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Characterisation of a preservative system in an oily preparation

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The influence of three variables, i.e. the concentrations of benzyl alcohol (BA), butylated hydroxytoluene (BHT) and *tert*-butyl-4-hydroxyanisol (BHA), on the preservative efficacy and antioxidant activity of an oily veterinary formulation was investigated using quantitative experimental designs and applying pharmacopoeial methods as part of the robustness-evaluation. Preservative Efficacy Tests (PETs) were performed using the validated European Pharmacpoeia (EP) methodology with 7 test-organisms over one month on lab-scale test-formulations. These were independently prepared according to a Box-Behnken experimental design with a triplicate central point at 0.75% m/V BA, 0.05% m/V BHT and 0.05% m/V BHA, and with an additional control-point outside the Box-Behnken cube containing no preservative ingredient. The preservative efficacies were evaluated against the USP and EP criteria for formulations for oral use, as well as by the statistical comparison of the slopes obtained by linear regression of the log of CFU/g versus time. The peroxide values were determined after two months storage at 50 °C, using the EP titrimetric method. No interactions between the preservatives were observed for any of the seven tested micro-organisms in the PETs. BA had a very significant preservative effect against several of the tested microorganisms, while no antimicrobial effect for BHT and BHA was observed. *Aspergillus niger* was the most preservative-resistant micro-organism, while *Staphylococcus aureus* was the most sensitive test-germ. Compliance with USP-PET criteria was found for all formulations tested, even those without preservatives, while the EP-PET criteria showed compliance for those formulations with the highest BA concentration only. Stored in glass vials, a statistically significant antioxidant effect was demonstrated for BA only, although all tested formulations showed acceptable anti-oxidative properties. No significant antioxidant effects were shown for BHT or BHA.

1. Introduction

The preservative behaviour of a product is determined by ingredients with known antimicrobial and antioxidant activity, i.e. the preservative system, as well as by the formulation ingredients and packaging. Each of these factors may contribute directly or indirectly to the antimicrobial and antioxidant activity of the product. Each ingredient in a product may thus have more than one function or effect on the properties of the formulation, the so-called multifunctional ingredients (Kabara and Orth 1997). During the formulation development of a pharmaceutical product which may be subject to microbial contamination and oxidative degradation, specific preservative ingredients are generally added: the choice and concentration is to be justified by the functional preservative efficacy and oxidation-marker tests (EMEA 2003; EMEA 1998). In commercial manufacturing, these specific preservatives are required to be identified and quantitatively determined as part of the obligatory analytical release control (ICH 1999). Although the general knowledge of multifunctional ingredients, there is currently no explicit formal regulatory requirement, nor is it standard practice, to investigate the relative importance as part of the ruggedness and robustness evaluation of the formulation composition.

Design of experiments (DOEs) are nowadays quite often used in food and pharmaceutical development studies, with a lot of examples published demonstrating the practical applicability. However, in the field of microbial and oxidation preservative efficacy investigations, DOEs are hardly used until now (De Spiegeleer et al. 2005).

Oily preparations are historically not only widely used in food and feed applications, but also in veterinary medicine and to a lesser extent, in human pharmaceuticals (Jerome 1972). Despite the widespread believe that these formulations only and explicitly need an antioxidant preservative system, it has been shown that benzyl alcohol at a concentration of 1.5% m/V in an oily preparation will inhibit microbial growth when tested according to the pharmacopoeial methods (Huyghe et al. 2003). The oxidative status of an oily system can be evaluated by different methods, depending on if primary and/or secondary oxidation products are measured. The peroxide value (PV) is a pharmacopoeial method measuring the amount of lipid hydroper-

Table 1: Concentration of the 3 preservatives in 16 batches of the formulation, expressed in % m/V, with antimicrobial preservative efficacy conclusions and peroxide value results (in meq/kg)

ID number	Conc. BA	Conc. BHT	Conc. BHA	Pharmacopoeial compliance[b]	PV
1	0.00	0.00	0.05	+ −	3.35
2	0.00	0.05	0.00	+ −	2.87
3	0.00	0.05	0.10	+ −	3.38
4	0.00	0.10	0.05	+ −	3.22
5	0.75	0.00	0.00	+ −	2.92
6	0.75	0.00	0.10	+ −	2.86
7	0.75	0.10	0.00	+ −	2.46
8	0.75	0.10	0.10	+ −	2.73
9	1.50	0.00	0.05	+ +	2.54
10	1.50	0.05	0.00	+ +	2.48
11	1.50	0.05	0.10	+ +	2.57
12	1.50	0.10	0.05	+ +	2.83
13[a]	0.75	0.05	0.05	+ −	3.16
14[a]	0.75	0.05	0.05	+ −	3.26
15[a]	0.75	0.05	0.05	+ −	3.00
16[c]	0.00	0.00	0.00	+ −	4.83

[a] ID numbers 13, 14 and 15 have identical composition of the central point in the design, used for variability assessment. Formulations were prepared and tested independently.
[b] Compliance to USP requirements is represented by the first +, while compliance with the Ph.Eur. is denoted by the second +. Non-compliance is given the symbol −.
[c] Additional experimental point at lowest level for the 3 variables, i.e. a corner-point of the Box-Behnken cube.

oxides as primary oxidation products. Results obtained with this method for samples stored up to 60 °C have been shown to be in agreement with sensory evaluation and analysis of volatiles by gas chromatography (Frankel 1993).

In this research, a newly developed veterinary pharmaceutical formulation for oral multi-use purposes (Derrieu et al. 2003) was used, where the antimicrobial and antioxidant preservative efficacy influence of three variables, benzylalcohol (BK), butylated hydroxytolnene (BHT), and tert-butyl-4-hydroxyanisol (BHA), was quantitatively investigated using a Box-Behnken experimental design and applying pharmacopoeial methodologies.

2. Investigations and results

The concentrations of the three preservative ingredients under investigation in the oil formulations are given in Table 1, together with the results of the pharmacopoeial compliance, while the full quantitative results are given in Table 2.

According to the EP requirements (EP 2002), an oral preparation is defined to be effectively preserved if the number of bacteria and fungi recovered per gram is reduced by a factor of 10^3, respectively 10^1, within 14 days of challenge, with no subsequent increase at the 28th day. The USP requirements are currently less stringent (Sutton and Porter 2002), although ICH efforts are on-going to eliminate these pharmacopoeial differences.

Only formulations 9, 10, 11 and 12 did comply with all pharmacopoeial requirements, whereas the other batches complied with the USP criteria but not with the EP criteria. The responsible microorganism for non-compliance was in all cases *Aspergillus niger*, while in only two formulations also *Pseudomonas aeruginosa* was marginally responsible for non-compliance.

The initial organism challenge was approximately 10^6 CFU · ml^{-1}. From the first evaluation timepoint, 7 days, until the end of the test, 28 days after inoculation, no viable cells were detected of *Staphylococcus aureus* or *Shewanella putrefaciens* in any of the 16 tested formulations. Depending upon the formulation, *Pseudomonas aeruginosa, Candida albicans, Lactobacillus parabuchneri, Escherichia coli* showed variable survival periods. *Aspergillus niger* showed a survival period exceeding 7 days, independent of the formulation.

To evaluate more quantitatively the results of the pharmacopoeial preservative efficacy tests, a kill rate parameter was calculated with the available data. The rate of kill (the log10 number of surviving microorganism cells versus time) was expressed as the slope of the linear curve when the log10 numbers of survivors (till no counts) were plotted against time within the experimental region. Table 3 provides the experimentally obtained slopes.

The underlying assumption of this parameter is that the relationship between the log 10 number of survivors and time is linear. This was experimentally confirmed by residuals analysis for the cases with still positive counts after 28 days. Although intrinsically, our slope represents a similar characteristic as the formal D-value, its expression is purposely different, as it was not meant as a predictor for responses beyond the experimental region (Sutton et al. 1991), but only as a comparative data-reduction value allowing to investigate more thoroughly the pharmacopoeial preservative efficacy test results.

The experimentally obtained slopes were all negative, and the magnitude corresponded generally well with the pharmacopoeial compliance conclusions.

Analysis of variance on the experimentally obtained slopes of the linear curves was used to evaluate the main and interaction effects of the concentrations of the three ingredients on the anti-fungal preservative efficacy. A significant two-way interaction between the different ingredients was only found between BA and BHT for *E. coli*, but visual examination of the plots of the estimated marginal means did not show a crossing of lines within the defined experimental region. Therefore, analysis of variance without interactions was applied for the evaluation of the main effects. The results for three representative microorganisms are given in the standard ANOVA summary table: *S. aureus*, representative for the least resistant (most vulnerable) microorganism, while *C. albicans* for the intermediate group and *A. niger* for the most resistant (Table 4).

The parameter estimates for the linear model of the pharmacopoeial germs gave following coefficients, with the standard errors of the coefficients between brackets:

$$\textit{E. coli:}\ \text{slope}_{calc} = -0.799(0.112) + 0.014(0.080)\ \text{BA} + 0.108(1.197)\ \text{BHT} + 0.195(1.197)\ \text{BHA}$$

$$\textit{S. aureus:}\ \text{slope}_{calc} = -0.831(0.015) + 0.006(0.011)\ \text{BA} + 0.050(0.164)\ \text{BHT} + 0.093(0.164)\ \text{BHA}$$

$$\textit{Ps. aerug.:}\ \text{slope}_{calc} = 0.939(0.963) - 3.886(0.683)\ \text{BA} + 0.836(10.248)\ \text{BHT} + 0.152(10.248)\ \text{BHA}$$

$$\textit{C. albicans:}\ \text{slope}_{calc} = -0.472(0.110) - 0.313(0.078)\ \text{BA} + 0.570(1.172)\ \text{BHT} + 1.685(1.172)\ \text{BHA}$$

$$\textit{A. niger:}\ \text{slope}_{calc} = -0.006(0.058) - 0.106(0.041)\ \text{BA} - 0.096(0.621)\ \text{BHT} + 0.534(0.621)\ \text{BHA}$$

Table 2: Preservative properties of the 16 formulations, each towards 7 individual strains

ID number	Organism	Control[a]	Results at culture medium days (CFU · ml^{-1})				
			0	7	14	21	28
1	*E. coli*	1.3 E + 06	1.4 E + 06	NR	NR	NR	NR
	S. aureus	6.3 E + 05	6.3 E + 05	NR	NR	NR	NR
	Ps. aeruginosa	4.0 E + 05	1.9 E + 04	2.9 E + 04	5.0 E + 02	5.5 E + 02	NR
	C. albicans	5.6 E + 05	5.6 E + 05	2.6 E + 03	5.0 E + 02	NR	NR
	A. niger	1.4 E + 05	2.0 E + 05	2.1 E + 05	1.8 E + 05	9.5 E + 04	6.5 E + 04
	S. putrefaciens	6.5 E + 04	2.5 E + 04	NR	NR	NR	NR
	L. parabuchneri	3.1 E + 05	1.5 E + 05	NR	NR	NR	NR
2	*E. coli*	1.3 E + 06	8.9 E + 05	NR	NR	NR	NR
	S. aureus	6.3 E + 05	5.2 E + 05	NR	NR	NR	NR
	Ps. aeruginosa	4.0 E + 05	1.3 E + 04	4.1 E + 03	NR	NR	NR
	C. albicans	5.6 E + 05	6.4 E + 05	1.6 E + 03	NR	NR	NR
	A. niger	1.4 E + 05	1.8 E + 05	1.8 E + 05	9.0 E + 04	6.0 E + 04	2.0 E + 04
	S. putrefaciens	6.5 E + 04	2.7 E + 04	NR	NR	NR	NR
	L. parabuchneri	3.1 E + 05	2.6 E + 05	NR	NR	NR	NR
3	*E. coli*	1.3 E + 06	6.0 E + 05	NR	NR	NR	NR
	S. aureus	6.3 E + 05	4.8 E + 05	NR	NR	NR	NR
	Ps. aeruginosa	4.0 E + 05	2.3 E + 04	2.8 E + 03	5.0 E + 01	NR	NR
	C. albicans	5.6 E + 05	5.0 E + 05	1.7 E + 03	1.0 E + 03	1.0 E + 02	NR
	A. niger	1.4 E + 05	2.1 E + 05	2.2 E + 05	1.4 E + 05	3.5 E + 04	3.0 E + 04
	S. putrefaciens	6.5 E + 04	1.3 E + 04	NR	NR	NR	NR
	L. parabuchneri	3.1 E + 05	1.5 E + 05	NR	NR	NR	NR
4	*E. coli*	1.3 E + 06	4.8 E + 05	5.0 E + 01	NR	NR	NR
	S. aureus	6.3 E + 05	5.3 E + 05	NR	NR	NR	NR
	Ps. aeruginosa	4.0 E + 05	2.0 E + 04	2.0 E + 04	2.5 E + 04	7.5 E + 03	2.6 E + 03
	C. albicans	5.6 E + 05	7.2 E + 05	6.0 E + 02	NR	NR	NR
	A. niger	1.4 E + 05	2.4 E + 05	1.4 E + 05	1.6 E + 05	3.5 E + 04	3.0 E + 04
	S. putrefaciens	6.5 E + 04	1.8 E + 04	NR	NR	NR	NR
	L. parabuchneri	3.1 E + 05	2.6 E + 05	5.0 E + 01	NR	NR	NR
5	*E. coli*	1.3 E + 06	3.3 E + 05	NR	NR	NR	NR
	S. aureus	6.3 E + 05	6.7 E + 05	NR	NR	NR	NR
	Ps. aeruginosa	4.0 E + 05	1.0 E + 04	NR	NR	NR	NR
	C. albicans	5.6 E + 05	3.8 E + 05	NR	NR	NR	NR
	A. niger	1.4 E + 05	2.2 E + 05	1.8 E + 05	2.1 E + 05	1.9 E + 05	2.0 E + 05
	S. putrefaciens	6.5 E + 04	1.5 E + 04	NR	NR	NR	NR
	L. parabuchneri	3.1 E + 05	2.2 E + 05	NR	NR	NR	NR
6	*E. coli*	1.3 E + 06	5.6 E + 05	NR	NR	NR	NR
	S. aureus	6.3 E + 05	8.9 E + 05	NR	NR	NR	NR
	Ps. aeruginosa	4.0 E + 05	2.3 E + 04	NR	NR	NR	NR
	C. albicans	5.6 E + 05	3.4 E + 05	1.0 E + 02	NR	NR	NR
	A. niger	1.4 E + 05	1.8 E + 05	1.5 E + 05	1.8 E + 05	1.3 E + 05	7.0 E + 04
	S. putrefaciens	6.5 E + 04	5.9 E + 03	NR	NR	NR	NR
	L. parabuchneri	3.1 E + 05	3.0 E + 05	NR	NR	NR	NR
7	*E. coli*	1.0 E + 06	8.0 E + 05	NR	NR	NR	NR
	S. aureus	8.9 E + 05	7.7 E + 05	NR	NR	NR	NR
	Ps. aeruginosa	4.1 E + 05	3.3 E + 03	NR	NR	NR	NR
	C. albicans	7.5 E + 05	7.1 E + 05	1.0 E + 02	NR	NR	NR
	A. niger	1.8 E + 05	2.4 E + 05	1.6 E + 05	1.5 E + 05	1.2 E + 05	9.0 E + 04
	S. putrefaciens	7.3 E + 05	1.5 E + 05	NR	NR	NR	NR
	L. parabuchneri	9.5 E + 03	6.3 E + 03	1.5 E + 02	NR	NR	NR
8	*E. coli*	1.0 E + 06	2.7 E + 05	NR	NR	NR	NR
	S. aureus	8.9 E + 05	6.3 E + 05	NR	NR	NR	NR
	Ps. aeruginosa	4.1 E + 05	2.0 E + 03	NR	NR	NR	NR
	C. albicans	7.5 E + 05	4.6 E + 05	5.0 E + 01	NR	NR	NR
	A. niger	1.8 E + 05	2.5 E + 05	1.2 E + 05	1.6 E + 05	8.5 E + 04	1.6 E + 05
	S. putrefaciens	7.3 E + 05	NR	NR	NR	NR	NR
	L. parabuchneri	9.5 E + 03	6.2 E + 03	NR	NR	NR	NR
9	*E. coli*	1.0 E + 06	6.6 E + 05	2.5 E + 02	NR	NR	NR
	S. aureus	8.9 E + 05	4.2 E + 05	NR	NR	NR	NR
	Ps. aeruginosa	4.1 E + 05	NR	NR	NR	NR	NR
	C. albicans	7.5 E + 05	3.8 E + 05	NR	NR	NR	NR
	A. niger	1.8 E + 05	2.6 E + 05	9.0 E + 02	4.0 E + 02	1.5 E + 02	2.0 E + 02
	S. putrefaciens	7.3 E + 05	NR	NR	NR	NR	NR
	L. parabuchneri	9.5 E + 03	1.5 E + 03	1.0 E + 02	NR	NR	NR
10	*E. coli*	1.0 E + 06	5.7 E + 05	NR	NR	NR	NR
	S. aureus	8.9 E + 05	6.9 E + 05	NR	NR	NR	NR
	Ps. aeruginosa	4.1 E + 05	NR	NR	NR	NR	NR
	C. albicans	7.5 E + 05	3.9 E + 05	NR	NR	NR	NR
	A. niger	1.8 E + 05	2.3 E + 05	2.0 E + 03	NR	NR	NR

Table 2: (continued)

ID number	Organism	Control[a]	Results at culture medium days (CFU · ml^{-1})				
			0	7	14	21	28
	S. putrefaciens	7.3 E + 05	NR	NR	NR	NR	NR
	L. parabuchneri	9.5 E + 03	8.0 E + 02	NR	NR	NR	NR
11	*E. coli*	1.0 E + 06	4.2 E + 05	NR	NR	NR	NR
	S. aureus	8.9 E + 05	3.8 E + 05	NR	NR	NR	NR
	Ps. aeruginosa	4.1 E + 05	NR	NR	NR	NR	NR
	C. albicans	7.5 E + 05	2.7 E + 05	NR	NR	NR	NR
	A. niger	1.8 E + 05	2.6 E + 05	2.1 E + 03	4.5 E + 02	2.0 E + 02	NR
	S. putrefaciens	7.3 E + 05	NR	NR	NR	NR	NR
	L. parabuchneri	9.5 E + 03	1.1 E + 03	NR	NR	NR	NR
12	*E. coli*	1.0 E + 06	7.0 E + 05	NR	NR	NR	NR
	S. aureus	8.9 E + 05	4.4 E + 05	NR	NR	NR	NR
	Ps. aeruginosa	4.1 E + 05	NR	5.5 E + 02	NR	NR	NR
	C. albicans	7.5 E + 05	2.8 E + 05	NR	NR	NR	NR
	A. niger	1.8 E + 05	2.3 E + 05	1.4 E + 03	7.0 E + 02	2.0 E + 02	1.0 E + 02
	S. putrefaciens	7.3 E + 05	NR	NR	NR	NR	NR
	L. parabuchneri	9.5 E + 03	6.0 E + 02	NR	NR	NR	NR
13	*E. coli*	1.4 E + 06	1.7 E + 06	NR	NR	NR	NR
	S. aureus	9.4 E + 05	9.0 E + 05	NR	NR	NR	NR
	Ps. aeruginosa	5.0 E + 05	8.5 E + 04	NR	NR	NR	NR
	C. albicans	7.8 E + 05	8.0 E + 05	NR	NR	NR	NR
	A. niger	2.2 E + 05	3.0 E + 05	1.5 E + 05	2.4 E + 05	1.0 E + 05	1.3 E + 05
	S. putrefaciens	7.0 E + 05	1.5 E + 03	NR	NR	NR	NR
	L. parabuchneri	1.1 E + 05	1.9 E + 04	NR	NR	NR	NR
14	*E. coli*	1.4 E + 06	4.3 E + 05	NR	NR	NR	NR
	S. aureus	9.4 E + 05	2.7 E + 05	NR	NR	NR	NR
	Ps. aeruginosa	5.0 E + 05	9.5 E + 04	NR	NR	NR	NR
	C. albicans	7.8 E + 05	5.6 E + 05	NR	NR	NR	NR
	A. niger	2.2 E + 05	2.4 E + 05	1.0 E + 05	1.5 E + 05	1.7 E + 05	1.2 E + 05
	S. putrefaciens	7.0 E + 05	NR	NR	NR	NR	NR
	L. parabuchneri	1.1 E + 05	8.6 E + 03	NR	NR	NR	NR
15	*E. coli*	1.4 E + 06	8.7 E + 05	NR	NR	NR	NR
	S. aureus	9.4 E + 05	3.8 E + 05	NR	NR	NR	NR
	Ps. aeruginosa	5.0 E + 05	2.5 E + 04	NR	NR	NR	NR
	C. albicans	7.8 E + 05	4.8 E + 05	NR	NR	NR	NR
	A. niger	2.2 E + 05	3.6 E + 05	1.8 E + 05	1.3 E + 05	1.4 E + 05	2.1 E + 05
	S. putrefaciens	7.0 E + 05	5.5 E + 02	NR	NR	NR	NR
	L. parabuchneri	1.1 E + 05	1.0 E + 05	NR	NR	NR	NR
16	*E. coli*	1.4 E + 06	2.1 E + 05	NR	NR	NR	NR
	S. aureus	9.4 E + 05	5.7 E + 05	NR	NR	NR	NR
	Ps. aeruginosa	5.0 E + 05	1.2 E + 05	3.0 E + 02	NR	NR	NR
	C. albicans	7.8 E + 05	1.5 E + 05	5.4 E + 03	4.5 E + 02	NR	NR
	A. niger	2.2 E + 05	4.8 E + 05	1.0 E + 05	6.0 E + 04	3.3 E + 03	2.2 E + 03
	S. putrefaciens	7.0 E + 05	1.3 E + 03	NR	NR	NR	NR
	L. parabuchneri	1.1 E + 05	1.2 E + 04	NR	NR	NR	NR

[a] Control on day 0.
NR: no recovery, *i.e.* no growth was detected (less than the detection limit of 50 CFU · ml^{-1})

where BA, BHT and BHA stand for the concentrations of benzyl acolhol, butylated hydroxytoluene and butylated hydroxy anisol respectively. The coefficients were estimated by minimizing the sum of the squared differences between $slope_{calc}$ and the experimental slope as given in Table 3. The absolute value of a coefficient, relative to its standard error, suggests the relative degree to which the corresponding variable contributes to the overall slope. A negative sign suggests that lower concentrations provide higher slopes.

For *S. aureus*, none of the investigated ingredients had a significant preservative effect and only the intercept was significant, indicating that the placebo formulation itself is sufficient antimicrobial and will not support the growth of *S. aureus*. This was confirmed by formulation 16, which was the experimental verification of the placebo preparation. The same conclusion is valid for *E. coli*. The opposite was found for *A. niger*, where the kill rate was significantly influenced by the BA concentration. Figs. a, b and c provide the box-plots: each box contains the pooled data of the two other ingredients.

The slopes clearly showed a more negative trend with increasing BA concentrations. No antibacterial preservative influence was observed for BHT or BHA.

The preservative BA has a statistically significant antimicrobial effect against some other organisms as well, like *Ps. aeruginosa* and *C. albicans*.

Last, for *S. putrefaciens* and *L. parabuchneri*, both non-pharmacopoeial germs, CFUs were not or hardly recovered already from the second time-point on. The slope values are in these cases thus dominantly influenced by the original quantity of CFUs added, and modelling is thus superfluous.

Table 3: Experimentally obtained slopes[a] of the linear regression results

ID number	*E. coli*	*S. aureus*	*Ps. aerug.*	*C. albicans*	*A. niger*	*S. putr.*	*L. parab.*
1	− 0.878	− 0.828	− 0.147	− 0.257	− 0.019	− 0.628	− 0.739
2	− 0.850	− 0.817	− 0.294	− 0.415	− 0.034	− 0.633	− 0.774
3	− 0.825	− 0.812	− 0.212	− 0.180	− 0.036	− 0.588	− 0.739
4	− 0.406	− 0.818	− 0.031	− 0.418	− 0.034	− 0.608	− 0.387
5	− 0.788	− 0.832	− 0.571	− 0.797	− 0.001	− 0.597	− 0.763
6	− 0.821	− 0.850	− 0.623	− 0.395	− 0.013	− 0.539	− 0.782
7	− 0.843	− 0.841	− 0.503	− 0.418	− 0.021	− 0.808	− 0.271
8	− 0.776	− 0.828	− 0.472	− 0.404	− 0.008	− −	− 0.271
9	− 0.416	− 0.803	− −	− 0.797	− 0.100	− −	− 0.227
10	− 0.822	− 0.834	− −	− 0.799	− 0.383	− −	− 0.415
11	− 0.803	− 0.797	− −	− 0.776	− 0.169	− −	− 0.434
12	− 0.835	− 0.806	− −	− 0.778	− 0.108	− −	− 0.397
13	− 0.890	− 0.851	− 0.704	− 0.843	− 0.013	− 0.454	− 0.611
14	− 0.805	− 0.776	− 0.711	− 0.821	− 0.005	− −	− 0.562
15	− 0.849	− 0.797	− 0.628	− 0.812	− 0.008	− 0.391	− 0.714
16	− 0.760	− 0.822	− 0.363	− 0.237	− 0.088	− 0.445	− 0.583

− − No value, as no CFU recovered from the zero time point on.
[a] Expressed as log CFU · (ml · day)$^{-1}$

Table 4: Analysis of variance of the slope-values from the linear regression for three representative microorganisms

Source	SS	df	MS	F	Significance (P-value)
S. aureus					
Corrected model	3.743 E-04	3	1.248 E-04	0.231	0.873
Intercept	1.563	1	1.563	2891.798	0.000
BA	1.531 E-04	1	1.531 E-04	0.283	0.605
BHT	5.000 E-05	1	5.000 E-05	0.093	0.767
BHA	1.711 E-04	1	1.711 E-04	0.317	0.585
Error	5.945 E-03	11	5.405 E-04		
Total	10.076	15			
Corrected total	6.319 E-03	14			
C. albicans					
Corrected model	0.505	3	0.168	6.131	0.010
Intercept	0.504	1	0.504	18.350	0.001
BA	0.442	1	0.442	16.089	0.002
BHT	6.498 E-03	1	6.498 E-03	0.237	0.636
BHA	5.678 E-02	1	5.678 E-02	2.068	0.178
Error	0.302	11	2.746 E-02		
Total	6.100	15			
Corrected total	0.807	14			
A. niger					
Corrected model	5.662 E-02	3	1.887 E-02	2.446	0.119
Intercept	7.303 E-05	1	7.303 E-05	0.009	0.924
BA	5.074 E-02	1	5.074 E-02	6.576	0.026
BHT	1.830 E-04	1	1.830 E-04	0.024	0.880
BHA	5.703 E-03	1	5.703 E-03	0.739	0.408
Error	8.487 E-02	11	7.715 E-03		
Total	0.202	15			
Corrected total	0.141	14			

3. Discussion

There exists a critical water activity a_w below which no microorganisms can grow (Kabara and Orth 1997). For most food preparations, this proliferation a_w limit is in the range of 0.4–0.6 (Fontana 1998). However, survival of spores can be extended to appreciable periods in a low a_w environment, e.g. a_w of 0.2. Water activity is a mean measure of the energy status of the water in a system and is controlled by colligative, capillary and surface effects (Fontana 2000). Although water activity is a continuum of energy states, it can be visualised into the extremes as dissolved (hydrogen bonded to the oil molecules), emulsified (available, supersaturated water solution in oil) or free (forming water droplets in oil) water. The water activity of the investigated complex formulation will thus be dependent on the oil characteristics, including the presence of components like lecithine in soybean oil, but also on the additional ingredients added to the formulation. Moreover, water activity is a mean value at equilibrium of a system, and does not consider inhomogeneities or places where locally a high accessible water is available, e.g. at solid particles. Last, the germs were added as an aqueous dispersion, limited to max. 1.0% V/V. This addition of a small quantity of water is however representative for the in-use situation of this multi-unit veterinary formulation, where water-contamination is an expected possible side-effect. While the water activity value for a newly opened

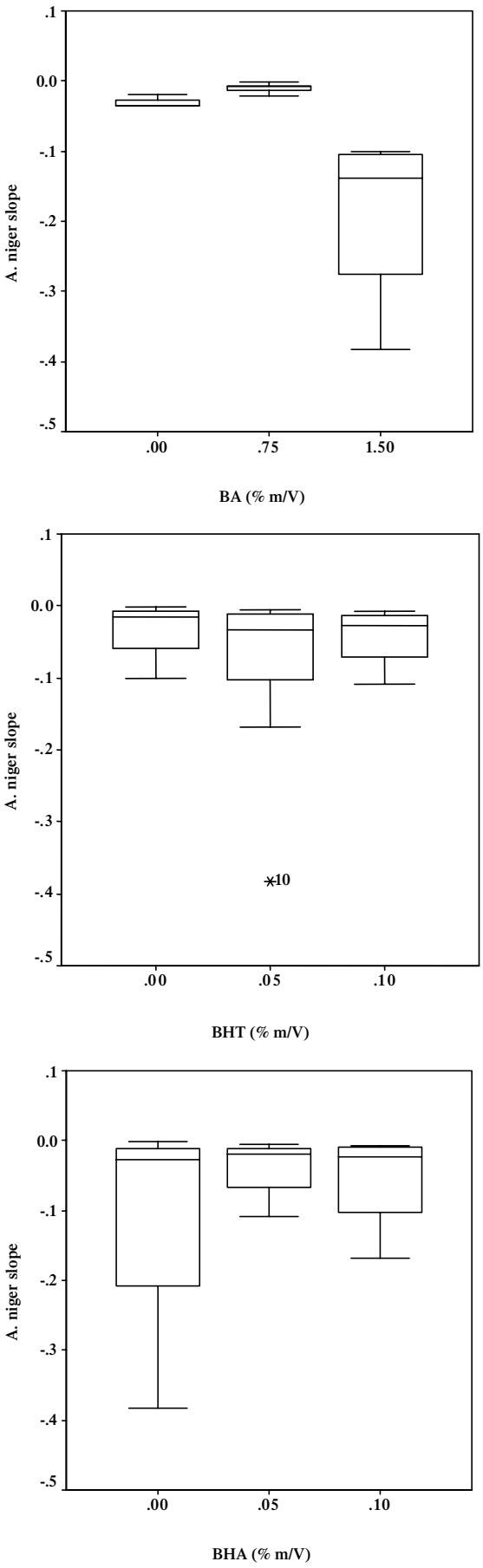

Fig.: Box-plots on the *A. niger* slopes for BA (a), BHT (b) and BHA (c)

bottle of vegetable oil is around 0.2 to 0.4, measured with an AquaLab water activity meter, the oil will absorb water over time and come to equilibrium with ambient humidity. Due to their low capacity for water, there is a large water activity change for a very small moisture content change: when 5 ml of fresh oil (with a water activity of 0.3) is mixed well with 0.05–0.1 ml of water, the water activity will increase sharply to above 0.9, due to the bulk like properties of water in oil (a_w information from personal communication with A. J. Fontana 2005). The formulation should be sufficiently robust so that this in-use water-contamination does not significantly adversely influence the quality and safety risks of the formulation.

The peroxide values obtained after two months storage at 50 °C ranged from 2.5 to 3.4 meq/kg (Table 1). These values are similar to those of samples stored in glass at 4 °C. Surprisingly, no statistically significant antioxidant effect of BHT and BHA was found, but a significant protective effect of BA was found under the test conditions ($P = 0.003$). No oxidative challenge, like metals, was introduced, as this test was conducted under accelerated temperature in otherwise normal-use conditions (e.g. glass packaging). However, in a separate comparative experiment using plastic polyethylene bottles instead of glass, the peroxide value obtained after two months at 50 °C for formulation 13 (i.e. the central point of the Box-Behnken cube) was 24.7 meq/kg, demonstrating a strong influence of the packaging despite the presence of intermediate concentrations of preservatives. Glass packaging preserved the oily preparation sufficiently well against oxidation: the EP limit applicable to oils as raw materials is a peroxide value below 10 meq/kg. This value is also suggested in edible food preparations (Frankel 1993).

The consequences of the initial studies by Huyghe et al. (2003) are still to be implemented in the current regulatory pharmaceutical context. The present study has extended the scope of this initial study, using an experimental design on a real-life formulation stored in glass vials. Antibacterial and anti-oxidative properties are simultaneously investigated with pharmacopoeial methods. There is a clear preservative effect of BA, while this was not the case for BHA and BHT.

This study demonstrated that oily preparations, like aqueous preparations, are also requiring robustness testing to characterise their preservative behaviour.

4. Experimental

4.1. Organisms and media

The following pharmacopoeial organisms were used in this study: *Escherichia coli* (ATCC 8739), *Staphylococcus aureus* (ATCC 6538), *Pseudomonas aeruginosa* (ATCC 9027), *Candida albicans* (ATCC 10231) and *Aspergillus niger* (ATCC 16404). Additionally, two food contaminants knowing to be resistant against low water-activity were included: *Shewanella putrefaciens* (LMG 2369) and *Lactobacillus parabuchneri* (LMG 12010). The strains were provided by BCCM/LMG (Gent, Belgium). Bacteria were grown overnight at 37 °C on Tryptone Soya Agar (TSA; Oxoid), while the two fungi were cultured on Sabouraud Dextrose Agar (SDA; Oxoid) at 25 °C. Organisms were harvested into peptone water, containing 0.1% (m/v) peptone (Oxoid) and 0.9% (m/v) NaCl (Oxoid), by gentle agitation, washed twice with peptone water and diluted to contain the required number of colony forming units per ml, i.e. approximately 10^8 CFU · ml^{-1}. The peptone water used for harvesting *A. niger* contained 0.05% (m/v) of Tween 80 (Sigma-Aldrich).

4.2. Chemicals

All chemicals used were of food and/or pharmacopoeial grade. Soybean oil was obtained from Soetenay. Both active drug substances, sodium closantel (hydrated sodium-5′-chloro-4′-(4-chloro-α-cyanobenzyl)-3,5-diiodosalicyl-*o*-toluidide) and ivermectine were supplied by Janssen Animal Health. Col-

loidal silicon dioxide was from Degussa, while benzyl alcohol (BA), butylated hydroxytoluene (BHT) and tert-butyl-4-hydroxyanisol (BHA) were from Sigma-Aldrich.

4.3. Preparation of the test formulations

The test formulations were independently prepared by adding weighed quantities of each of the ingredients to the soybean oil: 4% m/V total active drug substances and 2% m/V colloidal silicon dioxide, various concentrations of the three preservatives and soybean oil up to 100%. The density of the final formulations was 0.944 $g \cdot ml^{-1}$. The final concentrations of the three preservatives under investigation in these oily formulations are given in Table 1.

4.4. Preservative efficacy test (PET) methodology

The preservative efficacy was assessed by the European Pharmacopoeia (EP) microbial challenge test-method (EP 2002) concerning oral preparations. The samples were placed in sterile glass containers and separately inoculated with bacterial and fungal suspensions to give a final level of approximately 10^6 CFU $\cdot$ ml^{-1}. The preparations are thoroughly shaken to ensure a homogeneous microorganism distribution and incubated at 22.5 °C.

After a contact time of 0, 7, 14, 21 and 28 days, preparations were thoroughly shaken again and samples (1.0 ml) were removed and placed into 99.0 ml of neutralising medium MLEB (Difco). Cell viability was determined by the pour-plate count method in TSA or SDA plates, and CFUs were counted after a 3 and 5-day incubation at 37 °C and 30 °C for bacteria and fungi respectively. All counts were performed in duplicate.

A growth control with the medium alone at day 0 was always included for each formulation and organism. Moreover, days 7 and 21 were included, although these are not evaluation points according to the EP, to allow a weekly data-evaluation.

The viability of the inoculated cells and their ability to grow after dilution were evaluated by growth controls. The individual validation data are given in Table 5, indicating recoveries are in compliance with the pharmacopoeial criteria, as well as a low variability between the duplicate values.

4.5. Antioxidant efficacy test (AET) methodology

Antioxidant activity was determined following the EP method for peroxide value on 5 g sample (EP 2002). Results are expressed as meq per kg.

4.6. Statistical analysis

To effectively characterise the variables included in Table 1, a statistically designed experiment was used. The experimental design selected was a Box-Behnken design, with three levels for each of the three factors, since it is the most efficient design requiring only 13 unique experiments (Box and Behnken 1960). The geometric character of the Box-Behnken design is a cube with the individual experiments located at the midpoint factor levels (12 midpoints) and at the centre. This central point was repeated three times, to allow assessment of variability. A corner-point of the Box-Behnken cube with the lowest concentrations for all variables was added, as this design suffers potential model errors at the corners of the design space. In total, 16 formulations were thus independently prepared.

The linear regression model was applied on the first time points with non-zero CFU $\cdot$ ml^{-1} till and including the first time point with zero recovery, where the log-count was set at zero. Further zero recoveries were excluded from the linear regression analysis, which was performed in SPSS 11.0 (SPSS Inc.) software. The slopes of the obtained linear regressions, expressed as log CFU $\cdot$ $(ml \cdot day)^{-1}$ were statistically analysed using univariate ANOVA in SPSS 11.0, followed by post-hoc multiple comparison (Least Significant Difference) and graphical evaluations (box-plots and estimated marginal means). Boxplots, or box-and-whisker plots, were used to graphically represent the data: a line is drawn at the median, the rectangular box contains 50% of the observations (the interquartile range), while each of the whiskers spans 25% of the observations in the data set. Extreme outliers, i.e. individual values falling more than three times the interquartile range beyond the ends of the box, are indicated by an asterisk.

Table 5: Counting validation results

Test organism	Reference count	Product count[a]
E. coli	121/126	120/112/116/118
S. aureus	124/117	120/118/118/109
Ps. aeruginosa	18/24	18/23/20/18
C. albicans	88/94	87/92/90/88
A. niger	45/47	55/53/50/52
S. putrefaciens	18/18	20/21/20/14
L. parabuchneri	14/18	10/16/12/24

[a] Formulation number 12 of the Box-Behnken experimental cube was taken as preparation for validation purposes, as it contained the maximal concentration of BA. The first two results are those obtained at a 10^{-2} dilution, while the last two results are those obtained at a 10^{-4} dilution.

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