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Detection of frozen-thawed beef, pork and chicken meat

*Filip Beňo**, *Eliška Václavíková*, *Filip Hruška*, *Nell Melisa Heráková*, *Rudolf Ševčík*

Department of Food Preservation, Faculty of Food and Biochemistry Technology, University of Chemistry and Technology, Prague, Czech Republic

**Corresponding author: filip.beno@vscht.cz*

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Abstract: Thawed meat is usually of lower quality and less expensive than chilled meat, which some dishonest people may exploit by fraudulently marketing it as chilled. This study focused on methods for detecting and distinguishing frozen meat from chilled meat. The activity of the enzyme aconitase in the eluate was determined, and the mineral cations (Na^+, K^+, Mg^{2+}) as well as the concentrations of organic acids (acetic, citric, and lactic) were analysed in chilled and thawed meat stored for 7, 14, and 150 days at −18 °C. After 150 days of storage, aconitase activity increased from 47.2 ± 7.2 U·L^{-1} to 395.3 ± 59.2 U·L^{-1} in pork, from 45.8 ± 11.5 U·L^{-1} to 133.3 ± 31.8 U·L^{-1} in beef, and from 17.2 ± 8.6 U·L^{-1} to 143.6 ± 41.5 U·L^{-1} in chicken. The mineral content decreased during storage in meat samples, especially Na^+ and K^+ cations ($P < 0.05$). The results for organic acids were less conclusive. Principal component analysis (PCA) of the data confirmed a clear separation between chilled and thawed meat for all species, with a high variability of nearly 72%.

Keywords: thawed meat; aconitase; minerals; organic acids; PCA

Although freezing is one of the most effective methods of preservation, the processes of freezing and thawing cause damage to the microstructure and deterioration of the organoleptic properties of meat. Differences between fresh and frozen meat are also evident in the physical and chemical stability of muscle tissue (Rahman et al. 2014), with freezing altering the total water content. Fresh meat binds water more effectively and retains juiciness thanks to intact cell membranes. In contrast, freezing leads to fluid loss and ultrastructural damage, including disruption of the sarcolemma, fragmentation of sarcomeres, and release of myofibrils (Stafford et al. 2024). After thawing, the loss of juice is greater than in unfrozen meat, resulting in a drier texture after cooking, reduced palatability, and the loss of nutritionally valuable compounds with the eluate (Leygonie et al. 2012). Although frozen meat generally has a longer shelf life, thawed meat is more susceptible to spoilage. Freezing does not directly eliminate microorganisms but only prevents their growth (Dang et al. 2021). Defrosting causes the disruption of muscle structures, releasing water and nutrients to the surface, which can contribute to faster spoilage (Müller et al. 2012; Singha and Muthukumarappan 2015). In addition to reduced meat quality, there is also clear consumer deception. At the turn of the 21st century, studies in European countries found that 8–15% of meat samples were incorrectly labelled as chilled (Ballin and Lametsch 2008). According to Regulation (EC) No. 853/2004, fresh meat is defined as meat

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preserved solely by chilling or freezing, meaning that frozen meat is also classified as fresh. However, Regulation (EC) No. 1169/2011 requires that food which has been frozen prior to sale and is marketed in a thawed state be labelled as 'defrosted', particularly where thawing may affect its quality or safety.

Enzymatic methods for detecting frozen meat monitor the activity of intracellular enzymes such as aconitase released from cells damaged by ice crystals (Škorpilová et al. 2019). Aconitase is an iron-sulphur enzyme of the Krebs cycle with a [4Fe–4S] cluster that catalyses the conversion of citrate to isocitrate via cis-aconitate. In chilled meat, the activity is low because, unlike thawed meat, there has been no disruption of cell structures and no release of the enzyme into the eluate (the naturally released fluid from the meat) (Pipek et al. 2014; Ranjan and Dubey 2023). This was confirmed, for example, in a study by Škorpilová et al. (2014), where aconitase activity gradually increased in thawed chicken meat stored at –22 °C for 45 days.

Tissue destruction and the release of eluates during freezing may lead to changes in their mineral content, with the extent of these changes increasing over prolonged freezer storage. As ice crystal formation progresses, individual meat components, including minerals, become more concentrated (Leygonie et al. 2012). Although freezing can alter the mineral composition, short-term storage at temperatures just above the freezing point generally results in negligible losses (Fennema 2019). During long-term or frozen storage, the content of certain organic acids also changes. For example, Seçkin et al. (2011) found that the acetic acid content increased in goat cheese during frozen storage. In contrast, the study by Shu et al. (2024) mentions a decrease in citric acid content during long-term (250 days) storage of apples. Similarly, Mezey and Mezeyová (2018) confirmed a decrease in citrate in apples stored at 1–2 °C for 140 days, but no significant decrease. It is necessary to note that these are different matrices compared to meat tissue. There is a lack of scientific publications addressing the content of organic acids and their changes during frozen storage.

This study focused on verifying and comparing three different methods for detecting frozen and subsequently thawed meat (beef, pork, and chicken). The first method involved determining aconitase enzyme activity, the second was the analysis of selected mineral cations (Na^+, K^+, Mg^{2+}), and the third parameter examined was the content of organic acids (citric, lactic, and acetic), whose concentrations may be influenced by cellular changes, microbial activity, or degradation processes. For the purpose of detecting/distinguishing thawed meat, a PCA analysis incorporating all obtained qualitative variables was ultimately employed.

MATERIAL AND METHODS

Meat samples and storage conditions. For the purposes of this experiment, three types of meat were purchased: beef *musculus biceps femoris* (3 days *post mortem*) (Maso Uzeniny Polička, Plc., Czech Republic), pork *caro humeri* (3 days *post mortem*) (Masné Krámy Ltd., Czech Republic) and chicken *musculus pectoralis major* (2 days *post mortem*) (Vodňanská drůbež, Plc, Czech Republic). Before freezing, pieces of meat weighing approximately 150–200 g were prepared, packaged, and vacuum-sealed (95%) (S-100 BET; Technovac Ltd., Italy) in a polyamide bag. The meat was stored in a DK-9500 freezer (ELCOLD, Denmark) at a temperature of –18 ± 1 °C. This temperature was selected in accordance with several previous studies that applied comparable freezing conditions, including –20 °C (Cheng et al. 2015; Park et al. 2023), –18 °C (Ouyang et al. 2022; Yu et al. 2024), and –22 °C (Škorpilová et al. 2014). Although not all of these studies focused specifically on aconitase activity or mineral cations and organic acids content, freezing at approximately –18 °C represents a common experimental and practical approach. It is acknowledged that lower temperatures (below –40 °C) are considered optimal for minimising ice crystal formation, as only a very small fraction of water remains unfrozen at this point (Estévez 2011). Before determining the selected parameters, the meat was removed from the freezer 24 h in advance and left in a thermostat ST2B40 basic (POL–LAB, Poland) at a temperature of 4.0 ± 0.5 °C to be thawed. Measurements were performed on fresh (= chilled) samples at day 0 and on thawed samples after 7, 14, and 150 days of frozen storage.

Aconitase activity assay. For the aconitase enzyme activity, the Aconitase Activity Assay Kit (Merck Ltd., Czech Republic) was used in accordance with the manufacturer's technical bulletin, with minor modifications. The aconitase test kit measures the resulting isocitrate, which reacts with nicotinamide adenine dinucleotide phosphate (NADP) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). Isocitrate is oxidised to a product, producing NADPH. NADPH reduces MTT to purple formazan. A calibration

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curve was generated using isocitrate standards at concentrations of 0, 1 500, 3 000, and 5 000 µM. The reaction mixture consisted of the following components (volumes per single assay): 8 µL NADP/MTT, 1 µL enzyme A, 1 µL enzyme B, 5 µL substrate, and 70 µL assay buffer.

All exudates were collected and filtered through a 0.45 µm nylon filter. The reaction was conducted in narrow-path microcuvette at laboratory temperature. A volume of 20 µL of either standard or diluted sample was pipetted into the cuvette, followed by 80 µL of the reaction mixture. The cuvette contents were gently agitated to ensure proper mixing. Absorbance was measured spectrophotometrically using GENESYS 150 (Thermo Scientific, USA) at 565 nm after 10 min and again after 30 min of reaction. Standard curve values were expressed as the difference in absorbance between 30 min and 10 min, plotted against the isocitrate concentration. The slope of the resulting regression line was determined. Aconitase activity ($U \cdot L^{-1}$) was calculated using Equation 1:

$$Aconitase\ acitivity = \frac{(A_{565})_{30} - (A_{565})_{10}}{T \times slope} \quad (1)$$

where: $(A_{565})_{30}$ – the absorbance of the sample after 30 min; $(A_{565})_{10}$ – the absorbance of the sample after 10 min; T – the reaction time (20 min); *slope* – the value obtained from the calibration curve (μM^{-1}).

Unit definition. 1 unit (IU) of aconitase catalyses the conversion of 1 µmole of citrate to isocitrate per min at pH 7.4.

Mineral content. The contents of sodium, potassium, and magnesium cations were determined using an AAS Agilent 200 AA (Agilent Technologies, USA). Sodium determination was performed according to Bartáková et al. (2024) with slight modifications, while potassium and magnesium were analysed using the same analytical principle. Approximately 1 g of sample was transferred directly to a ceramic dish and then subjected to dry decomposition. The combustion process took place in four stages (Labotherm, Naberthem GmbH, Germany): (*i*) heating to 200 °C and holding for 8 h, (*ii*) heating to 300 °C and holding for 6 h, (*iii*) heating to 400 °C and holding for 6 h, and (*iv*) heating to 500 °C and holding for 28 h. After combustion, the sample container was left to cool to laboratory temperature. 3 mL of 1 M hydrochloric acid (Penta Ltd., Czech Republic) were pipetted into the obtained ash, and the solution was mixed and transferred to a 100 mL volumetric flask and filled with distilled water to the mark. To analyse the concentrations of Na^+ and K^+ cations, the appropriate volumes of solutions were taken and transferred to a 10 mL volumetric flask. Subsequently, 2 mL of caesium chloride (CsCl) (Penta Ltd., Czech Republic) solution was added, and the volume was filled to the mark with distilled water. Absorbance was recorded at a wavelength of 589 nm for Na^+ and 766.5 nm for K^+ cations. The Mg^{2+} cation content was determined in a similar way to Na and K, with the difference that instead of 2 mL of CsCl, a mixture of 1 mL of CsCl + 1 mL of $LaCl_3$ was used, and the absorbance was measured at a wavelength of 285.2 nm. Concentrations of $Na^+/K^+/Mg^{2+}$ ($mg \cdot kg^{-1}$) cations were calculated using Equation 2:

$$W = V \times f_z \times \rho / m \quad (2)$$

where: W – the content ($mg \cdot kg^{-1}$) of mineral; V – the extract volume (L); f_z – the dilution factor; ρ – the concentration of $Na^+/K^+/Mg^{2+}$ cations ($mg \cdot kg^{-1}$); m – the weight of the sample (g).

Organic acid content. The content of citric, lactic, and acetic acids was determined in the meat as an indicator of its quality (Kvasnička 2006). The determination was performed using an isotachophoresis (ITP) analyser EA 101 (Villa Labeco, Slovakia). Meat samples were cut into small pieces, and then 25 g was weighed into an A11 basic IKA (IKA–Werke GmbH & Co. KG, Germany). Approximately 100 mL of distilled water was added, and the mixture was blended for 30 s. The contents of the blender were then quantitatively transferred to a 250 mL measuring flask, which was filled with distilled water to the mark. A diluted filtrate was used for analysis. Standard solutions ($20\ mg \cdot L^{-1}$) of sodium citrate (Penta Ltd., Czech Republic), lithium lactate (Merck Ltd., Czech Republic), and sodium acetate were used (Penta Ltd., Czech Republic) to determine the content of acids based on the length (time) of the individual ion zones and using the molar concentrations and molar masses of the given substances. The limits of detection were as follows: citric acid, $20\ mg \cdot L^{-1}$; lactic acid, $15\ mg \cdot L^{-1}$; acetic acid, $12\ mg \cdot L^{-1}$.

Statistical analysis. Before statistical processing, the obtained data were subjected to the Dean–Dixon test to exclude outliers. This was followed by statistical processing in Statistica 14.0 software (StatSoft Inc., USA) to compare individual qualitative parameters (aconitase activity, mineral content, and organic acid content). First, the data were subjected to one-way ANOVA nonparametric analysis followed by a post hoc HSD Tukey test (P = 0.05).

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For principal component analysis (PCA), all individual measurements (n = 3 per sample) were included in the dataset, rather than group means, to preserve within-group variability. Prior to analysis, the variables were auto scaled (z-score standardisation) to eliminate the influence of different measurement units. PCA was performed on the correlation matrix, and the first two principal components were retained according to eigenvalues > 1 and the proportion of explained variance. The resulting score and loading plots were used to visualise the distribution of samples and the contribution of individual variables to their discrimination.

RESULTS AND DISCUSSION

Aconitase activity. Slow freezing produces larger crystals, which more severely disrupt sarcolemma and mitochondrial membranes, releasing enzymes such as aconitase into the eluate. Studies confirm that freezing induces structural damage to muscle cells and organelles, leading to mitochondrial enzyme leakage and elevated concentrations in the eluate of thawed meat (Tippala et al. 2021; Biswas et al. 2023). The results of aconitase activity measurements in this study are presented in Figure 1. It is evident that the lowest activities were recorded for all types of meat in the chilled condition, and the activity increased with prolonged frozen storage at –18 °C. The highest aconitase activity was observed in the eluate of pork meat after 150 days of frozen storage, showing an almost eightfold increase ($P < 0.05$) compared to chilled meat. In beef and chicken, such a pronounced increase was not detected. However, the activity was still significantly higher, with chicken meat showing a detectable increase as early as 14 days of frozen storage ($P < 0.05$). These results are consistent with Škorpilová et al. (2014) in monitoring the increasing activity of aconitase in chicken meat after frozen storage for 2 to 45 days. The authors explain this fact by saying that frozen meat can undergo the formation of new ice crystals, which damage cells and intracellular membranes and release enzymes into the exudate. A significantly higher increase in pork, similar to this study, was also confirmed by Biswas et al. (2023), where after thawing after 45 days at –10 °C, the value almost doubled. The study also tested the effect of storage temperature (–10, –20, and –40 °C), but these results do not clearly indicate that lower storage temperatures caused higher enzyme activity.

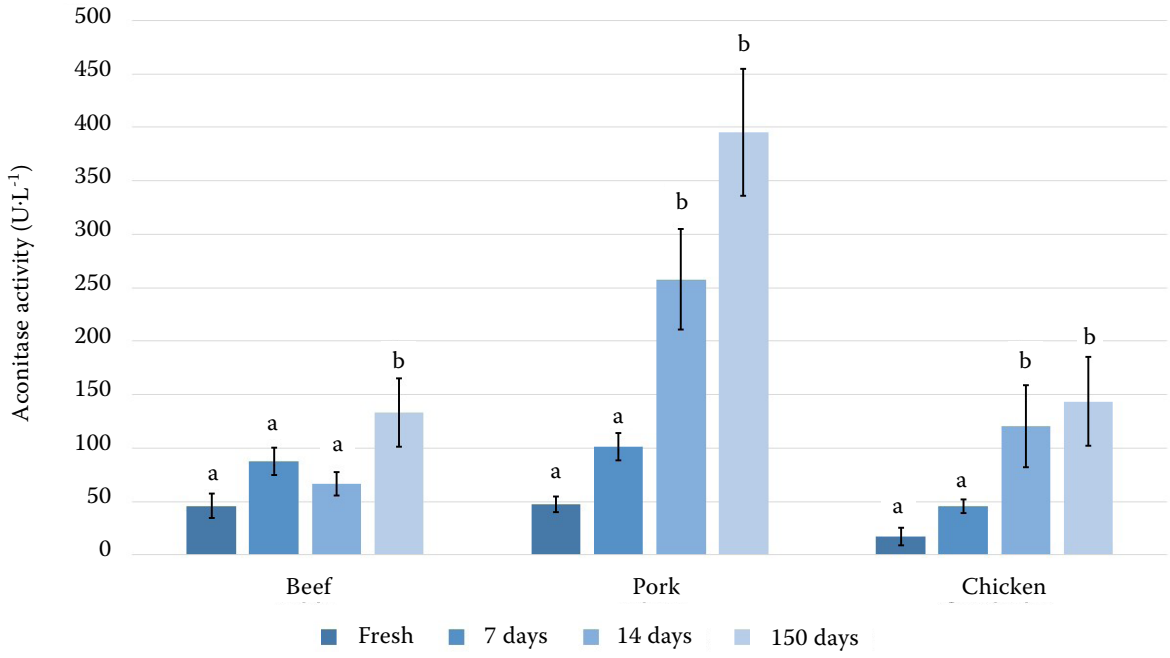

Figure 1. Aconitase activity in fresh and thawed meat samples after storage at –18 °C

[a–b]different letters indicate significant difference ($P < 0.05$) within a single meat species

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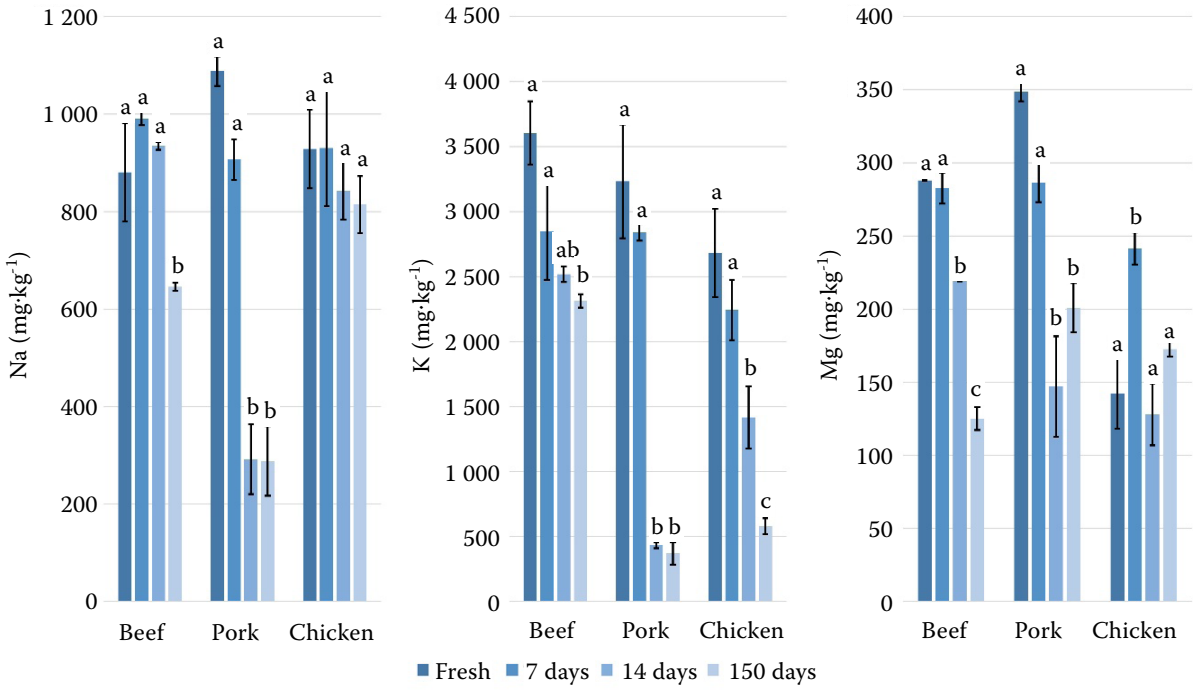

Figure 2. Sodium, potassium, and magnesium content in fresh and thawed meat stored at –18 °C

[a–c]different letters indicate significant difference ($P < 0.05$) within a single meat species

It is also necessary to take into account the type of animal and the anatomical part in question, as the structure of the meat itself is directly affected by freezing (Šimoniová et al. 2013). In summary, the results of this study and previous studies suggest that determining the activity of aconitase in meat eluate is a suitable method for detecting frozen and subsequently thawed meat.

Minerals. The concentrations of mineral cations in thawed meat undergo significant changes during storage (Figure 2). Prolonged frozen storage significantly affects the cationic composition of meat. While Na cation concentration remained relatively stable during the first week, they declined after 14 and 150 days ($P < 0.05$), likely due to cation and water loss via drip during thawing. K^+ cations exhibit the most pronounced decrease, particularly in pork, where levels drop sharply after extended storage (approximately 85% loss). Mg concentrations also decrease progressively across all meat types, possibly due to protein breakdown and cellular disruption. These findings are confirmed by Leygonie et al. (2012), who reported that meat eluate is rich in nutrients such as minerals, proteins, and vitamins. In addition, long-term frozen storage promotes ice crystal formation, leading to increased water exudation and subsequent significant nutrient loss (Wang et al. 2020). Overall, these results suggest that the determination of mineral cations may serve as a useful tool for the identification of thawed meat. However, it should be considered that cation concentrations may also vary depending on the anatomical part, and there are a number of other factors, such as the method of freezing (especially the speed) and thawing, or the initial mineral content, which may already be affected during the growth of the animal.

Future studies could also consider the simultaneous determination of meat sample dry matter and selected mineral cations in the eluate, which may further improve the interpretation of mineral losses associated with thawing.

Organic acids. Figure 3 illustrates the impact of freezing-thawing on the concentrations of citric, lactic, and acetic acids in beef, pork, and chicken. Citric acid shows the highest initial concentration in fresh beef (about 3 000 $mg \cdot kg^{-1}$), but declines significantly with longer freezing durations, especially after 150 days ($P < 0.05$). Pork follows a similar trend, while chicken maintains relatively stable citric acid levels. Lactic acid is most abundant in fresh beef (almost

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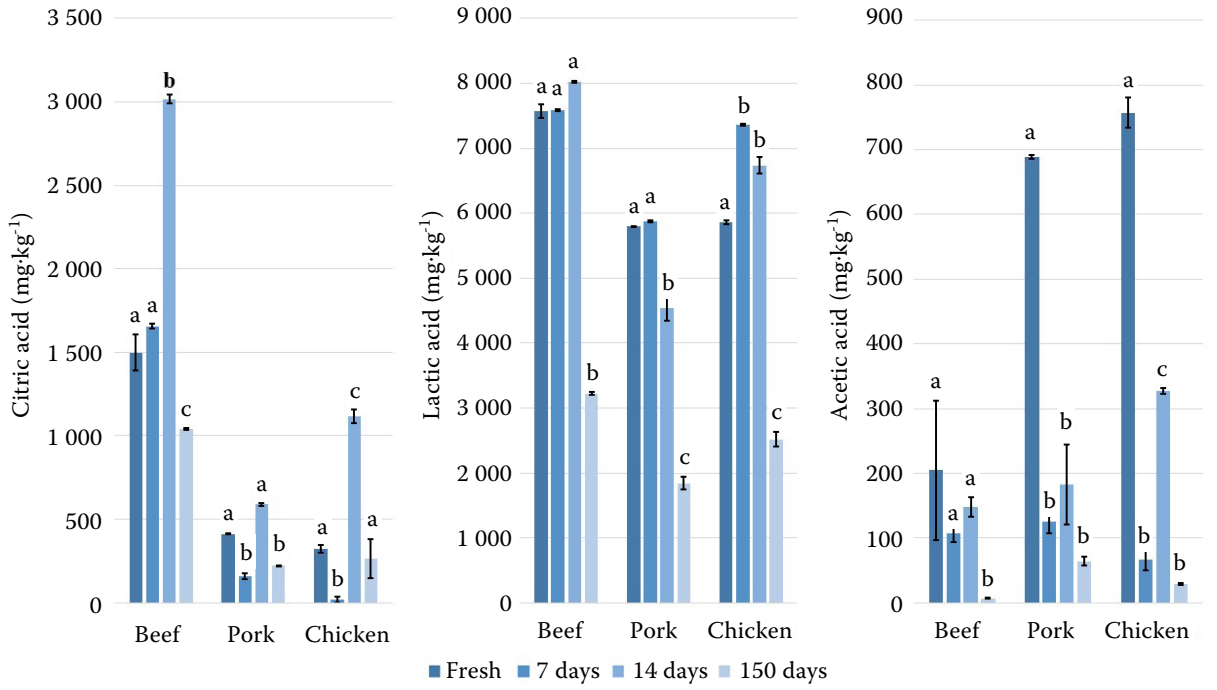

Figure 3. Organic acids content in fresh and thawed meat samples stored at –18 °C

[a–b]different letters indicate significant difference ($P < 0.05$) within a single meat species

8 000 mg·kg^{-1}), decreasing slightly over time. Pork mirrors this pattern at lower levels, whereas chicken starts with the lowest lactic acid content and slightly increases after extended freezing. Acetic acid is initially highest in chicken and decreases across all meat samples with time ($P < 0.05$). Park et al. (2004) tested the organic acid content in plain soft goat milk cheese and found that there was a decrease in acetic acid content at low temperatures (4 °C for 28 days). However, this could have been due to the natural microflora in the cheese. Similarly, González-Castro et al. (1997) confirmed, for example, a significant reduction in citric acid in green beans and peppers during storage for 30 days at –22 °C. It can be hypothesised that acids are eliminated from the matrix together with the substrate eluate.

Principal component analyses. The PCA score plot (Figure 4) shows a clear separation of fresh and thawed meat samples depending on storage duration. Fresh samples (●) are grouped on the negative side of Factor 1, whereas thawed samples are progressively shifted towards the positive axis according to the length of storage (■ – 1 week, ♦ – 2 weeks, ▲ – 150 days). This distribution indicates that the measured variables were able to discriminate between fresh and frozen-thawed meat. The most pronounced differentiation was observed for samples thawed after long-term storage (150 days), which formed a distinct cluster separated from both fresh and shortly frozen samples. The loading plot (Figure 5) provides insights into the variables contributing to this discrimination. Among the measured parameters, aconitase activity showed the strongest positive correlation with Factor 1, suggesting that this enzyme is a key indicator of meat subjected to freezing and thawing. In contrast, citric acid and acetic acid contributed negatively to Factor 1, reflecting their association with fresh meat. Lactic acid and the mineral cations (Na^+, K^+, Mg^{2+}) clustered closely near the origin, indicating only minor contributions to the discrimination along the first two factors. Overall, the PCA demonstrates that the combination of aconitase activity, organic acid profile, and mineral cation concentration can be effectively applied to differentiate fresh from thawed meat. The strong loading of aconitase suggests that enzymatic activity is particularly sensitive to freeze-thaw processes, whereas changes in citric and acetic acid contents also contribute to the separation. These findings highlight the potential of multivariate statistical approaches to authenticate meat freshness and to detect fraudulent substitution of thawed meat for fresh products.

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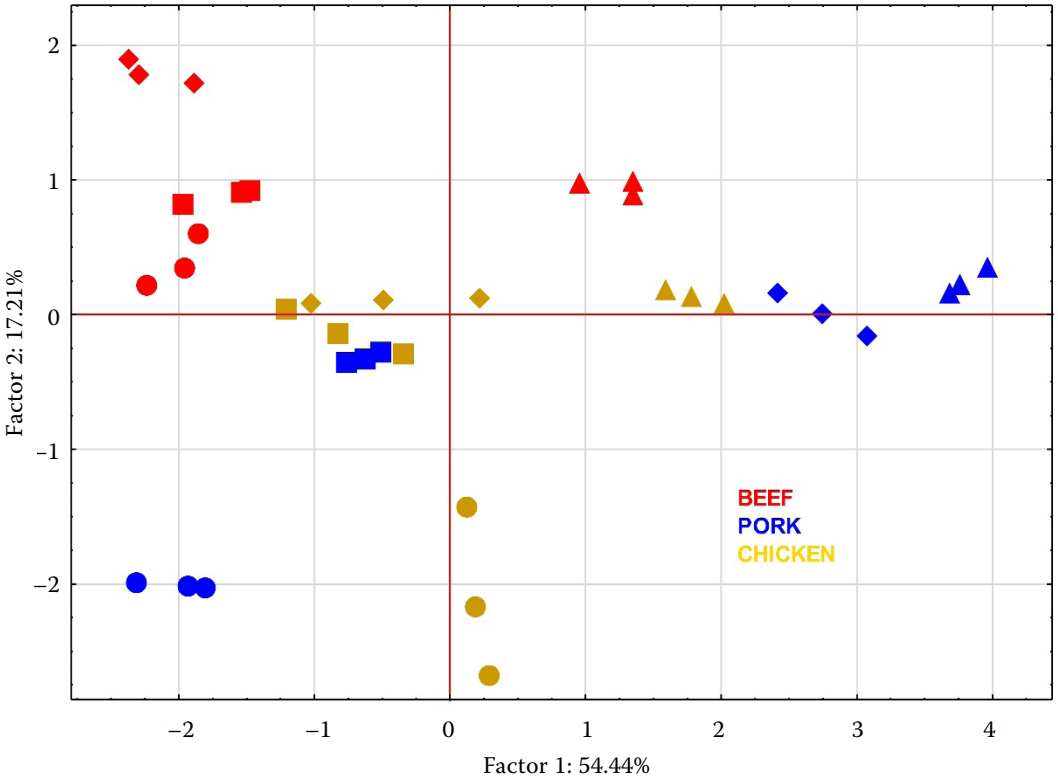

Figure 4. PCA of meat samples

● – fresh;
■ – thawed after 1 week;
♦ – thawed after 2 weeks;
▲ – thawed after 150 days

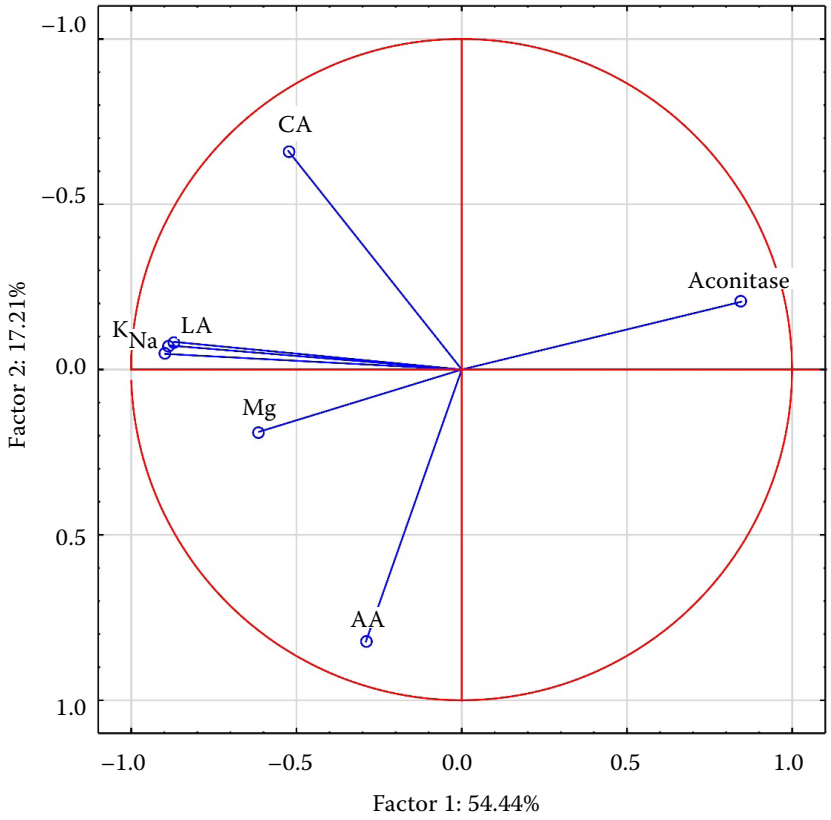

Figure 5. PCA of variables determined in fresh and thawed samples

AA – acetic acid; LA – lactic acid; CA – citric acid

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CONCLUSION

The results demonstrated that freezing and thawing significantly affect the biochemical and mineral parameters of meat, with the most reliable indicators being increased aconitase activity and changes in sodium and potassium concentrations. These parameters enabled clear differentiation between fresh and thawed meat across species, although the extent of changes was species-specific. Their potential can be applied in developing rapid and reliable methods for detecting meat fraud in practice. Future research should focus on validating these indicators under industrial conditions and exploring their combination with advanced chemometric approaches for improved detection accuracy.

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