South African Journal of Animal Science 2025, Vol 55(3)

An HPLC-MS/MS method for the quantification of heat stress-related milk metabolites in milk from Holstein-Friesian cross-bred cows in Tanzania

V. Habimana[1,2,3#], A.S. Nguluma[1], Z.C. Nziku[4], C.C. Ekine-Dzivenu[3], G. Morota[5], R. Mrode[3,6], & S.W. Chenyambuga[1]

[1]Department of Animal, Aquaculture, and Range Sciences, Sokoine University of Agriculture, Morogoro, Tanzania
[2]SACIDS Africa Centre of Excellence for Infectious Diseases, SACIDS Foundation for One Health, Sokoine University of Agriculture, Morogoro, Tanzania
[3]International Livestock Research Institute (ILRI), Nairobi, Kenya
[4]Tanzania Livestock Research Institute (TALIRI), Eastern Zone, Tanga, Tanzania
[5]Laboratory of Biometry and Bioinformatics, Department of Agricultural and Environmental Biology, Graduate School of Agricultural and Life Sciences, The University of Tokyo, Bunkyo, Tokyo 113-8657, Japan
[6]Animal and Veterinary Sciences, Scotland's Rural College, Easter Bush, Midlothian EH25 9RG, Scotland, United Kingdom

(Submitted 5 November 2024; Accepted 5 February 2025; Published March 2025)

Abstract

The main objective of this study was to develop and validate a high-performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS) method for the determination and quantification of three metabolites in raw milk samples: creatinine, citrate, and β-hydroxybutyrate (3-BHB). The effect of the temperature-humidity index (THI) on the concentrations of these metabolites was also investigated. The study used 29 Holstein-Friesian × Tanzanian Shorthorn Zebu cross-bred cows of two genotypes from the Tanzania Livestock Research Institute in Tanga, Tanzania. The cows were in their second or third parity and their second or third month of lactation. Chromatographic separation of the three metabolites was performed using a Supelco column (150 × 2.1 mm, 3 µm), with a mobile phase consisting of 1% formic acid and 10 mM ammonium formate in high-performance liquid chromatography (HPLC) water, and 1% formic acid in methanol. Creatinine, citrate, and 3-BHB were measured at 2.13, 2.33, and 2.48 minutes of run time, respectively. The calibration curves were linear and ranged from 0.0025 to 0.64 µg/mL for creatinine, 1.25 to 3.20 µg/mL for citrate, and 0.25 to1.28 µg/mL for 3-BHB. The creatinine (8.44–5.40 µg/mL), citrate (36.54–33.93 µg/mL), and 3-BHB (12.07–8.96 µg/mL) concentrations decreased with an increase in THI from 77 to 83. However, these results were complicated by significant interactions between the THI and the genotype, parity, and months in lactation. In conclusion, this study demonstrates the potential of an HPLC-MS/MS method to estimate creatinine, citrate, and 3-BHB concentrations in milk samples, and shows that heat stress affects the concentrations of these milk metabolites.

Keywords: β-hydroxybutyrate, citrate, creatinine, metabolic status, tandem mass spectroscopy

[#]Corresponding author: vincenthabimana5@gmail.com

URL: http://www.sasas.co.za
ISSN 0375-1589 (print), ISSN 2221-4062 (online)
Publisher: South African Society for Animal Science
http://dx.doi.org/10.4314/sajas.v55i3.07

Introduction

Heat stress (HS) has the potential to affect both the health and milk production traits of dairy cattle reared in tropical, sub-tropical, and temperate regions (Liu *et al.*, 2017). Lactating dairy cows under HS-inducing conditions limit their energy intake and are therefore unable to meet their bodies' requirements for milk production and the maintenance of good health. This results in reduced milk yield and quality and makes dairy cows susceptible to disease (Tian *et al.*, 2016). Considering that HS reduces feed intake, impairs fertility and pregnancy rate (Osei-Amponsah *et al.*, 2020; Yue *et al.*, 2020), and alters the metabolic status of dairy cows, it is expected that dairy cows exposed to HS will be affected by metabolic disorders such as subacute ruminal acidosis (Gantner *et al.*, 2016). High-yielding dairy cows exposed to HS-inducing environments appear to consume large amounts of high glycaemic carbohydrate feeds for fat mobilisation, but are often in a negative energy balance (NEB) status (Pires *et al.*, 2022; Wang *et al.*, 2022; Girma *et al.*, 2023). The mobilisation of body reserves during a NEB status causes an increase in the concentration of circulating long chain non-esterified fatty acids (NEFA), which are oxidised to acetyl-coenzyme A to produce energy, and are also used for fat synthesis in the mammary glands (Van *et al.*, 2020). If the liver is unable to oxidise fatty acids from NEFA to acetyl-coenzyme A and further to citrate through the tricarboxylic acid (TCA) cycle and respiratory chain reaction to produce energy as adenosine triphosphate, acetyl-coenzyme A is diverted to the production of ketones, which ultimately leads to ketosis (Turk *et al.*, 2020; Van *et al.*, 2020; Chen *et al.*, 2023). Dairy cows are susceptible to ketosis because of their increased maintenance and thermoregulation requirements, and decreased feed intake at two and seven weeks postpartum, secondary to poor feeding and management (Gantner *et al.*, 2016; Pires *et al.*, 2022). Ketosis is classified as subclinical ketosis when the blood β-hydroxybutyrate (3-BHB) concentration is between 1.2 and 1.4 mmol/L, and clinical ketosis when the blood 3-BHB concentration is between 2.6 and 3.0 mmol/L (Bonfatti *et al.*, 2019; Eom *et al.*, 2022).

Robust metabolite biomarkers are needed to diagnose the threshold for the onset of HS, monitor its progression, and provide insight into its physiological mechanisms, thereby enabling the implementation of timely interventions to protect dairy cows from diseases such as ketosis (Tian *et al.*, 2016). Several metabolites have been used to characterise the metabolism of dairy cows in the early stages of lactation (Koster *et al.*, 2019). Of the milk metabolites crucial for this purpose, creatinine and 3-BHB have been identified as molecules of interest in relation to ketosis, and citrate has been identified as an early indicator of a NEB (Grelet *et al.*, 2016; Eom *et al.*, 2022; Chen *et al.*, 2023). The ability to identify dairy cattle with high physiological imbalances because of HS is important for the early diagnosis of energetic and metabolic changes in high-producing dairy cows (Giannuzzi *et al.*, 2023).

Creatinine is excreted from the bloodstream into the milk and usually detected and measured in the milk of dairy cows (Giannuzzi *et al.*, 2023). Creatinine is the end product of creatine and creatine phosphate metabolism, and is associated with energy balance when a dairy cow requires the rapid provision of energy (Hu *et al.*, 2022). The creatinine metabolite is produced during muscle cell breakdown, and has been used in several studies as a biomarker for muscle mass (Joo *et al.*, 2021; Kim *et al.*, 2021). The plasma creatinine concentration declines during the early stages of lactation because of poor protein mobilisation and the loss of muscle mass (Pires *et al.*, 2022; Cattaneo *et al.*, 2023).

Citrate plays a crucial role in the udders of dairy cows as it regulates Ca^{2+} and H^{+} ion homeostasis and maintains milk fluidity by acting on casein micelles (Yue *et al.*, 2020). A decline in the citrate concentration in the udder causes the clumping of Ca^{2+} ions, which leads to damage to the parenchymatous tissues of the alveoli of the udder and, consequently, damage to the barriers between the milk and the blood, as well as inflammatory reactions in the alveolar tissue of the udder (Yue *et al*., 2020). Citrate also plays a central role as an intermediate in cellular energy metabolism in the TCA cycle, as well as in glycolysis and fatty acid synthesis, and is found in milk at higher levels than in other biofluids (Fan *et al.*, 2018; Zhang *et al.*, 2013; Kim *et al.*, 2021). Acetyl-coenzyme A and oxaloacetate are oxidised to produce citrate, which then undergoes various further metabolic reactions to generate energy (Van *et al.*, 2020). Citrate has been shown to be a milk biomarker for fat mobilisation and ketosis in dairy cattle during the early stages of lactation (Fan *et al.*, 2019), and a decrease in the citrate concentration may exacerbate ketosis by causing the disruption of the TCA cycle and amino acid deprivation (Zhang *et al.*, 2013).

β-hydroxybutyrate is a ketone body metabolite that has been used to diagnose ketosis in various studies (Kim *et al.*, 2021). Milk 3-BHB is a classical biomarker for ketosis and metabolic status (Zhang *et al.*, 2013; Pires *et al.,* 2022). The HS-induced change in 3-BHB is a sign of a disturbance in mitochondrial function (Fan *et al.*, 2019). Ketosis is diagnosed by measuring ketone body concentrations in the blood and milk, and occurs when ketone bodies from the adipose tissue, instead of glucose, are used for energy generation, when the energy generated by the body is insufficient (Grelet *et al.*, 2019; Foldager *et al.*, 2020). Analysis of milk metabolites may be useful in studies of body weight-related muscle synthesis and ketosis-related malnutrition (Kim *et al.*, 2021).

According to Yue *et al*. (2020), blood 3-BHB, creatinine, and citrate levels can be used as biomarkers to determine HS susceptibility and NEB in lactating dairy cows. Genetic variations between individual dairy cows that result in certain pathways functioning more efficiently than others can cause systematic variations in baseline levels of metabolites (Krogh *et al.*, 2020). Studies have shown that two different dairy cow genotypes exposed to similar HS conditions can respond very differently in terms of milk 3-BHB, citrate, and creatinine concentrations (Krogh *et al.*, 2020). Moreover, assessing dairy cow phenotypes according to their 3-BHB, citrate, and creatinine levels provides an alternative approach to implementing genetic and genomic selection for reduced ketosis and HS susceptibility (Lou *et al.*, 2022). Metabolomics, a useful tool for analysing the changes in metabolite concentrations in physiological fluids and tissues in response to internal and external stimuli (Hu *et al.*, 2022), has been successfully applied to identify biomarkers for milk quality, energy metabolism, and rumen health in dairy cows (Yue *et al.*, 2020).

A variety of methods, including high-pressure liquid chromatography, gas chromatography-mass spectrometry, and nuclear magnetic resonance, can be used for metabolite analysis (Eom *et al.*, 2021; Shi *et al*., 2021). The major advantages of mass spectrometry (MS) are its high sensitivity and its ability to measure hundreds of metabolites. However, its limitations include the need for large amounts of sample and a lack of reproducibility and reliability (Kim *et al.*, 2021). Reverse-phase liquid chromatography-tandem MS (LC-MS/MS) is a highly sensitive analytical technique that uses liquid chromatography to separate compounds within a sample and then uses MS to analyse the compounds. In a targeted format, this method can be used for the detection and quantification of known compounds or metabolites, which can be identified from the established metabolome database for that sample type (e.g., the milk metabolome) (Magan *et al.*, 2019). Multiple reaction monitoring (MRM), with its high sensitivity, specificity, and speed, can overcome the main drawbacks of traditional high-performance liquid chromatography (HPLC) (such as low sensitivity and overlapping peaks) and two-dimensional gel electrophoresis (such as difficult automation, poor dynamic range, and limited sensitivity) (Li *et al*., 2019; Le *et al.*, 2020).

Numerous studies conducted in temperate countries have quantified HS-related milk metabolites, using HPLC-tandem MS (HPLC-MS/MS) and proton nuclear magnetic resonance spectroscopy to identify metabolic differences in milk samples from mid-lactation cows (Tian *et al.*, 2016; Yue *et al.*, 2020). However, more information is needed on the mechanisms underlying the metabolic responses to HS exposure, particularly in high-producing dairy cattle breeds such as Holstein-Friesian cattle in sub-Saharan countries. Few studies have quantified the creatinine, citrate, and 3-BHB metabolites, which are important HS biomarkers in lactating dairy cows during the early stages of lactation. Therefore, the current study aimed to establish a reliable, accurate, and simple method for the determination and validation of creatinine, citrate, and 3-BHB metabolites in raw milk samples using HPLC-MS/MS. This is a new method for the determination of these three HS-related milk metabolites in a single chromatogram, and this method requires simple sample preparation, has a fast turnaround time, has interference-free analysis, and has traceability to other reference methods. The second objective of this study was to investigate the effects of HS – as measured using the temperature-humidity index (THI) – as well as the effects of genotype, parity, and months in lactation, and their interactions with the THI – on milk metabolite concentrations. The findings of this study will help to establish strategies to mitigate HS by using milk metabolites as biomarkers.

Materials and methods

Ethical approval for this research was granted by the institutional ethics committee of Sokoine University of Agriculture (SUA), College of Agriculture Guidelines (reference number: SUA/ADM/R.1/8/843).

The study was conducted in accordance with good scientific practices approved by SUA. During milk sampling, all animal welfare concerns were addressed to minimise discomfort.

Study site

This study was conducted at the Tanzania Livestock Research Institute (TALIRI) dairy farm in Tanga municipality. A comprehensive description of the study site, experimental farm characteristics, and the dairy cows used in this study and their management is available in our previous work, which evaluated the effects of HS on the milk yield, milk composition, and physiological parameters of the cows studied (Habimana *et al.*, 2024).

Experimental animals, milk sample collection, and THI computation

A total of 29 raw milk samples were collected from 29 Holstein-Friesian cross-bred dairy cows at the TALIRI Tanga farm during the last weeks of both the hot season (February 2022) and the cool season (July 2022). The cross-bred cows were the result of cross-breeding between Holstein-Friesian and Tanzania Shorthorn Zebu cattle. Milk samples were collected from cows belonging to two genotypes: 50% Holstein-Friesian (HF50, n = 18) and 75% Holstein-Friesian (HF75, n = 11). Second- or third-parity cows in their second to third months of lactation were used. Milk samples were collected in the afternoon between 14:30 and 17:00, and 16 milk samples were collected during the hot season (on 28 February 2022) and 13 milk samples were collected during the cool season (on 31 July 2022). Different lactating Holstein-Friesian cross-bred cows were sampled during the hot and cool seasons, based on the selection criteria stated above. However, the cows sampled in the hot and cool seasons were comparable in terms of their genetic compositions, months in lactation, and parities (Habimana *et al.*, 2024).

To assess the HS experienced by the dairy cattle, data on daily maximum and minimum temperatures (°C) and relative humidities (%) during the experimental periods were obtained from the Tanga meteorological station, which is located 500 m from the TALIRI Tanga dairy cattle farm. The daily THI was calculated using Equation 1, as described by Ekine-Dzivenu *et al.* (2020):

$$THI = (1.8 \times Tmax + 32) - [(0.55 - 0.0055 \times RHmin) \times (1.8 \times Tmax - 26.8)]$$

In the formula, *Tmax* represents the maximum daily dry bulb temperature (in degrees Celsius), and *RHmin* represents the minimum daily relative humidity (in per cent). The minimum, average, and maximum THI values were generated for each milk sampling day, as well as for the three previous days. This particular THI model was chosen as the most suitable for the equatorial climate of Tanzania, considering the impact of HS on animal performance (Ekine-Dzivenu *et al*., 2020). The temperature and THI trends during the study period are available in our previous work (Habimana *et al.*, 2024).

HPLC-MS/MS optimisation

The solvents and chemicals used for laboratory analysis included HPLC-grade methanol (Finar, India, 99.8%), HPLC-grade acetonitrile (Finar, India, 99.9%), analytical-grade formic acid (Finar, India, 98%), analytical-grade ammonium formate (Lober Chemie PVT, India, 98%), and HPLC-grade water (Carlo Erba, France). Ultrapure water for HPLC was obtained using a Milli-Q® UF-Plus apparatus (Millipore Corp., Burlington, MA, USA). Analytical standards of 3-BHB, creatinine, and sodium citrate, each with purities above 99% (Sigma Aldrich, USA), were used to quantify the metabolites in the raw milk samples.

Chromatography was performed using an Agilent 1100 HPLC system, which consisted of a micro-vacuum degasser (G1379A), binary pump (G1312A), autosampler (G1313A), thermostatted column compartment (G1316A), and diode array detector (G1315B). The chromatographic system was coupled with a Quattro Micro API triple quadrupole MS (Waters, Micromass Limited, UK), equipped with an electrospray ionisation (ESI) interface. System control, data acquisition, and the calculation of results for metabolites in milk were performed using MassLynx NT 4.1 software (Waters Corporation, USA). The nebulising and collision gases were nitrogen and argon, respectively.

Electrospray ionisation was operated in both positive and negative ion modes (ESI^+ and ESI^-) to select the mode that produced the most intense molecular ions. The optimisation of MS detection was

performed using the auto-tune function of the MassLynx software. Ideal MS tune settings and MRM conditions were found for each compound by direct infusion and flow injection of individual solutions of the analytes at 10 mg/mL. For each compound, the transition with the highest intensity was chosen for quantification and the next most intense transition was selected for reliable confirmation. Thus, the identity of each compound was determined by retention time and two MS/MS transitions.

Chromatographic conditions were optimised to improve separation, sensitivity, and selectivity, taking into account the targeted analytes. Five HPLC columns were tested: Restek Allure Acidix (100 × 2.1 mm, 5 µm), Supelco Ascentis (100 × 2.1 mm, 2.7 µm), Xterra C18 (150 × 2.1 mm, 3.5 µm), Supelco (150 × 2.1 mm, 3 µm), and YMCbasic (50 × 2.1 mm, 3 µm). Different mobile phase compositions and additives were tested to optimise chromatographic separation. For the aqueous phase, a formate/formic acid buffer and HPLC water with formic acid were tested, and for the organic phase, formic acid in either methanol or acetonitrile were tested. Different concentrations of methanol, acetonitrile, formic acid, and formate were also investigated. Furthermore, flow rate and column temperature were optimised to improve chromatographic resolution and peak shapes and reduce the total analysis time. Flow rates from 0.2 to 0.4 mL/min were tested, and an optimum flow rate of 0.25 mL/min was selected. Column temperatures of 30 °C to 45 °C were tested, and at 40 °C, peak shapes and chromatographic responses were improved and run time was shortened.

Accurately weighed, appropriate amounts of the citrate, 3-BHB, and creatinine standards were dissolved in methanol in 10 mL volumetric flasks to obtain individual stock solutions. The concentrations of citrate, 3-BHB, and creatinine were 1287, 2040, and 1597 ppm, respectively. For MS optimisation, working standard solutions were obtained by diluting the individual stock solutions to 64.4, 10.2, and 5 ppm for citrate, 3-BHB, and creatinine, respectively, in 0.1% formic acid in 10:90 methanol:water. To optimise sample preparation, a mixture of 400 ppm citrate, 200 ppm 3-BHB, and 4 ppm creatinine was prepared by mixing appropriate amounts of each stock solution and diluting with 10:90 methanol:water containing 1% formic acid and 10 mM ammonium formate. Calibration standards were prepared at concentrations of 320, 160, 80, 40, 20, 10, 5, 2.5, and 1.25 ppm for citrate; 128, 64, 32, 16, 8, 4, 2, 1, and 0.25 ppm for 3-BHB; and 0.64, 0.32, 0.16, 0.08, 0.04, 0.02, 0.01, 0.005, and 0.0025 ppm for creatinine.

Sample preparation involved extraction, evaporation, and filtration. Extraction was performed to isolate the analytes from the sample. Evaporation was carried out to reduce the organic modifier content prior to injection into the chromatographic system, to avoid band broadening and thus high variation in relative retention time. Filtration was performed to eliminate any particulates that would affect the column and HPLC performance. The influence of each step on recovery of the analytes was studied as described in the next sections.

The influence of filtration on the recoveries of the selected compounds was determined at two concentration levels: high (40 ppm citrate, 20 ppm 3-BHB, and 0.4 ppm creatinine) and low (10 ppm citrate, 5 ppm 3-BHB, and 0.1 ppm creatinine). Two sets of triplicate samples were prepared for each concentration. One set was filtered through a 0.22-µm MS nylon syringe filter, while the other set was not filtered. The peak areas for the filtered and unfiltered samples were used to calculate per cent recovery, as per Equation 2:

$$Recovery\ (\%) = \frac{Area_{filtered\ sample}}{Area_{unfiltered\ sample}} \times 100\ \%$$

The pre- and post-spiking approach was used to determine the influence of evaporation on the recoveries of the selected compounds at two concentration levels: high (40 ppm citrate, 20 ppm 3-BHB, and 0.4 ppm creatinine) and low (10 ppm citrate, 5 ppm 3-BHB, and 0.1 ppm creatinine).

For the pre-spiked samples at high concentrations, 100 µL of the standard mixture (400 ppm citrate, 200 ppm 3-BHB, and 4 ppm creatinine) was added to 4 mL of acetonitrile, then diluted to 5.0 mL with HPLC water and mixed by vortexing for 30 seconds. The mixture was evaporated in a water bath set to 85 °C until it reached approximately 500 µL in volume. The residue was quantitatively transferred to an Eppendorf tube and made up to 1 mL using the mobile phase. It was then mixed by vortexing and analysed. For the post-

spiked samples at high concentrations, 1 mL of HPLC water and 4 mL of acetonitrile were vortexed for 30 seconds and evaporated in a water bath set to 85 °C until a volume of approximately 500 µL was obtained. The residue was transferred to an Eppendorf tube, spiked with 100 µL of the standard mixture (400 ppm citrate, 200 ppm 3-BHB, and 4 ppm creatinine) and made up to 1 mL using the mobile phase. This was then mixed by vortexing and analysed. The pre- and post-spiked samples at low concentrations used 25 µL of the standard mixture (400 ppm citrate, 200 ppm 3-BHB, and 4 ppm creatinine), but otherwise the procedure used was the same as described above for the high concentration samples. The obtained peak areas were used to calculate the per cent recovery, as per Equation 3:

$$Recovery\ (\%) = \frac{Area_{pre-spiked\ sample}}{Area_{post-spiked\ sample}} \times 100\ \%$$

The pre- and post-spiking approach was also used to determine the influence of extraction on the recoveries of the selected compounds at two concentration levels: high (40 ppm citrate, 20 ppm 3-BHB, and 0.4 ppm creatinine) and low (10 ppm citrate, 5 ppm 3-BHB, and 0.1 ppm creatinine). For the pre-spiked samples at high concentrations, 100 µL of the standard mixture (400 ppm citrate, 200 ppm 3-BHB, and 4 ppm creatinine) was added to 1.0 mL of thawed and thoroughly vortexed milk sample. A total of 4 mL of acetonitrile was added and the mixture was vortexed for two minutes and sonicated for five minutes. The mixture was then centrifuged at 12000 × g for 15 minutes to remove large biomolecules such as proteins. The supernatant was transferred and left to evaporate in a water bath set to 85 °C until reduced to a volume of approximately 500 µL. The residue was transferred quantitatively to an Eppendorf tube, made up to 1 mL using the mobile phase, vortexed, filtered, and analysed. For the post-spiked samples at high concentrations, 1.0 mL of thawed and thoroughly mixed milk sample was added to 4 mL of acetonitrile and the mixture was vortexed for two minutes and sonicated for five minutes. The mixture was then centrifuged at 12000 × g for 15 minutes. The supernatant was transferred and evaporated in a water bath set to 85 °C until reduced to a volume of approximately 500 µL. The residue was transferred quantitatively to an Eppendorf tube, 100 µL of the standard mixture (400 ppm citrate, 200 ppm 3-BHB, and 4 ppm creatinine) was added, and the mixture was made up to 1 mL using the mobile phase. This was then vortexed, filtered, and analysed. To determine the influence of extraction on recoveries at low concentrations, the samples were pre- and post-spiked with 25 µL of the standard mixture (400 ppm citrate, 200 ppm 3-BHB, and 4 ppm creatinine), but the same procedure as for the high concentration samples was otherwise followed. The obtained peak areas were used to calculate the per cent recovery using Equation 3.

Sample preparation

Sample preparation involved thawing and thoroughly mixing a 1.0 mL milk sample, and then vortexing this for one minute. Thereafter, 4 mL of acetonitrile was added and the mixture was vortexed again, mixed for two minutes, and then sonicated for five minutes. The mixture was then centrifuged at 12000 × g for 15 minutes and the supernatant was transferred and left to evaporate in a water bath set to 85 °C until a volume of approximately 500 µL was reached. The residue was transferred quantitatively to an Eppendorf tube, made up to 1 mL using the mobile phase, vortexed, filtered, and analysed.

HPLC-MS/MS method validation

Validation of the developed method was performed according to the guidelines of the International Conference on Harmonization (Harron, 2013). The method was validated for specificity, linearity, precision, accuracy, limit of detection (LOD), and limit of quantification (LOQ).

Specificity is the ability of a method to discriminate between the analytes of interest and other components in the sample. In this study, specificity was determined by performing the procedure using blank, standard, and test samples.

Linearity was determined using different known concentrations of the citrate, 3-BHB, and creatinine standards in triplicate, which were obtained by diluting the standard stock solution. The standard solutions

were injected, the peak areas were measured, and linearity was evaluated by comparing the peak areas obtained to the known concentrations of the citrate, 3-BHB, and creatinine standards. For each analyte, a calibration curve was constructed by plotting the peak areas obtained against the known concentrations to obtain a linear equation. The correlation coefficient was also calculated.

Precision was determined by studying the repeatability of the analysis at a minimum of three different concentrations for each analyte. Repeatability was determined at 40 and 5 ppm for citrate, 16 and 2 ppm for 3-BHB, and 0.08 and 0.01 ppm for creatinine, using eight replicates. Precision was expressed as the percentage relative standard deviation (% RSD).

The accuracy of the method was tested by performing pre- and post-spiking recovery studies at two different concentrations, as described in the HPLC-MS/MS optimisation section for the extraction of selected compounds.

The sensitivity of the method was evaluated by determining the LOD and LOQ values. The LOD value was determined from the calibration curves, and was calculated as three times the ratio of the residual standard deviation of the linear regression to the slope. The LOQ value was calculated as ten times the same ratio.

Statistical analysis

The peak area values and chromatograms for the creatinine, citrate, and 3-BHB metabolites were obtained by manual integration using MassLynx NT 4.1 software. Statistical analysis of the data was performed using Excel. The THI values (77 and 83) calculated for the days of milk sampling (28 February 2022 and 31 July 2022) in our previous work (Habimana *et al.*, 2024) were used to investigate the effects of the THI on the milk metabolite concentrations. Moreover, the effects of genotype, parity, and months in lactation, and their interactions with the THI, on the creatinine, citrate, and 3-BHB concentrations were analysed using the PROC GLM procedure of SAS 9.2 (SAS Institute, 2003) through univariate analyses and a restricted maximum likelihood model. The model included fixed effects for the THI, genotype, parity, and months in lactation, as well as for the interactions between the THI and the genotype, parity, and months in lactation. The dependent variables were the creatinine, citrate, and 3-BHB concentrations in the milk samples. The final mixed linear model for each parameter was:

$$Y_{imjkln} = \mu + THI_i + G_m + P_j + L_k + (G * THI)_{ij} + (P * THI)_{ij} + (L * THI)_{ik}$$

where Y_{imjkln} is the phenotypic record for the creatinine, citrate, or 3-BHB concentration; μ is the overall mean; THI_i is the effect of the ith THI class (77 or 83); G_m is the effect of the mth genotype (HF50 or HF75); P_j is the effect of the jth parity (2nd or 3rd); L_k is the effect of the kth month in lactation (2nd or 3rd); $(G*THI)_{ij}$ is the effect of the genotype by THI interaction; $(P*THI)_{ij}$ is the effect of the parity by THI interaction; and $(L*THI)_{ik}$ is the effect of the month of lactation by THI interaction.

The significance of the fixed effects was analysed using the Tukey-Kramer test, and significance was set at $P \leq 0.05$. The results are presented as the least-squares means ± the standard errors of the means.

Results

HPLC-MS/MS optimisation

The optimal source parameters for HPLC-MS/MS were established as: capillary 3.2 kV, exit potential 3 V, radio frequency lens 0.2 V, source temperature 120 °C, desolvation temperature 450 °C, and desolvation and cone gas flow rates of 650 and 110 L/min, respectively (Table 1). Creatinine showed acceptable sensitivity in the positive mode (ESI^+), while citrate and 3-BHB showed acceptable sensitivities in the negative mode (ESI^-). The optimised MS/MS parameters for each compound are shown in Table 1. The dwell time was set at 100 ms for each channel.

Table 1 Optimised MS/MS parameters for the quantification of three metabolites in milk samples

Compound	Ion mode	Precursor ion (m/z)	Cone voltage (V)	Product ions[1] (m/z)	
				Quantification	Confirmation
Citrate	ESI^-	190.97	16	110.7 (11)	86.5 (17)
3-BHB	ESI^-	102.53	18	58.3 (9)	40.2 (20)
Creatinine	ESI^+	113.65	19	43.4 (12)	85.6 (11)

[1]Collision energy (in volts) used to obtain each product ion is shown in parentheses. 3-BHB: β-hydroxybutyrate

A Supelco column (150 × 2.1 mm, 3 µm) and a mobile phase consisting of 1% formic acid and 10 mM ammonium formate dissolved in HPLC water (aqueous phase - A) and 1% formic acid in methanol (organic phase - B) resulted in the best peak resolution and signal intensity with an injection volume of 5 µL, as shown in Figure 1. The retention times for all compounds were less than three minutes. Initially, a mobile phase composition of 5% phase B (organic phase) was held for 1.0 minute (0–1 min), and was then increased to 35% phase B in 1.5 minutes (1.0–3.5 min). The proportion of phase B was then further increased to 95% in 0.5 minutes (3.5–4.0 min), and held constant for 1.5 minutes (4.0–5.5 min). It was then decreased back to 5% phase B within 0.5 minutes (5.5–6.0 min), and equilibrated for a further 7.0 minutes (6–13 min). Figure 1 shows the extracted chromatogram for the separation of citrate (2.33 µg/mL), 3-BHB (2.48 µg/mL), and creatinine (2.13 µg/mL) in milk sample number 13 collected during the hot season, where the retention time was 2.50 minutes in the column.

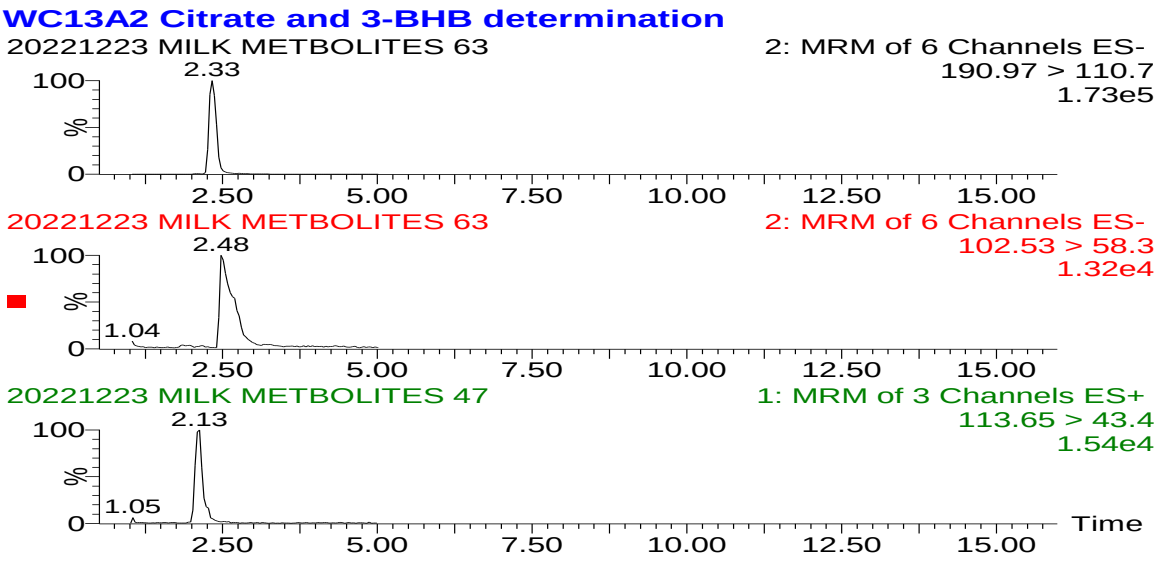


Figure 1 Extracted chromatograms for the separation of citrate, 3-BHB, and creatinine from a milk sample.

The filtration of the samples prior to HPLC analysis prevented the entry of unwanted particulate matter into the injector, and hence increased column life and optimised peak shape. However, filters release extractables into the sample and sometimes bind analytes. Therefore, the potential impact of filtration was evaluated based on analyte recovery. The average recovery percentages for the filtration step at high concentrations were 97% for creatinine, 93% for citrate, and 100% for 3-BHB. In contrast, the average recovery percentages at low concentrations were 106% for creatinine, 78%, for citrate, and 92% for 3-BHB (Figure 2).

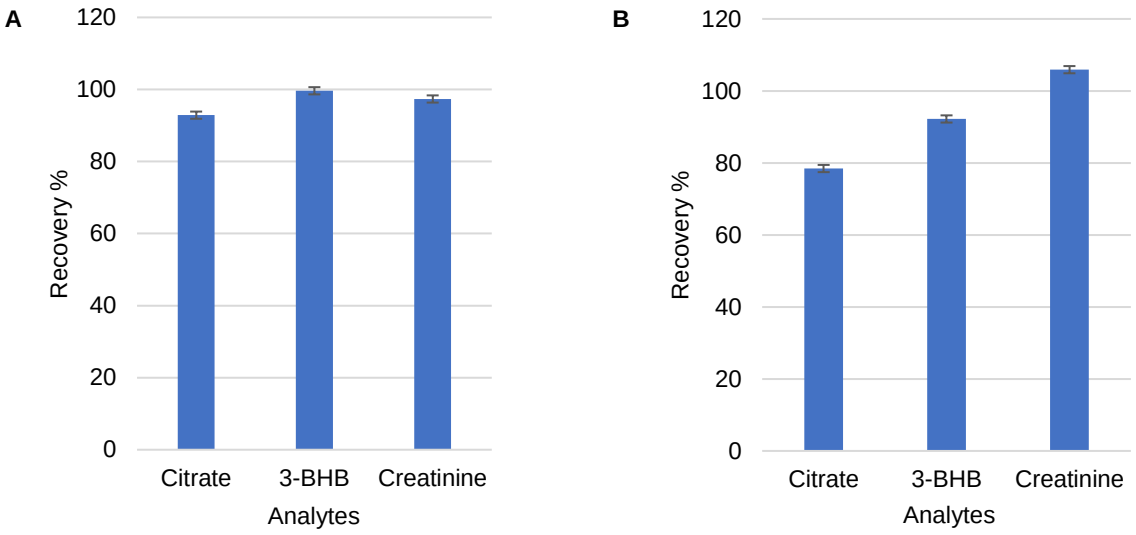


Figure 2 Recovery percentages for the filtration step at (A) high concentrations (40 ppm citrate, 20 ppm 3-BHB, and 0.4 ppm creatinine) and (B) low concentrations (10 ppm citrate, 5 ppm 3-BHB, and 0.1 ppm creatinine). 3-BHB: β-hydroxybutyrate.

Switching from a high proportion of organic solvent to a low proportion in which the analytes were extracted to a highly aqueous solvent was essential to obtain the best chromatographic separation. The effectiveness of the solvent exchange was evaluated based on the absolute recovery of the compounds. The average recovery percentages at high concentrations were 84% for creatinine, 107% for 3-BHB, and 99% for citrate. However, the average recovery percentages at low concentrations were 65% for creatinine, 80% for 3-BHB, and 75% for citrate (Figure 3).

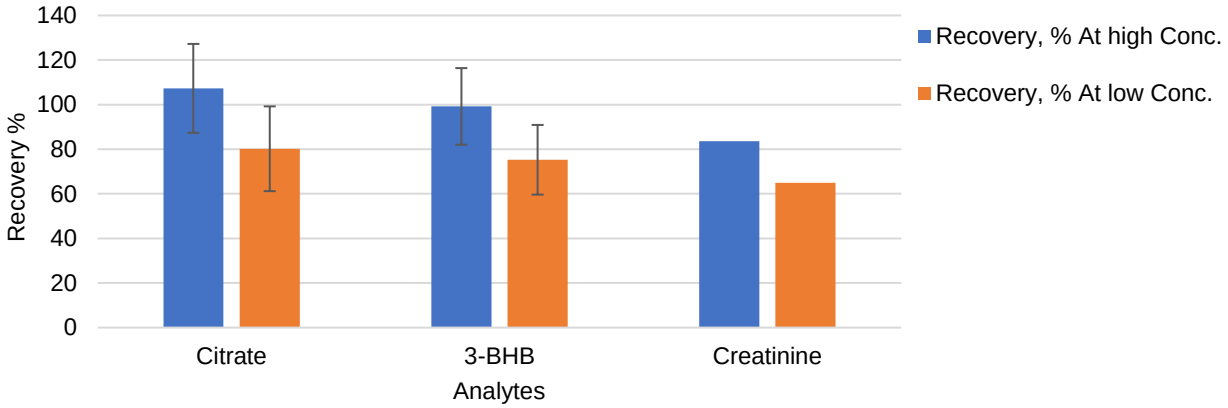


Figure 3 Influence of evaporation on recovery percentages at high concentrations (40 ppm citrate, 20 ppm 3-BHB, and 0.4 ppm creatinine) and at low concentrations (10 ppm citrate, 5 ppm 3-BHB, and 0.1 ppm creatinine). 3-BHB: β-hydroxybutyrate.

Pre- and post-spike recoveries were also used to assess the optimum conditions for the simultaneous extraction of analytes. The average recovery percentages at high concentrations were 96% for creatinine, 113% for citrate, and 77% for 3-BHB. However, the average recovery percentages at low concentrations were 94% for creatinine, 80% for citrate, and 80% for 3-BHB (Figure 4). These results showed that the developed method had good recovery rates and precision, and was able to meet the

requirements for the quantification of creatinine, citrate, and 3-BHB in raw milk samples from Holstein-Friesian cross-bred dairy cows.

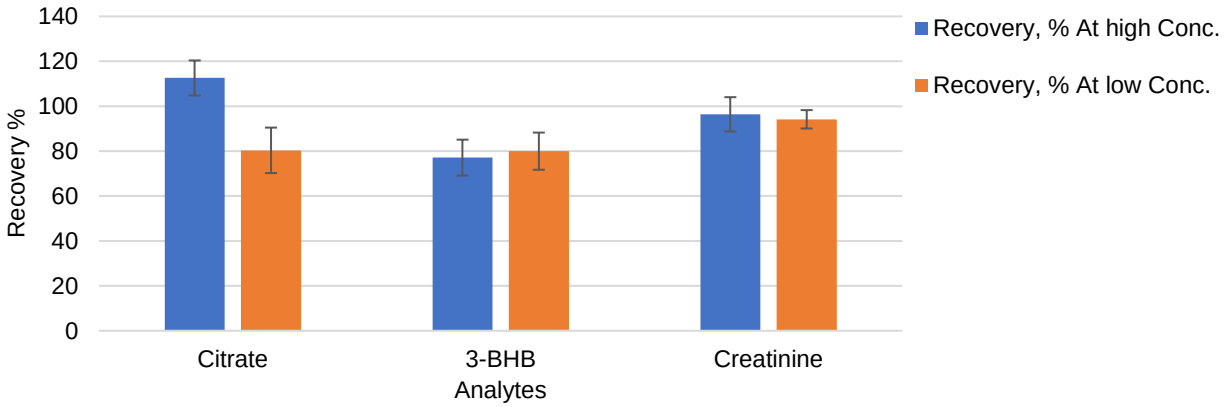
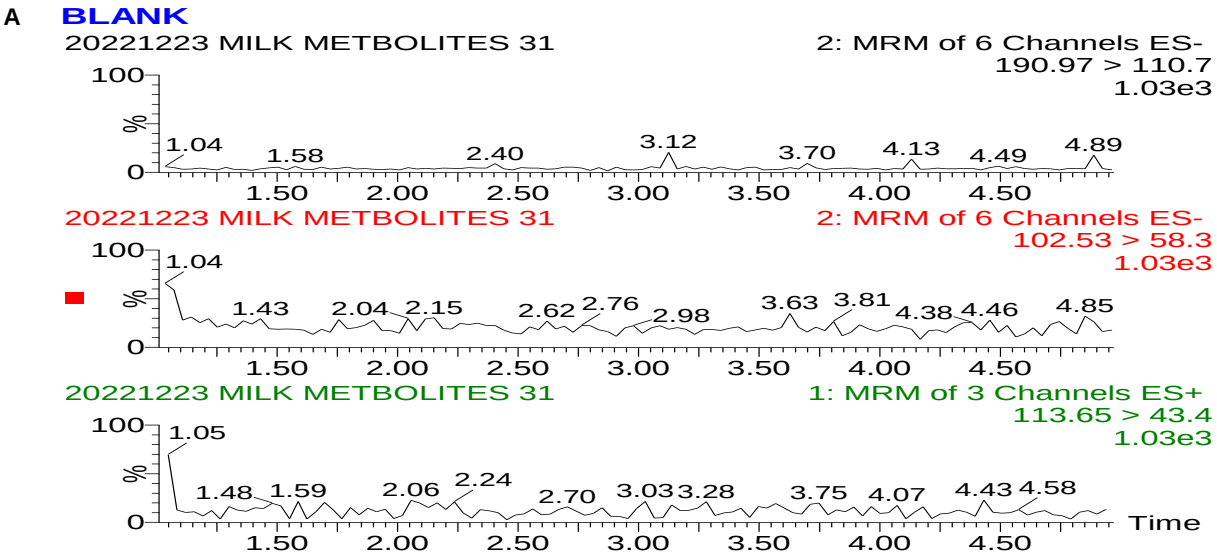


Figure 4 Spike recovery percentages for the extraction of the selected compounds at high concentrations (40 ppm citrate, 20 ppm 3-BHB, and 0.4 ppm creatinine) and at low concentrations (10 ppm citrate, 5 ppm 3-BHB, and 0.1 ppm creatinine). 3-BHB: β-hydroxybutyrate.

HPLC-MS/MS method validation

The specificity was determined by comparing the chromatograms obtained for the blank, standard, and test sample solutions (Figure 5). There was no clear separation of the analytes and identification was made possible by the MS. Briefly, the peak areas for the blank sample ranged from 1.04 µg/mL to 4.89 µg/mL for citrate, 1.04 µg/mL to 4.85 µg/mL for 3-BHB, and 1.05 µg/mL to 4.58 µg/mL for creatinine (Figure 5A). The peak areas for the standards were 2.30 µg/mL for citrate, 2.66 µg/mL for 3-BHB, and 2.06 µg/mL for creatinine (Figure 5B). In the test sample, the peak areas were 2.30 µg/mL for citrate, 1.07 µg/mL for 3-BHB, and 2.13 µg/mL for creatinine, with retention times in the column of 2.0 to 2.5 minutes (Figure 5C).



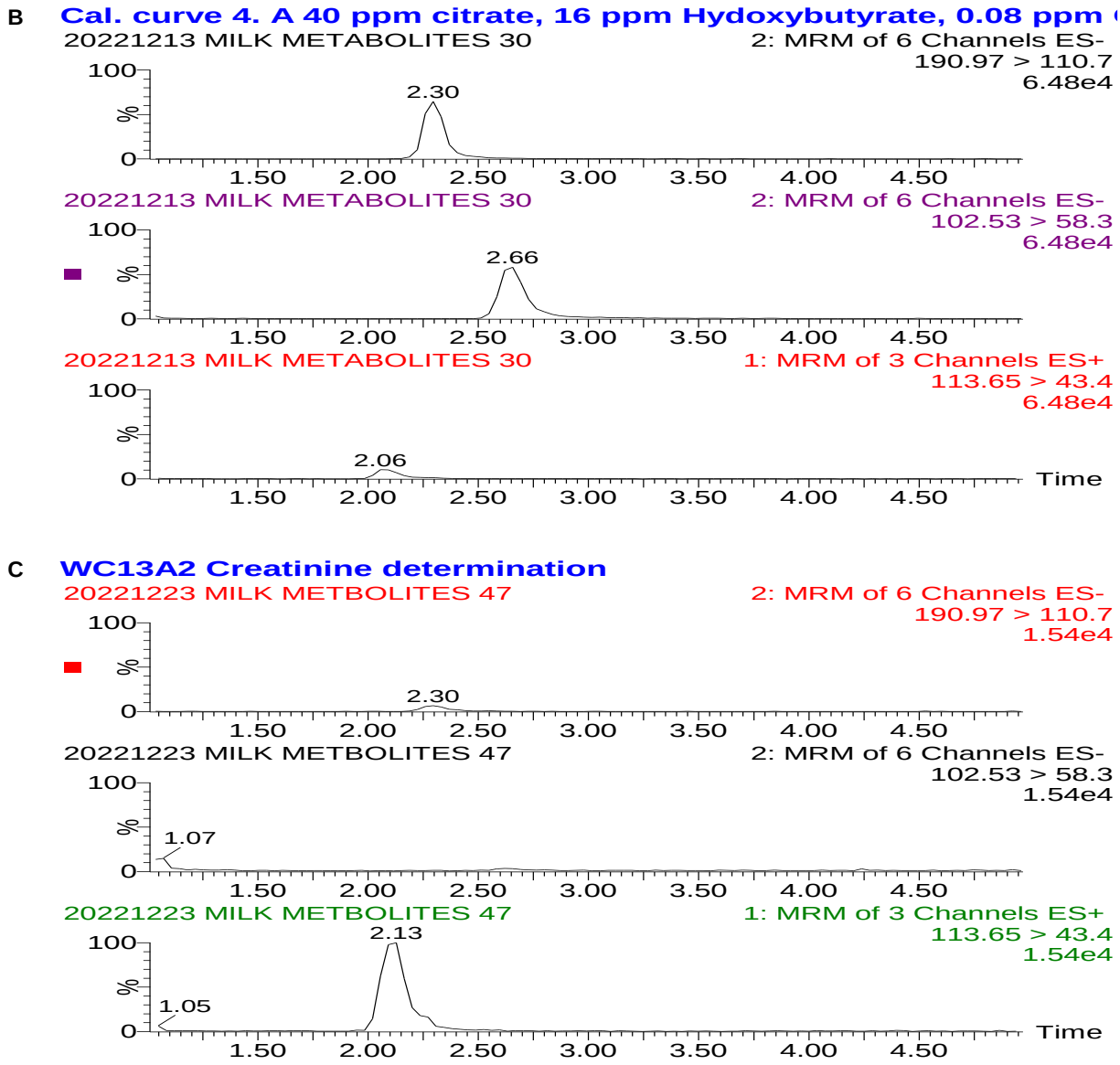


Figure 5 The specificity of the extracted chromatograms of (A) blank, (B) standard, and (C) test milk samples.

In this study, the calibration curves were linear (R^2 ≥0.995) over the range of 1.25–320 µg/mL for citrate, 0.25–128 µg/mL for 3-BHB, and 0.0025–0.64 µg/mL for creatinine. Additionally, the precision, expressed as the % RSD, ranged from 3.4% to 14% at low concentrations and from 3.2% to 10% at high concentrations. The per cent recovery of each analyte for the extraction of the selected compounds, obtained using the pre- and post-spiking recovery method as described, was 113% for citrate, 77% for 3-BHB, and 96% for creatinine at high concentrations, and 80% for citrate, 80% for 3-BHB, and 94% for creatinine at low concentrations.

The LOD and LOQ values obtained showed that all three milk metabolites analysed were detectable and quantifiable at the µg/mL level, as shown in Table 2. Briefly, the LOD values were 2.28 µg/mL for citrate, 1.72 µg/mL for 3-BHB, and 0.01 µg/mL for creatinine, while the LOQ values were 7.60 µg/mL for citrate,

5.73 µg/mL for 3-BHB, and 0.03 µg/mL for creatinine. For each metabolite, the slopes were significantly different from zero and the coefficient of determination (R^2) was greater than 0.99 (Table 2).

Table 2 Analytical method validation parameters, as indicated by the calibration equation, linearity (R^2), precision (expressed as the percentage relative standard deviation (% RSD)), limit of detection (LOD), and limit of quantification (LOQ)

Compound	Conc. range (µg/mL)	Slope	Intercept	R^2	%RSD		LOD (µg/mL)	LOQ (µg/mL)	Ret. time (min)
					Low conc.	High conc.			
Citrate	1.25–320	137.81440	−626.47601	0.9990	3.4	4.5	2.28	7.60	2.33
3-BHB	0.25–128	316.85761	257.13489	0.9966	4.1	3.2	1.72	5.73	2.48
Creatinine	0.0025–0.64	10264.66682	12.54313	0.9950	14.0	10.0	0.01	0.03	2.13

Conc.: concentration, Ret.: retention time; 3-BHB: β-hydroxybutyrate. Low conc.: citrate (5 µg/mL), 3-BHB (2 µg/mL), creatinine (0.01 µg/mL); high conc.: citrate (40 µg/mL), 3-BHB (16 µg/mL), creatinine (0.08 µg/mL).

Effects of the interactions between the THI and the genotype, parity, and month of lactation

Milk creatinine concentrations were significantly higher in the samples from the HF75 cows than the HF50 cows at a low THI. However, they were significantly higher for the HF50 cows than the HF75 cows at a high THI. Similar trends were observed for both citrate and 3-BHB, with higher concentrations found in the milk samples from the HF75 cows than the HF50 cows at a low THI, but higher concentrations in samples from the HF50 cows than the HF75 cows at a high THI (Table 3).

Table 3 Effects of the interactions between the temperature-humidity index (THI) and the genotype, parity, and month of lactation on the concentrations of three milk metabolites

Parameters		THI	Milk metabolites		
			Creatinine (µg/mL)	Citrate (µg/mL)	β-hydroxybutyrate (µg/mL)
Genotype	**HF50**	**77**	$7.63^{a} \pm 1.68$	$33.90^{a} \pm 7.44$	$8.79^{a} \pm 3.52$
		83	$5.67^{a} \pm 1.45$	$38.19^{b} \pm 6.41$	$8.00^{a} \pm 3.03$
	HF75	**77**	$9.53^{b} \pm 1.97$	$40.92^{b} \pm 8.73$	$16.44^{b} \pm 4.13$
		83	$4.69^{c} \pm 1.77$	$26.86^{c} \pm 7.81$	$4.17^{c} \pm 3.69$
		***P*-value**	<0.05	<0.05	<0.05
Parity	**2nd**	**77**	$8.25^{a} \pm 1.93$	$30.36^{a} \pm 8.50$	$12.15^{a} \pm 4.20$
		83	$5.38^{b} \pm 1.42$	$34.71^{b} \pm 6.24$	$6.79^{b} \pm 3.08$
	3rd	**77**	$8.60^{c} \pm 1.69$	$41.18^{b} \pm 7.45$	$12.05^{a} \pm 3.68$
		83	$5.36^{b} \pm 1.87$	$30.76^{c} \pm 8.25$	$7.22^{c} \pm 4.08$
		***P*-value**	<0.05	<0.05	<0.05
Month of lactation	**2nd**	**77**	$8.22^{a} \pm 1.41$	$41.92^{a} \pm 6.19$	$15.48^{a} \pm 3.06$
		83	$5.51^{b} \pm 1.32$	$34.68^{a} \pm 5.80$	$9.32^{b} \pm 2.87$
	3rd	**77**	$9.09^{c} \pm 2.52$	$25.32^{b} \pm 11.05$	$7.35^{c} \pm 5.46$
		83	$5.05^{d} \pm 1.99$	$36.46^{c} \pm 8.76$	$5.34^{c} \pm 4.33$
		***P*-value**	<0.05	<0.05	<0.05

$^{a–d}$ Means with different superscript letters in the same column within a parameter are significantly different (P <0.05). HF50: 50% Holstein-Friesian, HF75: 75% Holstein-Friesian (crossed with Tanzania Shorthorn Zebu cattle).

The milk creatinine concentration was significantly lower in the second parity than in the third at a low THI, but did not differ between the parities at a high THI. A similar trend was observed for the milk citrate concentration, which was lower in the second than the third parity at a low THI (P <0.0001). However, the citrate concentration also differed between the parities at a high THI, with the concentration being lower in the third parity than in the second under these conditions. In contrast, the milk 3-BHB concentration did not differ between the parities at a low THI, but was lower in the second parity than in the third at a high THI (Table 3).

Regarding the month of lactation, the milk creatinine concentration was significantly higher in samples from dairy cows in their third month of lactation than in samples from cows in their second month of lactation at a low THI. However, at a high THI, dairy cows in their second month of lactation had a higher milk creatinine concentration than those in their third month of lactation. The citrate concentration also showed a significant THI by month of lactation effect, but with contrasting trends to those found for creatinine. Dairy cows in their second month of lactation had a higher milk citrate concentration than those in their third month of lactation at a low THI, and the opposite effect was found at a high THI, with higher levels in third-month cows than second-month cows. The milk 3-BHB concentration showed yet another interaction effect, with significantly higher 3-BHB concentrations in cows in their second month of lactation than in those in their third month of lactation at both a low and a high THI (Table 3).

Effects of the THI, genotype, parity, and month of lactation

Considering the significance of the effects of the interactions between the THI and the genotype, parity, and month of lactation on milk metabolite concentrations, most of the main effects presented in Table 4 cannot be interpreted or discussed. However, it is notable that milk creatinine and 3-BHB concentrations consistently decreased as the THI increased, despite the effects of genotype, parity, and month of lactation. On average, the milk creatinine concentration decreased (P <0.0001) by 3.04 µg/mL when the THI increased from 77 to 83, and the milk 3-BHB concentration decreased (P <0.05) by 3.11 µg/mL when the THI increased from 77 to 83 (Table 4).

Table 4 Effects of the temperature-humidity index (THI), genotype, parity, and month of lactation on the concentrations of three milk metabolites

Parameters		Milk metabolites		
		Creatinine (µg/mL)	**Citrate (µg/mL)**	**β-hydroxybutyrate (µg/mL)**
THI	**77**	8.44 ± 1.27	36.54 ± 5.73	12.07 ± 2.77
	83	5.40 ± 1.13	33.93 ± 5.10	8.96 ± 2.47
	***P*-value**	<0.0001	<0.0001	<0.05
Genotype	**HF50**	6.81 ± 1.17	37.06 ± 5.26	9.03 ± 2.55
	HF75	7.03 ± 1.32	33.40 ± 5.93	10.00 ± 2.87
	***P*-value**	<0.0001	<0.0001	<0.05
Parity	**2nd**	6.84 ± 1.19	33.82 ± 5.36	9.43 ± 2.59
	3rd	7.00 ± 1.28	36.64 ± 5.74	9.61 ± 2.78
	***P*-value**	<0.0001	<0.0001	<0.05
Month of lactation	**2nd**	6.88 ± 0.96	38.02 ± 4.34	12.34 ± 2.10
	3rd	6.96 ± 1.52	32.45 ± 6.84	6.70 ± 3.31
	***P*-value**	<0.05	<0.0001	<0.05

HF50: 50% Holstein-Friesian, HF75: 75% Holstein-Friesian (crossed with Tanzania Shorthorn Zebu cattle).

Discussion

The development and validation of reliable analytical methods for milk metabolites, including creatinine, citrate, and 3-BHB, are essential for improving our understanding of the effects of HS, as measured using the THI, on the metabolic status of dairy cows. The present study describes a simple, rapid, and accurate HPLC-MS/MS method for the determination and quantification of these three important milk biomarkers for HS in lactating dairy cows. This method may be useful for the identification of lactating dairy cows that are physiologically imbalanced, and thus at risk of developing metabolic or infectious diseases. Furthermore, this study demonstrates the effects of HS on milk creatinine, citrate, and 3-BHB concentrations, as well as the effects of interactions between HS and genotype, parity, and month of lactation.

The developed and validated HPLC-MS/MS method for the quantification of creatinine, citrate, and 3-BHB in raw milk samples from Holstein-Friesian cross-bred dairy cows reared in the humid coastal region of Tanzania is simple, rapid, and accurate, and is specific for these three metabolites. Chromatographic separation was performed using an HPLC-MS/MS method with MRM, using a Supelco column (150 × 2.1 mm, 3 µm) and a mobile phase consisting of 1% formic acid and 10 mM ammonium formate dissolved in HPLC water, and 1% formic acid dissolved in methanol. This method allowed for both the detection and quantification of the targeted metabolites. The results showed that the run times for creatinine, citrate, and 3-BHB were within the run times observed in previous studies. Ou *et al*. (2015) used an LC-MS/MS method for the quantification of serum creatinine and found that the retention time ranged from 1.5 to 3.0 minutes, which is in agreement with the findings of this study. Izco *et al*. (2003) used capillary electrophoresis (CE) to optimise and validate a method for the determination and quantification of citrate and inorganic phosphate in milk, and reported a run time of 2.33 minutes for the separation of those compounds. This time of separation is in agreement with the findings of this study. In this study, the recoveries at high concentrations were high for creatinine, citrate, and 3-BHB, while the recoveries at low concentrations were low for creatinine, citrate, and 3-BHB. The recovery percentages obtained in this study are lower than those reported by Izco *et al*. (2003), who measured the organic acid concentration using CE and found that the recovery percentage for extraction with sulphuric acid was 100% in milk.

Nonetheless, the R^2-values for the regression lines for creatinine, citrate, and 3-BHB were remarkably high. This indicated that the responses for creatinine, citrate, and 3-BHB increased linearly with the concentrations of the standards added (Flores-Flores & González-Peñas, 2017). A similar validation coefficient after 999 random permutation tests yielded an R^2 of 0.291 in the study by Fan *et al*. (2018) and, thus, no significant interference of any compound could appear as a single peak in the chromatogram. In the study by Izco *et al.* (2003), the R^2 obtained for citrate using CE was 0.9967, which is in agreement with the findings of this study, although the determination and quantification methods used were different. This indicates the presence of various organic acids, such as formic acid, acetonitrile, acetic acid, or lactic acid, which are generally higher than the normal values observed in raw milk with citrate. The calculated concentrations of creatinine, citrate, and 3-BHB in the milk samples in the present study are within the range reported in other studies (Ou *et al*., 2015; Lamp *et al.*, 2015; Miranda *et al.*, 2020).

Once the HPLC-MS/MS method had been optimised and validated, the effects of the interactions between the THI and the genotype, parity, and month of lactation were assessed. Generally, under hot and humid weather conditions, high-yielding dairy cattle experience a NEB, as a result of their feed intake being insufficient to meet their energy requirements for milk production (Joo *et al.*, 2021). In this study, the milk creatinine concentration decreased as the THI increased in both the HF50 and the HF75 dairy cows. Moreover, the HF50 cows had lower milk creatinine concentrations than the HF75 cows at a low THI. Creatine and creatine phosphate metabolism produce creatinine as an end product, and this process is associated with energy balance when dairy cattle require the rapid provision of energy (Hu *et al.*, 2022). Therefore, the decrease in the milk creatinine concentration found with an increase in THI in this study suggests potential implications for creatinine phosphate metabolism in both the HF50 and HF75 cows in response to HS.

Milk citrate and 3-BHB are used as biomarkers of energy status in dairy cattle and are correlated with the presence of ketone bodies in the milk and the *de novo* synthesis of fatty acids (Tian *et al*., 2016). Chen *et al*. (2023) reported that citrate is a potential proxy for a NEB in dairy cattle, suggesting that the

genetic selection of dairy cattle with lower milk citrate levels could allow for the indirect selection of dairy cattle with a better energy balance at the beginning of lactation. Additionally, the genetic selection of lactating cows with lower milk citrate levels could potentially affect dairy cattle thermotolerance (Chen *et al*., 2023). Bjerre-Harpøth *et al*. (2012) identified milk citrate as a potential HS and physiological imbalance biomarker, which could be used for the monitoring of on-farm dairy cattle throughout lactation. This aligns with the findings of our study. Increases in milk 3-BHB and citrate concentrations reflect increases in the concentrations of these metabolites in the blood, and are indicative of excessive protein mobilisation and a reduced glucose supply to the mammary glands during milk synthesis (Tian *et al.*, 2016).

Regarding the effects of parity and THI on milk metabolite concentrations, a higher creatinine concentration was observed in the third-parity cows than in the second-parity cows at a low THI. This is in contrast with the findings of Wu *et al*. (2019), who reported that the serum creatinine concentration decreased from 62.5 mmol/L in second-parity cows to 57.2 mmol/L in third-parity cows. Moreover, in their study, the serum 3-BHB concentration also decreased, from 0.50 mmol/L in the second parity to 0.49 mmol/L in the third parity (Wu *et al*., 2019), which is also in contrast with the findings of this study. Nevertheless, the third-parity cows had higher milk 3-BHB concentrations than the second-parity cows at a high THI, which is in partial agreement with the findings of Kuczyńska *et al.* (2021) and Walter *et al*. (2022). Walter *et al*. (2022) reported that 3-BHB concentrations are always higher in multiparous cows than in primiparous cows. In our study, the milk citrate concentration was higher in the third-parity cows than in the second-parity cows at a low THI.

The month of lactation has also been found to effect milk metabolite concentrations (Walter *et al.*, 2022). In the present study, the examination of the month of lactation × THI interaction indicated that the milk creatinine concentration was significantly higher in cows in their third month of lactation than in those in their second month of lactation at a low THI. The opposite trend was observed at a high THI, with the creatinine concentration being higher in the cows in their second month of lactation than in those in their third month of lactation under these conditions. Moreover, in this study, the milk citrate concentration was higher in cows in their third month of lactation than in those in their second month of lactation at a high THI. These findings are in contrast with those of Garnsworthy *et al.* (2006), who observed higher milk citrate concentrations in dairy cattle in the early months of lactation than in dairy cattle in mid-lactation. Garnsworthy *et al.* (2006) also observed a daily decline in the milk citrate concentration as the months in lactation advanced, which is in partial agreement with the findings of this study for cows under low THI conditions. Furthermore, the finding that the milk citrate concentration decreased as the months in lactation increased at a low THI is consistent with the findings of Lim *et al*. (2020) in South Korea, who reported that the citrate concentration in milk from Holstein dairy cattle decreased from 6.08 mmol/L at three days in milk to 2.79 mmol/L at 30 days in milk. Moreover, milk 3-BHB concentrations were significantly higher in dairy cows in their second month of lactation than in those in their third month of lactation, at both low and high THI values. These findings concur with those of Van *et al.* (2020), who observed a decline in blood 3-BHB concentrations as the months in lactation increased, indicating extensive body reserve mobilisation during the early months of lactation. The decrease in the milk 3-BHB concentration observed in this study is also consistent with the findings of Sabek *et al*. (2021), who reported that days in milk significantly affected 3-BHB concentrations in cows in China. However, in their study, parity had no significant effect on the milk 3-BHB concentration (Sabek *et al.*, 2021), which is in contrast with the results of the present study. The findings of the current study are also in agreement with those of Lei & Simões (2021), who reported a decline in the milk 3-BHB concentration from the second to the third month of lactation.

Heat stress can also alter energy metabolism, as seen by the declines in the concentrations of creatinine and 3-BHB as the THI increased, despite the significant interaction effects. Similar findings were reported by Hu *et al.* (2022), in association with cold stress in Sanhe and Holstein cattle in China. β-hydroxybutyrate, a physiologically important ketone body, is generated from lipolysis in the liver mitochondria and can be used as source of energy during a period of low blood glucose levels (Yue *et al.*, 2020). The decreases in milk creatinine and 3-BHB concentrations in response to increases in the THI found in this study are in partial agreement with the findings of Wu *et al*. (2019) in China. The decrease in milk creatinine in this study is also in agreement with the findings of Joo *et al.* (2021), who observed a decline in blood creatinine, among other biochemical parameters, in response to HS in Holstein dairy cattle in South

Korea. Similar decreases in plasma 3-BHB concentrations due to HS were also reported by Yue *et al*. (2020), indicating that HS seriously impairs udder health. These findings are also consistent with those of Fan *et al.* (2019) on the effects of HS on milk metabolites, as they found that HS increased the concentration of glucose and galactose, but decreased the acetoacetate and 3-BHB concentrations. In contrast, Lamp *et al*. (2015) reported an increase in plasma creatinine and 3-BHB concentrations as a result of HS in dairy cattle in Germany, which contradicts the results of this study. Tian *et al*. (2016) similarly reported that HS increased blood 3-BHB (1.08–1.38, P <0.02) concentrations in dairy cattle, compared to a HS-free group of cows, providing a further contradiction to the results of our study. However, Safa *et al*. (2019) reported a significant decline in plasma glucose, NEFA, and 3-BHB concentrations in heat-stressed dairy cattle compared to dairy cattle under cool conditions in Iran, which concurs with the findings of our study. Yue *et al*. (2020) suggested that the lower milk and plasma 3-BHB concentrations found in heat-stressed dairy cattle was the result of the inhibition of the β-oxidation of fatty acids in dairy cattle experiencing HS.

Fan *et al*. (2019) used LC-MS/MS to investigate the effects of HS on milk composition traits and metabolites, and identified 34 milk metabolites that are potential indicators of HS in early lactating dairy cows. The identified metabolites included 3-BHB, citric acid, creatine, and creatinine. In an earlier study by Fan *et al*. (2018) on liver metabolic perturbations in heat-stressed lactating dairy cows, they identified 33 potential regulatory biomarkers of HS. These included creatine, citrate, creatinine, glucose, lactate, and acetoacetate, supporting the findings of our study. Fan *et al.* (2018) further suggested that these metabolites could be used to provide reliable information on the threshold of HS in dairy cattle, and may thus help in the genetic selection of dairy cattle genotypes and individual animals that are thermotolerant to an elevated THI. The present study confirmed that the selected milk metabolites (creatinine, citrate, and 3-BHB) are promising indicators of HS and metabolic status in lactating dairy cows. These milk metabolites, if combined with environmental parameters (such as ambient temperature, relative humidity, solar radiation, and THI), can provide accurate information about the HS thresholds of dairy cattle and may help in the genetic selection of dairy breeds that are heat-adaptive (Fan *et al.*, 2019).

The use of milk metabolites is advantageous because milk samples are easy to collect. Milk sampling is thus less invasive, requires minimal animal handling, and is well-suited for grazing dairy cows and behavioural studies as it does not require animal handling beyond standard management practices. Excessive handling can affect the research time budget and induce stress in cows (Pires *et al.*, 2022). Pires *et al.* (2022) recommended that standard milk sampling conditions need to be established and improved, in order for milk metabolites to be applied as HS biomarkers and metabolic status indicators for early stages of lactation in dairy cows.

Conclusions

The HPLC-MS/MS method developed in this study enabled the detailed analysis of metabolic profiles and the identification of three potential HS biomarkers in dairy cattle. The method used a Supelco column (150 × 2.1 mm, 3 µm), and a mobile phase consisting of 1% formic acid and 10 mM ammonium formate dissolved in HPLC water, and 1% formic acid dissolved in methanol. The method was found to be appropriate for the detection and quantification of creatinine, citrate, and 3-BHB metabolites and is highly advantageous over a wide analytical range. It can thus be used to diagnose dairy cattle with physiological imbalances caused by HS and metabolic diseases such as subclinical ketosis, hyperketonaemia, and a general NEB. Raw milk samples from lactating dairy cows in the early months of lactation can potentially be used to identify HS, but this will only be possible once appropriate threshold values for these metabolites have been determined. Furthermore, the significant effects of the interactions between the THI and the genotype, parity, and months in lactation on the concentrations of creatinine, citrate, and 3-BHB in the raw milk samples will have to be taken into account when determining these threshold values.

Further studies on other dairy cattle breeds reared in the warm and humid coastal regions of Tanzania or other countries with similar climatic conditions are needed to identify other HS-sensitive metabolites as potential biomarkers for HS using the developed HPLC-MS/MS method. The number of samples and farms should also be increased in future studies to maximise the included variation during the

calibration of this method. Other milk metabolites could also be analysed using the developed HPLC-MS/MS method, to further aid the determination of the physiological status of dairy cattle.

Acknowledgements

The authors acknowledge the assistance provided by Christopher Johnson Mwankuna of the Department of Chemistry and Physics, College of Natural and Applied Sciences, at SUA during the laboratory analyses and HPLC-MS/MS method development. The funders had no role in the study design, data collection and analysis, decision to publish, or preparation of the manuscript. The findings and conclusions of this study are those of the authors and do not necessarily represent the views of the funders.

Authors' contributions

Conceptualisation: VH, ASN, GM, and SWC; methodology: VH; formal analysis: VH, GM, and SWC; data curation: VH; writing (original draft preparations): VH; writing (review, editing, and supervision): ASN, ZCN, CC-ED, GM, RM, and SWC. All the authors have read and agreed to the published version of the manuscript.

Conflict of interest declaration

The authors have no conflicts of interest to declare.

Funding

This study was funded by the Regional Scholarship and Innovation Fund (RSIF) of the Partnership for Skills in Applied Sciences, Engineering, and Technology (PASET) grant (project grant number: P165581) to the SACIDS Africa Centre of Excellence for Infectious Diseases of Humans and Animals in Southern and East Africa (SACIDS-ACE) at the Sokoine University of Agriculture (SUA). VH is a recipient of an RSIF-PASET doctoral scholarship at SUA.

References

Bjerre-Harpøth, V., Friggens, N.C., Thorup, V.M., Larsen, T., Damgaard, B.M., Ingvartsen, K.L., & Moyes, K.M., 2012. Metabolic and production profiles of dairy cows in response to decreased nutrient density to increase physiological imbalance at different stages of lactation. *Journal of Dairy Science*, 95(5):2362–2380. DOI: 10.3168/jds.2011-4419

Bonfatti, V., Turner, S.A., Kuhn-Sherlock, B., Luke, T.D.W., Ho, P.N., Phyn, C.V.C., & Pryce, J.E., 2019. Prediction of blood β-hydroxybutyrate content and occurrence of hyperketonemia in early-lactation, pasture-grazed dairy cows using milk infrared spectra. *Journal of Dairy Science*, 102(7):6466–6476. DOI: 10.3168/jds.2018-15988

Cattaneo, L., Piccioli-Cappelli, F., Minuti, A., & Trevisi, E., 2023. Metabolic and physiological adaptations to first and second lactation in Holstein dairy cows. *Journal of Dairy Science*, 106(5):3559–3575. DOI: 10.3168/jds.2022-22684

Chen, Y., Hu, H., Atashi, H., Grelet, C., Wijnrocx, K., Lemal, P., & Gengler, N., 2023. Genetic analysis of milk citrate predicted by milk mid-infrared spectra of Holstein cows in early lactation. *Journal of Dairy Science*, 107(5):3047–3061. DOI: 10.3168/jds.2023-23903

Ekine-Dzivenu, C.C., Mrode, R., Oyieng, E., Komwihangilo, D., Lyatuu, E., Msuta, G., Ojango, J.M.K., & Okeyo, A.M., 2020. Evaluating the impact of heat stress as measured by temperature-humidity index (THI) on test-day milk yield of small holder dairy cattle in a sub-Sahara African climate. *Livestock Science*, 242:104314. DOI: 10.1016/j.livsci.2020.104314

Eom, J.S., Kim, E.T., Kim, H.S., Choi, Y.Y., Lee, S.J., Lee, S.S., Kim, S.H., & Lee, S.S., 2021. Metabolomics comparison of rumen fluid and milk in dairy cattle using proton nuclear magnetic resonance spectroscopy. *Animal Bioscience*, 34(2):213–222. DOI: 10.5713/ajas.20.0197

Eom, J.S., Park, D.S., Lee, S.J., Gu, B.H., Lee, S.J., Lee, S.S., Kim, S.H., Kim, B.W., Lee, S.S., & Kim, M., 2022. Metabolomic and transcriptomic study to understand changes in metabolic and immune responses in steers under heat stress. *Animal Nutrition*, 11:87–101. DOI: 10.1016/j.aninu.2022.06.012

Fan, C.Y., Su, D., Tian, H., Hu, R.T., Ran, L., Yang, Y., Su, Y.J, & Cheng, J.B., 2019. Milk production and composition and metabolic alterations in the mammary gland of heat-stressed lactating dairy cows. *Journal of Integrative Agriculture*, 18(12):2844–2853. DOI: 10.1016/S2095-3119(19)62834-0

Fan, C., Su, D., Tian, H., Li, X., Li, Y., Ran, L., Hu, R., & Cheng, J., 2018. Liver metabolic perturbations of heat-stressed lactating dairy cows. *Asian-Australasian Journal of Animal Sciences*, 31(8):1244–1251. DOI: 10.5713/ajas.17.0576

Flores-Flores, M.E. & González-Peñas, E., 2017. An LC – MS / MS method for multi-mycotoxin quantification in cow

milk. *Food Chemistry*, 218:378–385. DOI: 10.1016/j.foodchem.2016.09.101

Foldager, L., Gaillard, C., Sorensen, M.T., Larsen, T., Matthews, E., O'Flaherty, R., Carter, F., Crowe, M.A., Grelet, C., Salavati, M., Hostens, M., Ingvartsen, K.L., Krogh, M.A., McLoughlin, N., Fahey, A., Santoro, A., Byrne, C., Rudd, P., Hallinan, S., & Palma, V.S., 2020. Predicting physiological imbalance in Holstein dairy cows by three different sets of milk biomarkers. *Preventive Veterinary Medicine*, 179:105006. DOI: 10.1016/j.prevetmed.2020.105006

Gantner, V., Kuterovac, K., & Potočnik, K., 2016. Effect of heat stress on metabolic disorders prevalence risk and milk production in Holstein cows in Croatia. *Annals of Animal Science*, 16(2):451–461. DOI: 10.1515/aoas-2015-0097

Garnsworthy, P.C., Masson, L.L., Lock, A.L., & Mottram, T.T., 2006. Variation of milk citrate with stage of lactation and de novo fatty acid synthesis in dairy cows. *Journal of Dairy Science*, 89(5):1604–1612. DOI: 10.3168/jds.S0022-0302(06)72227-5

Giannuzzi, D., Mota, L.F.M., Pegolo, S., Tagliapietra, F., Schiavon, S., Gallo, L., Marsan, P.A., Trevisi, E., & Cecchinato, A., 2023. Prediction of detailed blood metabolic profile using milk infrared spectra and machine learning methods in dairy cattle. *Journal of Dairy Science*, 106(5):3321–3344. DOI: 10.3168/jds.2022-22454

Girma, M., van Knegsel, A.T.M., Heirbaut, S., Vandaele, L., Jing, X.P., Stefańska, B., & Fievez, V., 2023. Prediction of metabolic status of dairy cows in early lactation using milk fatty acids and test-day variables. *Journal of Dairy Science*, 106(6):4275–4290. DOI: 10.3168/jds.2022-22702

Grelet, C., Bastin, C., Gelé, M., Davière, J.B., Johan, M., Werner, A., Reding, R., Fernandez Pierna, J.A., Colinet, F. G., Dardenne, P., Gengler, N., Soyeurt, H., & Dehareng, F., 2016. Development of Fourier transform mid-infrared calibrations to predict acetone, β-hydroxybutyrate, and citrate contents in bovine milk through a European dairy network. *Journal of Dairy Science*, 99(6):4816–4825. DOI: 10.3168/jds.2015-10477

Grelet, C., Vanlierde, A., Hostens, M., Foldager, L., Salavati, M., Ingvartsen, K.L., Crowe, M., Sorensen, M.T., Froidmont, E., Ferris, C.P., Marchitelli, C., Becker, F., McLoughlin, N., Fahey, A., Matthews, E., Santoro, A., Byrne, C., Rudd, P., O'Flaherty, R., & Vera, S.P., 2019. Potential of milk mid-IR spectra to predict metabolic status of cows through blood components and an innovative clustering approach. *Animal*, 13(3):649–658. DOI: 10.1017/S1751731118001751

Habimana, V., Nguluma, A.S., Nziku, Z.C., Ekine-Dzivenu, C.C., Morota, G., Mrode, R., & Chenyambuga, S.W., 2024. Heat stress effects on physiological and milk yield traits of lactating Holstein Friesian crossbreds reared in Tanga region, Tanzania. *Animals*, 14(13):1914. DOI: 10.3390/ ani14131914

Harron, D.W.G., 2013. Technical requirements for registration of pharmaceuticals for human use: The ICH process. In: The Textbook of Pharmaceutical Medicine, 7th edition. Eds: Griffin, J.P., Posner, J., & Barker G.R., John Wiley & Sons, Ltd., West Sussex, UK. DOI: 10.1002/9781118532331.ch23

Hu, L., Brito, L.F., Zhang, H., Zhao, M., Liu, H., Chai, H., Wang, D., Wu, H., Cui, J., Liu, A., Xu, Q., & Wang, Y., 2022. Metabolome profiling of plasma reveals different metabolic responses to acute cold challenge between Inner-Mongolia Sanhe and Holstein cattle. *Journal of Dairy Science*, 105(11):9162–9178. DOI: 10.3168/jds.2022-21996

Izco, J.M., Tormo, M., Harris, A., Tong, P.S., & Jimenez-Flores, R., 2003. Optimization and validation of a rapid method to determine citrate and inorganic phosphate in milk by capillary electrophoresis. *Journal of Dairy Science*, 86(1):86–95. DOI: 10.3168/jds.S0022-0302(03)73587-5

Joo, S.S., Lee, S.J., Park, D.S., Kim, D.H., Gu, B.H., Park, Y.J., Rim, C.Y., Kim, M., & Kim, E.T., 2021. Changes in blood metabolites and immune cells in Holstein and Jersey dairy cows by heat stress. *Animals*, 11(4). DOI: 10.3390/ani11040974

Kim, H.S., Kim, E.T., Eom, J.S., Choi, Y.Y., Lee, S.J., Lee, S.S., Chung, C.D., & Lee, S.S., 2021. Exploration of metabolite profiles in the biofluids of dairy cows by proton nuclear magnetic resonance analysis. *PLoS ONE*, 16(1):1–18. DOI: 10.1371/journal.pone.0246290

Koster, J.D., Salavati, M., Grelet, C., Crowe, M.A., Matthews, E., Flaherty, R.O., Opsomer, G., Foldager, L., & Hostens, M., 2019. Prediction of metabolic clusters in early-lactation dairy cows using models based on milk biomarkers. *Journal of Dairy Science,* 102(3):2631–2644. DOI: 10.3168/jds.2018-15533

Krogh, M.A., Hostens, M., Salavati, M., Grelet, C., Sorensen, M.T., Wathes, D.C., Ferris, C.P., Marchitelli, C., Signorelli, F., Napolitano, F., Becker, F., Larsen, T., Matthews, E., Carter, F., Vanlierde, A., Opsomer, G., Gengler, N., Dehareng, F., Crowe, M.A., & Foldager, L., 2020. Between- and within-herd variation in blood and milk biomarkers in Holstein cows in early lactation. *Animal*, 14(5):1067–1075. DOI: 10.1017/S1751731119002659

Kuczyńska, B., Puppel, K., Gołębiewski, M., Wiśniewski, K., & Przysucha, T., 2021. Metabolic profile according to the parity and stage of lactation of high-performance Holstein-Friesian cows. *Animal Bioscience*, 34(4):575–583. DOI: 10.5713/ajas.20.0018

Lamp, O., Derno, M., Otten, W., Mielenz, M., & Nürnberg, G., 2015. Metabolic heat stress adaption in transition cows: differences in macronutrient oxidation between late-gestating and early-lactating German Holstein dairy cows. *PLoS ONE*, 10(5):1–24. DOI: 10.1371/journal.pone.0125264

Le, T.T., Poulsen, N.A., Kristiansen, G.H., & Larsen, L.B., 2020. Quantitative LC-MS/MS analysis of high-value milk

proteins in Danish Holstein cows. *Heliyon*, 6(9):e04620. DOI: 10.1016/j.heliyon.2020.e04620

Lei, M.A.C. & Simões, J., 2021. Milk beta-hydroxybutyrate and fat to protein ratio patterns during the first five months of lactation in holstein dairy cows presenting treated left displaced abomasum and other post-partum diseases. *Animals*, 11(3):1–11. DOI: 10.3390/ani11030816

Li, X., Li, Z., Xu, E., Chen, L., Feng, H., Chen, L., Deng, L., & Guo, D., 2019. Determination of lactoferrin in camel milk by ultrahigh-performance liquid chromatography-tandem mass spectrometry using an isotope-labeled winged peptide as internal standard. *Molecules*, 24(22). DOI: 10.3390/molecules24224199

Lim, D.H., Mayakrishnan, V., Lee, H.J., Ki, K.S., Kim, T. Il, & Kim, Y., 2020. A comparative study on milk composition of Jersey and Holstein dairy cows during the early lactation. *Journal of Animal Science and Technology*, 62(4):565–576. DOI: 10.5187/JAST.2020.62.4.565

Liu, Z., Ezernieks, V., Wang, J., Wanni Arachchillage, N., Garner, J.B., Wales, W.J., Cocks, B.G., & Rochfort, S., 2017. Heat stress in dairy cattle alters lipid composition of milk. *Scientific Reports*, 7(1):961. DOI: 10.1038/s41598-017-01120-9

Lou, W., Zhang, H., Luo, H., Chen, Z., Shi, R., Guo, X., Zou, Y., Liu, L., Brito, L., Guo, G., & Wang, Y., 2022. Genetic analyses of blood β -hydroxybutyrate predicted from milk infrared spectra and its association with longevity and female reproductive traits in Holstein cattle. *Journal of Dairy Science*, 105(4). DOI: 10.3168/jds.2021-20389

Magan, J.B., O'callaghan, T.F., Zheng, J., Zhang, L., Mandal, R., Hennessy, D., Fenelon, M.A., Wishart, D.S., Kelly, A.L., & McCarthy, N.A., 2019. Impact of bovine diet on metabolomic profile of skim milk and whey protein ingredients. *Metabolites*, 9(12). DOI: 10.3390/metabo9120305

Miranda, G., Bianchi, L., Krupova, Z., Trossat, P., & Martin, P., 2020. An improved LC–MS method to profile molecular diversity and quantify the six main bovine milk proteins, including genetic and splicing variants as well as post-translationally modified isoforms. *Food Chemistry: X*, 5:100080. DOI: 10.1016/j.fochx.2020.100080

Osei-Amponsah, R., Dunshea, F.R., Leury, B.J., Cheng, L., Cullen, B., Joy, A., Abhijith, A., Zhang, M.H., & Chauhan, S.S., 2020. Heat stress impacts on lactating cows grazing australian summer pastures on an automatic robotic dairy. *Animals*, 10(5). DOI: 10.3390/ani10050869

Ou, M., Song, Y., Li, S., Liu, G., Jia, J., Zhang, M., Zhang, H., & Yu, C., 2015. LC-MS/MS method for serum creatinine: Comparison with enzymatic method and Jaffe method. *PLoS ONE*, 10(7). DOI: 10.1371/journal.pone.0133912

Pires, J.A.A., Larsen, T., & Leroux, C., 2022. Milk metabolites and fatty acids as noninvasive biomarkers of metabolic status and energy balance in early-lactation cows. *Journal of Dairy Science*, 105(1):201–220. DOI: 10.3168/jds.2021-20465

Sabek, A., Li, C., Du, C., Nan, L., Ni, J., Elgazzar, E., Ma, Y., Salem, A.Z.M., & Zhang, S., 2021. Effects of parity and days in milk on milk composition in correlation with β-hydroxybutyrate in tropic dairy cows. *Tropical Animal Health and Production,* 53(2):4–11. DOI: 10.1007/s11250-021-02690-7

Safa, S., Kargar, S., Moghaddam, G.A., Ciliberti, M.G., & Caroprese, M., 2019. Heat stress abatement during the postpartum period: Effects on whole lactation milk yield, indicators of metabolic status, inflammatory cytokines, and biomarkers of the oxidative stress. *Journal of Animal Science*, 97(1):122–132. DOI: 10.1093/jas/sky408

Shi, W., Yuan, X., Cui, K., Li, H., Fu, P., & Rehman, S., 2021. LC-MS / MS based metabolomics reveal candidate biomarkers. *Animals*, 11(560). DOI: 10.3390/ani11020560

Tian, H., Zheng, N., Wang, W., Cheng, J., Li, S., Zhang, Y., & Wang, J., 2016. Integrated metabolomics study of the milk of heat-stressed lactating dairy cows. *Scientific Reports*, 6:24208. DOI:10.1038/srep24208

Turk, R., Rošić, N., Vince, S., Perkov, S., Samardžija, M., Beer-Ljubić, B., Belić, M., & Robić, M., 2020. The influence of heat stress on energy metabolism in simmental dairy cows during the periparturient period. *Veterinarski Arhiv*, 90(1):1–10. DOI: 10.24099/vet.arhiv.0856

Van, Q.C.D., Knapp, E., Hornick, J.L., & Dufrasne, I., 2020. Influence of days in milk and parity on milk and blood fatty acid concentrations, blood metabolites and hormones in early lactation Holstein cows. *Animals*, 10(2081):1–13. DOI: 10.3390/ani10112081

Walter, L.L., Gärtner, T., Gernand, E., Wehrend, A., & Donat, K., 2022. Effects of parity and stage of lactation on trend and variability of metabolic markers in dairy cows. *Animals*, 12(8). DOI: 10.3390/ani12081008

Wang, Z., Liu, L., Pang, F., Zheng, Z., Teng, Z., Miao, T., Fu, T., Rushdi, H.E., Yang, L., Gao, T., Lin, F., & Liu, S., 2022. Novel insights into heat tolerance using metabolomic and high-throughput sequencing analysis in dairy cow's rumen fluid. *Animal*, 16(3):100478. DOI: 10.1016/j.animal.2022.100478

Wu, X., Sun, H.Z., Xue, M., Wang, D., Guan, L., & Liu, J., 2019. Days-in-milk and parity affected serum biochemical parameters and hormone profiles in mid-lactation Holstein cows. *Animals*, 9(5):230. DOI: 10.3390/ani9050230

Yue, S., Ding, S., Zhou, J., Yang, C., Hu, X., Zhao, X., Wang, Z., Wang, L., Peng, Q., & Xue, B., 2020. Metabolomics approach explore diagnostic biomarkers and metabolic changes in heat-stressed dairy cows. *Animals*, 10(10):1741. DOI: 10.3390/ani10101741

Zhang, H., Wu, L., Xu, C., Xia, C., Sun, L., & Shu, S., 2013. Plasma metabolomic profiling of dairy cows affected with ketosis using gas chromatography / mass spectrometry. *BMC Veterinary Research,* 9:186.