71st ICoMST
GIRONA - CATALONIA - SPAIN
3rd-8th August - 2025

# APPLICATION OF METABOLITE PROFILING FOR THE DETECTION OF PREVIOUSLY FROZEN BEEF MEAT ACROSS DIFFERENT STORAGE CONDITIONS

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## I. INTRODUCTION

Food safety authorities in the European Union require previously frozen, unprocessed meat to be labeled as 'defrosted'. Although various analytical methods have been proposed to differentiate defrosted meat from fresh meat, a standardized approach for detecting freeze-induced damage has not yet been established [1]. A major challenge in detecting unlabeled previously frozen meat is that the specific freeze storage temperatures are unknown. Reliable detection methods must therefore be capable of identifying freeze-related damage across the broad range of freezing temperatures currently used in the meat industry. This includes relatively mild freezing (around -5°C), which does not meet the criteria for the EU's 'quick-frozen' label, as well as standard deep freezing below –18°C (Directive - 89/108 - EN - EUR-Lex). Typically, an established enzyme assay is used to detect previously frozen meat [2]. This assay measures the activity of the mitochondrial enzyme HADH, as a result of freeze-induced cellular damage. However, our previous studies have shown that the HADH assay is not reliable at temperatures above -14°C. These temperature-dependent limitations of the HADH assay, particularly within the range between chilling (2°C) and deep freezing (< -18°C), have been scarcely investigated to date. Additionally, we demonstrated that bioimpedance measurements – a less invasive technique – can also detect previous freezing for temperatures of -14°C or below [3, 4]. However, this method also failed to detect milder freezing conditions (e.g., -4°C). Therefore, alternative markers are needed to distinguish fresh from frozen-thawed meat across a broader temperature range and for different storage periods. In this study, we tested the capability of a non-targeted metabolite profiling approach using two-dimensional GC x GC-MS analysis to differentiate between freezing regimes and to identify metabolite markers associated with specific freeze storage temperatures.

## II. MATERIALS AND METHODS

Beef (*Bos taurus*) round cut muscle samples (N = 24 individuals) were obtained from a local slaughterhouse one day post mortem. From each animal, five replicate samples (5 × 5 × 4 $cm^3$) were subjected to different temperature treatments for storage: 2°C (refrigerated), –4°C, –14°C and –22°C. Control samples were directly frozen at –80°C. Following a 21-day storage period, samples were collected and prepared for metabolite and enzyme activity analyses. Prior to metabolite extraction, all meat samples were lyophilized and homogenized using a ball mill. The extraction was performed in two steps: initially, a polar extraction using 80% methanol, followed by a second extraction with a methanol:chloroform (2:1, v/v) mixture. The combined extracts were dried in glass vials using vacuum centrifugation. Before GC-MS analysis, samples were derivatized by methoximation, followed by silylation with MSTFA+1% TMCS. Two dimensional GC-MS analysis was conducted using a Shimadzu GCMS-QP2010 Ultra system equipped with a non-polar Rxi-5SilMS column (Restek, Bellefonte, USA) and a medium polar BPX50 column (SGE, Ringwood, Australia). Data acquisition and integration were performed using Shimadzu's GCMS Solution software. Subsequent statistical analysis and data visualization were carried out with SIMCA (Umetrics, Malmö, Sweden) and JMP (SAS, Heidelberg, Germany). The HADH assay was conducted following the method of Gottesmann and Hamm (1982), with modifications regarding volume reduction and dilution of the meat extracts.

## III. RESULTS AND DISCUSSION

Principal Component Analysis (PCA) revealed a distinct shift in the overall metabolite profile during storage at 2°C (Figure 1). This shift was primarily driven by the accumulation of free amino acids, which resulted from proteolytic activity during the ripening process. Additionally, degradation products associated with energy metabolism, such as hypoxanthine, accumulated throughout the refrigerated storage. In contrast, mild freezing at –4°C markedly slowed these breakdown processes. Storage at -14 and -22°C largely preserved the initial metabolite composition. Nonetheless, some differences were still observed, including modest increases in glycerol, several amino acids and malic acid (exemplarily shown in Figure 1). Among potential biomarkers to also detect effects of mild freezing was

phenylalanine, which showed a marked reduction for all freeze treatments as opposed to high levels for the chilled only group.

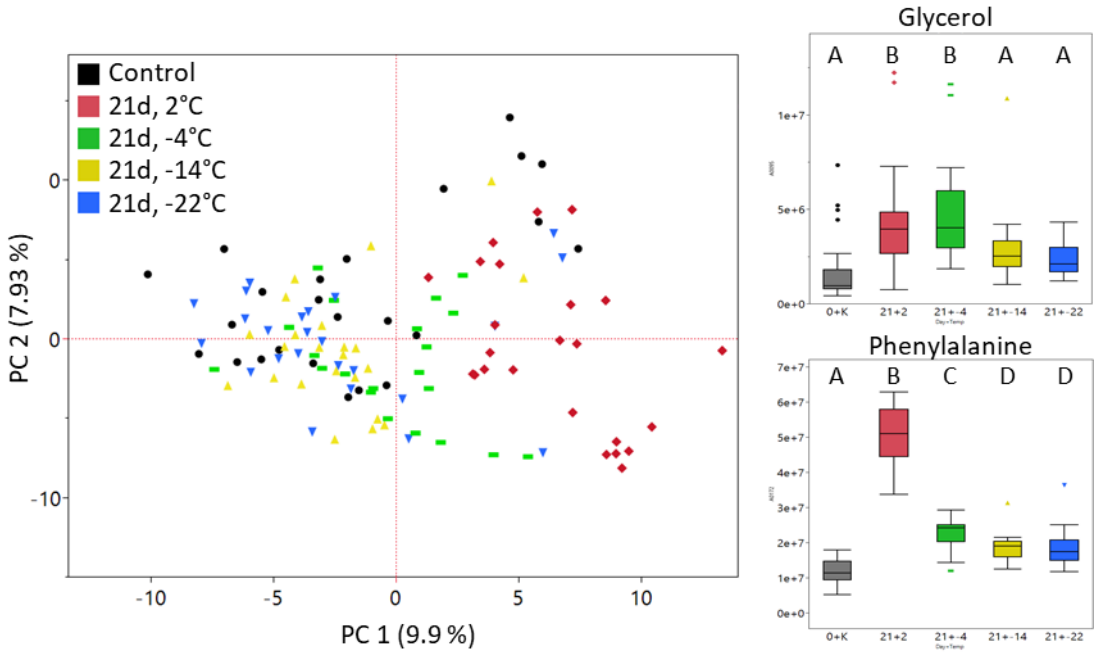



Figure 1. Principal component analysis after GC x GC-MS analysis of beef samples indicating a shift from fresh control samples to stored samples at 2°C (left). Overall pattern of samples stored at -14°C and -22°C was kept in relation to controls. Right: Examples for metabolite levels showing differences to fresh control at 1 day post mortem (0+K) and among different storage temperatures after 21 days.

Compared to pork [5], beef meat exhibited significantly fewer metabolic alterations following storage at 2°C, making the development of a reliable time–temperature classification map for metabolic markers more challenging. Beyond storage-related effects, substantial inter-individual variation in metabolite profiles was observed, likely driven by factors such as age, diet, gender, genotype, rearing conditions and quality defects.

## IV. CONCLUSION

Taking the time of slaughter into account, a defined set of specific biomarkers could improve the detection of previously frozen meat stored within the –4 to –14°C temperature range. To further investigate this hypothesis, future studies will incorporate a broader range of animals to examine potential confounding effects, including quality anomalies, age, gender, and diet in greater detail.

## ACKNOWLEDGEMENTS

This work was funded by the Norwegian Research Council (“Food Inspector”, #294767, “Meat Inspector”, #349910).

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