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# Effect of processing technologies on sugar profile of milk using UHPLC and milk protein analysis using gel electrophoresis

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## Abstract
Innovative processing technologies can sometimes modify the nutritional matrix of milk, thus leading to low bioavailability. It is essential that we moderate the processing parameters so that it does not have any degradable effect and also protect the milk from pathogenic microorganisms. Therefore, the aim of this study was to determine the changes in sugar content of raw milk samples when subjected to different treatments using UHPLC (Ultra High-Performance Liquid Chromatography) and comparative study of casein subunits of LTLT, US, UV treated and raw milk samples using SDS-PAGE was performed. SDS-PAGE technique is used to separate the milk proteins based on their molecular weight. Three varieties of cows were chosen that were locally available in southern part of India for collecting raw milk- Country (C), Jersey (J), and Holstein Friesian (HF). Further these raw milks were subjected to various thermal and non-thermal treatments. For thermal treatment the traditional low-temperature long-time (LTLT), was chosen and ultrasound (US), and ultraviolet light (UV) were selected under non thermal treatments. The sugar content changes incurred by milk when subjected to the three treatments were compared with untreated milk (control). The results demonstrate that the non-thermal treatments (US and UV) and thermal treatments (LTLT) have the potential to be used in the dairy industry and were successful in maintaining the sugar properties as well quality of milk.

**Keywords:** Sugar profiling, ultraviolet light, Ultra-sonification, UHPLC, SDS-PAGE

## Introduction
Since long back milk has been recognised as an excellent source of nourishment for all human, irrespective of age. The compounds necessary for sustaining and constructing the living body are present in balanced manner. Water makes up about 87 percent of milk's makeup, and nutrients like proteins, carbs, lipids, minerals, and vitamins make up the remaining 13 percent. It is a great source of vitamins, minerals, and high-quality protein. Vital amino acids essential for human nutrition all exist in milk. A great source of calcium and phosphorus for the development of bones and teeth is milk. The body can benefit from milk, which is the most nutrient-dense food, for growth, reproduction, energy, maintenance, and repairs. Alternative to mammal milk, plant-based milk is currently gaining popularity. This may be due to increased cases of lactose intolerance. Basava Prasad *et al*., (2022) [2] extracted milk from palmyra haustorium and further utilised it for making cake powder. Additionally, because it has a variety of qualities, it may be easily transformed into various milk products and used as a component in other food products. Proteins, carbs, fats, water, all the B vitamins, vitamins D and A, phosphorus, and calcium are among the nutrients found in milk that are necessary for human health (Ebing, 2006; Millogo *et al*., 2015) [7, 16].
In addition to milk, several dairy products have been manufactured and consumed for thousands of years, including cream, butter, yoghurt, kefir, and cheese. As a result, it is important to determine the effect of milk and milk products on human health and have the focus of research among scholars. Research on the possible health benefits of milk's fat fraction, which is primarily made up of SFAs, as well as some of its minor components, such as calcium and oligosaccharides, is ongoing (Visioli & Strata, 2014) [21].
On the other side, due to its neutral pH, large water content, and chemical composition, milk is also a perfect growth medium for numerous bacteria, including diseases. Heat treatment so enables spoilage bacteria inactivation and denaturation of enzymes to simply improve the quality and dairy products shelf life extension, taking into account the hazards to public health

connected with consuming raw milk (De Jong, 2008; Deeth & Lewis, 2017) [4]. Milk components may experience variable degrees of chemical and enzymatic change depending on the heat treatment settings (Bezie, 2019; Gathercole *et al*., 2017) [3, 9]. The use of thermal pasteurisation has historically been used to drastically reduce the amount of microorganisms in processed milk so that consuming it provides no health risks (Smith *et al*., 2002) [19]. Pasteurization is another crucial processing step that extends the shelf life of a product. Numerous research have measured the significance of pasteurisation and the heating effect on the organoleptic quality and nutritional attributes of milk (Walstra *et al*., 1984) [22]. Decrease in pH, calcium phosphate partial precipitation, whey protein denaturation and casein interaction with other sub components, lactose isomerization, Maillard reaction, and the casein micelle modification are the most significant modifications brought on by heat treatment of milk. In addition to making the milk safe to consume and lengthening its shelf life, the results also include changes that are linked to the sensory qualities of the treated milk, such as appearance, colour, flavour, and texture, as well as small modifications to its nutritional content.

Alternative to thermal treatment, shelf-life extension methods (ESL), like ultraviolet (UV) light, are currently gaining popularity (Donaghy *et al*., 2009; Keyser *et al*., 2008) [6, 14]. UVC germicidal wavelengths between 200 and 280 nm successfully lower microbe counts in water, fresh juices, and on surfaces that come into touch with both non-food items and food (Basaran *et al*., 2004; Hanes *et al*., 2002) [1, 11]. Koutchma (2009) asserts that UV treatment has a number of advantages over conventional thermal methods, including lower initial investment, low production and maintenance expenses, and much lower carbon emissions. Non-thermal technologies have come to light as a result of the hunt for such alternate techniques to prevent nutrient or flavour changes in food during production. Ultrasound is described as high frequency sound waves, over the threshold power of human hearing (20 kHz). Ultrasound creates cycles of compression and expansion (rarefaction) in the medium by producing alternating high- and low-pressures.

Casein represents about 75-80% of the milk protein (Fox *et al*., 2015) [8] and when it subjected to various processing technologies, it may interact with other protein moieties to form complexes. SDS-PAGE is a gel electrophoresis technique usually employed for studying the milk proteins. The electrophoresis is performed using a acrylamide gel, that acts as a sieve in the separation process.

Although the influence of thermal and non-thermal treatment on macronutrients of milk has been studied, especially for stability of dairy products, investigation on effect of various treatments on micronutrients such as sugar content is limited. This study presents the evaluation of impact of thermal and non-thermal treatments on sugar content of bovine milk and further comparative study between (LTLT, US, UV) treated and raw milk casein samples is done on SDS-PAGE.

## Materials and Methods

### Milk samples

For the LTLT (L), US (U), and UV (V) studies, raw milk from Jersey (J), Country (C), and Holstein Friesian (HF) cows was purchased from a milk society in Thanjavur (Tamil Nadu, India).

### Low Temperature Long Time (LTLT) Treatment

The temperature of the Igene Labserve (New Delhi, India) digital water bath was set to 65 °C, and closed containers containing raw milk from all three kinds were maintained inside (Guneser & Karagul Yuceer, 2012) [10]. The milk was held for 30 minutes and further cooled down to 4 °C using an ice bath.

### Ultra sonication (US) Treatment

40 millilitres of raw milk were processed in the US chamber. The sample was processed using a 200W, 26kHz UP200St Ultrasonic Processor (Hielscher, Germany) and an autoclavable titanium sonotrode. It comprises of a separate transducer (UP200St-T) and a generator (UP200St-G). All of the samples were subjected to pulsation (5 sec on and 2 sec off) at 60% amplitude for 10 minutes before being cooled in an ice bath to 4 °C.

### Ultraviolet Light (UV) Treatment

The raw milk samples were exposed to UV light using a UV-C lamp. A predefined volume of sample (20 ml) was collected in a glass plate, and it was left there for 30 minutes at a fixed distance from the UV-C lamp. A further ice bath is used to lower the sample's temperature to 4 °C.

### Extraction of sugar from milk

Bovine milk was diluted with 1.5 millilitres of distilled water before an incubation period of 60 °C for 10 minutes. Further, 0.25 mL of the 500 mM potassium ferrocyanide (aqueous)-containing Carrez I solution, 1 mL of acetonitrile, and 0.25 mL of the Carrez II solution were added. After being gently combined, the mixture was kept at room temperature unattended for one hour. By centrifuging the precipitate (10000 g, 8 min, 20 °C), the precipitate was obtained and discarded. The final cleared supernatant was fed into the UHPLC apparatus for analysis after filtering (Sharma *et al*., 2009) [18].

### Sugar profiling of milk samples by UHPLC (Ultra High-Performance Liquid Chromatography)

UHPLC (Agilent 1290 Infinity II, USA) with RI detector (Agilent Refractive Index -1260 Infinity II) equipped with column Zorbax (4.6 x 250mm x 5 mm) and guard Column Zorbax (4.6 x 12.5mm x 5 mm) was used for sugar profiling of all samples.

### Chemicals

Prestained protein ladder (10 to 245 kDa), acrylamide-bisacrylamide solution (29:1), laemmli loading buffer (sample buffer), ammonium persulphate (APS), sodium lauryl sulphate (SDS), Tetramethylethylenediamine (TEMED), β-mercaptoethanol, Coomassie Brilliant Blue R250 are procured from HiMedia Laboratories Pvt Ltd (Maharashtra, India)

### Preparation of caseins

1 M HCL was used for precipitating the casein from milk (Swaisgood, 1992) [20]. Obtained casein was kept at −30 °C for further analysis.

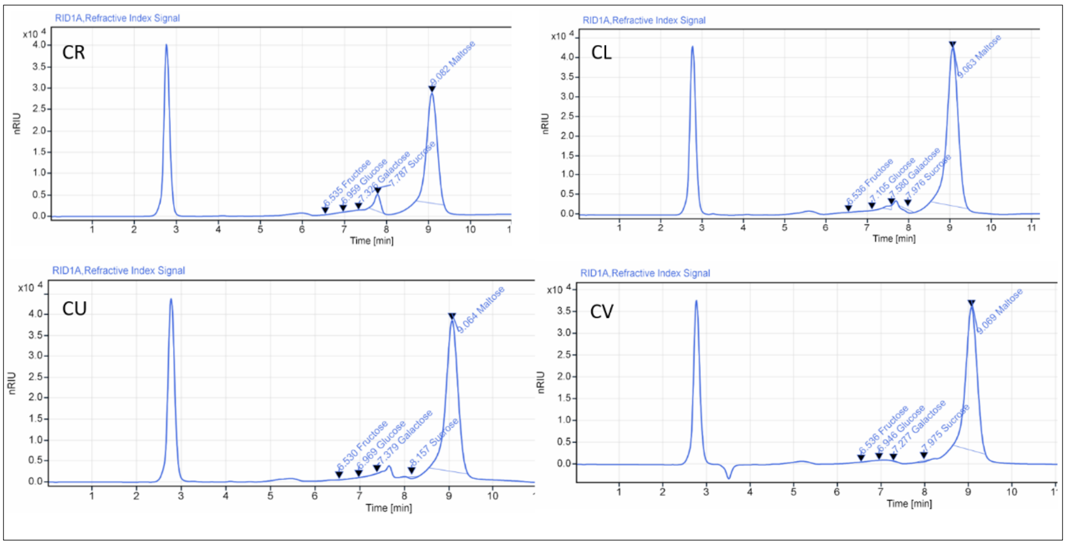
### Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE was used to study the casein profile of treated and untreated samples. Samples were analysed in vertical electrophoresis from BR Biochem Life Sciences Pvt. Ltd, New Delhi, India. Sample were diluted with Laemmli sample loading buffer (40µl), molecular biology grade water (150µl), and β-mercaptoethanol (10µl) and kept at 95 °C for 5 minutes in a water bath. At 60V the proteins were separated on 12% (v/v) resolving gel and 120V was utilised for the 4% (v/v) stacking the gel (Jin *et al.*, 2016) [13]. The proteins were stained using Coomassie Brilliant Blue R250 and de-stained using glacial acetic acid. Finally, the gel was scanned in gel documentation system (Azure Biosystems, Dublin, California).

## Results and Discussion

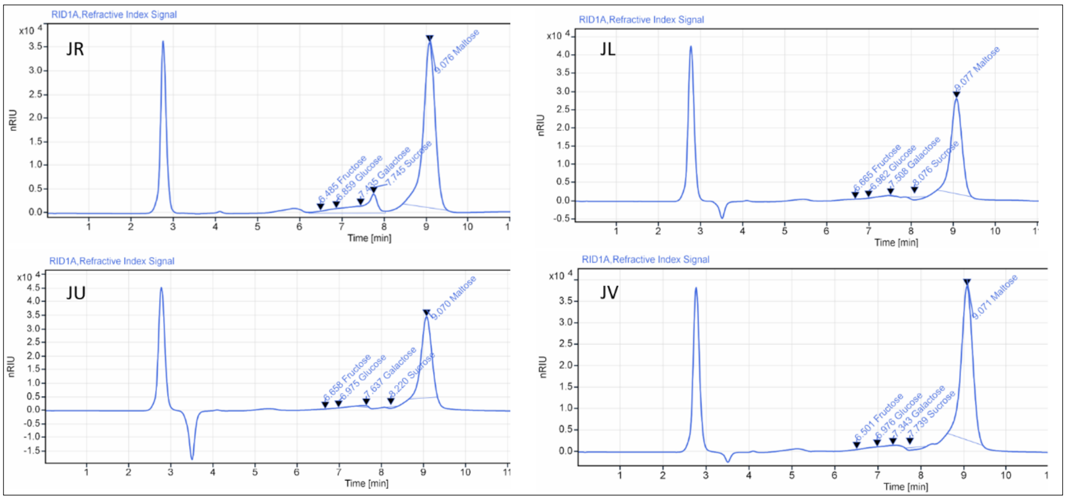
### Sugar profiling of milk samples by UHPLC (Ultra High-Performance Liquid Chromatography)

UHPLC–RI detection was used to determine galactose, sucrose, glucose, and fructose. Chromatograms of milk samples (Fig 1, Fig 2, and Fig 3) showed slight variations when subjected to the processing techniques. Maltose that is found to be higher in concentration consists of two glucose molecules that are joined by α-(1, 4) glycosidic bond. Lactose, the main carbohydrate present in milk sample is formed by linking a galactose and a glucose molecule. Both the monosaccharides were abundant in the milk samples and showed not much differences when subjected to various treatments. The Carrez solutions was used for precipitation of compounds. This simple and reproducible UHPLC–RI method was used to distinguish and evaluate the free sugar content. This technique is appropriate for routine analysis of mono- and disaccharides in milk and milk-based products in order to track the evolution of the compounds and check for probable adulteration and stability in the formulae's sugar fraction. The approach offers respectable recovery, sensitivity, and precision. Hurst *et al.*, (1979) [12] performed high performance liquid chromatography (HPLC) to characterise and quantify the simple sugars (lactose, maltose, glucose, sucrose, and fructose) present in a wide-ranging food item, both processed and fresh. Richmond *et al.*, (1982) [17] determined the carbohydrates commonly present in dairy products such as yoghurt and cultured butter milk. These results in line with these findings.



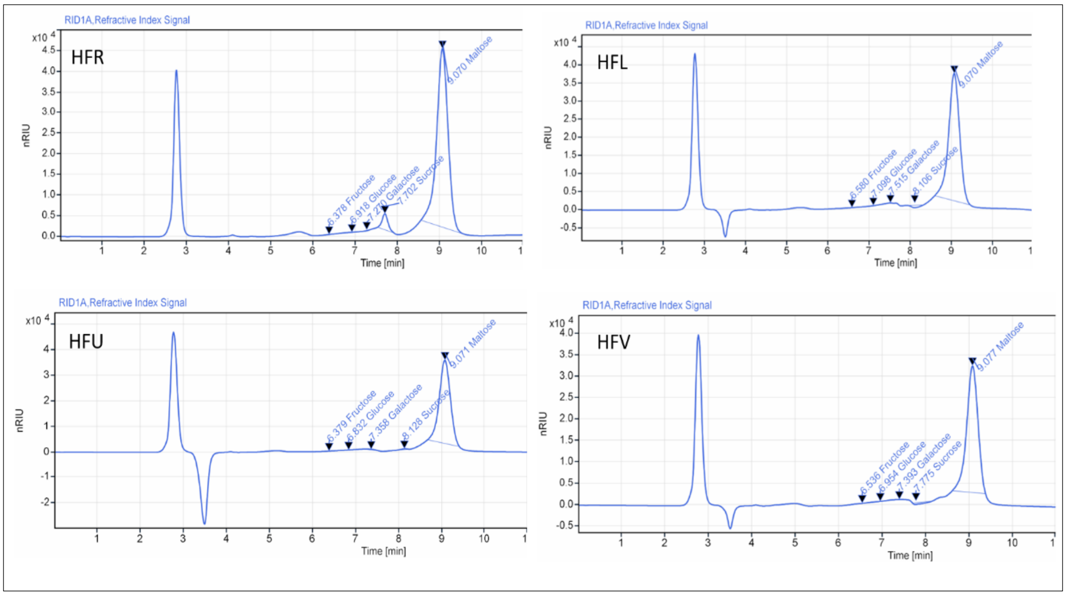

*Raw milk (CR), LTLT milk (CL), Ultra sonification milk (CU), Ultra violet light milk (CV)

**Fig 1:** Sugar profiling of country milk.




*Raw milk (JR), LTLT milk (JL), Ultra sonification milk (JU), Ultra violet light milk (JV)

**Fig 2:** Sugar profiling of raw and treated jersey milk

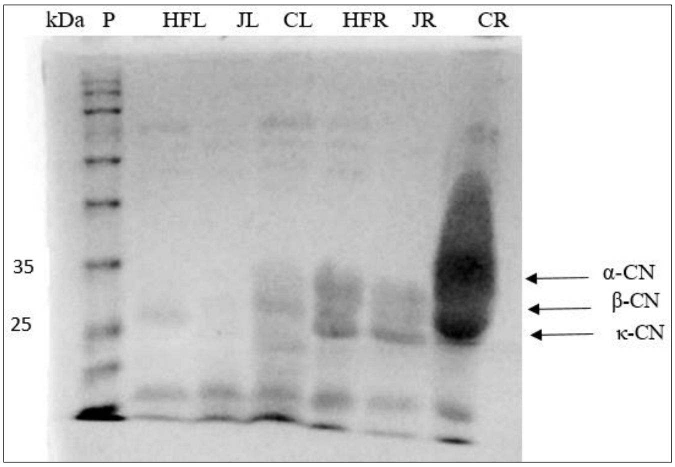



*Raw milk (HFR), LTLT milk (HFL), Ultra sonification milk (HFU), Ultra violet light milk (HFV)

**Fig 3:** Sugar profiling of raw and treated HF milk

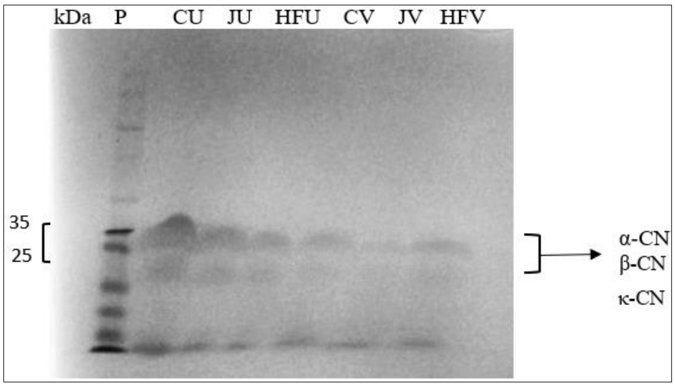
**Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)**

The casein of milk was analysed in SDS-PAGE for understanding the effect of heat treatment on subunits of casein and the results were depicted in in Fig 4 and Fig 5. Results showed that the three main components of casein protein including α-casein, β-casein, κ-casein were visualized in gel and were distributed between 25 and 35 kDa. The first three slots depict milk treated with LTLT -CL, JL, HFL and the last three slots are of raw milk samples- CR, JR, HFR. Based on the findings, it was found that the protein bands get fainter as the processing temperature rises. The casein subunits were unstable when the temperature was raised to 63 °C (held for 30 minutes), resulting in weak bands. Miao *et al*., 2020 [15] also observed similar faint protein lines when the donkey milk was subjected to higher temperature. Further research revealed that the temperature had a detrimental impact on the donkey milk supernatant fraction. In the second gel, the first three slots depict milk treated with US -CU, JU, HFU and the last three slots are of UV treated milk samples- CV, JV, HFV. The lines got fainter for ultraviolet treated milk samples.




P: Protein marker, CN: casein. Raw samples: CR, JR, HFR. LTLT treated milk samples: CL, JL, HFL

**Fig 4:** SDS-PAGE analysis of raw and LTLT treated milk samples




P: Protein marker, CN: casein. UV treated samples: CV, JV, HFV. US treated milk samples: CU, JU, HFU

**Fig 5:** SDS-PAGE analysis of UV and US treated milk samples

**Conclusion**

Production of dairy products is crucial for both economic development and life support. The macro- and micronutrients in milk have been thoroughly investigated, and the results are presented. The nutritional benefits of milk must not be sacrificed in order to use treatments that satisfy the criteria needed for safe and secure milk consumption. In this investigation, we found that low temperature long time (LTLT), ultra sonication, and ultraviolet (UV) treatments had no discernible effects on sugar content but slight variations were observed in the SDS-PAGE analysis of treated milk samples as compared to raw milk samples. It was found that when milk was subjected to heat treatment, the casein sub units may form complexes with other moieties present in the milk system and thus giving a fainter line in the SDS-PAGE gel. To expand the scope of the investigation into these effects, milk samples from three breeds-Country, Jersey, and Holstein Friesian-were selected.

**Compliance of Ethical standards**

The current article does not contain studies on animal or human subjects.

**Conflict of Interest**

The authors confirm no conflict of interest.

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