



Analytical Methods

Simultaneous determination of zearalenone and its derivatives in edible vegetable oil by gel permeation chromatography and gas chromatography–triple quadrupole mass spectrometry

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ABSTRACT

A sensitive gas chromatographic–triple quadrupole mass spectrometric (GC–QqQ MS) analytical method, for the determination of zearalenone and its five derivatives in edible vegetable oil, was developed. After the vegetable oil samples were prepared using gel permeation chromatography, the eluent was collected, evaporated and dried with nitrogen gas. The residue was silylated with N,O-bis-trimethylsilyltrifluoroacetamide, containing 1% trimethylchlorosilane. GC–QqQ MS was performed in the reaction-monitoring mode to confirm and quantify mycotoxins in vegetable oil. The limits of quantitation were 0.03–0.2 $\mu\text{g kg}^{-1}$ for the six mycotoxins. The average recoveries, measured at 2, 20 and 200 $\mu\text{g kg}^{-1}$, were in the range 80.3–96.5%. Zearalenone was detected in the range 5.2–184.6 $\mu\text{g kg}^{-1}$ in nine maize oils and at 40.7 $\mu\text{g kg}^{-1}$ in a rapeseed oil from the local market.

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1. Introduction

Zearalenone (ZON) [6-(10-hydroxy-6-oxo-trans-1-undecenyl)-resorcylic acid lactone] and its derivatives (shown in Fig. 1) are produced by several *Fusarium* fungi including *Fusarium graminearum*, *Fusarium culmorum*, *Fusarium crokwellense*, *Fusarium sambucinum* and *Fusarium equiseti*, which normally colonise grains including maize, barley, oats, wheat and sorghum (Placinta, D'Mello, & Macdonald, 1999). The structures of ZON and its derivatives resemble 17 β -estradiol and the potential for endocrine disruption has been evaluated using several models. The ability of some of these compounds to give an estrogen-like response has been reported. For example, uterine weight and epithelial cell height increased in a dose manner in rats after three-day oral exposure to the highest doses of ZON (Heneweer, Houtman, Poortman, Groot, Maliepaard, & Peijnenburg, 2007). ZON and α -zearalenol

(ZOL) at picomolar levels influenced negatively the chromatin structural stability and viability of swine spermatocytic cells (Benzoni, Minervini, Giannoccaro, Fornelli, Vigo, & Visconti, 2008). These compounds can act as potential endocrine disruptors, at the level of steroid receptor signalling, by altering hormone production as measured by the H295R steroidogenesis assay (Frizzell et al., 2011). ZON and its derivatives also demonstrate an estrogen-like effect inducing the proliferation of ER-positive human breast cells (MCF-7) (Minervini, Giannoccaro, Cavallini, & Visconti, 2005). Considering the capacity of these mycotoxins to interfere with the homeostasis (Massart, Harrell, Federico, & Saggese, 2005; Massart & Saggese, 2010), residues in agricultural products are a significant human health concern.

Many methods have been developed to evaluate ZON and its derivatives in agricultural products, including HPLC fluorescence (Trucksess, Fu, Oles, & White, 2011) and UPLC-MS/MS (Jin, Han, Cai, Wu, & Ren, 2010) in grain, high-field asymmetric waveform ion mobility spectrometry (McCooye, Kolakowski, Boison, & Mester, 2008) in maize, HPLC-FLD (Zhang et al., 2011) and UPLC-MS/MS (Han, Ren, Zhou, Luan, Cai, & Wu, 2011) in Chinese herbs, HPLC-MS/MS (Di Mavungu et al., 2009) in food supplements and

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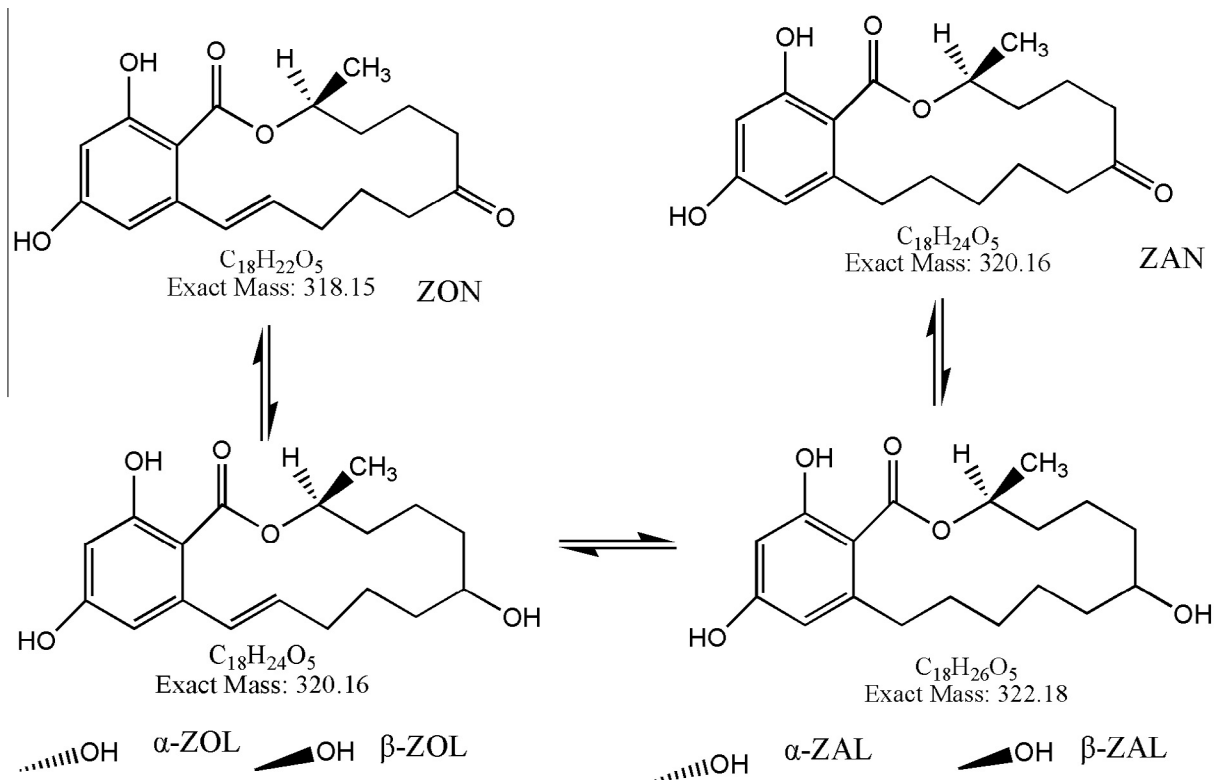


Fig. 1. The structure of zearalenone and its derivatives.

LC-MS/MS (Ediage, Di Mavungu, Monbaliu, Van Peteghem, & De Saeger, 2011) in cassava flour and peanut cake. The evaluation of ZON and its derivatives in environmental samples has been performed using GC-MS (Kinani, Bouchonnet, Bourcier, Porcher, & Aitaissa, 2008). But, only a few methods have been developed for the detection of ZON in edible vegetable oil. Siegel reported that the ZON in edible oil could be extracted and cleaned up based on dynamic covalent hydrazine chemistry and determined using LC-FLD and LC-MS/MS (Siegel et al., 2010). Kappenstein measured ZON in edible oil with size exclusion chromatography and LC-ESI-MS/MS (Kappenstein et al., 2005). In 2005, Cavaliere reported that ZON and its derivatives could be detected in maize samples collected from different farms in the north-central region of Italy (Cavaliere, D'Ascenzo, Foglia, Pastorini, Samperi, & Laganà, 2005), demonstrating that both ZON and its derivatives contaminate the raw materials of vegetable oil. This work developed a method for the detection of ZON and its derivatives in edible vegetable oil. It was convenient to remove fatty matrices using gel permeation chromatography (GPC), which has been used previously to purify edible oil samples for the detection of other residues including ZON (Kappenstein et al., 2005) and phthalate esters (Sun, Yang, Li, Zhang, & Sun, 2012). Because ZON and its derivatives have similar structures and molecular weights, in the range 318–322, GPC provided a simple way of separating target compounds from the triglyceride matrix, by analysing their hydrodynamic behaviour on the gel column, and collecting the eluent over narrow time intervals.

With advantages including high selectivity and specificity, triple quadrupole mass spectrometry combined with HPLC is a useful approach for the determination of mycotoxin in agriculture products (Di Mavungu, et al., 2009; Ediage et al., 2011; Han, Ren, Zhou, Luan, Cai, & Wu, 2011; Jin et al., 2010; Spanjer, Rensen, & Scholten, 2008; Zhang, et al., 2011). An alternative would be GC-MS after derivatisation to reduce the polarity of the target compounds (Cunha, Faria, & Fernandes, 2009; Kinani et al., 2008).

GC-MS and GC-MS/MS are the preferred tools for the detection of pesticide residues, especially for non-polar chemical compounds in agricultural products, and have been widely applied (Erger, Balsaa, Werres, & Schmidt, 2012; Koesukwiwat, Lehotay, & Leepipatiboon, 2011; Nardelli, Dell'Oro, Palermo, & Centonze, 2010; Vazquez-Quintal, Munoz-Rodriguez, Medina-Peralta, & Moguel-Ordonez, 2012; Xu et al., 2012). Gas chromatography triple quadrupole mass spectrometry (GC-QqQ MS) substantially improves the selectivity and sensitivity compared to single-stage MS because of the elimination of isobaric interference and the reduction of chemical noise (Lambropoulou & Albanis, 2007). Compared with ion-trap gas chromatography-tandem mass spectrometry, which has been applied for residue detection in agriculture products and foods (Jiang et al., 2011; Makabe, Miyamoto, Hashimoto, Nakanishi, & Hasegawa, 2010; Steiniger, Lu, Butler, Phillips, & Fintschenko, 2010; You, Wang, & Lydy, 2010; Zacharis, Rotsias, Zachariadis, & Zotos, 2012), GC-QqQ MS is more stable and provides better quantitation results, especially for low levels in complex matrices.

In our work, based on the optimum conditions described by Kinani (Kinani et al., 2008), GC-QqQ MS was five times more sensitive to ZON and its derivatives compared with HPLC-MS/MS at the same concentration. Combined with a simple GPC extraction technique, a sensitive method was developed for the detection of ZON and its derivatives including α -ZOL, β -ZOL, α -zearelanol (ZAL), β -ZAL and zearalanone (ZAN) in vegetable oil by GC-QqQ MS. The occurrence of these contaminants was evaluated in 40 samples from local markets.

2. Experimental

2.1. Reagents and materials

Standards including α -ZOL, β -ZOL, α -ZAL, β -ZAL, ZON and ZAN (purity $\geq 95\%$) were obtained from Sigma-Aldrich (St. Louis,

USA). HPLC-grade ethyl acetate, toluene and cyclohexane were obtained from Tedia (Fairfield, USA). N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) containing 1% trimethylchlorosilane (TMCS) was obtained from Sigma-Aldrich (St. Louis, USA). Standard stock solutions of the six compounds were prepared in methanol at a concentration at 100 mg L⁻¹. All of the solutions were stored in a refrigerator at 4 °C. Working standard solutions containing the six compounds, at concentrations of 0.1, 1 and 10 mg L⁻¹, were prepared in methanol and stored at 4 °C.

2.2. Optimisation of the sample preparation procedure by GPC

GPC (LC-Tech GPC Vario, Dorfen, Germany) equipped with an auto-sampler, a solvent delivery module and a fraction collector was used for the analyses. The standard sample loop was made of polytetrafluoroethylene and set to 5 mL. A common glass column (500 mm × 25 mm i.d.), packed with 50 g of 200–400 mesh Bio-Breads S-X3 resin, (Bio-Rad Laboratories GmbH, München, Germany) was used. The mobile phase was ethyl acetate-cyclohexane solution (1:1, v/v) at a flow rate of 5 mL min⁻¹. 5 mL of an ethyl acetate-cyclohexane solution (1:1, v/v) containing 5 µg of each mycotoxin was injected on to the GPC. For the first 10 min, the eluent was discarded but, for the subsequent 20 min, the eluent was collected every 1 min. Each fraction was evaporated at reduced pressure and dried under nitrogen. Mycotoxins in each fraction were derivatised and analysed using GC–QqQ MS.

2.3. Sample preparation

Aliquots (2 g) of vegetable oil samples were diluted to 10 mL with an ethyl acetate-cyclohexane solution (1:1, v/v), shaken vigorously and vortex-mixed at 2000 rpm for 1 min, before 5 mL was injected on to the GPC (LC Tech, German). The effective eluent was collected in a 110 mL glass tube and evaporated with automatic rotation (100 rpm) at 40 °C. After the residue was transferred to a glass tube and dried by a gentle nitrogen stream, it was reconstituted in 0.2 mL of toluene and added to a mixture of BSTFA + TMCS (99:1, 0.2 mL). For complete trimethylsilyl derivatisation, the mixture was vortex-mixed for 1 min, sealed and kept at 60 °C for 40 min. Finally, the solution was mixed with 0.1 mL of toluene, and 1 µL of the derivatised sample analysed by GC–QqQ MS.

2.4. GC analysis

Gas chromatographic analyses were performed on a Trace GC Ultra equipped with a split/splitless injector (Thermo Fisher Scientific, Waltham, USA). The analytical column used was a TR-5 MS (30 m × 0.25 mm, ID 0.25 µm film thickness) coated with a 5% phenylmethylpolysiloxane stationary phase (Thermo Fisher Scientific). High-purity helium (≥99.999%) at a constant flow rate of

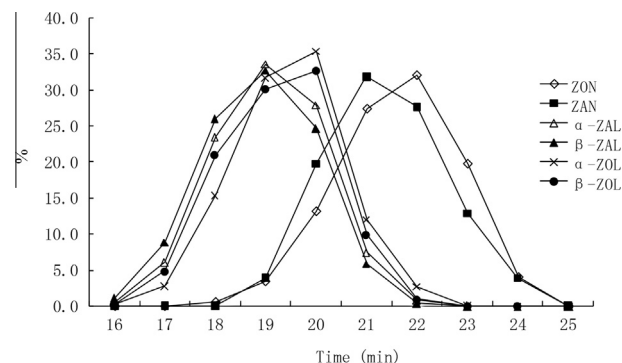


Fig. 2. The diagram for the recovery percent of six compounds in each fraction.

1 mL min⁻¹ was used as the carrier gas. Argon, at a pressure of 0.13 Pa, was used as the collision gas. The split/splitless injection port was maintained in the splitless mode for 1 min and set at a fixed temperature (280 °C). The oven temperature program was as follows: an initial temperature of 80 °C for 5 min, a temperature increase to 300 °C at a rate of 5 °C per min and finally isothermal at 300 °C for 10 min.

2.5. Mass spectrometric detection

A TSQ Quantum GC mass spectrometer (Thermo Fisher Scientific) operated in the electron impact (EI) mode was used. The ion source and transfer line temperatures were set at 250 °C and 280 °C, respectively. The emission current of the ionisation filament was set at 100 µA, generating electrons with 70 eV. The analyses were carried out with a filament-multiplier delay of 5 min. Instrument control and data acquisition were managed with a personal computer running Xcalibur (version 2.0.7).

Silylation is a nucleophilic substitution on the silicon atom of the silyl donor. In our work, BSTFA with 1% TMCS was used as the derivatisation reagent. The corresponding derivatives for the six compounds were ZON-2TMS, ZAN-2TMS, α,β-ZOL-3TMS and α,β-ZAL-3TMS. The retention times for the six derivatives were determined in the full scan mode. The *m/z* of the ion with the highest relative abundance in the mass spectrum was selected as the precursor ion for each derivative. For the determination of the six derivatives in the reaction-monitoring (SRM) mode, the collision energy was optimised for each precursor ion to produce two product ions with the highest sensitivity (peak area and S/N ratio), and the optimum parameters are listed in Table 1.

2.6. Validation procedure

The matrix matched calibration curves were constructed as follows: according to the sample preparation procedure, the blank

Table 1

Parameters of six derivatizations for GC–QqQ MS detection, matrix matched calibration (0.2–400 µg L⁻¹), LOD and LOQ in edible vegetable oil.

Compound	Molecular weight	Precursor ion (<i>m/z</i>)	Product ion (<i>m/z</i>)	Collision energy (eV)	Regression equation	R ²	LOD (µg kg ⁻¹)	LOQs (µg kg ⁻¹)
ZAN-2TMS	464	449	335	15	$y = 20965x + 3758$	0.9991	0.01	0.03
			361	15				
α-ZAL-3TMS	538	433	295	20	$y = 19729x + 4986$	0.9949	0.01	0.03
			389	10				
β-ZAL-3TMS	538	433	295	20	$y = 18884x + 4024$	0.9912	0.01	0.03
			389	10				
ZON-2TMS	462	462	317	20	$y = 2068.3x + 1061$	0.9962	0.06	0.2
			333	15				
α-ZOL-3TMS	536	446	317	15	$y = 4292.1x + 3039$	0.9917	0.03	0.1
			333	10				
β-ZOL-3TMS	536	446	317	15	$y = 18428x + 4385$	0.9950	0.01	0.03
			333	10				

vegetable oil samples were processed and these samples mixed with the standards and derivatised to create a series of matrix-matched calibrants in the concentration range 0.2–400 $\mu\text{g L}^{-1}$ for all six analytes.

The optimised method was subjected to validation based on a single-laboratory validation approach. The analytical performance characteristics, such as linearity (dynamic ranges and R^2), precision and limits of quantification (LOQ), were evaluated. The calibration curves were obtained by plotting the peak areas of the quantitative ion transition against the analyte concentrations with regression analysis. The linearity was expressed as a correlation coefficient. The limits of detection (LODs) were calculated as three times the signal-to-noise (peak to peak) ratio of the quantitative ion transition in vegetable oil. The LOQs were determined as 10-times the signal-to-noise ratio of the quantitative ion transition in vegetable oil.

Spiked samples were created by fortifying blank samples with the target mycotoxins at three concentrations (2, 20 and 200 $\mu\text{g kg}^{-1}$) and allowing them to equilibrate for 1 h. Recovery was calculated by comparing the mean of five parallel measurements with the fortified concentration in vegetable oil. The precision of the method was determined by the measuring the repeatability and reproducibility. To determine repeatability, the same operator analysed spiked vegetable oil samples five times on the same day using the same instrument, and the RSDs were calculated. To determine reproducibility, different operators analysed each spiked vegetable oil on three different days using same instrument, and the RSDs were calculated.

3. Results and discussion

3.1. Sample preparation procedure

5 mL of an ethyl acetate-cyclohexane solution (1:1, v/v) containing 5 μg of each mycotoxin was injected on to the GPC. According to the elution diagram (Fig. 2), the fractions from 17 min to 23 min were collected to ensure recoveries for the six compounds were >95%. The procedure, where fractions during the first 16 min were discarded and the fractions in the subsequent seven min were collected, was applied in all the experiments.

3.2. Fragmentation of ZON and its derivatives by GC-QqQ MS

Two steps should be performed to obtain precursor ions and daughter ions for detection using tandem mass spectrometry. In the first step, the precursor ion or an entire cluster of parent ions is isolated and, in the second step, the dissociation of the precursor ion is achieved by its collision with an inert gas. The M^+ at a m/z of 462 and $[M-15]^+$ at a m/z of 449 were selected as precursor ions for ZON-2TMS and ZAN-2TMS because they were produced in greater abundance with higher mass numbers in the EI ionisation mass spectrum at 70 eV. Considering the structural resemblance of ZON-2TMS and ZAN-2TMS, the double bond in ZON-2TMS may strengthen significantly the stability of M^+ . The $[M-90]^+$ at a m/z of 446 and $[M-105]^+$ at a m/z of 433 possessed the greatest intensities in the EI ionisation mass spectra, under 70 eV, for ZOL-3TMS and ZAL-3TMS. These were selected as precursor ions and produced similar intensities for the α - and β -isomers under the same conditions. Under SRM with transitions at a m/z from 433 to 295 and from 433 to 389, α -ZAL-3TMS and the β -isomer showed similar intensities at the same concentration. However, with transitions at a m/z from 446 to 317 and 446 to 333, α -ZOL-3TMS showed a significantly lower intensity than the β -isomer at the same concentration (see Fig. 3A). The full scan spectrum of the precursor ion could provide some explanation for this. The EI

ionisation mass spectrum at 70 eV for the α -ZAL-3TMS ion with a m/z of 433 was similar to that of the β -isomer (Fig. 3B.1.2.3), while the spectrum for the α -ZOL-3TMS ion at a m/z of 446 showed significantly more fragments than β -ZOL-3TMS (Fig. 3B.4.5.6). It was also noted that the α -ZOL-3TMS ion at a m/z of 446 was more liable to fragment than β -ZOL-3TMS under 70 eV. High-resolution mass spectrometry combined with gas chromatography was not available for this work. Information about the fragment ions structures was not acquired and more details about the different

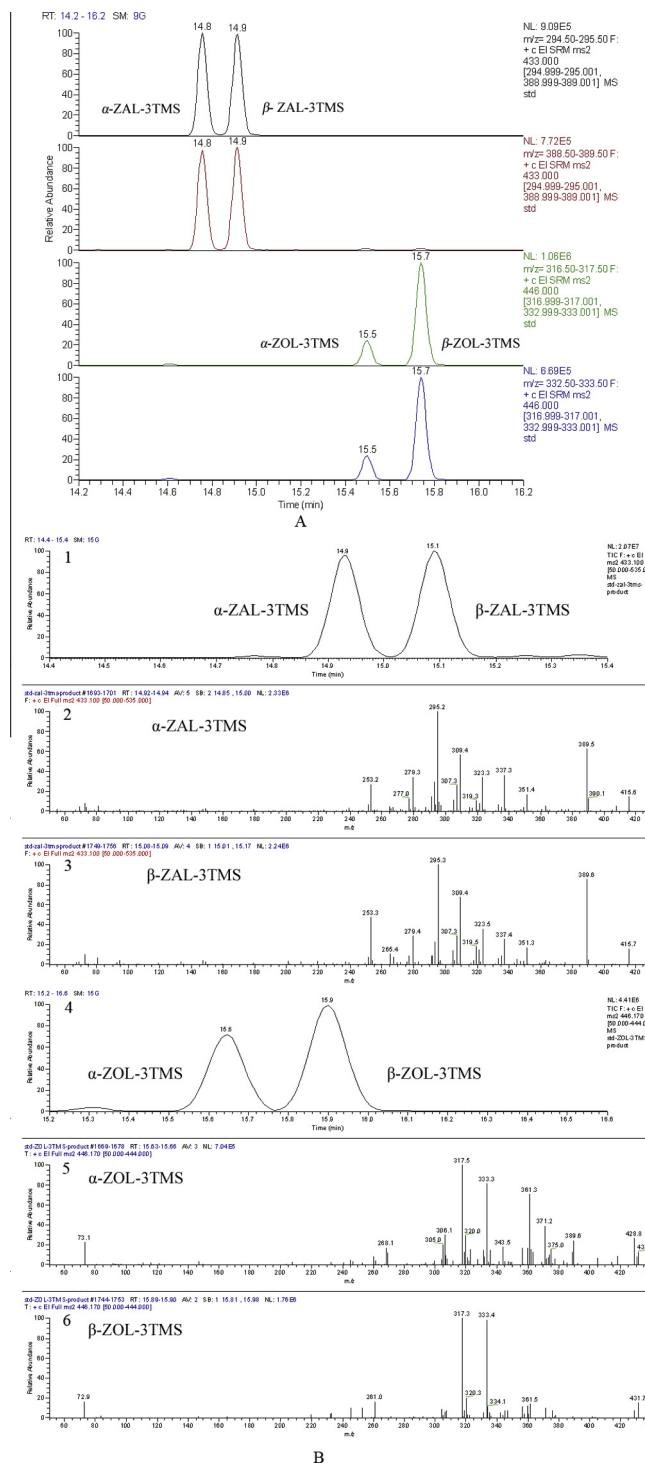


Fig. 3. SRM chromatograms for α,β -ZOL-3TMS and α,β -ZAL-3TMS at the same concentration (A). EI ionisation mass spectrum for m/z 433 of α,β -ZAL-3TMS under 70 eV (B 1.2.3). EI ionisation mass spectrum for m/z 446 of α,β -ZOL-3TMS under 70 eV (B 4.5.6).

fragmentation behaviours of α -ZOL-3TMS and β -ZOL-3TMS may be considered in future work.

3.3. Matrix effect

To compensate for matrix effects, which could cause errors in the quantification of the target mycotoxin, matrix-matched calibrations were analysed. The matrix effect was determined by comparing signals from ZON and its derivatives. In spiked vegetable oil samples ($20 \mu\text{g kg}^{-1}$), recoveries were in the range 86.7–96.3% using matrix-matched calibration curves. Higher recoveries were obtained, especially for β -ZOL-3TMS (128.6%), when pure standards were used for the calculations. So, to obtain more accurate results, matrix-matched calibrations were applied for the calculations.

3.4. Linearity LODs and LOQs

The linearity of the method was studied at the following six concentrations in triplicate: 0.2, 1, 10, 50, 200 and $400 \mu\text{g L}^{-1}$. With the established parameters for GC–QqQ MS, the peak areas of the quantification ion transition for ZON and its derivatives were linear in the range $0.2\text{--}400 \mu\text{g L}^{-1}$, and the correlation coefficients were

greater than 0.99 in all cases (Table 1). The LODs and LOQs of the six derivatives are listed in Table 1. The LOQs were in the range $0.03\text{--}0.2 \mu\text{g kg}^{-1}$ and were acceptable for the evaluation of ZON and its derivatives in vegetable oil.

3.5. Accuracy and precision

Accuracy and precision were assessed by spiking blank vegetable oil with the analytes at 2, 20 and $200 \mu\text{g kg}^{-1}$, respectively. Concentrations were calculated by measuring the peak areas for the ion transition and comparing these with matrix-matched standard calibrations. Data in Table 2 shows recoveries ranging from 80.3% to 96.5% with associated relative standard deviations (RSDs) $\leq 11.6\%$. The inter-day precision was determined by analysing spiked samples on three separate days. In general, the reproducibility was between 8.1% and 14.0%. Fig. 4 shows chromatograms for six compounds spiked at $0.2 \mu\text{g kg}^{-1}$ in vegetable oil.

3.6. Real samples

To assess the applicability of the proposed method for the detection of six mycotoxins in vegetable oil samples, 40 samples including 16 maize oils, six peanut oils, six rapeseed oils, six

Table 2

The recoveries, method accuracy and precision of ZON and its derivatives spiked at $2\text{--}200 \mu\text{g kg}^{-1}$ in blank vegetable oil when calculated with matrix solution calibration curve (A); spiked at $20 \mu\text{g kg}^{-1}$ in blank vegetable oil when calculated with solvent calibration curve (B).

Compounds	Recoveries (%) (RSD ^a %, RSD ^b %)			
	A			B
	2	20	200	20
ZAN-2TMS	86.8 (8.2,10.2)	86.7 (7.5, 9.5)	93.3 (9.8, 8.9)	90.5 (8.9,11.7)
α -ZAL-3TMS	80.3 (11.6, 13.7)	88.2 (7.9, 10.4)	95.7 (5.3, 8.7)	91.2 (7.8, 10.5)
β -ZAL-3TMS	82.3 (8.8, 14.0)	91.7 (6.8, 8.1)	96.5 (4.9, 10.3)	115.7 (6.4, 12.6)
ZON-2TMS	84.1 (6.7, 9.4)	90.5 (6.9, 9.7)	90.7 (5.4, 11.6)	104.6 (9.6, 13.7)
α -ZOL-3TMS	88.3 (8.4, 9.9)	87.0 (7.2, 10.3)	89.4 (7.6, 13.8)	127.3 (7.4, 9.2)
β -ZOL-3TMS	82.8 (6.2, 11.4)	96.3 (8.1, 12.2)	94.8 (3.5, 9.1)	128.6 (8.0, 9.5)

^a Intra-day ($n = 5$).

^b Inter-day ($n = 15$).

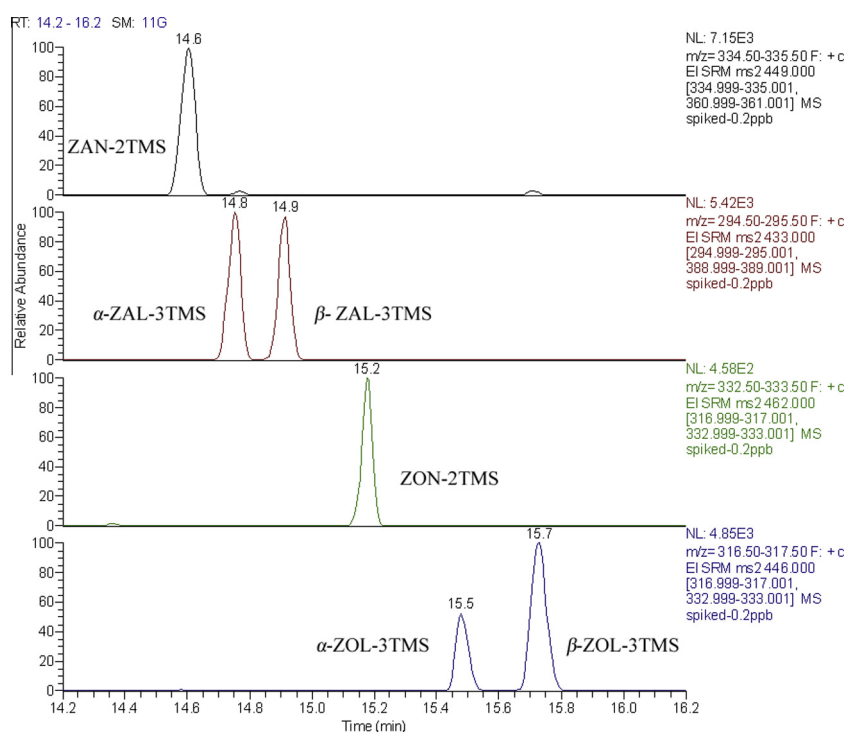


Fig. 4. SRM chromatograms for the derivatized ZON and its derivatives spiked at $0.2 \mu\text{g kg}^{-1}$ in blank vegetable oil sample.

soybean oils and six sunflower oils purchased from local markets, were tested. ZON was found at concentrations of 5.2, 11.6, 13.3, 24.2, 26.9, 58.3, 129.2, 132.5 and 184.6 $\mu\text{g kg}^{-1}$ in the nine maize oils and at 40.7 $\mu\text{g kg}^{-1}$ in a rapeseed oil. Based on the EU limit of 400 $\mu\text{g kg}^{-1}$ for ZON in refined maize oil, the residue levels found were acceptable.

4. Conclusions

A reliable analytical approach, based on GC–QqQ MS, was developed for the determination of ZON and its derivatives in edible vegetable oil. Convenient sample preparation was achieved by using GPC. Matrix-matched calibration was used to compensate for matrix effects and provide accurate results for the determination of ZON and its derivatives in vegetable oils. Recoveries were in the range 80.3–96.5% and associated with RSDs of 11.6% or less. The LOQs were 0.03–0.2 $\mu\text{g kg}^{-1}$ for the all six mycotoxins. This method is applicable to evaluation of vegetable oil contamination by these mycotoxins.

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