# Milk biomarkers to detect ketosis and negative energy balance using MIR spectrometry

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# Negative energy balance and ketosis

**In early lactation:**
**energy intake < energy output**

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**Negative energy balance**

**↘ fertility**
**↘ health**
(Collard et al., 2000; Butler, 2003)

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**Prevalence : 7 to 43%** (Suthar et al., 2013)

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**↘ reproductive performances**
**↗displaced abomasum**
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**BHB and Acetone known as biomarkers**

**(Enjalbert et al., 2001)**

# Citrate ?

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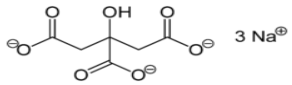
- **Present in milk**



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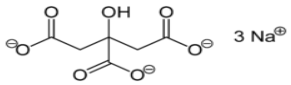


J. Dairy Sci. 95:2362–2380
http://dx.doi.org/10.3168/jds.2011-4419


**Metabolic and production profiles of dairy cows in response to decreased nutrient density to increase physiological imbalance at different stages of lactation**

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**Induced nutrient restriction**

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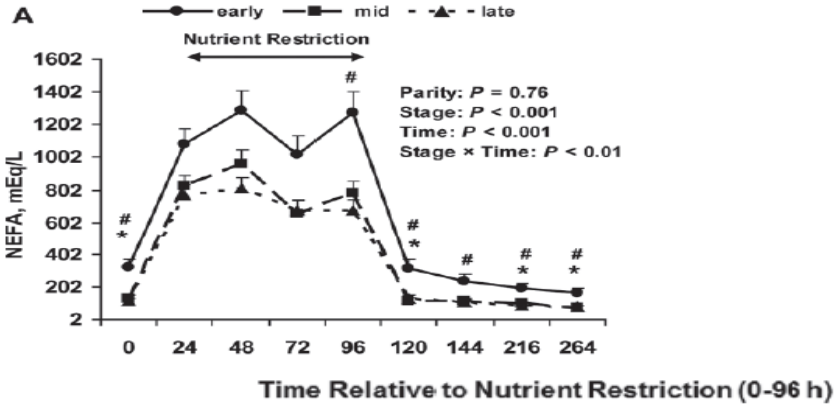

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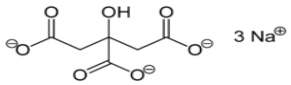


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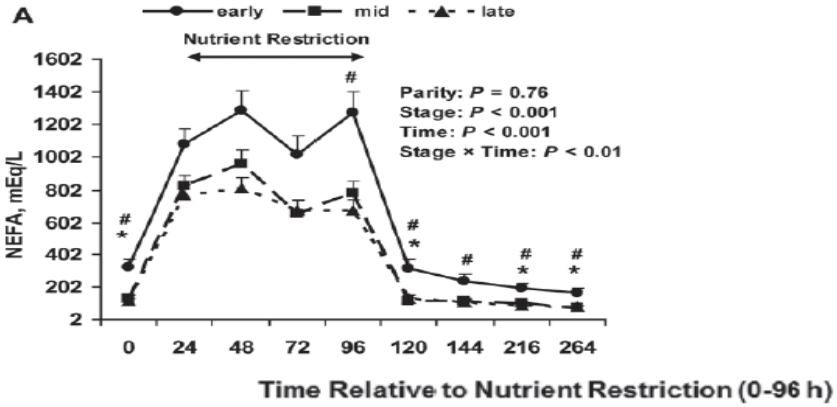
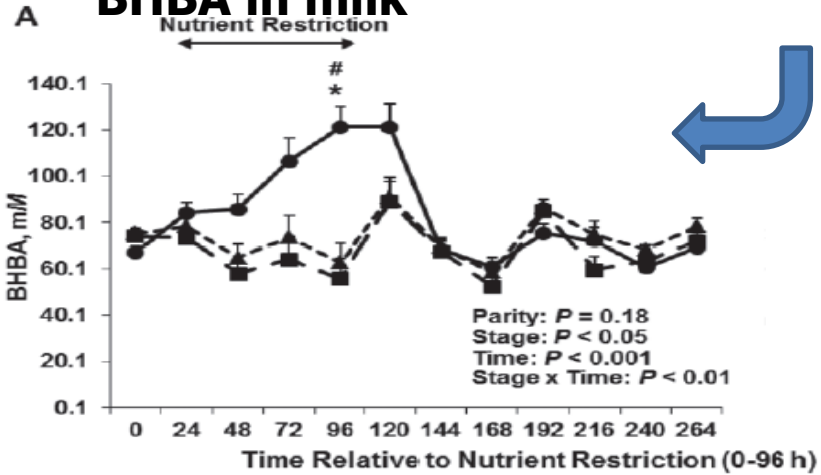
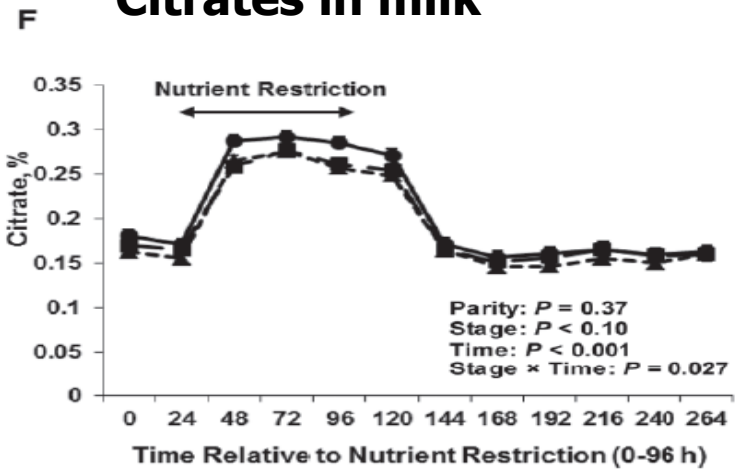



## BHBA in milk




## Citrates in milk



# Citrate ?

- **Bjerre-Harpoth (2012)**

  ***« …greatest increase (58%) during restriction for all cows »***

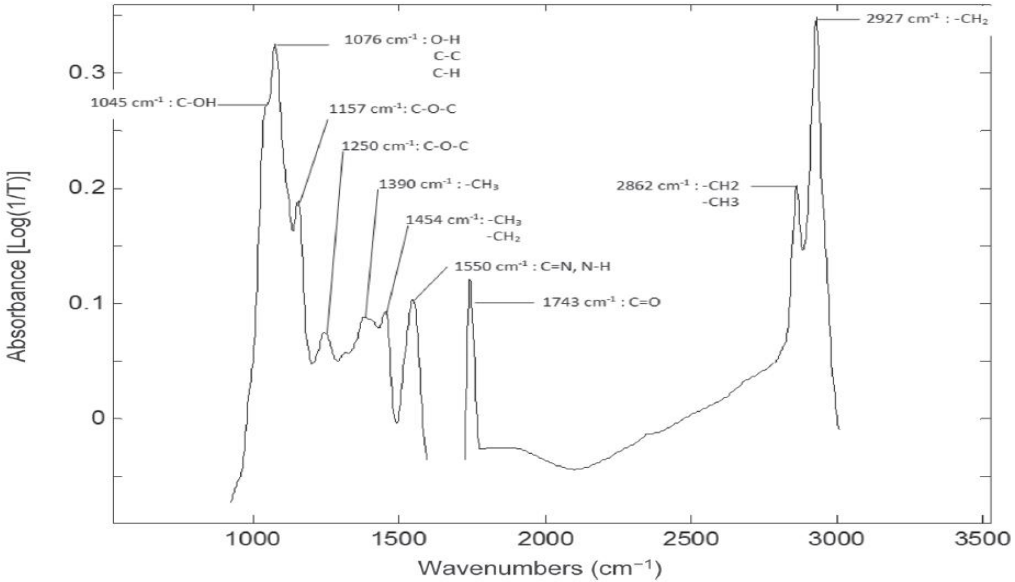
  ***« …promising <u>early</u> indicator of physiological imbalance »***

- **Baticz et al. (2002)**

  ***« Sodium citrate should be measured by easy and automated method such as FT-MIR technology to evaluate the energy status of cows »***

# Mid Infra Red (MIR)

- MIR spectrum reflect milk composition
- World-wide used for milk recording, payment
- Fast, cheap
- 1 sample → X predicted values
  - Fatty acids
  - Minerals
  - Methane
  - Cows state
  - Technical properties
  - …
- Limit of detection : 100 ppm (Dardenne, 2015)



# Previous studies in link with MIR

## Acetone: ketosis biomarker

| | | | Calibration | | | Cross validation | | | Validation | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Reference method | N | RMSE | $R^2$ | SECV | RMSE | $R^2$ | N | RMSE | $R^2$ |
| Hansen | 1999 | Vanilin test | 302 | - | - | - | 0.240 | 0.80 | 58 | 0.270 | 0.81 |
| Heuer | 2001 | Gas chromatography | 180 | - | - | 0.210 | - | - | - | - | - |
| De Roos | 2007 | Continuous flow analyer | 1063 | - | - | 0.184 | - | 0.72 | - | - | - |
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## Citrate: energy status of cow/physiological imbalance

- **Not very well documented, no target values or thresholds in the litterature**
- **No published MIR calibration (existing FOSS calibration)**

# Goals of the study

**In early lactation:**
**energy intake < energy output**

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**Negative energy balance**

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**Body fat mobilisation**

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**(1) Realize Optimir own MIR calibrations for BHB and acetone, with validation step**

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(2) Evaluate possibility to predict citrate via MIR

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(2) Evaluate possibility to predict citrate via MIR

(3) Use samples and spectra from severals countries

→ robust equations

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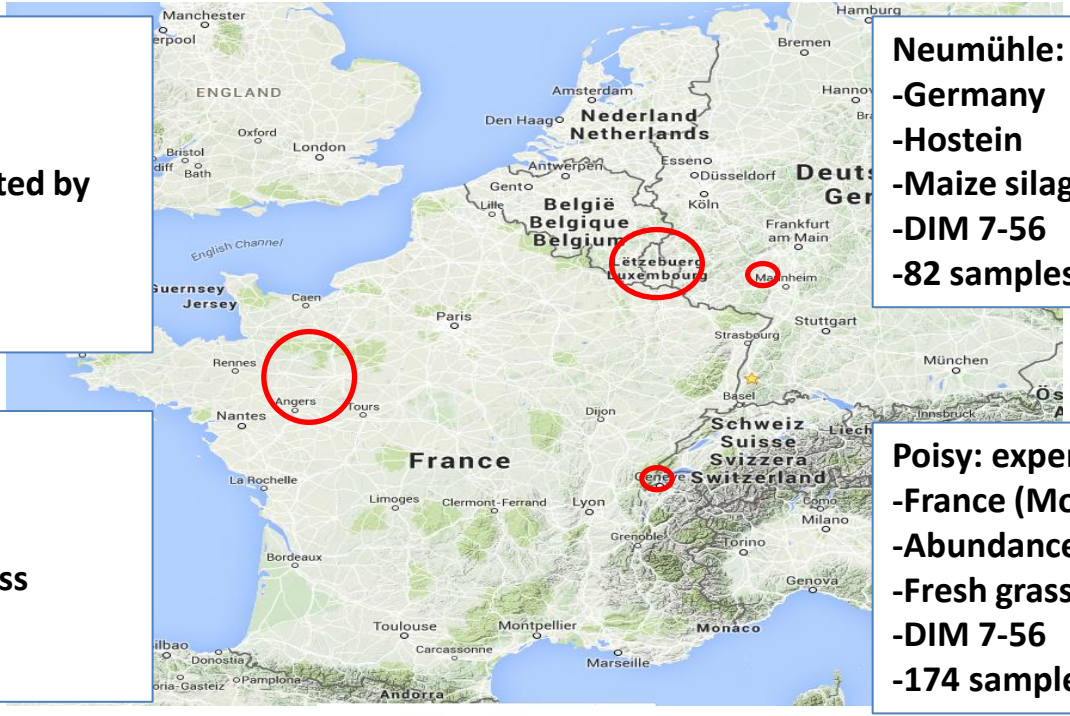
# Collect of samples

**Convis: MRO**
**-Luxembourg**
**-Hostein**
**-Maize silage supplemented by grazing in summer**
**-DIM 5-60**
**-110 samples**




**Neumühle: experimental farm**
**-Germany**
**-Hostein**
**-Maize silage**
**-DIM 7-56**
**-82 samples**

**CLASEL: MRO**
**-France**
**-Hostein and Normande**
**-Maize silage or fresh grass**
**-DIM 7-305**
**-200 samples**

**Poisy: experimental farm**
**-France (Montain area)**
**-Abundance and Montbéliarde**
**-Fresh grass or hay and maize silage**
**-DIM 7-56**
**-174 samples**

- Harmonized protocol by IDELE
- ICAR approved sampling systems
- Morning and evening samples pooled
- 566 * 2 identical samples generated → MIR and chemical analysis

# Analysis of samples

- Chemical analysis at CRA-W (Belgium)
- Continuous flow analyzer (Skalar, The Netherlands)
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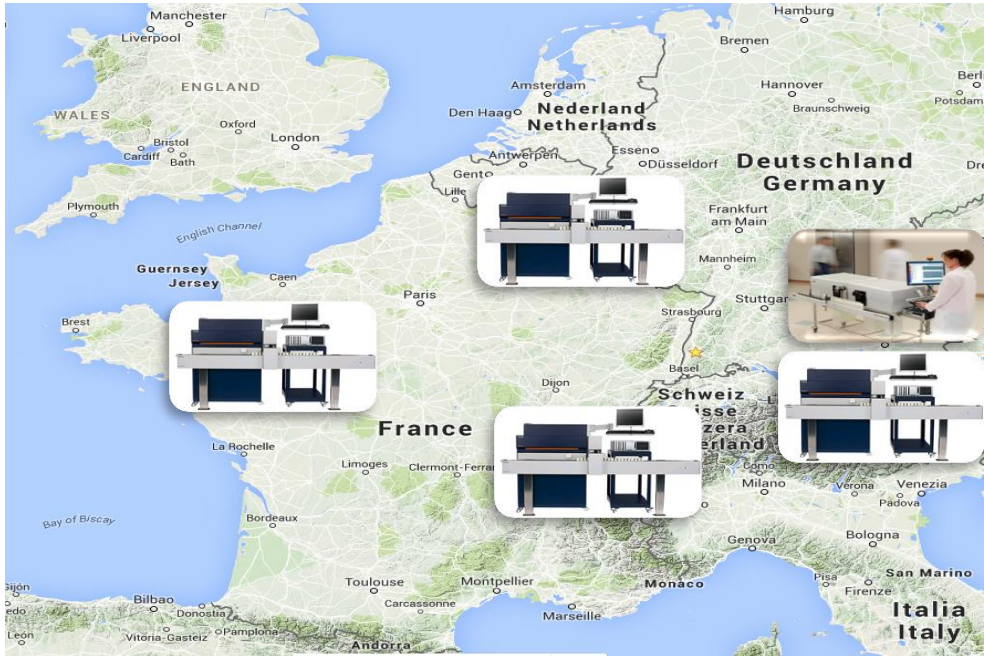
- Spectral analysis locally
- Foss and Bentley
- Standardization of spectra enabling a common database and a common use



# Results of chemical analysis

- **566 samples in total**
- **Removing of missing values**
- **Same ranges than litterature** (Denis-Robichaud et al., 2014; Garnsworthy et al., 2006)

| Component | Unit | N | Min | Max | **Mean** | SD | SEL |
|---|---|---|---|---|---|---|---|
| BHB | mmol/L | 558 | 0.045 | 1.596 | **0.215** | 0.174 | 0.005 |
| Acetone | mmol/L | 548 | 0.02 | 3.355 | **0.103** | 0.26 | 0.006 |
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- **Limit of detection with MIR: 100 ppm**

| | Concentration (mmol/L) | Molar mass (g/mol) | Concentration (ppm) | |
|---|---|---|---|---|
| BHB | 0.215 | 104.10 | 21.7 | → Indirect prediction |
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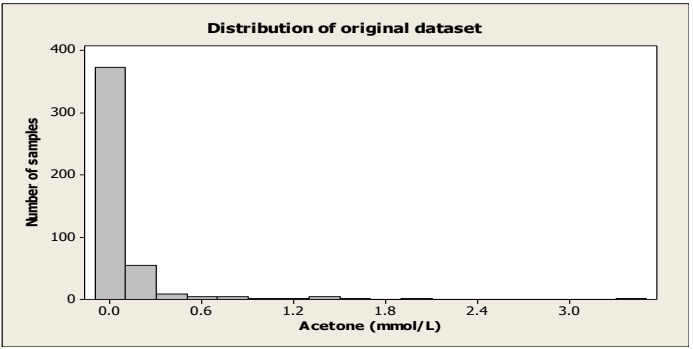
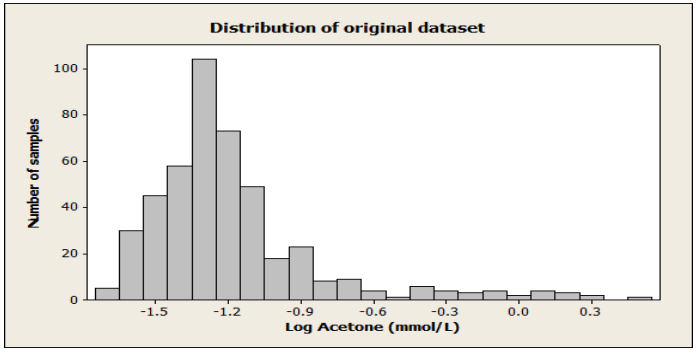
  **→ Use of Log (10) transformation**



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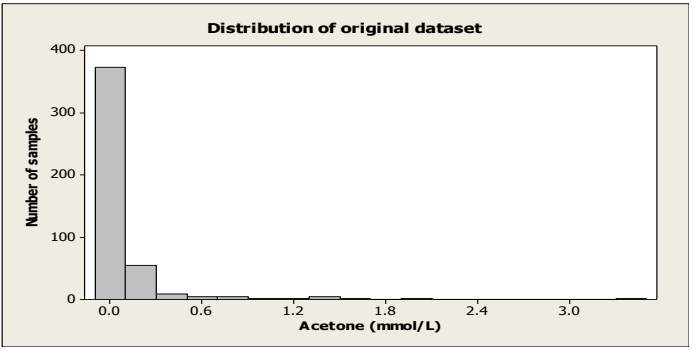


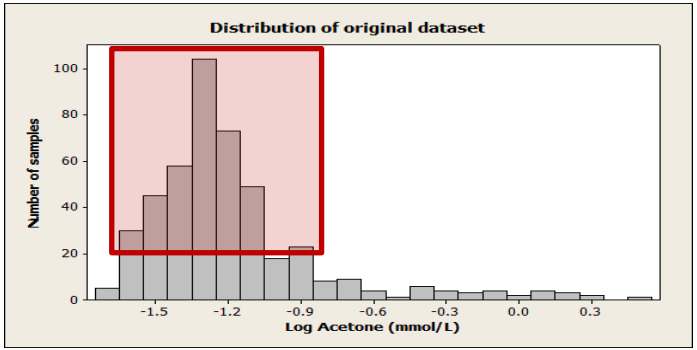

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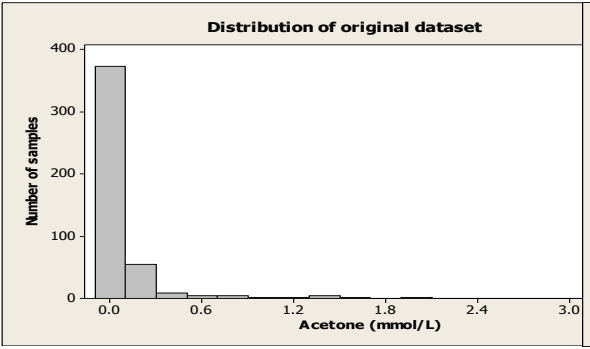


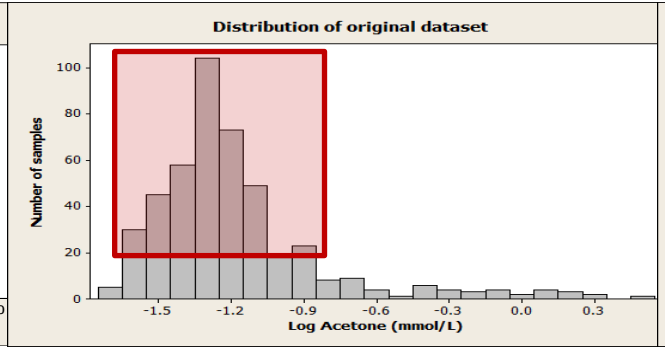


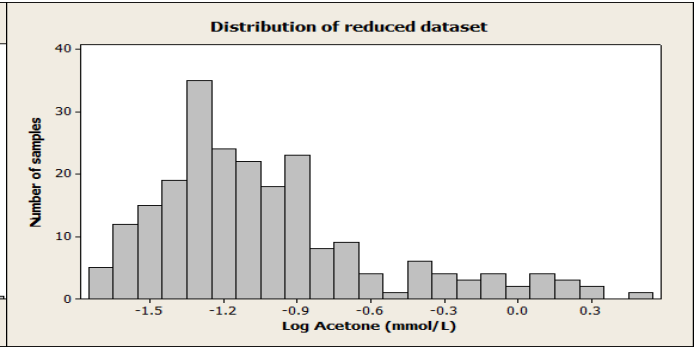


**558→ 433 samples for BHB**

**548 → 224 samples for acetone**

# MIR calibrations

- **Spectral pretreatment:**

  **Absorbance, Standardized, First derivative gap 5, Autoscale**

  **Area used : 968.1 - 1577.5, 1731.8 - 1762.6, 1781.9 - 1808.9 and 2831.0 - 2966.0 $cm^{-1}$**

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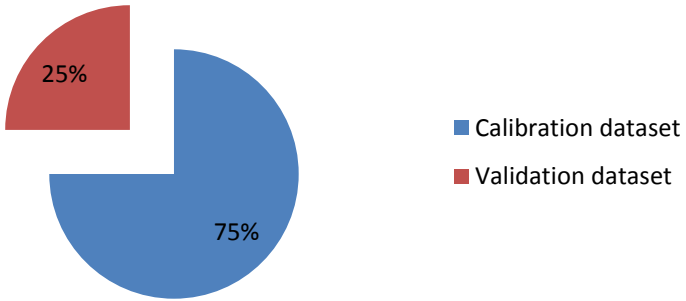
- **Partial Least Square (PLS) regression**
- **Cross-validation using 10 subsets**
- **Validation ¾ - ¼**

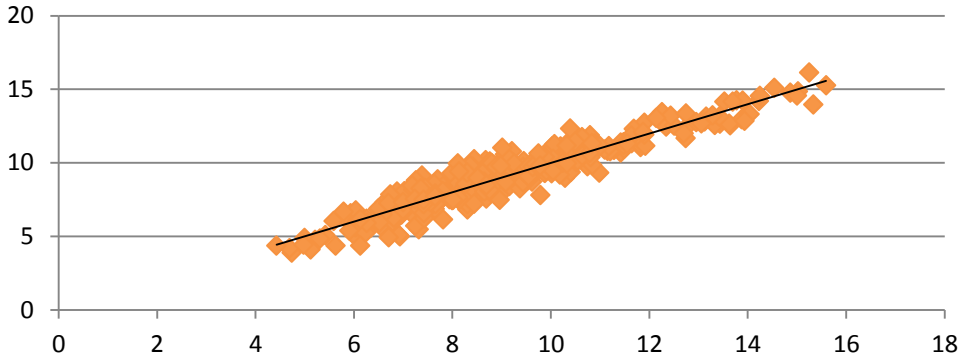



- **Use of Matlab and the PLS toolbox**

# MIR calibrations

- **Criteria observed**
  - **$R^2$ (but dependent of the range)**
  - **RMSE (Root Mean Square Error)**

$$\mathrm{RMSE} = \sqrt{\frac{\sum_{i=1}^{n} |\varepsilon_i|^2}{n}}$$



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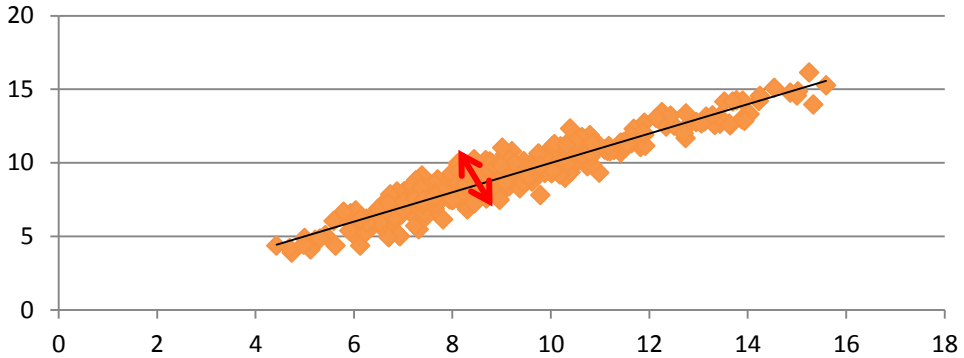

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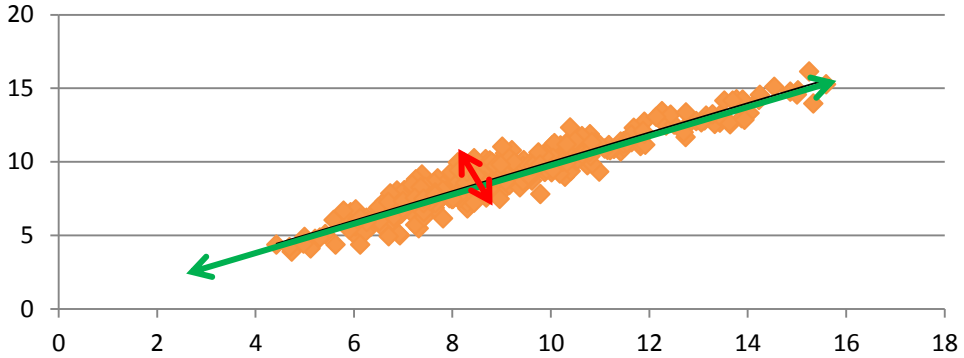


  - **RPD = SD (calibration) / RMSE**

| RPD | | Class | Application | Symbol |
|---|---|---|---|---|
| 0 | 2 | Very poor | Allows to compare groups of cows, distinguish high or low values | - |
| 2 | 3 | Poor | Rough screening | 0 |
| 3 | 5 | Fair | Screening | + |
| 5 | 6.5 | Good | Quality control | ++ |
| 6.5 | + | Excellent | As precise as reference value | +++ |

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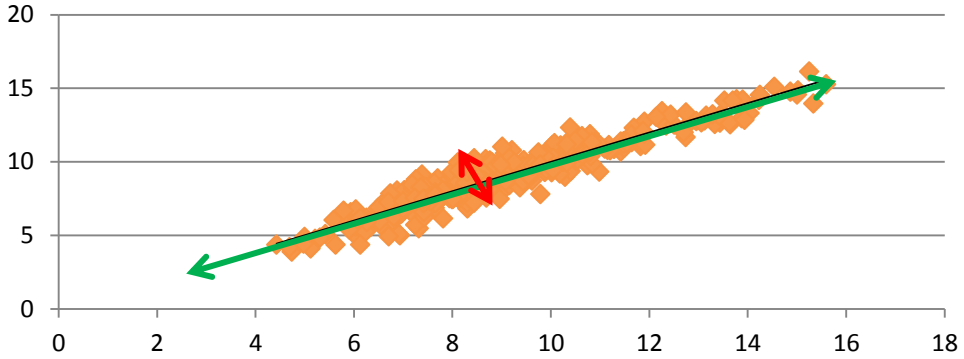


  - **RPD = SD (calibration) / RMSE**

- **Classification**
  - **0.20 mmol/L for BHB**
  - **0.15 mmol/L for acetone**

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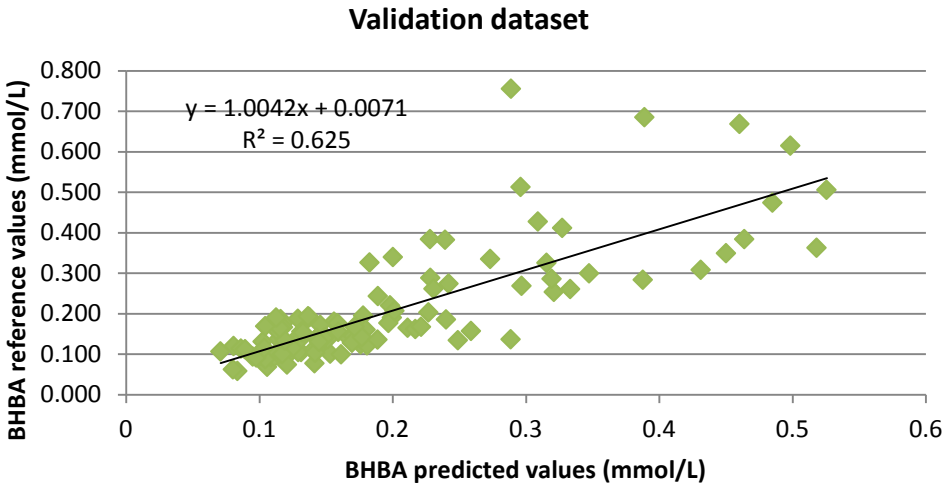
# Results – BHB

- **Statistics**

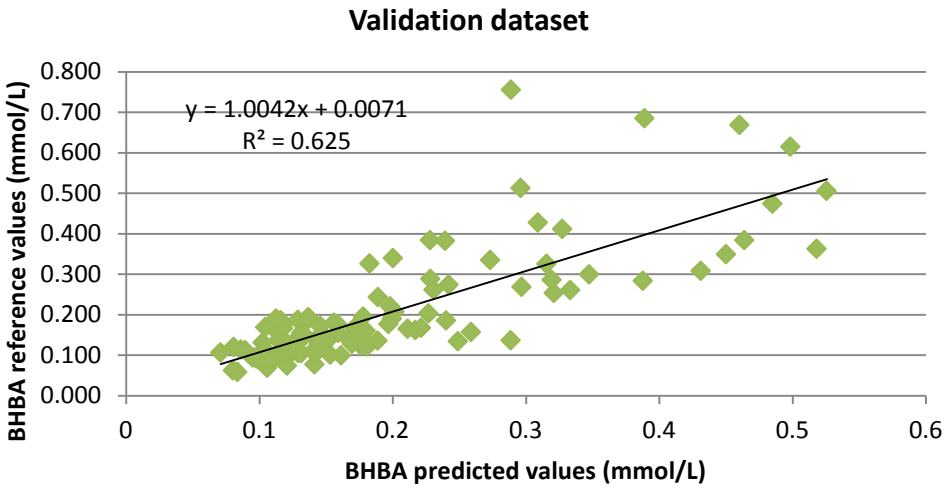
| Item | N | No. of LV | No. of Outliers | Min | Max | Mean | SD | RMSE | $R^2$ | RPD |
|---|---|---|---|---|---|---|---|---|---|---|
| **BHB (mmol/L)** | | | | | | | | | | |
| Cross-validation | 325 | 8 | 7 | 0.045 | 1.596 | 0.235 | 0.193 | 0.109 | 0.71 | 1.77 |
| Validation | 108 | - | - | 0.058 | 0.755 | 0.204 | 0.136 | 0.083 | 0.63 | 2.36 |



# Results – BHB

- **Statistics**

| Item | N | No. of LV | No. of Outliers | Min | Max | Mean | SD | RMSE | $R^2$ | RPD |
|---|---|---|---|---|---|---|---|---|---|---|
| **BHB (mmol/L)** | | | | | | | | | | |
| Cross-validation | 325 | 8 | 7 | 0.045 | 1.596 | 0.235 | 0.193 | 0.109 | 0.71 | 1.77 |
| Validation | 108 | - | - | 0.058 | 0.755 | 0.204 | 0.136 | 0.083 | 0.63 | 2.36 |




**Allows discriminate high or low levels**

# Results – BHB

- **Statistics**

| Item | N | No. of LV | No. of Outliers | Min | Max | Mean | SD | RMSE | $R^2$ | RPD |
|---|---|---|---|---|---|---|---|---|---|---|
| **BHB (mmol/L)** | | | | | | | | | | |
| Cross-validation | 325 | 8 | 7 | 0.045 | 1.596 | 0.235 | 0.193 | 0.109 | 0.71 | 1.77 |
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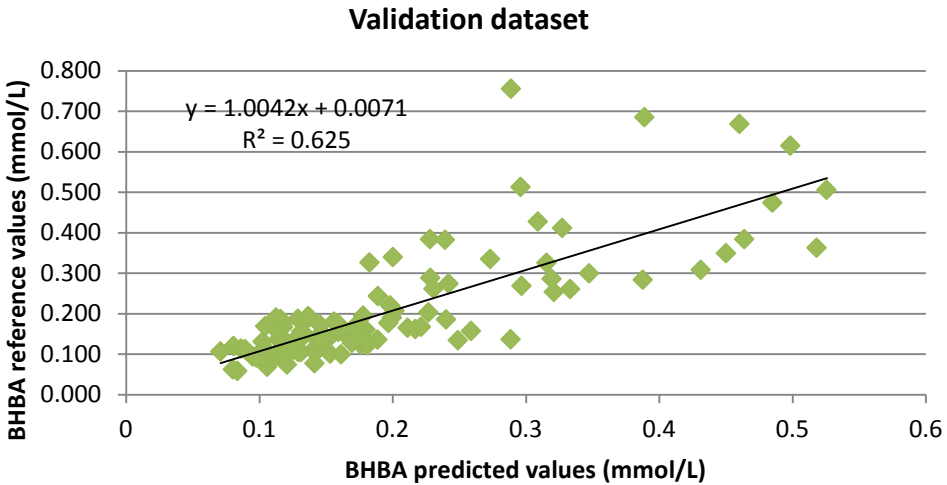


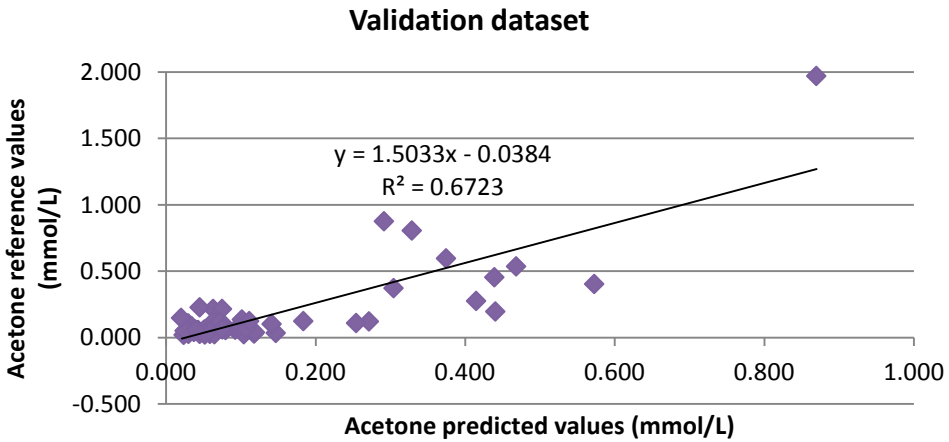

| | Low BHB content (<0.200mmol/l) | High BHB content (>0.200mmol/l) | Global good classification |
|---|---|---|---|
| **Validation** | n=77 | n=32 | |
| Predicted low | 90.90% | 9.40% | 90.80% |
| Predicted high | 9.10% | 90.60% | |

**Allows discriminate high or low levels**

# Results – Acetone

- **Statistics**

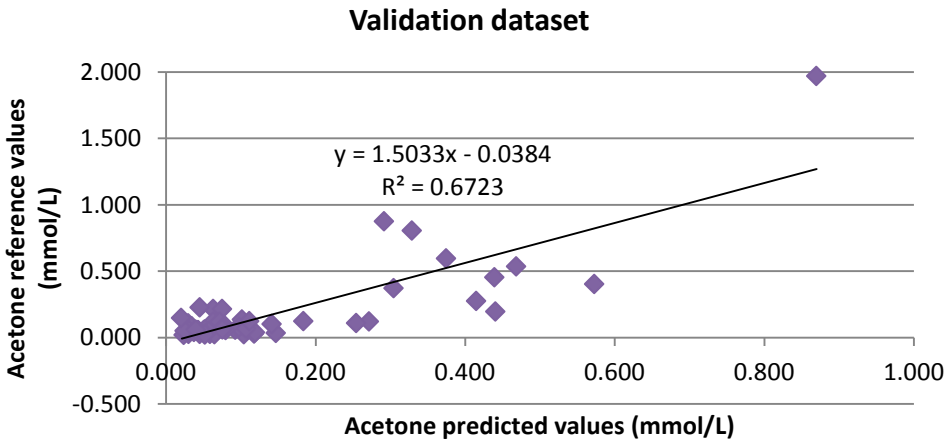
| Item | N | No. of LV | No. of Outliers | Min | Max | Mean | SD | RMSE | $R^2$ | RPD |
|---|---|---|---|---|---|---|---|---|---|---|
| **Acetone (mmol/L)** | | | | | | | | | | |
| Cross-validation | 168 | 7 | 2 | 0.02 | 3.355 | 0.19 | 0.397 | 0.248 | 0.73 | 1.6 |
| Validation | 56 | - | - | 0.021 | 1.968 | 0.179 | 0.306 | 0.196 | 0.67 | 2.03 |



# Results – Acetone

- **Statistics**

| Item | N | No. of LV | No. of Outliers | Min | Max | Mean | SD | RMSE | $R^2$ | RPD |
|---|---|---|---|---|---|---|---|---|---|---|
| **Acetone (mmol/L)** | | | | | | | | | | |
| Cross-validation | 168 | 7 | 2 | 0.02 | 3.355 | 0.19 | 0.397 | 0.248 | 0.73 | 1.6 |
| Validation | 56 | - | - | 0.021 | 1.968 | 0.179 | 0.306 | 0.196 | 0.67 | 2.03 |

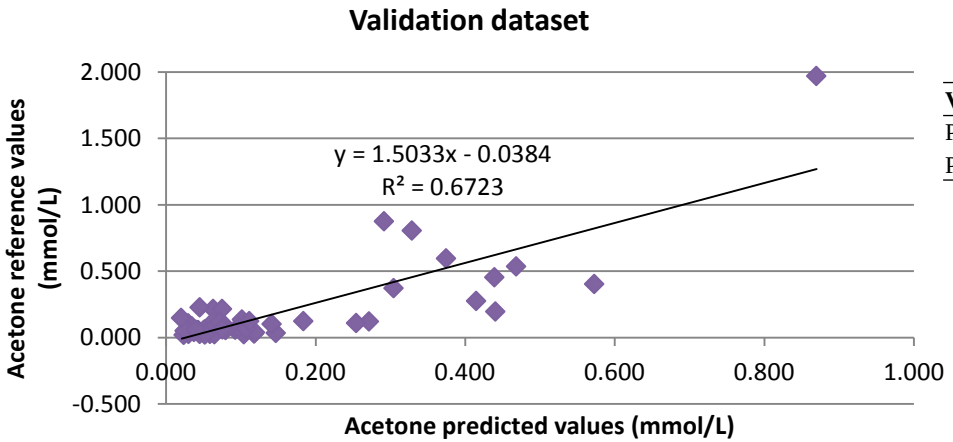



Allows discriminate high or low levels

# Results – Acetone

- **Statistics**

| Item | N | No. of LV | No. of Outliers | Min | Max | Mean | SD | RMSE | $R^2$ | RPD |
|---|---|---|---|---|---|---|---|---|---|---|
| **Acetone (mmol/L)** | | | | | | | | | | |
| Cross-validation | 168 | 7 | 2 | 0.02 | 3.355 | 0.19 | 0.397 | 0.248 | 0.73 | 1.6 |
| Validation | 56 | - | - | 0.021 | 1.968 | 0.179 | 0.306 | 0.196 | 0.67 | 2.03 |



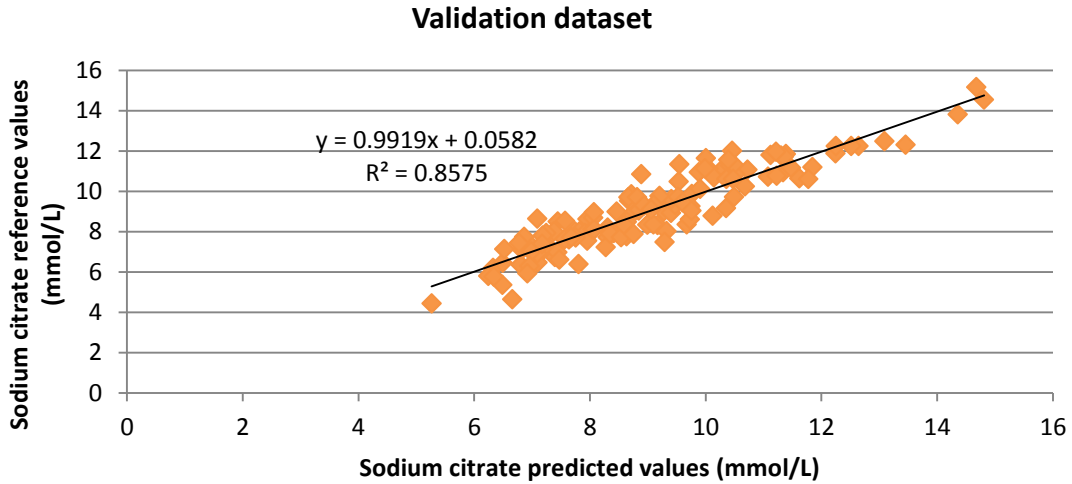

| | Low acetone content (<0.150mmol/l) | High acetone content (>0.150mmol/l) | Global good classification |
|---|---|---|---|
| **Validation** | n=43 | n=13 | |
| Predicted low | 93.00% | 23.10% | 89.30% |
| Predicted high | 7.00% | 76.90% | |

**Allows discriminate high or low levels**

# Results – Citrate

- **Statistics**

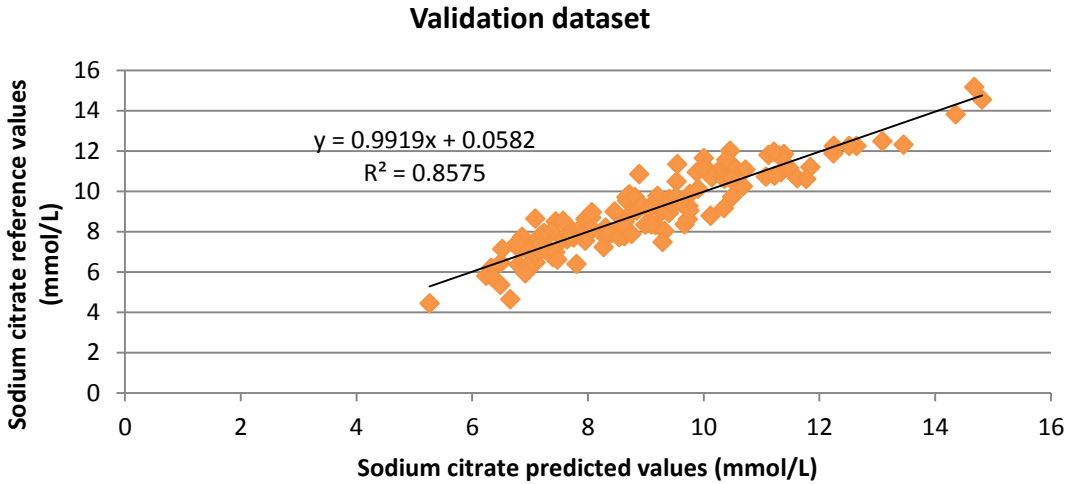
| Item | N | No. of LV | No. of Outliers | Min | Max | Mean | SD | RMSE | $R^2$ | RPD |
|---|---|---|---|---|---|---|---|---|---|---|
| **Sodium citrate (mmol/L)** | | | | | | | | | | |
| Cross-validation | 380 | 9 | 2 | 3.88 | 16.12 | 9.03 | 2.26 | 0.7 | 0.9 | 3.21 |
| Validation | 126 | - | - | 4.44 | 15.16 | 9.08 | 2.03 | 0.76 | 0.86 | 2.96 |



# Results – Citrate

- **Statistics**

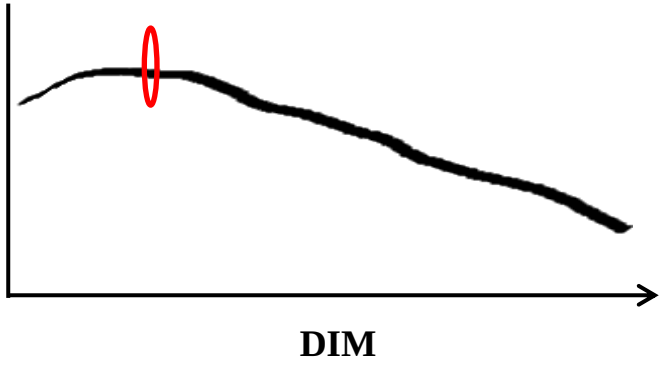
| Item | N | No. of LV | No. of Outliers | Min | Max | Mean | SD | RMSE | $R^2$ | RPD |
|---|---|---|---|---|---|---|---|---|---|---|
| **Sodium citrate (mmol/L)** | | | | | | | | | | |
| Cross-validation | 380 | 9 | 2 | 3.88 | 16.12 | 9.03 | 2.26 | 0.7 | 0.9 | 3.21 |
| Validation | 126 | - | - | 4.44 | 15.16 | 9.08 | 2.03 | 0.76 | 0.86 | 2.96 |



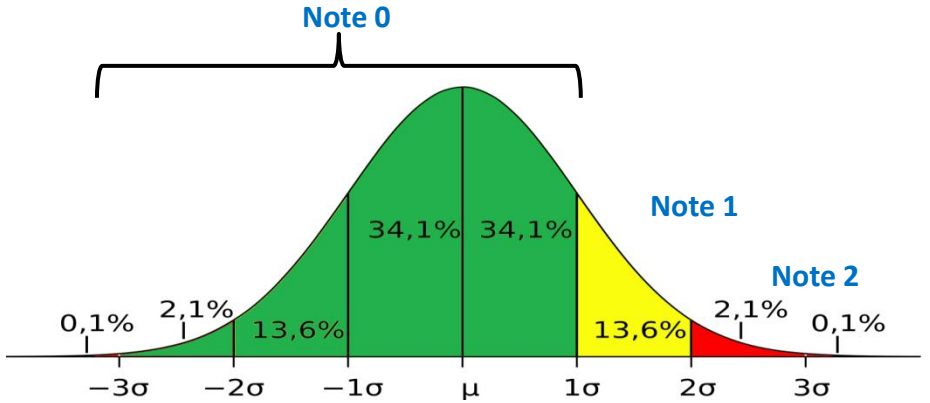

**Allows screening, quantitative information**

# Exemple of use by MROs (Baugnies, 2015)

- Walloon breeding association (AWE) tool
- BHB, acetone, citrate, C18:1 cis 9
- Relative approach
- Cow value compared to population values at same DIM




- Score 0,1 or 2 for each component



# Exemple of use by MROs (Baugnies, 2015)

- **Global score from 0 to 8 as a global approach of metabolic disorders**

| Exploitation | DATE CTRL | n° animal | n° lactation | JEL | Production (dl) | Cellules (*1000/mL) | Urée (mg/L) | Rapport TB/TP | Indice BHB | Indice acétone | Indice citrate | Indice c18:1cis9 | Indice GLOBAL |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| A | 17/02/2014 | 15146978 | 1 | 15 | 294 | 760 | 30 | 1.67 | 2 | 1 | 1 | 1 | 5 |
| A | 15/04/2014 | 14876705 | 2 | 59 | 376 | 400 | 179 | 1.36 | 0 | 2 | 0 | 1 | 3 |
| A | 18/11/2014 | 15012953 | 2 | 69 | 237 | 280 | 179 | 0.46 | | | | | |
| A | 16/12/2014 | 13904979 | 4 | 7 | 167 | 560 | 350 | 2.54 | 2 | 2 | 0 | 2 | 6 |
| B | 26/02/2014 | 15676607 | 1 | 115 | 275 | 10 | 290 | 1.12 | 2 | 0 | 2 | 1 | 5 |
| B | 23/05/2014 | 14022741 | 3 | 268 | 128 | 360 | 170 | 0.93 | 1 | 2 | 0 | 1 | 4 |
| B | 4/11/2014 | 14921815 | 2 | 212 | 203 | 60 | 310 | 1.39 | 2 | 0 | 2 | 0 | 4 |
| C | 9/08/2012 | 15180867 | 1 | 387 | 152 | 120 | 350 | 1.21 | 1 | 0 | 1 | 1 | 3 |
| C | 8/11/2012 | 15180793 | 5 | 11 | 258 | 300 | 50 | 1.59 | 2 | 2 | 1 | 2 | 7 |
| C | 6/04/2013 | 15180840 | 4 | 12 | 110 | 40 | 240 | 1.86 | 1 | 1 | 0 | 2 | 4 |
| C | 6/10/2013 | 14090385 | 3 | 14 | 226 | 560 | 170 | 1.75 | 1 | 1 | 1 | 2 | 5 |

- **Complex interpretation (ketosis, fat mobilization, fattening, feed effect, mastitis…)**
- **Preliminary tests in 4 farms**
- **Good feedback from breeders**
- **→ Cows to follow**

# Conclusions/Implications

- Calibrations for BHB and acetone → distinctions between high and low levels
- Citrate by MIR → good accuracy
- Standardisation of spectra: usable by all Optimir MROs

# Conclusions/Implications

- Calibrations for BHB and acetone → distinctions between high and low levels
- Citrate by MIR → good accuracy
- Standardisation of spectra: usable by all Optimir MROs

- USE ON FIELD
  - Complex interpretation
  - Different way to use it by MROs
  - Interest from breeders
  - Already used in France and Luxembourg
  - Tests in Germany, Belgium

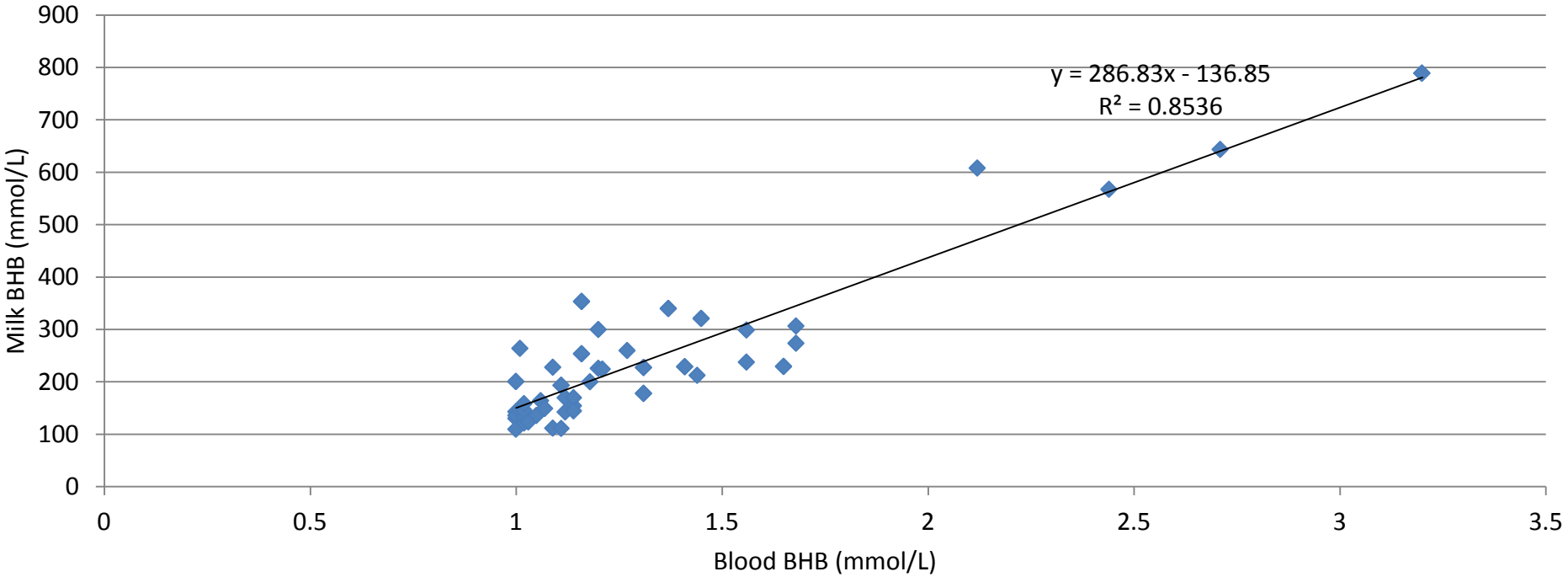
# Thank you for your attention

C. Grelet, C. Bastin, M. Gelé, J.-B. Davière, M. Johan, A. Werner, R. Reding, C. Darimont, S. Baugnies, J.A. Fernandez Pierna, F.G. Colinet, P. Dardenne, N. Gengler, H. Soyeurt, F. Dehareng

c.grelet@cra.wallonie.be

Centre wallon de Recherches agronomiques

Wallonie


900
800
700
600
500
400
300
200
100
0
Milk BHB (mmol/L)
0
0.5
1
1.5
2
2.5
3
3.5
Blood BHB (mmol/L)
y = 286.83x - 136.85
R² = 0.8536

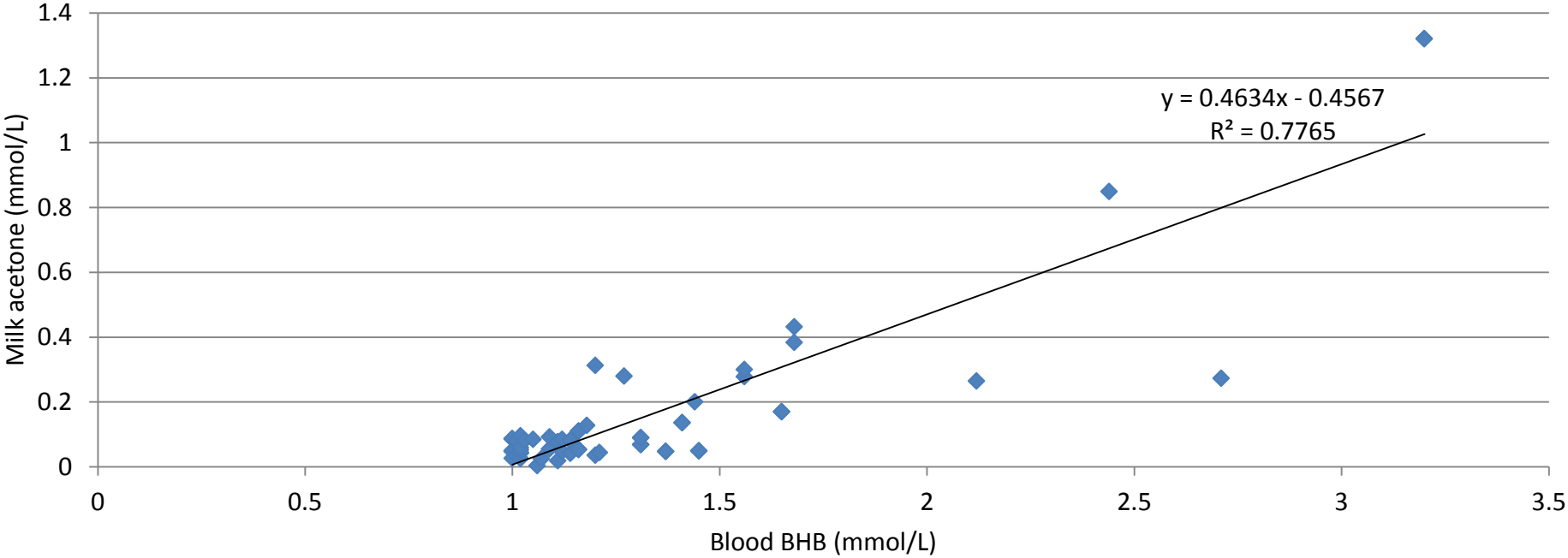

y = 0.4634x - 0.4567
R² = 0.7765
Milk acetone (mmol/L)
Blood BHB (mmol/L)
0
0.2
0.4
0.6
0.8
1
1.2
1.4
0
0.5
1
1.5
2
2.5
3
3.5

Tableau 17: Exemples d'alertes pouvant être générées

| Stade de lactation | Indice corps cétoniques I (BHB) | Indice corps cétoniques II (acétone) | Indice niveau énergétique (citrate) | Indice mobilisation (C18:1cis 9) | Alerte générée |
|---|---|---|---|---|---|
| Début (1-20) | 2 | 2 | 0 | 2 | Risque important d'acétonémie de type II |
| Début (20-60) | 2 | 2 | 0 | 2 | Risque important d'acétonémie de type I |
| Début (1-60) | 0 | 0 | 2 | 2 | Début de mobilisation des réserves |
| Tous | 0 | 0 | 0 | 1 | Mobilisation des graisses issues des tissus adipeux |
| Tous | 0 | 0 | 0 | 2 | Nette mobilisation des graisses issues des tissus adipeux |
| Fin (200-400) | 2 | 0 | 2 | 0 | Attention engraissement |
| Fin (200-400) | 2 | 0 | 2 | 1 | Attention vache grasse avec mobilisation vers foie ou vache maigre avec mobilisation des réserves |
| Fin (200-400) | 2 | 0 | 2 | 2 | Attention vache grasse avec forte mobilisation (!foie!) ou vache maigre avec mobilisation importante des réserves |

# Citrate ?

- **Bjerre-Harpoth (2012)**

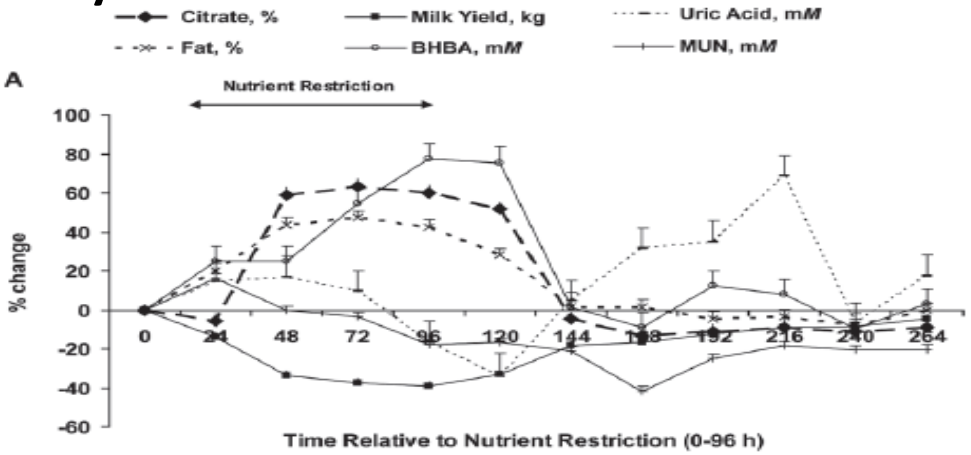
*« …greatest increase (58%) during restriction for all cows »*

*« …promising early indicator of physiological imbalance »*

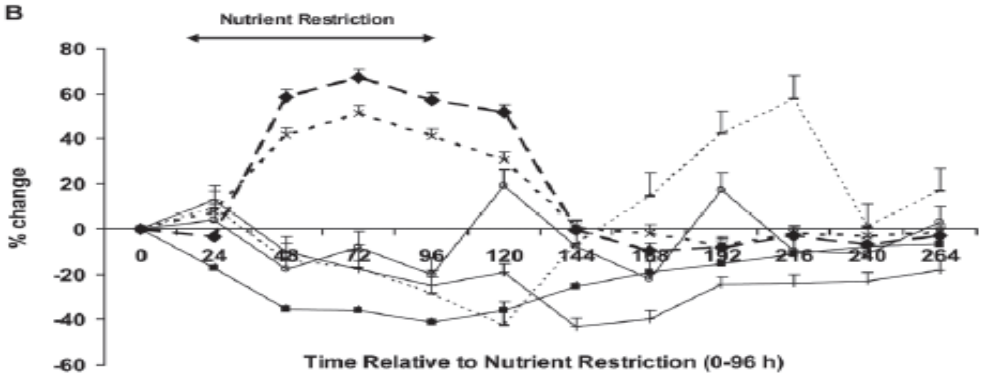
- **Baticz et al. (2002)**

*« Sodium citrate should be measured by easy and automated method such as FT-MIR technology to evaluate the energy status of cows »*

## Early Lactation




## Mid Lactation

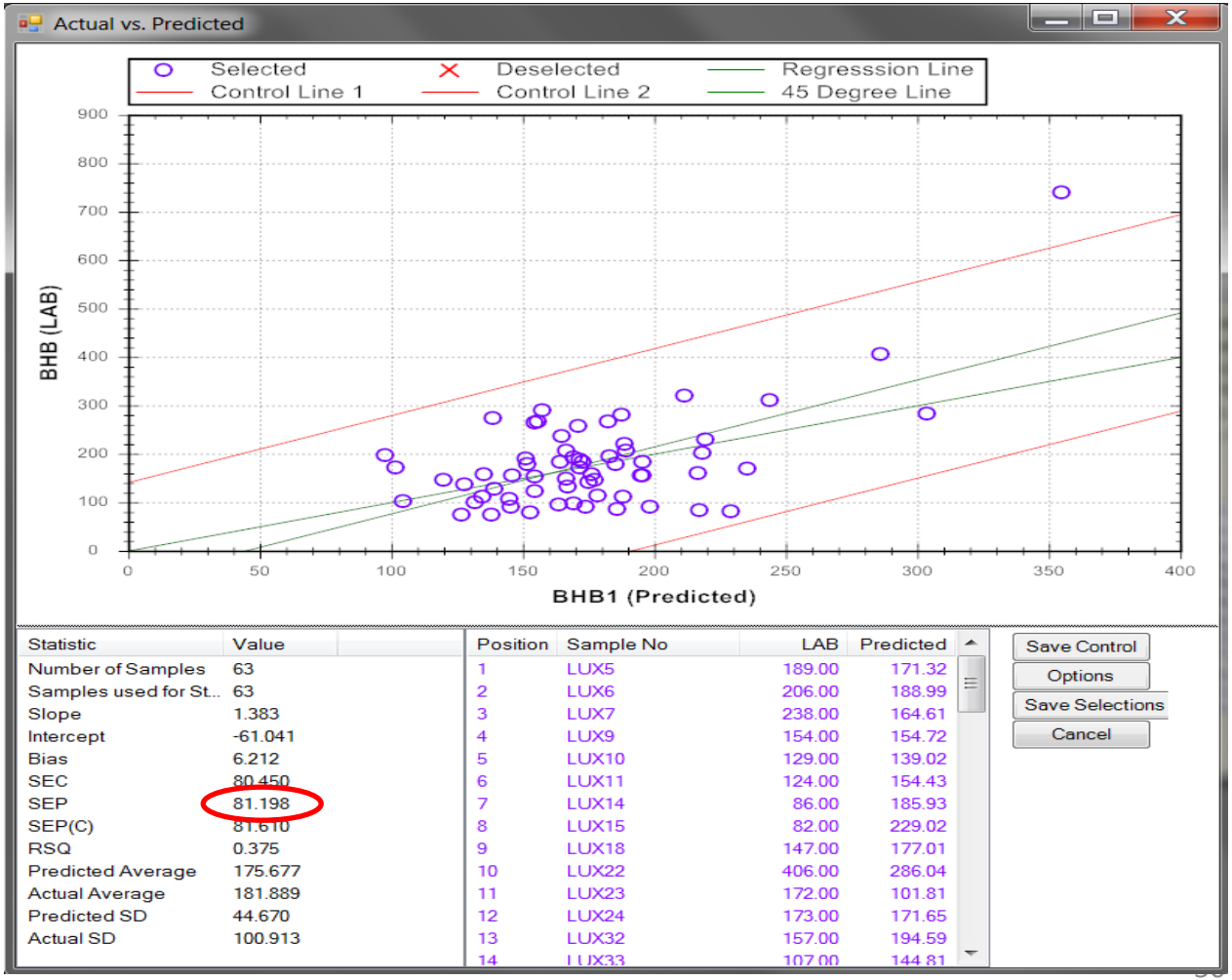


Modified PLS Regression Statistics

| | | | |
|---|---|---|---|
| Input File | 01luxcal.cal | REP File | None |
| Validation File | 01luxval.cal | Equation File | 01lux.eqa |
| Math Treatment | 1, 5, 1, 1 | Number of variables | 229 |
| Scatter Corrected | None | Downweight outliers | No |
| Constituent | LogBHB | Number of samples | 262 |
| Cross validation | By groups, no pre-sort, form groups by cycling, ignore duplicates | | |
| Mean 2.295 | | Range 1.52 - 3.20 | Standard deviation 0.298 |

| | SEC | AdjRSQ | F | SECV | 1-VR | SEV | BIAS | SEV(C) |
|---|---|---|---|---|---|---|---|---|
| 1 | 0.258 | 0.253 | 89.42 | 0.263 | 0.219 | 0.266 | 0.037 | 0.265 |
| 2 | 0.207 | 0.520 | 145.21 | 0.221 | 0.448 | 0.188 | -0.016 | 0.188 |
| 3 | 0.195 | 0.575 | 34.50 | 0.205 | 0.527 | 0.195 | -0.049 | 0.190 |
| 4 | 0.182 | 0.626 | 36.84 | 0.195 | 0.573 | 0.187 | -0.038 | 0.184 |
| 5 | 0.176 | 0.652 | 19.72 | 0.191 | 0.591 | 0.186 | -0.037 | 0.184 |
| 6 | 0.166 | 0.689 | 32.01 | 0.180 | 0.633 | 0.186 | -0.055 | 0.179 |
| 7 | 0.158 | 0.721 | 29.76 | 0.173 | 0.663 | 0.175 | -0.034 | 0.173 |
| 8 | 0.153 | 0.738 | 17.07 | 0.170 | 0.674 | 0.179 | -0.020 | 0.179 |

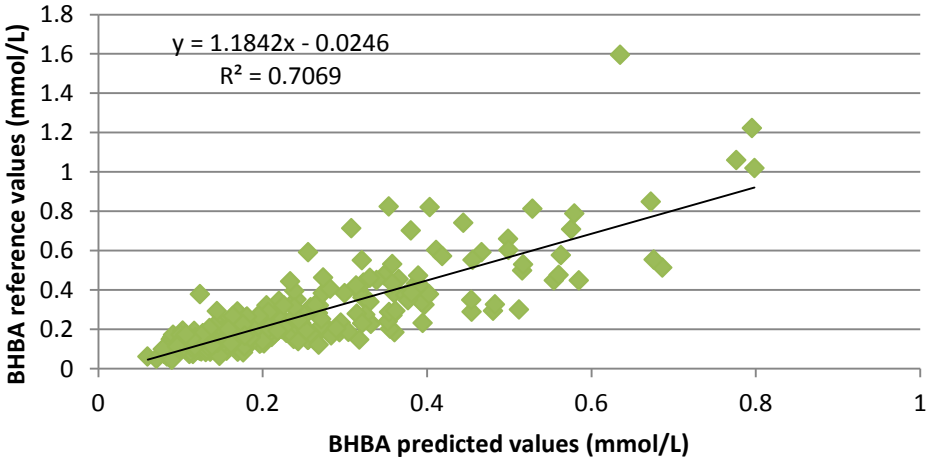
- **Calibrations with Clasel, Poisy and Neumuhle**
- **Validation on Luxembourg dataset**



# Results – BHB

- **Cross-Validation**

| Item | N | No. of LV | No. of Outliers | Min | Max | Mean | SD | RMSE | $R^2$ | RPD |
|---|---|---|---|---|---|---|---|---|---|---|
| **BHB (mmol/L)** | | | | | | | | | | |
| Cross-validation | 325 | 8 | 7 | 0.045 | 1.596 | 0.235 | 0.193 | 0.109 | 0.71 | 1.77 |

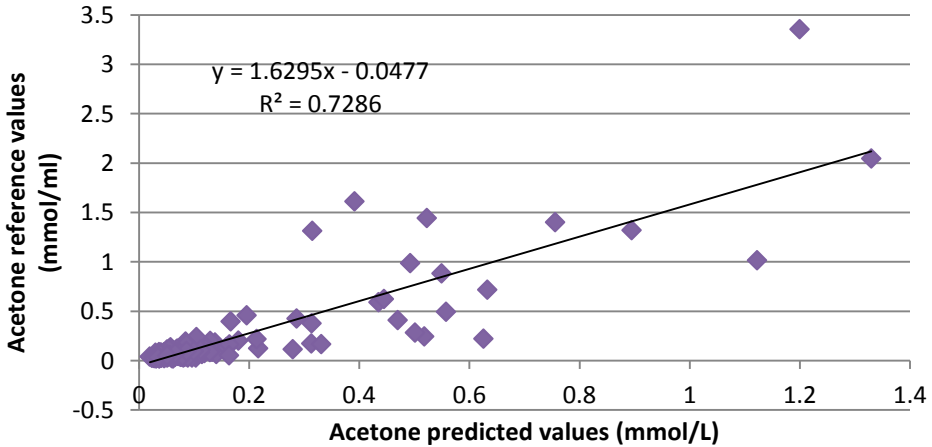



| | Low BHB content (<0.200mmol/ml) | High BHB content (>0.200mmol/ml) | Global good classification |
|---|---|---|---|
| **Cross-Validation** | n=198 | n=120 | |
| Predicted low | 87.40% | 15.00% | 86.50% |
| Predicted high | 12.60% | 85.00% | |

# Results – Acetone

- **Cross-Validation**

| Item | N | No. of LV | No. of Outliers | Min | Max | Mean | SD | RMSE | $R^2$ | RPD |
|---|---|---|---|---|---|---|---|---|---|---|
| **Acetone (mmol/L)** | | | | | | | | | | |
| Cross-validation | 168 | 7 | 2 | 0.02 | 3.355 | 0.19 | 0.397 | 0.248 | 0.73 | 1.6 |




| | Low acetone content (<0.150mmol/ml) | High acetone content (>0.150mmol/ml) | Global good classification |
|---|---|---|---|
| **Cross-Validation** | n=134 | n=32 | |
| Predicted low | 95.50% | 15.60% | 93.40% |
| Predicted high | 4.50% | 84.40% | |

# Results – Citrate

- **Cross-Validation**

| Item | N | No. of LV | No. of Outliers | Min | Max | Mean | SD | RMSE | $R^2$ | RPD |
|---|---|---|---|---|---|---|---|---|---|---|
| **Sodium citrate (mmol/L)** | | | | | | | | | | |
| Cross-validation | 380 | 9 | 2 | 3.88 | 16.12 | 9.03 | 2.26 | 0.7 | 0.9 | 3.21 |