37040 (170)**	**0.634 (0.003)**	**2.189 (0.036)**	**58490**	**1.000 (0.977–1.025)**
	KCl	**43410 (291)**	**0.582 (0.004)**	**1.673 (0.031)**	**78900**	**1.0589 (1.022–1.096)**
S. aureus 121	**NaCl**	**89600 (500)**	**1.533 (0.009)**	**2.190 (0.0476)**	**141500**	**2.421 (2.349 -2.498)**
	KCl	**114900 (554)**	**1.542 (0.007)**	**2.114 (0.0366)**	**184400**	**2.474 (2.411-2.539)**
S. aureus 6538	**NaCl**	**88100 (710)**	**1.508 (0.012)**	**2.176 (0.0603)**	**139500**	**2.388 (2.292-2.488)**
	KCl	**115700 (590)**	**1.552 (0.008)**	**1.984 (0.0363)**	**191500**	**2.569 (2.496-2.645)**
S. aureus 55	**NaCl**	**87650 (470)**	**1.500 (0.008)**	**1.875 (0.0341)**	**149400**	**2.557 (2.482-2.634)**
	KCl	**114400 (800)**	**1.534 (0.010)**	**1.817 (0.0408)**	**198400**	**2.661 (2.558-2.770)**

Table 2. Predicted and fitted parameters for the combined experiment of KCl and NaCl evaluated using equation 3.

Organism		Parameter	Predicted value	Fitted value
A. hydrophila	**NaCl**	**P_1**	**32290**	**32880**
		P_2	**1.000**	**1.018**
	KCl	**P_3**	**41870**	**43220**
		P_4	**0.918**	**0.942**
	Combined	**P_c**	**2.080**	**2.010**
E. sakazakii	**NaCl**	**P_1**	**48180**	**47670**
		P_2	**1.000**	**1.100**
	KCl	**P_3**	**62970**	**58880**
		P_4	**0.988**	**1.033**
	Combined	**P_c**	**1.606**	**1.526**

P_1 and P_3 values quoted are in mg l^{-1}

Legend to Figures

Figure 1. Effect of KCl on the rate to detection of *Enterobacter sakazakii* in TSB at 30^{o}C*.*

Figure 2. Comparison of the observed (symbols) and modelled (solid line) NaCl and KCl inhibition of *Enterobacter sakazakii*; □ NaCl; ○ KCL. The models for NaCl and KCl are essentially coincident and only that for NaCl is shown.

Figure 3. Comparison of the observed ln(time) to detection (mins) for NaCl (x) or KCl (○) inhibition of *Staphylococcus aureus* 121(ten replicates per humectant concentration).

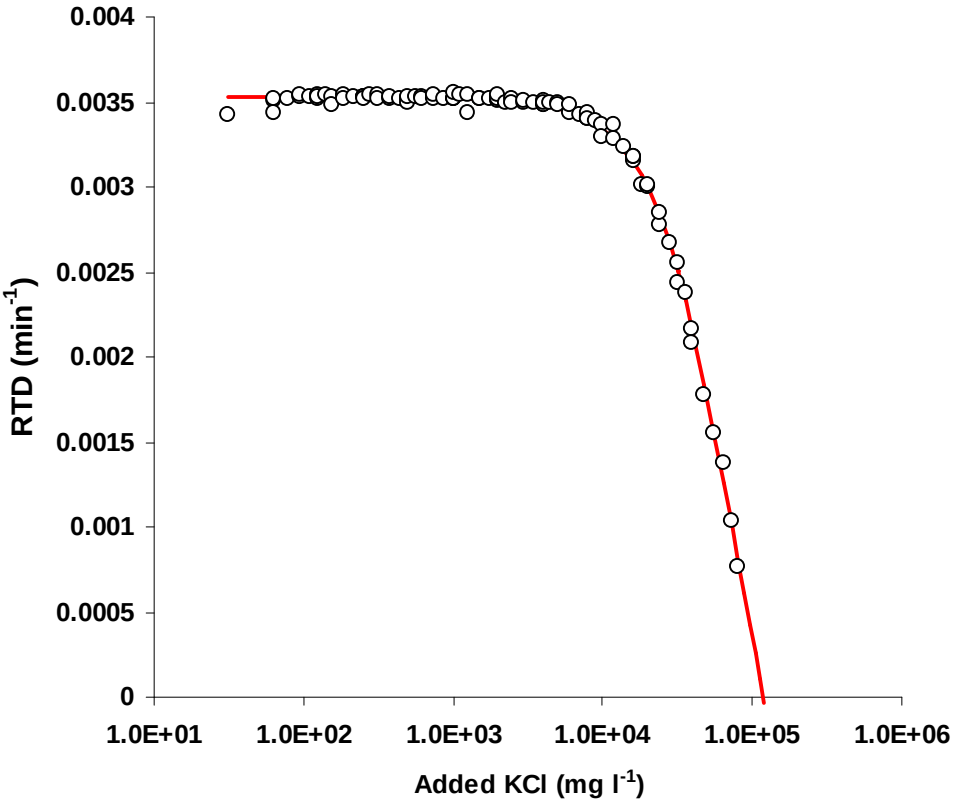
Figure 4. Observed RTD for mixtures of NaCl and KCl inhibition of *Aeromonas hydrophila*

Figure 5. Modelled RTD for mixtures of NaCl and KCl inhibition of *Aeromonas hydrophila*

Figure 6. A plot of the effective concentration against the observed (symbols) and modelled RTD (solid line) for combinations of NaCl and KCl against *Aeromonas hydrophila*.

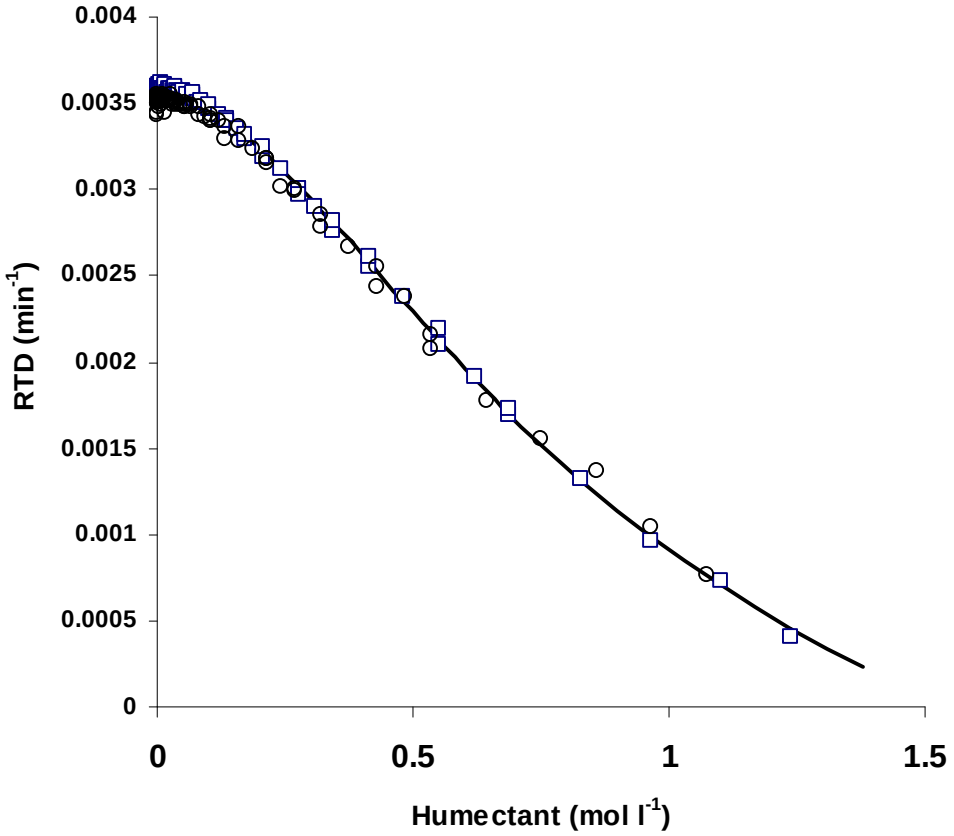
Figure 7. MIC boundaries for NaCl/KCl mixtures; ●, *Staphylococcus aureus* (Saur121); ○, *Enterobacter sakazakii*; ■*Shigella flexneri*; □, *Aeromonas hydrophila*; ▲, *Yersinia enterocolitica.*

321 *Figure 1.*



322

323 *Figure 2.*



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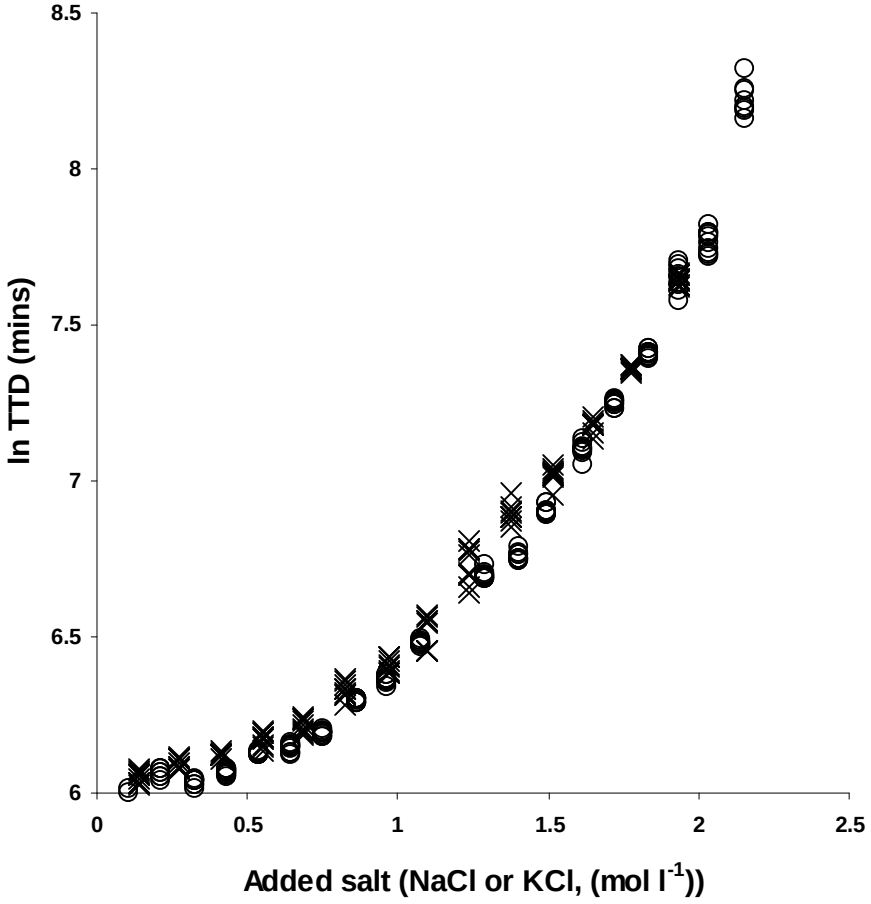
325 *Figure 3.*



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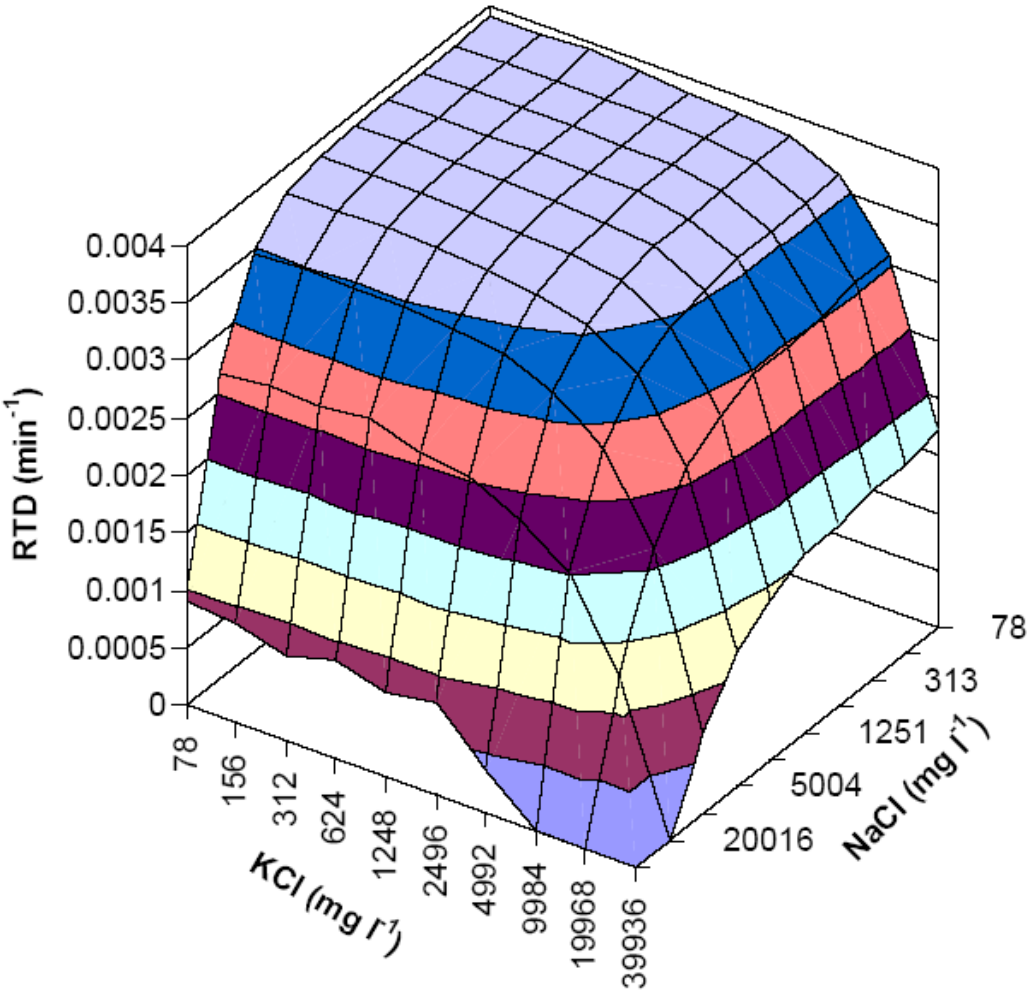
327 *Figure 4.*



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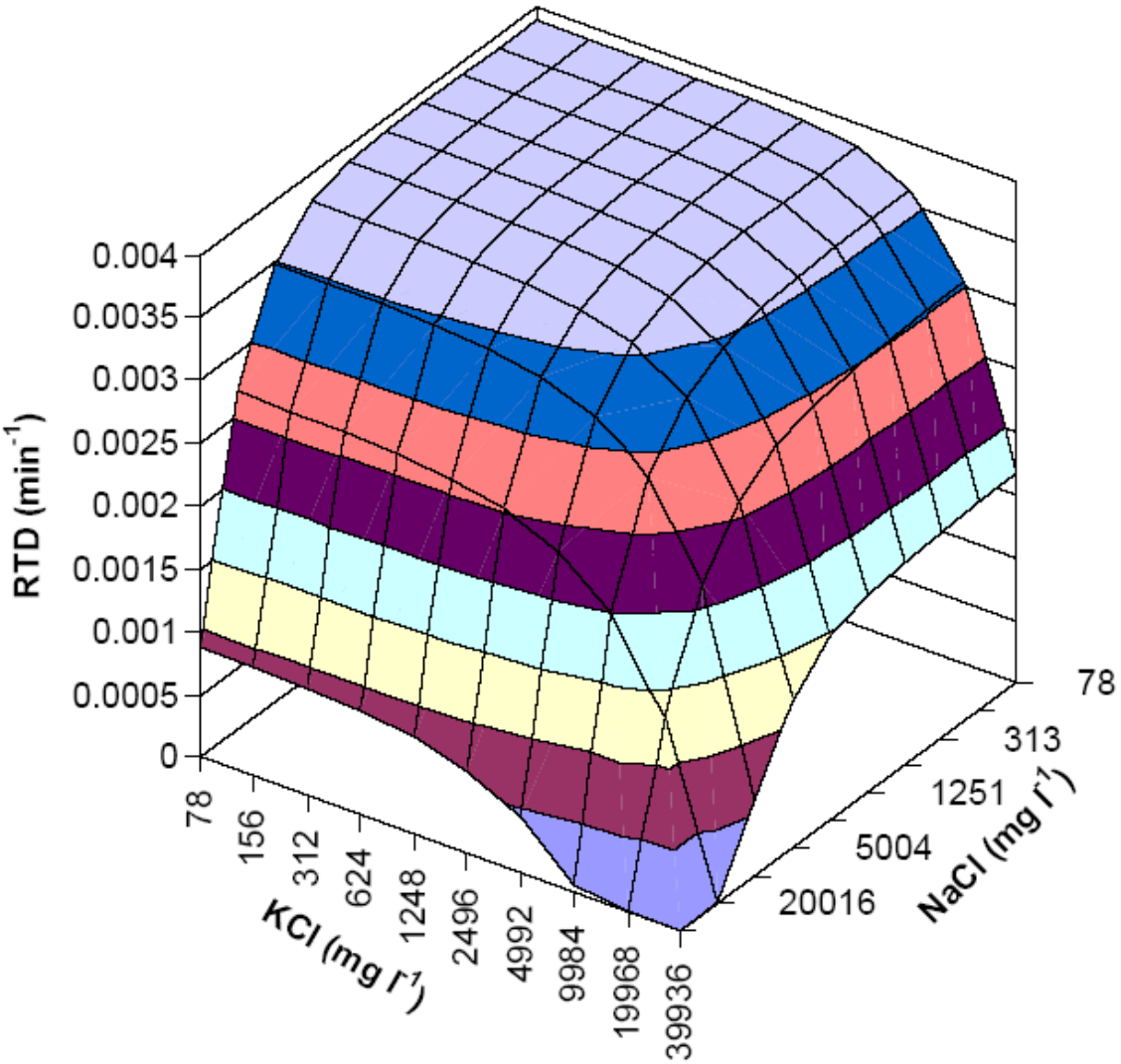
329 *Figure 5.*



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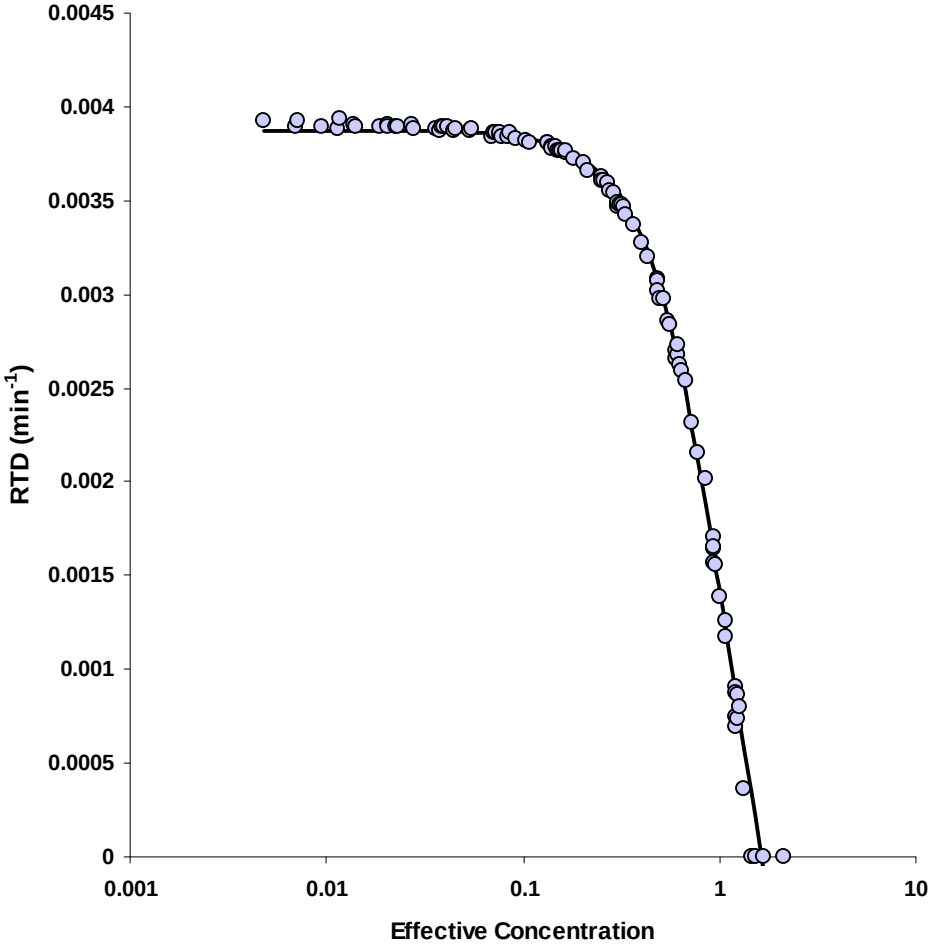
331 *Figure 6.*



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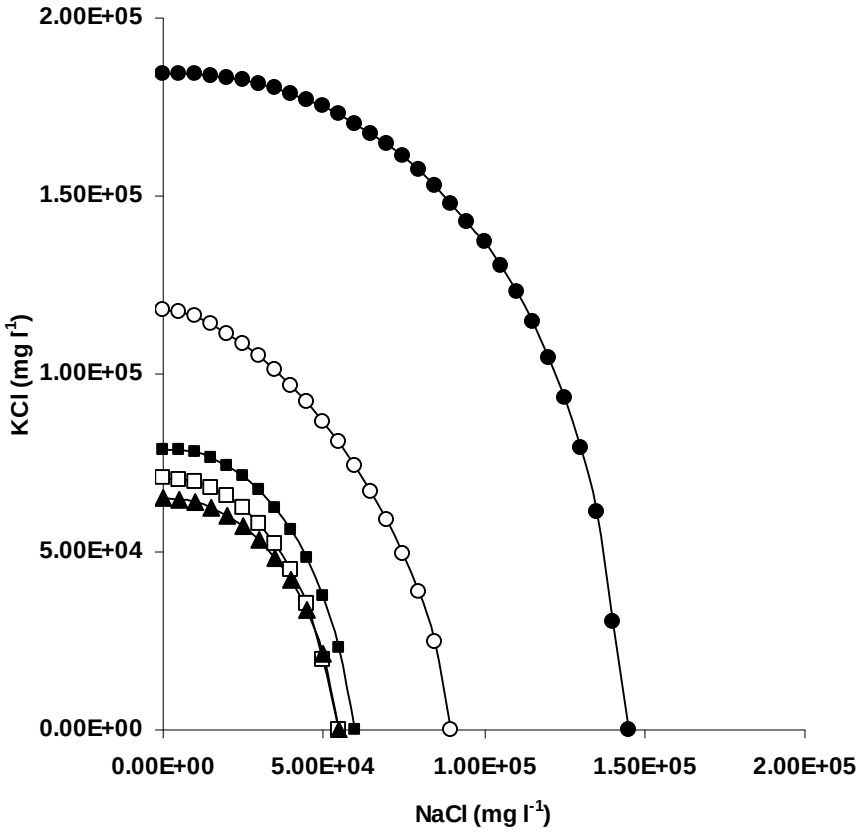
333 *Figure 7.*



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