Quantification of the Disaccharide Trehalose from Biological Samples: A Comparison of Analytical Methods

Gregory A. Hayner, ‡ Sudhir Khetan, ‡ and Margot G. Paulick*†

Department of Chemistry and ‡Bioengineering Program, Union College, 807 Union Street, Schenectady, New York 12308, United States

ABSTRACT: Trehalose is a disaccharide that is biosynthesized by many different organisms subjected to extreme conditions, such as dehydration, heat, oxidative stress, and freezing. This disaccharide allows organisms to better survive these environmental stresses; however, the mechanisms by which trehalose exerts its protective effects are not well understood. Methods to accurately measure trehalose from different organisms will help us gain better understanding of these protective mechanisms. In this study, three experimental approaches for the quantification of trehalose from biological samples were compared: an enzymatic trehalose assay (Trehalose Assay Kit; Megazyme International), a high-performance liquid chromatography coupled with refractive index detection-based assay, and a liquid chromatography–tandem mass spectrometry (LC–MS/MS)-based assay. Limits of detection and quantification for each assay were compared, as were the dynamic ranges for all three assays. The percent recoveries for known amounts of trehalose spiked into bacterial and mammalian cellular lysates were also determined for each of the assays. Finally, endogenous trehalose produced by Escherichia coli cells was detected and quantified using these assays. Results from this study indicate that an LC–MS/MS-based assay is the most direct and sensitive method for the quantification of low concentrations of trehalose from biological samples; however, the enzymatic assay is suitable for the rapid quantification of higher concentrations of trehalose when an LC–MS/MS is unavailable.

INTRODUCTION

The disaccharide trehalose, or \( \alpha-D\)-glucose(1→1)\( \alpha-D\)-glucose (Figure 1a), acts as a remarkable cellular protectant for many different organisms, including bacteria, fungi, plants, insects, and eukaryotic microorganisms. 1–9 When subjected to extreme conditions, such as cold, heat, desiccation, or reactive oxygen species, these organisms biosynthesize high concentrations of both intra- and extracellular trehalose, which allows them to better survive these environmental stresses. 1–9 For example, the desiccation-tolerant plant, Selaginella lepidophylla, accumulates intracellular trehalose at levels up to 12% of its dry weight during periods of drought. 10 High concentrations of trehalose are also found in Saccharomyces cerevisiae (a strain of yeast) that are subjected to heating; yeast mutants that are defective in the genes that encode for trehalose biosynthesis are unable to produce trehalose upon heat shock and are much less resistant to heating than wild-type yeast. 4,11 Mammals do not naturally produce trehalose; however, delivery of trehalose into mammalian cells improves survival rates after freezing, drying, or heat shock. 12–16 Furthermore, the administration of exogenous trehalose has been shown to provide neuroprotective effects in animal models of Huntington’s disease, Parkinson’s disease, and amyotrophic lateral sclerosis. 17–20 The ability of trehalose to protect cells from damage, along with its lack of cellular toxicity, has generated interest in using this disaccharide as a general cellular protectant.1,13–9,12–16

The exact mechanisms by which trehalose exerts its protective effects are not well elucidated; it is hypothesized that this carbohydrate partially replaces the water inside cells and around cellular components, including proteins and lipid membranes.3,4 By forming hydrogen bonds with proteins and lipids, trehalose helps maintain membrane integrity and enzyme structure. Trehalose is also reported to form a stable glass (a liquid of high viscosity) at room temperature, thus leading to a reduction in the rates of damaging biochemical reactions in a cell.3,4 Further investigation of the role of trehalose in cellular protection is necessary; therefore, it is crucial to be able to detect and accurately quantify trehalose from organisms exposed to various environmental conditions.

Several methods have been reported for the quantification of trehalose from biological sources. 21–30 One of the most commonly used methods for quantifying trehalose is an enzymatic assay that uses trehalase, an enzyme that cleaves trehalose into two glucose monomers (Scheme 1).22–24 The enzyme hexokinase then catalyzes the phosphorylation of...
glucose to glucose-6-phosphate (G-6-P), and G-6-P is subsequently oxidized to gluconate-6-phosphate. The oxidation of G-6-P is catalyzed by the enzyme glucose-6-phosphate dehydrogenase, and during the reaction, NADP+, the oxidized form of nicotinamide adenine dinucleotide phosphate, is reduced to reduced nicotinamide adenine dinucleotide phosphate (NADPH), the reduced form of this cofactor. NADPH absorbs light at a wavelength of 340 nm, which can be quantified by spectrophotometric means.24 Trehalose concentrations in biological samples can then be calculated on the basis of the NADPH absorbance from this series of enzymatic reactions.24 Another reported method for the quantification of trehalose has been developed by our laboratory.34 In this assay, trehalose is separated via liquid chromatography and quantified via tandem mass spectrometry using maltose as an internal standard.

Although all of the above methods can be used for quantifying trehalose from biological sources, the different methods have not been directly compared. Therefore, the relative advantages and limitations of these methods and the suitability of each method for a chosen application have not been established. The purpose of this study is to help trehalose researchers choose the most suitable method for trehalose quantification. In this study, three different assays for the quantification of trehalose were compared: the enzymatic trehalose assay (Trehalose Assay Kit; Megazyme International), an HPLC coupled to a refractive index detector (HPLC-RID)-

**Figure 1.** Structures of trehalose and trehalose ions discussed in this study. (a) Structure of trehalose, a disaccharide that is used as a cellular protectant by many different organisms subjected to environmental stresses. (b) Structures of the trehalose precursor ion (m/z = 360) and oxocarbenium fragment ion (m/z = 163) detected by the liquid chromatography–tandem mass spectrometry (LC–MS/MS)-based assay. (c) Structures of the 13C12-trehalose precursor ion (m/z = 377) and 13C6-glucose fragment ion (m/z = 209) detected by the LC–MS/MS-based assay.

**Scheme 1. Overview of the Enzymatic Trehalose Assay Used in This Study**

In the enzymatic trehalose assay (Trehalose Assay Kit), trehalose is converted to gluconate-6-phosphate through a series of enzymatic reactions. This coupled enzyme assay yields two reduced nicotinamide adenine dinucleotide phosphate (NADPH) for each trehalose input. NADPH absorbs ultraviolet light and thus allows for the quantification of trehalose by spectrophotometric means.
based assay, and an LC−MS/MS-based assay. The instrument limits of detection (LOD) and quantification (LOQ) for trehalose for each assay were compared, as were their dynamic ranges. The accuracy of each assay was determined by calculating the percent recoveries for known amounts of trehalose spiked into bacterial and mammalian cellular lysates. Finally, these assays were used to detect and quantify endogenous trehalose produced by *Escherichia coli* (E. coli) cells. Results from this study indicate that an LC−MS/MS-based assay is the most direct and sensitive method for the quantification of nanomolar concentrations of trehalose from biological samples; however, the enzymatic assay is suitable for the rapid quantification of micromolar concentrations of trehalose when an LC−MS/MS is unavailable.

## Results and Discussion

**Trehalose Assay Detection and Quantification.** The quantification of trehalose from biological samples can be accomplished using a variety of different assays, such as an enzymatic trehalose assay, an HPLC-RID-based assay, or an LC−MS/MS-based assay. To gain better understanding of the advantages and limitations of these different assays, it is necessary to demonstrate that all of them can specifically detect trehalose even in the presence of other disaccharides. The disaccharides trehalose, lactose, maltose, and sucrose (separately or as a mixture) were subjected to these three assays; for each assay, disaccharide concentrations were chosen to ensure a robust signal. For the enzymatic trehalose assay, samples containing a single disaccharide were subjected to Megazyme’s Trehalose Assay Kit. Only samples containing trehalose gave a significant absorbance signal above background, demonstrating that this assay is specific for the disaccharide trehalose (Figure 2a). For the HPLC-RID-based assay, a solution containing all four disaccharides was injected onto an Agilent 1100 series HPLC. The sugars were separated using a Waters high-performance carbohydrate column and then detected using an Agilent 1260 Infinity series RID. As shown in Figure 2b, trehalose is well separated from all three disaccharides, thus allowing for its reliable quantification in the presence of other disaccharides using this assay. The LC−MS/MS-based assay is based on an assay previously reported by our laboratory. In the present study, the assay has been modified to use $^{13}$C$_{12}$-trehalose, an isotopically labeled analogue of trehalose, in which all of the $^{13}$C atoms have been replaced by $^{13}$C atoms, instead of maltose as an internal standard for trehalose quantification. The use of $^{13}$C$_{12}$-trehalose allows for a shorter analysis time than the use of maltose; no significant differences in data quality have been observed. To determine the specificity of this LC−MS/MS-based assay, the four disaccharides were separated using a Waters high-performance carbohydrate column and detected using an Agilent 6410B triple quadrupole mass spectrometer. The mass spectrometer was operated in selected reaction monitoring (SRM) mode, monitoring for the trehalose transitions of 360−163 m/z and 360−85 m/z and for the $^{13}$C$_{12}$-trehalose transition of 377−209 m/z, all in positive mode. These transitions were chosen for each disaccharide using Agilent MassHunter Optimizer software. For trehalose, the m/z value of 360 corresponds to the [M + NH$_4$]$^+$ trehalose precursor ion (ammonium acetate was used in the LC−MS solvents to aid in the ionization of the disaccharides), the m/z value of 163 corresponds to the [M$^+$] oxocarbenium fragment ion, and the m/z value of 85 corresponds to a fragment ion with a molecular formula of [CO$_2$H$_2$ + K$^+$] (Figure 1b). For

![Figure 2. Specificity of assays for trehalose detection. (a) Corrected absorbances for lactose, maltose, sucrose, and trehalose for the enzymatic trehalose assay. All disaccharide concentrations were 1 mM, and the sample volume was 20 μL. For all corrected absorbance values, n = 3. (b) RI chromatogram showing the separation of trehalose (retention time = 32.2 min) from three other disaccharides, sucrose (retention time = 19.3 min), maltose (retention time = 25.5 min), and lactose (retention time = 30.0 min) for the HPLC-RID-based assay. Separation was achieved using a Waters high-performance carbohydrate column (4.6 × 250 mm$^2$, 4 μm) held at 35 °C. The mobile phase was composed of 16:84 water/acetonitrile with an isocratic elution over 40 min using a flow rate of 1.4 mL/min. All disaccharide concentrations were 20 mM, and the injection volume was 50 μL. (c) SRM chromatogram showing the separation of trehalose (retention time = 30.3 min) from three other disaccharides, sucrose (retention time = 18.2 min), maltose (retention time = 23.8 min), and lactose (not detected by this method) for the LC−MS/MS-based assay. Separation was achieved using a Waters high-performance carbohydrate column (4.6 × 250 mm$^2$, 4 μm) held at 35 °C. The mobile phase was composed of 16:84 2 mM ammonium acetate in water/2 mM ammonium acetate in acetonitrile with an isocratic elution over 35 min using a flow rate of 1.4 mL/min. All disaccharide concentrations were 25 μM, and the injection volume was 40 μL.](https://doi.org/10.1021/acsomega.7b01158)
interfere with trehalose quantification when using the LC−MS/MS-based assay.

Having determined that these three assays are selective for the detection of trehalose, calibration curves for this disaccharide were constructed for each assay. These curves reveal the range of trehalose concentrations suitable for each assay, as well as the precision of each assay. For the enzymatic trehalose assay, the calibration curve of 25−1000 μM trehalose is best fit by a linear curve (Figure 3a). The responses for the HPLC-RID-based assay also are fit best by a linear curve (Figure 3b); however, to detect trehalose, it is necessary to use concentrations that are much higher (1−100 mM trehalose) than those used for the enzymatic trehalose assay. In contrast to the enzymatic trehalose and HPLC-RID assays, the calibration curve of 0.1−100 μM trehalose using 13C12-trehalose as an internal standard for the LC−MS/MS-based assay is best fit by a single polynomial (Figure 3c). As previously reported, a single polynomial calibration curve is also the best way to model the LC−MS/MS responses to trehalose using maltose as an internal standard; a linear fit would introduce accuracy errors. This nonlinear response is most likely due to saturation; at the low end of the concentration range (1 μM trehalose and lower), the data are fit well by a linear curve, whereas higher concentrations of trehalose require a single polynomial curve. Moreover, there is precedent for single polynomial fits for LC−MS/MS data. For example, a single polynomial fit has been used to model the response of an LC−MS/MS instrument to a platinum anticancer drug in human plasma samples. For the LC−MS/MS-based assay used here, a single polynomial gives the best fit. The calibration curve for trehalose using this LC−MS/MS-based assay allows for very low concentrations of trehalose to be quantified, which is a significant advantage of this assay; as little as 0.1 μM trehalose is within its range. Moreover, these data demonstrate that all three assays are precise; the standard deviations (SDs) for the signals for each assay are low (Tables S1−S3, Supporting Information).

Trehalose Assay Sensitivities. Once it was determined that reproducible calibration curves for trehalose using these three assays could be generated, the assay sensitivities were established by determining the instrument limits of detection and quantification (LOD and LOQ, respectively). The instrument LOD and LOQ are defined as the smallest amount of trehalose that can be reliably detected and accurately quantified, respectively, using different assays. As shown in Table 1, the LC−MS/MS-based assay for trehalose is the most sensitive assay, giving an instrument LOD of 22 nM and an instrument LOQ of 28 nM for trehalose. These values are 2−3 and 4−5 orders of magnitude lower than those for the enzymatic assay and the HPLC-RID-based assay, respectively (Table 1). To confirm the validity of the instrument LODs, samples containing trehalose at the concentration corresponding to the LOD for each assay were prepared and analyzed. For all of the assays, the signal-to-noise ratios (S/N) at the instrument LOD are greater than 1.5, demonstrating that calculated values for the instrument LODs are able to be detected experimentally (Table 1 and Figure S2, Supporting Information).

More conservative measures of the sensitivity of a method are the method detection limit (MDL) and method quantification limit (MQL). The MDL and MQL are defined as the smallest amount of trehalose that can be reliably detected and quantified, respectively; they differ from the LOD and LOQ in that they include the error arising from sample preparation. As shown in Table 1, the LC−MS/MS-based assay gives an instrument MDL of 35 nM trehalose and an instrument MQL of 74 nM trehalose. These values are 2−3 and 4−5 orders of magnitude lower than those for the enzymatic assay and the HPLC-RID-based assay, respectively.
Table 1. Instrument Limits of Detection (LOD) and Quantification (LOQ), Method Detection Limits (MDL) and Quantification Limits (MQL), and Signal-to-Noise Ratios (S/N) for All Three Assays

| assay                  | LOD  | LOQ  | MDL  | MQL  | S/N at LOD |
|------------------------|------|------|------|------|------------|
| enzymatic trehalose    | 6.3 μM | 21 μM | 6.4 μM | 21 μM | 2.7        |
| HPLC-RID               | 0.6 mM | 2.2 mM | 0.9 mM | 2.9 mM | 1.8        |
| LC−MS/MS               | 22 nM | 28 nM | 35 nM | 74 nM | 177        |

(Tables 1). Such low values for the MDL and MQL demonstrate that the LC−MS/MS-based assay is the most sensitive method for the detection and quantification of trehalose, even when taking into account the error involved in sample preparation.

Having established the LOD, LOQ, MDL, and MQL for these assays, the dynamic ranges for all three assays were determined. For the enzymatic trehalose assay, the lower limit of quantification is 21 μM (Table 1). At trehalose concentrations above 4 mM, the enzymatic trehalose assay does not respond reliably to further increases in trehalose concentration; the corrected absorbance values do not change significantly as the concentration of trehalose increases from 4 to 100 mM (Figure S3, Supporting Information). On the basis of these data, it can be concluded that the dynamic range for the enzymatic trehalose assay spans slightly more than 2 orders of magnitude, from 21 μM to 4 mM trehalose. For the HPLC-RID-based assay, the lower limit for trehalose quantification is 2.2 mM (Table 1). At trehalose concentrations above 100 mM, the RI signal flattens out, suggesting that high concentrations of trehalose saturate the RI detector. The dynamic range for the HPLC-RID-based assay is slightly broader than that for the enzymatic trehalose assay, spanning approximately 2 orders of magnitude, from 2.2 to 100 mM trehalose. The dynamic range for the LC−MS/MS-based assay is the broadest of all three assays; it spans between 3 and 4 orders of magnitude, from 0.03 to 100 μM trehalose. At trehalose concentrations above 100 μM, the SRM peaks are very broad and exhibit two maxima, suggesting that such high concentrations of trehalose saturate the MS detector. Nevertheless, all three assays have good dynamic ranges, ranging from 2 to 4 orders of magnitude, allowing for the quantification of a broad range of trehalose concentrations in biological samples.

Trehalose Assay Accuracies. To determine the accuracies of the three assays for the quantification of trehalose in samples with known concentrations, solutions containing a variety of trehalose concentrations across the ranges of the different calibration curves were prepared and analyzed. Trehalose concentrations in these samples were calculated from the calibration curve obtained from each different assay. The percent recoveries for these samples for all three assays are within 10% of the expected concentrations, ranging from 98 to 110%. All three assays are therefore highly accurate for the calculation of trehalose concentrations from unknown samples (Table 2).

The accuracies of these three assays for the quantification of trehalose in more complex samples with known concentrations of trehalose were also evaluated. Known amounts of trehalose were spiked into lysates from either E. coli (strain DH5α) or Jurkat cells (a human T-cell line), and the trehalose concentrations in the samples were calculated from the calibration curves obtained from each trehalose assay. For the

Table 2. Calculated Trehalose Concentrations and Percent Recoveries from Trehalose Samples Prepared in Water or 1:1 Water/Acetonitrile for All Three Assays

| assay                  | expected [trehalose] | calculated [trehalose] | percent recovery |
|------------------------|----------------------|------------------------|-----------------|
| enzymatic trehalose    | 30 μM | 33 ± 3 μM | 110 % |
|                        | 80 μM | 80 ± 6 μM | 100 % |
|                        | 250 μM | 250 ± 5 μM | 100 % |
|                        | 800 μM | 800 ± 6 μM | 100 % |
|                        | 5 μM | 5 ± 0.2 μM | 100 % |
| HPLC-RID               | 40 mM | 40 ± 0.8 mM | 98 % |
|                        | 80 mM | 79 ± 1 mM | 99 % |
|                        | 0.3 μM | 0.303 ± 0.005 μM | 101 % |
|                        | 5 μM | 5.1 ± 0.1 μM | 102 % |
| LC−MS/MS               | 30 μM | 30.7 ± 0.2 μM | 102 % |
|                        | 80 μM | 78.0 ± 0.8 μM | 98 % |

*a *n* = 3–6.

LC−MS/MS-based assay, standards for the calibration curve were prepared in a mixture of 1:1 acetonitrile/cell lysates, with the lysates matching the lysates being analyzed; this was done to reduce the matrix effect often observed in MS-based assays.34,41 The matrix effect is a change in the response of the LC−MS/MS to a specific compound when complicated samples are analyzed; it is hypothesized to result from molecules originating from the sample mixture that coelute with the compound of interest, causing either ionization suppression or enhancement in the mass spectrometer.34 For all three assays, the percent recoveries from samples prepared in E. coli lysates are within 7% of the expected concentrations, ranging from 93 to 106% (Table 3). For samples prepared in

Table 3. Calculated Trehalose Concentrations and Percent Recoveries from Trehalose Samples Prepared in E. coli DH5α Cell Lysates or 1:1 E. coli DH5α Cell Lysates/ Acetonitrile for All Three Assays

| assay                  | expected [trehalose] | calculated [trehalose] | percent recovery |
|------------------------|----------------------|------------------------|-----------------|
| enzymatic trehalose    | 30 μM | 29 ± 2 μM | 97 |
|                        | 80 μM | 79 ± 4 μM | 99 |
|                        | 250 μM | 250 ± 20 μM | 96 |
|                        | 800 μM | 792 ± 5 μM | 99 |
|                        | 5 μM | 4.77 ± 0.05 mM | 95 |
| HPLC-RID               | 40 mM | 38.5 ± 0.4 mM | 96 |
|                        | 80 mM | 84.4 ± 0.7 mM | 106 |
|                        | 5 μM | 5.0 ± 0.2 μM | 100 |
| LC−MS/MS               | 30 μM | 28.3 ± 0.8 μM | 94 |
|                        | 80 μM | 74 ± 2 μM | 93 |

*a *n* = 3–5.

Jurkat cell lysates, the percent recoveries are also within 7% of the expected concentrations, ranging from 93 to 100% (Table 4). These high percent recoveries demonstrate that all three assays are very accurate for the quantification of trehalose, even when complex matrices, such as cell lysates, are analyzed. Furthermore, it can be concluded that all three assays can be used with a variety of biological samples because excellent recoveries in two different types of cell lysates are observed.

Quantification of Endogenous Trehalose from E. coli Cells. Finally, these three assays were evaluated for the detection and quantification of endogenous trehalose from bacterial cells that biosynthesize trehalose. For these experi-
ments, the arabinose-inducible OtsA/OtsB overproducer of the *E. coli* strain MC4100, which produces intracellular trehalose when arabinose is added to the medium used to grow the cells, was used.42 Lysates from these cells were subjected to the three different trehalose assays. For the LC−MS/MS-based assay, standards for the calibration curve were prepared using lysates from the trehalose knockout (Δ*otaA*) of the *E. coli* strain MC4100, which are unable to biosynthesize trehalose and therefore contain no endogenous trehalose to interfere with quantitative analysis.43 Trehalose was not detected in the lysates from the OtsA/OtsB overproducer *E. coli* cells by the enzymatic trehalose assay or the HPLC-RID-based assay. For the enzymatic trehalose assay, endogenous glucose in the lysates resulted in very high background absorbance values, which caused the corrected absorbance values for the lysates to be outside the dynamic range of the assay. For the HPLC-RID-based assay, no peak was detected for trehalose in the lysates. In contrast, a significant peak for trehalose was detected at the expected retention time for trehalose from the OtsA/OtsB overproducer *E. coli* cells (Figure S4, Supporting Information) when using the LC−MS/MS-based assay. The calculated trehalose concentrations from the four different *E. coli* lyse samples ranged from 5 to 10 μM. Such a low trehalose concentration in these lysates explains why trehalose was not detected by either the enzymatic trehalose assay or the HPLC-RID-based assay; 5−10 μM trehalose is below both the instrument LOQ and LOD for these two assays. Using the trehalose concentrations from the cell lysates (as calculated using the LC−MS/MS-based assay), as well as the total number of *E. coli* cells lysed (approximately 4 × 10^10 cells/sample), it was determined that the internal trehalose concentration inside one *E. coli* cell is 40 ± 10 mM (n = 4). These data demonstrate that the LC−MS/MS-based assay is exceptional when compared to the enzymatic trehalose assay or the HPLC-RID-based assay for the detection and quantification of low concentrations of trehalose in complex biological samples.

**Advantages and Limitations of the Trehalose Assays.** All three assays evaluated in this study, the enzymatic trehalose assay, the HPLC-RID-based assay, and the LC−MS/MS-based assay, can be used to accurately detect and quantify trehalose from a variety of biological matrices (Tables 3 and 4). Each assay, however, is best suited to a different range of trehalose concentrations (Figure 3 and Table 1). The LC−MS/MS-based assay is the most sensitive of the three assays, with an LOD of 22 nM and an LOQ of 28 nM for trehalose, and is optimal for quantifying nanomolar concentrations of trehalose. In contrast, the HPLC-RID-based assay has an LOD of 0.6 mM and an LOQ of 2.2 mM for trehalose; this assay is most suitable for biological samples containing millimolar concentrations of trehalose. With an LOD of 6.3 μM and an LOQ of 21 μM trehalose, the enzymatic trehalose assay is best used for trehalose concentrations in the micromolar range. The high sensitivity of the LC−MS/MS-based assay for trehalose is a significant advantage in that it allows for the detection and quantification of concentrations of trehalose in biological samples that are prohibitively low for the other two assays; specifically, it was the only assay able to detect and quantify endogenous trehalose from the OtsA/OtsB overproducer *E. coli* lysates evaluated in this study.

Beyond sensitivity, an additional benefit of the LC−MS/MS-based assay is that it conclusively identifies trehalose as the analyte being detected and quantified; MS signals are only observed for the trehalose precursor ion and its fragment ions. In contrast, the enzymatic trehalose assay does not quantify trehalose directly; the absorbance values obtained from this assay arise from NADPH, a product in the series of enzymatic reactions that converts trehalose to gluconate-6-phosphate (Scheme 1).24 Complex biological samples may contain molecules that interfere with this absorption; as observed in the present study, high levels of endogenous glucose in the OtsA/OtsB overproducer *E. coli* lysates resulted in high background absorbance values, causing a reduction in the actual signal for the enzymatic trehalose assay. The HPLC-RID-based assay also does not conclusively identify trehalose on the basis of its RI signal; trehalose can coelute with other biological compounds in the sample, thus causing an overestimation of trehalose concentration when using this assay. Therefore, the LC−MS/MS assay is advantageous for measuring trehalose concentrations in complex biological samples.

The LC−MS/MS-based assay could also be used to quantify radiolabeled trehalose or other trehalose analogues. Trehalose is an important nutrient for the bacterium *Mycobacterium tuberculosis*, the causative agent for tuberculosis; a better understanding of its uptake may lead to novel therapeutics.14 To differentiate endogenously biosynthesized trehalose from exogenously added trehalose, researchers often feed bacteria trehalose radiolabeled with 14C or 2H.15 The LC−MS/MS-based assay is a good approach for quantifying the exogenously added, radiolabeled trehalose because it can distinguish between trehalose and a radiolabeled analogue. Neither the enzymatic trehalose assay nor the HPLC-RID-based assay can differentiate between these different disaccharides. Esterified trehalose analogues have also recently been used to deliver trehalose into mammalian cells; for trehalose quantification, one study used the enzymatic trehalose assay and another study used the LC−MS/MS-based assay.15,16 The concentrations of esterified trehalose analogues from these cells could also be directly quantified using the LC−MS/MS-based assay; it is unlikely that the enzymatic trehalose assay or the HPLC-RID-based assay could detect these analogues.

Table 4. Calculated Trehalose Concentrations and Percent Recoveries from Trehalose Samples Prepared in Jurkat Cell Lysates or 1:1 Jurkat Cell Lysates/Acetonitrile for All Three Assays

| assay                  | expected [trehalose] | calculated [trehalose] | percent recovery |
|------------------------|----------------------|------------------------|------------------|
| enzymatic trehalose    | 30 μM                | 29 ± 2 μM              | 97 %             |
|                        | 80 μM                | 74 ± 9 μM              | 93 %             |
|                        | 250 μM               | 244 ± 9 μM             | 98 %             |
|                        | 800 μM               | 770 ± 10 μM            | 96 %             |
| HPLC-RID               | 5 mM                 | 4.9 ± 0.3 μM           | 98 %             |
|                        | 40 mM                | 39.9 ± 0.7 mM          | 100 %            |
|                        | 80 mM                | 79.0 ± 0.9 mM          | 99 %             |
|                        | 5 μM                 | 4.80 ± 0.07 μM         | 96 %             |
| LC−MS/MS               | 30 μM                | 29 ± 1 μM              | 97 %             |
|                        | 80 μM                | 78 ± 4 μM              | 98 %             |

*n = 3−5.*
the enzymatic trehalose assay or the HPLC-RID-based assay are less expensive than an LC−MS/MS system; a spectrophotometer equipped with a 96-well plate reader (for the enzymatic trehalose assay) costs approximately U.S. $50,000,46 and an HPLC system equipped with an RID (for the HPLC-RID-based assay) costs approximately U.S. $80,000.47

Although not directly evaluated in the present study, high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD) and gas chromatography−mass spectrometry (GC−MS) are also used for trehalose quantification.35−37 Both of these methods are highly sensitive for trehalose. HPAEC-PAD, like the trehalose enzymatic assay and the HPLC-RID-based assay, does not conclusively identify trehalose on the basis of its amperometric signal. In fact, one published report has concluded that HPAEC-PAD is not an appropriate method for the quantification of trehalose from complex biological samples such as plant extracts.36 Complex samples, like plant extracts, often contain other biological compounds that coelute with trehalose, leading to an overestimation of trehalose content.36 In contrast, GC−MS does allow for the identification of trehalose in biological samples; however, it requires the derivatization of trehalose with trimethylsilyl ethers, thus adding an extra, time-consuming step to this method and possibly introducing additional error.28,36 Therefore, HPAEC-PAD and GC−MS need to be used with caution when quantifying trehalose from complex biological samples.

In summary, the selection of a suitable assay for trehalose quantification will depend on the expected trehalose concentration in the sample (nanomolar, micromolar, or millimolar), the complexity of the sample, including the presence of interfering contaminants, and the type of instrumentation available. The work described herein thus provides guidance to trehalose researchers in their selection of the optimal method for their desired application.

■ CONCLUSIONS

In this study, three commonly used assays for the quantification of trehalose from biological samples were compared: an enzymatic trehalose assay, an HPLC-RID-based assay, and an LC−MS/MS-based assay. All assays were found to be accurate for the quantification of trehalose in a variety of biological matrices. The LC−MS/MS-based assay is the most sensitive assay, with an LOD of 22 nM and an LOQ of 28 nM trehalose, which are 2−3 and 4−5 orders of magnitude lower than those for the enzymatic trehalose assay or the HPLC-RID-based assay, respectively. Moreover, the LC−MS/MS-based assay was the only assay able to detect and quantify endogenous trehalose from OtsA/OtsB overproducer E. coli cells; the concentration of trehalose in lysates from these cells was too low to be detected by either the enzymatic trehalose assay or the HPLC-RID-based assay. Results from this study indicate that the LC−MS/MS-based assay is the most direct and sensitive method for the quantification of trehalose from biological samples; however, both the enzymatic trehalose assay and the HPLC-RID-based assay are reliable, cost-effective assays for applications not requiring the detection of nanomolar concentrations of trehalose. With the increasing interest in studying trehalose’s role in cellular protection, understanding the relative advantages and limitations of the different assays for quantifying trehalose from biological sources will enable researchers to select the method best suited to their desired application.

■ EXPERIMENTAL SECTION

Materials. D-(+)-Trehalose dihydrate (>99%) and LC−MS-grade ammonium acetate (>99%) were purchased from Fisher Scientific.13 C12-trehalose was purchased from Omicron Biochemicals (South Bend, IN). Solvents (acetonitrile and water) were of LC−MS/Optima grade and obtained from Fisher Scientific. Lysogeny broth (LB, Miller) and agar were obtained from Fisher Scientific. Roswell Park Memorial Institute (RPMI)-1640 medium was purchased from America Type Culture Collection (ATCC, Manassas, VA). Phosphate-buffered saline (PBS) was obtained from Fisher Scientific, and fetal bovine serum (FBS) was obtained from VWR. Trehalose Assay Kit (K-TREH) was purchased from Megazyme International (Ireland).

Enzymatic Trehalose Assay. The enzymatic trehalose assay (Trehalose Assay Kit) was completed in a 96-well plate following the manufacturer’s microplate assay procedure.44 Briefly, standard solutions containing varying concentrations of trehalose (25−100 μM) were prepared, and 20 μL of each of these solutions were used for creating the calibration curve. Next, 200 μL of distilled water, 20 μL of solution 1 (buffer), 10 μL of solution 2 (containing NADP+ and adenosine 5′-triphosphate), and 2 μL of solution 3 (containing the enzymes hexokinase and glucose-6-phosphate dehydrogenase) were added to the wells. The solutions were mixed, and the absorbance values at 340 nm (abs0) were obtained from these wells prior to addition of the enzyme trehalase. Suspension 4 (2 μL containing the enzyme trehalase) was then added to the wells, the solutions were mixed, and the reactions were allowed to incubate for 8 min at room temperature, after which the absorbance values were measured at 340 nm (abs1). Calibration curves were generated in Excel by plotting the background-corrected absorbance measurements (abs1−abs0) against trehalose concentration. Averages of the correct absorbance measurements for each standard were calculated and plotted against the known concentrations. These plots and the resulting best-fit equations were used for all further calculations (e.g., determining the LOD and LOQ, calculating the trehalose concentration of samples, etc.).

HPLC-RID Conditions. HPLC separation was achieved using a Waters high-performance carbohydrate column (250 × 4.6 mm², 4 μm) on an Agilent 1100 series HPLC system, including a degasser, quaternary pump, autosampler, and column compartment (maintained at 35 °C). The mobile phase was composed of water and acetonitrile. Isocratic elution conditions were varied to optimize the separation of trehalose, with an optimal flow rate of 1 mL/min and a percentage of acetonitrile of 77% over 30 min. The injection volume was 50 μL, and analyte detection was achieved using an Agilent 1260 Infinity series refractive index detector. RI signals for trehalose were analyzed using Agilent OpenLAB CDS ChemStation software; peaks were integrated manually for consistency. Calibration curves were generated in Excel by plotting the area of the RI signals against trehalose concentration. Averages of the areas for each standard were calculated and plotted against the known concentrations. These plots and the resulting best-fit equations were used for all further calculations (e.g., determining the LOD and LOQ, calculating the trehalose concentration of samples, etc.).

LC−MS/MS Conditions. LC separation was achieved using a Waters high-performance carbohydrate column (250 × 4.6 mm², 4 μm) on an Agilent 1200 series HPLC system, including
a degasser, binary pump, autosampler, and column compartment (maintained at 35 °C). The mobile phase was composed of water containing 2 mM ammonium acetate (A) and acetonitrile containing 2 mM ammonium acetate (B). Isocratic elution conditions were varied to optimize the separation of trehalose, with an optimal flow rate of 1 mL/min and a percentage of solvent B of 80–82% over 20 min. The injection volume was 40 μL, and analyte detection was achieved using an Agilent 6410B triple quadrupole mass spectrometer with an electrospray ionization source. The mass spectrometer was operated in selected reaction monitoring (SRM) mode, monitoring for the trehalose transitions of 360–163 m/z and 360–85 m/z and for the 13C12-trehalose transition of 377–209 m/z, all in positive mode. The MS/MS conditions are listed in Table S4 (Supporting Information).

SRM signals for trehalose and 13C12-trehalose were obtained and analyzed using Agilent MassHunter Quantitative Analysis software; peaks were integrated manually when necessary for consistency. Calibration curves were generated in the software by plotting the relative response (the trehalose signal divided by the maltose signal) against trehalose concentration. The software reports the accuracy for each point on the basis of the curve it produces. Only data points with accuracies between 80 and 120% were kept. Averages of the remaining responses for each standard were calculated and plotted against the known concentrations. These final plots and the resulting best-fit equations were used for any further calculations (e.g., determining the LOD and LOQ, calculating the trehalose concentration of samples, etc.).

*Escherichia coli* (E. coli) Lysate Preparation. *E. coli* cells (strain DHS\(\alpha\)) were a generous gift from K. Fox (Union College). *E. coli* cells were plated on LB (Miller)-agar plates and allowed to grow overnight at 37 °C. A single colony was picked from the plate, placed in 5 mL of sterile LB, and shaken (275 rpm) at 37 °C for 23 h. The cells were centrifuged (3000 rcf × 10 min), and the supernatant was removed. The cell pellet was resuspended in PBS (5 mL), centrifuged (3000 rcf × 10 min), and the supernatant was removed. This process was repeated one more time, after which the cell pellet was frozen at −80 °C until cell lysis.

The wild-type, trehalose knockout (Δ\(\alpha\)ts\(\alpha\)) of the *E. coli* strain MC4100 was created by Kaasen et al. and was a generous gift from P. Woodruff (University of Southern Maine). The Δ\(\alpha\)ts\(\alpha\) strain was plated on LB (Miller)-agar plates and allowed to grow overnight at 37 °C. A single colony was picked from the plate, placed in 5 mL of sterile LB, and shaken (275 rpm) at 37 °C for 23 h. The cells were centrifuged (3000 rcf × 10 min), and the supernatant was removed. The cell pellet was resuspended in PBS (5 mL), centrifuged (3000 rcf × 10 min), and the supernatant was removed. This process was repeated one more time, after which the cell pellet was frozen at −80 °C until cell lysis.

For cell lysis, DH\(\alpha\)ts\(\alpha\) cells (1.4 × 10^8 cells) or Δ\(\alpha\)ts\(\alpha\) cells (1.5 × 10^10 cells) were resuspended in 1.00 mL of water, and the OtsA/OtsB overproducer cells (4 × 10^10 cells) were resuspended in 150 μL of water. These cell suspensions were heated at 100 °C for 20 min, after which they were centrifuged (16 000 rcf × 10 min) at 4 °C. The supernatant was removed and kept frozen at −80 °C until further use.

**Jukrat Cell Lysate Preparation.** Jukrat cells (clone E6-1) were purchased from ATCC and grown in RPMI-1640 medium supplemented with 10% FBS. The cells were maintained in a 5% CO_2_, water-saturated environment at 37 °C. The cells were centrifuged (300 rcf × 5 min), and membrane-bound proteins were removed. The cell pellet was resuspended in PBS (5 mL), centrifuged (300 rcf × 5 min), and the supernatant was removed. This process was repeated one more time, after which the cell pellet was frozen at −80 °C until cell lysis.

**Method Sensitivities.** Enzymatic Trehalase Assay. To determine the instrument LOD and LOQ, for the enzymatic trehalase assay, standards ranging from 25 to 400 μM trehalose were prepared and subjected to the enzymatic trehalase assay as described above (20 μL sample volume) to generate a calibration curve. A solution of 20 μM trehalose was prepared and subjected to the enzymatic trehalase assay (20 μL sample volume). The absorbance values at 340 nm (abs_\(\lambda_{340}\)) for the 20 μM trehalose solution were measured eight times and then 2 μL of suspension 4 (containing the enzyme trehalase) was added to the wells. The solutions were mixed, and the reactions were allowed to incubate for 8 min at room temperature, after which the absorbance values at 340 nm (abs_\(\lambda_{340}\)) were measured eight times. The standard deviation (SD) of the corrected absorbance measurements (abs_\(\lambda_{340}\) − abs_\(\lambda_{340}\) for all eight replicates of the 20 μM trehalose sample was calculated. The instrument LOD was calculated using the equation 3 × SD/m, where m is the slope of the trehalose calibration curve. The instrument LOQ was calculated using the equation 10 × SD/m, where m is the slope of the trehalose calibration curve.

To determine the MDL and MQL for this method, standards ranging from 25 to 400 μM trehalose were prepared and
subjected to the enzymatic trehalase assay as described above (20 μL sample volume) to generate a calibration curve. A total of 10 solutions of 20 μM trehalose were prepared and subjected to the enzymatic trehalase assay (20 μL sample volume). The standard deviation (SD) of the corrected absorbance measurements for all 10 solutions of the 20 μM trehalose sample was calculated. The MDL was calculated using the equation 3 × SD/m, where m is the slope of the trehalose calibration curve. The MQL was calculated using the equation 10 × SD/m, where m is the slope of the trehalose calibration curve.

**HPLC-RID Assay.** To determine the instrument LOD and LOQ for the HPLC-RID assay, standards ranging from 1 to 100 mM trehalose were prepared in 1:1 acetonitrile/water and injected in triplicate (50 μL injection volume) onto the HPLC-RID to generate a calibration curve. Another solution of 2.5 mM trehalose was prepared in 1:1 acetonitrile/water and injected onto the HPLC-RID 10 times (50 μL sample volume). The standard deviation (SD) of the RI signals for all 10 replicates of the 2.5 mM trehalose sample was calculated. The instrument LOD was calculated using the equation 3 × SD/m, where m is the slope of the trehalose calibration curve. The instrument LOQ was calculated using the equation 10 × SD/m, where m is the slope of the trehalose calibration curve.

To determine the MDL and MQL for this method, standards ranging from 1 to 100 mM trehalose were prepared in 1:1 acetonitrile/water and injected in triplicate (50 μL injection volume) onto the HPLC-RID to generate a calibration curve. A total of 10 solutions of 2.5 mM trehalose in 1:1 acetonitrile/water were prepared and injected onto the HPLC-RID 10 times (50 μL sample volume). The standard deviation (SD) of the RI signals for all 10 solutions of the 2.5 mM trehalose sample was calculated. The MDL was calculated using the equation 3 × SD/m, where m is the slope of the trehalose calibration curve. The MQL was calculated using the equation 10 × SD/m, where m is the slope of the trehalose calibration curve.

**LC–MS/MS Assay.** To determine the LOD and LOQ for the LC–MS/MS method, standards ranging from 0.2 to 2.0 μM trehalose (all containing 5 μM 13C12-trehalose) were prepared in 1:1 acetonitrile/water and injected (40 μL injection volume) onto the LC–MS/MS to generate a calibration curve. Another solution of 0.5 μM trehalose and 5 μM 13C12-trehalose in 1:1 acetonitrile/water was prepared and injected six times onto the LC–MS/MS (40 μL injection volume). The standard deviation of the relative responses to trehalose for all six injections for the 0.5 μM trehalose sample was calculated. This standard deviation was multiplied by 3 (3SD), and the instrument LOD was calculated using 3SD and the equation from the trehalose calibration curve. The instrument LOQ was calculated by multiplying the standard deviation by 10 (10SD), and 10SD was then used in the equation from the trehalose calibration curve.

To determine the MDL and MQL for this method, standards ranging from 0.2 to 2.0 μM trehalose (all containing 5 μM 13C12-trehalose) were prepared in 1:1 acetonitrile/water and injected (40 μL injection volume) onto the LC–MS/MS to generate a calibration curve. A total of six solutions of 0.5 μM trehalose and 5 μM 13C12-trehalose were prepared in 1:1 acetonitrile/water, and each solution was injected onto the LC–MS/MS (40 μL injection volume). The standard deviation of the relative responses to trehalose for all six solutions of 0.5 μM trehalose was calculated. This standard deviation was multiplied by 3 (3SD), and the MDL was calculated using 3SD and the equation from the trehalose calibration curve. The MQL was calculated by multiplying the standard deviation by 10 (10SD), and 10SD was then used in the equation from the trehalose calibration curve.

**Method Validation.** To determine the accuracies of these methods for calculating trehalose concentrations in samples, a series of solutions containing known concentrations of trehalose (see Table 2 for trehalose concentrations) were prepared in water for the enzymatic trehalase assay or 1:1 acetonitrile/water for the HPLC-RID and LC–MS/MS assays. These solutions were treated as samples for the three assays, and the concentration of trehalose was calculated for each sample using the equation obtained from the trehalose calibration curve for each assay. The percent recovery was calculated using eq 1

\[
\text{% recovery} = \left( \frac{\text{calculated trehalose concentration}}{\text{known concentration}} \right) \times 100\% 
\]

(1)

The accuracies of these methods were also evaluated in both E. coli (strain DH5α) lysates and Jurkat cell lysates using a spike recovery test. Known amounts of trehalose (see Tables 3 and 4 for trehalose concentrations) were spiked into these lysates, and samples were prepared in either cell lysates (for the enzymatic trehalase assay) or in a mixture of 1:1 acetonitrile/cell lysates (for the HPLC-RID and LC–MS/MS assays). These spiked lysates were treated as samples for the three assays. For the enzymatic trehalase assay, the standards for the trehalose calibration curve were prepared in water. For the HPLC-RID assay, the standards for the trehalose calibration curve were prepared in a mixture of 1:1 acetonitrile/water. For the LC–MS/MS assay, the standards for the trehalose calibration curve were prepared in a mixture of 1:1 acetonitrile/cell lysates (E. coli or Jurkat, depending on the lysates that had been spiked with trehalose) to produce matrix-matched standards. The concentration of trehalose was calculated for each lysate sample using the equation obtained from the trehalose calibration curve for each assay. The percent recovery for each lysate sample was calculated using eq 1.

**Method Application.** To apply this method to the quantification of endogenous trehalose from a biological source, lysates from the arabinose-inducible OtsA/OtsB overproducer of the E. coli strain MC4100 were analyzed.

For the enzymatic trehalase assay, the cell lysates were used as is; for the HPLC-RID assay, the cell lysates were diluted 1:1 with acetonitrile; and for the LC–MS/MS assay, the cell lysates were diluted with acetonitrile and water and spiked with 13C12-trehalose to give lysates containing 10 μM 13C12-trehalose in 1:1 acetonitrile/lysates. For the enzymatic trehalase assay, the standards for the trehalose calibration curve were prepared in water. For the HPLC-RID assay, the standards for the trehalose calibration curve were prepared in a mixture of 1:1 acetonitrile/water. For the LC–MS/MS assay, the standards for the trehalose calibration curve were prepared in a mixture of 1:1 acetonitrile/lysates. To determine the extracellular trehalose concentration, the bacterial volume as calculated from the OD600 value (estimating the internal volume of an E. coli cell as 0.7 μm3).

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SRM chromatogram showing the absence of a signal for lactose for the LC–MS/MS-based assay (Figure S1); signals for trehalose at the instrument LOD for all three assays (Figure S2); trehalose calibration curve for 1–100 mM for the enzymatic trehalose assay (Figure S3); SRM chromatograms for $\Delta$otsA $E.\ coli$ lysates, $\Delta$otsA $E.\ coli$ lysates spiked with 5 $\mu$M trehalose, and OtsA/OtsB overproducer $E.\ coli$ lysates (Figure S4); trehalose concentrations, corrected absorbances, standard deviations (SD), and percent relative standard deviations (% relative SD) for trehalose standards using the three assays (Tables S1–S3); and MS/MS conditions used to monitor trehalose and $^{13}$C$_2$-trehalose for the LC–MS/MS-based assay (Table S4) (PDF)

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