A liquid chromatography-tandem mass spectrometry assay for the detection and quantification of trehalose in biological samples

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Abstract

Trehalose is an important disaccharide that is used as a cellular protectant by many different organisms, helping these organisms better survive extreme conditions, such as dehydration, oxidative stress, and freezing temperatures. Methods to detect and accurately measure trehalose from different organisms will help us gain a better understanding of the mechanisms behind trehalose's ability to act as a cellular protectant. A liquid chromatography – tandem mass spectrometry (LC-MS/MS) assay using selected reaction monitoring mode for the detection and quantification of trehalose using maltose as an internal standard has been developed. This assay uses a commercially available LC column for trehalose separation and a standard triple quadrupole mass spectrometer, thus allowing many scientists to take advantage of this simple assay. The calibration curve from 3 – 100 μ M trehalose was fit best by a single polynomial. This LC-MS/MS assay directly detects and accurately quantifies trehalose, with an instrument limit of detection (LOD) that is 2 – 1000 times more sensitive than the most commonly-used assays for trehalose detection and quantification. Furthermore, this assay was used to detect and quantify endogenous trehalose produced by *Escherichia coli* (*E. coli*) cells, which were found to have an intracellular concentration of 8.5 ± 0.9 mM trehalose. This method thus shows promise for the reliable detection and quantification of trehalose from different biological sources.

Keywords: trehalose; LC-MS/MS; biological samples; cellular protectant; quantification

1. Introduction

Trehalose, or α -D-glucose(1 $\rightarrow$ 1) α -D-glucose (**1**, Fig. 1a), is a disaccharide biosynthesized by many different organisms, including fungi, plants, insects, bacteria, and eukaryotic microorganisms [1-5]. Both intra- and extracellular trehalose allows these organisms to better survive environmental stresses such as reactive oxygen species, freezing temperatures, and almost complete drying [1-7]. It has been hypothesized that trehalose provides protection by replacing the water inside cells, thus stabilizing proteins and lipid membranes via hydrogen-bonding, and by forming a stable glass (a liquid of high viscosity) at room temperature, thus reducing the rates of damaging biochemical reactions [4, 8]. These protective properties of trehalose, coupled with its low cellular toxicity, have generated interest in using this carbohydrate as a general cellular protectant [1, 3-9].

To function as a cellular protectant, trehalose must be present inside cells [1-7]. Certain desiccation-tolerant plants, such as *Selaginella lepidophylla*, accumulate intracellular trehalose at levels up to 12% of their dry weight during periods of drought [10]. *Escherichia coli* (*E. coli*) cells increase their production of intracellular trehalose when exposed to cold temperatures, thus allowing these bacteria to better survive cold shock [11]. Mammalian cells, unfortunately, do not naturally biosynthesize trehalose. When mammalian cells are loaded with exogenous trehalose, however, they have greatly improved survival rates after freezing or drying compared to mammalian cells that contain no intracellular trehalose [7, 12]. Exogenously-administered trehalose has also been shown to provide neuroprotective effects in animal models of Huntington disease, Parkinson disease, and amyotrophic lateral sclerosis (ALS) [13-16]. Although there are several hypotheses that describe how trehalose exerts its protective effects, the exact mechanisms by which trehalose protects cells are not well characterized. To better understand the role of

trehalose in cellular protection, it is crucial to be able to detect and accurately quantify trehalose from organisms exposed to various environmental conditions.

A number of methods exist for the detection and quantification of trehalose from biological sources. One of these assays uses anthrone, a reagent that reacts with trehalose to give a colorimetric change that can be quantified spectrophotometrically [17]. However, anthrone reacts with sugars other than trehalose, which may lead to an overestimation of trehalose concentration [17-20]. Another method for detecting and quantifying trehalose uses the enzyme trehalase, which cleaves trehalose into two glucose monomers [20, 21]. An enzymatic assay is then used to determine the concentration of glucose, from which the original concentration of trehalose can be calculated after subtracting out the amount of endogenous glucose found in the sample. Unfortunately, this assay may also overestimate trehalose concentrations since one commonly-used trehalase enzyme from porcine kidney also hydrolyzes the disaccharide maltose as well as trehalose [20]. Moreover, both the anthrone reagent and the trehalase enzymatic assay do not measure trehalose levels directly. High performance liquid chromatography (HPLC), coupled with detection by a pulsed electrochemical detector or a refractive index detector, has also been used to detect and quantify trehalose [19, 22-25]. One problem with these HPLC methods is that there is no way to conclusively identify trehalose based on the refractive index or electrochemical signals. Furthermore, trehalose can coelute with other biological compounds, thus causing an overestimation of the trehalose concentration [20, 26]. Gas chromatography-mass spectrometry (GC-MS) provides high sensitivity and allows 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 [26, 27].

In this paper, a simple liquid chromatography-tandem mass spectrometry (LC-MS/MS)-based assay for the direct detection and accurate quantification of endogenous trehalose from biological samples using maltose (**2**, Fig. 1a) as an internal standard **is described**. LC-MS/MS is routinely used as a method for quantifying analytes from biological samples [28-30]. Moreover, LC-MS/MS analysis allows for the conclusive identification of the compound or compounds present in each chromatographic peak, thus ensuring that the signal used for quantification is produced by the target compound, rather than an unknown interfering compound. Although two LC-MS-based assays for the detection and/or quantification of trehalose have been reported in the literature, neither method detected nor quantified endogenous trehalose from a biological sample. Additionally, the reported methods rely either on a custom-produced, hand-packed silica column for trehalose separation or an expensive ion trap mass spectrometer [30, 31]. **This** LC-MS/MS-based assay for the detection and quantification of trehalose uses a commercially available LC column for HPLC separation and a standard triple quadrupole mass spectrometer, thus allowing many scientists to take advantage of this simple assay. **The method described here** detects and accurately quantifies trehalose with high sensitivity and selectivity, with an instrument limit of detection (LOD) of 28 nM (560 fmol) trehalose and an instrument limit of quantification (LOQ) of 110 nM (2.3 pmol) trehalose. These values are 2 – 400 times lower than those reported for almost all trehalose detection and quantification assays and will allow researchers to use fewer cells when measuring trehalose concentrations in biological samples. Furthermore, **this method is** used to detect and quantify endogenous trehalose from *E. coli* cells, which has never been accomplished using an LC-MS/MS-based assay. The LC-MS/MS assay reported in this paper will be useful for the detection and quantification of trehalose from a variety of biological sources, thus aiding in the study of the role that trehalose plays in cellular protection.

2. Methods and Materials

2.1. Materials

(D)-(+)-Trehalose dihydrate (>99%), D-(+)-maltose monohydrate (99%), and LC-MS grade ammonium acetate ($\geq 99\%$) were purchased from Fisher Scientific. Solvents (acetonitrile and water) were of LC-MS/Optima grade and obtained from Fisher Scientific. LB Broth (Miller), agar, ampicillin, (D)-(-)-arabinose, and tetracycline were obtained from Fisher Scientific. RPMI-1640 medium was purchased from America Type Culture Collection (ATCC, Manassas, VA). Phosphate-buffered saline (PBS) and fetal bovine serum (FBS) were obtained from Fisher Scientific.

2.2. *Escherichia coli* (*E. coli*) lysate preparation

E. coli cells (strain DH5 α) were a generous gift from K. Fox (Union College). *E. coli* cells were plated on LB Broth (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 broth, and shaken (275 rpm) at 37 °C for 23 h. The cells were centrifuged (3000 rcf $\times$ 10 min), and the supernatant was removed. The cell pellet was resuspended in PBS (5 mL), centrifuged (3000 rcf $\times$ 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 *otsA*) of the *E. coli* strain MC4100 was created by Kaasen *et al.* and was a generous gift from P. Woodruff (University of Southern Maine) [32]. The Δ *otsA* strain was plated on LB Broth (Miller)-agar plates containing 50 μ M tetracycline and

allowed to grow overnight at 37 °C. A single colony was picked from the plate, placed in 5 mL of sterile LB broth containing 50 µM tetracycline, and shaken (275 rpm) at 37 °C for 16 h. 1.5 mL of the *ΔotsA* strain starter culture were then used to inoculate 100 mL of sterile LB broth containing 50 µM tetracycline, and the culture was shaken (275 rpm) at 37 °C for 4 h. The cells were centrifuged (3000 rcf × 10 min), and the supernatant was removed. The cell pellet was resuspended in PBS (90 mL), centrifuged (3000 rcf × 10 min), and the supernatant was removed. The cell pellet was then resuspended in PBS (5 mL) and centrifuged (3000 rcf × 10 min) again. The supernatant was removed, and the cell pellet was frozen at -80 °C until cell lysis.

The arabinose-inducible OtsA/OtsB overproducer of the *E. coli* strain MC4100 was created by Frederick *et al.* and was a generous gift from P. Woodruff (University of Southern Maine) [33]. The arabinose-inducible OtsA/OtsB overproducer strain was plated on LB Broth (Miller)-agar plates containing 100 µM ampicillin and allowed to grow overnight at 37 °C. A single colony was picked from the plate, placed in 5 mL of sterile LB broth containing 100 µM ampicillin, and shaken (275 rpm) at 37 °C for 16 h. 200 µL of the starter culture were used to inoculate 5 mL of sterile LB broth containing 100 µM ampicillin, and the culture was shaken (275 rpm) at 37 °C until OD₆₀₀ ~ 0.6. Arabinose (1 mM) was used to induce the OtsA/OtsB overproducer cells, and the culture was shaken (275 rpm) at 37 °C for 22 h. The following day, another aliquot of arabinose (1 mM) was added to the OtsA/OtsB overproducer cells, and the culture was shaken (275 rpm) at 37 °C for 6 h longer. The cells were centrifuged (3000 rcf × 10 min), and the supernatant was removed. The cell pellet was resuspended in PBS (10 mL), centrifuged (3000 rcf × 10 min), and the supernatant was removed. The cell pellet was then resuspended in PBS (10 mL) and centrifuged (3000 rcf × 10 min) again. The supernatant was removed, and the cell pellet was frozen at -80 °C until cell lysis.

For cell lysis, DH5 α cells (1.4×10^9 cells), $\Delta otsA$ cells (1.5×10^{10} cells), or OtsA/OtsB overproducer cells (1.5×10^{10} cells) were resuspended in 1.00 mL of water, and the cell suspensions were heated at 100 °C for 20 minutes, after which they were centrifuged (16,000 rcf $\times$ 10 min) at 4 °C. The supernatant was removed and kept frozen at -80 °C until further use.

2.3. Jurkat cell lysate preparation

Jurkat 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% CO₂, water-saturated environment at 37 °C. Cells were centrifuged (300 rcf $\times$ 5 min), and the supernatant was removed. The cell pellet was resuspended in PBS (5 mL), centrifuged (300 rcf $\times$ 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. The cells (1×10^6 cells) were resuspended in 100 μ L of water, and the cell suspension was heated at 100 °C for 15 minutes, after which it was centrifuged (16,000 rcf $\times$ 10 min) at 4 °C. The supernatant was removed and kept frozen at -80 °C until further use.

2.4. LC-MS/MS conditions

HPLC separation was achieved using a Waters High Performance Carbohydrate Column (250 $\times$ 4.6 mm, 4 μ m) 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 and maltose, with an optimal flow rate of 1 mL/min and a percentage of solvent B of 80 - 85 % over 30 minutes. With extended column use, the retention times of the sugars decrease for the Waters

High Performance Carbohydrate column, which leads to a decrease in the resolution of the signals for trehalose and maltose [34]. To compensate for these decreases, the percentage of acetonitrile was increased as the age of the column increased. Injection volumes varied from 15 – 40 μL , depending on the concentration of trehalose in the standard or sample. Analyte detection was achieved using an Agilent 6410B triple quadrupole mass spectrometer with an electrospray ionization (ESI) source. The mass spectrometer was operated in selected reaction monitoring (SRM) mode, monitoring for the trehalose transition of 365 m/z to 203 m/z and for the maltose transition of 365 m/z to 203 m/z , both in positive mode. The MS/MS conditions are listed in Table 1.

SRM signals for trehalose and maltose 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) vs. trehalose concentration. The software reports the accuracy for each point based on the curve it produces. Only data points with accuracies between 80 – 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 (i.e., determining the limit of detection and the limit of quantification, calculating the trehalose concentration of samples, etc.).

2.5. Method Sensitivity

To determine the instrument limit of detection (LOD) and limit of quantification (LOQ) for this method, standards ranging from 0.1 to 2.0 μM trehalose (all containing 10 μM maltose) were prepared in 1 : 1 acetonitrile : water and injected in duplicate (20 μL injection volume) onto

the LC-MS/MS to generate a calibration curve. Another solution of 0.3 μM trehalose and 10 μM maltose in 1 : 1 acetonitrile : water was prepared and injected eight times onto the LC-MS/MS (20 μL injection volume). The standard deviation of the relative responses to trehalose for all eight injections for the 0.3 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 method detection limit (MDL) and the method quantification limit (MQL) for this method, standards ranging from 0.1 to 2.0 μM trehalose (all containing 10 μM maltose) were prepared in 1 : 1 acetonitrile : water and injected in duplicate (20 μL injection volume) onto the LC-MS/MS to generate a calibration curve. Five solutions of 0.3 μM trehalose and 10 μM maltose were prepared in 1 : 1 acetonitrile : water, and each solution was injected onto the LC-MS/MS (20 μL injection volume). The standard deviation of the relative responses to trehalose for all five solutions of 0.3 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.

2.6. Method Validation

In order to determine the accuracy of our method for calculating trehalose concentrations in samples, a series of solutions containing known concentrations of trehalose (5, 10, 20, 50, and 90 μM) and 10 μM maltose were prepared in 1 : 1 acetonitrile : water and injected in triplicate onto the LC-MS/MS (40 μL injection volume). The concentration of trehalose was calculated for each

sample using the relative response of trehalose and the equation obtained from the trehalose calibration curve. The percent recovery was calculated using equation 1:

$$\% \text{ recovery} = \left(\frac{\text{calculated trehalose concentration}}{\text{known trehalose concentration}} \right) \times 100 \quad (1)$$

The accuracy of the method was also evaluated in both *E. coli* (strain DH5 α) lysates and Jurkat cell lysates using a spike recovery test. Known amounts of trehalose and maltose were spiked into these lysates, giving trehalose concentrations of 5, 20, and 80 μ M and a maltose concentration of 10 μ M in a mixture of 1 : 1 acetonitrile : cell lysates. These spiked lysates were then injected onto the LC-MS/MS ($n = 3-14$, depending on the experiment, 40 μ L injection volume). In one set of experiments, the standards for the trehalose calibration curve were prepared in a mixture of 1 : 1 acetonitrile : water. In another set of experiments, 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). The concentration of trehalose was calculated for each lysate sample using the relative response of trehalose and the equation obtained from the appropriate trehalose calibration curve. The percent recovery for each lysate sample was calculated using equation 1.

2.7. 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 [33]. The lysates were diluted with acetonitrile and water to give lysates containing 10 μ M maltose in 1 : 1 acetonitrile : lysates. Standards for the calibration curve were prepared in a mixture of 1 : 1 acetonitrile: Δ otsA lysates, which contain no trehalose. The standards and lysates were injected onto the LC-MS/MS ($n = 8$, 40 μ L injection volume), and the concentration of

trehalose was calculated for the OtsA/OtsB overproducer lysates using the relative response of trehalose and the equation obtained from the trehalose calibration curve. The endogenous intracellular trehalose concentration for the OtsA/OtsB overproducer cells was calculated by taking the total moles of trehalose in the lysates calculated from the trehalose calibration curve and dividing by the total bacterial volume as calculated from the OD₆₀₀ value (estimating the internal volume of an *E. coli* cell as 0.7 μm^3) [33].

3. Results and Discussion

3.1. Development of the LC-MS/MS assay

To develop a straightforward LC-MS/MS-based assay for the detection and quantification of trehalose from biological samples, it was crucial to demonstrate that trehalose could be separated from other disaccharides and detected using a triple quadrupole mass spectrometer with electrospray ionization (ESI-MS/MS). A solution containing the disaccharides sucrose, maltose, lactose, and trehalose was injected onto a Waters High Performance Carbohydrate column using an Agilent 1200-series HPLC, and the sugars were detected using an Agilent 6410B triple quadrupole mass spectrometer, operated in selected reaction monitoring (SRM) mode, monitoring for the disaccharide transitions of 365 m/z to 203 m/z in positive mode. These transitions were chosen for each disaccharide using Agilent MassHunter Optimizer software. The trehalose transition of 365 m/z to 203 m/z was the only transition detected for trehalose using the MassHunter Optimizer software (Supplementary Material, Fig. S-1); the m/z value of 365 corresponds to the $[\text{M} + \text{Na}^+]$ trehalose parent ion, and the m/z value of 203 corresponds to the $[\text{M} + \text{Na}^+]$ glucose daughter ion (Fig. 1b). As can be seen in Fig. 2, these conditions give good detection and separation of all four disaccharides.

To account for the run-to-run variability of electrospray ionization, an appropriate internal standard for the accurate and reliable quantification of trehalose was chosen. Maltose, or α -D-glucose(1 $\rightarrow$ 4) α -D-glucose (**2**, Fig. 1a), was selected as an internal standard since it is similar in structure and polarity to trehalose. Maltose is formed when starch is hydrolyzed; therefore, plant samples can contain endogenous maltose, which may preclude the use of maltose as an internal standard for trehalose quantification in plants [35]. There are a variety of methods scientists can employ to overcome this issue; for example, the concentration of endogenous maltose in control plant samples can be quantified and subtracted from the maltose added as an internal standard. Alternatively, mass-labeled trehalose, such as trehalose containing ^2H (deuterium) or ^{13}C , can be used as an internal standard. One significant drawback to using mass-labeled trehalose is its cost; $^{13}\text{C}_{12}$ -trehalose costs \$4400/gram, whereas maltose only costs \$0.52/gram (U.S. Dollars) [36, 37].

As seen in Fig. 2, maltose is well separated from trehalose and is easily detected using this method, which allows for the reliable quantification of both disaccharides. Interestingly, commercial samples of trehalose and maltose are contaminated with the disaccharide sucrose (data not shown). Sucrose is a common disaccharide that also has a transition of 365 m/z to 203 m/z in positive mode (Fig. 2). Furthermore, commercial samples of trehalose and maltose are generally prepared from extracts of fungi (e.g., *S. cerevisiae*) or plants (e.g., potatoes) [38]. Sucrose is also found in these organisms; therefore, it can be found as a minor contaminant in commercial samples of trehalose and maltose. Since this method is able to easily separate these disaccharides from one another, the presence of small amounts of sucrose in the trehalose and maltose standards does not cause problems when quantifying trehalose concentrations.

Having determined that trehalose can be easily separated and detected using this LC-MS/MS method, a calibration curve for trehalose using maltose as an internal standard was constructed (Fig. 3). The relative responses for trehalose are all very similar, with relative standard deviations ranging from 1.5 – 14 % (Supplementary Material, Table T-1). Interestingly, the relative responses for trehalose using maltose as an internal standard are best fit by a single polynomial (Fig. 3). When the standards are divided into two ranges, a low range (0.5 - 10 μ M trehalose) and a high range (10 - 100 μ M trehalose), the low range responses are fit best by a single polynomial, but the high range responses are fit well by either a linear or a single polynomial (Supplementary Material, Fig. S-2). Although a linear calibration curve over the entire range of trehalose concentrations is desirable, automated quantification software tools, such as Agilent MassHunter Workstation Quantitative Analysis software, allow for the facile generation and use of single polynomial calibration curves. Furthermore, there is precedent for single polynomial fits for LC-MS/MS data [39-45]. 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, as well as the response of an LC-MS/MS instrument to glycopyrrolate in human plasma samples [40, 42]. For this assay, polynomial calibration curves are the best way to model the LC-MS/MS responses to trehalose using maltose as an internal standard. The polynomial fits produce R^2 values of greater than 0.996, whereas linear fits produce lower R^2 values (Supplementary Material, Fig. S-2). To force linear fits to the curved data would introduce accuracy errors [41, 42].

3.2. Sensitivity of the LC-MS/MS assay

Once it was determined that reproducible calibration curves for trehalose using maltose as an internal standard could be generated, the assay's sensitivity was 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 the LC-MS/MS instrument [30]. The LC-MS/MS is very sensitive for trehalose, giving an instrument LOD of 28 nM (560 fmol) and an instrument LOQ of 110 nM (2.3 pmol) for trehalose. The trehalose peak at the instrument LOD is symmetrical and robust, and the signal-to-noise ratio (S/N) at the instrument LOD is consistently greater than 60 (Supplementary Material, Fig. S-3). At the instrument LOQ, the S/N is consistently greater than 150, and again shows a robust, symmetrical peak for trehalose (Supplementary Material, Fig. S-3).

When compared to commonly-used assays for the detection and quantification of trehalose, the instrument LOD and instrument LOQ for this LC-MS/MS assay are exceptional. Spectrophotometric assays that use trehalase and other enzymes to determine the concentration of trehalose from a biological sample generally have an instrument LOQ of 50 - 100 μ M trehalose, which is more than three orders of magnitude greater than the instrument LOQ for the LC-MS/MS assay described in this paper [20, 21]. These assays also do not measure trehalose levels directly; they calculate trehalose concentrations from glucose concentrations measured from the hydrolysis of trehalose to glucose by the enzyme trehalase [20, 21]. An LC-MS/MS instrument is also more sensitive for trehalose detection and quantification than assays that use an HPLC coupled to a pulsed electrochemical detector or a refractive index detector, which can have instrument LOD values that range from 0.05 – 26 μ M trehalose, depending on the detector [19, 22-25]. The instrument LOD for this method is approximately 2 – 1000 times more sensitive than the instrument LODs for HPLC assays. Moreover, neither trehalase enzymatic assays nor HPLC assays conclusively identify trehalose as the compound being quantified. Detection of trehalose

using a GC-MS instrument gives a reported instrument LOD of 10 μM , which is orders of magnitude higher than the instrument LOD obtained using the LC-MS/MS method developed here [27]. Furthermore, the use of a GC-MS requires trehalose to be modified with trimethylsilyl ethers, which may not be quantitative, thus adding an additional step and a possible source of error to this method [26, 27]. Finally, there have been two reported methods for detecting and/or quantifying trehalose using LC-MS/MS instruments [30, 31]. In one method, the instrument LOD and LOQ values range from 0.26 - 0.52 μM , which are an order of magnitude higher than the values obtained using the method described in this paper. Furthermore, this method utilizes a less common, more expensive ion trap mass spectrometer [30]. In another method, the reported instrument LOD for trehalose is 8.5 nM, which is slightly lower than our instrument LOD; however, the reported assay requires the use of a specialized, hand-packed silica column for trehalose separation and an expensive ion trap mass spectrometer for trehalose detection [31]. Additionally, neither of these reported LC-MS/MS assays was able to detect or quantify endogenous trehalose from biological samples [30, 31].

More conservative measures of the sensitivity of a method are the method detection limit (MDL) and method quantification limit (MQL). The MDL and the MQL for trehalose were determined using this LC-MS/MS method. The MDL and MQL are defined as the smallest amount of trehalose that can be reliably detected and quantified, respectively, using the LC-MS/MS method developed here, which includes sample preparation. The MDL and MQL for this LC-MS/MS method are 73 nM (1.5 pmol) and 250 nM (5 pmol) for trehalose, respectively (see Materials and Methods for a detailed description for how these values were calculated). Such low values for the MDL and MQL demonstrate that this LC-MS/MS method is very sensitive for trehalose, even when taking into account the error involved in sample preparation.

Having establishing the LOD, LOQ, MDL, and MQL for this assay, the dynamic range was determined. Since it had already been demonstrated that this assay could generate reliable calibration curves for trehalose concentrations spanning the range of 0.5 – 100 μM trehalose, trehalose standards were prepared that ranged from 0.1 – 1 μM trehalose and 100 – 1000 μM trehalose. For the 0.1 – 1 μM trehalose calibration curve, a single polynomial fit gives an R^2 value of 0.9961, thus demonstrating that this method can reliably detect and quantify trehalose at these low concentrations (Supplementary Material, Fig. S-4). At trehalose concentrations above 100 μM , this assay does not respond reliably to further increases in trehalose concentration; the relative response of the LC-MS/MS does not change significantly as the concentration of trehalose increases from 100 – 1000 μM (Supplementary Material, Fig. S-4). In fact, at trehalose concentrations above 100 μM , the SRM peaks are very broad and exhibit two maxima, suggesting that such high concentrations of trehalose are overloading the carbohydrate column used to separate trehalose from other compounds. Based on these experiments, it was concluded that the dynamic range of this assay spans three orders of magnitude, from 0.1 – 100 μM trehalose.

3.3. Accuracy of the LC-MS/MS assay

To determine this method's accuracy for the quantification of trehalose in samples with known concentrations, solutions containing a variety of trehalose concentrations across the range of the calibration curve were prepared and injected them onto the LC-MS/MS instrument. Trehalose concentrations of these samples were calculated from a calibration curve. The percent recoveries for these samples are very high, ranging from 96 – 105 %, thus demonstrating the accuracy of this method for the calculation of trehalose concentrations from unknown samples (Table 2).

The method's accuracy for the quantification of trehalose in more complex samples with known concentrations of trehalose was also evaluated. Known amounts of trehalose, corresponding to 5, 20, and 80 μM trehalose, and 10 μM maltose were spiked into lysates from either *E. coli* (strain DH5 α) or Jurkat cells (a human T-cell line). These concentrations span the range of the trehalose concentrations from a typical calibration curve of our assay. These spiked samples were injected onto the LC-MS/MS and the trehalose concentrations were calculated in these samples using a trehalose calibration curve from samples prepared in 1 : 1 acetonitrile : water. Depending on the expected trehalose concentration, a low (1 - 10 μM) or high (10 - 100 μM) range of trehalose standards was used to prepare the calibration curve. As seen in Table 3, there are significant decreases in the expected trehalose concentrations when complex matrices such as cell lysates are analyzed using our LC-MS/MS method. For *E. coli* lysates, the percent recoveries are moderate to high, ranging from 71% for an expected trehalose concentration of 5 μM to 94% for an expected trehalose concentration of 80 μM . For Jurkat cell lysates, the percent recoveries are much lower, ranging from 38 – 39 % for expected trehalose concentrations of 5 μM and 20 μM to 83% for an expected trehalose concentration of 80 μM . The change in the response of the LC-MS/MS to a specific compound when complicated samples are analyzed is well-documented and is known as the matrix effect [46]. The matrix effect is hypothesized to result from molecules originating from the sample mixture that co-elute with the compound of interest, causing either ionization suppression or enhancement in the mass spectrometer [46]. In this method, molecules found in cell lysates appear to cause suppression of the ionization signal for trehalose, thus decreasing the calculated trehalose concentrations from these samples.

To reduce the matrix effect observed with this method, trehalose standards used for the calibration curve were prepared in a mixture of 1 : 1 acetonitrile : cell lysates, with the lysates

matching the lysates being analyzed. When the trehalose calibration curve is produced using standards prepared in this manner, the percent recoveries for the spiked lysate samples, containing 5, 20, and 80 μM trehalose, are much higher for both *E. coli* and Jurkat cell lysates (Table 4). As in previous experiments, depending on the expected trehalose concentration, a low (1 - 10 μM) or high (10 - 100 μM) range of trehalose standards was used to prepare the calibration curve. For the *E. coli* lysates, the percent recoveries are very high, ranging from 98 – 114 %, when the trehalose standards are prepared in 1 : 1 acetonitrile : *E. coli* lysates. For Jurkat cell lysates, the percent recoveries are also very high, ranging from 94 – 102 %, when the trehalose standards are prepared in 1 : 1 acetonitrile : Jurkat cell lysates. These high percent recoveries demonstrate that this LC-MS/MS method for the quantitative determination of trehalose concentrations is very accurate, even when complex matrices such as cell lysates are analyzed. This method is thus expected to be generalizable to many different types of biological matrices, since very good recoveries in two different types of cell lysates are observed when the trehalose standards are prepared using the lysates of interest.

3.4. Detection and quantification of endogenous trehalose from bacterial cells

Finally, the ability of this LC-MS/MS assay to detect and accurately quantify endogenous trehalose from cells that produce intracellular trehalose was assessed. For this experiment, the arabinose-inducible OtsA/OtsB overproducer of the *E. coli* strain MC4100, which biosynthesize intracellular trehalose when arabinose is added to the medium used to grow the cells, was used [33]. Lysates from these cells were diluted with acetonitrile, spiked with 10 μM maltose, and injected onto the LC-MS/MS. Trehalose standards for the calibration curve (5 - 100 μM trehalose) were prepared using lysates from the trehalose knockout (ΔotsA) of the *E. coli* strain MC4100,

which are unable to biosynthesize trehalose and therefore contain no endogenous trehalose to interfere with quantitative analysis [32, 33]. A peak for trehalose was detected at the expected retention time for lysates from the *E. coli* cells (Supplementary Material, Fig. S-5), and the trehalose concentration in the *E. coli* lysates was calculated to be $40 \pm 4 \mu\text{M}$ ($n = 8$). This value is accurate because ΔotsA lysates spiked with $56 \mu\text{M}$ trehalose were calculated to contain $59 \pm 4 \mu\text{M}$ trehalose ($n = 4$) using the same trehalose calibration curve. Using the trehalose concentration from the cell lysates, as well as the total number of *E. coli* cells lysed (1.5×10^{10} cells), it was determined that the internal trehalose concentration inside one *E. coli* cell is $8.5 \pm 0.9 \text{ mM}$ ($n = 8$). These data represent the first time that endogenous trehalose from a biological sample has been successfully detected and quantified using LC-MS/MS. The detection and quantification of endogenous trehalose from these *E. coli* cells demonstrates the suitability of this LC-MS/MS assay for detecting and quantifying trehalose concentrations in complex biological samples.

4. Conclusions

A highly sensitive, selective, and generalizable LC-MS/MS-based assay for the direct detection and accurate quantification of trehalose in biological samples using maltose as an internal standard has been developed. This method allows for the conclusive identification of trehalose as the analyte being detected and quantified, rather than an unknown interfering compound. Furthermore, this method uses a commercially available LC column for separation and a standard triple quadrupole mass spectrometer. The assay detects and accurately quantifies trehalose in complex biological samples with high sensitivity and selectivity. This LC-MS/MS assay has also been used to detect and quantify endogenous trehalose from a biological sample (*E. coli* cells), which has never been accomplished using LC-MS/MS before. As trehalose is an important

disaccharide produced by many different organisms, this assay will prove useful in helping determine the function of this sugar as a cellular protectant in many different biological systems.

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Figure legends

Fig. 1. (a) Structures of trehalose (**1**) and maltose (**2**). (b) Parent trehalose ion ($m/z = 365$) and daughter glucose ion ($m/z = 203$) detected by our LC-MS/MS method.

Fig. 2. SRM chromatogram showing the separation of the analyte, trehalose (retention time = 21.6 min) from three other disaccharides, sucrose (retention time = 14.0 min), maltose (retention time = 17.9 min), and lactose (retention time = 20.4 min). Separation was achieved using a Waters High Performance Carbohydrate column (4.6 × 250 mm, 4 μm) held at 35 °C. The mobile phase was

composed of 18: 82 Buffer A : Buffer B with an isocratic elution over 25 minutes using a flow rate of 1.4 mL/min. All disaccharide concentrations were 50 μ M, and the injection volume was 15 μ L.

Fig. 3. A trehalose calibration curve using maltose as an internal standard (3 - 100 μ M) is best fit by a single polynomial. Standards containing varying concentrations of trehalose (0.5 - 100 μ M) with 10 μ M maltose as an internal standard were injected onto the LC-MS/MS in quintuplicate (mobile phase = 15: 85 Buffer A : Buffer B, injection volume = 40 μ L), and the relative responses for trehalose were calculated. Error bars represent the standard deviation (n = 5).

Parameter	Condition Used
Fragmentor Voltage	130 V (trehalose, 365→203 m/z)
	190 V (maltose, 365→203 m/z)
Collision Energy	20 eV (trehalose, 365→203 m/z)
	20 eV (maltose, 365→203 m/z)
Capillary Voltage	3.0 kV
Source Gas Temperature	350 °C
Gas Flow	12 L/min
Gas Pressure	60 psi
Collision Gas	Nitrogen

Table 1. MS/MS conditions used to monitor trehalose and maltose.

Expected [trehalose] (μM)	Calculated [trehalose] (μM) ^b	Percent Recovery
5	5.2 $\pm$ 0.9	104%
10	10.5 $\pm$ 0.6	105%
20	21 $\pm$ 1	105%
50	48 $\pm$ 1	96%
90	94.4 $\pm$ 0.6	105%

Table 2. Calculated concentrations of trehalose and percent recoveries from trehalose samples.^a

^a n = 3 ^b Samples containing known concentrations of trehalose were injected onto the LC-MS/MS (mobile phase = 15 : 85 Buffer A : Buffer B, injection volume = 40 μL), and their average concentrations were calculated from the equation obtained from a trehalose calibration curve.

Lysates	Expected [trehalose] (μM)	Calculated [trehalose] (μM) ^b	Percent Recovery
<i>E. coli</i> DH5 α	5	3.56 $\pm$ 0.01	71%
	20	16.4 $\pm$ 0.2	82%
	80	74.9 $\pm$ 0.9	94%
Jurkat	5	1.95 $\pm$ 0.02	39%
	20	7.7 $\pm$ 0.1	38%
	80	66.2 $\pm$ 0.7	83%

Table 3. Percent recoveries for trehalose spiked into *E. coli* DH5 α lysates or Jurkat cell lysates using a calibration curve derived from standards prepared in 1 : 1 acetonitrile : water.^a

^a n = 3 ^b Lysates were spiked with known concentrations of trehalose, injected onto the LC-MS/MS (mobile phase = 18 : 82 Buffer A : Buffer B, injection volume = 40 μL), and the average concentrations of trehalose from these lysates were calculated from the equation obtained from a trehalose calibration curve.

Lysates	Expected [trehalose] (μM)	Calculated [trehalose] (μM) ^b	Percent Recovery
<i>E. coli</i> DH5 α	5	5.2 $\pm$ 0.2	105%
	20	23 $\pm$ 2	114%
	80	78 $\pm$ 4	98%
Jurkat	5	5 $\pm$ 1	100%
	20	20 $\pm$ 2	100%
	80	75 $\pm$ 4	94%

Table 4. Percent recoveries for trehalose spiked into *E. coli* DH5 α lysates or Jurkat cell lysates using a calibration curve derived from standards prepared in 1 : 1 acetonitrile : lysates.^a

^a n = 5-14 ^b Lysates were spiked with known concentrations of trehalose, injected onto the LC-MS/MS (mobile phase = 15 : 85 Buffer A : Buffer B, injection volume = 40 μL), and the average concentrations of trehalose from these lysates were calculated from the equation obtained from a trehalose calibration curve.

Figure 1

Figure 2

Figure 3