

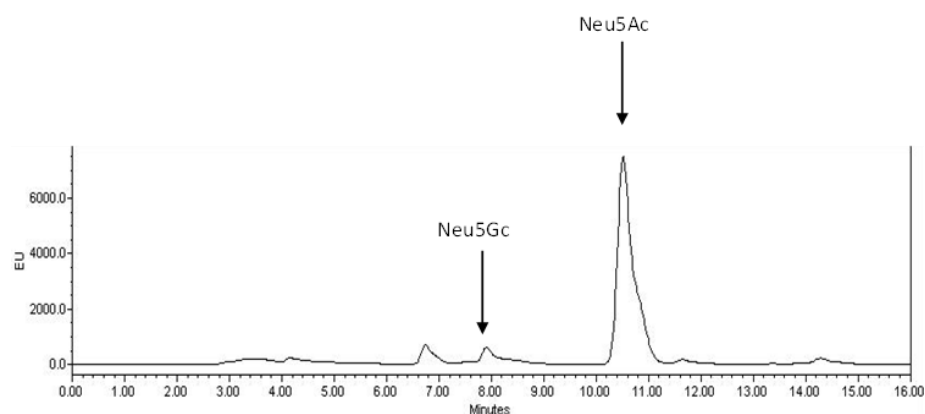
## Determination of N-acetylneuraminic and N-glycolylneuraminic acids in unprocessed milk of four cattle breeds

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### SUPPLEMENTARY FILE

#### Sialic acids determination protocol

Sialic acids (Neu5Ac and Neu5Gc) were determined following Spichtg and al (2010) procedure. 10 ml of milk was defatted by centrifuge at 4000 rpm at 4°C for 10 min. 5 ml of defatted milk was incubated with 2 ml of H<sub>2</sub>SO<sub>4</sub> 50mM at 80°C for 1 h. After cooling in the ice bath samples were centrifuged at 10000 rpm per 10 min at 4°C and a supernatant aliquot was filtered with an RC syringe filter with 0.22 µm of mesh and centrifuged at 14000 rpm per 10 min at 4°C. 50 µl of supernatant was incubated with 50 µl of DMB (4,5-methylenedioxy-1,2-phenylenediamine dihydrochloride) for 2 h and 30 min at 60°C and 10 µl was used for the HPLC analysis. Neu5Ac and Neu5Gc were detected in Water Alliance 2695 HPLC, with Fluorimeter Module 2475 (Water spa), on C18 Synergi (4u 250x4.6mm) column (Phenomenex Inc.). The column was equilibrated for 30 min, flow rate 1 ml/min, with start gradient condition: Pure Water:Methanol:Acetonitrile 84:9:7 (v/v/v); after that, the working procedure was: Pure Water:Methanol:Acetonitrile 84:9:7 for 20 min; 80:13:7 for 5 min; 75:18:7 for 5 min; 80:13:7 for 5 min; 84:9:7 for 5 min, plus 5 min of delay; oven at 40°C and detected by fluorescence em. 343 and ex. 447. The total sialic acid concentration expressed as µg/ml was obtained by comparing the peaks of the sample with the peaks of the Neu5Ac and Neu5Gc standards (Sigma Aldrich). A typical chromatogram is below reported.



**Figure legends:**

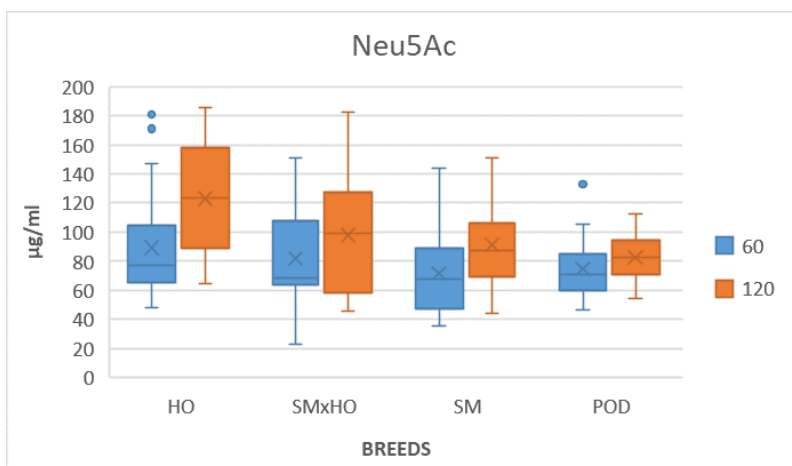
**Figure 1:**

Boxplot presenting distribution of Neu5Ac concentration ( $\mu\text{g/ml}$ ) in the four analysed cattle breeds at 60 and 120 days after calving. The ends of the whiskers of each boxplot represent the minimum and maximum of the data, the top and bottom of the box are the first and the third quartiles, the bold band is the median, and the cross is the mean.

**Figure 2:**

Boxplot presenting distribution of Neu5Gc concentration ( $\mu\text{g/ml}$ ) in the four analysed cattle breeds at 60 and 120 days after calving. The ends of the whiskers of each boxplot represent the minimum and maximum of the data, the top and bottom of the box are the first and the third quartiles, the bold band is the median, and the cross is the mean.

**Supplementary Figure 1:**



**Supplementary Figure 2:**

