Authentication of Hay Milk and its Dairy Products with Nuclear Magnetic Resonance Spectroscopy

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The EU product specification of Haymilk does not include an official methodology on its authenticity validation. Accordingly, this work proposed 1H-NMR for determination of cyclopropane fatty acids (CPFAs) in milk as a molecular marker for Haymilk authenticity. The presence of CPFAs was detected in milk samples from maize or grass silage feeding with 97% and 76% accuracy, respectively, whilst authenticity of Haymilk samples were confirmed with 100% accuracy. The results of this approach were validated by GC-MS analysis. NMR revealed advantageous compared to GC-MS due to accurate determination of CPFAs with unique signal in the up-field region in NMR spectra.

Autenticità di Latte Fieno e suoi derivati con spettroscopia di risonanza magnetica nucleare ad alta risoluzione

La specificazione “latte fieno” non include una metodologia ufficiale per la validazione della sua autenticità. Questo lavoro propone 1H-NMR per la determinazione di acidi grassi ciclopropanici (CPFAs) nel latte come marker molecolare per l’autenticità del latte fieno. La presenza di CPFAs è stata confermata nei campioni di latte da alimentazione insilata a base di mais o erba rispettivamente per 97% e 76%, mentre l’assenza nei campioni di latte fieno per 100%. Tutti i risultati sono stati validati tramite GC-MS. Rispetto GC-MS, NMR si è rivelata più vantaggiosa per la determinazione accurata di CPFA dovuta allo specifico segnale nello spettro NMR.

**Key words**: cyclopropane fatty acids, nuclear magnetic resonance, food authenticity.

# **1. Introduction**

 In accordance with the PhD thesis project previously described (Eltemur, 2022), this poster reports the main results of the first two activities concerning:

(A1) development of a 1H-NMR methodology for the determination of cyclopropane fatty acids (CPFAs) in milk as a molecular marker for Haymilk authenticity;

(A2) development of an untargeted 1H-NMR fingerprinting approach to validate the authenticity of Haymilk.

# **2. Materials and Methods**

Lyophilization of 245 milk samples was completed using a pilot plant (Martin Christ, Epsilon 2-6D LSC plus freeze-dryer, Osterode, Germany). 1H-NMR analysis of freeze-dried samples was performed both in CDCl3 and D2O solvents. All NMR experiments were carried out using a 600 MHz spectrometer (JNM-ECZ from JEOL Ltd., Tokyo, Japan), equipped with a “Royal” HFX/FGSQ probe. NMR spectra were acquired at room temperature 298 K (25 °C) with 32.000 complex points, using a 45° pulse length and 20 s of relaxation delay. A total of 1024 scans were acquired with a spectral width of 15 ppm and an acquisition time of 4 s. The spectra evaluation and processing have been carried out using Delta NMR processing and control software (v.5.3.1. Jeol Resonance Inc.). GC-MS analysis was performed on the fat extract of the milk samples after transesterification as a validation method using Shimadzu QP2010 SE GC-MS (Shimadzu, Kyoto, Japan).

# **3. Results and Discussion**

## **3.1 Determination of CPFAs in milk as a molecular marker using targeted 1H-NMR**

The CPFAs show a characteristic quartet signal in the chemical shift region between -0.30 and -0.36 ppm corresponding to the cis-methylene proton in the cyclopropane ring (Figure 1) (Knothe, 2006). Such upfield region does not overlap with any other signal arising from milk sample, therefore, it enables a precise detection and thus quantification of the CPFAs (Lolli *et al.*, 2018).



**Figure 1** 600 MHz 1H-NMR spectrum of CPFA standard (dihydrosterculic acid) in CDCl3. The signal of TMS internal standard was fixed to 0 ppm. The signal resonates from CPFA was expanded for identification.

The CPFA signal at -0.34 ppm was selected for quantification using the signal of TMS as reference. The concentration of CPFAs in freeze-dried milk sample (expressed mg of CPFA/kg of milk fat) was calculated as described by Eq.1:

$(CPFA)=\left[TMS\right]×\frac{A\_{CPFA}}{A\_{TMS}}×12×\frac{MW\_{CPFA}}{m\_{milk}} ×F$ (1)

Where, [TMS] is the concentration of the internal standard (2.20 mM), A**CPFA** and A**TMS** correspond to the areas of CPFA and TMS signals, respectively, MWCPFA is the molar mass of dihydrosterculic acid (294.489 g·mol−1), mmilk is the mass of freeze-dried milk sample (50 mg), and finally F is factor converting the concentration of CPFAs from mg/g freeze dried milk to mg/kg of milk fat. According to the calibration curve of CPFA standard solution (matrix effect was negligible), the area of the quartet at −0.34 ppm was linearly related to the concentration of CPFAs with a linearity of R2 = 0.99. Thus, the limit of detection (LOD) was found 230 mg of CPFA/ kg of milk fat. Whereas the GC-MS method resulted to a LOD of 7.5 mg of CPFA/ kg of milk fat.

***Table 1*** *Contingency table summarizing the results of the presence of CPFAs in milk samples analysed by ¹H-NMR and GC-MS.*

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
|  |  | **Haymilk** | **Grass silage** | **Maize silage** | **Chi-square test\*** |
|  GC-MS | CPFAs present | 0\* | 80 | 96 | < 0.005 |
| CPFAs absent | 49\* | 17 | 3 |
|  NMR  | CPFAs present | 0\* | 74 | 96 | < 0.005 |
| CPFAs absent | 49\* | 23 | 3 |
|  | Total number of samples | 49 | 97 | 99 |  |

\*Significantly different at p < 0.05.

Both methods confirmed the authenticity of Haymilk samples with 100% accuracy (Table 1). Moreover, ¹H-NMR was able to detect the presence of CPFAs at 97% accuracy for milk from maize silage feeding and 76% for grass silage feeding. Overall, the results of ¹H-NMR were in accordance with GC-MS analysis.

## **3.2 Development of an untargeted 1H-NMR fingerprinting approach to validate the authenticity of Haymilk**

According Untargeted 1H-NMR Haymilk fingerprinting analysis have been performed on the 245 milk samples. Multivariate data analysis resulted in a good separation between Haymilk and milk from both grass and maize silage feedings collected in winter. However, discrimination of milk samples collected on summer was challenging due to similar metabolic profiling of the milk samples caused by outdoor grazing of the cows on summer. More powerful discrimination analysis tools are to be used to obtain better results on summer milk samples.

# **4. References**

Knothe G (2006) NMR characterization of dihydrosterculic acid and its methyl ester. Lipids, 41(4), 393–396.

Lolli V, Marseglia A, Palla G, Zanardi E, Caligiani A (2018) Determination of cyclopropane fatty acids in food of animal origin by 1H NMR. Journal of Analytical Methods in Chemistry, 2018.