CHARACTERIZATION OF A SYSTEM USED TO EXPOSE ORGAN-LIKE TISSUES TO AEROSOLS: A BAYESIAN ANALYSIS OF A SPLIT-SPLIT-PLOT DESIGN

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Introduction

Philip Morris International is developing potential Reduced Risk Products[1] (RRPs) and is conducting a series of clinical and preclinical studies to assess the health risks associated with these products. Among these, studies are performed to investigate the biological response of organotypic systems (e.g. bronchial or nasal tissues) to exposure of aerosols generated from RRPs. In this context, the Vitrocell® aerosol exposure system is used and allows the simultaneous exposure of multiple organotypic tissue cultures to different doses of a given aerosol *in vitro*. As there are complex aerosol dynamics involved, it is of prime interest to characterize this exposure setup and understand its stability in order to ensure that organotypic tissue cultures can indeed be reproducibly exposed to the desired dose of the aerosol of interest.

In one such characterization study, we investigated the impact of aerosol particle sizes and concentrations on the aerosol delivery to the exposure chambers in the system. This study involved the generation of various fluorescently labelled glycerol aerosols of given particle sizes, exposure of the Vitrocell® system to specific concentrations of these aerosols, and the quantification of the delivered fluorescent activity. This experiment resulted in a split-split-plot error control design that we analyzed with a Bayesian approach.

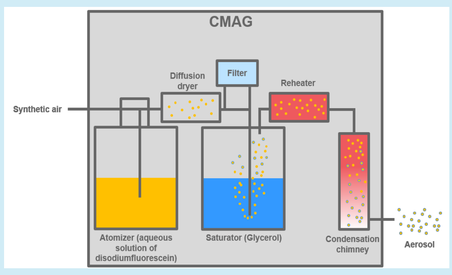
Experimental Methods

The experiment consisted of 3 main steps:

Aerosol generation of various particle sizes (Figure 1) → *Exposure to aerosols (Figure 2)* → *Quantification of aerosol delivery (Figure 3)*

Figure 1: Schematic representation of the generation of fluorescently labelled glycerol aerosols of various mean particle sizes.

A Condensation Monodisperse Aerosol Generator (CMAG) was used to generate fluorescently labelled glycerol aerosols. In brief, glycerol was condensed on disodium fluorescein nuclei. The particle size depends on the number of nuclei provided and the amount of glycerol vapor used. Continuous, stable generation of aerosols with a wide range of mean particle sizes (and controlled geometric standard deviation) can be achieved.



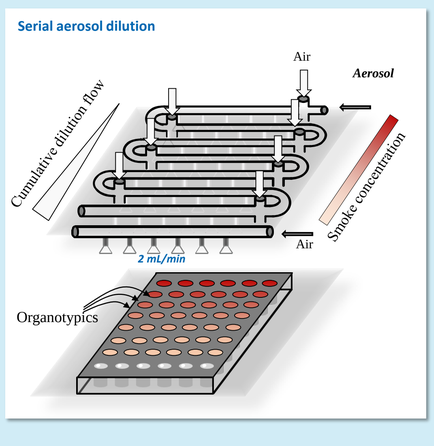


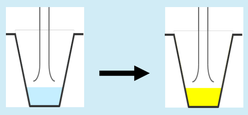
Figure 2: Schematic representation of the VITROCELL® 24/48 exposure system.

A climatic chamber houses an exposure module, which consists of a dilution/distribution system, on top of a cultivation base module. In the base module, up to 48 wells can be exposed simultaneously. The base module has a format of 8 rows x 6 columns.

The delivery of aerosol was achieved by individual trumpets, delivering the aerosols from the dilution/distribution system to the wells of the cultivation base module.

Figure 3: Schematic representation of the quantification of aerosol delivery.

In the cultivation base module, cell culture inserts were filled up with 100 µl of phosphate-buffered saline (PBS). During the 28 minutes of exposure (corresponding to our usual exposure duration of organotypic tissues), the disodium fluorescein was trapped in PBS and its concentration (quantity) was subsequently measured by a spectrophotometer.



Experimental study design:

In brief, over 5 different days (~run), 5 aerosols labeled A, B, C, D, E of various mean particle sizes were generated per day (the target mean particle sizes were < 0.5 µm, 0.83 µm, 1.13 µm, 1.41 µm, 1.63 µm respectively). Each of these aerosols was used as input to the Vitrocell® exposure system during 28 minutes, using 7 different concentrations simultaneously: 69%, 54%, 32%, 19%, 13%, 10%, and 7%. In each row of the Vitrocell® receiving a given concentration, 6 wells (number from 1 to 6) were exposed and separately measured for their disodium fluorescent content.

Run 1 (~Day 1)						← *Run or Day*
A	B	C	D	E		← *Aerosol*
69%	69%	69%	69%	69%		← *Concentration*
1 2 3 4 5 6	1 2 3 4 5 6	1 2 3 4 5 6	1 2 3 4 5 6	1 2 3 4 5 6		← *Well*
54%	54%	54%	54%	54%		
1 2 3 4 5 6	1 2 3 4 5 6	1 2 3 4 5 6	1 2 3 4 5 6	1 2 3 4 5 6		
32%	32%	32%	32%	32%		
1 2 3 4 5 6	1 2 3 4 5 6	1 2 3 4 5 6	1 2 3 4 5 6	1 2 3 4 5 6		
19%	19%	19%	19%	19%		
1 2 3 4 5 6	1 2 3 4 5 6	1 2 3 4 5 6	1 2 3 4 5 6	1 2 3 4 5 6		
13%	13%	13%	13%	13%		
1 2 3 4 5 6	1 2 3 4 5 6	1 2 3 4 5 6	1 2 3 4 5 6	1 2 3 4 5 6		
10%	10%	10%	10%	10%		
1 2 3 4 5 6	1 2 3 4 5 6	1 2 3 4 5 6	1 2 3 4 5 6	1 2 3 4 5 6		
7%	7%	7%	7%	7%		
1 2 3 4 5 6	1 2 3 4 5 6	1 2 3 4 5 6	1 2 3 4 5 6	1 2 3 4 5 6		

Figure 3: Schematic representation of the experimental design for Day 1.

The design was then replicated over 4 additional days (N = 5 days in total).

Study design expressed statistically:

This study design corresponds to a split-split-plot error control design without subsampling:

- 'Days' are (random) blocks;
- Whole-plot experimental units are assigned the levels of 'aerosol';
- Subplot experimental units are assigned the levels of 'concentration';
- Sub-subplot experimental units are assigned the levels of 'well'.

Statistical Methods

The considered statistical model was:

$$Y_{ijkl} = \mu + \alpha_i + \beta_j + (\alpha\beta)_{ij} + \gamma_k + \delta_l + (\alpha\beta\gamma)_{ijk} + \varepsilon_{ijkl}$$

where

- Y_{ijkl} is the response variable (disodium fluorescein amount) corresponding to aerosol *i* (*i* = 2, …, 5), run *j* (*j* = 1, …, 5), concentration *k* (*k* = 2, …, 7), and well *l* (*l* = 2, …, 6);
- μ is the intercept (corresponding to the average obtained for $\alpha_1, \gamma_1, \delta_1$);
- $\alpha_i, \gamma_k, \delta_l$ are fixed effects corresponding to aerosol, concentration, and well respectively;
- β_j, $(\alpha\beta)_{ij}$, $(\alpha\beta\gamma)_{ijk}$ are random effects corresponding to run, aerosol*run, and aerosol*run*concentration respectively with $\beta \sim N(0, \beta)$, $(\alpha\beta)_{ij} \sim N(0, \alpha\beta)$, $(\alpha\beta\gamma)_{ijk} \sim N(0, \alpha\beta\gamma)$;
- ε_{ijkl} are assumed to be independent and identically distributed with $\varepsilon_{ijkl} \sim N(0, \varepsilon)$ (and represent the aerosol*day*concentration*well interaction plus an error term).

Our Bayesian approach used the following 'vague' priors (since no prior knowledge was available for this first experiment):

- $\mu, \alpha_i, \gamma_k, \delta_l \sim N\left(0, \sqrt{10^{10}}\right)$ (default prior of the MCMCglmm package; $Y_{ijkl} \ll 100$)
- $\varepsilon \sim U(0, \infty)$
- $\beta, \alpha\beta, \alpha\beta\gamma \sim U(0, \infty)$ (prior type 1, main) or $\sim \text{half}-\text{Cauchy}\left(\sqrt{1000}\right)$ (prior type 2) or $\sim \text{inv}-\text{Gamma}(0.001, 0.001)$ (prior type 3)

The model was implemented using the MCMCglmm package and one chain was run with 80'000 iterations and a thinning parameter set to 10. The first 5'000 MCMC chain values were considered as 'burning steps'. Note that a classical frequentist approach was also used to estimate the model parameters by restricted maximum likelihood for comparison with the Bayesian approach.

Results

For each type of prior, the Bayesian model comprised 16 parameters representing the fixed effects, as well as 4 variance components induced by the topography of the design used. Examination of trace plots, autocorrelation plots, and density plots showed no strong evidence of convergence issue for the 20 model parameters (see Figure 4 for representative parameters).

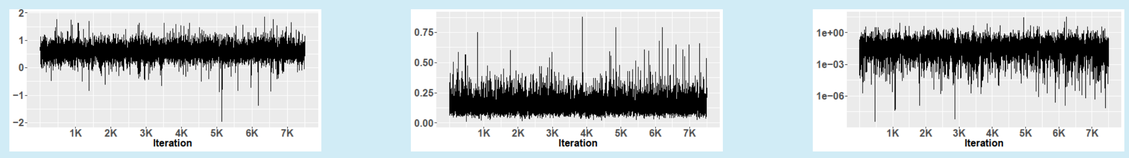


Figure 4: Representative trace plots (prior type 1).

Left panel: intercept μ, center: variance component $\alpha\beta$, right: variance component β (log-scale). The various trace plots showed a good mixing of the chain. Given the limited number of runs performed (N = 5), the variance component due to 'run', β, was the most difficult parameter to estimate and, given its amplitude, is adequately visualized on the log scale.

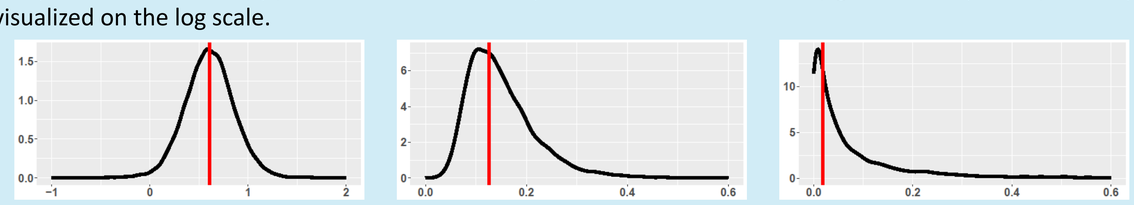


Figure 5: Representative density plots (prior type 1) and closeness with frequentist results.

Left panel: intercept μ, center: variance component $\alpha\beta$, right: variance component β. The red straight line highlights the frequentist estimate of the parameter. The representative density plots were usually in agreement with their frequentist counterparts. This result is less obvious for the variance component due to 'run', β.

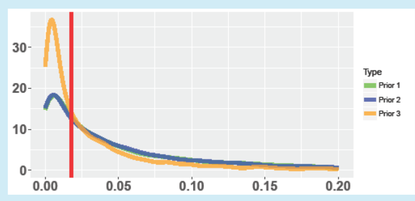


Figure 6: Influence of priors on β.

The figure contains the posterior probabilities of β given the 3 types of prior considered. As can be seen, the posteriors obtained under a uniform prior (type 1) and a half-Cauchy (type 2) closely overlap, while the one obtained under the inv-Gamma (type 3) tends to grant more weight to lower values, highlighting the impact of the choice of the priors on the posterior distribution of this parameter. It is worthwhile noticing that the other model parameters were almost not affected by the prior choice.

Discussion

- This poster describes an example of a Bayesian analysis of a split-split-plot design. The obtained results are largely in-line with the frequentists ones, but offer two major advantages. First, the results can be interpreted by means of Bayesian-probabilities with the focus being more on effect sizes rather than on p-values (note that p-values estimated under the Bayesian context are similar to the classical frequentist ones). Second, the posterior distributions obtained during this initial work of system characterization can be used as prior information when additional experiments will be performed, offering a natural way to use this historical information.
- Given the limited number of runs (N = 5), the posterior of the variance between runs is not dominated by the likelihood and thus still influenced by the choice of the prior. For future studies, increasing the number of runs should help. The choice of the priors do not seem to play a role in the posterior distributions of all other model parameters, meaning that the choice of the prior should have a minimal effect on the main conclusions regarding the characterization of the system (not discussed here).
- Additional model complexity could be considered, for instance additional interactions between fixed factors or autocorrelations between wells.
- Pertaining to the implementation, the model was also implemented in Stan (through the rstan package) and results revealed to be similar.

References

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[1]Reduced Risk Products ("RRPs") is the term we use to refer to products with the potential to reduce individual risk and population harm in comparison to smoking cigarettes. PMI's RRPs are in various stages of development and commercialization, and we are conducting extensive and rigorous scientific studies to determine whether we can support claims for such products of reduced exposure to harmful and potentially harmful constituents in smoke, and ultimately claims of reduced disease risk, when compared to smoking cigarettes. Before making any such claims, we will rigorously evaluate the full set of data from the relevant scientific studies to determine whether they substantiate reduced exposure or risk. Any such claims may also be subject to government review and authorization, as is the case in the USA today.

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COMPETING FINANCIAL INTERESTS
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