JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

pubs.acs.org/JAFC

# Matrix Binding of Ochratoxin A during Roasting

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**ABSTRACT:** The mycotoxin ochratoxin A is degraded during coffee roasting by up to 90%. During this process, the two known degradation products, 14*R*-ochratoxin A and 14-decarboxy-ochratoxin A are formed. However, there is still an unexplained loss of more than 50% ochratoxin A. Here, we describe the binding of ochratoxin A to coffee polysaccharides via esterification as a further thermal reaction. This ester formation was studied by heating ochratoxin A with methyl α-D-glucopyranoside, a model compound to mimic polysaccharides. From this experiment, (22 → 6′) ochratoxin A-methyl-α-D-glucopyranoside ester was isolated and characterized as a reaction product, showing the general ability of ochratoxin A for esterification with carbohydrates at roasting temperatures. Subsequently, a sample preparation protocol for the detection of ochratoxin A saccharide esters based on an enzymatic cleavage and purification using immunoaffinity chromatography was developed and applied. The detection was carried out by high-performance liquid chromatography−tandem mass spectrometry (HPLC−MS/MS). Using this method, it was possible to detect ochratoxin A polysaccharide esters formed during roasting of artificially contaminated coffee, confirming the results of the previous model experiments. Thus, the formation of ochratoxin A esters is a further explanation for the loss of ochratoxin A during coffee roasting.

**KEYWORDS:** *Ochratoxin A, roasting, coffee, masked mycotoxins, bound mycotoxins, ochratoxin A ester, 14R-ochratoxin A, ochratoxin A saccharide ester, enzymatic cleavage*

## INTRODUCTION

Ochratoxin A, (*N*-[(3*R*)-(5-chloro-8-hydroxy-3-methyl-1-oxo-7-isochromanyl)carbonyl]-L-phenylalanine) (**1**) (Figure 1), is a secondary metabolite produced by certain molds of the genera *Aspergillus* and *Penicillium*.[1] This mycotoxin was classified as a group 2B carcinogen by the International Agency for Research on Cancer (IARC) in 1993, being possibly carcinogenic to humans.[2] In addition, further toxic effects for ochratoxin A were described, such as nephrotoxicity,[3] teratogenicity,[4] and immunotoxicity.[5]

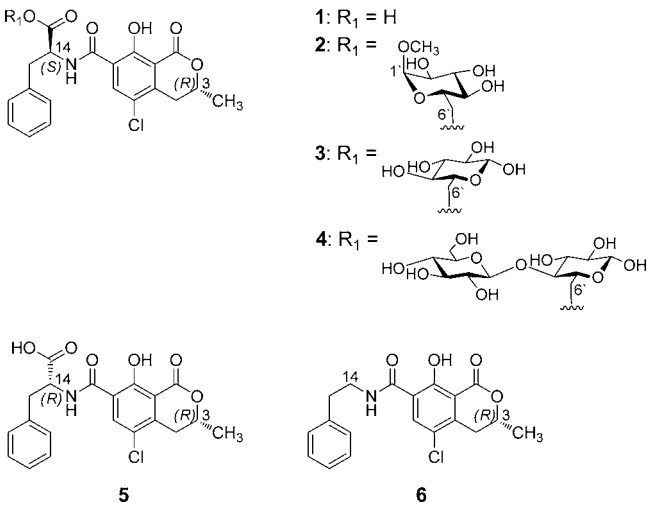


**Figure 1.** Structures of ochratoxin A (**1**), 14*R*-ochratoxin A (**5**), 14-decarboxy-ochratoxin A (**6**), (22 → 6′) ochratoxin A-methyl-α-D-glucopyranoside ester (**2**), ochratoxin A glucose ester (**3**), and ochratoxin A cellobiose ester (**4**).

Ochratoxin A occurs in a wide range of commodities, such as coffee beans, cocoa, cereals, and grapes. It can also be detected in processed food products, such as coffee, wine, beer, and grape juice.[6] Several studies reported that processing, such as roasting,[7] baking,[8] or extrusion cooking,[9,10] can decrease the ochratoxin A concentration in food products. With regard to this effect, especially the influence of coffee roasting on the reduction of the ochratoxin A content has been investigated in the last few decades, resulting in a broad set of data with different results. On the one hand, some studies reported only a low reduction of the ochratoxin A content;[7,11] meanwhile, most research groups found reduction rates of up to 90%.[12−14] However, it is unclear if ochratoxin A is degraded or bound to matrix compounds.

With regard to the chemical decomposition of ochratoxin A, two degradation reactions are known thus far, the isomerization to 14*R*-ochratoxin A (**5**) and the decarboxylation to 14-decarboxy-ochratoxin A (**6**) (Figure 1).[15] It could be shown that besides isomerization of the amino acid, ochratoxin A is nearly stable when heated to 200 °C.[15] Because the detected amounts of 14*R*-ochratoxin A in the analyzed coffees were only between 15 and 26% of ochratoxin A and 14-decarboxy-ochratoxin A being only detected in traces, further degradation reactions must occur to explain the loss of up to 90% ochratoxin A. A possible explanation for further losses of ochratoxin A could be the binding to matrix components, resulting in bound forms of ochratoxin A, which are not detectable by standard analytical methods. Such a covalent binding of a mycotoxin to the food matrix has already been

Received: September 6, 2013
Revised: November 29, 2013
Accepted: December 3, 2013
Published: December 3, 2013

described for fumonisins, a group of mycotoxins containing free carboxylic acid functions, such as ochratoxin A. As shown by Seefelder et al.,[16] the formation of esters between the mycotoxin and carbohydrates can result in bound fumonisins.

In our study, we investigated the formation of ochratoxin A saccharide esters during roasting of coffee beans. Starting with methyl α-D-glucopyranoside as a model compound to represent polysaccharides, we studied the reactivity of ochratoxin A with carbohydrates. Furthermore, we prepared appropriate references of ochratoxin A saccharide esters using cellulose and developed a method to isolate and detect bound ochratoxin A formed during roasting in artificially contaminated coffee beans.

## MATERIALS AND METHODS

**Safety Information.** Ochratoxin A is nephrotoxic and possibly carcinogenic to humans. It should be handled with care. Glassware was decontaminated in bleach solution.

**Chemicals and Materials.** All chemicals were purchased from Sigma-Aldrich GmbH (Seelze, Germany) or Carl Roth GmbH + Co. KG (Karlsruhe, Germany). Solvents were obtained in gradient-grade quality. Water for high-performance liquid chromatography (HPLC) separation was purified by a Milli-Q Gradient A 10 system (Millipore, Schwalbach, Germany).

Cellulase from *Trichoderma reesei* ATCC 26921 (lyophilized powder, ≥1 unit/mg of solid), hemicellulase from *Aspergillus niger* (powder, 0.3−3.0 unit/mg of solid), Driselase from *Basidiomycetes* sp., and cellulose (Sigmacell Type 50, 50 μm) were purchased from Sigma Aldrich.

Ochratoxin A was isolated from cultures of *Penicillium nordicum* BFE487 made available from the Max Rubner Institute (Karlsruhe, Germany), which were incubated at 25 °C for 14 days on YES medium (20 g/L yeast extract, 150 g/L sucrose, and 20 g/L agar) with an addition of sodium chloride (20 g/L), as described by Schmidt-Heydt et al.[17] The extraction was carried out as previously described, resulting in ochratoxin A with a purity of >98% [nuclear magnetic resonance (NMR)-spectroscopy].[15]

**High-Resolution Mass Spectrometry (HRMS).** The exact mass measurements and the higher energy collision dissociation (HCD) spectra were carried out on a LTQ Orbitrap XL mass spectrometer (Thermo Scientific, Bremen, Germany) with direct flow infusion of 10 μL/min. Data acquisition was performed with Xcalibur 2.07 SP I (Thermo Scientific). The mass spectrometer was operated in the negative mode, and the ionization was performed with heated electrospray ionization. Further conditions were as follows: capillary temperature, 225 °C; vaporizer temperature, 55 °C; sheath gas flow, 10 units; auxiliary gas flow, 5 units; and source voltage, 3 kV. For the HCD spectra, the optimal relative energy was determined for each compound in a range from 10 to 70%. The mass resolution was set to 30 000, and the ion injection time was set to 100 ms. The optimized tube lens and capillary voltage depending on the measured compound is shown in parentheses.

**NMR Spectroscopy.** $^{1}$H, $^{13}$C, and 2D NMR data were acquired on a 400 MHz Bruker DPX-400 (Bruker BioSpin, Rheinstetten, Germany). Signals are reported in parts per million relative to tetramethylsilane (TMS). For structural elucidation and NMR signal assignment, 2D NMR experiments, such as gradient-selected correlated spectroscopy (gs-COSY), heteronuclear multiple-quantum correlation (HMQC), and heteronuclear multiple-bond correlation (HMBC), were carried out. Pulse programs for these experiments were taken from the Bruker software library.

**Heating Experiment.** For the model heating experiment, 50 μL of a stock solution of ochratoxin A (1000 μg/mL in methanol), prepared by dissolving 20 mg of ochratoxin A in 20 mL of methanol using a volumetric flask, and 100 μL of methyl α-D-glucopyranoside (10 mg/mL in methanol), prepared by dissolving 100 mg of methyl α-D-glucopyranoside in 10 mL of methanol using a volumetric flask, were mixed and evaporated to dryness in a 1.5 mL autosampler vial under a stream of nitrogen at 40 °C. The vial was capped and heated at 225 °C for 5 min. After cooling, the reaction residue was dissolved in 500 μL of acetonitrile/water/formic acid (30:70:0.1, v/v/v), sonicated for 5 min, and analyzed by HPLC with fluorescence detection (HPLC−FLD).

**Analysis by HPLC−FLD.** For the HPLC−FLD analysis, 10 μL of the prepared solution was injected into an analytical HPLC system. A Jasco PU-2089 pump (Jasco GmbH, Groß-Umstadt, Germany) delivering a binary gradient of acetonitrile (A) and water with 0.1% formic acid (B) was connected to a Jasco AS-2057plus autosampler, and peaks were detected by a Jasco MD-2010plus diode array detector and a Jasco FP-1520 fluorescence detector set to the wavelengths of 333 nm for excitation and 460 nm for emission. The column used was a 250 × 4 mm inner diameter, 5 μm, ReproSilPur C18-AQ with a 4 × 4 mm inner diameter guard column of the same material (Dr. Maisch, Ammerbach, Germany). The following gradient was used: 0 min, 20% A; 1 min, 20% A; 30 min, 100% A; and 40 min, 100% A. After each run, the column was equilibrated at starting conditions for 10 min.

**Preparation of (22 → 6′) Ochratoxin A-methyl-α-D-glucopyranoside Ester.** A solution of 10.0 mg of ochratoxin A (24.8 μmol) in 4 mL of methanol and 100 mg of methyl α-D-glucopyranoside were mixed in a 4 mL screw capped vial, and the solvent was removed under a stream of nitrogen at 60 °C. The reaction mixture was heated at 200 °C for 30 min. After cooling, the residue was dissolved in 1.5 mL of 0.1% formic acid and extracted 3 times with 2 mL of *tert*-butyl methyl ether. The organic phases were combined and evaporated to dryness. The residue was dissolved in 2 mL of 0.1% formic acid/acetonitrile (55:45, v/v) and further purified by semi-preparative HPLC.

**Isolation of (22 → 6′) Ochratoxin A-methyl-α-D-glucopyranoside Ester.** The crude reaction mixture was separated on a 250 × 16 mm inner diameter, 4 μm, Phenomenex Synergi Fusion column with a 10 × 10 mm inner diameter guard column of the same material (Phenomenex, Aschaffenburg, Germany) using an isocratic mixture of 55% water containing 0.1% formic acid and 45% acetonitrile delivered by two Varian ProStar 210 HPLC solvent delivery modules (Varian, Darmstadt, Germany). The flow rate was set at 10 mL/min. Besides unreacted ochratoxin A, (22 → 6′) ochratoxin A-methyl-α-D-glucopyranoside ester was detected as a major peak with a retention time of 18 min by a Varian ProStar 325 UV/vis detector (Agilent Technologies, Böblingen, Germany) set at 330 nm. The peaks containing the desired product were collected over three runs; acetonitrile was removed; and the aqueous solution was extracted 3 times with 20 mL of *tert*-butyl methyl ether. The organic phases were combined and dried over sodium sulfate, and *tert*-butyl methyl ether was removed under reduced pressure. A total of 3 mg (5.2 μmol, 21%) of (22 → 6′) ochratoxin A-methyl-α-D-glucopyranoside ester was obtained.

*(22 → 6′) Ochratoxin A-methyl-α-D-glucopyranoside Ester.* ESI−MS negative mode (tube lens, −110 V; capillary voltage, −33 V), HRMS *m/z*: 578.1423 {calculated formula $[C_{27}H_{30}ClNO_{11}-H]^-$ (−0.025 ppm)}. MS/MS (HCD, 65%; $[M-H]^-$) *m/z* (%): 249.0196 (100), 340.0742 (44), 578.1424 (38), 211.0167 (22), 205.0300 (19), 296.0844 (18), 167.0271 (8), 487.0883 (6), 402.0746 (6). $^{1}$H NMR (400 MHz, $CD_3OD$) δ: 1.54 (3H, d, $J_{11,3}$ = 6.2 Hz, H-11), 2.90 (1H, dd, $J_{AB}$ = 17.3, $J_{3,4A}$ = 11.7 Hz, H-4A), 3.20 (1H, dd, $J_{AB}$ = 14.8, $J_{15A,14}$ = 7.2 Hz, H-15A), 3.26 (1H, dd, $J_{4',3'}$ = 9.7, $J_{4',5}$ = 9.2 Hz, H-4′), 3.28−3.32 (1H, m, H-15B), 3.33 (3H, s, C-1′-$CH_3$), 3.34−3.41 (2H, m, H-2′, H-4B), 3.60 (1H, dd, $J_{3',2'}$ = 9.2, $J_{3',4'}$ = 9.3 Hz, H-3′), 3.74 (1H, m, H-5′), 4.28 (1H, dd, $J_{AB}$ = 11.7, $J_{6'A,5'}$ = 6.6 Hz, H-6′A), 4.44 (1H, dd, $J_{AB}$ = 11.7, $J_{6'B,5'}$ = 2.1 Hz, H-6′B), 4.63 (1H, d, $J_{1',2'}$ = 3.2 Hz, H-1′), 4.84 (1H, m, H-3), 4.99 (1H, m, H-14), 7.21−7.30 (5H, m, H-17, H-18, H-19, H-20, H-21), 8.19 (1H, s, H-6), 8.68 (0.25 H, d, $J_{13,14}$ = 7.0 Hz, H-13). $^{13}$C NMR (100 MHz, $CD_3OD$) δ: 21.3 (C-11), 33.6 (C-4), 38.9 (C-15), 56.2 (C-14), 56.2 (C-1′-methyl), 66.7 (C-6′), 71.5 (C-4′), 72.5 (C-2′), 74.0 (C-3′), 75.6 (C-3), 77.9 (C-5′), 101.9 (C-1′), 112.6 (C-9), 122.0 (C-7), 124.5 (C-5), 128.8 (C-19), 130.2 (C-17, C-21), 131.1 (C-18, C-20), 138.1 (C-6), 139.1 (C-10), 169.8 (C-12), 172.0 (C-1), 173.0 (C-22).

**Reaction of Ochratoxin A with Cellulose.** A total of 4 g cellulose was suspended in 8.86 mL of a stock solution with an

ochratoxin A concentration of 226 μg/mL in acetonitrile (500 μg of ochratoxin A/g of cellulose), prepared by dissolving 45.2 mg of ochratoxin A in 200 mL of acetonitrile using a volumetric flask. After evaporation of the solvent under a stream of nitrogen at 40 °C, the mixture was heated at 240 °C for 9 min. The reaction product was suspended in 380 mL of sodium acetate buffer (50 mM, pH 4.8) containing 214 mg of cellulase from *T. reesei* and incubated at 40 °C for 48 h while stirring at 200 rpm. Non-hydrolyzed insoluble cellulose was removed by filtration using a Buchner funnel prepared with a filter paper (type 589[3] blue ribbon ashless; diameter, 90 mm) (Schleicher and Schuell, Dassel, Germany) and washed with water and methanol/3% sodium bicarbonate (50:50, v/v). The enzymatic degradation rate was determined gravimetrically by comparing the non-hydrolyzable residue with the starting material after drying the samples at 105 °C for 12 h. The obtained filtrate was acidified with formic acid to a pH of <3 and purified by solid-phase extraction (SPE) using a 2 g Strata C-18E, 55 μm, 70A Giga Tubes SPE column (Phenomenex, Aschaffenburg, Germany) preconditioned with 20 mL of methanol and 20 mL of water. The column was washed with 20 mL of water, and the ochratoxin A saccharide esters were eluted in two fractions. Using 20 mL of acetonitrile/0.1% formic acid (30:70, v/v), mostly ochratoxin A diglucose ester eluted from the column. In a second fraction of 20 mL of acetonitrile/0.1% formic acid (40:60, v/v), primarily ochratoxin A monoglucose ester could be isolated.

**Preparation of the Ochratoxin A Saccharide Ester Reference Solution.** From both fractions, the solvent was removed by a rotary evaporator and the residues were dissolved in 500 μL of acetonitrile. For the HRMS measurements, 50 μL of each reference material solution was diluted by adding 950 μL of acetonitrile/water (50:50, v/v).

**Exact Mass Measurements and HCD Spectra of Ochratoxin A Mono- and Diglucose Esters.** The exact mass measurements and the HCD spectra of the isolated ochratoxin A monoglucose ester and ochratoxin A diglucose ester were obtained on the HRMS system, as described above.

*Ochratoxin A Monoglucose Ester.* ESI−MS negative mode (tube lens, −140 V; capillary voltage, −47 V), HRMS *m/z*: 564.1276 {calculated formula $[C_{26}H_{28}ClNO_{11}-H]^-$ (0.865 ppm)}. MS/MS (HCD, 50%; $[M - H]^-$) *m/z* (%): 402.0748 (100), 444.0854 (71), 340.0744 (69), 249.0198 (63), 358.0849 (57), 504.1066 (25), 384.0642 (22), 564.1276 (14), 211.0169 (13).

*Ochratoxin A Diglucose Ester.* ESI−MS negative mode (tube lens, −140 V; capillary voltage, −47 V), HRMS *m/z*: 726.1790 {calculated formula $[C_{32}H_{38}ClNO_{16}-H]^-$ (0.062 ppm)}. MS/MS (HCD, 50%; $[M - H]^-$) *m/z* (%): 726.1790 (100), 340.0740 (79), 358.0846 (70), 296.0843 (61), 205.0299 (40), 666.1585 (34), 606.1374 (16), 249.0197 (11), 402.0748 (11), 564.1266 (11).

**Development of a Sample Preparation for Analysis of Ochratoxin A Saccharide Esters.** For the development of a sample preparation method, the cross-reactivity of the ochratoxin-A-specific antibodies of immunoaffinity columns from different suppliers against ochratoxin A mono- and diglucose esters was tested. Therefore, the immunoaffinity columns OchraTest (VICAM, Watertown, MA) and OtaCLEAN (LC-Tech GmbH, Dorfen, Germany) were compared. The testing solution was prepared by blending 2 μL of the ochratoxin A saccharide ester reference solution with 2 mL of acetonitrile/water/formic acid (95:5:0.1, v/v/v). A total of 200 μL of the mixture was diluted with 10 mL of phosphate-buffered saline (0.01 M phosphate buffer, 2.7 mM potassium chloride, and 0.137 M sodium chloride at pH 7.4 and 25 °C) and applied to the immunoaffinity columns, at a flow rate of 2 mL/min. After the columns were washed with 10 mL of distilled water, the bound compounds were eluted with 3 mL of methanol. The eluates were evaporated under a nitrogen stream at 40 °C, and the residue was dissolved in 200 μL of acetonitrile/water/formic acid (95:5:0.1, v/v/v). Afterward, the samples were analyzed by HPLC−MS/MS and compared to the testing solution before the immunoaffinity cleanup. The described preparation was carried out in duplicate with columns from each supplier [recovery rates were estimated on the basis of the peak areas of the testing solution for ochratoxin A glucose ester, 96% (VICAM) and 54% (LC-Tech GmbH), and ochratoxin A cellobiose ester, 74% (VICAM) and 31% (LC-Tech GmbH)].

**HPLC−MS/MS.** A QTRAP 5500 MS system (AB SCIEX, Darmstadt, Germany) coupled to a LaChrom Ultra HPLC system (VWR-Hitachi, Darmstadt, Germany) was used for the detection of the ochratoxin A saccharide esters in multiple reaction monitoring mode (MRM). Data acquisition was performed with Analyst 1.5.2 software. The chromatographic separation was carried out on a 150 × 2.1 mm inner diameter, 5 μm, Syncronis hydrophilic interaction liquid chromatography (HILIC) column (Thermo Scientific, Bremen, Germany) using a linear binary gradient at a column temperature of 40 °C. The injection volume was 20 μL, and the flow rate was set to 300 μL/min. Solvent A was acetonitrile, and solvent B was water, both containing 0.1% formic acid. The linear gradient was used as follows: 0 min, 95% A; 1.5 min, 95% A; 9.5 min, 20% A; and 13.5 min, 20% A. Afterward, the column was equilibrated at starting conditions for 4 min. The mass spectrometer was operated in the positive mode. For the electrospray ionization, the voltage was set to +5500 V. For fragmentation, the potentials were set as follows: declustering potential, 90 V; entrance potential, 10 V; and collision cell exit potential, 11 V. The choice of fragmentation of the molecular ions $[M + H]^+$ or $[M + Na]^+$ was compound-dependent. The MRM transitions and collision energies (CEs) were selected as follows: for ochratoxin A monosaccharide ester, $[M + H]^+$ 566.0 → 239.0 (CE of 51 V) and 566.0 → 358.0 (CE of 30 V), and for ochratoxin A disaccharide ester, $[M + Na]^+$ 750.0 → 260.9 (CE of 57 V) and 750.0 → 512.0 (CE of 53 V).

**Reaction of Ochratoxin A with Coffee Matrix.** A total of 6 g of whole green coffee beans (*Coffea arabica* from Guatemala) was weighed in an aluminum bowl and spiked with 665 μL of a standard solution with an ochratoxin A concentration of 45.2 μg/mL in acetonitrile (5 μg of ochratoxin A/g of coffee sample), prepared by the dilution of 2 mL of a stock solution containing 226 μg of ochratoxin A/mL with acetonitrile in a 10 mL volumetric flask. For spiking, small droplets of the spiking solution were applied on the individual coffee beans and the beans were allowed to dry at room temperature over a period of 2 h. Afterward, the samples were roasted at 240 °C for 9 min in a circulating air drying oven and ground to a particle size of 0.5 mm with a mill type A10 (Janke and Kunkel IKA-Labortechnik, Staufen, Germany). A total of 1 g of the ground roasted coffee was incubated with 80 mg of Driselase (Sigma-Aldrich GmbH, Seelze, Germany) in 40 mL of sodium acetate buffer (50 mM, pH 5.0) at 37 °C under constant stirring (200 rpm). After 24 h, 20 mg of Driselase was added and further incubated to achieve a whole incubation time of 48 h. The undigested material was precipitated by centrifugation at 440*g* for 10 min at room temperature and washed 2 times with 10 mL of distilled water. To improve polysaccharide saccharification, the residue of each coffee sample was additionally incubated with 80 mg of hemicellulase in 40 mL of sodium acetate buffer (50 mM, pH 5.0) at 40 °C for 48 h. During the incubation, 20 mg of hemicellulase was added to each sample to increase the digestion. After incubation, the separation and the washing step of the residue was performed, as described above. The obtained supernatants were combined.

**Isolation of Ochratoxin A Saccharide Esters from Roasted Coffee.** The combined supernatants were diluted to an acetonitrile content of 10% with water and acidified to pH of <3 with formic acid. To remove the insoluble compounds, the suspension was filtered through a Buchner funnel using a filter paper (type 602 H; diameter, 70 mm) (Schleicher and Schuell, Dassel, Germany). The filtrates were applied to a 2 g Strata C-18E, 55 μm, 70A Giga Tubes SPE column (Phenomenex, Aschaffenburg, Germany) preconditioned with 20 mL of methanol followed by 20 mL of water. When the column was washed with 20 mL of water, salts and polar coffee compounds, such as carbohydrates and amino acids, were removed. The fraction containing ochratoxin A disaccharide esters was eluted with 12 mL of acetonitrile/0.1% formic acid (30:70, v/v). Ochratoxin A monosaccharide esters were eluted with 12 mL of acetonitrile/0.1% formic acid (40:60, v/v). The achieved fractions were diluted with 150 mL of phosphate-buffered saline (PBS) at pH 7 and added to the OchraTest immunoaffinity column (VICAM, Watertown, MA) at a flow rate of

2 mL/min. After application, the column was washed with 10 mL of distilled water and the ochratoxin A saccharide esters were eluted with 3 mL of methanol. The solvent was evaporated under a nitrogen stream at 40 °C, and the residue was dissolved in 200 μL of acetonitrile/water/formic acid (95:5:0.1, v/v/v). The analysis was performed by HPLC−MS/MS, as described above. The injection volume was set to 50 μL.

## RESULTS AND DISCUSSION

**Model Experiments with Ochratoxin A and Methyl α-D-Glucopyranoside.** To investigate a possible binding of ochratoxin A to the coffee matrix and especially to polysaccharides during roasting, we performed different model experiments.

In a first experiment, the general ability of ochratoxin A to form ester linkages with carbohydrates was studied using methyl α-D-glucopyranoside, which is characterized through the methoxylated anomeric carbon atom. Thus, this compound provides a single subunit of polysaccharides and is therefore suitable for studies on the formation of carbohydrate esters with non-reducing sugars because it has already been used by Seefelder et al.[16] to prove the binding of fumonisins to polysaccharides. The model compound was heated in a mixture with ochratoxin A at 225 °C for 5 min, and the reaction residue was subsequently analyzed by HPLC with fluorescence detection. As seen in the chromatogram in Figure 2, besides

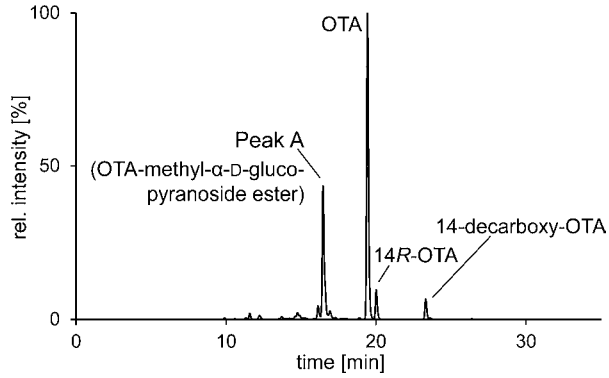


**Figure 2.** HPLC−FLD chromatogram of the reaction mixture of ochratoxin A and methyl α-D-glucopyranoside after heating at 225 °C for 5 min (see Figure 1 for chemical structures).

ochratoxin A and the two expected degradation products 14*R*-ochratoxin A and 14-decarboxy-ochratoxin A, a larger peak (peak A) with two smaller side peaks with a retention time of 16.5 min was detectable. When the experiment was repeated on a larger scale and a semi-preparative HPLC-UV system was used, this unknown peak A was isolated and the structure was elucidated using NMR and MS experiments.

The obtained NMR data of the unknown compound show the characteristic NMR signals of all ochratoxin A protons and carbon atoms. Furthermore, the signals corresponding to a methyl-glucose substructure could be detected. However, in comparison to methyl α-D-glucopyranoside, the protons at position 6′ of the isolated product (**2**) (Figure 1) showed a low field shift by 0.63 and 0.59 ppm, respectively. The resulting signals at 4.28 and 4.45 ppm correspond to typical protons in the α position to esters. Also, the signal for the proton H′-5 was shifted to a low field because of the ester in the β position. All other $^{1}$H signals for methyl α-D-glucopyranoside changed only marginally. These proton shifts clearly show an esterification between the carboxylic function of ochratoxin A and C-6 of methyl α-D-glucopyranoside and prove the structure of the unknown peak A as (22 → 6′) ochratoxin A-methyl-α-D-glucopyranoside ester (**2**) (Figure 1).

To confirm the NMR experiments and allow for a mass spectrometric identification of further ochratoxin A carbohydrate ester, MS experiments were performed. For the isolated substance, an accurate mass of 578.1423, corresponding to a sum formula of $[C_{27}H_{30}ClNO_{11}-H]^-$ could be determined. Furthermore, the characteristic isotopic pattern of a chlorine-containing compound with a relative signal intensity of 29% for the $^{37}$Cl-isotopologue at *m/z* 580.1399 was observed. The fragmentation pattern of the product ion spectrum of the $[M-H]^-$ ion *m/z* 578.1424 represented in Figure 3 shows the

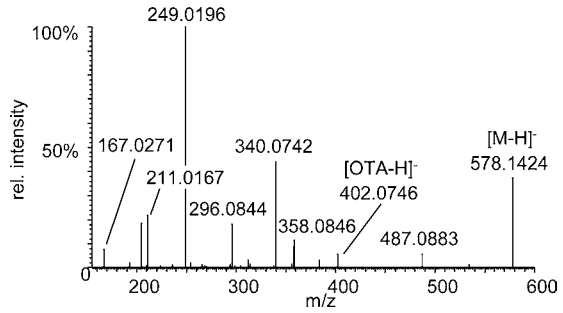


**Figure 3.** HCD spectrum of (22 → 6′) ochratoxin A-methyl-α-D-glucopyranoside ester (*m/z* 578.1424) in the negative mode.

deprotonated ion of ochratoxin A *m/z* 402.0746 as the fragment generated by the loss of the sugar side chain. In addition to that, the $[M-H]^-$ ion *m/z* 578.1424 revealed the typical product ions of ochratoxin A *m/z* 358.0846, 211.0167, and 167.0271. The fragment *m/z* 358.0846 indicates the decarboxylation of ochratoxin A. The fragment *m/z* 211.0167, which results from the cleavage between C-8 and C-12 and the product ion *m/z* 167.0271 are characteristic for the dihydroisocoumarin moiety. This fragmentation pattern agrees with the described fragmentation pathway of ochratoxin A.[18] Furthermore, fragments at *m/z* 340.0742 and 249.0196 were observed with a high intensity, which were generated in a second fragmentation pathway of the precursor ion. In detail, the fragment *m/z* 340.0742 results from the loss of the bound methyl α-D-glucoside molecule and an additional loss of the $CO_2$ group of the dihydroisocoumarin component. The observed fragment at *m/z* 249.0196 was generated by a further loss of the benzyl group, resulting from the cleavage between C-14 and C-15 of the ochratoxin A backbone. These fragments are characteristic for ochratoxin A carbohydrate ester.

With this model experiment, we could demonstrate that ochratoxin A is generally able to form an ester linkage to carbohydrates during heating. On the basis of the data obtained from the reaction of ochratoxin A with methyl α-D-glucopyranoside, the primary hydroxyl group at the C-6′ atom could be identified as the preferred binding position.

**Model Experiments with Ochratoxin A and Polysaccharides.** On the basis of the observations described above, we investigated the reactivity of ochratoxin A with polysaccharides in coffee beans during roasting. Therefore, we first performed a simplified model experiment with cellulose, which is one of the predominant polysaccharides found in coffee beans.[19] Cellulose was spiked with ochratoxin A and heated under typical roasting conditions at 240 °C for 9 min.[21]

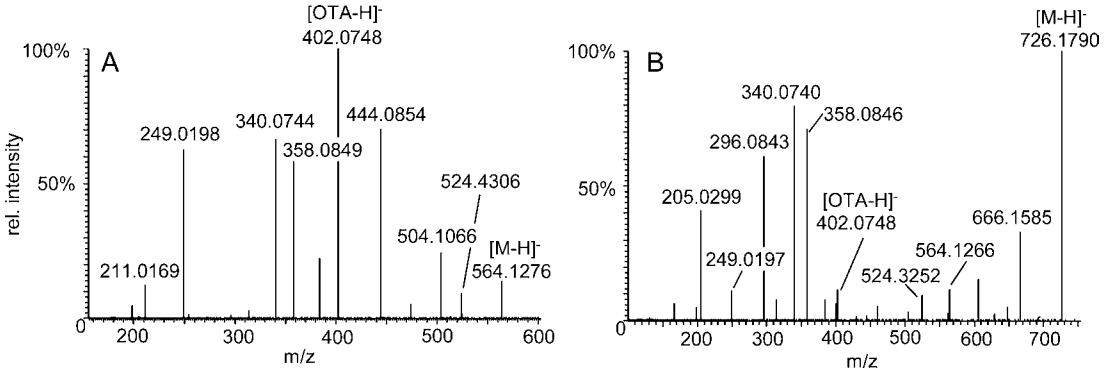


**Figure 4.** HCD spectra of (A) ochratoxin A glucose ester ($m/z$ 564.1276) and (B) ochratoxin A cellobiose ester ($m/z$ 726.1790) in the negative mode.

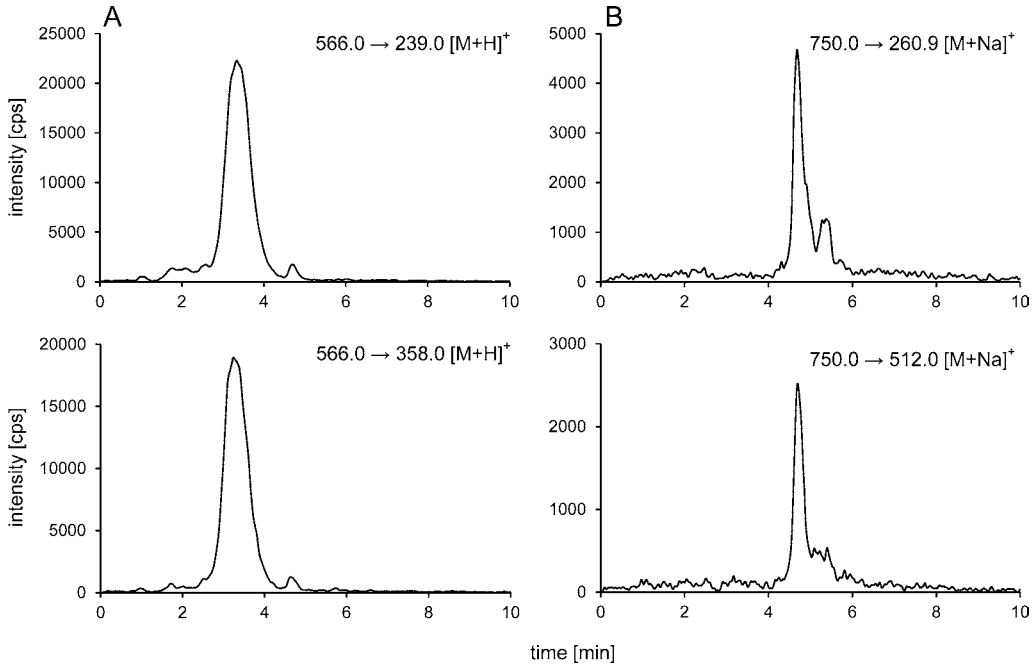


**Figure 5.** HPLC–MS/MS chromatogram of roasted coffee spiked with 5 $\mu$g/g of ochratoxin A after enzymatic hydrolysis: (A) MRM transitions of protonated ochratoxin A monosaccharide ester and (B) MRM transitions of the sodium adduct of ochratoxin A disaccharide ester in the positive mode.

Afterward, the cellulose backbone was cleaved enzymatically to obtain low molecular saccharides bound to ochratoxin A. For the cellulose cleavage, we performed an enzymatic hydrolysis with cellulase from *T. reesei*, as described by Medve et al.,[21] to keep the ester linkage between the saccharides and ochratoxin A intact. The achieved degradation rate of the thermal-treated cellulose was 26.1%. In the hydrolyzed sample, we detected signals for the deprotonated molecules $[M - H]^-$, at $m/z$ 564.1276 and 726.1790, which indicate a covalent binding of ochratoxin A with one (**3**) or two (**4**) glucose molecules, respectively (Figure 1). These two compounds were isolated in small quantities as reference material for further studies. The exact mass measurements of these references confirmed the proposed structures **3** and **4** shown in Figure 1. In detail, for reference A, the accurate mass of the deprotonated molecular ion $[M - H]^-$ at $m/z$ 564.1276 provided the molecular formula of $C_{26}H_{27}ClNO_{11}$, and from the accurate mass $m/z$ 726.1790 of reference B, a molecular formula of $C_{32}H_{37}ClNO_{16}$ was calculated. These sum formula are consistent with the calculated formula of the deprotonated molecular ion $[M - H]^-$ of ochratoxin A linked to glucose for reference A and ochratoxin A bound to cellobiose for reference B. For both compounds, a HCD spectrum (Figure 4) was acquired by HRMS in the negative mode and compared to the product ion spectrum of the synthesized (22 → 6′) ochratoxin A-methyl-$\alpha$-D-glucopyranoside ester (Figure 3). Each product ion spectra of the deprotonated molecular $[M - H]^-$ ions showed the molecular $[M - H]^-$ ion of ochratoxin A at $m/z$ 402.0748 as a fragment, generated by the loss of the sugar side chain. In accordance with the product ion spectrum of the synthesized (22 → 6′) ochratoxin A-methyl-$\alpha$-D-glucopyranoside ester (Figure 3), both references revealed, besides the typical fragment ions of ochratoxin A at $m/z$ 358.0849 and 211.0169, the fragment ions $m/z$ 249.1098 and 340.0744.[18] The intensities of these fragments differ between the product ion spectra. Resulting from the same fragmentation pattern, the structural similarity of these compounds could be confirmed. Furthermore, for both references, the characteristic isotopic pattern of one chlorine atom could be observed. Thus, we identified the isolated reference A as ochratoxin A glucose ester

(3) and reference B as ochratoxin A cellobiose ester (4), shown in Figure 1. In conclusion, the model experiment with cellulose showed that a binding of ochratoxin A to larger carbohydrates can occur and that a release of glucose and cellobiose molecules bound to ochratoxin A is possible after the enzymatic cleavage with cellulase from *T. reesei*.

**Model Experiments with Coffee Beans Spiked with Ochratoxin A.** After we proved the binding of ochratoxin A to cellulose, we applied this analytical approach to whole coffee beans spiked with ochratoxin A to a level of 5 μg/g and roasted at 240 °C for 9 min[20] to investigate if bound ochratoxin A is also formed under typical coffee roasting conditions. Because of the high complexity of the coffee matrix containing mannan, arabinogalactan, cellulose, and xylan, a different protocol for the enzymatic cleavage was applied.[19,22] Thus, the samples were incubated with Driselase, an enzyme mixture of several carbohydrolases, such as cellulase, xylanase, galactanase, arabinanase, and polygalacturonase, to achieve the cleavage of the numerous glycosidic linkages in the different polysaccharides. In previous studies, the treatment of plant material with Driselase to investigate ester linkages of compounds to polysaccharides has been shown to be a helpful experimental technique.[23−25] In a second step, hemicellulase containing among others xylanase and mannase activities was applied to increase the degradation rate. With this enzyme cocktail, we obtained the best results for the enzymatic hydrolysis of the ground roasted coffee. Nevertheless, only a degradation rate of about 2% of the coffee matrix was achieved. This low hydrolysis rate results, on the one hand, from the presence of lignin, which interferes with the interaction of the enzymes with the substrate, and on the other hand, from crystallinity of the polysaccharides that hinders the binding of the enzymes on the substrate.[26,27]

To enrich the amount of the ochratoxin A saccharide esters, the coffee hydrolyzate was concentrated by a SPE with C18 material. Afterward, a selective purification of the ochratoxin A saccharide esters was necessary to enable the detection of these compounds in the complex matrix. Therefore, immunoaffinity columns were tested for the cross-reactivity of their ochratoxin A antibodies with ochratoxin A saccharide esters. The immunoaffinity column provided from the manufacturer VICAM was able to bind the ochratoxin A glucose ester and ochratoxin A cellobiose ester in nearly quantitative yields with a recovery rate of 96% for ochratoxin A glucose ester and 74% for ochratoxin A cellobiose ester. Thus, the fractions achieved from the SPE, which may contain the ochratoxin A saccharide esters, could be further purified by the immunoaffinity column clean up and were then analyzed by HPLC−MS/MS in the positive mode. Figure 5 shows the chromatograms of the roasted coffee sample spiked with 5 μg/g of ochratoxin A after the enzymatic hydrolysis with the measured MRM transitions for ochratoxin A mono- and disaccharide esters. As seen, we obtained signals for ochratoxin A monosaccharide esters and ochratoxin A disaccharide esters in the enzymatically hydrolyzed coffee sample. The broad signals result from the mixture of different hexose stereoisomers, which were derived from the various polysaccharides in the coffee matrix, e.g., galactomannan, cellulose, or mannan.[19,22] With this experiment, we could show that ochratoxin A is able to bind to the coffee matrix during roasting.

In summary, we demonstrated that ochratoxin A is able to bind to carbohydrates under thermal processes. The form of this binding could be elucidated as an ester linkage between the primary hydroxyl group of the carbohydrates and the carboxyl group of ochratoxin A. These ochratoxin A saccharide esters could also be detected in artificially contaminated coffee beans after roasting. Thus, we proved the formation of ochratoxin A esters with the coffee polysaccharides as a further thermal reaction, which takes place during roasting of coffee. Consequently, further studies using naturally contaminated coffee beans are necessary in the future to determine the amount of bound ochratoxin A in roasted coffee and the influence of different conditions, such as roasting time and temperature, on the formation of these ochratoxin A saccharide esters. In addition, there is still a lack of knowledge concerning the stability of these ester linkages. Resulting from the findings of Studer-Rohr et al.,[7] who observed lower amounts of ochratoxin A in roasted coffee beans compared to the coffee brew, a cleavage of the ester linkage between ochratoxin A and the polysaccharides during the coffee preparation might be possible and can release ochratoxin A into the coffee brew. Because of the fact that roasting of coffee beans results in an increased solubility of the polysaccharide content caused by the hydrolysis of the polysaccharide backbone and the removal of side chain sugars, the ochratoxin A saccharide esters can also be transferred into the coffee brew.[20] For this reason, the toxicity of these bound forms of ochratoxin A has to be studied in the future.

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### Funding

The authors thank the Deutsche Forschungsgemeinschaft (HU 730/10-1) for financial support.

### Notes

The authors declare no competing financial interest.

## ACKNOWLEDGMENTS

The authors thank AB Sciex for supplying us with a QTRAP 5500 mass spectrometer, Merck VWR for supplying us with a VWR Hitachi LaChrom Ultra HPLC system, and Rolf Geisen for providing *P. nordicum* BFE487.

## REFERENCES

(1) van der Merwe, K. J.; Steyn, P. S.; Fourie, L.; Scott, D. B.; Theron, J. J. Ochratoxin A, a toxic metabolite produced by *Aspergillus ochraceus* Wilh. *Nature* **1965**, *205* (976), 1112−1113.

(2) International Agency for Research on Cancer (IARC).. Some naturally occurring substance: Some food items and constituents, heterocyclic aromatic amines and mycotoxins. *IARC Monogr. Eval. Carcinog. Risks Hum.* **1993**, *56*, 489.

(3) Krogh, P. Role of ochratoxin in disease causation. *Food Chem. Toxicol.* **1992**, *30* (3), 213−224.

(4) Brown, M. H.; Szczech, G. M.; Purmalis, B. P. Teratogenic and toxic effects of ochratoxin A in rats. *Toxicol. Appl. Pharmacol.* **1976**, *37* (2), 331−338.

(5) Petzinger, E.; Weidenbach, A. Mycotoxins in the food chain: The role of ochratoxins. *Livestock Prod. Sci.* **2002**, *76* (3), 245−250.

(6) European Food Safety Authority (EFSA).. Opinion of the scientific panel on contaminants in the food chain on a request from the commission related to ochratoxin A in food. *EFSA J.* **2006**, *365*, 1−56.

(7) Studer-Rohr, I.; Dietrich, D. R.; Schlatter, J.; Schlatter, C. The occurrence of ochratoxin A in coffee. *Food Chem. Toxicol.* **1995**, *33* (5), 341−355.

(8) Valle-Algarra, F. M.; Mateo, E. M.; Medina, A.; Mateo, F.; Gimeno-Adelantado, J. V.; Jimenez, M. Changes in ochratoxin A and type B trichothecenes contained in wheat flour during dough fermentation and bread-baking. *Food Addit. Contam., Part A* **2009**, *26* (6), 896−906.
(9) Scudamore, K. A.; Banks, J. N.; Guy, R. C. Fate of ochratoxin A in the processing of whole wheat grain during extrusion. *Food Addit. Contam.* **2004**, *21* (5), 488−497.
(10) Castells, M.; Pardo, E.; Ramos, A. J.; Sanchis, V.; Marin, S. Reduction of ochratoxin A in extruded barley meal. *J. Food Prot.* **2006**, *69* (5), 1139−1143.
(11) Tsubouchi, H.; Yamamoto, K.; Hisada, K.; Sakabe, Y.; Udagawa, S. Effect of roasting on ochratoxin A level in green coffee beans inoculated with *Aspergillus ochraceus*. *Mycopathologia* **1987**, *97* (2), 111−115.
(12) Viani, R. Fate of ochratoxin A during processing of coffee. Cooperative work by PEC (Physiological Effects of Coffee committee). *Food Addit. Contam.* **1996**, *13* (Supplement), 29−33.
(13) Blanc, M.; Pittet, A.; Munoz-Box, R.; Viani, R. Behavior of ochratoxin A during green coffee roasting and soluble coffee manufacture. *J. Agric. Food Chem.* **1998**, *46* (2), 673−675.
(14) Romani, S.; Pinnavaia, G. G.; Dalla Rosa, M. Influence of roasting levels on ochratoxin A content in coffee. *J. Agric. Food Chem.* **2003**, *51* (17), 5168−5171.
(15) Cramer, B.; Königs, M.; Humpf, H. U. Identification and in vitro cytotoxicity of ochratoxin A degradation products formed during coffee roasting. *J. Agric. Food Chem.* **2008**, *56* (14), 5673−5681.
(16) Seefelder, W.; Knecht, A.; Humpf, H. U. Bound fumonisin B1: Analysis of fumonisin-B1 glyco and amino acid conjugates by liquid chromatography−electrospray ionization−tandem mass spectrometry. *J. Agric. Food Chem.* **2003**, *51* (18), 5567−5573.
(17) Schmidt-Heydt, M.; Graf, E.; Stoll, D.; Geisen, R. The biosynthesis of ochratoxin A by *Penicillium* as one mechanism for adaptation to NaCl rich foods. *Food Microbiol.* **2012**, *29* (2), 233−241.
(18) Schmidt-Heydt, M.; Cramer, B.; Graf, I.; Lerch, S.; Humpf, H. U.; Geisen, R. Wavelength-dependent degradation of ochratoxin and citrinin by light in vitro and in vivo and its implications on *Penicillium*. *Toxins* **2012**, *4* (12), 1535−1551.
(19) Bradbury, A. G. W.; Halliday, D. J. Chemical structures of green coffee bean polysaccharides. *J. Agric. Food Chem.* **1990**, *38* (2), 389−392.
(20) Redgwell, R. J.; Trovato, V.; Curti, D.; Fischer, M. Effect of roasting on degradation and structural features of polysaccharides in Arabica coffee beans. *Carbohydr. Res.* **2002**, *337* (5), 421−431.
(21) Medve, J.; Karlsson, J.; Lee, D.; Tjerneld, F. Hydrolysis of microcrystalline cellulose by cellobiohydrolase I and endoglucanase II from *Trichoderma reesei*: Adsorption, sugar production pattern, and synergism of the enzymes. *Biotechnol. Bioeng.* **1998**, *59* (5), 621−634.
(22) Jooste, T.; Garcia-Aparicio, M. P.; Brienzo, M.; van Zyl, W. H.; Gorgens, J. F. Enzymatic hydrolysis of spent coffee ground. *Appl. Biochem. Biotechnol.* **2013**, *169* (8), 2248−2262.
(23) Smith, B. G.; Harris, P. J. Ferulic acid is esterified to glucuronoarabinoxylans in pineapple cell walls. *Phytochemistry* **2001**, *56* (5), 513−519.
(24) Bunzel, M.; Allerdings, E.; Sinwell, V.; Ralph, J.; Steinhart, H. Cell wall hydroxycinnamates in wild rice (*Zizania aquatica* L.) insoluble dietary fibre. *Eur. Food Res. Technol.* **2002**, *214* (6), 482−488.
(25) Ralet, M. C.; Thibault, J. F.; Faulds, C. B.; Williamson, G. Isolation and purification of feruloylated oligosaccharides from cell walls of sugar-beet pulp. *Carbohydr. Res.* **1994**, *263* (2), 227−241.
(26) Mansfield, S. D.; Mooney, C.; Saddler, J. N. Substrate and enzyme characteristics that limit cellulose hydrolysis. *Biotechnol. Prog.* **1999**, *15* (5), 804−816.
(27) Hall, M.; Bansal, P.; Lee, J. H.; Realff, M. J.; Bommarius, A. S. Cellulose crystallinity—A key predictor of the enzymatic hydrolysis rate. *FEBS J.* **2010**, *277* (6), 1571−1582.