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Fast Screening and Secure Confirmation of Milk Powder Adulteration with Maltodextrin via Electrospray Ionization−Mass Spectrometry [ESI(+)−MS] and Selective Enzymatic Hydrolysis

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Direct-infusion electrospray ionization−mass spectrometry [ESI(+)−MS] of several milk powder samples, confiscated by the Brazilian Federal Police, showed ions accounting for sodiated and potassiated molecules of disaccharides (*m*/*z* 365 and 381) as well as trisaccharides (*m*/*z* 527 and 543), whereas monosaccharide ions were not detected. The trisaccharide ions were not detected in samples of genuine milk powder, raising the suspicion that their presence indicates adulteration by the addition of maltodextrin. In control samples, maltose and maltotriose were hydrolyzed by α-glucosidase and not β-galactosidase, whereas lactose was resistant to α-glucosidase but was hydrolyzed with β-galactosidase. Samples suspected of being adulterated behaved in the same fashion, confirming the presence of maltose and maltotriose or maltodextrin. Direct-infusion ESI−MS is shown therefore to provide rapid screening of milk powder for adulteration with maltodextrin, whereas its combination with selective enzymatic hydrolysis provides highly reliable confirmation for unambiguous results.

KEYWORDS: ESI−MS; fraud; glycosidases; lactose; maltodextrin; mass spectrometry; milk powder

INTRODUCTION

The authenticity of foods is currently of major concern for consumers, industries, and policymakers at all levels of the production process (*1*). Dairy products form a group of foods of high interest because they play important roles in human nutrition and are essential for women, children, and the elderly. Milk is not an expensive raw material, but the large amount commercialized worldwide makes it, from an economic point of view, an attractive target for criminal elements to generate milk-like compositions by mixing cheaper raw materials with other dairy or non-dairy ingredients and selling the product as "instant" milk. Replacing, for instance, genuine milk fat, protein, or carbohydrates with components of different origins is a practice that is considered as fraud, regardless whether this substitution may even enhance the nutritional value of the final product.

Progress in dairy chemistry and technology has led to the manufacture of specialized milk products, which unfortunately has also provided new opportunities for sophisticated types of manipulations. Ramos and Juárez (*2*) reviewed some 25 years ago the main possible frauds in dairy products and the corresponding analytical procedures for their detection. Since then, significant advances in analytical dairy science have been achieved, mostly for protein analysis, owing to further developments in chromatography, electrophoresis, enzyme-linked immunosorbent assay (ELISA), polymerase chain reaction (*3*−*9*), and in particular, mass spectrometry (MS) (*10*−*17*). A recent example with worldwide awareness was the "melamine" scandal, resulting in enormous health damages and even a number of fatalities in China, which triggered the development of new methods of trace analysis by MS (*18*, *19*).

Electrospray ionization (ESI) is a soft and wide-ranging ionization technique that has revolutionized the way molecules are ionized and transferred to mass spectrometers for mass measurement and characterization (*20*). ESI has greatly expanded the applicability of MS to a variety of new classes of molecules with thermal instability and high polarity and mass. We and others have also used direct-infusion ESI−MS for fast fingerprinting and quality control of complex mixtures, such as plant extracts (*21*), propolis (*22*), wine (*23*), whisky (*24*), and cachaça (*25*−*27*). ESI−MS with direct sample introduction therefore

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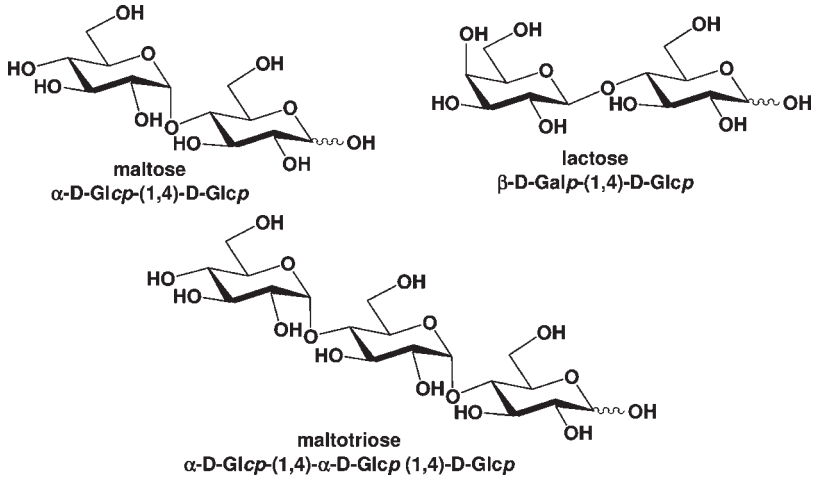

Figure 1. Structures of the di- and trisaccharides investigated.

seems to us as a promising technique for reliable fingerprinting and fast quality control of milk powder with little sample manipulation.

A common milk fraud in Brazil is the addition of small amounts of maltodextrin to milk powder adulterated with, e.g., whey protein and fat, to adjust the density and cryoscopy of the liquid milk prepared thereof and to offer the product to customers as "integral" milk powder (*28*). Some milk products, such as infant formula, may however legally contain starch and the derivatives maltose and maltodextrin (for a reference, see ref *29*). Adulteration of milk powder with maltodextrin is, therefore, a rather sneaky practice. To prove is quite a challenge, which, to the best of our knowledge, has not been addressed in the literature. Existing methods for carbohydrate analysis in dairy products by liquid and gas chromatography are time-consuming and may suffer from rather low sensitivity. With the aim to establish a fast and reliable method for routine detection of maltodextrin in adulterated milk powder, we have developed a protocol for a combined enzymatic hydrolysis−direct-infusion ESI−MS analysis.

MATERIALS AND METHODS

Chemicals. Carbohydrates, i.e., lactose, maltose, isomaltose, maltotriose, isomaltotriose, panose, and maltodextrin as well as α-glucosidase (maltase) from *Bacillus stearothermophilus* (specific activity, according to the supplier: 79 units/mg of solid) and *β*-galactosidase from *Aspergillus oryzae* (specific activity, according to the supplier: 10 units/mg of solid) were purchased from Sigma (St. Louis, MO). High-performance liquid chromatography (HPLC)-grade methanol and formic acid solutions were purchased from Sigma-Aldrich (Sigma-Aldrich, St. Louis, MO) and used without further purification. Deionized water was obtained on a Milli-Q system (Millipore, Billerica, MA).

Sample Preparation. Milk powder samples were provided by the Brazilian Federal Police Department. A total of 13 g of milk powder was suspended in 100 mL of deionized water. Milk fats and proteins were precipitated with the addition of 5 mL of a solution of potassium ferrocyanide in water (15%, w/w) and 5 mL of a solution of zinc acetate in water (30%, w/w). Filtered samples were then diluted (1:1000, v/v) in a solution of methanol and water (50:50, v/v).

Enzyme Assays. Authentic (code MP350) and adulterated (code MP364) whole milk powder samples were used for the enzyme assays, which were adapted from protocols provided by Sigma (*30*, *31*). Stock solutions were prepared in deionized water (Milli-Q). For assays with α-glucosidase, 20 μL of a solution of 3 mM glutathione (reduced form, GSH) and 20 μL of a solution of 1.0 mg mL^{-1} (79 units mL^{-1}) α-glucosidase were mixed with 500 μL of a 67 mM potassium phosphate buffer, prepared from KH_2PO_4, and adjusted to pH 6.8 with 1 M NaOH. The final substrate concentrations were 0.47 mg mL^{-1} disaccharides, 0.78 mg mL^{-1} trisaccharides, 1.0 mg mL^{-1} milk powder sample MP350, or 1.24 mg mL^{-1} sample MP364, respectively.

For assays with *β*-galactosidase, 50 μL of a solution of 2.2 mg mL^{-1} (22 units mL^{-1}) *β*-galactosidase was mixed with 400 μL of sodium phosphate−citrate buffer, prepared from 20 mM Na_2HPO_4, and adjusted to pH 4.5 with a 100 mM citric acid solution. Solutions of oligosaccharides or milk powder (50 μL) were added to give final substrate concentrations of 0.30 mg mL^{-1} disaccharides, 0.50 mg mL^{-1} trisaccharides, 0.64 mg mL^{-1} milk powder MP350, or 0.80 mg mL^{-1} MP364, respectively. Blanks were prepared by replacing either the enzyme, the reference saccharide, or the milk powder solutions by equal volumes of H_2O. For control experiments, milk powder solutions were spiked with lactose, maltose, or maltotriose to give the same concentration of saccharide as in the reference hydrolysis experiments. The buffer and enzyme solutions were mixed by means of a Vortex apparatus and left for 5 min at ambient temperature (22−25 °C) before the addition of substrate solutions. The assay solutions were mixed again and kept for 60 min at room temperature before aliquots were analyzed by direct-infusion ESI−MS.

Direct-Infusion ESI−MS. The characterization of lactose and maltodextrin standard samples was performed in a hybrid 9-T Fourier transform ion cyclotron resonance mass spectrometer (LTQ FT, Thermo Scientific, Bremen, Germany), equipped with a chip-based direct-infusion nanoelectrospray ionization source (Triversa, Advion Biosciences, Ithaca, NY). Nanoelectrospray conditions comprised a 200 nL min^{-1} flow rate, 0.3 psi backing pressure, and 1.5−2.0 kV electrospray voltage for 120 s, controlled by ChipSoft software (version 8.1.0, Advion Biosciences). A conventional SIM scan mode was employed. Mass resolution was fixed at 100 000 (defined for an ion of m/z 400) throughout. Data were obtained as transient files (scans recorded in the time domain). All of the samples were evaluated in the positive-ion mode, and mass spectra were acquired and accumulated over 10 s and scanned in the range between m/z 150 and 2000.

A Q-TOF mass spectrometer (Micromass, Manchester, U.K.) was used for fingerprinting ESI−MS analysis. The general conditions were a source temperature of 100 °C, capillary voltage of 3 kV, and cone voltage of 35 V. For ESI(+)−MS, 10.0 μL of concentrated formic acid aqueous solution was added to the sample mixture to a total volume of 1000 μL, yielding 0.1% as the final concentration. ESI(+)−MS was performed by direct infusion with a flow rate of 10 μL min^{-1} using a syringe pump (Harvard Apparatus). Mass spectra were acquired and accumulated over 60 s and scanned in the range between m/z 50 and 1500. Data were evaluated from m/z ranges covering Hex (m/z 200−230), Hex_2 (m/z 350−400), and Hex_3 (m/z 520−560). The threshold value for peak annotation was set to 2% of the base peak.

RESULTS AND DISCUSSION

Lactose, the main sugar in milk, is 4-*O*-(*β*-D-galactopyranosyl)-D-glucopyranose, with a molecular mass of 342 Da (**Figure 1**). The ESI(+)−MS of lactose (**Figure 2A**) shows disaccharide ions of m/z 365 for $[M + Na]^+$ and m/z 381 for $[M + K]^+$. The sodiated dimer of lactose $[2M + Na]^+$ of m/z 707 is also detected.

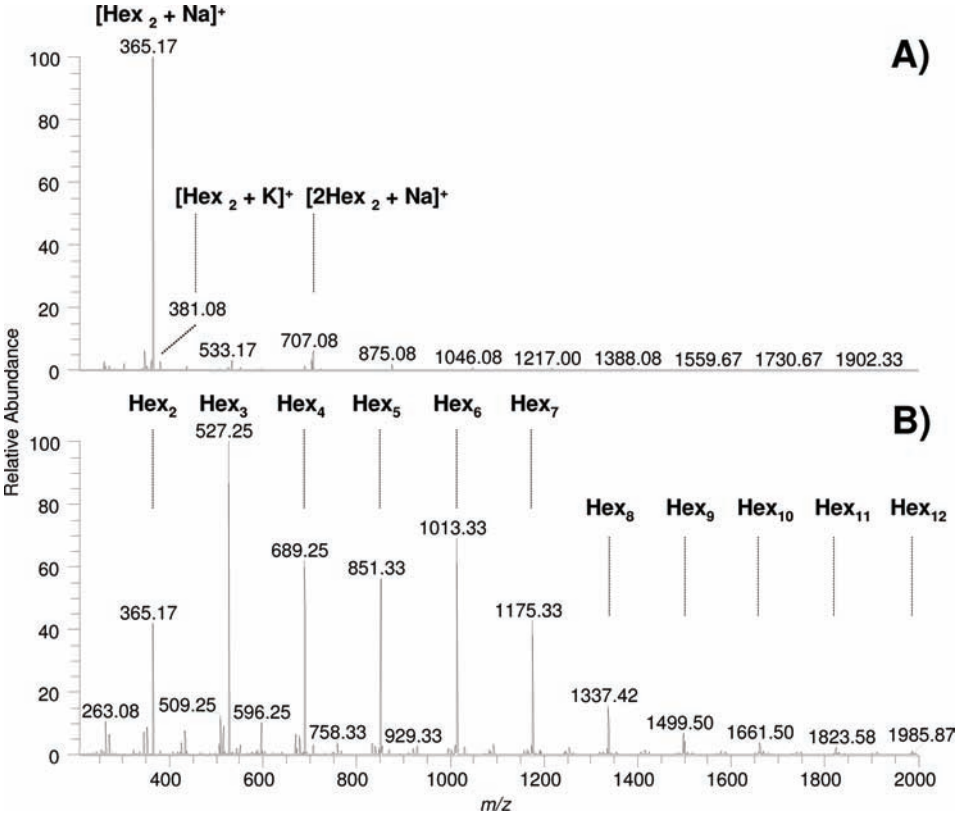

Figure 2. ESI(+)−MS (LTQ FT) fingerprints of (A) lactose and (B) maltodextrin. Only ions of sodiated molecules $[Hex_n + Na]^+$, with $n = 2-12$, are labeled.

Table 1. Calculated *m/z* Values of Hexose Oligosaccharides

oligosaccharide	$[M + H]^+$	$[M + Na]^+$	$[M + K]^+$
Hex	181	203	219
Hex_2	343	365	381
$2Hex_2$	685	707	723
Hex_3	505	527	543
Hex_4	667	689	705
Hex_5	829	851	867
Hex_6	991	1013	1029
Hex_7	1153	1175	1191
Hex_8	1315	1337	1353
Hex_9	1477	1499	1515
Hex_{10}	1639	1661	1677
Hex_{11}	1801	1823	1839
Hex_{12}	1963	1985	2001

Maltodextrin, which is industrially obtained by acidic or enzymatic hydrolysis of starch, is a mixture of oligosaccharides of α-(1,4)-linked D-glucopyranose. The disaccharide maltose (**Figure 1**) is an isomer of lactose with the same molecular mass of 342 Da. The ESI−MS of maltodextrin (**Figure 2B**) displays a characteristic set of $[M + Na]^+$ ions mainly of *m/z* 365, 527, 689, 851, 1013, 1337, 1499, 1175, and 1337, which are assigned to a series of oligomers of glucose (**Table 1**). Because those are absent in the ESI−MS of lactose, a characteristic chemical signature is provided by ESI−MS for both lactose and maltodextrin.

The ESI−MS of samples of defatted and deproteinized genuine milk powder (**Figure 3A**) shows ions consistent with the presence of a disaccharide: *m/z* 381 for $[Hex_2 + K]^+$, *m/z* 365 for $[Hex_2 + Na]^+$, *m/z* 707 for $[2Hex_2 + Na]^+$, and *m/z* 723 for $[2Hex_2 + K]^+$. The additional ions of *m/z* 223, 441, 453, 533, and 783 are not assigned but arise probably from other components present in the highly complex matrix of milk powder. There is no indication for the presence of a hexose and a trihexose, the ions of which would be of *m/z* 203 $[Hex + Na]^+$, 219 $[Hex + K]^+$, 527 $[Hex_3 + Na]^+$, and 543 $[Hex_3 + K]^+$, respectively.

The ESI−MS of several other milk powder samples, suspected to be adulterated, reveals the absence of a hexose. A small but diagnostic $[M + K]^+$ ion of *m/z* 543 of a trihexose is, however, clearly detected in some samples, as shown in **Figure 3B**. The expansion of the spectra in the *m/z* 500−600 range reveals a second diagnostic ion corresponding to $[Hex_3 + Na]^+$ of *m/z* 527 (inset of **Figure 3B**). Assuming that the trihexose (Hex_3) is maltotriose, a sample giving a mass spectrum as shown in **Figure 3B** would be classified as milk powder adulterated with maltodextrin. The major ion of *m/z* 381 assigned to a disaccharide would then be actually a mixture of $[M + K]^+$ adducts of the isomeric lactose and maltose. Fingerprinting by direct-infusion ESI(+)−MS of the defatted and deproteinized sample thus provides evidence of adulteration of milk powders with sugars derived from starch. Screening by ESI(+)−MS is fast and requires little sample workup or no pre-chromatographic separation, allowing high-throughput analysis of milks for routine quality control.

The detection of a trihexose in milk powder by MS alone must not necessarily indicate the presence of maltotriose because the presence of other isomeric neutral trisaccharides, such as galactosyl lactoses, cannot be securely excluded (*32*, *33*). The proof of the occurrence of small amounts of maltose together with relatively large amounts of the isomeric lactose is more complex. To perform this challenging task of isomer distinction via MS, we have envisaged the use of specific glycosidases, which would allow for discrimination between α- and β-glycosidic linkages of non-reducing-end glucosyl and galactosyl residues, respectively. Because none of the known natural milk oligosaccharides contains a non-reducing-end α-glucose (*32*, *33*), treatment with α-glucosidase should yield a hexose (glucose) from maltose and maltotriose present in adulterated milk powder, whereas the saccharide

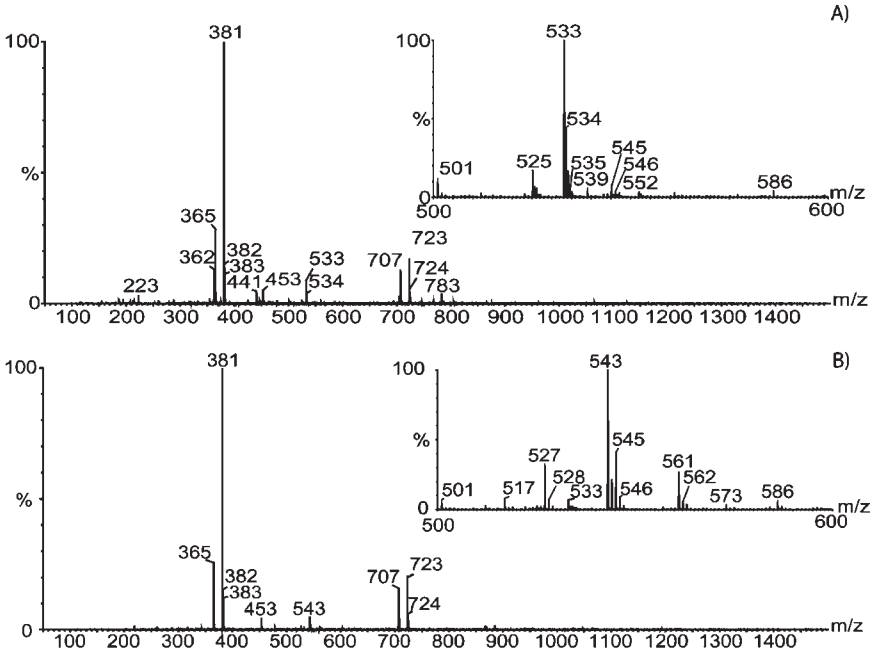

Figure 3. ESI(+)−MS (LTQ FT) fingerprints of (A) non-adulterated and (B) adulterated milk powder. The insets show an expansion in the *m/z* 500−600 range. Note the absence in both samples of ions of *m/z* 203 $[Hex + Na]^+$ and 219 $[Hex + K]^+$. The ions of *m/z* 527 $[Hex_3 + Na]^+$ and 543 $[Hex_3 + K]^+$ are detected in adulterated milk powder only.

profile of "natural" milk powder should remain unchanged. However, β-galactosidase should cleave lactose and not maltose or maltotriose.

In our approach, the enzymatic reaction mixtures, including blanks and controls, were subjected to direct-infusion ESI(+)−MS without previous desalting or chromatographic cleanup, although this will yield spectra with a rather high background and possibly also cause a decrease in sensitivity. Because of the buffers used, ESI(+)−MS experiments performed with α-glucosidase show predominantly $[M + K]^+$ ions, whereas $[M + Na]^+$ ions prevail in experiments with β-galactosidase. The monoisotopic mass of glutathione (GSH), which is a component of the buffer used for assays with α-glucosidase, is 307.08 Da. The corresponding ions are *m/z* 308 $[M + H]^+$, *m/z* 330 $[M + Na]^+$, and *m/z* 346 $[M + K]^+$, none of which would interfere with the analysis.

Analysis of Reference Saccharides with Glycosidases. $[M + H]^+$ ions and B-type fragments, i.e., $C_{6n}H_{12n-2}O_{6n-1}$ ($n = 1-3$), are not observed in the ESI(+)−MS of assays with α-glucosidase (for data, see Figure S1 in the Supporting Information). The blank, i.e., KH_2PO_4/NaOH buffer with α-glucosidase, shows no ions that would interfere with the analysis of Hex, Hex_2, and Hex_3. Hydrolysis of maltose is detected by the $[Hex + K]^+$ ion of *m/z* 219 with a trace amount of disaccharide remaining after 20 min. The ESI(+)−MS of maltotriose in the absence of enzyme shows the $[M + K]^+$ ion of *m/z* 543 and a low intensity ion of Hex_2 of *m/z* 381 for $[M + K]^+$, which could be either a C/Y-type fragment ion or a contaminant present in the commercial trisaccharide sample. Maltotriose is completely hydrolyzed with α-glucosidase, with no Hex_2 and Hex_3 remaining. The non-reducing-end α-(1,6)-linked trisaccharides isomaltose [α-D-Glc*p*-(1,6)-D-Glc*p*], isomaltotriose [α-D-Glc*p*-(1,6)-α-D-Glc*p*-(1,6)-D-Glc*p*], and panose [α-D-Glc*p*-(1,6)-α-D-Glc*p*-(1,4)-D-Glc*p*] are not hydrolyzed by the α-glucosidase from *B. stearothermophilus* (data not shown), confirming that the enzyme is specific for non-reducing-end α-(1,4)-glucosyl residues (*34*). Lactose is not hydrolyzed by α-glucosidase, and accordingly the ESI−MS recorded in the presence of the enzyme shows only the disaccharide $[M + Na]^+$ ions of *m/z* 365 and $[M + K]^+$ of *m/z* 381, confirming that the commercial enzyme is not contaminated with β-galactosidase activity (see Figure S1 in the Supporting Information).

Protonated molecules and B-type fragments are also not observed in the ESI(+)−MS of assays with β-galactosidase (see Figure S2 in the Supporting Information). There is no ion that could interfere with the analysis of the Hex $[M + Na]^+$ ion of *m/z* 203, although the enzyme preparation contains contaminants giving ions of *m/z* 365 and 527, which would interfere with small amounts of maltotriose. The ESI(+)−MS of the reaction mixture containing lactose and β-galactosidase shows an abundant $[M + Na]^+$ ion for Hex of *m/z* 203, which is consistent with the hydrolysis of lactose to galactose and glucose. As expected, treatment of maltose and maltotriose with β-galactosidase fail to cause the detection of a hexose ion by ESI(+)−MS, confirming that the commercial enzyme is not contaminated with α-glucosidase activity (see Figure S1 in the Supporting Information).

Analysis of Milk Powders. ESI(+)−MS of genuine milk powder sample MP350 in K_2HPO_4 buffer shows before and after treatment with α-glucosidase $[M + Na]^+$ ions of *m/z* 365 and $[M + K]^+$ of *m/z* 381 for Hex_2 (panels **A** and **B** of **Figure 4**). Monosaccharide, expected to give an ion of *m/z* 219, is not detected. A weak but significant $[M + K]^+$ ion for Hex_3 of *m/z* 543.17 is found in the ESI−MS of the adulterated sample MP364 in the absence of α-glucosidase (**Figure 4C**). Treatment of MP364 with α-glucosidase results in the complete disappearance of the Hex_3 ion of *m/z* 543.17 and in the appearance of a $[M + K]^+$ hexose ion of *m/z* 219.05 (**Figure 4D**). The combination of enzymatic hydrolysis and ESI(+)−MS indicates therefore that the trisaccharide present in MP364 is maltotriose and the disaccharides are obviously a mixture of lactose and maltose. Considering the relative abundance of the ions of *m/z* 381 (100%) and *m/z* 543 (ca. 5%), it is estimated that the disaccharide consists of > 95% lactose and < 5% maltose.

Ions for Hex_3 are found in the absence of β-galactosidase in the ESI−MS of buffer solutions of MP364 but not of MP350 (see Figure S3 in the Supporting Information). Treatment of both MP350 and MP364 with β-galactosidase results in the appearance

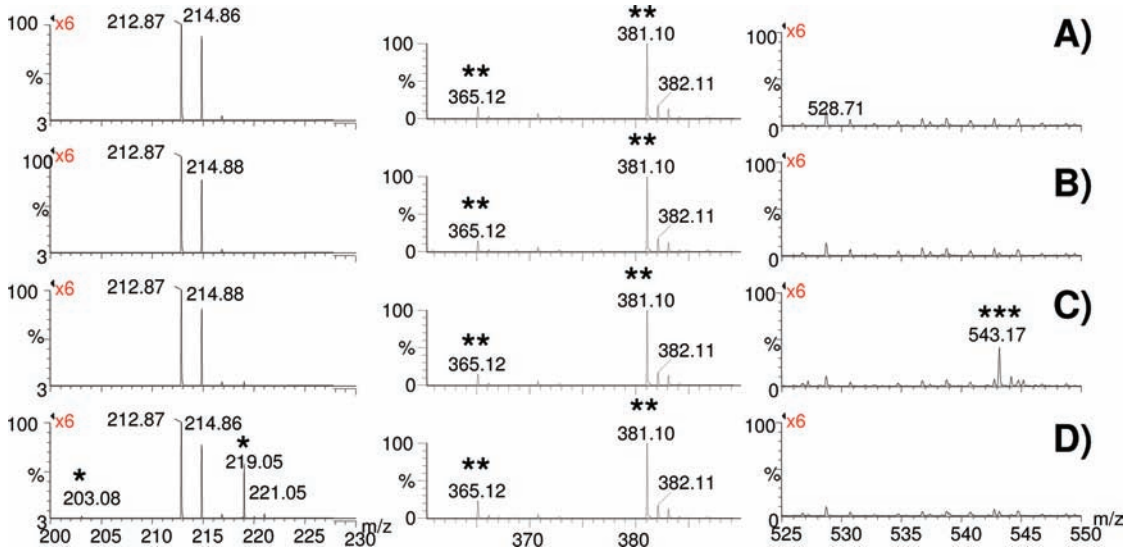

Figure 4. ESI(+)−MS (Q-TOF) of mixtures containing milk powder samples in potassium phosphate buffer: (A) genuine MP350 without α-glucosidase, (B) MP350 with α-glucosidase, (C) adulterated MP364 without α-glucosidase, and (D) MP364 with α-glucosidase. Ions of saccharides are indicated by asterisks: (∗) Hex, (∗∗) Hex_2, and (∗∗∗) Hex_3. The ion of *m/z* 543 $[Hex_3 + K]^+$ is detected in the sample MP364 only. It disappears completely after treatment of MP364 with α-glucosidase to give the ion of *m/z* 219 $[Hex + K]^+$.

of the $[M + Na]^+$ ion for Hex of *m/z* 203. A comparison of the relative ion intensities of the background ions of *m/z* 215 and 378 to those of *m/z* 365 suggests that Hex is formed indeed as a mixture of galactose and glucose from lactose in both milk powder samples MP350 and MP364.

Glycosidase Treatment of Milk Powders Spiked with Saccharides. To support the analytical concept, both milk powder samples were spiked with reference saccharides, followed by enzymatic hydrolysis and ESI(+)−MS. To simulate adulteration, maltose or maltotriose was added to genuine milk powder sample MP350 and treated with α-glucosidase, yielding an abundant $[Hex + K]^+$ ion of *m/z* 219 for glucose (see Figure S4 in the Supporting Information). The addition of maltose or maltotriose to sample MP364 and the subsequent treatment with α-glucosidase give essentially the same result. In both cases, the $[Hex_3 + K]^+$ ion of *m/z* 543 was not detected, indicating complete hydrolysis of the maltotriose. The lactose-spiked samples MP350 and MP364 yielded no monosaccharide ion of *m/z* 219 after treatment with α-glucosidase.

With β-galactosidase, both milk powder samples spiked with lactose, maltose, or maltotriose gave abundant $[Hex + Na]^+$ ions of *m/z* 203, as expected from hydrolysis of lactose (see Figure S5 in the Supporting Information). As mentioned before, the appearance of ions of *m/z* 365 and 527 is ambiguous, because of contaminants in the enzyme preparation, although a comparison of the relative intensities of mono-, di-, and trisaccharides confirms that maltooligosaccharides are resistant to hydrolysis with β-galactosidase.

In forensic cases, with samples classified as suspicious by ESI(+)−MS monitoring, adulteration can be confirmed with high confidence by checking the chemical identity of maltose and maltotriose, which is proven by selective enzymatic hydrolysis of glucose with α-glucosidases followed by ESI(+)−MS. Obviously, the critical point of proof by a ESI(+)−MS plus enzymatic assay is that the milk powder to be analyzed is essentially free of monohexoses, as confirmed by ESI−MS (**Figures 3** and **4**).

Supporting Information Available: Mass spectra of blanks with peak assignments and interpretation of hydrolysis of reference oligosaccharides with α-glucosidase and β-galactosidase, milk powder samples with β-galactosidase, and milk powder samples spiked with reference oligosaccharides. This material is available free of charge via the Internet at http://pubs.acs.org.

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