

Mass Spectrometric Investigation of the Chemical Composition of Caramel Formed upon Heating of Disaccharides

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Abstract: Disaccharides are a very important group of carbohydrates, being main components of many daily food products. The heating of these biomolecule causes the formation of caramel, an extremely complex material. The dominant fraction of non-volatile compounds, responsible for both color and flavor of food products, has been studied on a few occasions. Herein, the composition of caramels obtained by heating of sucrose, lactose and maltose were studied using combined mass spectrometry techniques. High resolution electrospray mass spectrometry was applied followed by targeted multi-stage LC-tandem mass spectrometry (ESI-MSⁿ) and MALDI-MS. Novel graphical interpretation strategies such as van Krevelen and Kendrick mass analysis have been applied to study the composition of caramels. Products of caramel include oligomerization, depolymerization, hydration and dehydration products. Oligomers with up to eight carbohydrate units and dehydrated oligomers losing up to eight water molecules have been identified.

Key words: Browning, caramelization, carbohydrates, disaccharides, sucrose, complex mixtures, mass spectrometry.

1. Introduction

Heating of mono and disaccharides by thermal food processing generates the brown color and pleasurable aroma of caramel [1, 2]. Thermally processed sugars are consumed at an estimated level of 50 Mt annually, thus representing one of mankind most important dietary materials [3]. Despite its importance, the detailed chemical composition of non-volatile fraction, corresponding to around 98% of its dry weight, has remained largely mysterious, mainly due to its high complexity. Several studies have been carried out on melting, thermodynamic properties of disaccharides [4-7] and demonstrate an enhanced interest in the chemistry of heated carbohydrates.

Caramels have been analyzed particularly by gas chromatography/mass spectrometry focusing on volatile compounds such as hydroxymethylfurfural

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(HMF) and hydroxyacetylfuran (HAF), hydroxydimethylfuranone (HDF), dihydroxydimethylfuranone (DDF), maltol and hydroxymaltol [8-10]. The volatile compounds formed investigated caramelization have been comprehensively, whereas the chemistry non-volatile components has been studied only on a few occasions [11, 12]. The reaction products of caramelization were classified into three particular classes: Caramelans-Tetramers of hexoses (C₂₄H₃₆O₁₈), Caramelens-Hexamers of glucose (C₃₆H₅₀O₂₅) and Caramelins-Polymers of glucose ($C_{125}H_{188}O_{80}$) [13]. That classification exists without any further structure suggestions due to the complexity of created products during caramelization. Mass spectrometry (MS) has been applied for structural investigation of saccharides, using mainly electrospray ionization (ESI) [14-16] and matrix-assisted laser desorption/ionization (MALDI) [17-19]. Tandem mass spectrometry (MS/MS) is demonstrated to be a valuable tool in the structural

characterization of carbohydrates to achieve thorough information regarding their structure [20, 21].

We have recently reported on the application of mass spectrometry strategies novel for the characterization of caramel obtained from glucose and fructose. Following these reports, we report here on the characterization of caramel products obtained from disaccharides. We aim to unravel the complexity of caramel, formed when these carbohydrates are heated. The most dietary relevant disaccharides are sucrose (or saccharose) [22, 23], lactose a reducing disaccharide abundant in milk and dairy products and maltose, present in malt and beer. While heating of sucrose produces classical caramel used in traditional cooking, baking and as food additive for coloring of beverages or baking goods, heating of lactose occurs frequently in traditional baking recipes with added dairy products or the production of the Hispanic "dulce de leche" and maltose is heated prior to traditional beer brewing. In this study, we applied the novel graphical interpretation strategies such as Kendrick and van Krevelen for complex mass spectrometric data of caramels. These data interpretation techniques have visualized wide spectrum of the product formed during caramelization process and led to more specify characterization.

2. Materials and Methods

Solvents (analytical grade) and all chemicals were purchased from Sigma-Aldrich (Bremen, Germany).

2.1 Sample Preparation

All sugar samples (1 g) were heated in an oven for 2 h at 180 °C (sucrose, maltose) and at 200 °C (lactose). The heated samples were stored at room temperature. Heated carbohydrates (1 mg) were dissolved in methanol/water (1:1, v/v, 1 mL) and directly used for micrOTOF and direct infusion ION TRAP.

2.2 High Resolution MS

Bruker Daltonics micrOTOF instrument from methanolic/water solutions were used for high resolution mass spectra, employing the negative and positive electrospray ionization mode. MicrOTOF focus mass spectrometer (Bruker Daltonics) fitted with an ESI source and internal calibration was achieved with 10 mL of 0.1 M sodium formate solution. Calibration was carried out using the enhanced quadratic calibration mode. All MS measurements were performed in the negative and positive ion mode. It should be noted that the intensities of the measured peaks in a TOF calibration influenced the magnitude of the mass error with high-intensity peaks resulting in detector saturation displaying larger mass errors. Usually this problem can be overcome by using spectra averaging on the side flanks of a chromatographic peak or by taking more diluted samples.

2.3 Data Analysis

Molecular formulas were calculated using Bruker software Data Analysis 4.0. Data was subsequently exported to Excel to carry out simple mathematical operation such as determination of H/C and O/C ratios or Kendrick analysis. All graphs were created using Origin 7.5.

$2.4 LC-MS^n$

The LC equipment (Agillent 1100 series, Bremen, Germany) comprised a binary pump, an auto sampler with a 100 µL loop, and a DAD detector with a light-pipe flow cell (recording at 320 and 254 nm and scanning from 200 to 600 nm). This was interfaced with an ion-trap mass spectrometer fitted with an ESI source (Bruker Daltonics HCT Ultra, Bremen, Germany) operating in an Auto MSⁿ mode to obtain fragment ion m/z. As necessary, MS^2 , MS^3 , and MS^4 fragment-targeted experiments were performed to focus only on compounds producing a parent ion at m/z143, 161, 179, 197, 287, 305, 323, 341, 359, 449, 431, 467, 485, 503, 521, 611, 629, 647, 665, 683, 773, 791, 809, 827 and 845. Tandem mass spectra were acquired in an Auto-MSⁿ mode (smart fragmentation) using a ramping of the collision energy. Maximum fragmentation amplitude was set to 1 V, starting at 30% and ending at 200%. MS operating conditions (negative mode) have been optimized using glucose with a capillary temperature of $365\,^{\circ}\text{C}$, a dry gas flow rate of $10\,\text{L/min}$ and a nebulizer pressure of $12\,\text{psi}$.

2.5 HPLC

Separation was achieved on a 250 \times 4.6 mm i.d. column containing diphenyl 5 μ m and 5 \times 4.6 mm i.d. guard column of the same material (Varian, Darmstadt, Germany). Solvent (water/formic acid 1000:0.05 v/v) was delivered at a total flow rate of 850 μ L/min by 25 min isocratic.

2.6 MALDI-TOF-MS

All experiments were performed in the positive ion mode using an Ultraflex II MALDI TOF/TOF mass spectrometer (Bruker Daltonics, Bremen) equipped with a pulsed 50 Hz N_2 laser (wavelength 337 nm). Laser intensity was varied in the range from 30% to 45%. Sample (0.5 μ L) was deposed on top of a layer of crystals of 2,5-dihydroxybenzoic acid (DHB) formed by decomposition of 0.5 μ L of DHB solution on the MALDI plate and allowing it dry at ambient temperature. A MALDI matrix was prepared by dissolving 5 mg of DHB in 1 mL mixture of acetonitrile/methanol/aqueous trifluoroacetic acid (1%, v/v) (1:1:1, v/v/v).

2.7 Thermogravimetric Analysis (TGA)

TGA were performed using a TA instruments SDT Q600 instrument. The temperature was ramped from 25 to 180 °C for sucrose, maltose and to 200 °C for lactose at a rate of 5 °C/min and kept at final temperature for 2 h using a nitrogen atmosphere.

2.8 Infrared (IR) Analysis

Infrared spectra were recorded on KBr pellets using a Nicolet Avatar 370 spectrometer.

$2.9^{-1}HNMR$

¹H NMR spectra were acquired on a JEOL ECX-400

spectrometer operating at 400 MHz, at room temperature in D_2O , using a 5 mm probe. The chemical shifts (δ) are reported in parts per million (ppm).

3. Results and Discussion

The chemical composition of the non-volatile fraction of caramel is largely unknown due to its complexity combined with a lack of analytical strategies so far able to characterize extremely complex material comprising several thousand individual components. Modern LC-mass spectrometry combines the best of four worlds, ultra high resolution combined with high sensitivity, high selectivity due to targeted ion isolation and isomer separation based on LC-techniques. These features allow finally a chemically meaningful analysis of processed food materials.

In this contribution, we characterize the products obtained by thermal processing from [a space deleted] the three dietary most relevant disaccharides sucrose 1, maltose 2 and lactose 3 using a recently developed approach called domino tandem mass spectrometry, in which high resolution direct infusion MS data are acquired and interpreted yielding several structural hypothesis, one of which is in a second step (therefore domino) is confirmed by targeted LC-tandem MS experiments.

In term to find the optimized heating conditions for further analysis, the mass spectra were recorded for samples obtained at different temperatures. The studied samples were thus heated from room temperature until 180 °C for sucrose and maltose, until 200 °C for lactose and were subsequently kept for 2 h at the final temperature. Thermogravimetric curves showed around 10%-12% weight loss, which statistically correspond to one water molecule per monosaccharide. Heating procedures caused the formation of brown color, which was observed during the roasting procedure of coffee beans or heating of sugar-rich food products [24]. It is worthy to mention that heating procedure caused melting of sucrose and maltose, while lactose remained solid. So we decided to carry

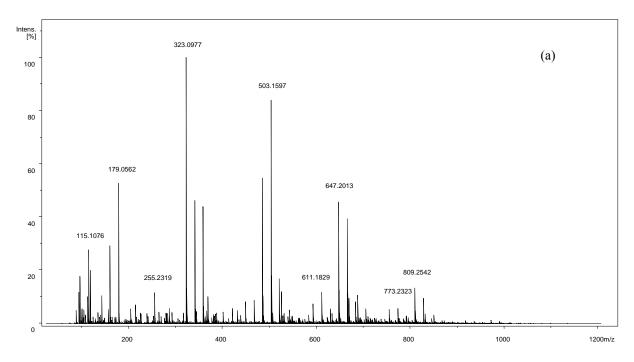
out further characterization work using these aforementioned conditions.

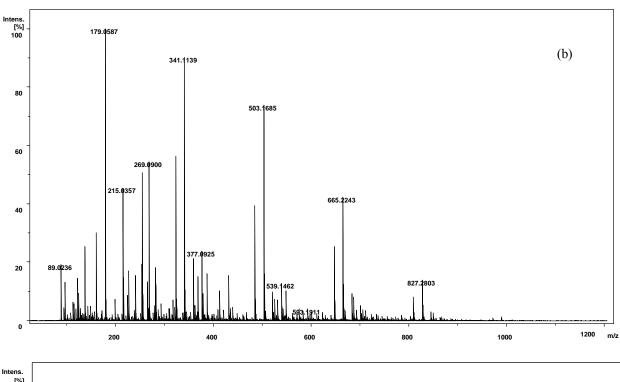
Mass spectrometric data were obtained in both negative and positive ion modes from caramelized sucrose (1), maltose (2) and lactose (3). Fig. 1 illustrates mass spectra of all three disaccharides in the negative ion mode. Mass spectra in the positive ion mode were dominated by sodiated molecular ions. In Table 1, the mass/charge ratio (m/z) of the product ions, elemental composition and average mass error for caramelized sucrose as the representative disaccharides are presented (positive mode data and further signal assignments are provided in the supplementary information). The exploration of mass spectra of caramelized disaccharides indicated mostly oligomerization, dehydration and hydration reactions of carbohydrates. Oligomers of disaccharides are present with up to hexamers, dehydration products of those oligomers, hydration and some redox disproportionation reaction products. Furthermore, the decomposition of oligomers to monomers appeared with the formation of their dehydrated products. Subsequently, MALDI-MS techniques were applied to observe a possible forming of further oligomers. The

MALDI spectrum with DHB (2,5-dihydroxybenzoic acid) of caramelized sucrose was recorded and resulting oligomers up to octamer at m/z 1,337.6. Among them, hydrated and dehydrated ions have been observed which consequently confirm the results obtained with electrospray ionization technique.

3.1 Chemometric Data Interpretation

graphical data Novel mass spectrometric interpretation strategies might be applied to the complex mixture as is caramel. The van Krevelen and Kendrick type of analyses, first time used by petroleum researchers [25], were employed to caramelized disaccharides. These techniques visualized complex data and were used as the inspiration for further characterization of particular groups of the compounds. The aforementioned graphical techniques have been utilized only for dietary materials, such as wine [26], black tea thearubigins [27, 28] and caramel [29]. The van Krevelen diagrams constructed with the atomic ratios of H/C and O/C were calculated from the list with molecular formulas of the high resolution mass data of caramelized carbohydrates, as shown in Fig. 2. Different groups of the compounds have typical atomic





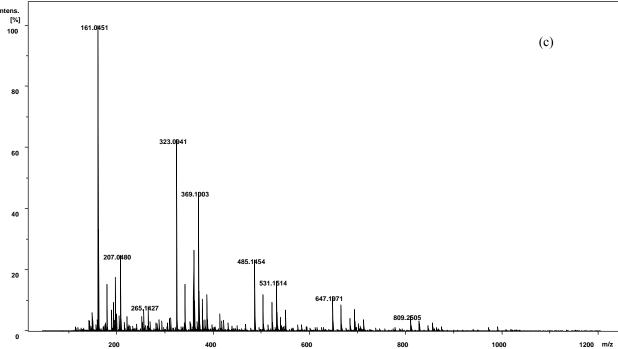


Fig. 1 Mass spectra of caramelized (a) sucrose, (b) maltose and (c) lactose in the negative ion mode using a direct infusion into an ESI-TOF-MS instrument.

ratios and consequently appear at characteristic area of the plot [30], for example, the atomic ratios for carbohydrates are H/C-2 and O/C-1. The profiles of figures for 1, 2 and 3 are similar and the following groups of the compounds were found.

The group I represents the points characteristic for carbohydrates. The points in the middle of the graphs belong to dehydrated products (II). The products of disproportionation redox reactions are present in the upper left corner (III). The group of the points in the

Table 1 High resolution mass (MS-TOF) data of caramelized sucrose and their parent ions (M-H) in the negative ion mode.

Peak numbering	Assignment	Mol. formula	Experimental m/z (M-H)	Theoretical <i>m/z</i> (M-H)	Relative error (ppm)
1		$C_6H_{12}O_2$	115.0765	115.0765	0.1
2		$C_4H_8O_4$	119.0351	119.0350	1.4
3		$C_8H_{16}O_2$	143.1081	143.1078	2.2
4		$C_9H_{18}O_2$	157.1226	157.1234	4.9
5	Glu-H ₂ O	$C_6H_{10}O_5$	161.0461	161.0455	3.5
6	Gluor Fru	$C_6H_{12}O_6$	179.0562	179.0561	0.3
7		$C_{14}H_{28}O_2$	227.2010	227.2017	3.0
8		$C_{15}H_{30}O_2$	241.2164	241.2173	3.6
9		$C_{16}H_{32}O_2$	255.2324	255.2330	2.2
10		$C_{15}H_{22}O_4$	265.1448	265.1445	1.1
11		$C_{18}H_{36}O_2$	283.2629	283.2643	4.8
12		$C_{12}H_{16}O_{8}$	287.0767	287.0772	2.0
13		$C_{17}H_{26}O_4$	293.1756	293.1758	0.8
14	Glu-Fru-2 \times H ₂ O	$C_{12}H_{18}O_9$	305.0866	305.0878	3.9
15	Glu-Fru-H ₂ O	$C_{12}H_{20}O_{10}$	323.0977	323.0984	2.2
16	Glu-Fru	$C_{12}H_{22}O_{11}$	341.1083	341.1089	1.7
17	Glu - $Fru + H_2O$	$C_{12}H_{24}O_{12}$	359.1159	359.1195	10.0
18	$3 \times M-4 \times H_2O$	$C_{18}H_{24}O_{12}$	431.1155	431.1195	3.9
19	$3 \times M$ - $3 \times H_2O$	$C_{18}H_{26}O_{13}$	449.1280	449.1301	4.6
20	$3 \times M-2 \times H_2O$	$C_{18}H_{28}O_{14}$	467.1391	467.1406	3.2
21	$3 \times M-H_2O$	$C_{18}H_{30}O_{15}$	485.1494	485.1512	3.7
22	$3 \times M$	$C_{18}H_{32}O_{16}$	503.1597	503.1618	4.2
23	$3 \times M + H_2O$	$C_{18}H_{34}O_{17}$	521.1698	521.1723	4.8
24	$4 \times M\text{-}5 \times H_2O$	$C_{24}H_{32}O_{16}$	575.1613	575.1618	0.7
25	$4\times M\text{-}3\times H_2O$	$C_{24}H_{36}O_{18}$	611.1798	611.1829	5.0
26	$4 \times M-2 \times H_2O$	$C_{24}H_{38}O_{19}$	629.1903	629.1935	4.9
27	$4 \times M-H_2O$	$C_{24}H_{40}O_{20}$	647.2013	647.2040	4.2
28	$4 \times M$	$C_{24}H_{42}O_{21}$	665.2129	665.2146	2.6
29	$4 \times M + H_2O$	$C_{24}H_{44}O_{22}$	683.2243	683.2251	1.2
30	$5 \times M-4 \times H_2O$	$C_{30}H_{44}O_{22}$	755.2228	755.2251	3.1
31	$5 \times M\text{-}3 \times H_2O$	$C_{30}H_{46}O_{23}$	773.2323	773.2357	4.4
32	$5 \times M-2 \times H_2O$	$C_{30}H_{48}O_{24}$	791.2478	791.2463	1.9
33	$5 \times M-H_2O$	$C_{30}H_{50}O_{25}$	809.2536	809.2568	4.0
34	$5 \times M$	$C_{30}H_{52}O_{26}$	827.2645	827.2674	3.5
35	$5 \times M + H_2O$	$C_{30}H_{54}O_{27}$	845.2775	845.2780	0.5
36	$6 \times M-4 \times H_2O$	$C_{36}H_{54}O_{27}$	917.2748	917.2780	3.4
37	$6 \times M\text{-}3 \times H_2O$	$C_{36}H_{56}O_{28}$	935.2860	935.2885	2.7
38	$6 \times M-2 \times H_2O$	$C_{36}H_{58}O_{29}$	953.2968	953.2991	2.4
39	$6 \times M-H_2O$	$C_{36}H_{60}O_{30}$	971.3095	971.3097	0.1
40	$6 \times M$	$C_{36}H_{62}O_{31}$	989.3201	989.3202	0.1

M—designates monosaccharide, Glu—glucose and Fru fructose.

bottom left corner (IV) belongs to products with condensed heterocyclic ring structures after successive dehydration. Both heterocyclic condensed ring structures and disproporionation products appear as minor products in the mass spectrum at a concentration

that does not allow further structural investigation by tandem MS. The second graphical technique, which we used to study the composition of caramelized disaccharides, was Kendrick type analyses. High resolution mass data obtained from TOF-MS experiments

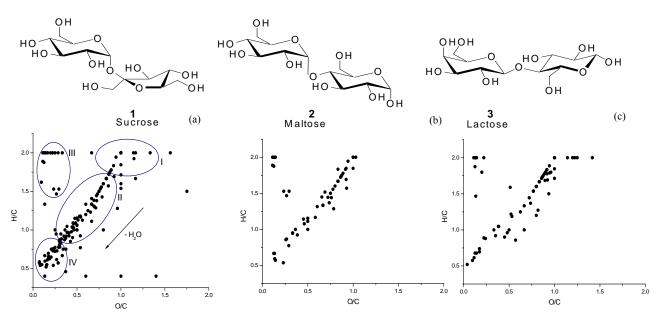


Fig. 2 Two dimensional van Krevelen plot (elemental ratio plot) showing the O/C ratio versus H/C ratio for caramelized (a) sucrose, (b) maltose, (c) lactose in the negative ion mode in m/z range between 50 and 1,200. I-carbohydrates; II-dehydrated products; III-disproportionation redox reactions products; IV-products with condensed heterocyclic ring structures after successive dehydration.

with Kendrick mass defect and nominal Kendrick mass values were recorded. The Kendrick plots were created with H₂O increment as illustrated in Fig. 3. The compounds, which belong to the same homologous series, have identical Kendrick mass defect and lie on the horizontal lines in the graphs [31]. The plots of caramelized disaccharides possess some homologous series of the compounds. This type of graph showed the formation of oligomers with up to six monomeric hexoses in the case of heated disaccharides. Hydrated and dehydrated ions were visualized as well. Applied high-resolution mass spectrometry data interpretation techniques showed the whole range of products and reactions occurring during the heating procedure of disaccharides. In the following parts, the characterization of predominant products of caramelization of disaccharides will be disclosed.

3.2 Characterization of Monomeric Hexoses

In the direct infusion MS data for heated 1 and 2 strong peaks and for 3 a weak peak were apparent at m/z 179.1 corresponding to monomeric hexose units. LC-tandem MS experiments confirmed the monomeric

hexoses as the α , β anomeric pairs for glucose 4 and fructose 5 for 1, the α , β anomeric pairs for glucose 4 for 2 and the α , β anomeric pairs for glucose 4 and galactose 6 for 3, respectively (Fig. 4), by comparison of retention times and MS data with authentic reference standards. For example, for the ion at m/z 179.1, a neutral loss of 18 as the base peak was observed, for heated 1 and 3 and a neutral loss of 90 as the base peak for 2, which correspond to one water molecule and three formaldehyde molecules derived from the cross-ring cleavage, respectively. Presumably water as a reaction product of dehydration processes hydrolyses the disaccharides into their monomeric counterparts. For galactose, a dehydration process leading to a galactose based ion at m/z 161.1 is favoured (see later for detailed discussion). Epimerization was shown to be not observed for monomeric hexoses previously [23], as it has been reported in quinic acid and chlorogenic acid chemistry in food processing chemistry at identical temperatures [32, 33].

3.3 Characterization of Oligomers of Hexoses

The MSⁿ data of oligomers of hexose generated upon

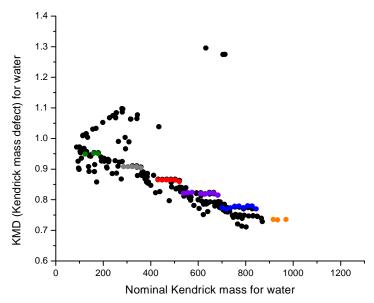


Fig. 3 Two dimensional Kendrick plot for mass increment H_2O showing the distribution of the Kendrick mass defect plotted against the nominal Kendrick mass of pseudo-molecular ions for caramelized sucrose in the negative ion mode. The colors indicate formation of homologous series of monomers (green), dimers (gray), trimers (red), tetramers (violet), pentamers (blue), hexamers (orange).

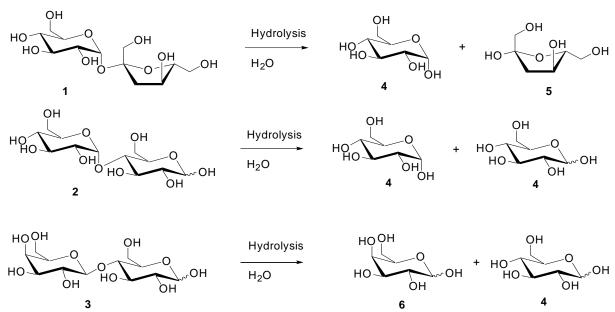


Fig. 4 Formation of monomeric hexoses from disaccharides 1-3 during heating.

heating of 1, 2 and 3 has been acquired in the negative ion mode using a direct infusion into ESI ion-trap mass spectrometer. Fragmentation of precursor ions of disaccharides ($C_{12}H_{22}O_{11}$) at m/z 341.1 gave the main peaks at m/z 178.9 for heated sucrose, at m/z 160.9 for heated maltose and at m/z 323.0 for heated lactose. They came from the glycosidic bond cleavage between

two monosacharides, which might occur in either one side or both sides of the bridging oxygen atom [34] and the removal of one water molecule.

Tandem LC-MS measurements under optimized chromatographic conditions revealed three resolved chromatopygraphic peaks in extracted ion chromatograms (EICs) at m/z 341 for heated 1, one

peak for heated 2 and two peaks for heated 3. Hence for both heated maltose and lactose, the intact disaccharides are observed. For heated sucrose 1 in contrast hydrolysis into monomers occurs, followed by recombination to yield a third major disaccharides, whose similarity of the MS² fragment spectra allows tentative assignment as a dimer of fructose [35, 36]. Presumably fructose yields preferentially the more stable fructosyl cation, if compared to the glycosyl cation, serving as a glycosyl acceptor due to the increased number substituents at the sp² carbon centre.

Disaccharides ions corresponding to oligomers containing three to six hexose units were observed at m/z 503.2, 665.2, 827.3 and 989.3 in the negative ion mode. The mass spectra of the precursor ion at m/z 503.2, trimeric hexoses showed a neutral loss of 162 as the main peak for all studied caramel samples, arising from the cleavage of the glycosidic bond without bridging oxygen atom in the product ion. Tandem LC-MS investigations revealed in the EICs at m/z of 503.2 twelve resolved chromatographic peaks for heated sucrose 1 (of which only six were sufficiently strong to provide MS^n data), and four resolved chromatographic peaks for maltose and lactose

respectively (Fig. 5). Tentative reaction products are shown in Fig. 6.

It must be assumed that not all isomeric compounds can be chromatographically resolved here. The tandem MS fragment spectra and retention times for the four observed peaks for heated maltose 2 were identical to four MS² spectra of caramelized glucose and have been tentatively assigned as trimers of glucose formed through non-selective glycosidic bond formation. A similar interpretation is suggested for heated lactose 3. For heated sucrose 1 three of the compounds identified resemble based on similarities of MS² fragment spectra kestose or inulin type structures reported previously [35-37] with a saccharide sequence of Glu-Fru-Fru through non-selective glycosidic bond formation using the fructosyl cation intermediate. The other three peaks can be tentatively assigned as either Fru-Glu-Fru or Glu-Glu-Fru, for which no reference data are available (Fig. 6). The detailed regio- and stereochemistry of the reaction products remain open. It has to be noted that sucrose constitutes a special case among the disaccharides since the two anomeric centres are directly connected to one another and therefore sucrose can not act as a glycosyl acceptor directly.

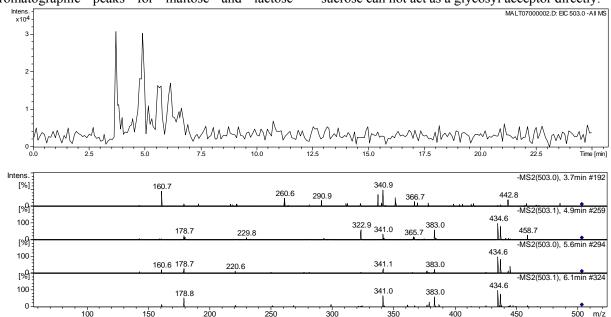


Fig. 5 Extracted ion chromatogram and MS^2 spectra of pseudomelecular ion at m/z 503.0 ($C_{18}H_{32}O_{16}$) for four selected chromatographic peaks of caramelized maltose in the negative ion mode.

Fig. 6 Formation of oligomeric saccharides from 1-3 during heating (regio- and stereochemistry of oligosaccharides remains open).

 MS^2 spectra of the ion at m/z 665.2, tetramer of hexoses gave the most abundant fragment at m/z 485 of (1, 2) and at m/z 503 (3), indicated the cleavage of the external glycosidic bond with and without bridging oxygen atom in the product ion, respectively. A neutral loss of 162 ($C_6H_{10}O_5$) as the main peak for all studied caramel samples was present in MS² spectra of the ion at m/z 827.3, originating from the cleavage of the glycosidic bond in the external side of the molecule without bridging oxygen atom in the product ion, as it was observed for the aforementioned trimers of hexoses. MS^2 spectrum of m/z 989.3, a hexamer of glucose gave a base peak at m/z 827 (1, 3), coming from the cleavage of the external glycosidic bond and at m/z 665.2 (2), coming from the cleavage of the internal glycosidic bond. Fragmentation studies of oligomeric ions of disaccharides in the negative ion mode for studied caramel samples are consistent with cleavages of glycosidic bonds on the external part of the molecules without and in rare cases with bridging oxygen atom. Targeted tandem-LC-MS measurements were carried out for all caramelized disaccharides and the extracted ion chromatograms (EICs) of oligomeric

hexoses were generated. Each EIC showed various numbers of chromatographic peaks (see supplementary information).

3.4 Characterization of Dehydration Products

A direct infusion into ESI ion-trap mass spectrometer measurements in the negative ion mode has been performed for dehydrated monomers and oligomers, which were the major products of caramelized disaccharides.

Dehydrated monosaccharides were observed at m/z 161.1 and tentative product structures 7 and 8 are shown in Fig. 7. Dehydrated disaccharides ($C_{12}H_{20}O_{10}$) were observed at m/z 323.1. Fragmentation of such precursor ions gave the fragments at m/z 160.9 (1, 2) and at m/z 178.9 (3). Both of them rose from the cleavage of the glycosidic bond leaving the bridging oxygen atom in the product ion and excluding the bridging oxygen atom in the product ion, respectively. The elimination of water molecule from glucose at C1-C4 positions forms an enol which tautomerises to its carbonyl form, as expected according to NMR and IR data analysis (Fig. 8). Moreover, monomeric hexoses

Fig. 7 Formation of dehydrated hexoses from heated disaccharides 1-3 (regiochemistry of water elimination was chosen randomly in 7).

Fig. 8 Dehydration products of heated sucrose 1 and maltose (regiochemistry of water elimination was chosen randomly in 10-13).

undergo dehydration reactions and product ions appeared at m/z 161.1. Fragmentation data of this ion gave different main fragments for studied caramel samples, including the main fragment at m/z 113 (1, 3) and 101 (2) arising from the cross-ring cleavage.

Dehydrated oligomers of caramel samples derived from a loss of a single water molecule were observed at m/z 485.1, 647.2, 809.2 and 971.3. The mass spectra of fragment ion 485 showed a loss of 162 (C₆H₁₀O₅) as the main peak for all caramels samples and derived from the cleavage of the glycosidic bond leaving the bridging oxygen atom in the product ion. Fragmentation of monodehydrated tetramers of hexoses at m/z 647.2 gave the main fragment at m/z 485 with a neutral loss of 162 ($C_6H_{10}O_5$) for 1 and 3, arising from dehydrated dimers and at m/z 467 with a neutral loss of 180 ($C_6H_{10}O_5$) for 2. MS^2 spectra of the ion m/z809.3, dehydrated pentamer of hexoses showed the main peak at m/z 647 and it is consistent for all analyzed samples with a neutral loss of 162 (C₆H₁₀O₅). MS^3 spectra of an ion 485 gave the base peak at m/z323 for all analyzed samples. Fragmentation of monodehydrated hexamers of hexoses at m/z 971.3 gave the main fragment at m/z 809.3 with a neutral loss of 162 (C₆H₁₀O₅) for heated 1 and 3, arising from dehydrated dimers. The ion at m/z 647 with a neutral loss of 324 (C₆H₁₀O₅) is present as a main fragment of MS² spectrum of an ion 971 for 2, coming from the cleavage of the second internal glycosidic bond. ESI-MS experiments with applied graphical techniques, showed the formation of dehydration products with one, two and three water molecules. Didehydrated disaccharide appeared at m/z 305.1 and followed didehydrated oligomers of hexoses at m/z 467.1, 629.2, 791.2 and 953.3, respectively. When it was possible, fragmentation studies in the negative ion mode have been performed for mentioned ions and didehydrated pentamer of hexoses at m/z 791.1 of maltose gave in MS^2 spectrum the main fragment at m/z 449 with a neutral loss of 342 (C₁₂H₂₂O₁₁), which correspond to the cleavage of the glycosidic bond. Although more

intense peaks belong to dehydration reactions products with three water molecules, which appeared at 287.1 for disaccharides and 449.1, 611.2, 773.2, 935.3 for oligomers, respectively. MS^2 spectra of the ion m/z 287 gave a neutral loss of 126 (C₆H₆O₃) as the main fragment for 1. It could thus suggest the presence of hydroxymethylfurfural and supports the elimination of all three water molecules at one saccharide moiety. MS^2 spectra of the ion m/z 449.1 gave ions 323.1 for all caramelized disaccharides with a neutral loss of 126 (C₆H₆O₃). This is consistent with the behavior of the aforementioned tridehydrated disaccharide showing the presence of hydroxymethylfurfural. Targeted tandem-LC-MS experiments have been performed for dehydrated products and resulted with their EICs. As an example, EIC of the ion at m/z 161 of an anhydro glucose in case of caramelized sucrose gave a total of 11 resolved chromatographic peaks, three of high, four of medium and four of low intensities. The formation of all 11 possible isomers of anhydro glucose could thus be explained. EICs of further dehydrated oligomers where generated and showed the formation of possible isomers. MS² spectra of particular showed an identical or different compounds fragmentation pattern, and demonstrated non-selective formation of dehydrated oligomers of hexose.

Targeted tandem LC-MS measurements producing EICs of the ion at m/z 323.1 revealed 10 chromatographically resolved peaks for heated sucrose 1, six for heated maltose 2 and 10 for heated lactose 3 (see supplementary information). For sucrose one chromatographic peak was showing retention time and MS^2 spectra identical to difructosedianhydride 11, two compounds showed data resembling dehydration products 9, 10 and 12 of heated maltose 2, as well previously observed for heated glucose [23]. The remaining seven signals showed tandem MS not previously observed and are tentatively assigned as dehydration products of sucrose loosing water at the fructose or glucose moiety. Similarly multiple peaks were observed in targeted LC-tandem MS experiments

in EICs at *m/z* 485.2 and 647.2 (up to 16 resolved peaks). Hence, it can be concluded that non-selective loss of water from disaccharides generates a multitude of different carbonyl reaction products. At this point, it should be noted that such carbonyl reaction products generated during the heating of carbohydrates should be considered in Maillard type reactions with amino acids and peptides as electrophilic reagents.

3.5 Characterization of Hydration Products

Hydrated disaccharides appeared in the negative ion mode at m/z 359.2. Hydrated products of further oligomers are also formed and were detected at m/z 521.2, 683.2, 845.3 and 1,007.3 in the negative ion mode. The observation of such hydration products is highly unusual and could alternatively be explained assuming the formation of non-covalent hydrated adducts. For this purpose we investigated a sample of beer containing various maltose derivatives under identical mass spectrometrical conditions [38]. These data show that hydrated oligomers are absent and should therefore be considered as genuine reaction products in caramel formation. Tandem MS data of mentioned ions showed similar fragmentation behavior of hydrated ions, which involves a loss of 36 (2H₂O), for example in MS² spectrum of 359 and 521 the base peaks at m/z 323 and m/z 485 were observed, respectively. When it was possible, the MS³ spectra of precursor ions 323 and 521 were recorded to obtain the main peak with neutral losses of 162 coming from the cleavage of the glycosidic bonds. MS² spectra of hydrated ions contained also the peaks with neutral losses of 180.1 which are consistent with hydration at any of anomeric centers producing an open chain hemiacetal structure. Analogously to oligomers and dehydration products targeted tandem-LC-MS experiments for all caramelized disaccharides were carried out and generating the extracted ion chromatograms (EICs) of hydrated ions. For example, EIC of hydratated disaccharide ion at m/z359.2 gave a total of 13 resolved chromatographic peaks, two of high, two of medium and nine of low intensities.

The multiple peaks for hydrated ions suggest the formation of isomeric structures.

3.6 Comparison of Three Disaccharides

In the previous section, we have described the identification of individual components formed in caramelization of disaacharides. In all three cases for sucrose, lactose and maltose, the same type of reaction products has been observed and classified mainly as oligomers and dehydration products. However, the three different mass spectra in Fig. 1 clearly show large variations in intensities of the individual ions pointing towards significant differences in the reaction kinetics observed in the three disaccharides. Therefore, we like to discuss in this section the main differences observed.

To graphically visualize differences in product formation, we decided to generate radar plots usually employed in sensory analysis. Within the plots, the individual ions are shown on the vertices, the relative intensity is shown on a scale from the center of the plot to its vertices and all three different disaccharides investigated are shown as colored lines within the plot. The first plot (Fig. 9a) shows difference in intensities of various oligomeric structures. The plot clearly shows that maltose favors oligomer formation followed by sucrose, whereas lactose is the most reluctant to form oligomers. The second plot (Fig. 9b) shows the relative intensities of various dehydrated ions. Here the opposite trend is apparent with lactose preferentially forming a monomeric dehydrated product at m/z 161. For higher molecular weight ions sucrose is more prone to dehydration followed by maltose. In the third plot (Fig. 9c), the ratio of intensities of oligomers versus dehydrated parent compounds is shown. Again for a monomeric hexose lactose displays the highest degree of dehydration followed by sucrose and finally maltose. For higher molecular weight derivatives the trend is reversed e.g., at m/z 665/647 or 827/809 sucrose shows the highest degree of dehydration followed by maltose and finally lactose. The preferential formation of dehydrated monomers of lactose can be readily

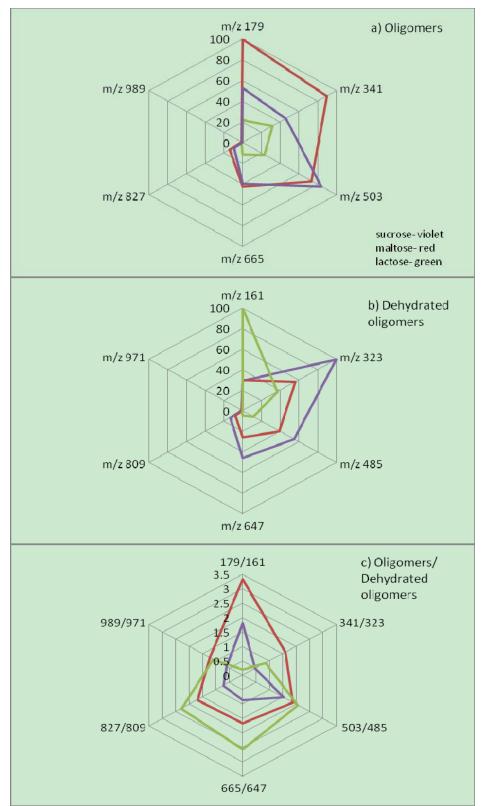


Fig. 9 Radar plots of classes of ions detected in direct infusion negative mode mass spectra of heated disaccharides 1-3 (a) oligomeric hexoses; (b) dehydrated oligomeric hexoses; (c) ratio of oligomeric to dehydrated hexoses (colored lines are coding samples 1-3).

understood in terms of its stereochemistry. The axial 4-OH substituent in the galactose moiety of lactose allows for an intramolecular S_N2-like substitution reaction producing most likely a bicylic structure 8 (Fig. 7). The substitution can be classified according to Baldwin's rules as a 5-exo-tet cyclisation and is hence favored as demonstrated previously [39]. This fragmentation behavior has been previously proposed for galactose derivatives [40, 41]. This means as well that lactose readily breaks into its monosaccharide units at elevated temperatures, whereas as sucrose and maltose are thermally more stable. Dehydration at the disaccharide stage is favored for sucrose, which can be rationalized by assuming that the new olefinic double bond formed in a five membered ring furanose is favored over a double bond formed in a pyranose. Consecutive elimination of water yields an even more stable aromatic furan derivative.

4. Conclusions

In summary, we have solved the complexity of caramelized disaccharides including sucrose, maltose and lactose using advanced mass spectrometric techniques with novel data interpretation strategies. We described the majority of formed compounds and compared to each others. A high resolution mass spectrometry with fragmentation studies led to the assignment of formulas for around 40 compounds. Caramel formed from heated disaccharides consists of thousand of the compounds formed by a small number unselective and chemoselective Oligomerization, dehydration and hydration reaction products are predominant. The oligomers of hexoses are created with up to eight carbohydrates unit through the unselective glycosidic bond formation. Monomeric hexoses are created with their dehydrated products as well. The fragmentation studies showed the similar behavior for studied disaccharides; therefore, novel data interpretation strategy such as van Krevelen and Kendrick plots are really useful to visualize a complex matrix like caramel. The work presented herein gives a

valuable contribution to the complex composition of one of the most popular dietary material such as caramel.

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Supporting Information Available

Additional EICs, MS² + MS³ data of all compounds mentioned in the text, table of high resolution MS-TOF data for compounds identified, NMR and IR data. This material is free of charge via the Internet.

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