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Stable Isotope Dilution Assays of Alternariol and Alternariol Monomethyl Ether in Beverages

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Stable isotope dilution assays (SIDAs) for the determination of the most important mycotoxins of the black mold *Alternaria*, namely, alternariol and alternariol monomethyl ether, have been developed. For this purpose, deuterated alternariol and alternariol methyl ether were synthesized by palladium catalyzed protium−deuterium exchange from the unlabeled toxins. Reaction conditions were chosen in such a manner that the formation of the [$^2H_4$]-isotopologues was favored. The synthesized products were characterized by LC-MS, NMR, and UV-spectroscopy. On the basis of the use of [$^2H_4$]-alternariol and [$^2H_4$]-alternariol methyl ether as internal standards, SIDAs were developed and applied to the determination of alternariol and alternariol methyl ether in beverages using LC-MS/MS. Method validation revealed a high sensitivity, i.e., low limits of detection (alternariol, 0.03 μg/kg; alternariol methyl ether, 0.01 μg/kg) and limits of quantitation (alternariol, 0.09 μg/kg; alternariol methyl ether, 0.03 μg/kg), respectively. Recovery from spiked apple juice was 100.5 ± 3.4% for alternariol (range 0.1−1 μg/kg) and 107.3 ± 1.6% for alternariol methyl ether (range 0.05−0.5 μg/kg). Interassay precision (expressed as coefficient of variation, CEV) for alternariol was 4.0% (7.82 ± 0.31 μg/kg; vegetable juice, naturally contaminated) and 4.6% (1.04 ± 0.05 μg/kg; grape juice, naturally contaminated). For alternariol methyl ether, a CEV of 2.3% (0.79 ± 0.02 μg/kg; vegetable juice, naturally contaminated) was obtained. Analysis of fruit juices showed low contamination with alternariol and alternariol methyl ether in general, but higher values of both toxins were found in wine and vegetable juices. The values for alternariol were higher than those for alternariol methyl ether in nearly any case. However, the developed SIDA has proven to be optimally suited for further studies on alternariol and alternariol methyl ether content in food samples to obtain further insight into possible health hazards for the consumer.

KEYWORDS: *Alternaria*; mycotoxins; alternariol; alternariol monomethyl ether; stable isotope dilution assay; LC-MS/MS

INTRODUCTION

Food spoilage by micro organisms is a severe problem in food hygiene and food safety. In many cases, molds are responsible for large-scale infestations of both raw materials and processed foods and, therefore, render them unsuitable for human or animal consumption. Besides economical losses, mold growth on food is sometimes accompanied with the production of toxic substances, the so-called mycotoxins. The most important pathogenic molds are species from the genera *Fusarium*, *Penicillium*, *Aspergillus*, and *Alternaria*. While *Fusarium* can be clearly characterized as a field fungus and *Penicillium* as a storage fungus, the situation is much less simple for the genus *Alternaria*. Most species of *Alternaria* are plant pathogens responsible for a number of diseases of economic plants such as carrots (*1*) or citrus fruits (*2*). However, *Alternaria* is also involved in the postharvest decay of fruits, grains, and vegetables. As *Alternaria* tolerates relatively low temperatures, spoilage can also occur during refrigerated transport and storage (*3*). All pathogenic species of *Alternaria* produce a series of mycotoxins including alternariol, alternariol monomethyl ether, altenuene, altertoxin I and II, and L-tenuazonic acid. The two first mentioned metabolites have already been isolated in 1953 (*4*) and are phenolic compounds with a coumarin-like structure (**Figure 1**).

Alternaria mycotoxins were detected in wheat (*5*), apple juice (*6*), and other fruit juices (*7*), citrus fruits (*8*), carrots (*9*), tomato products (*10*), and sunflower grains (*11*). Although tomatoes in particular were affected, grains such as wheat, barley, oats, and sorghum were contaminated to the same degree (*12*).

From a toxicological point of view, there is strong evidence that alternariol and alternariol methyl ether are mutagenic (*13*−*16*). Although the acute toxicity of alternariol and alternariol methyl ether in mice was low ($LD_{50} \gg 400$ mg/kg bw), both compounds showed remarkable cytotoxicity in cell culture (*17*). In-vitro,

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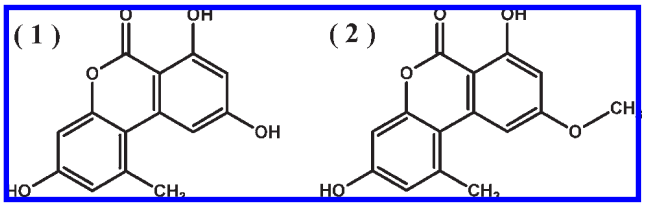

Figure 1. Structures of alternariol **1** and alternariol monomethyl ether **2**.

alternariol and alternariol methyl ether were shown to induce DNA strand break in cell line systems (*18*). Furthermore, it has been suggested that alternariol and alternariol methyl ether produced by *Alternaria alternata* on grain might be responsible for the increased occurrence of human esophageal cancer in the county of Linxian in China (*14*).

Analytical methods for the determination of *Alternaria* toxins, which have been reviewed in ref (*12*), are based on thin-layer chromatography, gas chromatography after derivatization, and liquid chromatography (LC) with UV, fluorescence, or electrochemical detection. Although until now only few methods have been published that analyze *Alternaria* toxins with LC-MS (*7*, *8*, *19*, *20*), it can be expected that there will be a great increase in the following years. LC-MS generally suffers from ion-suppression, and thus, quantitative determinations have to be corrected by the use of suitable internal standards that compensate for this effect (*21*). Additionally, *Alternaria* infestation of a broad variety of possible substrates expands the range of analytical samples that require different sample preparation and separate method validation. To avoid these analytical challenges, the use of stable isotope labeled internal standards is a useful tool that counterbalances both analyte losses during sample preparation and ion-suppression in the ESI interface of the LC-MS instrument.

Different approaches toward the total synthesis of alternariol and alternariol methyl ether have been published (*22*−*24*); however, none of them was promising enough in terms of yield and expenditure of time to be used for the synthesis of labeled isotopologues. Thus, efforts were undertaken to obtain labeled alternariol and alternariol methyl ether via a semisynthetic route using H/D exchange. On the basis of the access to these alternariol and alternariol methyl ether isotopologues, the goal of this study was to develop stable isotope dilution assays (SIDAs) for these mycotoxins in beverages.

MATERIAL AND METHODS

Chemicals and Reagents. Alternariol and alternariol monomethyl ether were purchased from Sigma-Aldrich (Steinheim, Germany). Deuterium oxide, 99 atom % D and 1,4-dioxane-D_8, 99 atom % D as well as acetonitrile Chromasolv, 99.9%, 1,4-dioxane, anhydrous, 99.8%, ethanol, gradient grade, 99.8%, and hexane Chromasolv, 97% were also from Sigma-Aldrich (Steinheim, Germany). Palladium, 5% Pd on barium sulfate and methanol Chromasolv, ≥99.9% were available from Fluka (Buchs, Switzerland). Water for HPLC was purified by a Milli-Q-system (Millipore GmbH, Schwalbach, Germany).

Synthesis of Deuterated Alternariol (D-Alternariol) and Deuterated Alternariol Methyl Ether (D-Alternariol Methyl Ether). Commercial alternariol or alternariol methyl ether (5 mg) was dissolved in the original flask using 2.5 mL of dioxane yielding a concentration of approximately 2 mg/mL and stored in a refrigerator at −20 °C until further use. For synthesis, 0.5 mL of these solutions (equivalent to 1 mg; 3.9 mmol alternariol or 3.8 mmol alternariol methyl ether) was transferred into a culture tube with screw cap and PTFE sealing (10 cm, ϕ 1.8 cm), and the solvent was removed under reduced pressure. Subsequently, 1.5 mL of D_8-dioxane, 1.5 mL of deuterium oxide, and the catalyst (250 mg Pd on barium sulfate) were added. The tube was inserted into a tailor-made metal block (aluminum, height 7 cm, ϕ 13 cm with a central bore, ϕ 2 cm, 5 cm deep) and placed on a magnetic heating stirrer. The temperature was controlled by means of an electronic thermostat with the sensor placed into a second bore (ϕ 0.2 cm, 5 cm deep) of the metal block. The reaction was carried out with stirring at 160 °C for several days. From time to time, the tube was removed from the heating block and cooled, and aliquots of the reaction mixture were analyzed using LC-MS. In order to terminate the reaction, the tube was cooled, the reaction mixture decanted into two 2 mL polypropylene micro test tubes (Eppendorf AG, Hamburg, Germany), and the catalyst removed by centrifugation (10 min, 13200 rpm, 25 °C). The supernatants were concentrated to dryness under reduced pressure and the residues dissolved in acetonitrile and water (30:70; v/v).

Preparative HPLC-UV. HPLC-UV was performed on a HPLC-system (BIO-TEK Instruments, Eching, Germany) equipped with a two pump (type 552) gradient mixer (M 800), a Rheodyne injector (7725i), and an UV-detector (type 535). A 250 × 4.6 mm, i.d., 5 μm, Hyperclone RP column (Phenomenex, Aschaffenburg, Germany) was used as the stationary phase, and variable mixtures of acetonitrile (solvent B) and water (solvent A) served as the mobile phase. Two different linear gradient programs at a flow of 1 mL/min were used for the preparative isolation of D-alternariol and D-alternariol methyl ether from the solution obtained above. For the chromatography of D-alternariol, the initial content of B was 30%, which was held for 3 min, until the content of B was raised within 2 min to 100% 5 min. One hundred percent B was held for 3 min and then braught back to 30% within 4 min. For the separation of D-alternariol methyl ether, the gradient started with 50% B. After 3 min, the content of B was brought to 100% within 6 min and held for 2 min. The content of B returned to 50% within 1 min, and both gradients were followed by an equilibration phase of 8 min to restore initial conditions. The eluting peaks were collected and the solvents removed in vacuo. The yield was determined spectrophotometrically as described below and was 73% for D-alternariol and 80% for D-alternariol methyl ether.

NMR. The synthesized products were characterized by ^{1}H NMR in D_8-dioxane at 600 MHz on a Varian NMR System 600 MHz (Varian, Darmstadt, Germany). D-alternariol, ^{1}H NMR, δ/ppm (TMS): 11.81 (s, 1H), 8.76 (s, 1H), 8.43 (s, 1H), 7.29 (s, 0.01H), 6.59 (s, 0.42H), 2.72 (s, 2.46H). D-alternariol methyl ether, ^{1}H NMR, δ/ppm (TMS): 11.81 (s, 1H), 8.24 (s, 1H), 7.25 (s, 0.02H), 7.15 (s, 0.07H), 6.55 (s, 0.1H), 6.46 (s, 0.02H), 3.82 (s, 3H), 2.69 (s, 2.36H).

LC-MS. MS-spectra of the synthesized products were obtained during LC-MS runs on LCQ Classic Mass Spectrometer (Finnigan MAT, Bremen, Germany). The LC equipment was a ThermoSeparations HPLC System with an auto sampler AS 3000 and an UV-detector UV 1000 (Finnigan MAT, Bremen, Germany). HPLC conditions were chosen analogous to those described above for HPLC-UV. The parameters of the ion source were source voltage, 5 kV; source current, 80 μA; sheath gas flow, 80 arb (arbitrary unit, instrument parameter); aux gas flow, 20 arb; capillary temperature, 200 °C; capillary voltage, 4 V. Only electrospray ionization (ESI) in the positive mode was used. MS-spectra were recorded in the full-scan mode (range m/z 100−300) as well as in the zoom-mode (defined as $m/z \pm 5$ amu) for better resolution.

LC-MS/MS. A hybrid triple quadrupole/linear ion trap mass spectrometer (API 4000 QTrap; Applied Biosystems INC, Foster City, CA, US) was used for the analysis of alternariol and alternariol methyl ether in food samples. The ion source (Turbo Ion Spray) was operated in the negative ESI mode exclusively. The source parameters were set as follows: curtain gas, 10 psi; temperature, 550 °C; spray gas (GS1), 50 psi; dry gas (GS2), 70 psi; ion spray voltage, −4500 V. For MS/MS measurements, the mass spectrometer was operated in the MRM (multiple reaction monitoring) mode. The following transitions were monitored (in parentheses, collision energy, CE; collision cell exit potential, CXP; the declustering potential, DP was −60 V for all transitions): alternariol: m/z 257 → 147 (CE −46 V, CXP −5 V) and m/z 257 → 213 (CE −36 V, CXP −15 V); D_4-alternariol: m/z 261 → 150 (CE −46 V, CXP −5 V) and m/z 261 → 217 (CE −32 V, CXP −9 V); alternariol methyl ether: m/z 271 → 256 (CE −34 V, CXP −9 V) and m/z 271 → 228 (CE −42 V, CXP −1 V); D_4-alternariol methyl ether: m/z 275 → 260 (CE −30 V, CXP −17 V) and m/z 275 → 232 (CE −44 V, CXP −11 V). Both quadrupoles were set at unit resolution.

For HPLC separation, a Shimadzu LC-20A prominence HPLC system (Shimadzu, Kyoto, Japan) was linked to the mass spectrometer. The mobile phase consisted of variable mixtures of water (solvent A) and methanol (solvent B). A 150 × 2 mm, 4 μm, Synergi Polar RP (Phenomenex, Aschaffenburg, Germany) was used as the stationary

phase. The linear binary gradient started with 40% B that was held for 2 min. Afterward, the content of B was raised to reach 100% 5 min after injection. These conditions were continued until the end of the run after 12 min. Injection volume was 10 μL, flow rate 0.2 mL/min, and equilibration time between two runs 15 min. Data acquisition was carried out using Analyst 1.4.2 software (Applied Biosystems).

UV-Spectroscopy. The UV spectrometer U-2000 (Hitachi, Maidenhead, Great Britain) was calibrated using potassium dichromate (*25*). For the determination of extinction coefficients, defined amounts of alternariol and alternariol methyl ether (1.00 mg each) were weighed and dissolved in 5 mL of acetonitrile. Dilutions of 10 μg/mL, 5 μg/mL, and 1 μg/mL were prepared in acetonitrile. The purity of the alternariol and alternariol methyl ether reference substances was checked by full scan UV−vis spectra and by comparison with literature data (*23*) as well as by HPLC-DAD and LC-MS analysis. The absorptions of the respective toxin dilutions were recorded at the absorption maximum of 256 nm. The extinction coefficients were calculated using the equation ε = (absorption × 1000)/concentration in mmol/L.

Preparation of Standard Solutions. A commercial reference substance (5 mg) was dissolved in the original flask with dioxane (2.5 mL), and the solution was transferred into a 10 mL volumetric flask. The original flask was repeatedly flushed with dioxane, which was completely transferred into the 10 mL volumetric flask. After the flask was brought up to volume, an aliquot (1 mL) was transferred into another 10 mL volumetric flask. The dioxane was removed in a stream of nitrogen, and the residue was taken up in acetonitrile. Further dilutions were prepared with acetonitrile. The respective concentrations were calculated on the basis of their UV absorption using the extinction coefficients determined. The concentrations of solutions containing labeled standards were determined identically. All solutions were stored in the dark at −20 °C.

Sample Preparation and Cleanup. Sample preparation was carried out by following a simplified procedure described elsewhere (*6*). In short, 10 g of the liquid sample was weighed into a 100 mL flask. In order to facilitate interpretation and comparison, gravimetric weighing was favored over volumetric sampling as the different beverages varied in their specific density. Wine was diluted with 4 mL of water before the addition of labeled standards, whereas alcohol-free products were spiked with 1 mL of ethanol to adjust similar conditions. Labeled standards of deuterated alternariol and deuterated alternariol methyl ether were added in amounts of 20−40 ng as 0.2 μg/mL solution and of 5−10 ng as 0.1 μg/mL solution, respectively, in acetonitrile. Samples were stirred for 30 min for equilibration. Cloudy samples were centrifuged (15 min, 4600 rpm, 25 °C) by means of a Heraeus Multifuge 3 L-R (Thermo Fisher Scientific Inc., Waltham, MA, USA), and the supernatant was used for further cleanup. For this purpose, the complete sample was passed through a 6-mL C_{18}-SPE column (1000 mg, 55 μm, 140 A, Strata C18-T, Phenomenex, Aschaffenburg, Germany), attached to a vacuum manifold and preconditioned with 4 mL of hexane, 4 mL of methanol, and 4 mL of water, at a flow rate of about 1 drop/s by gentle vacuum. The column was washed with 5 mL of water and 3 mL of acetonitrile + water (30:70; v/v) and rapidly dried by aspirating air after the last washing step. The toxins were eluted with 5 mL of acetonitrile. The solvent was removed in a stream of nitrogen and the residue taken up in 200 μL of acetonitrile and water (30:70; v/v) for LC-MS/MS analysis.

Calibration and Quantitation. Calibration method I: solutions of deuterated standard (S) and analyte (A) for both alternaria toxins in acetonitrile were mixed in molar ratios [n(S)/n(A)] between 0.11 and 9 (1:9; 2:8; 5:5; 8:2; 8.5:1.5; 9:1) and diluted with water to obtain a total toxin concentration of 0.1 μg/mL (sum of deuterated and unlabeled toxin) before LC-MS/MS analysis. Calibration method II: constant amounts of deuterated standard (S) were mixed with varying amounts of analyte (A) in molar ratios between 0.10 and 10 (0.1:1; 0.2:1; 0.5:1; 1:1; 2:1; 5:1; 10:1). All concentrations of solutions containing deuterated toxins or analytes were verified by UV spectroscopy before mixing. After dilution with water to a final concentration of 0.1 μg/mL of the deuterated standard, the solutions were measured by LC-MS/MS, and peak area ratios [A(S)/A(A)] were determined. Response factors (Rf) of each n(S)/n(A) value were calculated from the obtained A(S)/A(A) ratio for both methods. Both data sets of response factors were further analyzed using Student's t-test after passing Kolmogorov−Smirnov's test for confirming a normal distribution for both sets. Because both data sets were statistically not distinguishable (90% and 99% confidence interval), the data were combined (data set 3). Calibration functions were obtained for all three data sets using simple linear regression. To check whether the chosen range of n(S)/n(A) = 0.1 to 10 was within the linear range of the SIDA method, analysis of the residuals after linear regression was performed. Regardless of the calibration method, the residuals were homogeneous and normally distributed indicating linearity of the data that could successfully be described by simple linear regression. The obtained y-intercepts of the calibration lines were negligibly small, and thus, it was possible to perform quantitation over the Rf value only, i.e., the slope of the calibration line. The stability of the response was regularly checked by measuring a randomly chosen n(S)/n(A) value in the linear range, and the calculated Rf value had to fall into the 95% confidential interval of the Rf value taken from the calibration curve.

Stability of Labeled Standards. Buffers of different pH (pH 2−pH 10) were prepared according to standard laboratory protocols. Solutions of D-alternariol and D-alternariol methyl ether in acetonitrile were mixed with their unlabeled analogues to yield an equimolar ratio. Aliquots of this solution were added to the nine buffer solutions as well as to deionized water (adjusted toxin concentration 0.1 μg/mL). The obtained samples were analyzed regularly by LC-MS/MS during the following three weeks. From the peak area ratio obtained, the molar ratio was calculated for each toxin as described above. Alterations of the n(S)/n(A) from the original value were interpreted as decomposition of the standard by pH catalyzed H/D exchange.

Limits of Detection (LODs) and Quantification (LOQs). LODs and LOQs for alternariol and alternariol methyl ether were determined according to ref (*26*). Self-made apple juice as blank matrix was prepared from whole, intact apples by means of a commercial juicer (Gastroback GmbH, Hollenstedt, Germany). Apples were washed, peeled, pitted, and sliced, and the juice obtained from the juicer was centrifuged, filtered, and stored at −20 °C until use. LC-MS/MS analysis confirmed that the apple juice neither contained alternariol or alternariol methyl ether. For determination of LODs and LOQs, the apple juice was spiked (each in triplicate) with alternariol and alternariol methyl ether at four different concentration levels (0.05−1 μg/kg). After the addition of the respective deuterated internal standards, all samples underwent sample preparation and cleanup as described above and were finally analyzed by LC-MS/MS. LODs and LOQs were derived statistically from the data according to a published method (*26*).

Precision. Interassay precision was determined by analyzing two different naturally contaminated samples three times in triplicate during three weeks. For this purpose, a vegetable juice containing alternariol and alternariol methyl ether and a red grape juice containing alternariol were chosen.

Recovery. Blank apple juice was spiked (each in triplicate) with three different amounts of alternariol and alternariol methyl ether above their respective LODs and analyzed by LC-MS/MS. The recovery was calculated as the mean of the addition experiments.

RESULTS AND DISCUSSION

Synthesis of Labeled Alternariol and Alternariol Methyl Ether. Synthesis of deuterated alternariol (D-alternariol) and alternariol monomethyl ether (D-alternariol methyl ether) was performed by H/D exchange of the unlabeled toxins. From the many varieties of possible reaction protocols (*27*) H/D exchange by heterogeneous metal catalysis was chosen. This method has certain advantages as the appropriate catalysts are readily available, easy to handle, and can be removed at the end of the reaction simply by filtration. Different catalysts, mostly metal elements from the d-block of the periodic table, can be used in H/D exchange reactions. The most common catalysts are palladium and platinum, which are commonly applied in elemental form on a chemically inert substrate to increase the catalytically active surface. However, many different protocols are described in the literature that include different reaction conditions (time and temperature) and sources of deuterium (deuterated solvents or gaseous deuterium). For the preparation of D-alternariol

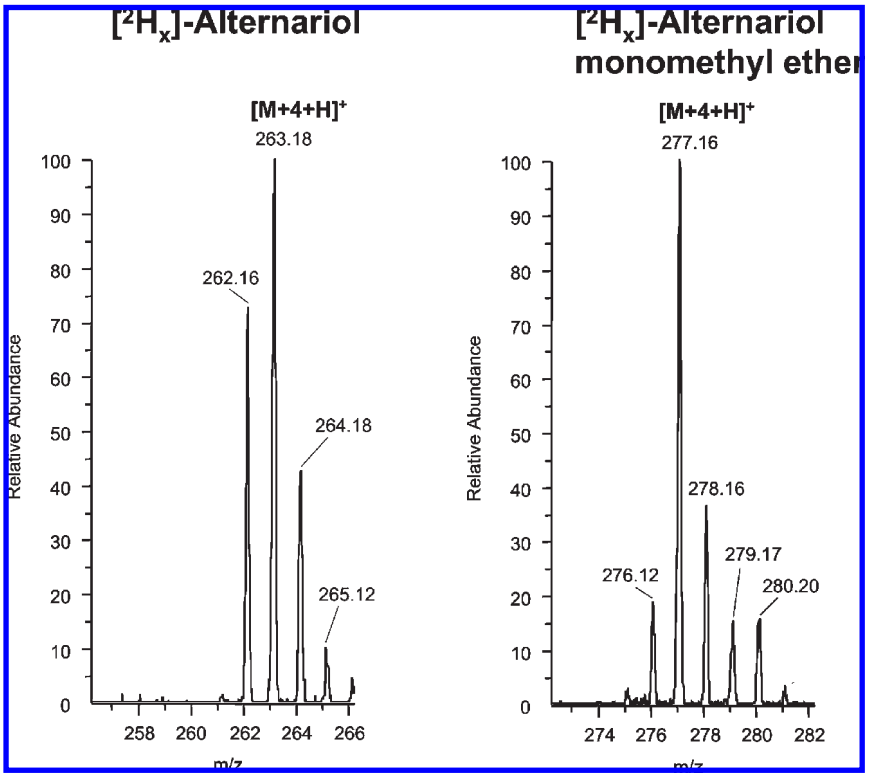

Figure 2. ESI-(+)-LC-MS spectra (zoom scan) of deuterated alternariol and alternariol methyl ether.

and D-alternariol methyl ether, palladium as catalyst and deuterium oxide as deuterating agent were chosen. This method has successfully been used for the synthesis of labeled folate vitamers (*28*) and aroma active compounds (*29*). However, this kind of reaction requires vigorous conditions that reduce yields and seldomly lead to a complete exchange of protium to deuterium. In the case of D-alternariol and D-alternariol methyl ether, the H/D exchange reaction had to be stopped after about 4 days because the formation of thermal decomposition products increased. Neither with D-alternariol nor with D-alternariol methyl ether was the H/D exchange complete at this point as can be seen in the respective LC-MS spectra (**Figure 2**). Considering the elemental formula and the molecular structure (**Figure 1**) of alternariol ($C_{14}H_{10}O_5$) and alternariol methyl ether ($C_{15}H_{12}O_5$), both molecules possess 7 protons that can be exchanged by deuterium (4 protons bound to the aromatic ring system and 3 benzylic protons situated at the ring-bound methyl group), while neither the 3 (alternariol) nor 2 (alternariol methyl ether) phenolic OH-groups nor the methyl ether (alternariol methyl ether) play a major role for persistent H/D exchange (*27*). According to the latter study (*27*), aromatic protons are rapidly exchanged by deuterium in the presence of palladium, which led to the formation of the respective [$^2H_4$]-isotopologues that were observed as base peaks in the LC-MS spectra of D-alternariol and D-alternariol methyl ether after 4 days (**Figure 2**). However, especially for D-alternariol, but to a smaller degree also for D-alternariol methyl ether, distinctive amounts of the [$^2H_3$]-isotopologues were also detected, which indicates that the exchange of the aromatic protons was incomplete at this time. On the contrary, the LC-MS spectra of D-alternariol and D-alternariol methyl ether also showed signals of isotopologues up to the [$^2H_7$]-species indicating a minor exchange most probably of the three benzylic hydrogen atoms at the methyl group bound to the aromatic ring.

These mass spectrometric observations were confirmed by ^{1}H NMR spectroscopy. In both the ^{1}H NMR spectrum of D-alternariol and D-alternariol methyl ether, the signals of the aromatic protons in the region between 6 and 8 ppm were strongly reduced because of the incorporation of deuterium. Integration of the signals was found to be not trivial. In the ^{1}H NMR spectrum of D-alternariol methyl ether, integration of signals was referred to the 9-*O*-methyl ether group as those hydrogen atoms are exposed to H/D exchange to a much smaller extent than aromatic or benzylic hydrogen atoms (*27*). Hence, integration resulted in values of 3H for the *O*-methyl group, the two phenolic protons each with 1H, but only 2.69H for the ring-bound methyl group. The four observed signals in the aromatic region sum up to about 0.2H altogether. Thus, in agreement with the LC-MS spectrum of D-alternariol methyl ether it can be concluded that the H/D exchange of the aromatic protons was nearly complete and that the exchange of the benzylic hydrogen atoms had already started.

In the case of D-alternariol, all proton signals are susceptible to an H/D exchange during synthesis. Therefore, integration of signals in the ^{1}H NMR spectrum was referred to the phenolic protons. Of course, these phenolic protons are also exchanged with deuterium during synthesis, but due to their acidity, they rapidly back-exchanged with protium during preparative HPLC. In aprotic solvents such as [$^2H_8$]-dioxane used for NMR measurements, no H/D exchange of the phenolic protons was observed neither with D-alternariol nor with D-alternariol methyl ether, anyway. However, the ^{1}H NMR spectrum of D-alternariol showed only two signals in the region between 6 and 8 ppm, one of them with 0.01H and the other with 0.42H. Recent evidence (*23*) suggests that the correct assignment of the signals to discrete proton positions in the structure is impossible by ^{1}H NMR. Two dimensional experiments failed because of the low amount of substance available. However, it was obvious that incomplete H/D exchange of only one aromatic proton led to the remainder of the [$^2H_3$]-isotopologue in the LC-MS spectrum of D-alternariol.

UV Spectroscopy. Literature data of molar extinction coefficients of alternariol and alternariol methyl ether are both ambiguous and partly questionable: for alternariol, two different molar extinction coefficients of 5.37×10^4 L/mol/cm (*30*) and

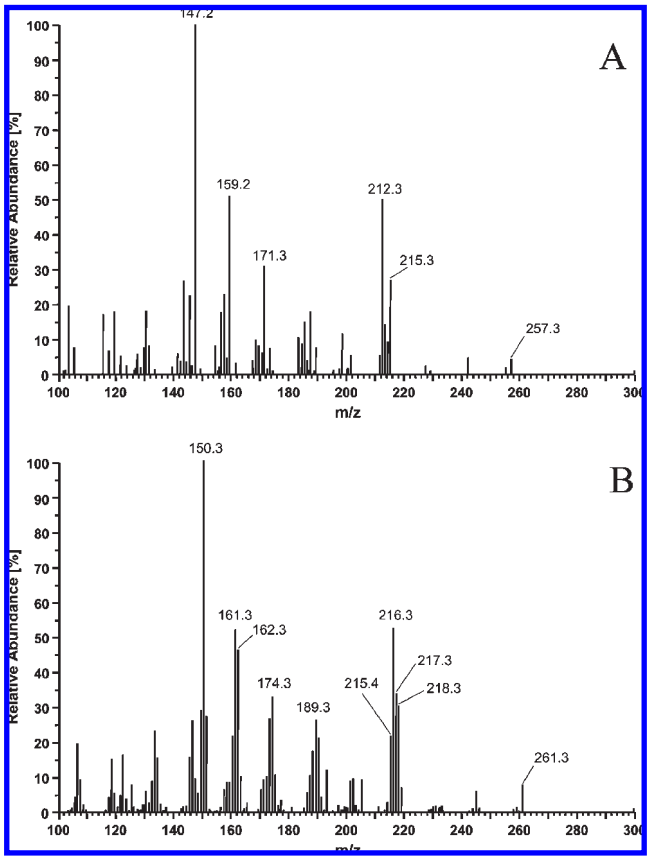

Figure 3. ESI-(−)-LC-MS/MS spectrum of (**A**) alternariol (parent, m/z = 257, $[M - H]^-$; CE = −50 V) and (**B**) $[^2H_4]$-alternariol (parent, m/z = 261, $[M - H]^-$; CE = −50 V).

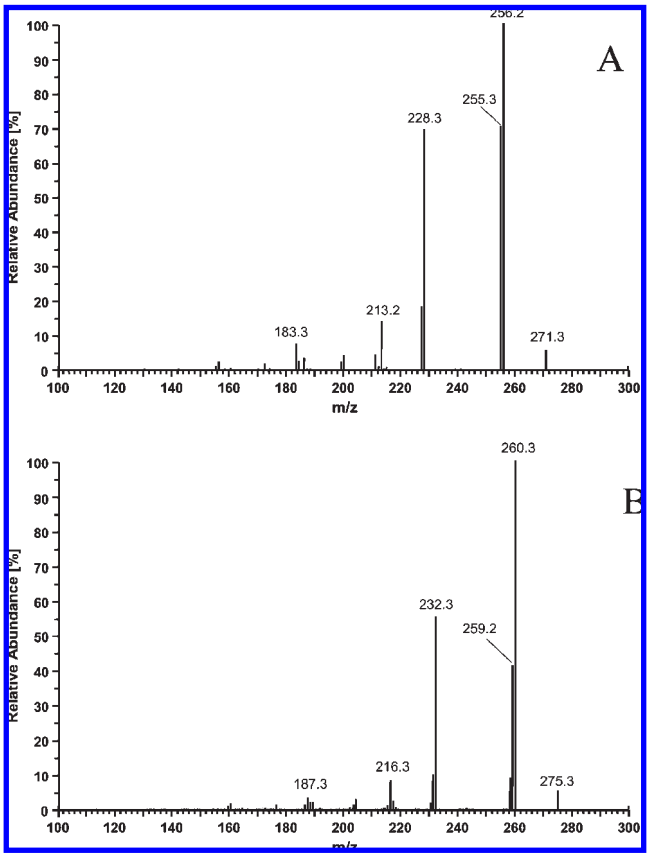

Figure 4. ESI-(−)-LC-MS/MS spectrum of (**A**) alternariol methyl ether (parent, m/z = 271, $[M - H]^-$; CE = −40 V) and (**B**) $[^2H_4]$-alternariol methyl ether (parent, m/z = 275, $[M - H]^-$; CE = −50 V).

4.17×10^4 L/mol/cm (*31*) have been reported. For alternariol methyl ether, a molar extinction coefficient of 4.79×10^4 L/mol/cm can be found in the literature (*31*). All molar extinction coefficients have been determined in ethanol. Newer studies used mixtures of acetonitrile and water at different pH values for UV measurement (*32*), but the molar extinction coefficients obtained differed significantly from the above-mentioned molar extinction coefficients of alternariol and alternariol methyl ether. In the present study, acetonitrile was used as the preferred solvent for both the deuterated standards and the unlabeled compounds of alternariol and alternariol methyl ether. Thus, knowledge of molar extinction coefficients for both compounds in acetonitrile was required in order to determine the precise concentration in the respective solutions. For alternariol, a molar extinction coefficient of $(4.06 \pm 0.19) \times 10^4$ L/mol/cm and for alternariol methyl ether, a molar extinction coefficient of $(4.76 \pm 0.06) \times 10^4$ L/mol/cm (mean ± standard deviation) in acetonitrile was calculated. These values of the molar extinction coefficients of alternariol and alternariol methyl ether in acetonitrile were quite similar to the literature data obtained in ethanol (*31*).

Stability of D-Alternariol and D-Alternariol Methyl Ether. Both D-alternariol and D-alternariol methyl ether were stable over three weeks at room temperature as solutions in both, acetonitrile and water and acetonitrile and buffer in the pH range between pH 2−9. Beyond pH 9, stability was limited, especially for alternariol. Equimolar mixtures of standard and analyte were used in this study. Thus, the chosen test conditions compensate for general decomposition, for example, opening of the lactone moiety that might affect both the standard and the analyte. Instability of the label at pH 10 by alkaline catalyzed H/D exchange was deduced from the reduction of the $n(S)/n(A)$ ratio, which was observed only at pH 10 after 3 weeks.

Development of a Stable Isotope Dilution Assay. Analysis of alternariol and alternariol methyl ether in beverage samples was performed by LC-MS/MS after sample cleanup on RP-18 SPE cartridges. SPE was performed according to the method reported by Delgado et al. (*6*) with the modification that the additional cleanup on an aminopropyl column was omitted. Chromatographic separation of both compounds was easily achieved using a linear gradient and methanol/water as the mobile phase. Detection of the analytes was carried out by MS/MS in the multiple reaction monitoring (MRM) mode measuring respective product ions obtained from the parent ions by collision induced dissociation (CID). Full scan product ion spectra were recorded for alternariol, alternariol methyl ether, and their deuterated standards. As the $[^2H_4]$-isotopologues of D-alternariol and D-alternariol methyl ether were the most intense ions in the LC-MS spectra of the labeled compounds (**Figure 2**), $[^2H_4]$-alternariol and $[^2H_4]$-alternariol methyl ether were chosen as parent ions for MS/MS fragmentation of the internal standards.

The recorded MS/MS-spectra of the unlabeled compounds were identical to recently published data in general (*7*, *8*). The respective MS/MS-spectra of the $[^2H_4]$-isotopologues revealed similar fragmentation (**Figures 3**−**4**). Generally, during CID the labeling was maintained in the fragments. However, the deduction of possible fragmentation pathways was difficult, especially for alternariol showing extensive fragmentation. The hypothetic fragmentation scheme is shown in **Figure 5**. It has been proposed (*7*) that m/z 213 is derived from m/z 257 (alternariol, $[M - H]^-$) by elimination of CO_2 (−44 amu) and m/z 215 by elimination of CH_2CO (−42 amu). The preferred formation of

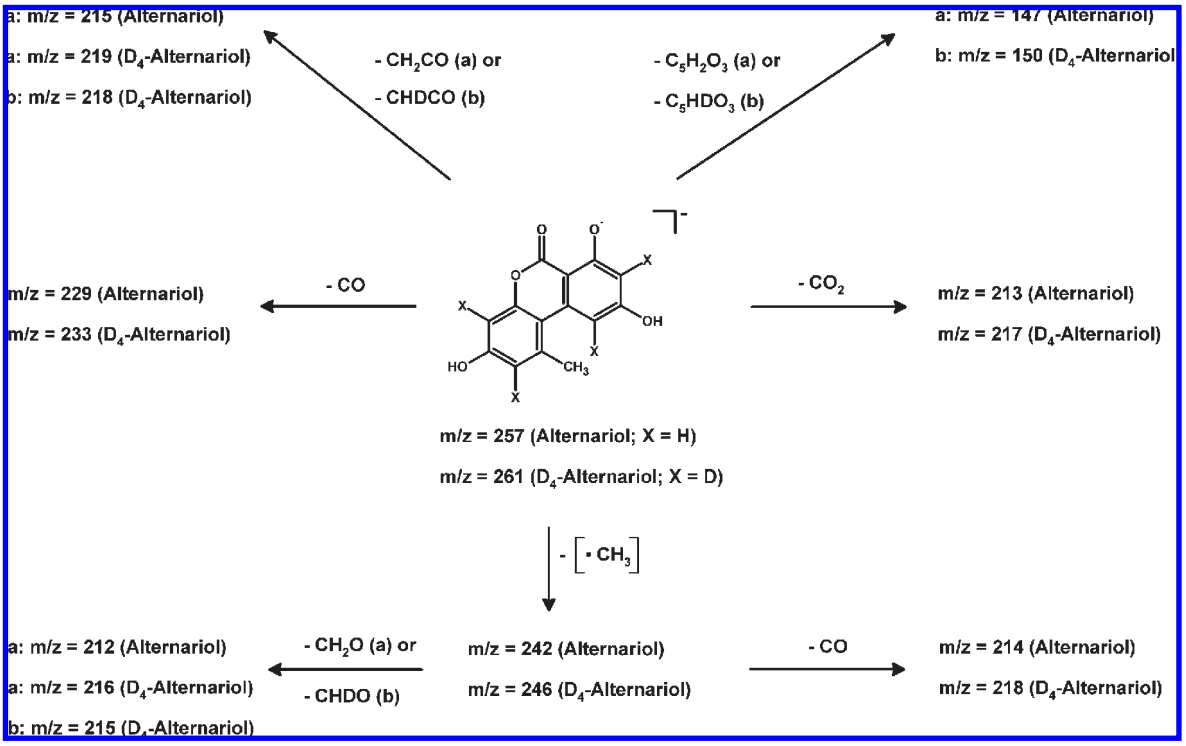

Figure 5. Proposed fragmentions of labeled and unlabeled alternariol in LC-MS/MS.

the ion at *m*/*z* 212 can only be attributed to the elimination of a methyl radical (−15 amu) from the parent ion and consecutive elimination of CH_2O (−30 amu). For [$^2H_4$]-alternariol, isotopic clustering in the region from *m*/*z* 215−*m*/*z* 218 was observed (**Figure 3A**). Thus, several different mass transitions are supposed to occur simultaneously. Elimination of CO_2 from the parent ion (*m*/*z* 261; [$^2H_4$]-alternariol, $[M - H]^-$) results in *m*/*z* 217, while the ion *m*/*z* 219 formed by elimination of CH_2CO was only marginally observable. Elimination of CHDCO (leading to *m*/*z* 218), CD_2CO (leading to *m*/*z* 217), or HCOOH (leading to *m*/*z* 215) may occur in this case. However, the latter two transitions seem unlikely due to the molecular structure of [$^2H_4$]-alternariol, which neither contains two protons nor two deuteriums in the immediate neighborhood. Another plausible mechanism is an initial fragmentation of *m*/*z* 261 ([$^2H_4$]-alternariol, $[M - H]^-$) starting with the elimination of a methyl radial (−15 amu), thereby generating *m*/*z* 246 (only observed in traces). From *m*/*z* 246, the fragments *m*/*z* 218 can be derived by elimination of CO, *m*/*z* 216 by elimination of CH_2O (−30 amu), and *m*/*z* 215 by elimination of CHDO (−31 amu). Hence, because of several possible formation pathways, especially in the case of [$^2H_4$]-alternariol, the fragment ions between *m*/*z* 215 and *m*/*z* 218 were not generated in reproducible intensity. However, the desired criteria were fulfilled by the product ions *m*/*z* 147 (alternariol) and *m*/*z* 150 ([$^2H_4$]-alternariol). Advanced fragmentation has taken place in the formation of *m*/*z* 147 (alternariol) and *m*/*z* 150 ([$^2H_4$]-alternariol) including ring cleavage and loss of one label in the case of [$^2H_4$]-alternariol. Again, theoretical structures of fragmentation products are highly speculative. The transition *m*/*z* 257 → 147 ([$^2H_4$]-alternariol *m*/*z* 261 → 150) was used as a quantifier for LC-MS/MS analysis of alternariol because it has already been shown to be both, sensitive and reproducible (*7*). Additional confirmation (qualifier) of alternariol signals was based on the intense transition *m*/*z* 257 → 213 ([$^2H_4$]-alternariol *m*/*z* 261 → 217).

For alternariol methyl ether and [$^2H_4$]-alternariol methyl ether, fragmentation of the parent ions was less extensive than for alternariol and [$^2H_4$]-alternariol. Both alternariol methyl ether and [$^2H_4$]-alternariol methyl ether showed identical fragmentation in general. According to the literature (*7*), the intense fragment ions *m*/*z* 255 and *m*/*z* 256 of alternariol methyl ether (parent *m*/*z* 271) were produced by elimination of a methyl radical (−15 amu) or an oxygen radical (−16 amu), respectively. In the case of [$^2H_4$]-alternariol methyl ether (parent *m*/*z* 275), the ions with *m*/*z* 259 and *m*/*z* 260 were generated by the same transitions. From the product ions *m*/*z* 256 (alternariol methyl ether) or *m*/*z* 260 ([$^2H_4$]-alternariol methyl ether), the ions at *m*/*z* 228 (alternariol methyl ether) and *m*/*z* 232 ([$^2H_4$]-alternariol methyl ether) may originate from the elimination of CO. Taking these considerations into account, the intense transitions *m*/*z* 271 → 256 (alternariol methyl ether) and *m*/*z* 275 → 260 ([$^2H_4$]-alternariol methyl ether) were chosen for quantitation, whereas the transitions *m*/*z* 271 → 228 (alternariol methyl ether) and *m*/*z* 275 → 232 ([$^2H_4$]-alternariol methyl ether) were used for additional confirmation.

Calibration and Quantitation. SIDA is recognized as a primary analytical method requiring no calibration. However, the relationship between analyte (A) and standard (S) is not linear in general (*33*), and the determination of the linear working range of the method has to be performed by recording a response function. Hence, calibration was performed by determination of the response factors between the molar ratio *n*(S)/*n*(A) and the peak area ratio *A*(S)/*A*(A) obtained from mass spectrometric analysis at different values of *n*(S)/*n*(A). Two different approaches for calibration were tested: first, standard (S) and analyte (A) were mixed in alternating molar ratios yielding an absolute toxin concentration of 0.1 μg/mL (calibration method I). Second, concentration of the standard was kept constant (0.1 μg/mL), and the analyte concentration varied (calibration method II). Response factors of each *n*(S)/*n*(A) value were calculated for both methods. After statistical evaluation of the data, it became obvious that both methods were not distinguishable (99% confidence interval) and can be combined. Calibration curves were obtained by simple linear regression showing linearity in the working range of *n*(S)/*n*(A) = 0.1−10. Quantitation was based on

Table 1. Concentrations of Alternariol and Alternariol Monomethyl Ether in Different Beverages

		alternariol		alternariol methyl ether	
no. of samples	beverage	no. of positive samples	conc. in [μg/kg] min − max (med)[a]	no. of positive samples	conc. in [μg/kg] min − max (med)[a]
6	white wine	6	0.10 − 7.59 (1.13)	1	+[b]
5	red wine	5	0.36 − 7.50 (4.50)	5	+[b] − 0.15 (0.08)
2	mulled wine	2	2.04 − 2.70	1	+[b]
1	fruit punch	1	0.27	1	0.04
5	grape juice	5	0.10 − 1.05 (0.11)	2	+[b]
4	apple juice	3	0.16 − 0.22 (0.17)	3	+[b]
2	orange juice	2	0.16 − 0.24 (0.20)	2	0.18 − 0.27 (0.23)
2	tomato juice	2	0.52 − 1.99 (1.26)	2	0.23 − 0.38 (0.31)
1	vegetable juice	1	7.82	1	0.79

[a] Min = minimum value; med = median value; max = maximum value. [b] + = detected, but below the limit of quantitation.

the response factor, i.e., the slope of calibration functions with negligible *y*-intercepts. While commonly a response factor value of 1.0 is found in the linear range of SIDA, in this case response factors were only 0.39 for alternariol versus [$^2H_4$]-alternariol and 0.61 for alternariol methyl ether versus [$^2H_4$]-alternariol methyl ether. Calculated from the signal intensities in the LC-MS spectrum (**Figure 2**), [$^2H_4$]-alternariol occurs with 43% and [$^2H_4$]-alternariol methyl ether with 59% of the sum over all isotopologues of the respective labeled materials. Therefore, response factors for alternariol versus [$^2H_4$]-alternariol and alternariol methyl ether versus [$^2H_4$]-alternariol methyl ether are far below 1.0 because the [$^2H_4$]-isotopologues are only a fraction of total standard concentration.

Limit of Detection (LOD) and Limit of Quantification (LOQ). For both alternariol and alternariol methyl ether, the respective LODs and LOQs were determined by the previously reported method (*26*), which is comparable to DIN EN standard 32645. However, this method requires a blank matrix for spiking purposes. As none of the analyzed beverage samples was devoid of alternariol or alternariol methyl ether, apple juice was homemade from whole and sound apples in a laboratory scale and confirmed as blank by LC-MS/MS analysis. Using this matrix, alternariol and alternariol methyl ether were spiked at different concentration levels and analyzed by LC-MS/MS after the addition of labeled standards. LODs and LOQs were statistically deduced from the resulting data using a 95% confidence limit as suggested by the method. Alternariol and alternariol methyl ether could be detected with high sensitivity as highlighted by low LODs (alternariol, 0.03 μg/kg; alternariol methyl ether, 0.01 μg/kg) and LOQs (alternariol, 0.09 μg/kg; alternariol methyl ether, 0.03 μg/kg), respectively. Similar sensitivity was previously estimated from the signal-to-noise ratio of the LC-MS/MS detection of alternariol and alternariol methyl ether in beverages (*7*).

Recovery. Recovery of alternariol and alternariol methyl ether was calculated from spiking experiments of the blank apple juice at different addition levels. For alternariol, recovery was 100.5 ± 3.4% from 0.1−1 μg/kg and for alternariol methyl ether 107.3 ± 1.6% from 0.05−0.5 μg/kg, and thus, recovery was complete even at low analyte concentrations.

Precision. Interassay precision was determined by triple analysis of two different naturally contaminated samples over 3 weeks. For this purpose, a vegetable juice, naturally containing alternariol and alternariol methyl ether, and a red grape juice, containing alternariol, were chosen. For the vegetable juice, coefficients of variation (CEVs) of 4.0% for alternariol (7.82 ± 0.31 μg/kg) and of 2.3% for alternariol methyl ether (0.79 ± 0.02 μg/kg) were obtained. The CEV of the determination of the alternariol content in red grape juice (1.04 ± 0.05 μg/kg) was calculated to be 4.6%. Compared to the literature (*8*), this is an improvement of precision by SIDA compared to conventional LC-MS/MS analysis by a factor of 3.

Analysis of Beverages. A series of commercially available beverages was analyzed for alternariol and alternariol methyl ether. The variety of samples ranged from fruit juices (apple, grape, and orange juice), to vegetable juices (tomato juice and mixed vegetable juices), and to several different kinds of wine (**Table 1**). Most of these samples contained alternariol and alternariol methyl ether, but significant differences were observed for the analyzed products.

Apple juice was contaminated only moderately with median values of 0.17 μg/kg alternariol. The content of alternariol methyl ether was negligibly low. These values were significantly lower than the content of alternariol and alternariol methyl ether in apple juice reported in ref (*7*). In orange juice, nearly equal values of alternariol and alternariol methyl ether (about 0.2 μg/kg each) were found. Knowledge about *Alternaria* toxin content in orange products is rather limited, but there are reports of the natural occurrence of alternariol and alternariol methyl ether in tangerines (*8*) and in other citrus fruits inoculated with *Alternaria citrii* (*34*). However, in the latter report the equal concentration of alternariol and alternariol methyl ether in rotten oranges and lemons was remarked. As long as comprehensive data of secondary metabolites of *Alternaria* species are deficient, this feature may be characteristic for *A. citrii*.

Concerning red grape juice and red wine, similar results were obtained as reported previously (*35*). Both beverages contained distinct amounts of alternariol. Whereas red grape juice was contaminated with a maximum of 1 μg/kg (n = 5), values ranged up to a maximum of 7.5 μg/kg in red wine (n = 5). However, the median values were much lower. Alternariol methyl ether was only detected in red wine at median values of 0.08 μg/kg. White wine was reported to contain only small amounts of alternariol (*35*). However, analysis of white wine samples produced in Germany (n = 6) exhibited no difference between both types of wine. The maximum value of alternariol in a white wine of the variety Riesling was 7.6 μg/kg, thus exceeding the maximum value of red wine. Concerning the median values, white wine contained lower amounts of alternariol (median 1.1 μg/kg) than red wine (median 4.5 μg/kg). In contrast to red wine, alternariol methyl ether was detected only in traces in white wine. The alternariol concentrations in mulled wine (> 2 μg/kg) and fruit punch (0.3 μg/kg), both seasonally consumed beverages, followed the toxin distribution observed for wine and fruit products, in general.

Both tomato and vegetable juices (consisting of tomatoes, carrots, celery, beetroot, and red pepper) contained elevated amounts of both alternariol and alternariol methyl ether. Vegetable juice, especially as a composite food product, accumulates

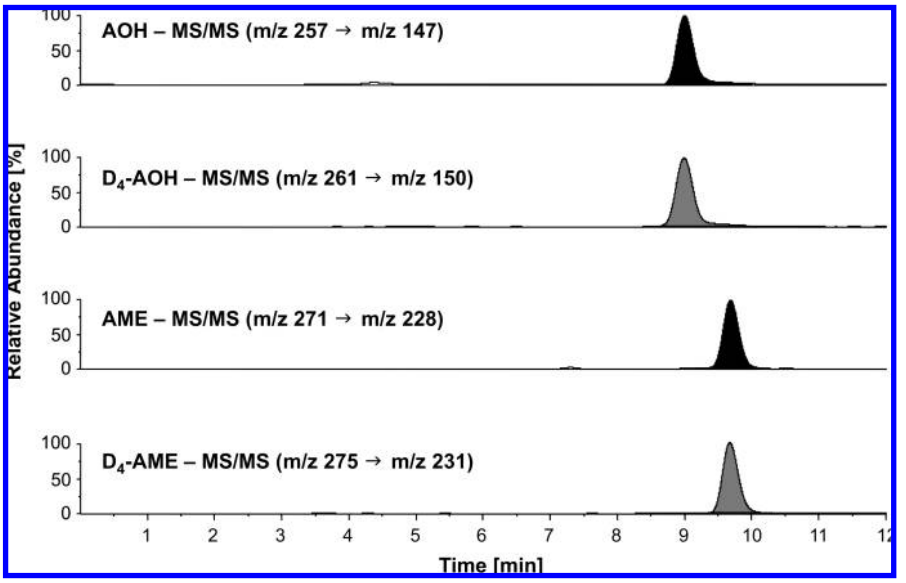

Figure 6. LC-MS/MS chromatograms of SIDA analyses of a vegetable juice containing alternariol (AOH, 7.8 μg/kg) and alternariol monomethyl ether (AME, 0.8 μg/kg). Only mass traces of transitions used for quantitation are shown.

toxins from different sources, probably produced by different *Alternaria* species. An LC-MS/MS chromatogram of a vegetable juice is shown in **Figure 6**. It is known that tomatoes are rapidly colonized by *Alternaria* (*36*), and thus, alternariol and alternariol methyl ether were found in tomato puree (*37*) in the mg/kg range. However, quantitative data of alternariol and alternariol methyl ether contamination of other vegetables are lacking.

Risk Evaluation. Risk evaluation of alternariol and alternariol methyl ether is hindered as it is still unclear if alternariol and alternariol methyl ether have to be regarded as carcinogenic substances. Nevertheless, the detected amounts of alternariol and alternariol methyl ether in fruit juices were low in general. Presumably, even at regular consumption they do not pose a major threat to the health of the consumer. However, in nonstaple foodstuff such as wine, values of alternariol of up to 7.5 μg/kg were found, but since only marginal amounts of these beverages are commonly consumed, adverse effects can be considered unlikely. But especially in view of possible carcinogenity, the sum uptake of alternariol and alternariol methyl ether from different sources has to be considered, and although it might be an exceptional case, the occurrence of elevated values of both alternariol and alternariol methyl ether in a vegetable juice in this study points out the need of further surveys about the content of both toxins in vegetables and vegetable products.

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