Determination of Enzyme/Substrate Specificity Constants Using a Multiple Substrate ESI-MS Assay

Na Pi and Julie A. Leary

Department of Chemistry, University of California at Berkeley, Berkeley, California, USA

The traditional method used to investigate the reaction specificity of an enzyme with different substrates is to perform individual kinetic measurements. In this case, a series of varied concentrations are required to study each substrate and a non-regression analysis program is used several times to obtain all the specificity constants for comparison. To avoid the large amount of experimental materials, long analysis time, and redundant data processing procedures involved in the traditional method, we have developed a novel strategy for rapid determination of enzyme substrate specificity using one reaction system containing multiple competing substrates. In this multiplex assay method, the electrospray ionization mass spectrometry (ESI-MS) technique was used for simultaneous quantification of multiple products and a steady-state kinetics model was established for efficient specificity constant calculation. The system investigated was the bacterial sulfotransferase NodH (NodST), which is a host specific *nod* gene product that catalyzes the sulfate group transfer from 3'-phosphoadenosine 5'-phosphosulfate (PAPS) to natural Nod factors or synthetic chitooligo-saccharides. Herein, the reaction specificity of NodST for four chitooligosaccharide acceptor substrates of different chain length (chitobiose, chitotriose, chitotetraose, and chitopentaose) was determined by both individual kinetic measurements and the new multiplex ESI-MS assay. The results obtained from the two methods were compared and found to be consistent. The multiplex ESI-MS assay is an accurate and valid method for substrate specificity evaluation, in which multiple substrates can be evaluated in one assay. (J Am Soc Mass Spectrom 2004, 15, 233–243) © 2004 American Society for Mass Spectrometry

Recently, substrate specificity investigations along with specificity constant (k_{cat}/K_M) measurements have provided significant insights into a number of biologically important enzymes [1–15]. Specificity studies of an enzyme for different natural or synthetic substrates can help determine the enzyme structure and physiological functions [1, 4–7] as well as address the key residues involved in substrate binding and catalysis [2, 8]. Specificity studies can also help to unravel the reaction pathway or catalytic mechanism [9], and guide substrate structure-based inhibitor development and drug design [3, 10, 11]. For example, Hara et al. examined the specificity of three isoenzymes of human 3(20) α-hydroxysteroid dehydrogenase and successfully addressed their roles in neurosteroid metabolism [1]. Griffith and coworkers evaluated the influence of divalent metal ions on the substrate specificity of γ-glutathione synthetase, elucidating the enzyme substrate binding interactions [2]. The novobiocic acid noviosyl transferase activity of NovM and its specificity for various substrate analogs in designing novel coumarin-based antibiotics was recently characterized by Walsh and coworkers [3]. In addition, efforts have been made to alter enzymes by site-directed mutagenesis in order to manipulate substrate specificity [12–14] or enhance enzyme catalysis [15].

Traditionally, the general method of evaluating the specificity of a given enzyme is to measure the specificity constant of a group of substrates individually followed by comparison of measured values, regardless of the assay used for measurement [1–15]. This requires large amounts of material and considerable effort to study substrate libraries. Consequently, a simple and rapid methodology for evaluating enzyme specificity and obtaining k_{cat}/K_M values would be of great interest for kinetic analyses and would dramatically expedite the process of understanding biologically important enzymatic systems.

Recently, the potential of mass spectrometry in studying enzyme catalysis has been widely demonstrated [16–29]. The development of soft ionization techniques, such as electrospray ionization (ESI) and matrix-assisted laser desorption (MALDI), has made mass spectrometry an excellent complementary technique to conventional spectrophotometric methods for

studying enzyme kinetics [23–29]. With the improvements of resolving power, sensitivity, and versatility, mass spectrometry has become a highly competitive analytical method for detection and characterization of biochemical reaction intermediates that can be used to elucidate enzymatic reaction pathways and catalytic mechanisms [16–22]. Esaki et al. reported a novel catalytic mechanism for L-2-Haloacid dehalogenase involving a cyanoalanine intermediate revealed by LC/MS monitoring of the enzymatic reaction [16]. Likewise, Leary and coworkers reported the identification of a sulfated NodH sulfotransferase (NodST) intermediate formed in a hybrid Ping-Pong mechanism using Fourier transform ion cyclotron resonance (FT-ICR) mass spectrometry [17]. A facile and broadly applicable ESI-MS assay developed by Leary and coworkers has been used for kinetic analyses on several enzyme systems such as glutathione S-transferase (GST) [27], hexokinase [28], and NodH sulfotransferase (NodST) [29]. The mass spectrometry assay is especially useful because traditional spectrophotometric assays are not applicable to NodST kinetics since no shift in absorption accompanies sulfuryl group transfer. Using this assay, precise and accurate kinetic data were generated for all the enzymes above, and for the first time, a hybrid Ping-Pong Bi-Bi mechanism was unraveled for NodST [17]. A distinct feature of the ESI-MS assay is that product quantification is realized by monitoring the product ion in relation to an internal standard ion in a quenched reaction solution, allowing different product ions of different mass to charge ratio to be monitored and quantified simultaneously in one reaction system. Consequently, development of a method to calculate the specificity constants of multiple substrates based on the concentrations of multiple products in one reaction system would make it feasible to utilize the ESI-MS technique, thus avoiding the redundant "one-at-a-time" measurements in the traditional method. The ability of mass spectrometric assays to monitor many reaction products in one sample makes the investigation of multiple substrate kinetics fast and easy, something that is difficult or even impossible by spectrophotometric assays.

The multiplex ESI-MS assay method reported herein demonstrates a successful combination of the steady-state kinetics calculation model and the ESI-MS multiple product quantification technique for the streamlined specificity constant measurements of multiple competing substrates in one reaction. It is rather unique in that only one reaction system and one ESI-MS measurement are required to obtain the enzyme specificity information for multiple substrates. The validity of this approach was tested on the specificity study of the bacterial carbohydrate sulfotransferase, NodH (NodST) for a library of four chitooligosaccharide substrates.

NodH is one of the host specific *nod* gene products that is necessary for diverse modification of the core lipochitooligosaccharide (Nod factor) structure in *Rhi-*

zobium meliloti [30–34]. As a GlcNAc-6-O-carbohydrate sulfotransferase, NodH catalyzes the transfer of a sulfuryl group from 3'-phosphoadenosine 5'-phosphosulfate (PAPS) to the reducing terminal 6-O position of the Nod factor [30, 31]. The presence of the sulfate group is critical for biological activity on the host plant alfalfa, while its absence renders the Nod factor active on the non-host plant vetch [31]. Therefore, the sulfotransferase activity of NodST is a major determinant of host specificity and much of the focus of recent research has been on the substrate specificity of this enzyme [30, 31, 35, 36]. Employing a thin layer chromatography assay, Long et al. [30] and Kondorosi and coworkers [31] discovered that NodST can sulfate biosynthetic chitooligosaccharides in addition to natural Nod factors, although with a lower reaction activity. Kinetic analyses with both Nod factors and chitooligosaccharides of different chain lengths revealed the highest binding affinity and best reaction activity of NodH for the appropriate tetrameric substrates, which guarantees the modification of the tetrameric Nod factor-the most active one in *Rhizobium meliloti*, and thus contributes to host specificity [31, 37]. Kinetic constants for various chitooligosaccharides have been determined individually by Kondorosi and coworkers [31] and Wong et al. [37], which indicated that chitotetraose is a better substrate than chitotriose, chitopentaose, or chitohexaose, while NodST's activity for chitobiose is more than one order of magnitude lower than that for the other four.

In this study, NodST's specificity for four biosynthetic chitooligosaccharide substrates was investigated. Despite different oligosaccharide chain length, all four acceptor substrates accommodate the transferred sulfate group using the reducing end GlcNAc residue, as shown in Scheme **1**. Their specificity constants were determined first using individual kinetic measurements and then by the newly-developed multiplex assay. The results of the two methods were found to be consistent with each other as well as with those reported in the literature, thus demonstrating that the multiplex ESI-MS assay is a valid method for substrate specificity studies for NodST. We believe the simplicity and rapidity of this assay will make it a viable alternative to the more commonly used individual assays.

Experimental

General Materials and Methods

All chemical reagents were obtained from commercial suppliers and used without further purification. NodST, chitobiose, and chitopentaose were purchased from Calbiochem (San Diego, CA). Chitotriose and chitotetraose were purchased from Seikagaku America Inc. (Iamsville, MD). All the other compounds were purchased from Sigma Co. (St. Louis. MO), including 3'-phosphoadenosine 5'-phosphosulfate (PAPS), 3'-phosphoadenosine 5'-phosphate (PAP) and α-ΔUA-
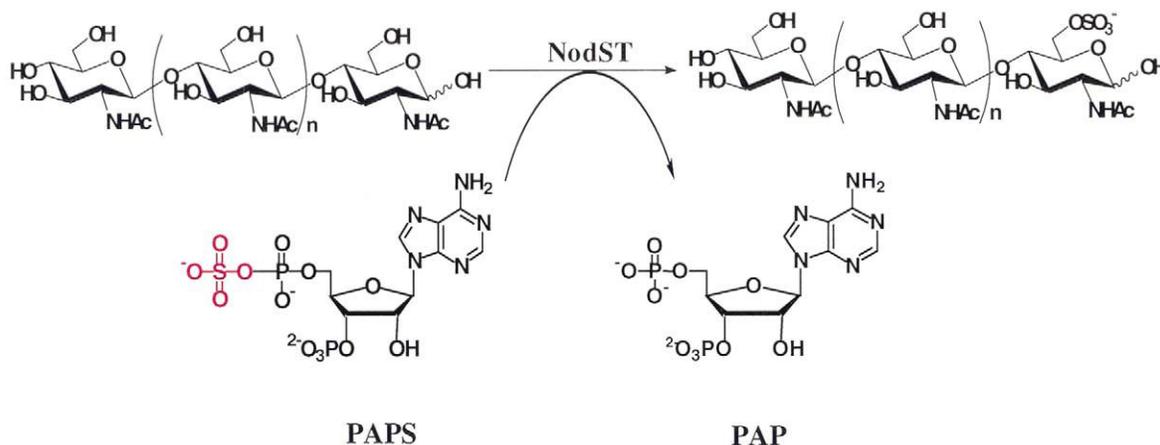
Scheme 1. NodST catalyzes the sulfation of chitooligosaccharide, generating PAP and chitooligosaccharide-6-OSO_3^- as products. (Chitobiose, $n = 0$; chitotriose, $n = 1$; chitotetraose, $n = 2$; chitopentaose, $n = 3$.)

[1→3]-GalNAc-6S (ΔD_i-6S). Commassie plus protein assay reagent (950 mL) and albumin standard (10 × 1 mL) were purchased from Pierce (Rockford, IL). All the mass spectrometric measurements were performed at 22 °C in 10 mM NH₄OAc (pH = 8.0) (Buffer A).

Instrumentation

A Finnigan LCQ ion trap mass spectrometer equipped with an ESI source and a HPLC pump (Thermo-Finnigan, San Jose, CA) was used in this study. The capillary temperature and the spray voltage were kept at 200 °C and 3.2 kV, respectively. Approximately 20 μL of each sample solution was injected through a 5 μL injection loop and delivered via a LC pump at a flow rate of 20 μL/min. Four product ions (m/z 503, 706, 909, 1112) and the internal standard (ΔD_i-6S) ion (m/z 458) were monitored in the negative ion mode using selected ion monitoring (SIM). The signals for the ions of interest were optimized by using the automatic tuning option on the instrument. The optimized conditions were then applied in subsequent experiments. When the signal intensity for one sample decreased from approximately 5×10^5 detector counts per scan to 5×10^3 detector counts per scan, indicating the consumption of the former injection, the next sample was introduced. The total ion chromatogram of the Qual Browser program was used to monitor the processing of the sample versus time. An average of $17 \times 3 = 51$ scans were taken to obtain a spectrum list for each sample, which provides the absolute intensities for the monitored ions along with the relative abundance. The sums of the intensities within 0.8 mass units around the center of

each product ion and the internal standard ion were used to determine the intensity ratio (I_P/I_{IS}).

Individual Kinetic Measurements

Methodology for product quantification. A stock solution of NodST (200 nM) (NodST stock 1) was prepared in 10 mM NH₄OAc (pH = 8.0) (Buffer A). Stock solutions of chitobiose (250 μM), chitotriose (250 μM), chitotetraose (250 μM), chitopentaose (250 μM), and PAPS (750 μM) were prepared in Buffer A. A pre-reaction solution (total volume = 25 μL) for each of the chitooligosaccharides was prepared by mixing 5 μL PAPS stock solution (750 μM) with 5 μL chitooligosaccharide stock solution (250 μM) and 15 μL Buffer A. All four reactions were initiated upon addition of 25 μL of NodST stock 1. The concentrations of PAPS and chitooligosaccharide in each reaction system were 75 μM and 25 μM respectively, along with 100 nM NodST. The four samples were kept at rt overnight and the enzymatic reaction was allowed to proceed to completion. At this point, the acceptor substrate in each reaction system had been completely consumed as evidenced by the absence of a peak corresponding to the chitooligosaccharide in the ESI-MS spectra, providing a sulfated-chitooligosaccharide concentration of 25 μM. A 20-μL aliquot of each reaction solution was quenched in 80 μL of MeOH containing 6.25 μM internal standard, ΔD_i-6S (Figure 1a). The resulting four quenched sample solutions were analyzed by selected ion monitoring (SIM) to obtain the single-point normalization factors (see below) for the four sulfated chitooligosaccharide products (chitobiose-6-OSO_3^-, chitotriose-6-OSO_3^-, chitotetraose-6-OSO_3^-, and chitopentaose-6-OSO_3^-) (Figure 1b). The four single-point normalization factors were subsequently used for product quantification.

The single-point normalization factor (R) is determined from eq 1, through which the relative ion intensity ratio of the product and the internal standard (I_P/I_{IS}) is related to the ratio of their concentrations. R can be obtained by analyzing a mixture of the internal standard and the product of known concentration [27–29].

$$R = (I_P/I_{IS})/([Product]/[Internal\ standard]) \quad (1)$$

In this kinetic study, a chondroitin disaccharide, α-ΔUA-[1 $\rightarrow$ 3]-GalNAc-6S (ΔD_i-6S) was chosen as an internal standard. The linear response of the R factor was verified by measuring the ion intensity ratio for various concentrations of the product and internal standard (see "supporting information"). To obtain the single-point normalization factor for each product, two ions were monitored: the internal standard ion, $[\Delta D_i$-6-S-H]$^{1-}$, at m/z 458 and the corresponding product ion, which is [chitobiose-6-OSO$_3$]$^{1-}$ at m/z 503, [chitotriose-6-OSO$_3$]$^{1-}$ at m/z 706, [chitotetraose-6-OSO$_3$]$^{1-}$ at m/z 909, or [chitopentaose-6-OSO$_3$]$^{1-}$ at m/z 1112.

For each sample analyzed in the subsequent kinetic measurements, the product concentration can be calculated via eq 2 using the ESI-MS data (I_P/I_{IS}) and the normalization factor R determined above.

$$[Product] = (I_P/I_{IS}) \times [Internal\ standard]/R \quad (2)$$

Kinetic constant (K_M, k_{cat}, and k_{cat}/K_M) determination. A stock solution of NodST (100 nM) (NodST stock 2) was prepared in Buffer A. For each of the four acceptor substrates studied, a series of seven pre-reaction solutions were prepared by mixing the PAPS stock solution (250 μM), chitooligosaccharide stock solutions (0.5, 1, 2, 3, 4, 6, 8 mM for chitobiose; 50, 100, 200, 300, 400, 600, 800 μM for chitotriose, chitotetraose, and chitopentaose) in Buffer A. After initiating each reaction with 25 μL of NodST stock 2, the chitooligosaccharide concentrations in the seven reaction solutions were 50 to 800 μM for chitobiose, and 5 to 80 μM for chitotriose, chitotetraose, and chitopentaose, while the concentration of PAPS and NodST in each reaction system was 25 μM and 50 nM respectively. The reaction was allowed to proceed for 10 min for chitobiose, and 4 min for chitotriose, chitotetraose, and chitopentaose, after which a 20-μL aliquot of each reaction solution was quenched in 80 μL of MeOH with 6.25 μM internal standard (ΔD_i-6S). The seven quenched samples were analyzed by ESI-MS and the amount of product in each sample was quantified using the corresponding single-point normalization factor. The K_M, k_{cat}, and k_{cat}/K_M values for each chitooligosaccharide were determined by plotting the calculated initial velocity versus chitooligosaccharide concentration using the GraFit program (Version 4.0.12, from Erithacus Software Ltd., Horley, Surrey, UK). Error in these calculations was determined from four replicate experiments.

Substrate Specificity Evaluation Using the Multiplex Approach

Methodology for product quantification. Stock solutions of chitobiose (1 mM), chitotriose (1 mM), chitotetraose (1 mM), chitopentaose (1 mM), and PAPS (1.5 mM) were prepared in Buffer A. Four individual pre-reaction solutions (50 μL) for the four acceptor substrates were prepared by mixing 10 μL PAPS stock solution and 30 μL Buffer A with 10 μL chitobiose, chitotriose, chitotetraose and chitopentaose stock solution respectively. Each reaction was initiated with the addition of 50 μL of NodST stock 1. The concentrations of PAPS and chitooligosaccharide in each reaction system were 150 μM and 100 μM respectively, along with 100 nM NodST. The samples were kept at rt overnight to allow the enzymatic reactions to proceed to completion. As evidenced by the absence of a peak corresponding to the chitooligosaccharide in the ESI-MS spectra, the acceptor substrate in each reaction system had been completely converted into the corresponding sulfated chitooligosaccharide product, giving a product concentration of 100 μM. At this point, 75 μL of each completed reaction solution were mixed together, generating a 300 μL solution mixture (Solution 1) in which the concentrations of the four sulfated chitooligosaccharide products: chitobiose-6-OSO$_3^-$, chitotriose-6-OSO$_3^-$, chitotetraose-6-OSO$_3^-$, and chitopentaose-6-OSO$_3^-$ were all 25 μM. Solution 1 was subsequently diluted with Buffer A in seven different ratios, generating Solutions 2 through 8, in which the concentrations of the four products are 20 μM, 15 μM, 10 μM, 5 μM, 2.5 μM, 1 μM, and 0.5 μM respectively. A 20-μL aliquot of each solution (1 through 8) was quenched in 80 μL MeOH containing 6.25 μM internal standard, and the resulting series of eight quenched sample solutions was used to construct the corresponding calibration curves for the four sulfated products.

Selected ion monitoring (SIM) analysis was performed on each of the eight quenched sample solutions. In this particular study, the five ions were monitored simultaneously, the four sulfated products ([chitobiose-6-OSO$_3$]$^{1-}$, [chitotriose-6-OSO$_3$]$^{1-}$, [chitotetraose-6-OSO$_3$]$^{1-}$, and [chitopentaose-6-OSO$_3$]$^{1-}$) at m/z 503, 706, 909, and 1112, respectively, and the internal standard ($[\Delta D_i$-6S-H]$^{1-}$) at m/z 458. Consequently, four relative ion intensity ratios were obtained for each quenched sample solution, which fall between the internal standard ion and each of the four sulfated-product ions. For each sulfated product, a calibration curve was generated by plotting the ion intensity ratios of the product and the internal standard (I_P/I_{IS}) versus their concentration ratios. The slope of each calibration curve (normalization factor) was used for quantification of the product of each competing substrate in one reaction system.

Specificity constant (k_{cat}/K_M) measurement and substrate specificity evaluation. A 50-μL pre-reaction solution containing multiple chitooligosaccharide substrates was prepared by mixing 10 μL PAPS stock solution (250 μM), and the four chitooligosaccharide stock solutions (10 μL each) (3 mM for chitobiose, and 300 μM for chitotriose, chitotetraose, and chitopentaose). After initiating each reaction with 50 μL of NodST stock 2, the chitooligosaccharide concentrations in the multi-substrate reaction solution were 300 μM for chitobiose and 30 μM for chitotriose, chitotetraose, and chitopentaose, while the concentration of PAPS and NodST in each reaction system was 25 μM and 50 nM respectively. At 2, 4, 6, and 8 min, a 20-μL aliquot of reaction solution was quenched in 80 μL of MeOH containing the internal standard. Upon analysis by ESI-MS, the four sulfated chitooligosaccharide products in each quenched sample were quantified using the normalization factors determined above and the conversion of reaction was plotted versus the reaction time to generate a reaction progress curve. The relative reaction specificity of the four substrates, represented by the ratios of their individual specificity constant (k_{cat}/K_M), was obtained using their product concentration ratios at 2 min. Furthermore, the value of the specificity constant for each substrate can be calculated based on the determined ratios and the k_{cat}/K_M value of one of the substrates obtained in the individual kinetic measurement.

## Results and Discussion

Individual Kinetic Measurements

Before the individual kinetic measurement for each substrate, several preliminary experiments with a limited number of chitooligosaccharide concentrations were performed to determine the proper substrate concentration range and quench time. As revealed by the preliminary experiments, chitobiose is the weakest binder to NodST and its K_M value is expected to be much higher than that of the other three chitooligomers. Therefore, a substrate concentration range of 50 to 800 μM was used for the kinetic measurement of chitobiose, which is one order of magnitude higher than that used for the other three chitooligomers (5 to 80 μM). Additionally, the chitobiose reaction was allowed to proceed for 10 min compared to 4 min for chitotriose, chitotetraose, and chitopentaose, since chitobiose reacts much slower than the other three acceptor substrates. For the individual measurements of each chitooligosaccharide, PAPS was kept at a fixed, saturating concentration of 25 μM. An average single-point normalization factor was determined to be 2.4 for chitobiose-6-OSO_3^-, 3.0 for chitotriose-6-OSO_3^-, 4.0 for chitotetraose-6-OSO_3^-, and 2.7 for chitopentaose-6-OSO_3^-. The initial reaction velocity was calculated and plotted versus the chitooligosaccharide concentration, generating four saturation plots for the four acceptor substrates (Figure 1) (Figure 2a, b, c, d). A nonlinear regression analysis of each saturation

(a)

(b)
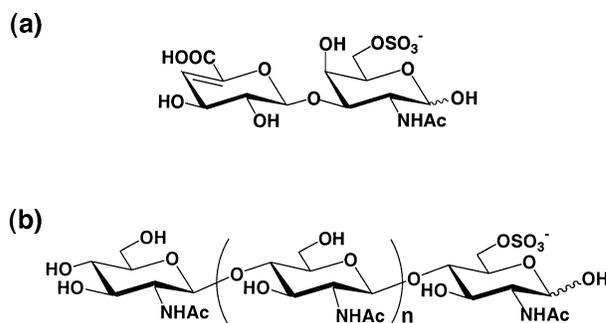
Figure 1. Structure of the internal standard and the four products monitored. (**a**) Internal standard, α-ΔUA-[1 $\rightarrow$ 3]-GalNAc-6S (ΔD_i-6S), with m/z = 458; (**b**) Chitobiose-6-OSO_3^- (n = 0) with m/z = 503, chitotriose-6-OSO_3^- (n = 1) with m/z = 706, chitotetraose-6-OSO_3^- (n = 2) with m/z = 909, chitopentaose-6-OSO_3^- (n = 3) with m/z = 1112.

plot was subsequently performed to obtain the kinetic constants. Average K_M, k_{cat}, and k_{cat}/K_M values for four replicate analyses of chitobiose, chitotriose, chitotetraose, and chitopentaose are summarized in Table 1. There is an obvious 10-fold decrease in the K_M value from chitobiose to chitotriose. Chitotetraose, which has the lowest K_M value of 14.9 μM, turns out to have the highest binding affinity to NodST among the four chitooligosaccharides. Although chitopentaose was revealed to have the highest reaction turn over number (k_{cat} value), chitotetraose was the most preferred substrate of NodST among the four, as indicated by the specificity constant values (k_{cat}/K_M). The k_{cat}/K_M ratios were calculated to be 1:12:24:21 with respect to the k_{cat}/K_M value of chitobiose. Chitobiose was arbitrarily chosen as the integer value because of its low specificity constant with respect to the other three chitooligomers. The relative reaction specificity of the four substrates determined by our assay was in excellent agreement with that reported in the literature [31, 37], which suggests that in the pathway of Nod signal biosynthesis, the lipochitotetrasaccharide Nod factor is preferably sulfated by NodH.

The method of elucidating the reaction specificity of NodH for multiple competing substrates by individual kinetic measurements was successfully performed using ESI-MS, as demonstrated above. However, a major problem of this technique is that all substrates must be studied individually and a group of varied concentrations of each substrate is necessary to obtain the specificity constant. In these individual measurements, seven reaction solutions were used for each of the four substrates tested and a total of 28 samples were generated. In order to radically simplify this procedure, a multiplex ESI-MS assay was developed and applied to NodST, in which multiple competing reactions of different substrates are handled in one reaction system to obtain specificity constants by ESI-MS analysis.
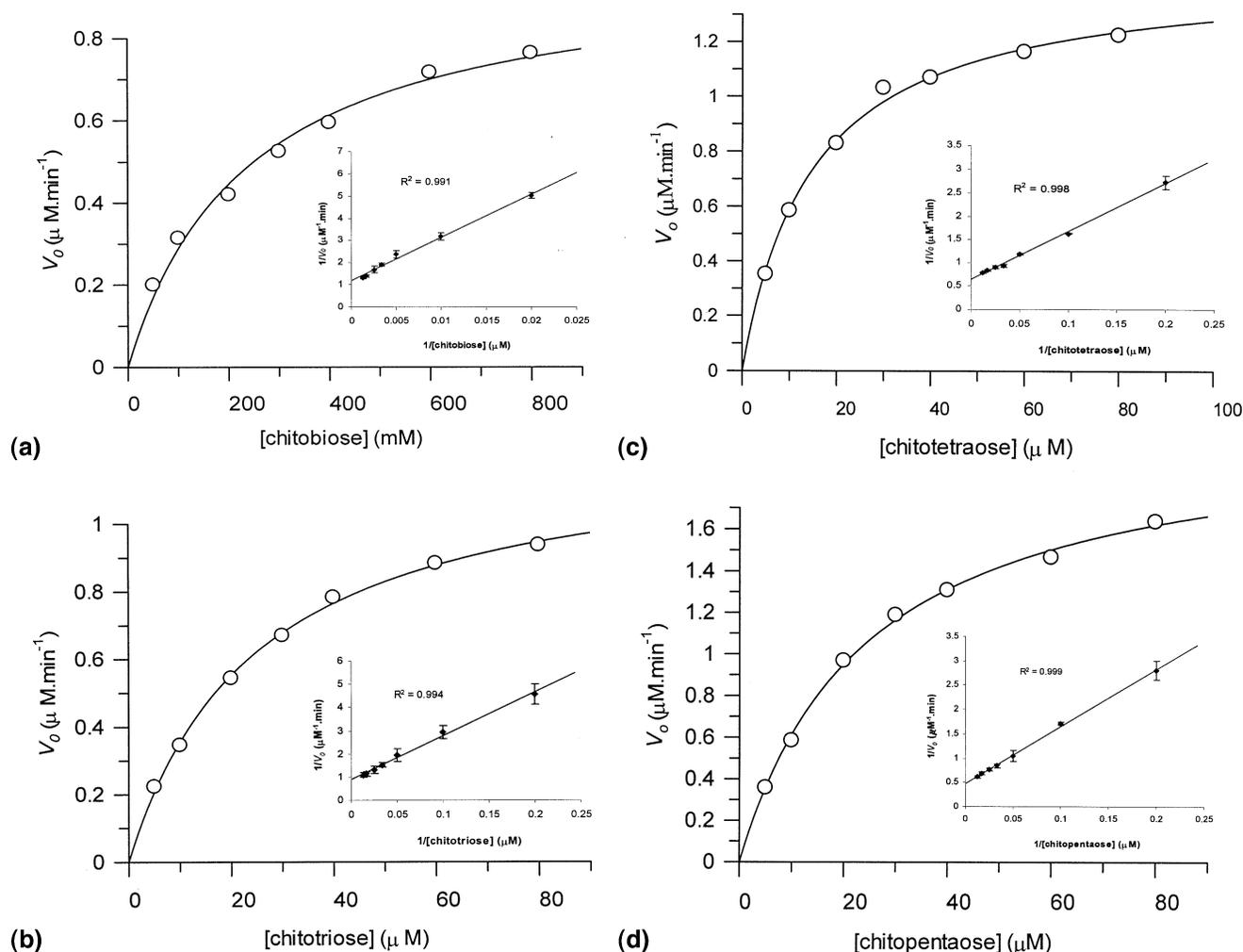
Figure 2. Determination of the kinetic constants for chitooligosaccharide substrates via individual kinetic measurements. (**a**) Saturation plot: V_0 vs. [chitobiose]. The inset is double reciprocal plot: $1/V_0$ vs. 1/[chitobiose]. ([Chitobiose] = 50, 100, 200, 300, 400, 600, 800 μM). (**b**) Saturation plot: V_0 vs. [chitotriose]. The inset is double reciprocal plot: $1/V_0$ vs. 1/[chitotriose]. ([Chitotriose] = 5, 10, 20, 30, 40, 60, 80 μM). (**c**) Saturation plot: V_0 vs. [chitotetraose]. The inset is double reciprocal plot: $1/V_0$ vs. 1/[chitotetraose]. ([Chitotetraose] = 5, 10, 20, 30, 40, 60, 80 μM). (**d**) Saturation plot: V_0 vs. [chitopentaose]. The inset is double reciprocal plot: $1/V_0$ vs. 1/[chitopentaose]. ([Chitopentaose] = 5, 10, 20, 30, 40, 60, 80 μM). [PAPS] was fixed at 25 μM for all the four chitooligosaccharides. [NodST] = 50 nM, pH = 8.0. Each saturation plot was generated from the average data of three replicate experiments.

Multiplex Substrate Specificity Evaluation

A theoretical model for calculation. If several substrates are mixed together and compete for the same active site of an enzyme, the initial reaction rate of each competing substrate (v_n) can be calculated using eq 3,

$$v_n = (k_{cat}/K_M)_n \cdot [S_n]/(1 + \sum [S_n]/(K_M)_n) \qquad (3)$$

where $(K_M)_n$ and $(k_{cat}/K_M)_n$ are the Michaelis-Menten constant and specificity constant, respectively, for each particular substrate (S_n).

Since the reaction rate is determined by more than just the concentrations and kinetic parameters of a single competing substrate, we can not obtain the

Table 1. Kinetic constants determined for chitooligosaccharide substrates in individual kinetic measurements

Acceptor substrate	K_M (μM)	k_{cat} (min^{-1})	k_{cat}/K_M (μM$^{-1}\cdot$min^{-1})	Relative k_{cat}/K_M[c]
Chitobiose	240 ± 10[a]	19.5 ± 0.7[b]	0.082 ± 0.005	1
Chitotriose	25 ± 5	25 ± 3	1.0 ± 0.1	12 ± 2
Chitotetraose	14.9 ± 0.2	29 ± 2	2.0 ± 0.1	24 ± 2
Chitopentaose	23 ± 4	42 ± 1	1.7 ± 0.1	21 ± 2

[a]The standard deviations were calculated based on 4 sets of replicate experiments for each substrate.
[b]The k_{cat} values are calculated using the V_{max} value with NodST concentration of 50 nM.
[c]The relative k_{cat}/K_M is represented by the ratios of k_{cat}/K_M values of the four substrates, which were calculated in respect to k_{cat}/K_M value of chitobiose.

specificity constant of the substrate by solely plotting the initial rate versus the substrate concentration. However, the ratio of reaction rates for competing substrates in a mixture is related to their specificity constants and their concentrations, as shown in eq 4.

$$\nu_1 : \nu_2 : \ldots \ldots :$$
$$\nu_n = \{(k_{cat}/K_M)_1 \cdot [S_1]\} : \{(k_{cat}/K_M)_2$$
$$\cdot [S_2]\} : \ldots \ldots : \{(k_{cat}/K_M)_n \cdot [S_n]\} \tag{4}$$

Hence, the specificity constant ratio of multiple competing substrates can be determined in one reaction using eq 5, given the initial reaction rate of each substrate.

$$(k_{cat}/K_M)_1 : (k_{cat}/K_M)_2 : \ldots \ldots : (k_{cat}/K_M)_n$$
$$= (\nu_1/[S_1]) : (\nu_2/[S_2]) : \ldots \ldots : (\nu_n/[S_n]) \tag{5}$$

In the multi-substrate reaction system, all the competing reactions are initiated and quenched at the same time and the reaction rates in eq 5 can be substituted by the corresponding product concentrations, resulting in eq 6.

$$(k_{cat}/K_M)_1 : (k_{cat}/K_M)_2 : \ldots \ldots : (k_{cat}/K_M)_n$$
$$= ([P_1]/[S_1]) : ([P_2]/[S_2]) : \ldots \ldots : ([P_n]/[S_n]) \tag{6}$$

Using mass spectrometry, the investigator has the advantage of monitoring and quantifying multiple species with different molecular weights without further separation, and multiple products of different molecular weights generated in the multi-substrate reaction system can be quantified simultaneously by monitoring the corresponding product peaks in relation to an internal standard peak. Since we know the starting concentration of each substrate and we can measure the concentration of each product, the k_{cat}/K_M ratios of multiple substrates can be easily determined and the substrate specificity of an enzyme can be rapidly evaluated. Therefore, as long as we know the k_{cat}/K_M value of one substrate, the k_{cat}/K_M values for the rest of the competing substrates can be calculated using the k_{cat}/K_M ratios.

Methodology for product quantification. In the individual kinetic measurements for each acceptor substrate, only one reaction was involved and one product was quantified. The product quantification was carried out using a single-point normalization factor, since there is a linear response between the concentration ratio and ion intensity ratio of the product being quantified and the internal standard. However, the multi-substrate reaction system contains multiple competing reactions and results in formation of multiple products, hence the

parallel quantification of all the products is required. In particular, the linear responses between the concentration ratio and ion intensity ratio of all the products and the internal standard in the multi-substrate reaction system need to be carefully monitored. In order to check this, four calibration curves were constructed for the four sulfated chitooligosaccharide products in the multi-substrate reaction system of NodST. As demonstrated in Figure 3, excellent linearity ($R^2 > 0.99$) was obtained for all four calibration curves over a wide range of product and internal standard concentration ratios (0.02 to 1), and the lowest product concentration tested in each calibration curve was 0.1 μM. The results strongly indicate a linear response between the concentration ratio and ion intensity ratio of each product and the internal standard. Furthermore, this linear response is not affected by the presence of other substrates and products in the sample. Thus, the quantification of multiple products formed in the multi-substrate reaction system is feasible. The slope of each calibration curve was determined to be 2.8 for chitobiose-6-OSO_3^-, 4.0 for chitotriose-6-OSO_3^-, 4.0 for chitotetraose-6-OSO_3^-, and 3.1 for chitopentaose-6-OSO_3^-, which were subsequently used as the normalization factors to quantify the corresponding product in the multi-substrate reaction system.

Specificity constant (k_{cat}/K_M) measurement and substrate specificity evaluation. Since the specificity constant value of chitobiose is one order of magnitude lower than the other three acceptor substrates, as revealed in our individual kinetic measurements, we adjusted the starting concentration of chitobiose (300 μM) to be ten times the concentration of the other three (30 μM) in the multi-substrate reaction system to facilitate quantification.

As shown in Figure 4, five peaks in the quenched reaction sample were monitored simultaneously using the SIM mode of ESI-MS. They are the internal standard peak at m/z 458, and the four sulfated chitooligosaccharide peaks at *m/z* 503, 706, 909, and 1112. Parallel quantification of the four sulfated products was performed at different reaction times to generate the reaction progress curves, which indicated that following two minutes of reaction time the reaction conversion for chitobiose, chitotriose, chitotetraose, and chitopentaose were 0.1%, 1.5%, 3.5%, and 2.4%, respectively. Since the sulfation percentage of all the substrates was lower than 5% after 2 min of reaction, a reaction time of 2 min should reside in the initial period of all the four reactions. It is important to note that to obtain the correct specificity constant ratios using eq 6, the product concentrations at the initial reaction stage must be used. This is a direct result of the conditions required in eq 3, which describes the initial reaction velocity of each competing substrate in a multi-substrate enzymatic system, from which eq 6 is derived. Hence, the product concentrations at a two minute reaction were used to calculate the k_{cat}/K_M ratios of the four acceptor sub-
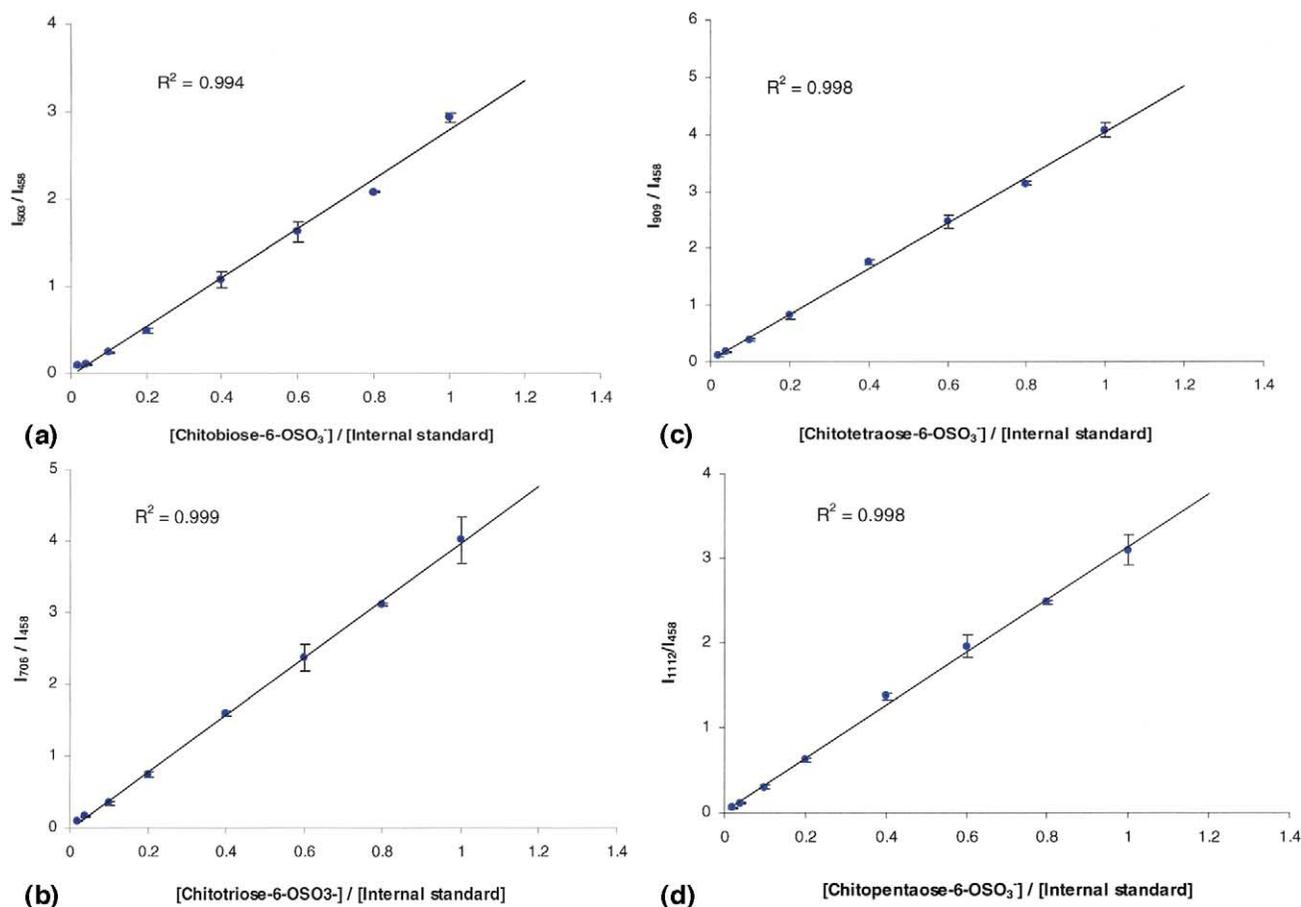
Figure 3. Calibration curves for the four sulfated chitooligosaccharide products in the multi-substrate reaction system. (**a**) I_{503}/I_{458} vs. [chitobiose-6-OSO$_3^-$]/[internal standard]. (**b**) I_{706}/I_{458} vs. [chitotriose-6-OSO$_3^-$]/[internal standard]. (**c**) I_{909}/I_{458} vs. [chitotetraose-6-OSO$_3^-$]/[internal standard]. (**d**) I_{1112}/I_{458} vs. [chitopentaose-6-OSO$_3^-$]/[internal standard]. A mixed completed reaction solution was used for calibration curve construction, in which [PAPS] = 50 μM, [chitobiose-6-OSO$_3^-$] = [chitotriose-6-OSO$_3^-$] = [chitotetraose-6-OSO$_3^-$] = [chitopentaose-6-OSO$_3^-$] = 25 μM. [NodST] = 50 nM, pH = 8.0. Each calibration curve was generated from the average data of three replicate experiments.

strates based on the theoretical calculation model. The average results of three replicate experiments are listed in the first column of Table 2, which are in good agreement with the ratios obtained from the individual measurements (Table 1). The consistency of the results strongly supports the fact that the multiplex ESI-MS assay, based on the theoretical steady-state kinetics model, is valid for accurate enzyme specificity determination with multiple competing substrates. Given the k_{cat}/K_M ratios of the four substrates obtained in the multiplex assay, and having the k_{cat}/K_M value for any one substrate determined in the individual measurement, we can subsequently calculate the k_{cat}/K_M values for all the other substrates. As listed in the second column of Table 2, the k_{cat}/K_M values of chitotriose, chitotetraose, and chitopentaose were calculated based on the k_{cat}/K_M value of chitobiose obtained in the individual study.

For substrate specificity studies, our multiplex assay is clearly competitive with the traditional methodology.

One main advantage is that only one reaction system and one quenched sample is required: all the substrates tested are mixed together and their specificity information can be rapidly obtained in only one measurement. Another important advantage of the multiplex assay is that small substrate concentrations ($\leq 0.2 K_M$), which are necessary in the individual kinetic measurement to generate accurate saturation plot, are not required in this new methodology. Since a small starting concentration of substrate will result in formation of very small amount of product, the instrument needs to have a high sensitivity and low detection limit to achieve accurate product quantification. However, in the multiplex assay, there are no specific requirements for the starting concentration of each substrate. A considerable amount of each product will be formed in the multi-substrate reaction system when all the competing reactions are quenched in the initial reaction period. Therefore, accurate specificity constant ratios can be obtained by quantifying multiple products.
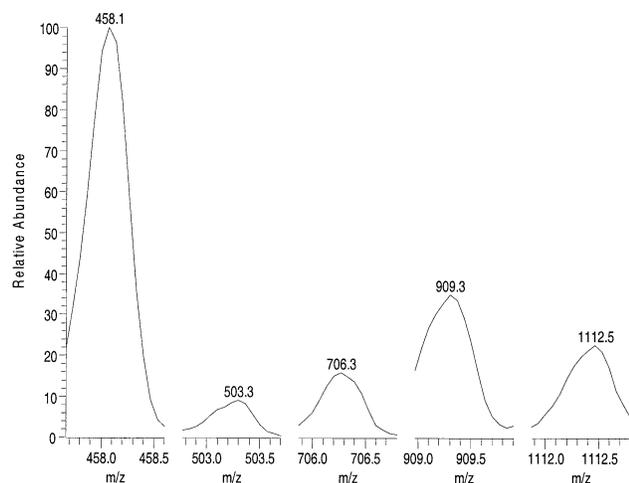
Figure 4. Selected ion monitoring (SIM) of five peaks in the multi-substrate reaction system. The internal standard peak is at m/z 458 and the four product peaks are at m/z 503, 706, 909, 1112. [PAPS] = 25 μM, [chitobiose] = 300 μM, [chitotriose] = [chitotetraose] = [chitopentaose] = 30 μM. [NodST] = 50 nM, pH = 8.0.

Conclusions

A novel multiplex ESI-MS assay for determining specificity constants was developed and applied to the study of NodST, a GlcNAc-6-O-sulfotransferase. A substrate library consisting of four chitooligosaccharides (chitobiose, chitotriose, chitotetraose, and chitopentaose) was used, and the specificity constant (k_{cat}/K_M) of each library component was first determined by individual kinetic measurements. Subsequently, a multiplex assay was used to rapidly determine the specificity constant ratios for a mixture of the four chitooligosaccharide substrates. The specificity constant ratios determined from the multiplex assay were found to be in

Table 2. Substrate specificity evaluation and specificity constant measurement using the multiplex ESI-MS assay

Acceptor substrate	Relative k_{cat}/K_M [a]	k_{cat}/K_M (μM$^{-1} \cdot$ min^{-1})
Chitobiose	1	0.082
Chitotriose	12.3 ± 0.2[b]	1.0 ± 0.0
Chitotetraose	27.6 ± 0.6	2.3 ± 0.1
Chitopentaose	21 ± 2	1.8 ± 0.2

[a]The ratios of k_{cat}/K_M values of the four substrates were calculated in respect to chitobiose.
[b]The standard deviations were calculated based on 3 sets of replicate experiments for each substrate.
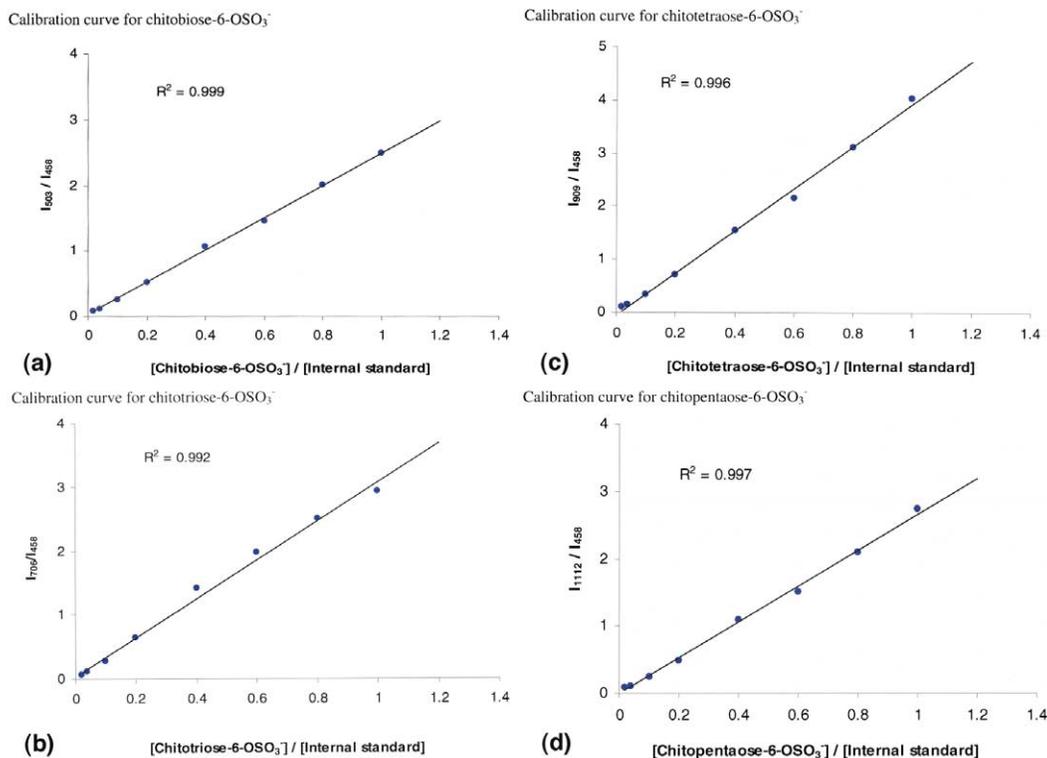
good agreement with those obtained from the individual kinetic measurements. The consistency of the results demonstrated the multiplex ESI-MS assay to be an accurate and valid method for substrate specificity evaluation of NodST. Using this assay, multiple substrates of different sizes can be tested in one reaction system and their reaction specificity can be revealed by one measurement in which all the products are quantified simultaneously. Compared with the traditional method for substrate specificity study, this new assay requires fewer experimental materials, shorter analysis time, and is expected to be an efficient and promising methodology to study other enzymes of therapeutic interest. Current efforts are focused on applying this multiplex methodology to the specificity study of a synthetic substrate library of HS sulfotransferase.

Acknowledgments

Appendix

Supporting information: Calibration curves for each sulfated chitooligosaccharide product in the single substrate reaction system

Calibration curve for chitobiose-6-OSO$_3^-$

$R^2 = 0.999$

(a)   [Chitobiose-6-OSO$_3^-$] / [Internal standard]

Calibration curve for chitotetraose-6-OSO$_3^-$

$R^2 = 0.996$

(c)   [Chitotetraose-6-OSO$_3^-$] / [Internal standard]

Calibration curve for chitotriose-6-OSO$_3^-$

$R^2 = 0.992$

(b)   [Chitotriose-6-OSO$_3^-$] / [Internal standard]

Calibration curve for chitopentaose-6-OSO$_3^-$

$R^2 = 0.997$

(d)   [Chitopentaose-6-OSO$_3^-$] / [Internal standard]

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