Talanta 85 (2011) 2352–2360

Contents lists available at ScienceDirect

Talanta

journal homepage: www.elsevier.com/locate/talanta

Determination of post-culture processing with carbohydrates by MALDI-MS and TMS derivatization GC–MS

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ARTICLE INFO

Article history:
Received 19 May 2011
Received in revised form 20 July 2011
Accepted 21 July 2011
Available online 30 July 2011

Keywords:
Gas chromatography–mass spectrometry
Matrix-assisted laser desorption/ionization
Carbohydrate additives
Trimethylsilyl derivative

ABSTRACT

Biological materials generally require stabilization to retain activity or viability in a dry form. A number of industrial products, such as vaccines, probiotics and biopesticides have been produced as dry preparations. The same methods and materials used for stabilizing commercial microbial products may be applicable to preserving biothreat pathogens in a dry form. This is a likely step that may be encountered when looking at samples from terrorism attempts since only spores, such as those from *Bacillus anthracis*, are inherently stable when dried. The stabilizers for microbial preparations generally include one or more small carbohydrates. Different formulations have been reported for different industrial products and are often determined empirically. However sugar alcohols (mannitol and sorbitol) and disaccharides (lactose, sucrose and trehalose) are the common constituents of these formulations. We have developed an analytical method for sample preparation and detection of these simple carbohydrates using two complementary analytical tools, MALDI-MS and GC–MS. The native carbohydrates and other constituents of the formulation are detected by MALDI-MS as a screening tool. A longer and more detailed analysis is then used to specifically identify the carbohydrates by derivatization and GC–MS detection. Both techniques were tested against ten different types of stabilization recipes with *Yersinia pestis* cell mass cultured on different media types used as the biological component. A number of additional components were included in these formulations including proteins and peptides from serum or milk, polymers (e.g. poly vinyl pyrrolidone – PVP) and detergents (e.g. Tween). The combined method was characterized to determine several figures of merit. The accuracy of the method was 98% for MALDI-MS and 100% for GC–MS. The repeatability for detection of carbohydrates by MALDI-MS was determined to be 96%. The repeatability of compound identification by GC–MS was determined by monitoring variation in retention time, which is vital for identification of isomeric carbohydrates. The figures of merit illustrate an effective and accurate method for mono and disaccharide detection independent of formulation. This meets our primary goal for method development as small carbohydrates are among the most common stabilizers employed.

1. Introduction

The Amerithrax attacks of 2001 remain a vivid memory and an important case study in microbial forensics. Compared to many priority biological threat agents, *Bacillus anthracis* represents a unique situation as *Bacillus* endospores are intrinsically stable as dried preparations [1]. The remaining bacterial threat agents are largely Gram negative bacteria and do not create this stable structure [2]. Gram negative bacteria will typically lose viability after drying [3], so preservatives are typically required to retain stability [4]. This same challenge is faced when producing vaccines, immunoglobulins or probiotics in a dried form [5–10]. Additives or stabilizers may play a variety of roles as thermoprotectants during freezing and drying processes as well as lyoprotectants for long term dry storage.

In addition to increased shelf-life of many products, stabilized dry preparations have been used to make vaccines suitable for aerosol dissemination [11,12]. The aerosol route is still considered a likely route for mass dissemination of biological weapons in a mass casualty scenario [2]. The materials used for stabilizing commercial biological materials might also be used to create stable dry preparations of otherwise labile pathogens. If a Gram negative

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doi:10.1016/j.talanta.2011.07.073

bacteria or viral pathogen is intentionally disseminated in a dry form, it is also likely that additives will be used to maintain viability or infectivity.

The formulation to stabilize vaccines and probiotics may vary, but they often employ a thermoprotectant and lyoprotectant such as mannitol, sucrose, trehalose or lactose as well as water-soluble carbohydrate polymers, such as alginate [4,8,13–15]. These stabilizer compounds are not commonly intrinsic to the organisms. Therefore, methods to detect key formulation components added to provide stability may provide vital information on whether and how a preparation was processed following culture. These signatures may be key pieces in understanding the sophistication of the preparation method.

The analysis of carbohydrate monomers by gas chromatography (GC–MS) has been routinely used for bacterial profiling [16–18]. The method employs hydrolysis, reduction of native aldoses to alditols followed by acetylation with acetic anhydride [19,20]. This method provides semi-quantitative information on cellular content of neutral and amino sugars. Alternative carbohydrate derivatives for GC–MS analysis, such as trimethylsilyl (TMS), are commonly used [21,22]. A number of examples exist where TMS-derivatized carbohydrates from plants and food stuffs have been analyzed by GC–MS [21–25]. However these methods have not been widely applied to bacterial samples.

Gas chromatography is an effective tool for resolving the many isomeric forms of sugars found in biological systems such as the commonly encountered glucose, galactose and mannose. This is particularly important because the mass and fragmentation information provided by mass spectrometry cannot easily differentiate isomers, thus chromatographic resolution is critical. However derivatization is required to make mono and disaccharides amenable to GC–MS analysis. Other techniques have been proposed for sensitive analysis of complex carbohydrate mixtures including capillary electrophoresis (CE) and high performance liquid chromatography (HPLC) [26–30]. Only high performance anion exchange chromatography (HPAEC) and CE have been widely used for resolving a variety of sugar isomers.

Electrospray ionization (ESI) is the most common used soft ionization technique for native labile biomolecules prior to online MS analysis. ESI–MS has been widely used for the analysis of carbohydrates, although the ions are often detected as adducted species with lithium, sodium or acetate adducts [29–33]. While adduct formation is often critical for efficient detection, excessive ionic content competes for ionization and suppresses the analyte intensity, making confident sugar detection and characterization difficult. Unfortunately, the high ionic content required for separation of carbohydrates by CE or HPAEC creates a challenge for sensitive detection in combination with ESI–MS [29]. This challenge makes GC–MS of derivatized carbohydrates the more attractive choice to provide sufficient chromatographic resolution and sensitive detection for identification of mono and disaccharide mixtures.

Carbohydrate detection using adducting species has also been investigated using matrix-assisted laser desorption/ionization (MALDI-MS) [34]. MALDI-MS of the underivatized carbohydrates requires only a few minutes to prepare samples and perform analysis. Another advantage of this technique is that it requires only low microliter sample volumes for analysis. In addition to native carbohydrates, MALDI-MS has also been widely applied to the detection of other labile and low volatility compounds such as proteins, peptides, phospholipids and synthetic polymers [35–38]. As a result, this technique was chosen as a complementary technique to GC–MS that would also allow detection of other components present in a stabilization recipe, such as detergents.

Several example stabilization formulations have been drawn from the literature describing methods for creating dry stable preparations of biopesticides [39], probiotics [15,40], and vaccines or therapeutics [5–7,9,12,14]. These stabilization recipes use similar formulation stabilizers for lyophilization and cryopreservation of bacterial strains [3,4,41]. These example formulations are used as surrogates for stabilization of biothreat agents. The biological component used for the method development was an attenuated strain of *Yersinia pestis.* A combination of MALDI-MS and GC–MS based methods were developed for characterizing and differentiating stabilization recipes. The combination of these two techniques has been tested against several variables including varied growth media, cell purification methods and sample size. The resulting combination of analytical methods provided complementary information for detecting the presence and profile of carbohydrate stabilizers.

2. Methods and materials

2.1. Organism culture

An attenuated strain of *Y. pestis* was used for preparation of samples. The KIM D27 strain used lacks the chromosomal DNA region responsible for hemin storage. The hemin storage proteins are also part of the region responsible for the pigmentation negative phenotype (pgm−) and necessary for iron acquisition [42]. Isolated colonies were picked from a tryptic soy agar (TSA) without dextrose plate (Difco, Lansing MI) and transferred to 3 mL of media in a 15 mL snap-cap tube to create starter cultures. Four media recipes were used: Tryptic Soy Broth (TSB) without dextrose, Luria Bertani (LB) broth and Brain Heart Infusion (BHI) broth from Difco, Lansing MI and Glucose (G) broth. The latter recipe was prepared by combining 2 g/L of yeast extract (Difco, Lansing MI) with 1 g/L dextrose 2 g ammonium sulfate, 5 mg copper sulfate, 400 mg magnesium sulfate, 100 mg manganese sulfate, 1.2 g dibasic potassium phosphate, 100 mg zinc sulfate, 80 mg of calcium chloride and 1 mg iron sulfate per liter of water. The last two components were added following medium sterilization using filter sterilization.

Cultures were incubated overnight at 28 °C and 150 rpm. One hundred microliters of the starter culture was transferred into 3 replicate 1 L flasks containing 300 mL of the same medium. The flasks were incubated at 28 °C, shaken 150 rpm and monitored using absorbance readings at 10, 13.5 and 15.5 h. Colony counts were performed using serial dilutions in phosphate buffered saline at dilutions of 10^{-5} to 10^{-11}. The remainder of each culture was autoclaved. The cell mass calculation was based on the average cellular dry mass estimate of 660 fg per cell (for *E. coli*) which corresponds to 1.0×10^9 cfu equal to 0.66 mg [43,44]. Cell mass was harvested by centrifugation for 10 min at $5000 \times g$ with the culture medium decanted. This step was repeated using water, 10% glycerol or phosphate buffered saline with Tween detergent. The later two wash buffers were applied only to recipes five through ten. The concentration was adjusted to 2.5 mg/mL or approximately 3.8×10^9 cfu/mL in the indicated wash buffer.

2.2. Recipe formulation

The cell mass was combined into ten recipes typically used for stabilization of commercial products. The formulations are listed in Table 1 along with the reference. All chemicals were obtained from Sigma (St. Louis MO). The formulations one to five were mixed using cell mass cultured only on TSB medium and washed two times in water. The remainder of the stabilization recipes (six through ten) were prepared using cell mass grown on each of the four types of growth medium and washed with one of three types of solutions, water, 10% glycerol or phosphate buffered saline containing Tween detergent (PBST). All of the recipes 6–10 prepared with cell mass washed with water were analyzed by derivatization and GC–MS. A

Table 1
Formulation recipes used in study.

Recipes		Form	Calculated amount	Product class and physical form	Ref.
1)	10% cell preparation	vol/vol	5 mg (2 mL of 2.5 mg/mL)	Biopesticide	Adapted from Terra-Arunsiri et al. 2003 [39]
	10% gelatinized tapioca starch	wt/vol	5 mg	Dry	
	10% sucrose	wt/vol	5 mg	Dry	
	38% tapioca starch	wt/vol	19 mg	Dry	
	20% milk powder (contains lactose)	wt/vol	10 mg	Dry	
	10% silica fume	wt/vol	5 mg	Dry	
	2% polyvinyl alcohol	wt/vol	1 mg	Dry	
	Water	vol/vol	16.6 mL	Mix wet and dry	
	Total volume		20 mL	Mix Wet and Dry	
2)	3% Trehalose in water	wt/vol	30 mg	Vaccine	Corbanie, et al. 2008 [12]
	1% PVP (polyvinylpyrrolidone)	wt/vol	10 mg	Mix in water and spray dry	
	1% BSA (Bovine serum albumin)	wt/vol	10 mg		
	10^8 cells (~0.1 mg) attenuated virus or organism in PBS	vol	0.040 mL of 2.5 mg/mL		
	Water	vol	0.960 mL		
	Total volume		1 mL		
3)	5% Mannitol in water	wt/vol	50 mg	Vaccine	Corbanie, et al. 2008 [12]
	10^8 cells (~0.1 mg) attenuated virus or organism in PBS	vol	0.040 mL of 2.5 mg/mL	Mix in water and spray dry	
	Water	vol	0.960 mL		
	Total volume		1 mL		
4)	50% sucrose	wt/vol	500 mg	Vaccine	Pisal et al. 2006 [5]
	10% galactose	wt/vol	100 mg	Mix in water and lyophilize	
	0.6% polyethylene glycol	wt/vol	6 mg		
	0.25% microbial preparation (in PBS) = 5 mg*	wt/vol	2 mL of 2.5 mg/mL		
	Total volume		2 mL		
5)	3% mannitol	wt/vol	30 mg	Pharmaceutical	Maa et al. 2004 [6,7]
	3% trehalose	wt/vol	30 mg	Mix in water and spray dry	
	4% dextran	wt/vol	40 mg		
	0.25% protein or cell mass	wt/vol	1 mL of 2.5 mg/mL		
	Total volume		1 mL		
6)	20% Reconstituted Skim Milk (RSM contains lactose)	wt/vol	400 mg	Probiotic	Gardiner et al. 2000 [40]
	0.5% yeast extract	wt/vol	5 mg	Spray dry	
	1% sucrose	wt/vol	10 mg		
	~0.07% microbial preparation	vol	2 mL of 0.7 mg/mL		
	Total volume		2 mL		
7)	6% Reconstituted skim milk (RSM contains lactose)	wt/vol	120 mg	Probiotic	Juarez-Tomas et al. 2009 [15]
	12% lactose	wt/vol	240 mg	Lyophilize	
	~0.07% microbial preparation	vol	2 mL of 0.7 mg/mL		
	Total volume		2 mL		
8)	50% sucrose	wt/vol	1 g	Vaccine	Pisal et al. 2006 [5]
	10% NZ Amine	wt/vol	200 mg	Lyophilize	
	3% PVP (polyvinylpyrrolidone)	wt/vol	60 mg		
	~0.07% microbial preparation	vol	2 mL of 0.7 mg/mL		
	Total volume		2 mL		
9)	10% DMSO	vol	0.2 mL	Cryopreservation	Hubaalek 2003 [41]
	5% sorbitol	wt/vol	100 mg		
	~0.07% microbial preparation	vol	2 mL of 0.7 mg/mL		
	Total volume		2.2 mL		
10)	95% leucine (4 mg/mL)	vol/vol	1.8 mL	Vaccine	Garcia-Contreras et al. 2008 [46]
	5% cell mass 2.5×10^9 CFU/mL = 0.084 mg	vol/vol	0.1 mL	Lyophilize	
	1% glycerol (0.01 mg/mL)	vol/vol	0.02 mL		
	Water	vol	0.08 mL		
	Total volume		2.0 mL		

portion of those samples washed with glycerol or PBST were also analyzed with this method. Most recipes were produced using a total volume of 2 mL. The liquid preparations were then frozen at −70 °C before drying under vacuum (<10 mTorr) for one to three days until visibly dry using a lyophilizer (Virtis Corp).

All ten recipes in Table 1 were lyophilized for one to three days below 10 mTorr following overnight freezing. No temperature program was used during lyophilization. The physical appearance of the dry recipes varied somewhat, but most obtained a dry powdery or glassy appearance. A number of the formulations were very hygroscopic and easily returned to a liquid or gelatinous consistency if not stored in a desiccator. In particular those formulations with at least 10% sucrose (recipes 1, 4, 8) reverted to a liquid state during room temperature storage. The biopesticide (recipe 1) and cryopreservation (recipe 9) recipes never reached a visibly dry state during lyophilization using the published recipes. Some modifications were made to the published recipe 1 in order to get a dried preparation. The rice bran oil, antifoam and Tween detergent were eliminated from the final recipe resulting in a dry sample following lyophilization when these additives were not present.

Each recipe was prepared using cell mass cultured in four different growth media. These samples were also washed using different buffers (i.e. water, glycerol or PBST). As a result, they all appeared to have different physical forms depending on which cell mass was added. For instance, those washed with 10% glycerol did not dry completely and retained a moist appearance. This characteristic appeared independent of the medium the cells were cultured on or the final recipe used for the cell mass. Thus, glycerol washed samples of recipes 6–10 did not easily reach a dry form. As a result, they were not the subject of extensive characterization by GC–MS. The formulations made with cells washed in water were the subject of full characterization by both MALDI-MS and GC–MS.

2.3. Sample preparation for mass spectrometry

Approximately 10 mg of sample was weighed out and combined with 50% acetonitrile (ACN) solution and mixed at 60 °C, 300 rpm for 1 h. The sample was split into two separate 200 μL samples (replicates A and B) and put through a Durapore PVDF 0.22 μm filter to remove cell debris. Samples were centrifuged at 10,000 rpm for 6 min to collect filtrate. Following drying to roughly 50 μL the sample was reconstituted to 200 μL with 0.1% trifluoroacetic acid (TFA) and 20 μL of this sample was taken for MALDI-MS analysis. The remaining sample was processed further first by running through a C18 spin column to remove peptides, proteins and other possible interferents. The sample flow through was then derivatized and analyzed by gas chromatography (GC) mass spectrometry (MS).

2.4. Protocol for Matrix Assited Laser Desorption/Ionization Mass Spectrometry (MALDI-MS)

A 1 M dopant stock solution of NaCl was created and then serial diluted to obtain 10 pM solution. A 2,5-dihydroxybenzoic acid (DHB) (FW = 154.12 g) matrix solution was then prepared at 10 mg/mL concentration in 50% ACN. Fifty μL of 10 mg/mL DHB and 50 μL of 10 pM NaCl were combined in an Eppendorf tube for use as the matrix solution. A Peptide Calibration Standard II (Bruker Daltonics, Billerica MA) was reconstituted in 250 μL of 0.1% TFA + 25% ACN. Finally the matrix and each analyte were mixed using a 1:10 matrix:analyte ratio (1 μL matrix to 10 μL analyte) in a clean Eppendorf tube and vortexed for 10 s.

A peptide calibration standard was used for external calibration on separate MALDI sample spots using matrix related peaks *m*/*z* 155, *m*/*z* 177, *m*/*z* 273, and peptides peaks *m*/*z* 757 (Bradykinin), and 1047 (Angiotensin II) for linear calibration. A minimum of three 1 μL droplets were used for MALDI-MS analysis. Data was collected in positive ion, reflector mode with a mass range of *m*/*z* 80 to 2000. The data was collected with a 19.7 kV extraction voltage, 130 ns pulsed ion extraction delay and matrix suppression turned off.

Data was acquired from each of the sample spots using 100 shots in 4 locations within the spot. Samples were diluted with 50% ACN at 1:1000 if no ions are observed in the first analysis. MALDI-MS data was collected and examined for key monosaccharide and/or disaccharide ion(s) at *m*/*z* values indicated below. This step provided preliminary evidence of carbohydrate presence in a preparation. The primary carbohydrate ions monitored for by MALDI-MS were the monoisotopic sodium adduct ions: *m*/*z* 203.05 for hexose (e.g. mannose), *m*/*z* 205.07 for hexitol (e.g. mannitol), and *m*/*z* 365.11 for disaccharides (e.g. lactose). Monoisotopic masses detected within 300 part per million (ppm) of the predicted mass and having a signal to noise (S/N) ratio of greater than 5 were recorded as positive samples.

2.5. Derivatization

Toluene rinsed glassware and pipettes were used to transfer all reagents and samples. First the glass reacti-vials were presilanized with 1 mL Tri-Sil reagent. (Thermo Fisher). A 50 μL sample from the C-18 purification step was used for derivatization. Three external standard mixtures were prepared using 5, 100 and 500 μL of external standard mixture added to reaction vials. The external standard mixture contained glycerol, galactose, sorbitol, mannitol, *myo*-inositol, sucrose, lactose, maltose and trehalose at 0.22 mg/mL. A 125 μL volume of internal standards containing methyl glucose and gentiobiose at 1 mg/mL was added to external standards and unknown samples. In recipes 1–5, a reduction step was used where 50 μL of 100 mg/mL sodium borodeuteride ($NaBD_4$) was added to the reaction mixture and allowed to incubate at 4 °C overnight. The reduction step was found to introduce variability in the yield and so was not used for analysis of recipes 6–10. In all recipes samples were dried using 0.5 mL of acetone added to each sample in heat block under a stream of N_2. After the acetone had reached dryness, a 0.5 mL volume of toluene was then added to each sample and allowed to dry down and then held at temperature under N_2 for ∼30 min. A 150 μL volume of pyridine and 150 μL of N,O-bis(Trimethylsilyl)trifluoroacetamide (BSTFA) w/1% Trimethylchlorosilane (TMCS) (Thermo Fisher) was added to each vial before capping and vortexing for a few seconds. The reaction was placed in heat block at 60 °C for 1 h. Following derivatization 50 μL of sample was added to a labeled target vial with insert.

2.6. Gas Chromatography Mass Spectrometry (GC–MS)

An Agilent 7890 GC-5975 MS analyzer (Agilent Technologies, Inc., Santa Clara, CA, USA) equipped with a CTC Combi PAL autosampler (LEAP Technologies, Carrboro, NC, USA) was used for GC–MS data acquisition. A 1 μL sample injection using a 20:1 split inlet held at 275 °C was used. The derivatized compounds were separated on a DB-5 or HP-5MS column (30 m × 250 μm × 0.25 μm) using a constant helium flow at 1.2 mL/min. The temperature program proceeded over 58 min from 50 to 250 °C. The MS monitored a mass range of 50–550 *m*/*z* to collect identifying fragments from the electron impact (EI) source. The order of analysis was randomized with each sample set including three separate vials of derivatized external standards.

3. Results

3.1. MALDI-MS detection of carbohydrate additives

The detection of carbohydrates by MALDI-MS was not developed as a quantitative method. Instead this approach was used to

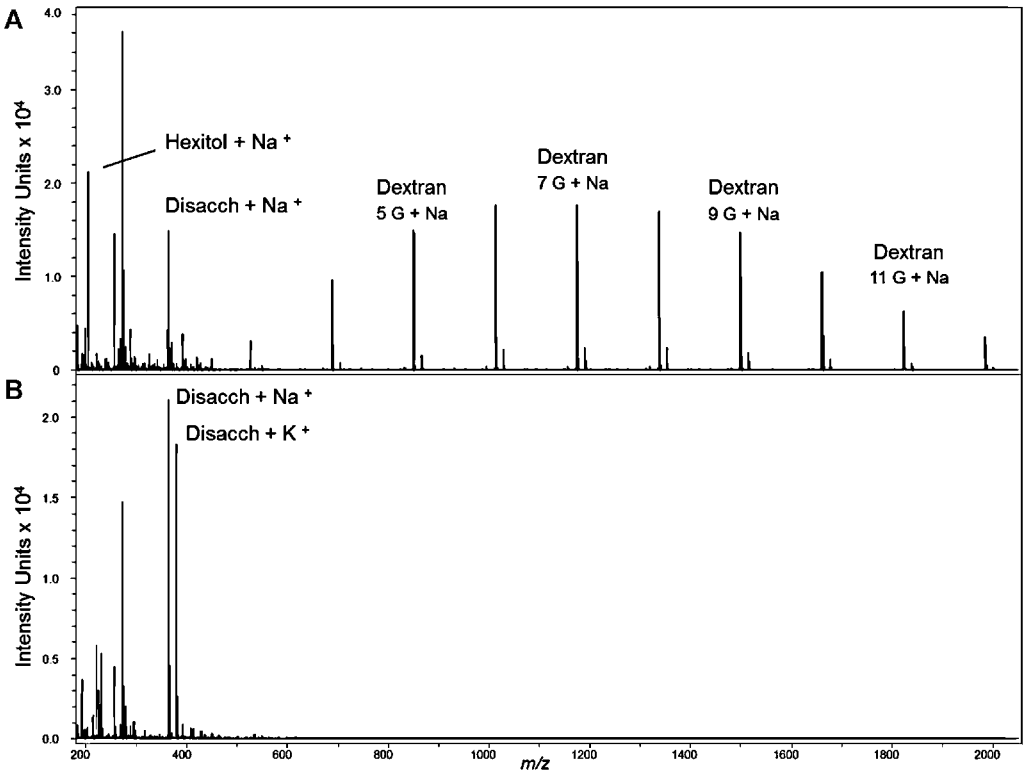

Fig. 1. MALDI-MS spectra of carbohydrates from recipe 5 (A) and recipe 7 (B). The mass spectrum contains mass information for primarily the Na^+ adduct ion for each compound (m/z 205 for hexitols and m/z 365 for disaccharides). The K^+ for lactose adduct ion (m/z 381) can also be observed in panel B. Note the oligomeric carbohydrates such as dextran can also be observed as Na^+ adduct ions with this technique. The number of glucose (G) residues present in each peak is indicated.

screen for carbohydrate and other stabilizing additives. The method was focused on detection of carbohydrate masses alone, so information was limited to determining if the sodium adduct mass for a hexose, hexitol or disaccharide was present. The presence of potassium as an impurity is common, and so K^+ adducts were also observed. However these were not used for reliable detection of the mono or disaccharides. The performance parameters of accuracy, sensitivity, selectivity and range were tested for this method across the stabilization formulations. An example of the data obtained for recipes 5 and 7 are presented in Fig. 1.

The accuracy of a technique refers to the closeness of an analytical result to the true or accepted reference value. The accuracy of the method was assessed by examining the number of MALDI-MS data files where all carbohydrate masses that were expected were also detected within the parameters stated above. Accuracy was assessed across all sample recipes, cell mass wash methods and concentrations tested. Table 2 contains the results based on recipe and ion type. Several of the recipes are not distinguishable by MALDI-MS detection of carbohydrates alone because the presence of only disaccharides is common to recipes 1, 2, 6, 7 and 8. As a result, the accuracy of this method is determined only for correct detection of all distinct masses anticipated in each sample. Over all the accurate detection of disaccharide, mannitol/sorbitol or galactose was estimated at 98.7%. Data was also collected on glycerol detection; however, this compound was not the focus of method development by MALDI-MS. Blank places in Table 2 indicate samples where the ions were not observed based on the recipe components.

The limit of detection (LOD) of an analytical procedure is the lowest amount of analyte in a sample that can be detected, but not necessarily quantitated. Lyophilized samples of Recipes 2, 5 and 7 were weighed out for this estimate in 0.5, 1.0 and 5.0 mg quantities used for sample cleanup and analysis. Typically 10 mg of sample were prepared for analysis, so these amounts represent 5–50% of that mass. Each recipe resulted in a known mass of each component in the final product after water was removed by lyophilization.

The LOD for the MALDI-MS method was estimated for lactose, trehalose and mannitol in the three different stabilizer recipes. The ion indicative of trehalose and lactose (m/z 365) was detected in recipes 2 and 7 respectively for all twelve data files from all sample concentrations examined. Ions indicative of mannitol and trehalose (m/z 205 and m/z 365 respectively) were not detected for two of the twelve replicate data files in the 0.5 mg samples of recipe 5 (data included in Table 2). This suggests that 0.5 mg of sample mass is a lower limit for this method. It is also likely that sample sizes smaller than 0.5 mg level would be hard to manipulate and aliquot.

The analyte mass range for detection of carbohydrates by MALDI-MS was estimated from these experiments. The range of carbohydrate masses deposited on the MALDI sample plate was estimated to be between 4 ng and 150 ng. A minimum of 8 ng was estimated for reliable detection of these analytes from any of the recipes examined. MALDI-MS was not used quantitatively and no intensity information was utilized to determine the linearity of the instrument response.

3.2. MALDI-MS of polymeric additives

A number of components are present in several recipes in addition to the carbohydrate additives. Oligosaccharides, peptides, amino acids, polymers and detergents are also amenable to analysis by MALDI-MS. In addition to stabilizing additives, the buffer used to wash the cell mass introduced detectable ions such as masses from Tween detergent present in PBST. A series of ions from m/z 500 to m/z 1600 with spacing of 44 Da was observed in the PBST washed samples. A comparison of recipe 7 samples with cell mass cultured on TSB and washed with either water or PBST is provided in

Table 2
Accuracy of carbohydrate detection using all MALDI-MS data files.

Recipe	Sucrose/lactose/trehalose *m/z* 365	Mannitol/sorbitol *m/z* 205	Galactose *m/z* 203	Glycerol *m/z* 115	Accuracy
1	8/8				100%
2	44/44				100%
3		8/8			100%
4	8/8		8/8		100%
5	41/42	40/42			95.2%
6	90/92				97.8%
7	102/104				98.1%
8	82/82				100%
9		64/64			100%
10				28/70	100%
Total	375/380 (98.6%)	112/114 (98.2%)	8/8 100%	28/70 (40%)	446/452 (98.7%)

Fig. 2. Two distributions of ions with a peak spacing of *m/z* 44 were observed. This mass difference corresponds to different numbers of ethoxy groups present in each molecule detected and is consistent with the presence of the polymer.

3.3. GC–MS detection of carbohydrate additives

The repeatability of the GC–MS measurement was determined by reproducible detection of peaks in the external standard mixture using 37 ng injected for each derivatized carbohydrate. No more than 0.1 min variation was observed between corresponding peaks in the external standard and samples. The mass spectrum for each chromatographic peak was also inspected to ensure the identity of each compound. The variation in retention time was determined for the carbohydrate standard mixture within three different data sets for a single instrument. The average standard deviation in retention time was less than 0.02 min (less than 0.04%) for all data sets.

The LOD for GC–MS detection of derivatized carbohydrates was determined from standard mixtures analyzed at different concentrations over five separate datasets. The calculated mass of each carbohydrate injected on to the column (factoring in a 1/20 injector split) for each standard was 0.18, 3.70 and 18.50 ng respectively. The monosaccharides glycerol, mannitol and sorbitol were detected at all three levels while none of the disaccharides (sucrose, lactose, and trehalose) were detected at the 0.18 ng level. The minimum mass tested for confident detection by derivatization and GC–MS was established at 3.7 ng for all carbohydrates of interest.

The range and linearity of carbohydrate detection by derivatization and GC–MS was determined from the LOD and standard curve experiments. The range of sample masses where all carbohydrate targets were detected was 3.7 ng up to 444 ng. The peak area was calculated for all carbohydrates relative to an internal standard over the 7.4–390 ng range. The R^2 values and estimated LOD was calculated for each compound (Table 3). A graphical display of the mannitol and sorbitol standard curves is provided in Fig. 3.

Accurate detection of carbohydrate additives by derivatization and GC–MS was determined by the ability to identify the complete set of carbohydrates present in the sample. All ten recipes had unique combinations of simple carbohydrates and so can be distinguished from one another by this method. Without includ-

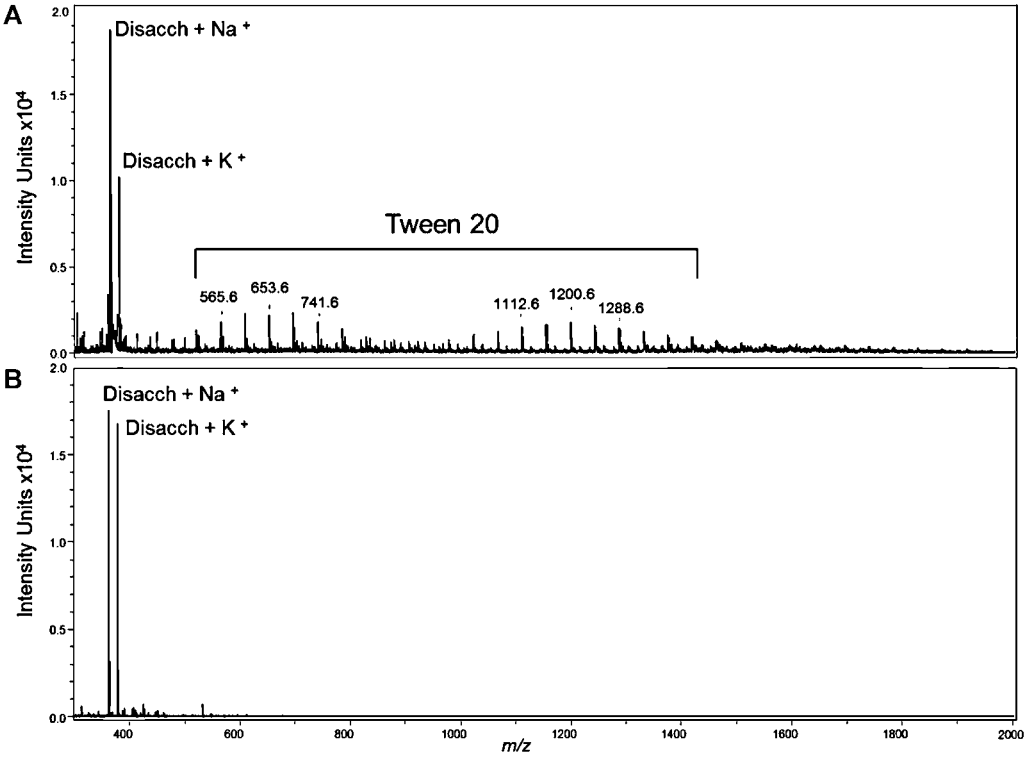

Fig. 2. Comparison of MALDI-MS spectra from recipe 7 samples washed in PBST (A) and water (B) are aligned. The presence of the disaccharide sodium (Disacch + Na^+) and potassium (Disacch + K^+) adducted species are indicated. Peaks derived from the Tween 20 component of PBST are also indicated with alternating peaks within two distributions are labeled.

Table 3
Figures of merit for the standard curves for each TMS derivatized compound. The standard curve covered a range of concentrations from 7.4 to 390.0 ng of each compound. The deviation from a linear response (R^2) and estimated limit of detection (LOD) in ng is provided for each.

	Sucrose	Lactose	Trehalose	Maltose	Mannitol	Sorbitol	Galactose	*myo*-Inositol
LOD	3.70	3.70	3.70	3.70	0.18	0.18	ND	0.18
R^2	0.988	0.997	0.992	0.994	0.996	0.998	0.981	0.996

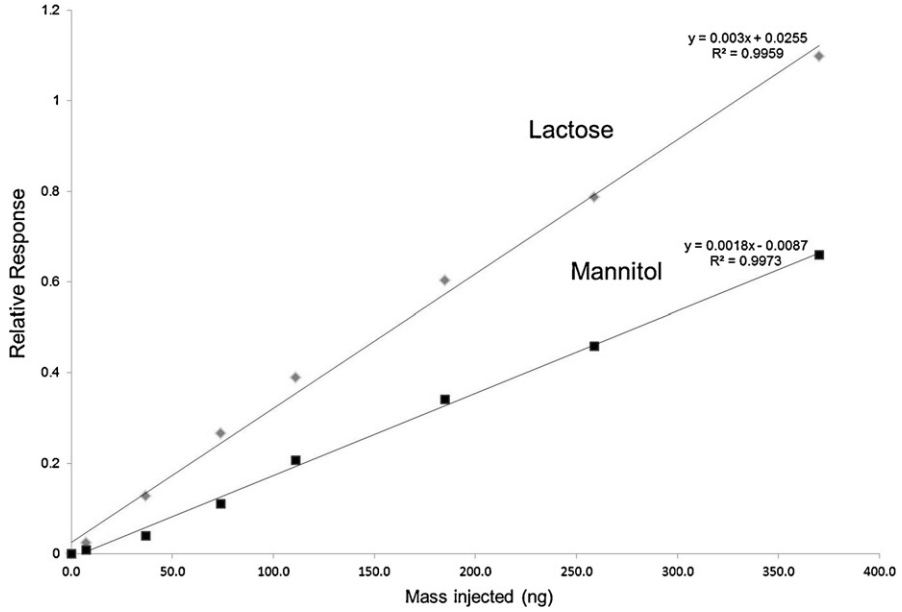

Fig. 3. Examples of standard curves generated for Mannitol (black squares) and lactose (gray diamonds). The instrument response determined by peak area over a range of concentrations from 7.4 to 390 ng injected on column was found to be linear as described by the equations and corresponding R^2 values.

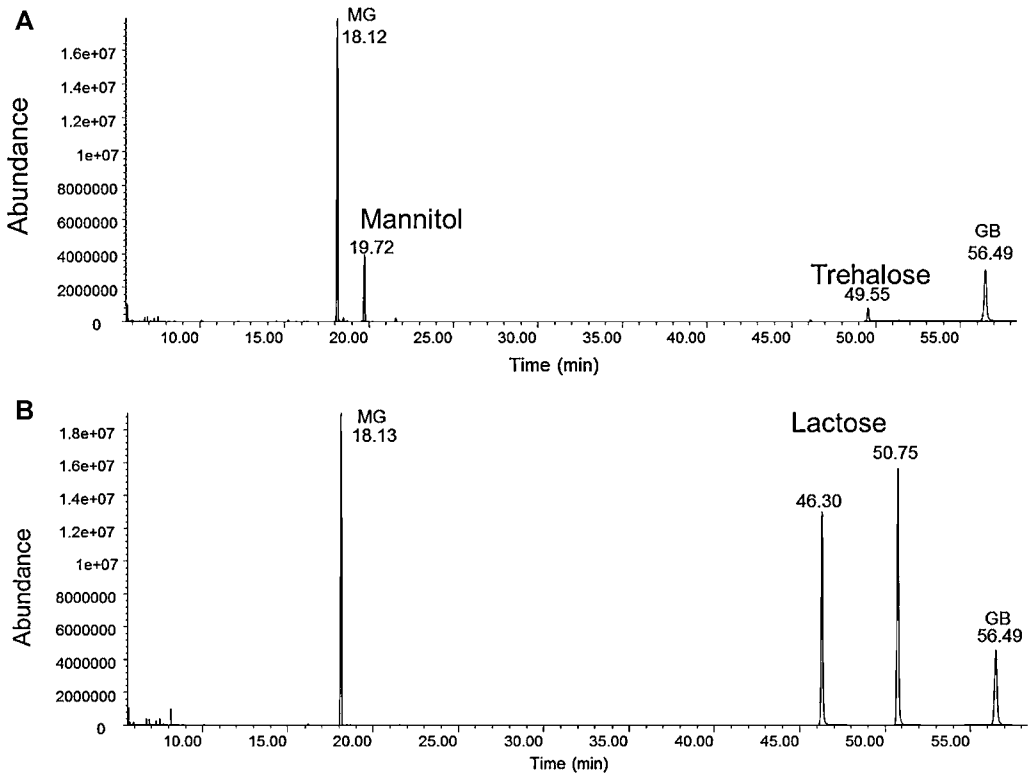

Fig. 4. GC–MS chromatographs of TMS derivatized carbohydrates prepared from recipes 5 (A) and recipe 7 (B). The detected recipe carbohydrates are indicated in each panel with two peaks appearing for lactose. The internal standards methylglucose (MG) and gentiobiose (GB) are indicated in each panel at 18.1 and 56.5 min respectively. Electron impact mass spectra are collected at each time point as well (not shown).

Table 4
Accuracy of carbohydrate identification by GC–MS using the number of samples where each TMS derivatized carbohydrate was detected at least in the quantifiable range relative to the total number of samples analyzed. Accuracy determined by detection and identification of all anticipated carbohydrate additives. *Select samples of recipes 6–9 with cell mass washed in glycerol were examined by GC–MS.

Recipe	Sucrose	Lactose	Trehalose	Mannitol	Sorbitol	Glycerol*	% Accuracy
2			12/12				100%
5			12/12	12/12			100%
6	6/6	6/6				1/1	100%
7		16/16				1/1	100%
8	7/7					1/1	100%
9					6/6	1/1	100%
10						4/4	100%
% Total	100%	100%	100%	100%	100%	100%*	100%

ing a reduction step prior to derivatization, carbohydrates with anomeric centers (such as those with aldehyde group at C1) can produce stereoisomers that will be resolved by the GC separation. As a result, polyols such as glycerol, mannitol, sorbitol and inositol as well as the internal standard methyl glucose produced single peaks in the chromatogram. The hexose galactose produced three peaks. The disaccharides sucrose, trehalose and internal standard gentiobiose produced single peaks while lactose and maltose in the standard mixtures produced two peaks. Ten mg of sample was used for preparation and analysis for the data sets evaluated. Examples of GC–MS data for recipes 5 and 7 are provided in Fig. 4.

The number of times each carbohydrate was detected relative to the total number of data files is provided in Table 4. The method accurately detected all the anticipated mono and disaccharide carbohydrate additives in the recipes. One of the samples for recipe 10 had glycerol detected at a level near the LOD in a sample with cell mass washed with PBST. As a result, this detection is considered tenuous. Again, glycerol was not a focus of this method development but reported for future information if additional target recipe components are considered in future method development. This finding was in contrast to the detection of other mono and disaccharides in the samples. All other peaks in samples from each recipe were easily detected and appeared at or above the linear range for quantitation. Dilution of the samples was generally required for accurate quantitation.

The GC–MS portion of the method was able to distinguish all recipes with different types and numbers of carbohydrate additives. The challenges encountered by GC–MS related to detection of sucrose that could be partially obscured in the raw data by relatively large amounts of the earlier eluting lactose peak. However the different mass spectra for each compound allowed specific detection of sucrose using an extracted ion chromatogram to specifically integrate only the peak area attributed to sucrose.

4. Discussion

We have demonstrated an assay for determining the presence of carbohydrate additives in bacterial preparations. This assay combines two analytical techniques, MALDI-MS and derivatization followed by GC–MS. The performance characteristics of each method have been evaluated for several different formulations used in biotechnology for stabilizing biological products. These formulations represent potential surrogates for stabilization of pathogens in a dry form prior to dissemination of a biological weapon. In addition to formulation, the growth medium used for bacterial culture as well as method of cell mass washing has been examined to ensure the methods were robust against these variables.

The MALDI-MS portion of the method is most applicable for rapid sample screening for carbohydrates, but limited in its ability to differentiate between carbohydrates of different mass. All three disaccharides (sucrose, lactose and trehalose) have identical mass, but were used in different recipes. As a result, the MALDI-MS data set can identify the presence of a mass consistent with a disaccharide as additive but cannot distinguish among these three disaccharides and thus cannot distinguish between five of the recipes used here. Combinations of mono and disaccharides or presence of mannitol alone could be distinguishing features. The detection of glycerol was also challenging using this technique. However this approach was an effective method for rapid screening and was able to distinguish between hexose, hexitol and disaccharides.

Different instrument responses were observed depending on the mass of carbohydrate in a given recipe. However, confident detection of each carbohydrate was made using the combination of analytical techniques in the method. The combination of analytical techniques used in the method appears effective for sample amounts down to 0.5 mg. This mass of sample is 1/20th of the amount originally used in the method. While information on other compounds, including dextrans, was captured in this experiment, the minimum sample requirement was only determined using trehalose, lactose and mannitol from three different recipes using only cell mass cultured on one media type and washed in water. However this minimum sample requirement should be extendable to all of the analytes.

The initial GC–MS analysis used a derivatization method that included a reduction step prior to creating a trimethylsilyl derivative. Sodium borodeuteride was used to convert the aldehyde group into an alcohol and leave a deuterium on carbon one of each sugar. The goal was to create a single anomer following derivatization which would result in a single peak for each compound and simplify data interpretation. This approach has previously been used for detection of acetate derivatives [45]. However the TMS derivatization produced inconsistent results until the reduction step was removed from the method (Data not shown). A possible problem was that steps used to remove borodeuteride (which would inhibit the TMS derivatization) from the sample included addition of a methanol/acetic acid solution. The residual sodium acetate was also likely competing for the derivatization reagent and limited reproducibility.

The removal of this step increased the reproducibility of the method and so was used on all of the recipe samples. One outcome of this modification was a change in data analysis. Several of the target carbohydrates with aldehyde groups (e.g. glucose and mannose) produced multiple anomeric forms and multiple GC–MS peaks upon TMS derivatization, while those without reducible groups (e.g. sorbitol and mannitol) produced only a single peak. This compromise was acceptable for this application because a small number of carbohydrates were present in each sample. The derivatization method also became shorter and can be accomplished in a single day.

The two portions of the method provide different yet complementary types of information. MALDI-MS provides information on

the presence of monosaccharides (differentiating between aldoses and alditols) and disaccharides in samples using a small amount of sample. The speed of the technique makes it well suited for screening samples as well. Once the samples were filtered dried and reconstituted, the entire process for data collection and analysis required less than 30 min. The analysis of multiple samples on the same plate add 5 min or less per additional sample.

The complementary GC–MS method was used to provide identifying information on all the isomeric carbohydrates within each class (e.g. differentiating between sucrose and lactose) using both retention time and fragmentation data. This method required more time for sample cleanup and derivatization (about six hours per batch) than MALDI-MS and over an hour per GC–MS run, requiring an overnight analysis for a batch of twelve. The GC–MS method appeared to have very little interference; however it detected and identified only compounds that were volatile. All of the mono or disaccharide carbohydrate additives, as well as compounds like leucine, were derivatized and detected by this method. As a result GC–MS is very selective and blind to additional types of additives that may be present.

By contrast, the MALDI-MS was able to detect a variety of additional compounds including the NZ-amine peptides, and dextran oligosaccharide. These stabilizers provide additional constituents that can distinguish formulations. Furthermore, the method of cell washing also impacted the MALDI-MS spectra with Tween detergent detectable despite the minor contribution of cell mass to the overall sample. This tool has demonstrated the ability to detect a variety of analytes from within a complex sample.

Using the two detection methods together provided accurate and comprehensive description of common additives in samples formulated to be stable as dry preparations. If the same types of preservation methods were used for stabilizing desiccation-sensitive pathogens, it would be vital to detect the presence of additives. Taken together the multiple pieces of data would serve as useful indicators of the formulation used to preserve biological materials in a dry form.

Acknowledgments

Funding for this work was provided through contract AGRHSHQDC07X00451 to Pacific Northwest National Laboratory by the Department of Homeland Security Science and Technology Directorate. Battelle Memorial Institute operates Pacific Northwest National Laboratory for the U.S. DOE under Contract DE-AC06-76RLO.

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