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Photochemical *trans*-/*cis*-Isomerization and Quantitation of Zearalenone in Edible Oils

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Supporting Information

ABSTRACT: The emphasis of the present work was to investigate the photochemical conversion of *trans*- to *cis*-zearalenone in edible oils under real-life conditions. For quantitation purposes a *cis*-zearalenone standard was synthesized and characterized for its identity and purity (≥95%) by ^{1}H NMR, X-ray crystallography, HPLC fluorescence and mass spectrometric detection. In a sample survey of 12 edible oils (9 corn oils, 3 hempseed oils) from local supermarkets all corn oils contained *trans*-zearalenone (median 194 μg/kg), but no *cis*-zearalenone was detected. For alteration studies *trans*-zearalenone contaminated corn oils were exposed to sunlight over 4 and 30 weeks, revealing an obvious shift toward *cis*-zearalenone up to a *cis/trans* ratio of 9:1 by storage in colorless glass bottles. Irradiation experiments of *trans*-zearalenone in different organic solvents confirmed the preferred formation of *cis*-zearalenone possibly caused by entropic effects rather than by enthalpic entities as investigated by quantum chemical and classical force field simulations.

KEYWORDS: *Fusarium mycotoxins, food, analysis, occurrence*

INTRODUCTION

Zearalenone is a nonsteroidal mycotoxin produced by a variety of plant pathogenic *Fusarium* species, including *F. graminearum* (*Gibberella zeae*), *F. culmorum*, *F. cerealis*, *F. equiseti*, *F. rookwellense* and *F. semitectum* in zones of moderate climate.[1,2] Despite a relatively low acute toxicity after oral administration zearalenone was shown to be hyperestrogenic, hepatotoxic, hematotoxic, immunotoxic, genotoxic, teratogenic and carcinogenic.[3]

Zearalenone is one of the worldwide most common mycotoxins in cereal grains and animal feed. Consequently, humans as well as animals are at risk of being exposed to zearalenone by consuming contaminated food products and feed.[3−5] Recent findings indicate that edible oils, particularly from corn, significantly contribute to the zearalenone intake.[6−8] Thus, an EU maximum level for zearalenone in refined corn oil of 400 μg/kg is currently in force.[9]

Because of the ethylenic double bond between C_{11} and C_{12} in the 14-membered macrocyclic lactone ring two stereoisomeric forms are generally possible: *trans*- and *cis*- zearalenone (Figure 1). With the exception of very few studies[10] only the *trans*-isomer could be isolated so far from different *Fusarium* spp. This led to the assumption that a highly isomer-specific pathway is involved in the fungal biosynthesis of zearalenone. In 1966 the structure of *trans*-zearalenone was elucidated using classical chemical, NMR and mass spectrometric analysis.[11] Since the 1970s it is known that a photochemical conversion of *trans*- to *cis*-zearalenone can easily be achieved by UV light irradiation as well as by sunlight.[12,13] The structure of *cis*-zearalenone was primarily confirmed by NMR[13,14] and recently by X-ray crystallography.[15] Reports on the occurrence of *cis*-zearalenone as natural product[10,16] may be due to exposure of the *trans*-isomer to light.[13]

In mammals zearalenone is converted to α- and β-zearalenol by reductive metabolism. It was shown that α-*trans*-zearalenol exhibits an estrogenic potency about 70 times higher than *trans*-zearalenone and is slightly less potent than 17β-estradiol.[17] Substantial estrogenicity is retained in *cis*- analogues like *cis*-zearalenone and its metabolites α- and β-*cis*-zearalenol. However, not entirely consistent results were obtained comparing several toxicological studies.[14,18−20]

According to IUPAC, zearalenone is chemically defined as (3*S*,11*E*)-14,16-dihydroxy-3-methyl-3,4,5,6,9,10-hexahydro-1*H*-2-benzoxacyclotetradecine-1,7(8*H*)-dione representing only the *trans*-isomer. Consequently, worldwide all established maximum levels for zearalenone in food and feed apply to *trans*-zearalenone, whereas the *cis*-isomer is not considered.

Because only little is known about the occurrence, fate, and risks associated with *cis*-zearalenone entering the food chain, a major problem could arise for the official control of foodstuffs and consumer protection.[21] Most of the various analytical methods for the determination of zearalenone in food and feed, based on HPLC coupled to fluorescence or mass spectrometric detection,[22−24] were not developed and optimized to distinguish between *trans*- and *cis*-zearalenone. The separation and reliable quantitation of one or both zearalenone isomers strongly depends on the chromatographic conditions, posing a

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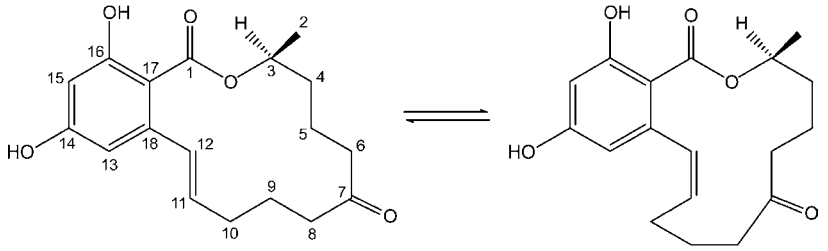

Figure 1. Stereoisomerization of *trans*-zearalenone (**1**) and *cis*-zearalenone (**2**).

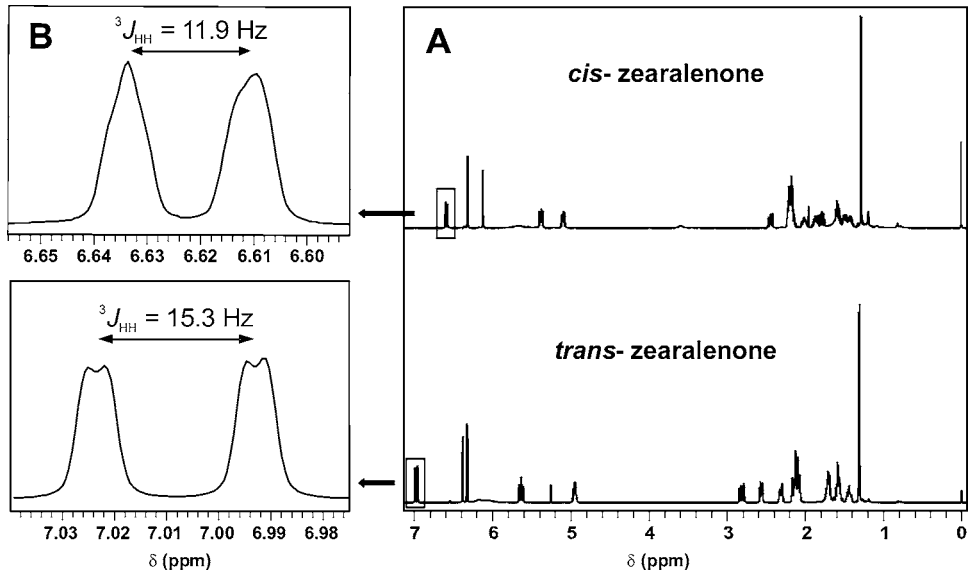

Figure 2. (A) Full ^{1}H NMR spectra of *cis*- and *trans*-zearalenone (500 MHz, $CDCl_3$). (B) Signal splitting of the proton at C_{12} position showing the vicinal coupling constant $^3J_{HH}$.

risk of noncomparable results and, consequently, leading to consumer health risks and economic problems.

For all these reasons, investigations into the formation, occurrence and quantitation of *cis*- zearalenone in food and feed are urgently needed. Thus, the objective of this work was to investigate the sunlight-induced conversion of *trans*- to *cis*-zearalenone in edible oils and to quantitate *cis*-zearalenone by using a synthesized and characterized pure *cis*-zearalenone standard. To our knowledge this is the first report on the formation and quantitation of *cis*-zearalenone in edible oils.

MATERIALS AND METHODS

Chemical Reagents. All solvents and chemicals were of analytical grade and used without further purification. Solid *trans*-zearalenone (purity 99.8%) used for photochemical isomerization was obtained from AppliChem GmbH (Darmstadt, Germany). Certified solutions of *trans*-zearalenone (100.7 ± 0.8 μg/mL) and fully $^{13}C_{18}$ isotope labeled zearalenone (25.1 ± 0.7 μg/mL) in acetonitrile (MeCN) for calibration purposes were supplied by Biopure, Romer Labs Diagnostic GmbH (Tulln, Austria). Ultrapure water provided by a Seralpur PRO 90CN (Ransbach- Baumbach, Germany) was used throughout the experiments.

Synthesis and Characterization of *cis*-Zearalenone. *Synthesis.* Twenty-five milligrams (78.5 μmol) of *trans*-zearalenone (purity 99.8%) were dissolved in 18 mL of ethyl acetate and irradiated for 8 h with ultraviolet light (λ = 350 nm) by using an universal UV-Lamp, type TL-900 (CAMAG, Muttenz, Switzerland). The irradiated zearalenone solution was redissolved in MeCN/H_2O (38:62, v:v) for the chromatographic separation of *cis*-zearalenone and residual *trans*-zearalenone. This purification was achieved by applying the solution on a 1200 series HPLC (Agilent, Böblingen, Germany) equipped with an automatic fraction collector. The column used was a 150 mm × 2 mm i.d., 3 μm, Gemini-NX C_{18} (Phenomenex, Aschaffenburg, Germany) operated under isocratic conditions (MeCN/H_2O, 38:62, v:v) containing 0.1% formic acid at a flow rate of 0.3 mL/min.

The purity of the isolated white powder (yield 16 mg; 64%) was determined being ≥95% by analytical HPLC-FLD and HPLC-MS/MS. In addition, ^{1}H NMR and X-ray crystallography have also been used to identify *cis*-zearalenone and to evaluate its purity.

^{1}H NMR Spectroscopy. A *cis*-zearalenone solution (13 mg/mL) was measured by ^{1}H NMR (500 MHz, $CDCl_3$) on an AVANCE III 500 (Bruker Daltonik, Bremen, Germany). The chemical shifts (δ) in Figure 2 are given in parts per million, relative to tetramethylsilane.

X-ray Crystallography. Colorless crystals of *cis*-zearalenone, grown by slow evaporation of solvent (dichloromethane/*n*-hexane, 1:2 v/v) were measured on an APEX CCD area-detector diffractometer (Bruker, Karlsruhe, Germany) at 296 K using graphite monochromatized Mo Kα radiation (λ = 0.71073 Å). The structure of *cis*-zearalenone, solved by direct methods and refined by full-matrix least-squares calculation, was recently published.[15]

Edible Oil Samples. A total of 12 edible oils (9 corn oils and 3 hempseed oils) from different geographical origins produced in 2011/2012 were purchased in German retail markets in the spring of 2012 (Table 1). The sealed oil bottles each containing 0.25−1 L were stored at ambient temperature (23 °C) in a dark place until analysis.

Alteration of Edible Oils by Sunlight Exposure. A naturally and a fortified contaminated corn oil were exposed to sunlight on a window sill indside the laboratory (4 and 30 weeks, respectively) to examine the isomerization process from *trans*- to *cis*-zearalenone

Table 1. Determination of *trans*-Zearalenone in Edible Oils from Retail Markets in Germany

			trans-zearalenone[a] (μg/kg)	
no.	type	produced	Method A	Method B
1	corn oil	2011	221.3 ± 14.5	230.4 ± 1.2
2	corn oil	2011	259.6 ± 10.0	263.8 ± 0.7
3	corn oil	2011	322.7 ± 14.9	309.1 ± 2.4
4	corn oil	2011	257.9 ± 8.7	249.0 ± 3.4
5	corn oil	2011	187.0 ± 10.7	205.8 ± 1.5
6	corn oil	2011	170.3 ± 7.2	185.7 ± 4.2
7	corn oil	2012	90.2 ± 2.1	n.a.[b]
8	corn oil	2012	92.7 ± 1.3	n.a.[b]
9	corn oil	2012	26.5 ± 0.1	n.a.[b]
10	hempseed oil	2011	n.q.[b]	n.a.[b]
11	hempseed oil	2011	n.q.[b]	n.a.[b]
12	hempseed oil	2011	22.8 ± 1.0	n.a.[b]

[a]The mean values and their corresponding standard deviations are given based on three independent replicates (n = 3). [b]n.q.: not quantifiable, i.e., lower than LOQ (3 μg/kg); n.a.: not analyzed.

depending on the exposure time and the bottle material. Subsamples of 2 mL were withdrawn after defined time intervals and stored at 4 °C. After completing the time-driven stability studies, all subsamples were extracted and analyzed in a single HPLC sequence to minimize method-based uncertainties.

Natural Corn Oil for Alteration. For this study the oil sample no. 3 (Table 1) was used due to its high natural *trans*-zearalenone content of 323 μg/kg. It was stored in thin amber and colorless laboratory glass vials as well as in the original thick colorless bottle for 4 weeks. A weekly sampling was performed.

Fortified Corn Oil for Alteration. A corn oil sample containing a low natural *trans*-zearalenone content of 30 μg/kg was gravimetrically spiked with 600 μg/kg *trans*-zearalenone. This fortified corn oil was stored in a bottle of colorless laboratory glass for 30 weeks. After one month of weekly sampling every second week subsamples were withdrawn.

Analysis of Zearalenone. *Method A (HPLC-MS/MS). Extraction.* 50 μL internal standard (IS) solution was evaporated to dryness followed by addition of 0.5 mL of edible oil and 0.5 mL of *n*-hexane. Zearalenone was extracted from oil with 5 mL of methanol/water (9:1, v:v) for 30 min using a horizontal shaker (300 oscillations/min). After centrifugation (1378*g*) 1 mL of the upper aqueous layer was evaporated in a gentle stream of nitrogen at 30 °C and redissolved in 0.4 mL of HPLC-MS/MS eluent. All given volumes were gravimetrically controlled.

Measurement. The liquid chromatographic system consisted of an 1200 series HPLC (Agilent, Böblingen, Germany) equipped with a vacuum degasser, a binary pump, a thermostatted column compartment set to 50 °C, and a 4000 Q TRAP mass spectrometer (AB Sciex, Darmstadt, Germany). Solutions containing *trans*-/*cis*-zearalenone were applied (10 μL) to HPLC using the same chromatographic conditions as stated above for *cis*-zearalenone preparation supplemented by a guard column (4 mm × 2 mm i.d., 3 μm) of the same material. MS/MS detection was performed by negative electrospray ionization (ESI^-) using the multiple reaction monitoring (MRM) acquisition mode with a dwell time of 50 ms for each transition. The transitions monitored for native *trans*-/*cis*-zearalenone were (m/z) 317.1 → 131.1 (quantifier), 317.1 → 175.0 (qualifier) and 335.2 → 140.2 for the $^{13}C_{18}$-labeled *trans*-zearalenone. The optimized MRM-MS instrument parameters for each monitored transition were: ion spray voltage: −4000 V; declustering potential: −80 V; desolvation temperature: 500 °C; ion source gas 1: 50 arbitrary units (a.u.); ion source gas 2: 50 a.u.; curtain gas: 20 a.u. and collision energy: −42 eV (qualifier: −40 eV).

Calibration and Quantitation. Certified standards of native and isotope labeled *trans*-zearalenone as well as synthesized *cis*-zearalenone were used for calibration. The *trans*- and *cis*-zearalenone contents in the edible oil samples were quantitated applying a six point calibration curve after linear regression established in a range of 10−520 μg/kg (R^2 = 0.9997 for *trans*-zearalenone, R^2 = 0.9990 for *cis*-zearalenone) by analyzing each calibration level in duplicate. According to Liao et al.[25] the limit of detection (LOD) was estimated being 0.5 μg/kg based on the peak area ratio of signal-to-noise (S/N) of 3, the corresponding limit of quantitation (LOQ) was 3 μg/kg (S/N = 10). The absolute zearalenone recovery from edible oil, controlled by spiking experiments, was found to be within the required range of 70−120% according to EC 401/2006.[26] Since there is no ^{13}C-labeled analogue available for *cis*-zearalenone, the analyte was determined by using the internal standard technique with $^{13}C_{18}$-*trans*-zearalenone as IS.

Method B (DCHC-HPLC-FLD). Extraction. A recently published method,[27] initially developed for *trans*-zearalenone, was incorporated to extract and quantitate *trans*- and *cis*-zearalenone in edible oils based on dynamic covalent hydrazine chemistry (DCHC) using a hydrazine-functionalized macroporous polymer resin (Sigma-Aldrich, Steinheim, Germany). Zearalenone is selectively extracted from oil by shaking the sample (0.2 g), diluted in 0.8 mL of methanol, with 100 mg of hydrazine-functionalized polymer resin for 2 h. After covalent coupling the hydrazone bond formed allows a thorough washing of the solid-phase with methanol and *n*-heptane. Decoupling of zearalenone from the resin is achieved by applying an acetone containing elution mixture leading to highly purified extracts suited for HPLC fluorescence detection (FLD) analysis.

Measurement. The chromatographic analysis was performed on a 1200 series HPLC equipped with vacuum degasser, binary pump, oven thermostat, diode array detector (DAD) and FLD (Agilent, Böblingen, Germany). Chromatographic run parameters were as follows: oven temperature: 50 °C, injection volume: 10 μL, flow rate: 0.3 mL/min, solvent A: water + 0.1% (v) formic acid, solvent B: MeCN + 0.1% (v) formic acid. The following linear gradient was used: 30−40% B in 21 min, followed by 100% B for 7 min and 30% B for 7 min (re-equilibration). The HPLC column used was as stated in the section of *cis*-zearalenone characterization, FLD parameters were set to λ_{em} = 276 nm; λ_{ex} = 456 nm. This method separates *trans*- and *cis*-zearalenone (t_R = 21.7 min and t_R = 22.8 min, respectively) as the DCHC approach is applicable to *cis*-zearalenone as well.

Calibration and Quantitation. In contrast to Method A external calibrations without ^{13}C-labeled IS were established for HPLC-FLD analysis. The linear regression lines of *trans*- and *cis*-zearalenone in the range of 10−520 μg/kg (R^2 = 0.9998 for *trans*-zearalenone, R^2 = 0.9994 for *cis*-zearalenone) were used for quantitation of zearalenone in edible oil samples.

Molecular Simulations. *Classical Force Field Simulations.* Two strategies with different classical force fields and physical conditions were employed in order to investigate their suitability for the estimation of the steady state distribution of *trans*- and *cis*-zearalenone after interconversion. At first, the isomers were parametrized according to the Merck molecular force field (mmff)[28] and simulated with implicit water at 1500 °C using the Hybrid Monte Carlo algorithm,[29] which is known for an efficient sampling of the conformational space.[30] Afterward, all geometries were minimized using the conjugate gradient method.[31,32] In addition, the generalized amber force field (GAFF)[33] was used for molecular dynamics simulations with Gromacs[34−36] including explicit ffamber_tip4pew water molecules and starting sampling from the most favorable energy minimized structure (global minimum) of each zearalenone isomer as provided by mmff above. Charges were assigned with the AM1-BCC method[37,38] and the temperature of the canonical ensemble was coupled to 293 °C.

Quantum−Chemical Simulations. All quantum chemical analyses including geometry optimizations, single point energy calculations and pK_a value estimations were carried out using Gaussian 09. The density functional theory (DFT) was employed using Becke's three-parameter hybrid exchange functional along with the Lee−Yang−Parr correlation (B3LYP)[39,40] in combination with the 6-31+G* basis set.[41] Both acetonitrile and water were chosen as implicit solvents with a self-consistent reaction field (SCRF)[42] computed on the basis of the integral equation formalism for the polarizable continuum model (IEFPCM).[43,44]

RESULTS AND DISCUSSION

***cis*-Zearalenone Calibration Standard.** In order to ensure a reliable quantitation of *cis*-zearalenone in edible oils a corresponding calibration standard is necessary, which is commercially not available. Thus, the first aim was to prepare *cis*-zearalenone and to characterize this compound for its chemical identity and purity. On the basis of previous studies,[12,14] a suitable method is the photochemically induced isomerization of the double bond of the macrocyclic lactone ring. Beside irradiation energy and reaction time, the *trans*-/*cis*-isomerization may also depend on the solvent. Different polar solvents were tested for their suitability to achieve a high conversion rate by irradiation of *trans*-zearalenone solutions with UV-light (Table 2).

Table 2. Isomerization of *trans*-Zearalenone in Different Solvents (232 μg/kg) by Irradiation with UV-Light (λ = 350 nm) after 48 h

		cis-/*trans*-zearalenone ratio[a]		
solvent	polarity E^0 (Al_2O_3)	t = 0 h	t = 48 h	total zearalenone recovery[b] (%)
methanol	0.95	0.01	2.49	78.1
acetonitrile	0.65	0.02	1.39	44.2
ethyl acetate	0.58	0.01	4.80	73.8
n-hexane	0.00	0.03	5.18	77.3

[a]Results are means based on measurements in duplicate. [b]Sum of *trans*- and *cis*-zearalenone.

Although a clear correlation between solvent polarity and *cis*-/*trans*-isomerization rate could not be concluded, it was obvious that *n*-hexane representing the solvent with the lowest dipole moment, yielded the highest isomerization rate (5.18). In contrast, acetonitrile, often used as solvent for commercial zearalenone standards, showed the lowest *cis*-/*trans*-conversion rate (1.39) and, additionally, only 44% remaining zearalenone after irradiation. It is supposed that further light-induced reaction products were preferably formed in the presence of acetonitrile. The highest zearalenone recovery was obtained by using methanol showing, however, a rather low isomerization rate (2.49). Because zearalenone is only slightly soluble in *n*-hexane, ethyl acetate was used as the best alternative for the photochemical synthesis of *cis*-zearalenone based on the results of Table 2. In addition, the irradiation time for synthesis was set to 8 h to avoid side-reaction products. Purified *cis*-zearalenone was subjected to ^{1}H NMR for structure elucidation measured in comparison to pure *trans*-zearalenone (Figure 2).

cis-Zearalenone was identified by the vicinal H−H coupling constant $^3J_{HH}$ of the proton at C_{12} position ($^3J_{HH}$ = 11.9 Hz) being in the typical range of *cis* double bonds ($^3J_{HH}$: 6−14 Hz). For comparison, the $^3J_{HH}$ of *trans*-zearalenone was measured $^3J_{HH}$ = 15.2 Hz (typical range of *trans* double bonds: $^3J_{HH}$ 11−18 Hz). The doublet−doublet splitting of the H_{12} proton of *trans*-zearalenone (Figure 2) is caused by a long-range coupling to the proton at C_{13} position ($^4J_{HH}$ = 1.6 Hz) which is lower in the case of *cis*-zearalenone, indicating a torsion of the *cis*-zearalenone ring system. In addition, due to the change in the molecular shape the protons of the *cis* double bond and also other protons of *cis*-zearalenone undergo a shielding effect leading to an upfield shift compared to *trans*-zearalenone, e.g., *cis*/*trans* (δ H_{11}: 5.43/5.68 ppm; δ H_{12}: 6.62/7.01 ppm) confirming the results of other studies.[12,13]

A further unambiguous confirmation of the chemical composition and stereochemical structure of *cis*-zearalenone was obtained by single crystal X-ray structural analysis. Crystals of both zearalenone isomers belong to the centrosymmetric monoclinic $P2_1$ space group with two molecules in the elementary cell ($V_{cis\text{-zearalenone}}$ = 851.7 Å^3; $V_{trans\text{-zearalenone}}$ = 819.0 Å^3). In the crystal structures, an intramolecular hydrogen bond between the C_{16} hydroxyl group and the C_1 carbonyl oxygen helps to stabilize the molecular conformation.

Sample Survey. Ten out of 12 analyzed edible oils (83%) from German retail markets revealed measurable contents of *trans*-zearalenone. However, none of them exceeded the EU maximum level of 400 μg/kg (Table 1). The results indicate that corn oils tend to have elevated zearalenone contents compared to hempseed oils. All nine corn oils were found positive (median 194 μg/kg), confirming a previous study on zearalenone in corn oil reporting an average content of 170 μg/kg (total samples: 38, pos. samples: 38).[6] In addition, an obvious year-specific zearalenone contamination of the corn oils was observed, showing lower zearalenone contents for the oil samples produced in 2012.

The isotope dilution mass spectrometry ID-HPLC-MS/MS (Method A) was defined by CCQM (Consultative Committee for Amount of Substance − Metrology in Chemistry) as primary (ratio) method representing a high level of metrology.[45] However, the expensiveness caused by the instrumental equipment and use of a ^{13}C-labeled internal standard makes this method applicable as a reference method rather than as a routine method. Reliable alternative methods

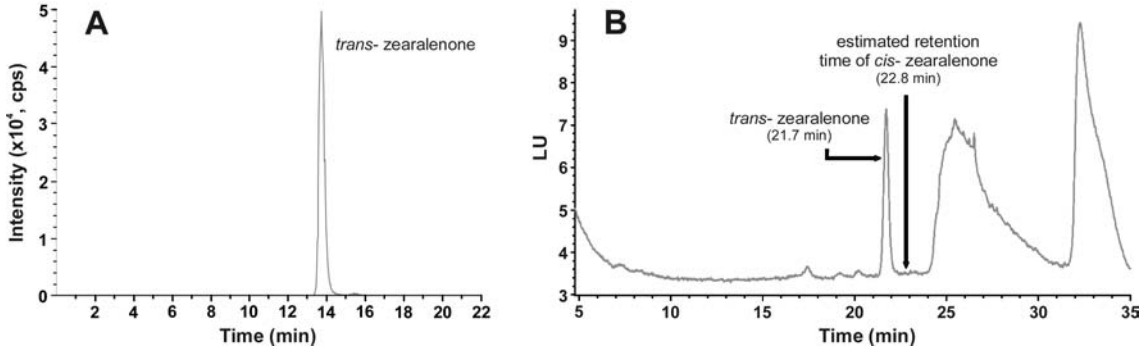

Figure 3. Chromatograms of corn oil no. 3 analyzed by (A) HPLC-MS/MS (Method A) and (B) DCHC-HPLC-FLD (Method B). The broad signals beginning at 24 min (Method B) are not caused by oil matrix but related to the HPLC gradient (fast increase of solvent B (MeCN) and reconditioning, respectively).

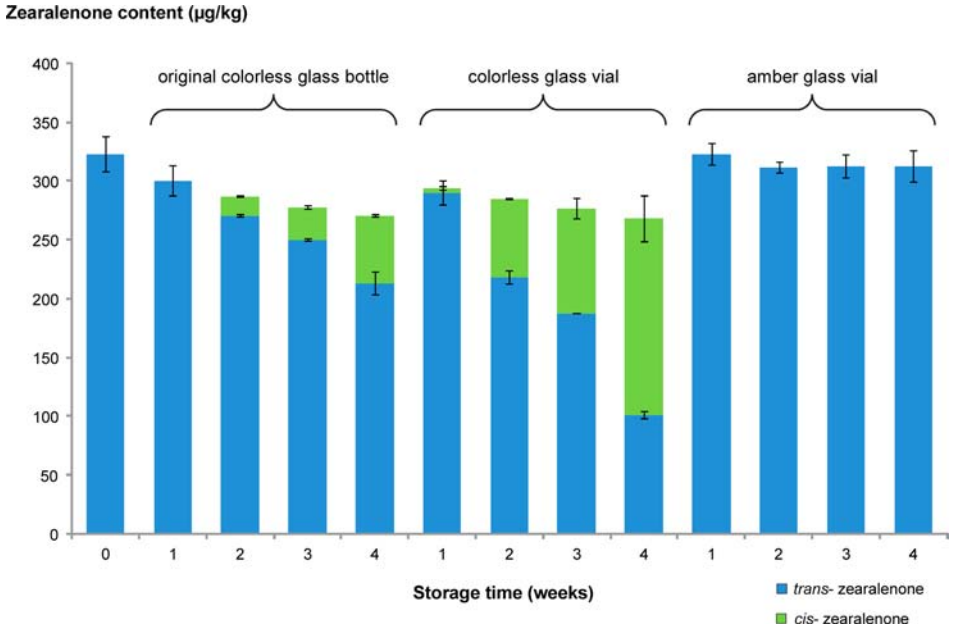

Figure 4. Isomerization of a naturally *trans*-zearalenone contaminated corn oil (no. 3) over 4 weeks of sunlight exposure (day−night rhythm), analyzed by ID-HPLC-MS/MS (Method A). The mean values and their corresponding standard deviations are given based on three independent replicates (n = 3).

are therefore needed, but currently there is no standard method available for the determination of zearalenone in edible oil. A recently published method[27] based on a selective extraction of zearalenone and effective extract purification using hydrazine-functionalized polymer resins could be promising for standardization combining cost-efficiency and accuracy. To underpin its suitability all corn oil samples from 2011 were additionally analyzed by this Method B (Table 1). While comparable mean values of Methods A and B were obtained, quite different measurement uncertainties were observed, leading to a failed F-test and consequently, preventing the applicability of a mean value t test. It must be kept in mind that the extracts analyzed by Method A contained much more matrix components due to a simple liquid−liquid partitioning step possibly causing a poorer precision even by applying ID-HPLC-MS/MS. However, due to the selective MRM mode the integration of zearalenone in the chromatogram of Method A (Figure 3) is not influenced by matrix compounds. The almost matrix-free chromatogram of Method B demonstrates the high extraction selectivity and efficient cleanup of the DCHC procedure.

Zearalenone is produced by *Fusarium* spp. on cereal plants during the period of growth and, in this time exposed to sunlight. Because of the light-sensitivity of zearalenone at least small amounts of *cis*-zearalenone could be expected in zearalenone contaminated samples. Surprisingly, *cis*-zearalenone was not detected in any of the tested oil samples not even in oil no. 3 with the highest *trans*-zearalenone contamination. These findings suggest that the preharvest stage as well as the harvesting and oil production conditions do not contribute to the conversion of *trans*- to *cis*-zearalenone. However, many edible oils are packed and stored in colorless bottles exposing a risk of light-induced isomerization. Therefore, the (in)stability of *trans*-zearalenone during the storage and use phase of an edible oil was investigated.

Alteration of Edible Oils by Sunlight Exposure. The alteration experiments were done under real-life conditions by storing the oils on a window sill inside the laboratory exposed to sunlight (day−night rhythm).

Short-Term Stability Study. In a first step it was tested if a naturally *trans*-zearalenone contaminated corn oil (no. 3, Table 1) is principally susceptible for *trans*/*cis* isomerization during storage in different glass bottles over 4 weeks. The results of the weekly sampling in Figure 4 show a conversion of zearalenone strongly depending on storage time and bottle material. In both experiments using colorless glass containers a weekly increase of the *cis*-zearalenone content was observed reaching 21.4% (original oil bottle) and 62.4% (laboratory glass vial) after 4 weeks. Furthermore, a decrease of the total zearalenone content (sum of *trans*-and *cis*-zearalenone) was detected when using colorless glass containers. This was independent of the isomerization rate. At the end of the study only about 83% of total zearalenone compared to zearalenone at t = 0 was quantitated, confirming the outcome shown in Table 2.

Quite different results were found when using amber glass vials. Here, neither an isomerization of *trans*-zearalenone nor a significant degradation over time was detected (Figure 4) leading to the conclusion that the isomerization as well as the degradation process of zearalenone are clearly related to a (sun)-light exposure which can be avoided by using amber glassware. While different processes took place in the oil depending on bottle material and storage time, no changes of oil color and turbidity were visible over the whole period of time.

Long-Term Stability Study. After finding that *cis*-zearalenone can generally be derived from *trans*-zearalenone contaminated oils during real-life storage at sunlight, the formation of a *trans*-/*cis*-zearalenone equilibrium during a long-term storage was investigated in a second step. For that purpose a corn oil was spiked with *trans*-zearalenone to 630 µg/kg containing no detectable amounts of *cis*-zearalenone. The *trans*-zearalenone content, exceeding the EU maximum level of 400 µg/kg, was confirmed by an interlaboratory comparison study (median 627 µg/kg) including 15 participants,[46] so a well

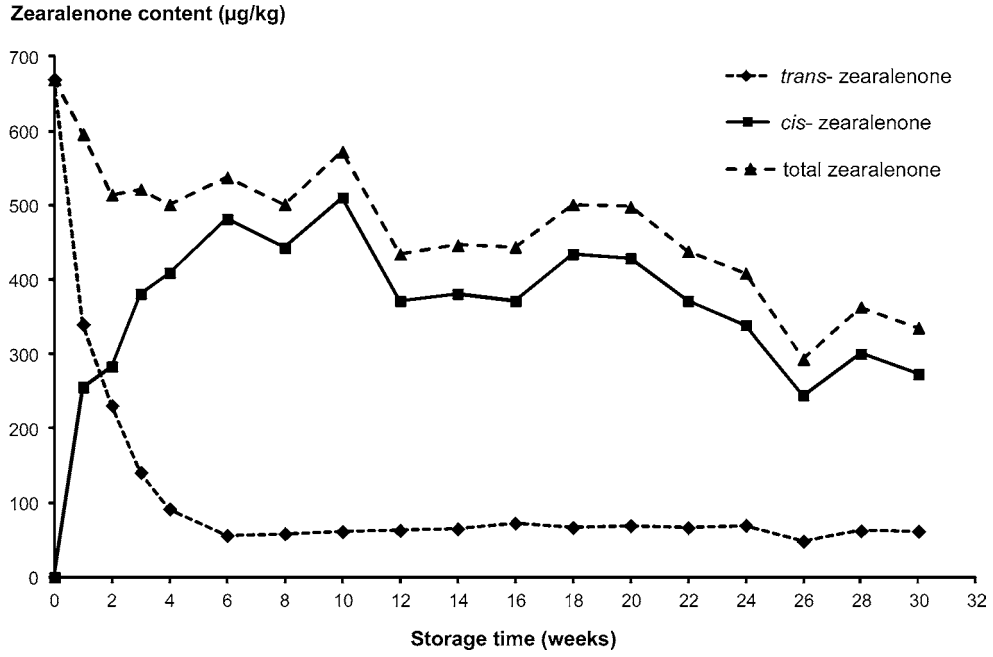

Figure 5. Light-induced isomerization of a corn oil fortified with *trans*-zearalenone (630 µg/kg) over 30 weeks of sunlight exposure (day−night rhythm), analyzed by ID-HPLC-MS/MS (Method A).

characterized reference material could be used for this stability study. The *trans*-zearalenone reference value determined at t = 0 was slightly increased (Figure 5) but within the range of uncertainty, confirming the accuracy of Method A. During the first weeks a considerable decrease of *trans*-zearalenone and, consequently, an increase of *cis*- zearalenone occurred leading to a *cis*-to-*trans* zearalenone ratio of about 9:1 after 6 weeks. That indicates either a preferred formation of the *cis*-isomer or a hindered back-reaction of *cis*- to *trans*-zearalenone. The reaction starting phase can easily be described by kinetics of pseudo first order $\ln(c/c_0) = -kt$ (c = *trans*-zearalenone content at $t > 0$; c_0 = *trans*-zearalenone content at $t = 0$; k = reaction rate). The obtained reaction rate of k = 0.485 weeks^{-1}, represented by the slope of the linear regression line, is slightly higher than the reaction rates resulting from the short-term stability study, k(original bottle) = 0.102 weeks^{-1} and k(colorless glass vial) = 0.276 weeks^{-1} possibly caused by using a different type of glass. After the sixth week the *trans*-zearalenone content remained stable (∼63 µg/kg; ∼10% of the reference value at t = 0, Figure 5) until the end of the study, and with that, falling below the EU maximum level. On the other hand, after the first phase of increasing *cis*-zearalenone values until the sixth week (482 µg/kg) a continuous decrease over the subsequent 24 weeks was observed leading to a final *cis*-zearalenone content of 273 µg/kg. The total zearalenone content significantly decreased up to the end of the study (335 µg/kg), i.e., a loss of about 50% compared to the zearalenone content at t = 0. Because this work was not intended to investigate the identity and/or the mechanism of (light-induced) degradation products, that issue should be the focus of a future study. Several fluorescent degradation products with shorter retention times than zearalenone were detected by RP-C_{18} HPLC after irradiation of *trans*-zearalenone in organic solvents depending on the irradiation energy. However, the photochemical fate of zearalenone in a natural triglyceride matrix could be more complex, ranging from chemical degradation products to masking effects, e.g., matrix adsorption or conjugation.

A general conclusion drawn from the presented results is an obvious shift of the isomeric zearalenone ratio toward *cis*-zearalenone, possibly caused by an enhanced thermodynamic stability. Thus, quantum-chemical and classical force field simulation data were calculated in order to answer the question whether the formation of *cis*-zearalenone is preferred over the natural occurring *trans*-zearalenone.

Molecular Simulation Results. Electron density computations of *trans*- and *cis*-zearalenone were in general consensus with the X-ray structure results showing an intramolecular hydrogen bond. However, whereas calculations revealed blurred boundaries between the electrons of *cis*-zearalenone a clear electron boundary was obtained for *trans*-zearalenone. This result possibly indicates keto−enol tautomerism and suggests resonance-stabilization in case of *cis*-zearalenone. This assumption was supported by comparing pK_a values computed for both zearalenone isomers and their tautomeric counterparts. These results indicate a significantly larger entropy of *cis*-zearalenone, possibly explaining its higher concentration at equilibrium.

Mean and minimal potential energies have been computed for artificial zearalenone derivatives by reducing the lactone ring size in order to see the enthalpic/sterical effect on the equilibrium distribution of *trans*- and *cis*-zearalenone after interconversion. The GAFF and mmff simulations demonstrate that a *cis*-isomer is favored only for small rings (<10 atoms). On the basis of enthalpic entities resulting from classical force field simulations, it is not possible to explain the substantially higher preference of *cis*-zearalenone. This observation as well indicates the dominance of *cis*-zearalenone due to entropic reasons.

With this study it could be shown that edible oils from retail markets, especially corn oils, may contain remarkable amounts of *trans*-zearalenone depending on the year of production. A fast conversion to the possibly more stable *cis*-zearalenone isomer occurs if the oils are stored in colorless glass bottles

exposed to sunlight. Official maximum levels currently only exist for *trans*-zearalenone despite studies revealing an estrogenic potential of *cis*-zearalenone. A chromatographic separation of *cis*- and *trans*-zearalenone requires an optimization of the HPLC conditions which is not part of the existing standard methods. Because of the almost identical fluorescence and mass spectrometric properties, it can be assumed that routine laboratories as well as official control laboratories are not always aware of analyzing *trans*- or *cis*-zearalenone.

Overall, the issue of *cis*-zearalenone formation and occurrence in edible oils may gain significant importance for researchers, industry, and legislative bodies to control official maximum levels by developing reliable analytical methods to unambiguously analyze *trans*- and *cis*-zearalenone. Certified reference materials can contribute to the quality assurance and therefore to food safety and consumer protection.

■ ASSOCIATED CONTENT

Supporting Information

Pseudo-first-order kinetics for the isomerization of *trans*- to *cis*-zearalenone in a fortified corn oil exposed to sunlight (Figure S1). Electron densities of *trans*- and *cis*-zearalenone computed with Gaussian (Figure S2). Minimal potential energies of *trans*- and *cis*-zearalenone as well as artificial derivatives, simulated according to the generalized amber force field and the Merck molecular force field (Figure S3). This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS USED

DCHC, dynamic covalent hydrazine chemistry; ID, isotope dilution; MRM, multiple reaction monitoring

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