CHAPTER 23

Combined Assays for Lactose and Galactose by Enzymatic Reactions

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23.1 Introduction

Lactose (milk sugar) is a disaccharide that is found primarily in the milk of various mammals and milk products (Linko 1982). It is formed from the condensation of two monosaccharides, galactose and glucose, which form a β-1→4 glycosidic (covalent) linkage.

The systematic name of lactose is β-D-galactopyranosyl-(1→4)-D-glucose. The glucose can be in either the α-pyranose form or the β-pyranose form, whereas the galactose can only have the β-pyranose form: hence α-lactose and β-lactose refer to anomeric form of the glucopyranose ring alone.

Lactose concentration in the milk of various mammals range 4 to 9%. Human milk has the highest lactose percentage at around 9%. Cow’s milk has ~5% lactose and similar values are found in buffalo, yak and sheep milk, whereas those in goat’s milk (4.4–4.7%) are somewhat lower, but are not sufficiently low to prevent the problem of lactose intolerance (Silanikove et al. 2010).
Galactose (derived from the Greek word *galaktos*, which means milk) occurs in milk, plants and microbiological products (Sunehag *et al.* 2002). Milk galactose is less sweet than glucose. However, it is considered a nutritive sweetener because it has food energy. Galactose exists in both open-chain and cyclic form. The open-chain form has a carbonyl at the end of the chain. Four isomers are cyclic; two of them with a pyranose (six-membered) ring are predominant in eukaryotes, and two with a furanose (five-membered) ring occurs in bacteria, fungi and protozoa.

### 23.2 Methodology for the Determination of Lactose and Galactose

Lactose and galactose concentrations, and in fact most organic components in nature, can be determined in biological fluids and food samples by means of HPLC, paper chromatography or gas chromatography, and those are considered in different chapters in this book. These techniques are valuable, particularly in situations where many metabolites can be analyzed simultaneously. However, these techniques typically require either expensive chemicals or instrumentation, are time-consuming and sometimes may lack appropriate sensitivity (Kleyn 1985).

Analysis of lactose and galactose concentration by enzymatic reactions coupled to spectrometric detection of the reaction products is simple, specific and inexpensive (Kleyn 1985). This chapter is associated with the presentation of its principle, recent improvements that make it more effective and of unique advantageous associated with the enzymatic detection in testing dairy foods.

### 23.3 Principles of the Simultaneous Determination of Lactose and Galactose in Dairy Products by Enzymatic Reactions

Milk lactose is hydrolysed into \(\beta\)-D-glucose and \(\beta\)-D-galactose in infant mammals’ small intestines by enzyme called lactase (\(\beta\)-D-galactosidase; \(\beta\)-D-galactoside galactohydrolase, EC 3.2.1.23), which is secreted by the villi of epithelial cells. Splitting of lactose by lactase in a similar manner to the way it occurred in the intestine forms also the first step in the enzymatic determination of lactose (Reaction [1]). Lactase enzymes similar to those produced in the small intestines of humans are produced industrially by fungi of the genus *Aspergillus*, and are available at much lower price (Seyis and Aksoz 2004). Thus, industrially produced enzymes are mostly applied in the enzymatic determination of lactose (Kleyn 1985; Shapiro and Silanikove, 2010, Shapiro *et al.* 2002). Another important factor is the need to use \(\beta\)-lactose as a standard; lactose isolated from cow’s milk most likely would fit for that.
Most organisms metabolize galactose to glucose 6-P by the Leloir pathway (Frey 1996). However, few species of bacteria, most notably *Pseudomonas saccharophila* and *Pseudomonas fluorescens* have been shown to possess a unique enzyme pathway, called the DeLey-Doudoroff pathway (Wong and Yao 1994). The first reaction in this pathway converts the pyridine nucleotides coenzyme, nicotinamide adenine dinucleotide, commonly abbreviated NAD, into its reduced forms NADH in direct proportion to oxidation of galactose to D-galactono-\(\gamma\)-lactone (D-galactono-1,4-lactone) in the presence of \(\beta\)-galactose dehydrogenase (systemic name, D-galactose:NAD\(^+\) 1-oxidoreductase; E. C. 1.1.1.48) as described below (Reaction [2]).

\[
\text{D-galactose} + \text{NAD}^+ \xrightarrow{\beta\text{-galactose dehydrogenase}} \text{D-galactono-1,4-lactone} + \text{NADH} + \text{H}^+ \]

Thus, by adding \(\beta\)-galactose dehydrogenase and NAD\(^+\) to reaction mixture that contain galactose it is possible to quantify it by measuring the increase in NADH absorption at 339, 334 or 365nm (Kleyn 1985). The simultaneous determination of lactose and galactose requires the determination of the samples in two sets. Set 1 starts with Reaction (1) and followed by Reaction (2). Set 2 starts with Reaction (2). Galactose concentration is determined from Reaction (2), using galactose as a standard, whereas lactose concentration from (Reaction [1] + [2]) – Reaction (1), using lactose as a standard (Kleyn 1985; Shapiro *et al.* 2002).
23.4 Simplification of the Colorimetric Assay by Replacing NAD with Thio-NAD

The original above procedure was carried out in single-well spectrophotometers (Kleyn 1985), which is much more time consuming in comparison to determination with microplate readers. Microplate readers have been widely used for the determination of many materials by ELISA, but also have been proved useful in measuring various colorimetric reactions. Theoretically, NAD$^+$ can be replaced by thio-NAD$^+$, as described by Reaction (3), with the advantage that the accumulation of thio-NADH can be monitored by the increase in absorbance in the visible range at 405 or 415 nm (Buckwalter and Meyerhoff 1994).

Reaction (3)

\[
\text{D-galactose} + \text{thio-NAD}^+ \xrightarrow{\beta\text{-galactose dehydrogenase}} \text{D-galactono-1, 4-lactone} + \text{thio-NADH} + \text{H}^+
\]

The results described by Shapiro et al. (2002) point out that the procedure for simultaneous detection of lactose and galactose which is based on detection of thio-NADH in the visible range were very similar to those based on detection of NADH in the UV range both in milk and in mammary secretion during involution, in which lactose concentration was only \(\sim 10\%\) of its normal value. Plate readers in the UV range are much more expensive than plate readers in the visible range, particularly when using the wavelength (405 nm) most widely available on inexpensive microplate readers used for ELISA determinations in which the indicator enzyme is alkaline phosphatase.

However, whereas the modification described above simplified and improved the rate of analysis of lactose and galactose, it still suffers from the deficiency of applying colorimetric procedure for the determination of metabolites in complex materials such as milk and dairy products. This was the drive for further development.

23.5 The Challenge of Quantifying Metabolites in Milk and Dairy Products

Applying monochromatic absorbance photometry for analysis of foods is frequently difficult and problematic (Nielsen 2003). This is particularly so when relating to products such as milk and yogurt. The following challenges emerge when the aim is to measure metabolites such as lactose and galactose by colorful reactions in these substances: (i) they contain fat droplets of varying size that scatters light in an unpredictable way; (ii) they are opaque and colloidal solution of proteins that scatter and absorb light; (iii) that frequently contain intense colorant (e.g., cacao drink, yogurts with fruits or colorant) that interfere with the monochromatic absorbance.
Overcoming these problems necessitates the use of various pretreatment procedures of samples to minimize the above described problems. However, frequently such pretreatments only partially resolve the problem, in addition to the fact that many of these procedures are cumbersome and time consuming.

### 23.6 Fluorometry Coupling to Formation of Chromophore and Enzymatic Cycling as a Solution for the Determination of Lactose and Galactose in Milk and Dairy Products

Fluorometry refers to measurement of fluorescence emitted by certain compounds when exposed to intense radiant energy, usually in the ultraviolet range. The atoms of such substances emit fluorescence light when excited. The response of a fluorescent chromophore is specific to the excitation light and emit fluorescent light with a characteristic color and wavelength, allowing identification and quantification of the chromophore in biologic specimens. Fluorometry is a highly sensitive method of analysis, which is free to a large degree from the deficiencies of monochromatic absorbance photometry outlined above, making it suitable for detection of metabolites such as milk and other dairy products (Shapiro and Silanikove 2010).

Fluorescent chromophore (fluorochromophore) may be indigenous (biological) or synthetic molecules. As already mentioned, NAD$^+$ and NADH absorb strongly in the ultraviolet range due to the adenine base. In addition, when NADH in solution is excited at 340 nm, it emits fluorescence with a peak at 460 nm and a lifetime of 0.4 nanoseconds, whereas the oxidized form of the coenzyme (NAD) does not fluoresce. These properties of NADH have been used to measure dissociation constants, which are useful in the study of enzyme kinetics (Lakowicz et al. 1992) and changes in the redox state of living cells, by fluorescence microscopy (Kasimova et al. 2006). Fluorescent emission of NADH has been also used to measured metabolites in NAD-depended reaction; however, the accuracy of this method when applied to biological specimens is less satisfactory (Shapiro and Silanikove 2010) most likely because many indigenous molecules emit fluorescence in this rage, which increases the background noise.

A potential solution to this problem of specificity is to use synthetic fluorochromophore, which emits florescence at specific excitation/emission wavelength. One such candidate is resazurin (7-Hydroxy-3H-phenoxazin-3-one 10-oxide). Resazurin is a blue dye, which itself is nonfluorescent until it is reduced to the pink colored and highly red fluorescent resorufin (7-Hydroxy-3H-phenoxazin-3-one) (Figure 23.1).

The main applications of these substances are their use as an oxidation-reduction indicator in cell viability assays for bacteria and mammalian cells. Resazurin was already in use in 1929 to quantify bacterial content in milk (Pesch and Simmert 1929). It is also widely used as an indicator for cell viability
in mammalian cell cultures (Anoopkumar et al. 2005). The conversion of resazurin to resorufin can be used to measure NADH, metabolites derived from NAD-dependent reactions, and the kinetics of the enzyme involved of such reactions (Lakowicz et al. 1992). For this, we need an enzyme that will be able to catalyze the following reaction:

\[
\text{NADH} + \text{H}^+ + \text{acceptor (resazurin)} \rightleftharpoons \text{NAD}^+ + \text{reduced acceptor (resorufin)}
\]

One such candidate is the oxidoreductase diaphorase (EC 1.6.99.1) also known as NADPH dehydrogenase; this enzyme has many other commonly used names related to its mode of action. From its common name NADPH dehydrogenase its also appears that it can oxidize NADPH to NADP+. However, this does not form a problem of specificity for our case because the conversion of resazurin to resorufin is specific for the presence of NADH (Shapiro and Silanikove 2010). Diaphorase has the ability to reduce chromogenic acceptors, such as decolorization of 2, 6-dichlorophenolindophenol, tetrazolium dye and resazurin; the last two become chromogenic on reduction. Diaphorases are a ubiquitous class of flavin-bound enzymes, and members of this group are also produced by bacteria such as Cl. Kluyveri. Bacterial diaphorase are commercially available and can serve as relatively cheap source for the enzyme (Shapiro and Silanikove 2010). Consequently, following reaction 1, galactose can be quantified by reaction 4 and lactose as explained for the colorimetric reaction. A schematic presentation of the methodology is presented in Figure 23.2. As can be seen in Figure 23.2, the reaction system occurs according to the principles of enzymatic cycling.

For the simultaneous determination of lactose and galactose by enzymatic reaction coupled to formation of fluorochromophore, stage 1 (described by reaction 2), whereas stage 2 is described by Reaction (4):

\[
\text{NADH} + \text{H}^+ \xrightarrow{\text{resazurin diaphorase}} \text{NAD}^+ + \text{resorufin}
\]

In order to start the reaction in the desired direction, the reaction medium needs to contain excess of NAD$^+$ and appropriate concentration of β-galactose dehydrogenase, whereas the coupling of the conversion of resazurin to resorufin with the conversion of NADH to NAD$^+$ requires the presence of
appropriate concentrations of resazurin and diaphorase. An example for appropriate reaction conditions is detailed in Shapiro and Silanikove (2010).

The benefits of enzyme cycle reaction are well known (Lowry 1980) and related to improved sensitivity, which allow to analyze minute amount of substances, or to carry out the reaction in small volume, like the wells of microplate, and thus in a rapid and potentially automatic mode. This principle is exemplified in its successful application in measuring lactose and galactose in milk in yogurts applying minimal preparation of the samples (Table 23.1), attaining accuracy which is not falling or even exceeding those attained by HPLC and in much faster way (Shapiro and Silanikove, 2010).

23.7 Conclusions

Enzymatic methods were found particularly valuable in characterizing the fate of lactose and galactose in yogurts, because their metabolism is stereoisomer

![Figure 23.2](image)
The principle of enzymatic cycling reaction in the fluorimetric determination of galactose.

### Table 23.1
Concentrations (mean) of L-lactate, D-lactate and lactose in commercial plain (classical) yogurts and modern bio-yogurts.*

<table>
<thead>
<tr>
<th>Product</th>
<th>L-lactate, mM</th>
<th>D-lactate, mM</th>
<th>D/L ratio</th>
<th>Lactose, mM</th>
<th>D-l/L Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Plain Yogurts</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>75.1</td>
<td>122.2</td>
<td>1.64</td>
<td>89.1</td>
<td>0.84</td>
</tr>
<tr>
<td>B</td>
<td>116.1</td>
<td>73.0</td>
<td>0.63</td>
<td>92.1</td>
<td>0.79</td>
</tr>
<tr>
<td>C</td>
<td>83.1</td>
<td>54.2</td>
<td>0.63</td>
<td>98.1</td>
<td>0.55</td>
</tr>
<tr>
<td>D</td>
<td>79.2</td>
<td>29.0</td>
<td>0.37</td>
<td>100.2</td>
<td>0.29</td>
</tr>
<tr>
<td>E</td>
<td>87.1</td>
<td>90.1</td>
<td>1.01</td>
<td>85.5</td>
<td>1.05</td>
</tr>
<tr>
<td><strong>Bio-yogurts</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>F</td>
<td>96.2</td>
<td>2.0</td>
<td>0.02</td>
<td>128.5</td>
<td>0.02</td>
</tr>
<tr>
<td>G</td>
<td>123.2</td>
<td>5.2</td>
<td>0.04</td>
<td>173.0</td>
<td>0.03</td>
</tr>
<tr>
<td>H</td>
<td>101.5</td>
<td>6.6</td>
<td>0.04</td>
<td>129.3</td>
<td>0.03</td>
</tr>
<tr>
<td>I</td>
<td>123.0</td>
<td>12.0</td>
<td>0.03</td>
<td>130.3</td>
<td>0.02</td>
</tr>
</tbody>
</table>

D/L ratio = D-lactate/L-lactate ratio; D-l/L ratio = D-lactate/lactose ratio.

*Rearranged from Shapiro and Silanikove (2010). The product codes were fully identified in the paper. Briefly, the plain yogurts and the bio-yogurts were produced in Israel; however, the bio-yogurts under license of international brands: F and H by DANONE, G by Muller, and I by Yoplait.
specific and thus applying stereoisomer specific enzymes is the only way to distinguish between the isomer products and the final nutritional quality of the product (Shapiro and Silanikove 2010). An example for the efficiency of this method in analyzing classical yogurts and common modern bio-yogurts that contains additions of probiotic bacterial cultures is provided in Table 23.1, rearranged from Shapiro and Silanikove (2010). The results clearly indicate that in the set measured degradation of lactose is more extensive in yogurts then in bio-yogurts and that the level of D-lactate (measured by similar fluorimetric principle) is higher in yogurts then in bio-yogurts. Only enzymatic reactions can distinguish between the presence of D-and L- lactate.

The versatility of present fluorimetric methodology allows applying the same principle for analyzing other test substances in various types of biological fluids and foods (Larsen 2010, 2011; Shapiro and Silanikove 2011).

**Summary Points**

- Lactose and galactose are important carbohydrate resources and their main dietary source is milk and dairy products.
- It is possible to determine the concentration of lactose (after its splitting to glucose and galactose) and galactose colorimetrically by NAD/NADH depended reaction.
- Use of thio-Nad instead of NAD is a modification allowing the reaction to be carried out in plate reader in the visible range.
- However, applying monochromatic colorimetric analysis in complex media such as milk and dairy products is inherently difficult.
- A solution based on fluorometry, which applies formation of synthetic chromophore and enzymatic cycling has been shown as effective solution for analyzing lactose and galactose in milk and dairy products.
- The main advantages are minimal sample preparation, low reaction volume, high rate of analysis and low cost.
- This approach is versatile and may be applied for a broad spectrum of metabolites, and some recent applications were noted.

**Key Facts**

- Use of enzymatic reaction for the determination of lactose and galactose is particularly important when the fate of their metabolism is stereoisomer specific.
- An example was provided by comparing the composition of classical (plain) yogurts and modern bio-yogurts (Table 23.1). The results suggest that bio-yogurts are inferior in comparison to classical yogurt in important aspects, such as lower lactose concentration.
Combined Assays for Lactose and Galactose by Enzymatic Reactions

References


