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Enzymatic degradation and electrospray tandem mass spectrometry as tools for determining the structure of cationic starches prepared by wet and dry methods

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Abstract—Cationic starches from various semi-technical processes, two 'wet' (slurry and paste modification) and two 'dry' procedures (dry modification and extrusion), each type in a DS range from 0.03 to 0.1, were investigated by electrospray ionisation mass spectrometry (ESIMS) and tandem mass spectrometry ($ESIMS^2$) after enzymatic degradation with α-amylase and subsequent glucoamylase digestion. For comparison, chemically derived cationic oligosaccharides were also analysed by ESIMS. The cationisation pattern in the glucosyl units was analysed by GLC after methanolysis, permethylation and Hofmann elimination. Results from ESIMS are discussed and interpreted with respect to enzyme susceptibility, monomer composition and physical properties of the different types of cationic starches.

Keywords: Cationic starch derivatives; Enzymatic degradation; Electrospray ionisation mass spectrometry; Tandem mass spectrometry; Substituent distribution

1. Introduction

The properties of starch derivatives are influenced by many structural features such as the molecular mass distribution, degree of branching, degree of substitution (DS), type and distribution of substituents. In order to establish the relationship between structure and properties, a number of methods have been developed to determine the substituent distribution in the glycosyl units, but it is a more complex task to obtain information about the substitution pattern in the polymer chain and over the starch granule with its fascinating architecture formed by amylose and amylopectin. Cationic starch is widely used in paper manufacturing. Thus *O*-(2-hydroxy-3-trimethylammonium)propyl starch (**1**) is usually prepared by base catalysed addition of the 2,3-epoxypropyl-trimethylammonium chloride in an aqueous granular suspension of starch (slurry) or in a 'dry' or 'semi-dry' process, where the starch is directly mixed with the reagents.[1,2] Since the structure of a starch granule with the linear amylose and the branched amylopectin is very complex including crystalline and amorphous lamellae,[3] the distribution of cationic groups must be considered on different structural levels. The pattern in the monomer unit has been investigated by Wilke and Mischnick[4,5] and Goclik and Mischnick.[6] Mass spectrometry, especially using 'soft' ionisation techniques such as fast atom bombardment (FAB), matrix assisted laser desorption/ionisation (MALDI) and electrospray ionisation (ESI), is a valuable method for gathering structural information on oligosaccharides. Collision-induced dissociation (CID) experiments with FAB- and ESIMS or post-source decay (PSD) in MALDIMS have been proven to provide even more details on composition and branching of oligosaccharides. ESI in combination with CID and an ion-trap detector is a powerful tool due to the capability of performing several consecutive steps of isolation and

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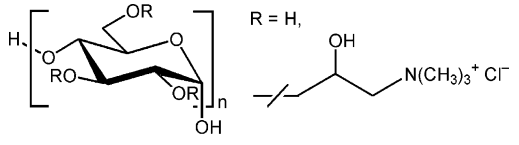
fragmentation (MS^n). There are also a few examples where the mentioned methods have been applied to oligosaccharide mixtures obtained from starch or cellulose derivatives.[7–13] Degradation to oligosaccharides can be performed in a random manner by partial methanolysis or hydrolysis, if required after appropriate derivatisation, or by a selective method, like enzymatic degradation. On the basis of the enzyme specificity or by statistical evaluation after random cleavage, respectively, the substitution pattern in the polymer chain can be characterised.[7–13] Debranching and enzymatic degradation of cationic starches by β-amylase has been carried out in combination with chromatographic separation by Manelius et al.,[2,14] and digestion with α-amylase and glucoamylase by Wilke and Mischnick[5] and in combination with mass spectrometry by Richardson et al.[7] The amount of liberated glucose, the degree of debranching and the β-limit value have been discussed. From these investigations, it was concluded that the wet modification leads to preferred substitution in the branching regions and the amorphous lamellae of the granule, while dry-modified cationic starch preferably is modified at the outer surface. By tandem mass spectrometry such as $ESIMS^n$, more detailed information about the molecular structure of oligosaccharides is gained due to the influence of the position of glycosidic linkages or the location of substituents. But here is in fact the weak spot of these approaches. The situation often is so complex and the knowledge about the enzymes' specificity and the fragmentation behaviour with respect to the location of substituents is so poor that progress beyond the stage of mere description and speculative interpretation is difficult.



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We now report on the comprehensive and systematic investigation of 12 cationic starches prepared under four different semi-technical reaction conditions, each set in a DS range of 0.03–0.12. As analytical tools monomer analysis, enzymatic degradation and comparison of the product pattern by ESIMS and $ESIMS^n$ are used, a combination of complementary methods, which has not been applied before to cationic starches. For the first time, the relative ions intensities in ESIMS mother and daughter mass spectra are interpreted on a molecular level and related to the original location of cationic groups in the starch granule. The positions of substituents, which do not interfere with the enzymes' activity were determined, and the interpretation is also based on our recent systematic investigations of the ESIMS-CID fragmentation behaviour of regioselectively etherified maltotrioses.[15]

2. Results and discussion

Cationic starches (**1**) prepared by various processes were investigated. They had been modified in a slurry (CS-SL), paste (CS-PA), dry (CS-DR) or extrusion process (CS-EX). DS values were in the range of 0.03–0.12. Preparation and properties are described by Radosta et al. in more detail.[16]

2.1. Substituent distribution in the glucosyl unit

First, the distribution of cationic groups in the glucosyl units was determined for each type of process according to the method developed in our group.[4,5] This knowledge is important, since the position of substitution influences the fragmentation behaviour in ESIMS[2] and therefore might cause differences for the various sets of CS.[15] Briefly: The cationic starch is submitted to a reaction sequence including methanolysis, permethylation and Hofmann elimination. The resulting methyl *O*-(2-methoxy-2-propenyl)-*O*-methyl-α,β-D-glucosides are then analysed by GLC. Results are summarised in Table 1. The nomenclature of Spurlin is used for the mole fractions and partial DS values.[17] Nearly all samples show strongly preferred O-2-substitution. About 86–90% of the cationic groups are usually located in this most acidic position, which is well known for the addition of oxiranes to α-glucans at the low concentration of catalytically acting base. Modification of the O-3- and O-6-position are similar with a slight preference for the secondary position, a phenomenon, which is not well understood until now, since the bulkiness of the cationic reagent as well as the usually higher reactivity of primary compared to secondary OH-groups should favour the reactivity of O-6 over O-3. Due to the low DS values, no or only traces of disubstituted compounds were determined, while trisubstituted ones could not be detected in any case. However, the cationic starches from the extrusion process (CS-EX) do not follow this general pattern just outlined. First, the regioselectivity is much less pronounced and changes with DS. Only 61% of the substituents are located in position 2 at DS 0.04, increasing to 68% at a DS of 0.07, and to 73% at a DS of 0.11. With 24% the relative amount of O-3-substitution is surprisingly high at the lowest DS, but decreases to 16% for DS 0.11. For the CS-EX samples, the preference of O-3-compared to O-6-reactivity is therefore more obvious with 14% at O-6 at DS 0.04 and 12% for DS 0.11. In contrast to other procedures, a thermal plastification process occurs during the transport in the winding and completely destroys the granular structure,

Table 1. Substituent distribution in the glucosyl unit of cationic starches (CS) from different processes SL = slurry, EX = extruder, DR = dry, PA = paste modified

Cationic starch (mol%)	CS-SL-010	CS-PA-008	CS-EX-004	CS-EX-007	CS-EX-011	CS-DR-003	CS-DR-006	CS-DR-010
DS	0.10	0.08	0.04	0.07	0.11	0.030	0.06	0.10
s_0	90.00	92.00	97.01	93.03	89.09	97.01	94.00	90.02
s_2	8.86	6.48	2.43	4.70	7.92	2.66	5.31	8.62
s_3	0.65	0.92	0.96	1.32	1.67	0.19	0.36	0.71
s_6	0.49	0.60	0.58	0.93	1.24	0.14	0.33	0.62
s_{23}	n.d.	n.d.	0.012	0.014	0.044	0.002	n.d.	0.013
s_{26}	n.d.	n.d.	0.007	0.006	0.037	0.003	n.d.	0.011
s_{36}	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
s_{236}	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
c_0	90.00	92.00	96.01	93.03	89.09	97.01	94.00	90.02
c_1	10.00	8.00	3.97	6.95	10.83	2.99	6.00	9.95
c_2	n.d.	n.d.	0.019	0.02	0.081	0.005	n.d.	0.024
c_3	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
2 (%)	88.6	81.0	61.0	67.5	72.8	88.8	88.5	86.5
3 (%)	6.5	11.5	24.3	19.1	15.6	6.5	6.1	7.2
6 (%)	4.9	7.5	14.7	13.4	11.6	4.7	5.4	6.3

Number behind the letters refer to the DS values. For example: CS-SL-010 = cationic starch from slurry process with a DS of 0.10.
$c_0 = s_0$.
$c_1 = \sum s_i$ $(i = 2, 3, 6)$.
$c_2 = \sum s_{i,j}$ $(i, j = 2, 3, 6)$.
$c_3 = s_{2,3,6}$.

accompanied by strong chain degradation.[16,18] A further difference observed for CS-EX samples is the occurrence of disubstitution even at such a low DS as 0.04, which also indicates a higher local density of substitution or heterogeneity in this process. The paste-modified cationic starches also show a higher O-3-substitution compared to slurry- and dry-modified samples. At a DS of 0.08, a relative ratio of 81.0 (2) : 11.5 (3) : 7.5 (6) was found, while an earlier investigated CS-PA with a DS of 0.04 had shown an average pattern of 80.2 (2) : 13.4 (3) : 6.4 (6).

2.2. Enzymatic degradability

All cationic starches were submitted to exhaustive enzymatic degradation with a combination of α-amylase (EC 3.2.1.1) from *B. licheniformis* and glucoamylase (EC 3.2.1.3) from *A. niger* as described.[5,19] The amount of glucose liberated by the enzymes was then determined photometrically with an enzyme kit. It was between ca. 60% at DS 0.10 and ca. 90% at DS 0.03. α-Amylase is an *endo*-enzyme, which cleaves α-(1 → 4) linkages in the glucan chain to yield maltose and maltodextrins in a DP range up to about 7, depending on the source of the enzyme and therefore on the structure of the active site. Then glucoamylase as an *exo*-enzyme cuts glucosyl units from the nonreducing end and hydrolyses (1 → 6) as well as (1 → 4) linkages. With this set of enzymes, starch is completely degraded to glucose under the conditions applied. *B. licheniformis* amylase was chosen since it is not stabilised with glucose as many other α-amylases. In the case of derivatives the digestibility by the enzymes is inhibited, and the extent of inhibition is influenced by the DS, the substituent distribution along the polymer chain and with regard to the branching points, and by the location in the monomer unit. We localised the cationic substituents in the products of enzymatic degradation with respect to the three different types of glucosyl units: the terminal nonreducing glucosyl units, the (1 → 4)-linked inner and the reducing-end glucosyl units. Details of the complex procedure will be reported elsewhere. Surprisingly, the terminal nonreducing glucosyl units were substituted to a similar extent as the inner residue (CS-EX-011). While no cationic groups could be detected for the reducing-end glucosyl unit, the terminal nonreducing units could bear a (2-hydroxy-3-trimethylammonium)propyl residue in positions 2 and 6. This result is in agreement with the enzyme selectivity determined for methyl amyloses,[19] but nevertheless surprising due to the much higher steric requirement and the charged nature of the cationic residue. Substitution in positions 2, 3 and 6 was found for the inner glucosyl residues, as expected. For samples with a similar DS, the amount of glucose is expected to correlate with the average block length of unsubstituted glucosyl units and should therefore indicate differences in the homo- or heterogeneity of the substituent distribution in and/or over the polymer chains. Although slight differences were found for the cationic starches from various processes with the highest average digestibility for paste-modified (CS-PA), followed by slurry-modified (CS-SL) and extruder- (CS-EX), and lowest for dry-modified cationic starch (CS-DR), these differences are not significant and reproducible enough to deduce reliable

information about the homo- or heterogeneity due to the low DS range investigated. It might also be influenced by the solution state achieved by pressure-cooking. CS-DR and CS-EX were not really dissolved at 130 °C, but the suspensions became clear during enzymatic degradation. For methyl amyloses, we could clearly demonstrate that under our conditions of enzymatic degradation O-6-methylation favours digestibility, while O-3-methylation inhibits it.[19] Since we found similar partial DS values in positions 3 and 6 for the cationic starches, these opposite effects could overlay the influence of homo- or heterogeneity, making a straightforward interpretation impossible. The location of substituents with respect to the branching points might also influence the digestibility as will be discussed later.

2.3. ESIMS of cationic starches after enzymatic degradation

Oligosaccharides bearing permanently cationic groups show a number of advantages compared to neutral oligosaccharides in mass spectrometry. Since they are already charged, no formation of sodium or potassium adducts as pseudomolecular ions is necessary, which often does not occur selectively and therefore causes more than one signal for a certain compound. Multiply charged oligosaccharides with more than one substituent are detected at lower and therefore more appropriate m/z values. The mixture of products obtained by enzymatic digestion was analysed by ESIMS. Figure 1a shows the ESI mass spectrum of CS-SL-010 after digestion with α-amylase and glucoamylase. The peak with the by far highest intensity is related to the monosubstituted trisaccharide (DP/n: 3/1), followed by the monosubstituted disaccharide (2/1), the disubstituted tetra- (4/2) and the disubstituted oligosaccharides (3/2–6/2). The same products were reported by Richardson et al.[7] for cationised amylopectin potato starches, treated with α-amylase from *A. oryzae* and amyloglucosidase from *A. niger*, however with a different oligomer composition. They found the monosubstituted tetrasaccharide (4/1) together with the monosubstituted trisaccharide (3/1) to be the most intensive ions, which is in agreement with the requirement of at least two unsubstituted glucosyl units left from the attacked linkage for α-amylase from *A. oryzae* as reported by Takeda et al.[20,21] Due to the susceptibility of the enzymes applied by us (see above) and the nearly exclusively observed monosubstitution/glucosyl unit (see Table 1), monomers should be unsubstituted, disaccharides mono-, and trisaccharides mono- or disubstituted. Although the relative signal intensities in the mass spectra do not simply represent the quantitative composition of the mixture, the patterns obtained for different samples can be compared. Figure 2a shows the

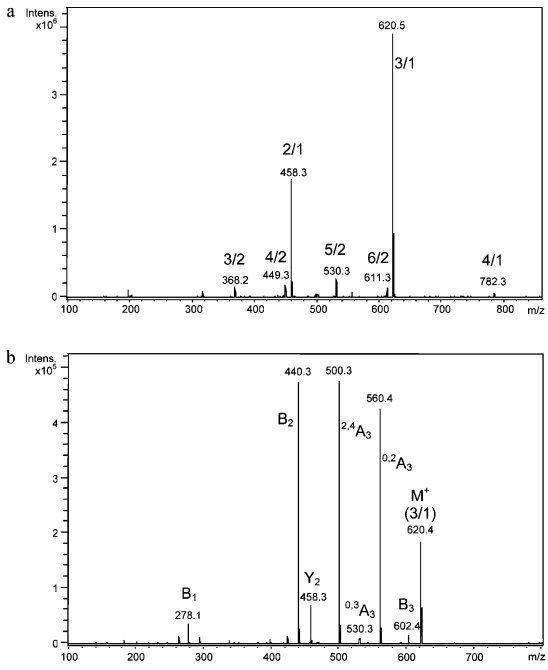


Figure 1. ESI mass spectrum of CS-SL-010 (a) and MS^2 spectrum of the monosubstituted trimers (3/1) (b).

relative intensities of the oligosaccharide signals in ESIMS obtained for the cationic starches with the highest DS (0.10–0.12) from all processes. Figure 2b–e compares the patterns obtained for the CS with various DS values for each process. The corresponding m/z values are listed in Table 2. For a certain procedure, the relative composition is nearly identical up for the main products 3/1 and 2/1, while the amount of higher substituted oligomers up to 5/2 and 6/2 always increases with the DS, as expected. The oligosaccharide patterns show significant differences for samples from various procedures. The relative amount of 3/1 is highest for dry-modified cationic starches (CS-DR), followed by the CS from the extrusion (CS-EX), the paste (CS-PA) and the slurry process (CS-SL). The various extent of disaccharide formation (2/1) reflects the ability to cleave a glucosyl linkage on the left side of a substituted unit, generating a new modified terminal nonreducing unit. This ability is highest for CS-SL and lowest for CS-DR, both showing the same regioselectivity of substitution (see Table 1). Therefore, these differences cannot easily be explained by the various substitution patterns, but parameters of influence are more complex. However, there is a correlation with the amount of glucose set free by the enzymes, which is not surprising, since formation of 2/1 from higher maltodextrins is accompanied by liberation of glucose. There is one further aspect, which should be considered. In their investigation of a wet and a dry cationised potato starch, Manelius et al.[2] found indications that the cationisation reagent attacks the crystalline lamellae in the amylopectin growth rings of

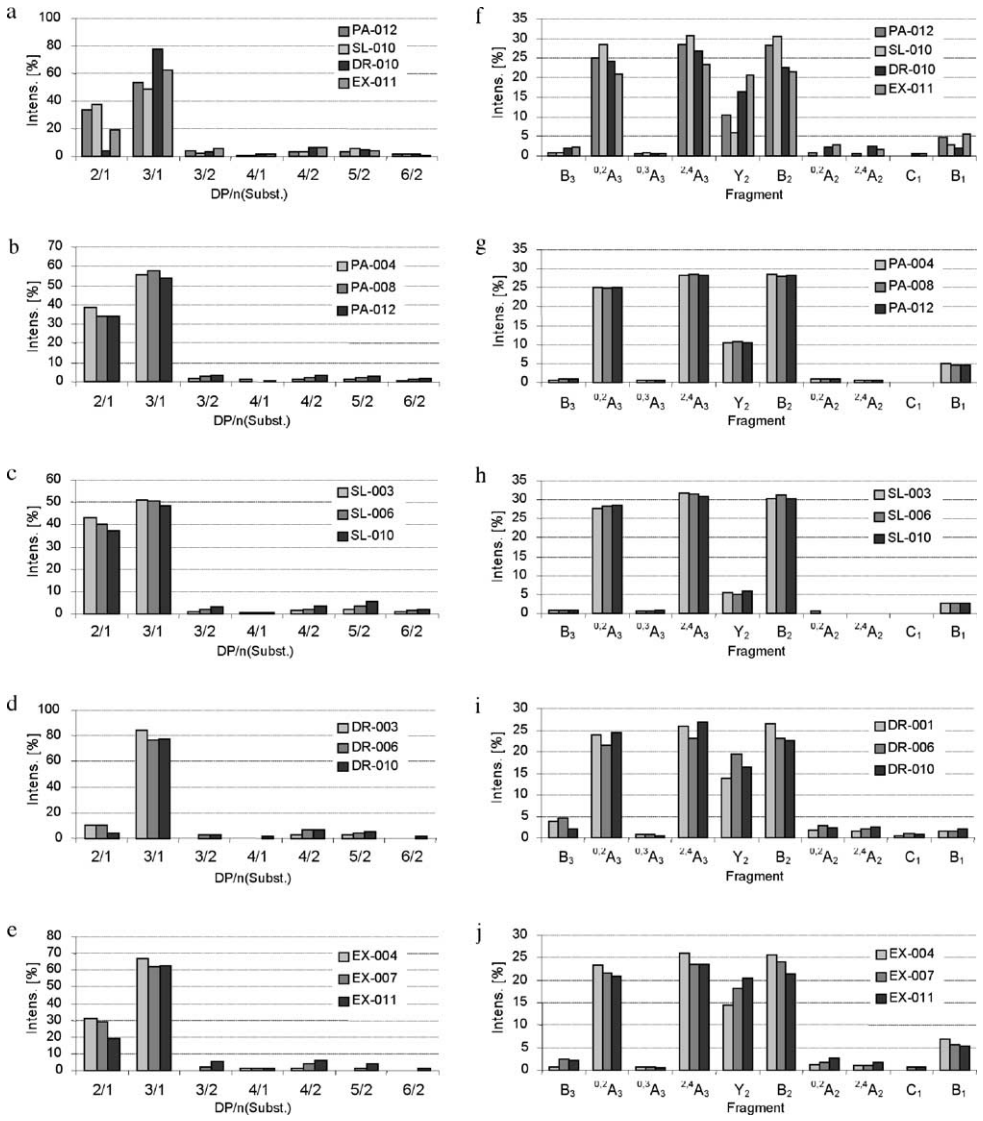


Figure 2. Left: relative intensities of the oligosaccharide signals in ESIMS of the CS with the highest DS (0.10–0.12) from all processes (a) and comparison of the patterns obtained for the CS with various DS values for each process: paste (b), slurry (c), dry (d) and extruder (e). Right: relative intensities of the fragment ions obtained from monosubstituted trisaccharides (3/1, m/z 620) for CS from all processes with the highest DS (f) and comparison of the results of various DS values for each CS series: paste (g), slurry (h), dry (i) and extruder (j).

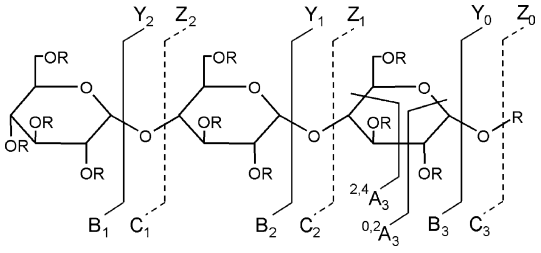
Table 2. Fragment ions of monosubstituted trimers of CS (M^+: m/z 620)

Fragment	m/z
B_3	602
$^{0,2}A_3$	560
$^{0,3}A_3$	530
$^{2,4}A_3$	500
Y_2	458
B_2	440
$^{0,2}A_2$	398
$^{2,4}A_2$	338
C_1	296
B_1	278

the intact granule from both sides, that is the amorphous branching regions and the ends of the side chains involved in helix formation. As a consequence, the higher ratio of substituents at the nonreducing terminal units of the oligosaccharides from the CS-SL might already have been present in the original cationic starch. In this case, 3- and also O-4-substitution should occur at the terminal nonreducing glucosyl units, which might be the matter of further investigations.

2.4. ESIMS^2 spectra of oligosaccharides from enzymatic degradation

Further fragmentation of isolated molecular ions by tandem mass spectrometry as CID in an ion trap has become a powerful tool in structure analysis of oligosaccharides in the last decade. Fragmentation is



Scheme 1. Fragmentation pattern of oligosaccharides: nomenclature according to Domon and Costello.[22]

preferred at the glycosidic linkages, while diagnostic valuable cross-ring cleavages are influenced by the linkage positions in the oligosaccharides. Domon and Costello have published a systematic nomenclature for carbohydrate fragmentations in MS/MS, which is also used in this paper[22] (see Scheme 1). Studies were mainly applied to neutral oligosaccharides in the native or permethylated[23] form. Cross-ring cleavages require a free reducing end of the oligosaccharide and can be formulated as retro-aldol cleavages of the aldehyde form, directly or after tautomerisation.[24–27]

For all enzymatically degraded cationic starches, the monosubstituted maltotrioses as the most abundant constituents were isolated and fragmented in the collision cell. Table 2 lists the m/z values of the corresponding fragment ions assigned. Figure 2f shows the relative intensities of the assigned fragment ions obtained from monosubstituted trisaccharides (3/1, m/z 620) for CS from all processes with the highest DS. In Figure 2g–j, the results for various DS values are compared for each CS series. When interpreting ESIMS^n spectra of oligosaccharides with a free reducing end, it must be considered that the fragment series B/Y and C/Z are of the same mass/charge ratio (Scheme 1). From the absence of C or Z ions in the fragment spectra of the methanolysis products (see below), we assumed that they would not appear for the hydrolysis products, either. On the other side, Z-fragments have been observed for oligosaccharides labelled with a permanently charged tag at the nonreducing end,[28] which might be relevant in our case of permanent cationic substituents, too. MS^3 of the isolated daughter ion from MS^2 at m/z 440 did not show any cross-ring cleavage product and could therefore be assigned to B_2, since Z_2 with its reducing end would have shown A-fragments. However, we were surprised to detect traces of an ion at m/z 296 corresponding to Y_1/C_1, since the reducing ends should not be substituted due to the observed enzyme selectivity, with the exception of oligosaccharides involving an original reducing chain end with an cationic residue. Therefore, we performed the enzymatic degradation of CS-EX-011 in ^{18}O-labelled water. Since no shift of the ion of interest (m/z 296) was observed in the daughter spectrum of the ^{18}O-labelled monosubstituted trisaccharide (m/z 622), it represents the nonreducing end of the trisaccharide and therefore must be assigned as C_1.

Assignment of fragment ion Y_2 was confirmed by a shift of two mass units (m/z 460). The relative intensity of the nonshifted signal (m/z 458) was only slightly higher than calculated from the also partially isolated unlabelled saccharide (m/z 620) corresponding to less then 5% C_2. No Z-fragments were observed, since m/z 278 and m/z 440 were not shifted and therefore could undoubtedly be assigned to B_1 and B_2, respectively, confirming the result of MS^3 experiments. In addition to m/z 602 (B_3, $M-H_2{}^{18}O$) a small signal was detected at m/z 604 ($M-H_2{}^{16}O$), indicating unspecific loss of water from the molecule. For the monosubstituted tetrasaccharide (4/1, m/z 784, results not shown) we found exclusively C_1 and no Y_1. The ratios of C_2/Y_2 and C_3/Y_3 were similar as found for 3/1 with a preference (>95%) for Y. Generally, MS^2 spectra of isolated ions showed 2-fold loss of $C_2H_4O_2$ (60). To clear up the way of formation of these cross-ring cleavage fragments, the acidic protons were changed against deuterium. In the MS^2 spectrum of the monosubstituted trimers (3/1, Fig. 1b) now the primary loss of 62 (a fragment carrying two deuterium atoms) occurred, according to a cleavage of the acetal bond and the C-2–C-3 bond, resulting in an $^{0,2}A_3$-fragment. The secondary loss of 61 mass units generates a $^{2,4}A_3$-fragment. The deuterium labelling experiments also confirmed the transfer of an acidic hydrogen from the B- to the Y-fragment ($m/z+1$) during the dissociation process of the glucosidic linkage. By model studies on permethylated maltotriose[15] we also observed a transfer of a proton, like Viseux et al. had observed for permethylated oligosaccharides.[29] This led to the conclusion that an acidic proton is abstracted preferably, but in case of unavailability a hydrogen from a carbon, presumably from C-2, is transferred. Besides the already mentioned cross-ring cleavage products and the Y_2 fragment, B_1, B_2 and B_3 ions were observed with the highest intensity for B_2. Low amounts of ions corresponding to m/z of $^{0,2}A_2$ and $^{2,4}A_2$ fragments could also be detected, which inevitably must be formed from Y_2 as a precursor, since a free reducing end is required and significant C fragment ion formation could be excluded by ^{18}O-labelling. It should be mentioned that in principle no secondary fragmentation processes are expected under CID-conditions, since the excitation is specific for the isolated ion, but not for the fragment ions. However, it is known from the literature[30] that sometimes the fragment ions have energy enough to undergo further fragmentation or elimination of water or MeOH, for example. In their study of ^{18}O-labelled cellopentaose by nano-ESIMS and ESIMS^2 Friedl et al.[30] observed B and Y fragments in the positive-ion-spectrum, but C and B-fragments in the negative-ion-spectrum. In the latter spectra, they also observed

cross-ring cleavage products from C fragment ions as the precursor, although only the mother ion was specifically excited. As has been observed earlier,[7,31] trimethylamine can be eliminated in the fragmentation step. However, this should not change the relative fragment ion intensities, since elimination should occur with the same probability for all positions.

As can be seen from Figure 2f–j, there are significant and reproducible differences for CS from the slurry, paste, dry and extruder modification. The ratio of B_2 and Y_2 is most distinct. It decreases in the order CS-SL > CS-PA > CS-DR $\geqslant$ CS-EX at DS 0.1 from 6.3 to about 1. While relative fragment ion intensities are constant over the whole DS range for slurry- and paste-modified starches, they show a clear trend for the CS-EX series, which corresponds to the change of the substitution pattern (see Table 1). For CS-DR similar differences depending on DS are also observed, but these do not show such a clear tendency. All CS-DR exhibit a noticeable high relative intensity of an ion corresponding to m/z of B_3, but which also might be caused by unspecific loss of H_2O from the mother ion. The CS from the various procedures can also be clearly distinguished by the relative intensities of the B_1 fragment decreasing in the order CS-EX > CS-PA > CS-SL > CS-DR. Other heterogeneously slurry-modified cationic starches[5] with higher DS fitted excellently within these groups. The pattern of a sample prepared in solution was close to the starches from the paste modification, which is also a more homogeneous process (Fig. 3). Although the differences in the fragmentation pattern of the monosubstituted trisaccharides obtained by enzymatic degradation are such significant and reproducible, it is difficult to interpret them on a molecular level. There is always a correlation of the relative intensities of the A_3 and the B_2 fragments. Intensity of Y_2 is complementary to that of B_2. A_2 fragments show a similar trend as Y_2, which further confirms that this must be the precursor ion of these cross-cleavage products. Small amount of $^{0,3}A_3$ ion were observed for all samples with no significant differences. These fragments represent (1 → 6) linkages in a (1 → 4)–(1 → 6) sequence, because the cross-ring cleavage between C-3 and C-4 by retro-aldol-reaction requires a free 4-OH group of the reducing sugar. The ratio of B_2 to Y_2 decreases when the ratio of 3/1:2/1 in the mother spectrum increases, which will be discussed below. MS^2 spectra of monosubstituted maltose (2/1) and maltotetraose (4/1) are in accordance with those from 3/1 and do not give significant new information. For comparison, we also recorded the ESI-mass spectra (direct MS and MS^2) after α-amylase digestion, only. The main difference is a strong preference for Y_2 and a decrease of B_2 (Y_2:B_2 is between 4.5:1 for CS-EX-010 and 2.5:1 for CS-SL). B_1 is also observed indicating that already the α-amylase accepts modification at subsite +1. Before discussing the MS^2 data in more detail, the pattern obtained after methanolysis and acid hydrolysis shall briefly be reported, to compare the pattern obtained by unselective linkage cleavage.

2.5. ESIMS^n of methanolysis products

Partial methanolysis of starch derivatives leads to oligomeric methyl glycosides. For naturally occurring oligosaccharides the predominant appearance of B/Y fragments (see Scheme 1) has already been described. The $ESIMS^2$ spectra of the monosubstituted trisaccharides (m/z 634) show that B and Y fragments are formed exclusively. From the fragmentation pattern, it can be derived that in the mixture of these trimers the substituent is located on either of the terminating glycosyl units (appearance of Y_1 and B_1 fragments). Assuming a random process, the charged substituent should be located at either of the three glucosyl units with the same probability. Cationisation of the terminal nonreducing residue will preferably give B_2 and no Y_2, while substitution at the reducing end enables to observe Y_2 only, but no B_2. Modification of the inner glucosyl residue obviously favours Y_2 formation over B_2. This is due to the preferred substitution at O-2, since the lack of OH at C-2 strongly disfavours B_i-cleavage. In the case of cationic starches intramolecular acetal formation via the 2-hydroxy group of the substituent might be an alternative process, however, it is kinetically unfavourable. For our discussion, it should be noted that the intensity of Y_2 is more than 2-fold of that of B_2.

2.6. ESIMS^n spectra of acid hydrolysis products

For comparison, all cationic starches with highest DS and an unmodified amylose were partially hydrolysed by trifluoroacetic acid. $ESIMS^2$ of the monosubstituted

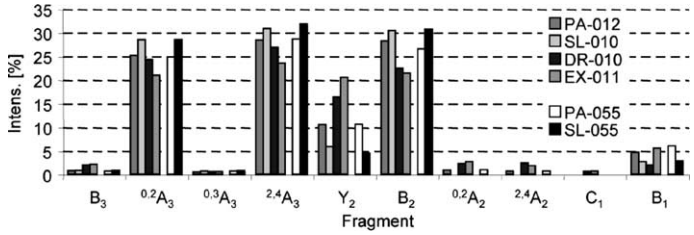


Figure 3. Relative intensities of daughter ions obtained from monosubstituted trimers of CS of low (0.1) and high DS (0.55).

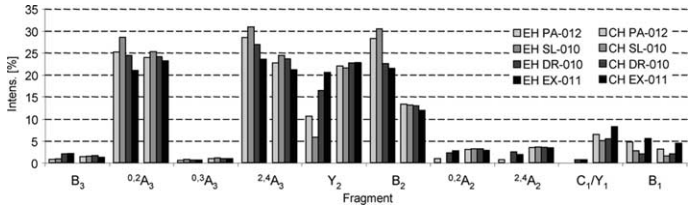


Figure 4. Relatives intensities of daughter ions of monosubstituted trimers derived from CS by enzymatic (EH, each left set of columns) and chemical hydrolysis (CH, each right set of columns).

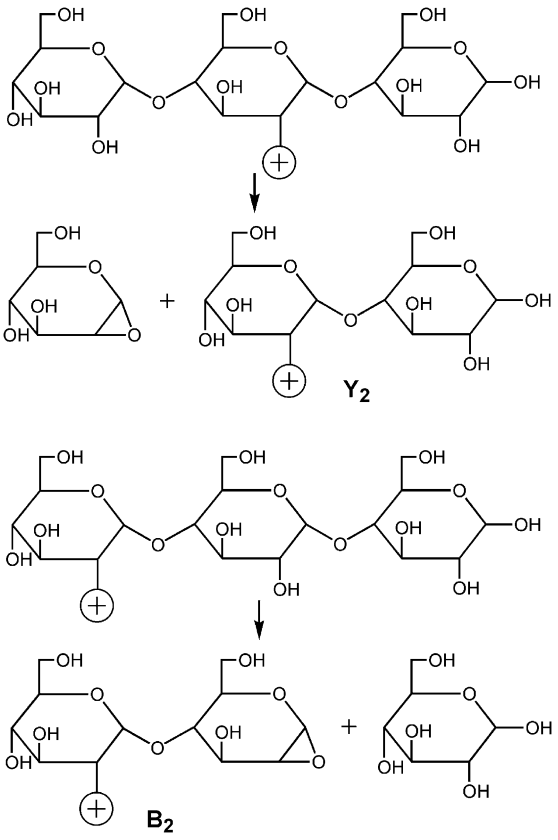
trimers (3/1) now again showed strong cross-ring cleavage fragments $^{0,2}A_3$ and $^{2,4}A_3$, and the dimeric fragments Y_2 and B_2 with a ratio of about 1.8:1. The difference to the methyl glycosides might be caused by the chance of additional cross-ring cleavage. Some Y_2 is further degraded to A_2 fragments, therefore lowering the Y_2/B_2 ratio. Monomeric fragments (B_1, Y_1/C_1) were present with lower intensities. From the graph in Figure 4 it is obvious that nearly no differences for the CS from various cationisation procedures can be observed with respect to B_2 and Y_2. The biggest differences are observed for B_1, which correspond to the partial DS in position 2 (x_2). The lower x_2, the higher the intensity of B_1. In addition, these results indicate that the partial hydrolysis is a random process. For comparison, for unmodified maltotriose equal intensities of Y_2 and B_2 were detected.

2.7. Discussion of the MS^2 spectra

From the data presented above, it is obvious that B_2 and Y_2 are the key fragment ions in the interpretation of structural correlations. Compared to a random cleavage, the specificity of enzymatic degradation changes the composition of the monosubstituted trimers in a way that formation of Y_2 in the daughter spectrum strongly decreases. At the same time, the relative intensity of B_2 increases. The effect is most pronounced for slurry-modified cationic starches and least for CS-EX-011, where B_2:Y_2 is about 1:1. From our knowledge of monomer composition (Table 1) and enzyme susceptibility (see Section 2.2), we can conclude that the main constituent of the isomeric monosubstituted trisaccharides is substituted at O-2 of the middle glucosyl residue. Blocking of 2-OH in the glycosyl unit usually disfavours formation of B_i, since no acidic proton is available for transfer on the leaving glycosyl residue. From the randomly cleaved product, we have seen that proton transfer from the 2-hydroxy group of the substituent under intramolecular acetal formation is not an efficient alternative. Therefore, the differences can only be explained by a correlation of the extent of B_2 formation with substitution at the nonreducing glucosyl unit, and of Y_2 with modification of the inner glucosyl residue. This interpretation is in accordance with the oligomer composition as shown in Figure 2. From CS-SL a relatively high amount of monosubstituted disaccharides (2/1) is formed, which proves a high ability to form products with a substituent at the terminal nonreducing glucosyl unit. In contrast, from the very low amount of 2/1 for CS-DR substituted with the same regioselectivity as CS-SL, it can be deduced that mainly oligosaccharides with nonmodified nonreducing glucosyl groups are formed. This correlation is also in line with the DS-dependent changes observed for CS-EX. With increasing DS the relative amount of 2/1 strongly decreases, indicating a decreasing contribution of oligosaccharides modified at the terminal nonreducing unit. Consequently, B_2 should decrease and Y_2 should increase with DS, which is in fact the case. The CS-PA samples also fit into this scheme. Differences for B_1, which do not simply correlate with these explanation are additionally affected by regioselectivity of substitution. While blocking of 2-OH inhibits B_1 formation, it is possible for O-6-substituted residues. So the order of relative intensities of B_1 (CS-EX-004 > CS-EX-007 > CS-EX-011 > CS-PA > CS-SL $\geqslant$ CS-DR) is in agreement with the order of increasing partial DS in position 2 (x_2) as mentioned above for the chemically hydrolysed samples. The most important fragmentation pathways outlined above are summarised in Scheme 2.

2.8. Correlation of MS results with the structural differences of the various cationic starches

After understanding the structural differences that affect the typical MS^2 patterns for cationic starches from various processes, the question has still to be answered why and how they are produced by the enzymes. Since the differences in regioselectivity of substitution cannot explain the product pattern of enzymatic degradation, we have to consider relations to amylose and amylopectin. Therefore, we analysed amylopectin and amylose enriched fractions obtained from CS-SL-010 by various procedures as is reported elsewhere by Radosta et al.[16] There was a slight but clear difference between the amylose enriched and the amylopectin enriched fractions showing a higher B_2/Y_2 ratio for the latter (ca. 10) compared to about 4 for the amylose enriched ones (original CS-SL: about 6). That means that more ter-



Scheme 2. Preferred formation of the Y_2 fragment ion from a maltotriose derivative with a cationic substituent at position 2 of the inner glucosyl residue, and of the B_2 fragment ion when the cationic substituent is located at position 2 of the non reducing terminal glucosyl residue.

minally substituted trisaccharides are derived from the amylopectin fraction than from substituted amylose. The structural differences of amylopectin and amylose are related to additional (1 → 6) linkage for the amylopectin and an equivalent amount of nonreducing ends of the branches. Bearing the assumption of Manelius et al.[2] in mind that slurry modification of potato starch (B-type) yields preferred substitution at the branching points and the nonreducing ends of the amylopectin side chains, the enhancement of terminal substitution found for CS-SL can be partially understood. However, it is obvious from the change of oligomer composition and of the Y_2/B_2 ratio before and after additional glucoamylase digestion that most of the terminally substituted trisaccharides (S–U–U∼) are not originally present in the cationic starch. For those the precursor contains the sequence U_n–S–U–U∼ with two unsubstituted glucosyl units (U) counted from the reducing end, since this part of the oligosaccharide precursors is preserved during glucoamylase digestion. It is close at hand that the differences between wet and dry cationised samples are caused by the occurrence of 1,6-linkages in the precursors of the 3/1-fraction and therefore are indicative of preferred substitution around the better accessible branching points. The exact structure is not yet clear, but it can be assumed that one of the U units in the S–U–U∼ motif is branched at O-6 [S–U(U)$_n$–U∼], but other structural features are also thinkable and must be further investigated. The branched glucosyl residue is then cleaved off by the *endo*-enzyme. Preferred modification in the vicinity of branching points is in accordance with the conclusions reported from other studies.[2,7,16,32,33]

2.9. Structure–property relationships

In the following, the mass spectrometric data and our interpretation will be discussed in context with the properties of the CS samples investigated by Radosta et al.[16] In the slurry process, the corn structure is retained and topochemical selectivity is most probable for this heterogeneous procedure. Radosta et al. found no decrease in crystallinity, poor cold water solubility, but complete solubility at 95 °C for the CS-SL samples independent of DS. Therefore, a high preference for the amorphous regions regularly distributed over the whole starch granule can be assumed, resulting in a preferred cationisation at the less compact structure in the vicinity of the branching points, at the nonreducing ends. Interestingly, Manelius et al.[2] found 91% of the original nitrogen content in the amylopectin framework of the granule retained after lintnerisation of wet modified cationic starch. In contrast, nitrogen was completely lost for the dry-modified starch by this selective hydrolysis procedure, which mainly affects the outer amorphous regions of native granules. The corn structure of the dry-modified CS-DR was partially damaged and the chains strongly degraded. Dependent on DS, they could only partly be dissolved in cold (6–26%) as well as in hot water (33–56%), indicating that only part of the starch, presumably at the surface, has reacted. Mainly the low molecular mass fraction was detected by HPSEC of the soluble cationic part, which includes primarily the amylose.[16] These different preferences for branching points and end groups over the whole granule in the case of CS-SL and the outer amylose in the dry process are in complete accordance with the B_2/Y_2 ratios of these samples. The pattern of the MS^2 spectra obtained for the paste-modified cationic starches lies between the slurry- and the dry-modified starches and is similar to that of a CS modified in solution. Crystallinity was completely lost by the gelatinisation process. Interruption of hydrogen bonds by gelatinisation obviously enhances reactivity of OH at C-3. The products were highly swollen in cold water, but completely soluble in hot water. Therefore, no pronounced preference with respect to the corn architecture can be observed. Amylopectin and amylose should be evenly affected.

During extrusion, the corn structure of the potato starch is completely destroyed by thermal plastification

under the alkaline conditions. The reaction seems to start in the outer region of the corn, since a higher density of substituents can be deduced from the monomer analysis (disubstituted glucosyl units already at DS 0.03) as for CS-DR. However the higher extent of 2/1 for CS-EX after enzymatic digestion indicates a higher participation of amylopectin in line with our interpretation model developed so far, while the situation becomes more similar to the CS-DR at increasing DS. Due to the lower regioselectivity, data of CS-EX are most difficult to interpret. Molecular mass is strongly degraded and nearly complete cold and hot water solubility is achieved at low DS. However, solubility decreases to 53% (25 °C) and 77% (95 °C) for CS-EX-011. From HPSEC analysis, it can be concluded that cross-linking had occurred, as was also observed for the other dry process.[16]

3. Conclusion

ESIMS and ESIMS^2 are shown to be powerful tools to analyse structural differences of cationic starches from various wet and dry processes. Reproducible and significant differences were observed and evaluated for the MS^2 spectra of the fraction of monosubstituted trisaccharides obtained by digestion with α-amylase from *B. licheniformis* and subsequently glucoamylase from *A. niger*. By a comprehensive investigation with respect to monomer composition, location of substituents in the terminal nonreducing, the inner-linked, and reducing glucosyl units of the enzymatically degraded cationic starch, and comparison with the MS^2 spectra of randomly cleaved cationic starches, it was possible to interpret the data on a molecular level and with respect to topochemical control in the starch granule. The fragmentation pattern in MS^2 could be explained on the basis of isotopic labelling experiments and mechanisms reported in the literature. The ratio of the relative intensities of the fragment ions B_2 and Y_2 from the fraction of the monosubstituted maltotrioses are indicative for the preference of cationisation in the branched regions of the amylopectin or the amorphous amylose lamellae. By this independent method, it could be confirmed that slurry modification of potato starch preferably occurs in the amorphous lamellae of the amylopectin growth rings of the starch granules, that is in the branched regions and at the end groups of side chains. Preferred cationisation at O-2 was observed (89%), followed by O-3- and O-6. Paste modification showed no such preference and an enhanced reactivity at O-3. Cationic starches from the dry process exhibited the same regioselectivity as slurry-modified ones, but a completely different pattern after enzymatic digestion and hence in the MS^2 spectra, indicating that mainly amylose has been affected. In connection with data and properties reported by other authors this can be understood as the result of a surface reaction under extensive damaging of corn structure. Cationic starch from the extrusion process showed the lowest regioselectivity, and a very strong DS-dependence. As for dry-modified cationic starch, disubstitued glucosyl moieties could be detected, indicating a higher substituent density for the products from the two dry processes compared to the two wet procedures. At low DS, more substituted terminal glucosyl units are found in the trisaccharide fraction from enzymatic treatment compared to dry-modified starch, which might be a result of better digestibility by glucoamylase, which accepts substitution at O-6, and presumably less appropriate at O-2.

4. Experimental

4.1. Materials

The *O*-(2-hydroxy-3-trimethylammonium)propyl starch (cationic starch, CS) was produced from native potato starch and epoxypropyltrimethylammonium chloride from Degussa (QUAB 151) as described in detail by Radosta et al. from the Fraunhofer Institute of Applied Polymer Research, Golm, Germany.[16] Derivatives in a DS range between 0.03 and 0.12 were prepared according to each process. The graded DS values were adjusted by application of different amounts of etherification reagent. The amount of sodium hydroxide was selected in such a way that it was consumed during the reaction. DS values were determined by ^{13}C NMR by the supplier.

All chemicals were of analytical grade, except water ('bidest.' quality by Nanopure™ device) and MeOH (HPLC grade, purchased from Fluka). HCl and NH_3 (aq) were purchased from Riedel-de Haën, $CaCl_2$, glacial acetic acid and D_2O from E. Merck and MeOH-d_4 from Deutero. Unless otherwise indicated, all materials were obtained in the highest purity and used without further purification. Methanolic HCl was made by dripping the appropriate amount of acetyl chloride (E. Merck) into iced dry MeOH. The mixed bed resin (TMD-8) and α-amylase (no. 4551, *B. licheniformis*, 620 U/mg) was purchased from Sigma, amyloglucosidase (no. 102857, *A. niger*, 15 U/mg) from Boehringer. Heating/stirring and evaporating with nitrogen were done in a Barkey device. ^{18}O-Labelled water was from Sigma Aldrich (95%) and a kind gift of Prof. Höfle, GBF Braunschweig.

4.2. Monomer analysis

The distribution of cationic substitutents in the glucosyl unit was determined as described earlier.[4,5]

4.3. Enzymatic hydrolysis

About 20 mg of derivatised starch was suspended in 2.5 mL of an aq $CaCl_2$ soln (0.02%) in a 2.5 mL-V-vial. After heating to 130 °C for 30 min and cooling to room temperature, a soln of 186 U α-amylase (EC 3.2.1.1) in 20 μL water was added and the mixture was incubated for 3 h at 52 °C in a water bath. Then the pH was adjusted to 4.7 with HCl (1 mmol/L) and 20 μL of a suspension containing 3 U glucoamylase (EC 3.2.1.3) was added. The incubation was continued at 52 °C for 72 h. The reaction mixture was held at 100 °C for 30 min, cooled and made up to a volume of 5 mL with water. For ESIMS measurement this soln was microfiltrated and diluted in the ratio 1:10 with MeOH and water in same amounts. For differentiation of C/Y and B/Z fragments, enzymatic digestion of CS-EX-011 (10 mg) was performed in 1 mL of ^{18}O-labelled water.

4.4. H/D-exchange

About 200 μg of oligosaccharides was dissolved in 500 μL of D_2O. After 24 h, the soln was evaporated in a stream of nitrogen and the residue solved again in 500 μL of D_2O. This was repeated three times. For ESIMS measurement, the product was dissolved in 500 μL of MeOH-d_4 and of D_2O in same amounts.

4.5. Partial methanolysis

About 2–3 mg of the derivatised starch was treated with 1 mL methanolic HCl (0.1 mol/L) at 90 °C for 75 min in a 1 mL-V-Vial. Mixed bed resin was added until the reaction mixture was neutral. For ESIMS measurement, the soln was microfiltrated and diluted in the ratio 1:10 with MeOH.

4.6. Partial hydrolysis

About 2 mg of the derivatised starch was treated with 1 mL TFA (2 mol/L) at 100 °C for 1 h in a 1 mL-V-Vial. The acid was removed by co-destillation with $C_6H_5CH_3$. For ESIMS measurement, the soln was microfiltrated and diluted in the ratio 1:5 with methanol and water in the same amounts.

4.7. ESI mass spectrometry

The ESI mass spectra were recorded with a Bruker EsquireLC mass spectrometer (ion trap, CID). The sample was injected by a syringe pump at a flow rate of 240 μL/min. Electrospray ionisation was done in positive mode (capillary 4500 V, end plate offset −500 V). Source parameters: nebuliser 10 psi, dry gas 4 L/min, dry temperature 300 °C. The capillary exit was set at 120 V. The amplitude of the resonance frequency, which excites the ions before fragmentation was optimised for every ion and was between 0.7 and 1.1 V. Two hundred spectra were accumulated and averaged.

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