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

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► To cite this version:

Fabienne Cortade, Virginie Gaillard, Béatrice Chabi. Optimization of permeabilized fibers preparation for mitochondrial respiration measurements using Design of Experiments methodology.. MITOEAGLE 2017, Jul 2017, Obergurgl, Austria. , 2017. hal-02941628

HAL Id: hal-02941628

<https://hal.inrae.fr/hal-02941628>

Submitted on 17 Sep 2020

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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

Y₁: PMS_L level → to be maximized (at least 40 pmol O₂/s*mg fibers)
Y₂: variability of RCR_{PMS}, estimated by coefficient of variation of 4 repeated experiments → to be minimized

Design of Experiments

The influence of 6 factors on Y₁ and Y₂ 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 Y₁ response, each experiment has been replicated 4 times. To evaluate experimental variance for Y₂ response, one experiment (n°6) has been replicated 4 additional times. In total, 36 experiments have been performed.

Experimental domain

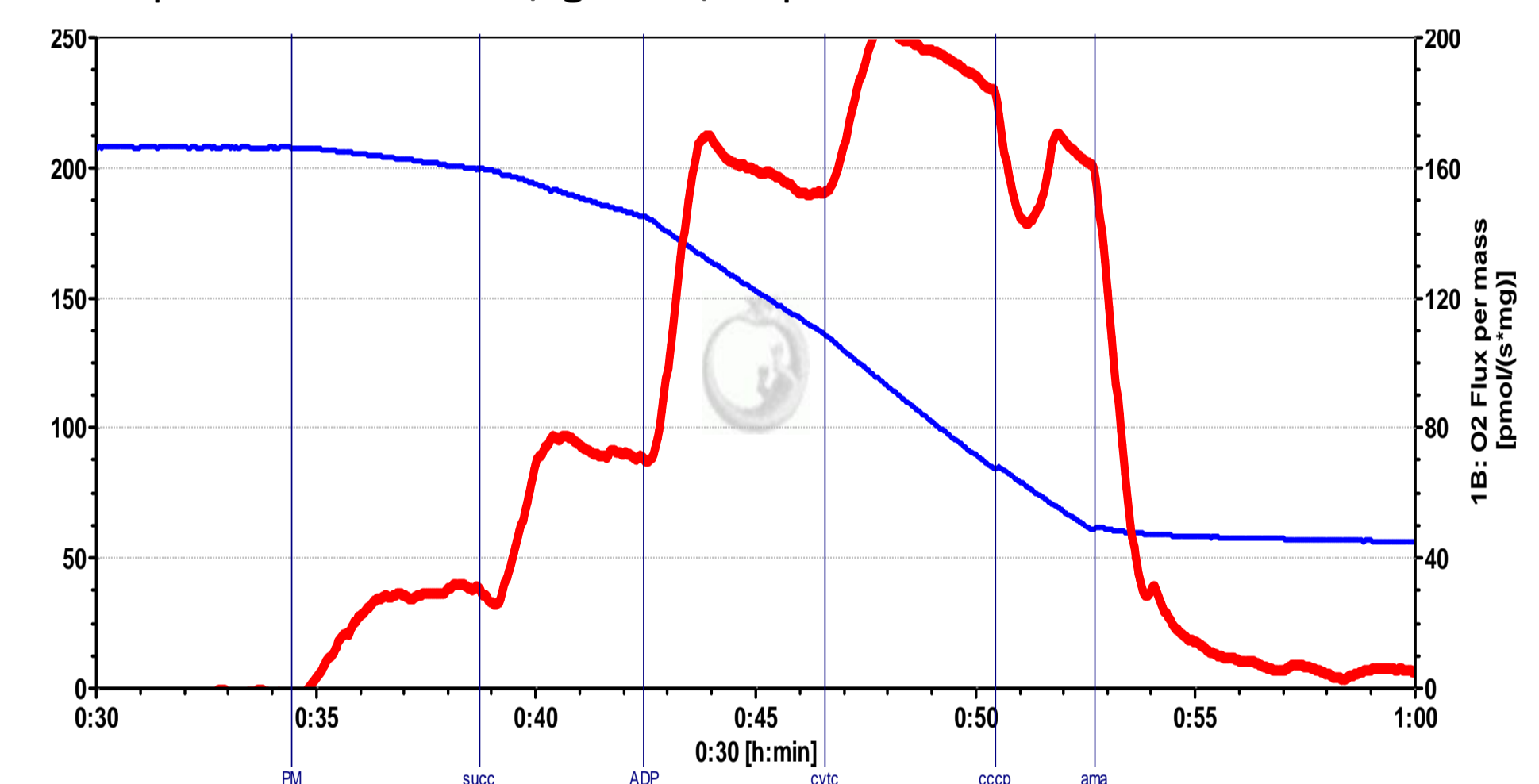
Factors evaluated	Level -1	Level +1
Fiber Types	Red	White
Manual Teasing	Gentle	Rough
Saponin content	8-25% (S1)	20-35% (S2)
Saponin concentration for permeabilization	25 µg/ml	50 µg/ml
Permeabilization time	10 mn	30 mn
Resting period before permeabilization	0 hour	6 hours

Results / Interpretations

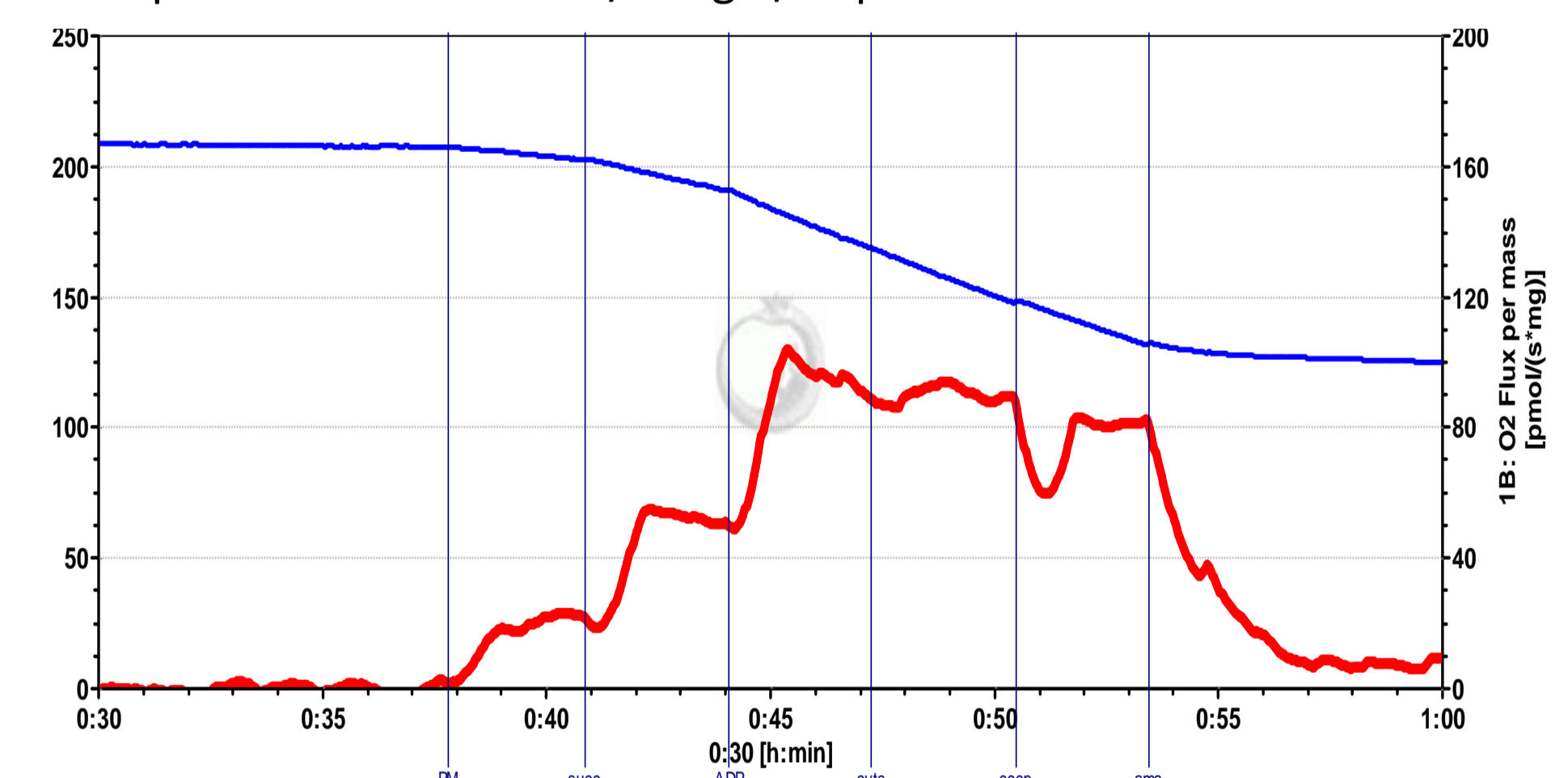
Experimental matrix and results

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
							1	2	3	4	
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

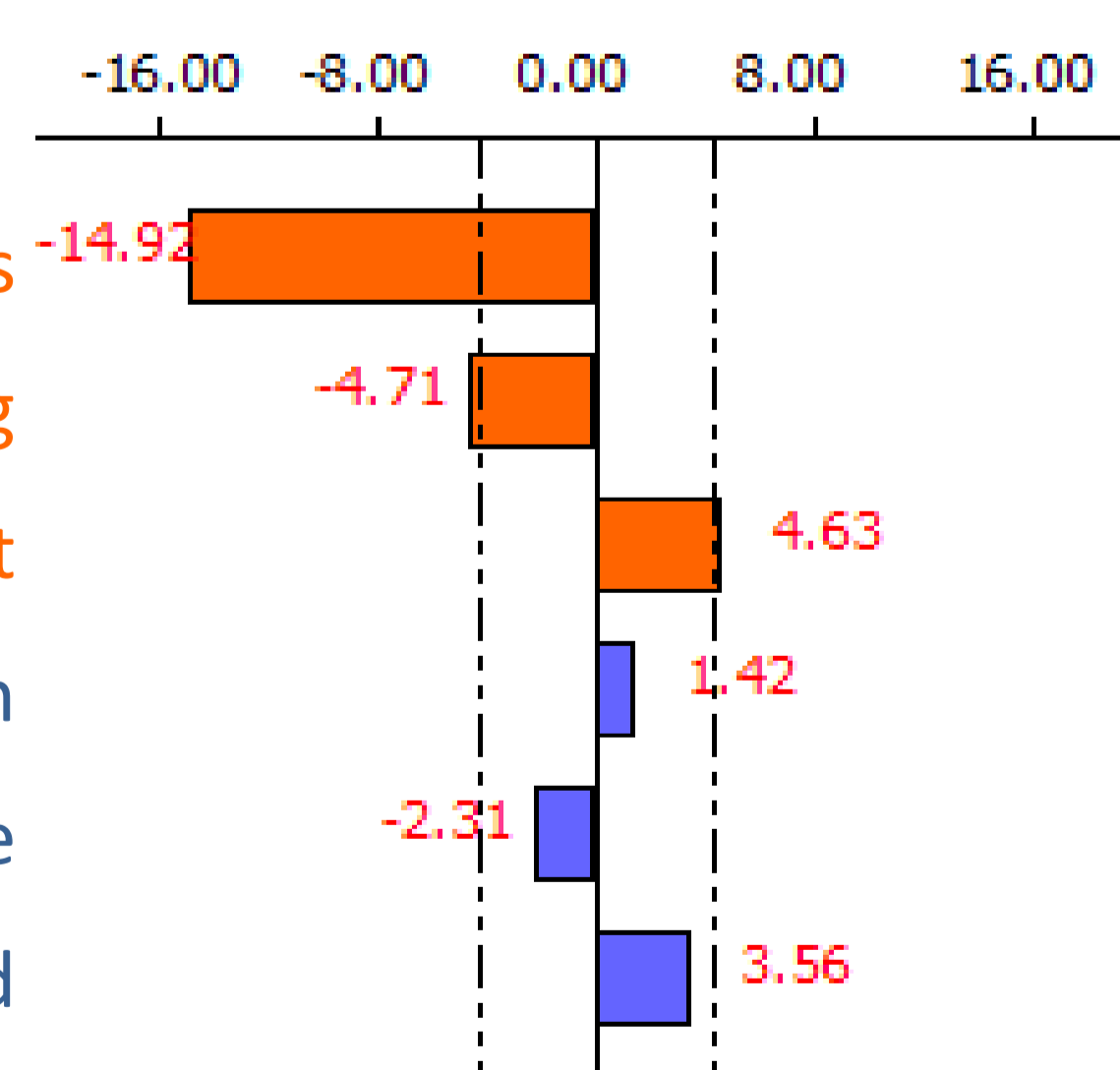
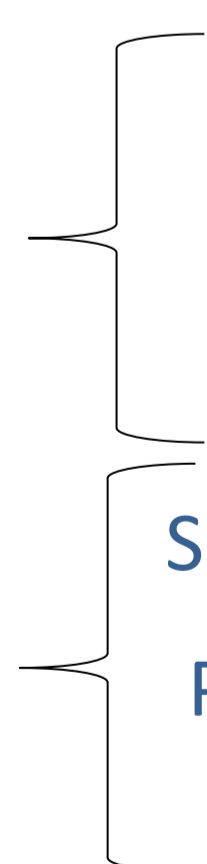


Influencing factors

Non influencing factors

Y₁: PMS_L level

Y₂: RCR_{PMS} variability



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 O₂/s*mg fibers is reached with both saponin content allowing to choose between the more practical/less toxic mode of preparation.

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.