Cortisol determination in dairy cows hairs by Near-infrared (NIR), Mid-infrared (MIR) and Raman spectroscopy.

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Introduction

Biomarkers of chronic stress?

Grelet et al 2022 has determine two biomarkers of chronic stress

- hair cortisol
- glycated protein (fructosamine)

- 1047 cows hair samples were collected and analysed by ELISA and spectroscopy technique → Cortisol determination
- 134 coming from a stress experimentation → 3 spectroscopy techniques
- 913 without experimentation and coming from “Supposed stress farm” → best spectroscopy technique

The objective of this work is to evaluate the possibility of hair cortisol determination through vibrational spectroscopy
And maybe helping to directly classify if a cow is stressed or not

Experiment

Stress group (severe overstocking for 4 weeks) + punctual unusual events

<table>
<thead>
<tr>
<th></th>
<th>Number of cows</th>
<th>Place</th>
<th>Feed bunk</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stressed</td>
<td>15</td>
<td>&lt;5 m²</td>
<td>7 places</td>
</tr>
<tr>
<td>Unstressed</td>
<td>15</td>
<td>&gt;10 m²</td>
<td>&gt;15 places</td>
</tr>
</tbody>
</table>

Control group normal situation for 4 weeks

Experiment: Hair preparation

Sampling
Shaving cow’s tail

Sieving
Porosity 400 → 250 → 200µm

Cleaning
3.5ml isopropanol
Vortex: 2min 5d drying

Spectroscopic analysis

Cortisol extraction
1) 50 mg of Hair+1.5ml methanol
2) Extraction 18h 30°C
3) Centrifuge 7000 RPM 2min
4) 0.75ml of supernatant
5) Dry under vacuum
6) Complete with 0.25mL of Elisa Buffer

Grinding
Ball: 20mm
Time: 5min
Frequency: 22Hz
ELISA: Cortisol determination

Enzyme-Linked Immuno Sorbent Assay

After acidic condition: =NH $\rightarrow$ =NH$_2^+$ $\rightarrow$ Yellow colour
Optical reading at 450nm
Cortisol inhibit Horseradish Peroxidase $\rightarrow$ [Cortisol] is inversely proportional to the appearance of the color
Spectroscopy: analysis

Vibrationnel spectroscopy

Focus on infrared wavelength

Near infrared (NIR): $12500 < f < 4000 \text{ cm}^{-1}$

Mid infrared (MIR): $4000 < f < 200 \text{ cm}^{-1}$

Raman: $4000 < f < 50 \text{ cm}^{-1}$

NIR is higher in energy so it will be more focus groupment, skeleton vibration

MIR & Raman less energy focus on the bonding vibration
Spectroscopy: Apparatus

Bruker MPA spectrometer

Bruker VERTEX 70

→ ATR Platinium

→ RAM II
MIR and Raman has approximately the same excitation energy → focus on molecular structure
Both techniques will accept different vibration modes
• MIR will be related to the dipolar moment of the molecule
• Raman will be related on the polarizability of a molecule

MIR \rightarrow \text{Heteronuclear bond: C=O, C-N, C-Cl}
Raman \rightarrow \text{Homonuclear bond: C-C, C=C, C-S}

Complementary information of MIR and Raman on molecular levels
MIR: Spectra

Absorbance

N-H def C-N str
amide II band

C=O stretch, amide I band

Keratine reference (nail)

N-H str + O-H str

Wavenumber (cm$^{-1}$)
MIR: PCA

Samples/Scores Plot of MIR

- Scores on PC 2 (1.58%)
- Scores on PC 3 (0.16%)
- Stressed
- Unstressed
- 95% Confidence Level
MIR: PLS

SNV + first derivative 5% of outliers

- Stress
- Unstressed
- 1:1 Fit

\[ R^2 = 0.656 \]
- 6 Latent Variables
- \( \text{RMSEC} = 7.6954 \)
- \( \text{RMSECV} = 8.6867 \)
- Calibration Bias = 0.0021742
- CV Bias = 0.16642

Interreg North-West Europe
HappyMoo

Wallonie recherche CRA-W
NIR: Spectra

2 Major Peaks of NIR → Amides groupment
NIR: PCA

Scores on PC 2 (0.07%)
Scores on PC 3 (0.01%)
Samples/Scores Plot of NIR_2021

Stressed
Unstressed
95% Confidence Level
NIR: PLS

- SNV + first derivative of spectra and delete 5% of outliers
- $R^2 = 0.505$
- 5 Latent Variables
- $RMSEC = 8.2856$
- $RMSECV = 9.3731$
- Calibration Bias = -0.043183
- CV Bias = 0.16062
Raman: Spectra

- C-C stretch
- C-H bend
- C=O stretch, amide I band
- C-H stretch

**Wavenumber (cm\(^{-1}\))**

**Absorbance**
Raman: PCA

Samples/Scores Plot of Raman

- Scores on PC 2 (1.18%)
- Scores on PC 3 (0.15%)

Stressed
Unstressed

95% Confidence Level

Scores on PC 3 (0.15%) vs. Scores on PC 2 (1.18%)
Raman: PLS

$R^2 = 0.642$

5 Latent Variables

RMSEC = 6.7392

RMSECV = 9.0638

Calibration Bias = 1.0658e-014

CV Bias = -0.12501

Autoscale + delete 6% of outliers
### Large-scale sampling descriptive statistics

- 1047 Hair samples were collected in 12 DHI
- Analyzed in MIR analysis and ELISA
- Different farm were selected where different stress factor were supposed
- Any experimentation were done expect for CRA-stressed

<table>
<thead>
<tr>
<th>Hair Cortisol(pg/mg)</th>
<th>Count</th>
<th>Mean</th>
<th>Std</th>
<th>Min</th>
<th>25%</th>
<th>50%</th>
<th>75%</th>
<th>Max</th>
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<tbody>
<tr>
<td>Global</td>
<td>1047</td>
<td>17.34</td>
<td>10.32</td>
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<td>10.04</td>
<td>14.95</td>
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<tr>
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<td>8.04</td>
<td>3.70</td>
<td>6.02</td>
<td>7.16</td>
<td>8.60</td>
<td>28.91</td>
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<tr>
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<td>7.65</td>
<td>1.02</td>
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<td>13.29</td>
<td>19.24</td>
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<tr>
<td>Convis</td>
<td>37</td>
<td>19.29</td>
<td>6.92</td>
<td>2.04</td>
<td>14.84</td>
<td>20.18</td>
<td>24.01</td>
<td>33.23</td>
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<tr>
<td>Cra stressed</td>
<td>58</td>
<td>36.22</td>
<td>19.01</td>
<td>8.60</td>
<td>22.63</td>
<td>31.55</td>
<td>49.32</td>
<td>91.50</td>
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<tr>
<td>Cra unstressed</td>
<td>75</td>
<td>20.09</td>
<td>6.74</td>
<td>8.30</td>
<td>14.60</td>
<td>19.10</td>
<td>24.25</td>
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<tr>
<td>DOU</td>
<td>120</td>
<td>13.69</td>
<td>4.63</td>
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<td>15.23</td>
<td>21.53</td>
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<td>52.08</td>
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<tr>
<td>LKVAT</td>
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<td>11.24</td>
<td>7.70</td>
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<td>7.94</td>
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<td>PDD</td>
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<td>20.69</td>
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<td>SNO</td>
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<td>8.17</td>
<td>11.79</td>
<td>16.11</td>
<td>32.23</td>
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Large-scale prediction: modelling

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<tr>
<th>Model</th>
<th>PCR R²</th>
<th>PLS R²</th>
<th>ElasticNet R²</th>
<th>KRR R²</th>
<th>SVM-R R²</th>
<th>R²_{cal}</th>
<th>R²_{cv}</th>
<th>R²_{val}</th>
<th>RMSE_{cal}</th>
<th>RMSE_{cv}</th>
<th>RMSE_{val}</th>
<th>RPD_{cal}</th>
<th>RPD_{cv}</th>
<th>RPD_{val}</th>
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</thead>
<tbody>
<tr>
<td>PCR</td>
<td>0.48</td>
<td>0.46</td>
<td>0.61</td>
<td>0.53</td>
<td>0.79</td>
<td>0.48</td>
<td>0.44</td>
<td>0.56</td>
<td>6.43</td>
<td>6.71</td>
<td>7.18</td>
<td>1.39</td>
<td>1.33</td>
<td>1.24</td>
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<tr>
<td>PLS</td>
<td>0.44</td>
<td>0.41</td>
<td>0.47</td>
<td>0.47</td>
<td>0.52</td>
<td>0.44</td>
<td>0.41</td>
<td>0.56</td>
<td>6.25</td>
<td>6.58</td>
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<td>1.22</td>
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<td>ElasticNet</td>
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<td>0.44</td>
<td>0.58</td>
<td>0.57</td>
<td>0.63</td>
<td>0.56</td>
<td>0.44</td>
<td>0.56</td>
<td>5.51</td>
<td>6.47</td>
<td>7.02</td>
<td>1.60</td>
<td>1.37</td>
<td>1.26</td>
</tr>
<tr>
<td>KRR</td>
<td>0.53</td>
<td>0.47</td>
<td>0.58</td>
<td>0.57</td>
<td>0.63</td>
<td>0.53</td>
<td>0.47</td>
<td>0.56</td>
<td>6.10</td>
<td>6.49</td>
<td>7.06</td>
<td>1.46</td>
<td>1.38</td>
<td>1.26</td>
</tr>
<tr>
<td>SVM-R</td>
<td>0.79</td>
<td>0.52</td>
<td>0.63</td>
<td>0.63</td>
<td>1.44</td>
<td>0.79</td>
<td>0.52</td>
<td>0.63</td>
<td>4.23</td>
<td>6.49</td>
<td>6.57</td>
<td>2.20</td>
<td>1.44</td>
<td>1.42</td>
</tr>
</tbody>
</table>

SVM-R is better with an external validation
Similar tendency and performances are found than before
Conclusion & outlook

• Conclusion
  - This preliminary work has demonstrated that the hair cortisol could be determine by the spectroscopy technique
  - MIR spectrum seems to be the most accurate method to determine cortisol
  - Enlarge the dataset keep the same performance modelling
  - The chronic stress could have an effect to the structure of the hair

• Outlook
  - Enlarge the dataset: Stressed cows
  - Applied on meat cows
  - Classification with different algorithms
  - Do the analysis without any treatment of the Hair
  - With portable spectrometer

Thank you for your attention
Any questions?
o christophe@cra.wallonie.be
Week 0

Start of stress, Separation of cows

Week 1

Week 2

Week 3

Week 4

Week 5

End of stress, Grouping of both groups

Behavior observations

Saliva & blood sampling, weight & BCS recording, heart monitoring

Hair sampling

Additional stress

All cows together (n=30)

Stress group (n=15)

Control group (n=15)

All cows together

Results: ELISA

Hair cortisol

<table>
<thead>
<tr>
<th>(pg/mg)</th>
<th>Week 0</th>
<th>Week 1</th>
<th>Week 2</th>
<th>Week 3</th>
<th>Week 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unstressed</td>
<td>19.3</td>
<td>21.5</td>
<td></td>
<td></td>
<td>21.5</td>
</tr>
<tr>
<td>Stressed</td>
<td>16.5</td>
<td>24.8</td>
<td>43.3</td>
<td>23.1</td>
<td>52.0</td>
</tr>
</tbody>
</table>

- Intro: Différence stress aigue et chronique
- Intro: Explication Elisa NIR MIR Raman
- M&M Préparation des poils et schema experimental du stress
- Resultats: PCA: NIR, MIR Raman
- Outlook validation externe

- https://www.researchgate.net/figure/The-different-Amide-contributions-to-the-IR-bending-region-are-reported-with-different_fig3_268748998
"stress is the non-specific response of the body to any demand made upon it" (Selye, 1976)

- susceptibility to metabolic, inflammatory and infectious diseases (Moberg et al., 1980; Romero, 2004).
- fertility troubles (Dobson and Smith, 2000; Walker et al., 2008)
- growth disturbances (Elsasser et al., 1995)
- weight (Mormède et al., 2007)
- milk production (Tallo-Parra et al., 2018)
- production and economics of farms,
- welfare of cows
- societal perception of dairy production

Figure of General adaption syndrome (from A.C. Brown, C.I. Waslien, in Encyclopedia of Food Sciences and Nutrition (Second Edition), 2003)
Scores on PC 2 (0.08%)  
Scores on PC 3 (0.01%)  
Samples/Scores Plot of NIR_2020_1  

Stressed  
Unstressed  
95% Confidence Level  

NIR 2020  

Week 1 Stress  
Week 2 Stress  
Week 3 Stress  
Week 4 Stress  
Week 5 Stress  
Unstressed  
1:1 Fit  

R\textsuperscript{2} = 0.661  
6 Latent Variables  
RMSE = 7.9519  
RMSECV = 9.0765  
Calibration Bias = -0.0021409  
CV Bias = 0.023254
ELISA: Cortisol determination
$R^2 = 0.617$

6 Latent Variables

RMSEC = 6.4305

RMSECV = 6.765

Outlier: 21

SD: 10.9

RPD: 1.61

Pretreatment

SNV+Savgol derivative
$R^2 = 0.548$

- 6 Latent Variables
- RMSEC = 6.4305
- RMSECV = 6.765
- RMSEP = 8.6328
- Prediction Bias = -1.3142