Optimization of permeabilized fibers preparation for mitochondrial respiration measurements using Design of Experiments methodology.

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Aim of the study

To optimize the permeabilized fibers (pf) preparation from mouse *Tibialis anterior* in our lab, we used the Design of Experiments (DoE) methodology that evaluates the impact of 6 experimental conditions or factors, on the pf respiration parameters (Pyruvate Malate Succinate respiration (PMS leak) and respiratory control ratio (RCR_{PMS})), to provide a maximum of information using a limited number of experiments and animals.

Materials and Methods

Test system

Animals: C57BL/6 mice, 25 week-old, male and female (n=18)
Muscle: *Tibialis anterior,* n=2 per mice
Device: High-resolution Oxygraph-2k (OROBOROS Instruments)
DoE software: NemrodW®, version 2015, NewrodW SAS, Marseille, France

Muscle fiber bundle (source : DMEM)

Fixed experimental conditions

Resting rate (PMS leak): 5 mM pyruvate, 5mM malate and 10 mM succinate
ADP-stimulated rate (PMS_P): addition of 5 mM ADP
Respiratory Control Ratio (RCR_{PMS}) set as the ratio of oxygen consumption at PMS leak (PMS_L) over oxygen consumption at PMS_P .

Responses studied

Y_1: PMS_L level ➡ to be maximized (at least 40 pmol O_2/s*mg fibers)
Y_2: variability of $RCR_{PMS,}$ estimated by coefficient of variation of 4 repeated experiments ➡ to be minimized

Experimental domain

Factors evaluated	Level -1	Level +1
Fiber Types	Red	White
Manual Teasing	Gentle	Rough
Saponin content	8-25% (S1)	20-35% (S2)
Saponin concentration for permeabilization	25 µg/ml	50 µg/ml
Permeabilization time	10 mn	30 mn
Resting period before permeabilization	0 hour	6 hours

Design of Experiments

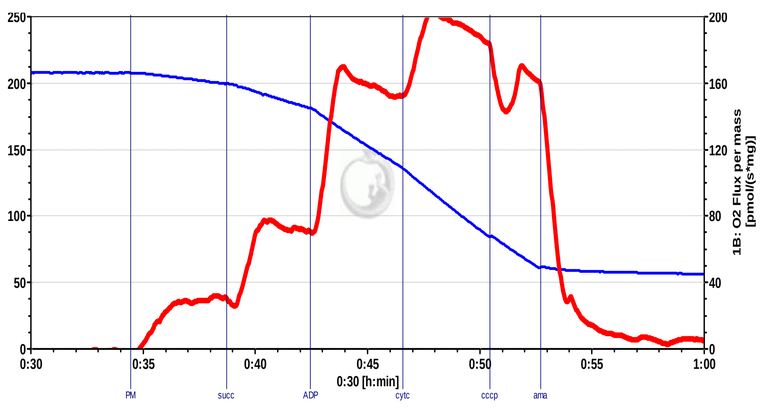
The influence of 6 factors on Y_1 and Y_2 responses has been evaluated using a Hadamard matrix with 8 experiments (instead of 64 experiments if all combinations had been tested with a « One-Factor-At-A-Time » (OFAT) method), see below. To evaluate experimental variance for Y_1 response, each experiment has been replicated 4 times. To evaluate experimental variance for Y_2 response, one experiment (n°6) has been replicated 4 additional times. In total, 36 experiments have been performed.

Results / Interpretations

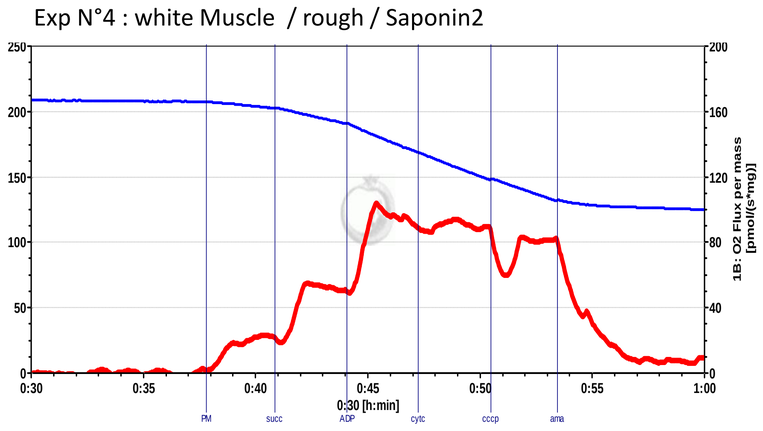
Experimental matrix and results

Experiments	Fiber types	Manual teasing	Saponin content	Saponin concentration (µg/ml)	Permeabilization time (min)	Resting period (h)	Y_1: PMS_L level (pmol O_2/s*mg)				Y_2: RCR_{PMS} variability
N°1	White	Gentle	S1	25	30	0	30.7	24.6	12	33.5	0.196
N°2	Red	Gentle	S1	50	10	6	80.1	56.1	81	61.2	0.194
N°3	Red	Rough	S1	50	30	0	56.4	63.8	68.6	90.2	0.084
N°4	White	Rough	S2	50	30	6	31.3	42.9	27.6	39.5	0.204
N°5	Red	Gentle	S2	25	30	6	51.2	53.4	53.3	63.5	0.096
N°6	White	Rough	S1	25	10	6	32.8	30.3	44.8	43.1	0.094
							67.9	81.9	35.2	49.5	0.099
N°7	White	Gentle	S2	50	10	0	31.8	24.3	25.1	22.2	0.120
N°8	Red	Rough	S2	25	10	0	62.4	61.4	69.6	45.9	0.202

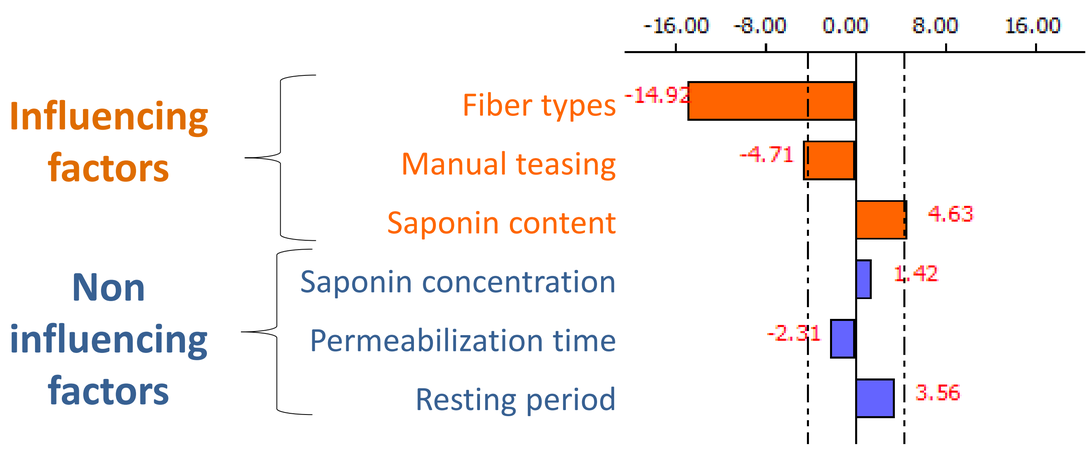
Exp N°2 : red Muscle/ gentle / Saponin1



Exp N°4 : white Muscle / rough / Saponin2



Y_1: PMS_L level



Best experimental conditions to maximize PMS_L level are red fibers, rough manual teasing and 20-35% saponin content.

To be noticed:

- ✓ Six hours resting period had no deleterious impact on PMS_L level, allowing a more convenient organization of the protocole schedule.
- ✓ Objective level of 40 pmol O_2/s*mg fibers is reached with both saponin content allowing to choose between the more pratical/less toxic mode of preparation.

Y_2: RCR_{PMS} variability

Evaluation of experimental variance with only one replicate of one experiment over 8 was not accurate enough to discriminate with confidence which of the 6 tested factors are really influencing RCR_{PMS} variability. Nevertheless, it seems that levels of influencing factors that maximize PMS_L level were not deleterious in minimizing RCR_{PMS} variability.

Conclusion

Using a DoE analysis, we were able to optimize pf assay conditions with a reduced number of experiments and animals, and rapidly obtain valuable data in accordance with ethical recommendations (3Rs). The optimization of pf preparation by DoE will be pursued with two objectives (i) studying the possible interactions existing between the 3 factors related to saponin (saponin content, saponin concentration and incubation time), (ii) calculating the optimal sample size (n) needed to observe statistically significant differences between two animal groups.

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