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Validated UPLC-MS/MS Methods to Quantitate Free and Conjugated *Alternaria* Toxins in Commercially Available Tomato Products, Fruit and Vegetable Juices in Belgium

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ABSTRACT

Ultra-performance liquid chromatography tandem mass spectrometry and Quick, Easy, Cheap, Effective, Rugged and Safe based analytical methodologies to quantitate both free (alternariol (**1**), alternariol monomethyl ether (**2**), tenuazonic acid (**3**), tentoxin (**4**), altenuene (**5**), altertoxin-I (**6**)) and conjugated (sulfates and glucosides of **1** and **2**) *Alternaria* toxins in fruit and vegetable juices and tomato products were developed and validated. Acceptable limits of quantitation (0.7-5.7 µg/kg), repeatability (RSD_r < 15.7%), reproducibility (RSD_R < 17.9%) and apparent recovery (87.0-110.6%) were obtained for all analytes in all matrices investigated. 129 commercial foodstuffs were analyzed, and **3** was detected in 100% of tomato product samples (<LOQ to 333 µg/kg), while **1**, **2**, **4** and **5** were also frequently detected (21-86%, <LOQ to 62 µg/kg). Moreover, low levels (<LOQ to 9.9 µg/kg) of modified *Alternaria* toxins (sulfates of **1** and **2**) were repeatedly detected. A deterministic dietary exposure assessment revealed the possible risk for human health related to the presence of **1** and **2** in tomato based foodstuffs, whereas **3** is unlikely to be of human health concern.

KEYWORDS: *Alternaria*, (modified) mycotoxins, UPLC-MS/MS, method development and validation, dietary exposure assessment

Introduction

Alternaria fungal species are omnipresent in the environment. A widespread natural occurrence of *Alternaria* mycotoxins, i.e. toxic secondary metabolites produced by these fungi, has been reported in various fruits and vegetables as well as their derived products, such as juices, beverages, sauces and concentrates[1-7], thus designating them as susceptible commodities. The most prevalent *Alternaria* toxins are **1**, **2**, **3** and **4**, whereas occurrence of **5** and altertoxins is reported to be rather scarce, mainly due to shortcomings in current analytical methodologies. Maximum concentrations of *Alternaria* toxins reported in commercial food products were in the range of 1-10^3 µg/kg[1], while higher levels were found in samples visibly infected with *Alternaria* rot, i.e. in products obviously not suitable for human consumption.[8] About three decades ago, extremely high levels of **3** (> 100 mg/kg), and high levels of **1** (58 mg/kg) and **2** (2.3 mg/kg) were reported in apples and tomatoes visibly affected by *Alternaria* rot.[9,10] Later, high **1** and **3** levels (> 50 mg/kg) were found in a tomato sample with a typical decay due to *Alternaria* spoilage, leading to the assumption that a single *Alternaria*-infested tomato within a large batch of tomatoes may be enough to measurably contaminate a certain derived product.[5,11] Indeed, due to the limitations of the current industrial processes to completely eliminate the rotten tissues[8], and the reported stability of **1**, **2** and **6** in fruit juices[12] and during tomato processing[13], it is obvious that these mycotoxins are likely to be present in commercial end products.[14]

Mycotoxins, like other xenobiotics, can be partly metabolised in living plants leading to the formation of conjugated toxins.[15] Conjugation of the parent mycotoxins to glucose, sulfates and other sugar moieties has been reported. Shortly after transformation of zearalenone (ZEN) to the β-D-glucopyranoside conjugate was demonstrated in maize cell cultures[16], the term “masked mycotoxins” first appeared to define a mycotoxin derivative that may be

cleaved during digestion in mammals to release its parent form.[17] Based on a more recent comprehensive classification[18], these conjugated mycotoxins are now referred to as "modified mycotoxins".[19] A rather important discussion with respect to modified mycotoxins, is whether they can be hydrolysed and absorbed in the gastrointestinal (GI) tract, thereby further contributing to the overall exposure. Currently information on the bioavailability of modified mycotoxins is very limited.[19] Therefore, the European Food Safety Authority (EFSA) has recommended national agencies to gather occurrence data on these modified forms using properly validated and sensitive routine analytical methods.[19] Obviously, the availability of reference standards is a prerequisite to realize these recommendations. Consequently, over the last two decades, bio(organic) synthesis has been successfully applied to obtain mycotoxin conjugates, including *Alternaria* toxin conjugates, to be used as reference standards[15,20-24], which self-evidently has led to the inclusion of toxin conjugates in multi-mycotoxin analytical methodolgies.

Although the conversion of zearalenone (ZEN), deoxynivalenol (DON), T-2 and HT-2 toxins, fusarenon-X (FUS-X), nivalenol (NIV), diacetylscirpenol (DAS), neosolaniol (NEO) and ochratoxin A (OTA) to their modified forms has been reported so far[15,19,25], the occurrence of modified forms has only frequently been described for DON and ZEN in naturally infected maize, cereals and derived cereal products.[25-28] Furthermore, natural occurrence of modified forms of minor trichothecenes such as NIV[29], as well as the regulated T-2 and HT-2 toxins[30], has only recently been described. Also, our research group recently described a validated UPLC-MS/MS method for the simultaneous determination of free and conjugated (glucosides and sulfates of **1** and **2**) *Alternaria* toxins in cereal-based foodstuffs, but did not detect toxin conjugates in any of the 24 samples analyzed.[31] So far, natural occurrence of modified forms of *Alternaria* toxins has never been reported. In this study, UPLC-MS/MS

based sample preparation methodologies were developed and validated for the simultaneous determination of free and conjugated *Alternaria* toxins (depicted in Figure 1) in fruit and vegetable juices and tomato products. Additionally, the occurrence of these (conjugated) mycotoxins was investigated in foodstuffs commercially available on the Belgian market using the validated methods. Finally, these occurrence data were combined with previously reported consumption data[32] to assess the dietary exposure to **1**, **2** and **3** in tomato products.

Materials and methods

Chemicals and reagents

1 (1 mg, standard) was procured from Fermentek (Jerusalem, Israel) and dissolved in 1 mL of methanol:dimethylformamide (MeOH:DMF, 60:40, v/v). Certified reference standards of **2**, **3** and **4** (101.3, 100.5 and 100.3 µg, respectively, dried down) were obtained from Romer Labs Diagnostic GmbH (Tulln, Austria). **3** and **4** were dissolved in 1 mL of acetonitrile (AcN), while 2 was dissolved in 1 mL of MeOH:DMF (60:40, v/v). **5** (1 mg/mL, in methanol) was procured from the Institut für Organische Chemie (Karlsruher Institut für Technologie, Germany), while Dr. Michele Solfrizzo (ISPA-CNR, Bari, Italy) attentively provided **6** (200 µg/mL, in AcN). Reference standards of conjugated *Alternaria* toxins (**7**, **8**, **9** and **10**) and isotopically labeled internal standards [$^{13}C_6$,^{15}N]-**3** and [$^{2}H_4$]-**2** were provided by the Institute of Applied Synthetic Chemistry (University of Technology, Vienna, Austria) and the Chair of Analytical Food Chemistry (Technische Universität München, Freising, Germany), respectively. Synthesis and characterization (Nuclear Magnetic Resonance spectroscopy/high resolution MS) of conjugated *Alternaria* mycotoxins (sulfates and glucosides of **1** and **2**), labeled **2** ([$^{2}H_4$]-**2**) and **3** ([$^{13}C_6$,^{15}N]-**3**) were previously described.[3,24,31,33] A Milli-Q SP Reagent water

system (Merck Millipore, Darmstadt, Germany) was used to obtain ultra-pure water. AcN (absolute, LC-MS grade) and acetic acid (ULC/MS) were procured from BioSolve BV (Valkenswaard, The Netherlands) and AcN (HiPerSolv Chromanorm HPLC grade) was acquired from VWR International (Leuven, Belgium). Sodium sulfate (Na_2SO_4 anhydrous), sodium chloride (NaCl) and acetic acid (glacial, 100%) were provided by Merck (Darmstadt, Germany), whereas magnesium sulfate ($MgSO_4$, anhydrous) was procured from Sigma-Aldrich (Bornem, Belgium). Bondesil-C_{18} (40µm) bulk sorbent was obtained from Agilent Technologies (Diegem, Belgium).

Commercially available foodstuffs: sample collection

A total of 129 commercially available fruit and vegetable juices (apple, *n*=24; grape, *n*=14; carrot, *n*=8) and tomato products (juice, *n*=28; sauce, *n*=28; concentrate, *n*=27) were collected from local supermarkets in Belgium between February 2013 and February 2015. In accordance with Commission Regulation (EC) N° 401/2006, laying down the method of sampling for the official control of the maximum levels established for mycotoxins in foodstuffs, the weight of the aggregate sample (representing the combined total of all the incremental samples) at retail stage must be at least 1 kg or 1 L.[34] Therefore, several retail units (with identical batch number) were combined to obtain a total sample size of at least 1 kg or 1 L. Prior to analysis, aggregate samples of fruit and vegetable juices were thoroughly homogenized, after which a laboratory sample was weighed and stored (4 °C) until analysis. After homogenization of the aggregate sample, individual tomato products were transferred to a Petri dish and subsequently subjected to lyophilisation using a Lyobeta 25 device (Telstar, Terrassa, Spain). The lyophilised product was immediately vacuum-packed and stored (4 °C) until analysis.

Sample preparation and extraction methodology

Homogenized sample (fruit and vegetable juices: 2.0000 ± 0.0020 g; lyophilised tomato products: 0.5000 g ± 0.0020 g) was fortified with labeled internal standards [$^{13}C_6$,^{15}N]-**3** and [$^{2}H_4$]-**2** at concentrations of 7.5 µg/kg and 10 µg/kg (fruit and vegetable juices) or 60 µg/kg and 30 µg/kg (tomato products), respectively. After 10 s of vortex-mixing, samples were kept in the dark for 15 min. Prior to extraction, 5 mL of ultra pure water was added to the lyophilised tomato products, followed by vortex-mixing and soaking for 15 min. Samples were extracted for 30 min with 10 mL of extraction solvent (AcN, HPLC grade) using an overhead shaker. Sample extracts were briefly centrifuged (1 min, 3200xg) and pre-weighed $MgSO_4$ anhydrous salt (2.00 ± 0.05g) and NaCl (0.50 ± 0.05g) (fruit and vegetable juices) or Na_2SO_4 anhydrous salt (2.00 ± 0.05 g) (tomato products) were added. Subsequently, the tubes were vortex-mixed for 30 s, placed on an overhead shaker for 15 min and centrifuged (10 min, 3200xg). An aliquot (6.00 mL) of the supernatant was evaporated to dryness using a Turbovap LV module (Biotage AB, Uppsala, Sweden) maintained at 40 °C. Finally, the residue was redissolved in 100 µL of injection solvent (ultra pure water/AcN (LC-MS grade), 70/30, v/v), vortex-mixed for 30 s and subjected to centrifugation (Ultrafree-MC centrifugal filter units, 0.22 µm; Merck Millipore, Darmstadt, Germany) for 10 min at 10000xg prior to analysis.

LC-MS/MS methodology

Analysis was performed on an Acquity UPLC-Quattro Premier XE mass spectrometric system (Waters, Milford, MA). Data acquisition and processing was performed with MassLynx and

QuanLynx version 4.1. software (Micromass, Manchester, UK). Chromatographic and mass spectrometric operating conditions have been previously described.[31]

Method validation

Because of unavailability of certified reference material, optimization and validation of the analytical methodologies were performed using fortified blank (lyophilised in case of tomato products) samples. The analytical parameters specificity, linearity, apparent recovery, repeatability (intraday precision; RSD_r), reproducibility (intermediate precision; RSD_R) and expanded measurement uncertainty (*U*) were investigated to be compliant with the requirements stipulated in legislative documents.[34,35] Limit of detection (LOD) and limit of quantitation (LOQ) were assessed according to International Conference on Harmonisation (ICH) guidelines.[36] All parameters were calculated using the response (relative peak area) defined as the ratio of the peak area of the analyte to the peak area of the internal standards [$^{13}C_6$,^{15}N]-**3** (used for **3**, and also for **1** and **4**-**10**) and [$^{2}H_4$]-**2** (used for **2**).

Specificity involved the analysis of 12 representative blank samples per investigated matrix. Signal suppression/enhancement (*SSE*) due to matrix effects and extraction efficiency (*EE*) were evaluated according to Sulyok et al.[37] To evaluate linearity, matrix matched calibration (MMC) curves were constructed (in triplicate) by fortification of representative blank samples at five concentration levels (5-100 µg/kg in case of fruit and vegetable juices, and 50-1000 µg/kg in case of lyophilised tomato products). Besides calculation of regression coefficients (R^2), lack-of-fit tests (IBM SPSS 21) were performed to evaluate the linearity of the chosen regression model. Furthermore, assessment of homoscedasticity (homogeneity of variance)[38], as well as non-parallelism (the necessity to use matrix specific MMC curves for quantitation purposes) through visual inspection and *t*-test confirmation[39] was carried out.

To determine LOD and LOQ, MMC curves were constructed (in triplicate), by fortification of blank samples at 8 concentrations levels (0.1-10 µg/kg in case of fruit and vegetable juices, and 1-80 µg/kg in case of lyophilised tomato products). The linest function (Microsoft Excel 2013) was applied to calculate both the standard error of the y-intercept and the slope of the corresponding calibration curve (lower level equaled concentration for which S/N ≥ 3 for both product ions, and upper concentration level equaled 10 µg/kg in case of fruit and vegetable juices, and 80 µg/kg in case of lyophilised tomato products). Finally, LOD and LOQ equaled the concentration corresponding to respectively three and ten times the standard error of the y-intercept divided by the slope of the calibration curve.[36]

For each investigated matrix, apparent recovery, RSD_r, RSD_R and *U* were determined upon analysis of fortified representative blank samples (five concentration levels, in triplicate on three consecutive days) and subsequent quantitation by plotting the response into corresponding MMC curves separately constructed on each day of validation (five concentration levels, 5-100 µg/kg in case of fruit and vegetable juices, and 50-1000 µg/kg in case of lyophilised tomato products). One-way ANOVA was used to calculate RSD_r and RSD_R. Finally, *U* was obtained by multiplying the combined standard uncertainty (u_c, estimated standard deviation combining RSD_R and bias of an analytical methods) by a coverage factor of 2 (95% confidence level). The validation protocol for the assessment of the performance criteria of the different validation parameters has been previously described.[31]

The potential influence of the lyophilisation process on the accuracy of quantitation was assessed in a separate experiment. For every type of tomato product, six representative blank samples were fortified with a mixture of all target analytes (10 µg/kg) prior to lyophilisation. After lyophilisation, the apparent recovery was determined using MMC curves

constructed in representative blank lyophilised matrix (five concentration levels, 50-1000 µg/kg).

Dietary exposure assessment

A deterministic exposure assessment was performed to assess the risk associated with the dietary exposure to *Alternaria* toxins **1**, **2** and **3**. Commonly, mycotoxin dietary exposure is estimated by integration of contamination and consumption data obtained through sample analysis and dietary surveys, respectively.[40] Regarding the contamination data obtained in this study, two different scenarios (lower [LB] and upper bound [UB]) related to the treatment of the non-detects (NDs) and values below the limit of quantitation (<LOQ) were applied.[40] Consumption data were obtained from the Belgian food consumption survey (conducted in 2004) and its resulting food consumption database stemming from daily food intake data from two 24-h food recalls.[32] Only the consumption data from the adult population (18-64 years old; *n* = 1304) were selected to be used in this study. Furthermore, consumption data were extracted from the database based on the food name and facet strings, the output being a combination of all derived tomato products (tomato concentrate, ketchups, sauces, peeled canned tomatoes and purees). Finally, the usual food intake (expressed as kg/kg body weight (b.w.)/day) was determined using the Multiple Source Method (MSM) program (German Institute of Human Nutrition).[13] Dietary exposure to **1**, **2** and **3** was assessed based on the combination of the fixed mean toxin concentration with the mean, median, minimum, maximum and the percentiles (P75, P90, P95 and P99) of the other exposure component (consumption), considering LB and UB scenarios with regard to the data treatment.[40]

Statistical analysis

Microsoft Office Excel 2013 and IBM SPSS Statistics 21 were used for calculations and further data processing.

Results and discussion

Optimization of the sample preparation and extraction methodology

Fruit and vegetable juices

Optimization of the sample preparation and extraction protocol was based on a QuEChERS methodology. An experimental plan consisting of eight different sample preparation conditions (in triplicate) was set up for every juice matrix. Factors subjected to optimization were extraction solvent composition, liquid-liquid partitioning through salting out and aliquot volume of the supernatant to be evaporated. For this, representative blank samples (n=24 per juice matrix) were fortified with a mixture of ten free and conjugated *Alternaria* toxins at a concentration of 20 µg/kg. Finally, after evaporation, the residue of all samples (n=24) of every juice matrix (n_{total}=72) was redissolved in 100 µL of injection solvent fortified with labeled internal standards [$^{13}C_6$,^{15}N]-**3** and [$^{2}H_4$]-**2** (both at 2 µg/L), vortex-mixed and subjected to centrifugation prior to LC-MS/MS analysis. Results showed that extraction with pure AcN, addition of $MgSO_4$ together with NaCl and evaporation of a 6 mL aliquot of the supernatant led to highest relative peak area values for the majority of target analytes in every juice matrix.

SSE (signal suppression/enhancement), expressed as percentage of the signal recovered, and *EE* (extraction efficiency) were assessed for this sample preparation methodology. *EE* proved to be satisfactory, as *EE* values varied from 84%-108%, 92%-107% and 82%-107% in apple, carrot and grape juice respectively. Slightly lower *EE* values could be observed for **3** in carrot

and grape juice (74% and 75%, respectively), and **6** in grape juice (61%). Strong signal suppression (<25% of signal recovered) was observed for **7** in all three juice matrices, for **2** (24%) in carrot juice and for **9** (33%) and **6** (34%) in grape juice. Only limited signal suppression (>70% of signal recovered) could be observed for **1**, **4** and the sulfates (**8**, **10**). Finally, the optimization phase was concluded by performing pre-validation quantitation experiments, in which for every juice matrix the apparent recovery and RSD_r of nine quality control (QC) samples (representative blank samples fortified at low, medium and high concentration level, in triplicate) was determined using MMC curves (five concentration levels, 5-100 µg/kg).

Tomato products

Preliminary experiments using a previously developed sample preparation and extraction methodology[31] resulted in extracts most likely detrimental to the LC-MS/MS device, especially for the tomato concentrate matrix. Additional hexane defatting prior to centrifugation slightly improved the quality of the analytical sample. Substitution of the filtration step by solid phase extraction (SPE) using either aminopropyl or Oasis HLB cartridges further reduced interfering matrix components, but resulted in considerable losses of **3** and the modified mycotoxin conjugates, respectively. Therefore, similar to fruit and vegetable juices, a QuEChERS based experimental plan consisting of four different sample preparation conditions (in triplicate) was set up for every tomato product matrix. Extraction with pure AcN and addition of Na_2SO_4 led to highest relative peak area values for the majority of target analytes in every tomato product matrix. Further clean-up through the implementation of a dispersive SPE (d-SPE) step was investigated by adding anhydrous $MgSO_4$ (150 mg/mL) and C_{18} sorbent (50 mg/mL) to the supernatant, followed by vortex-

mixing, shaking and centrifugation prior to the evaporation step. Whereas signal intensities for the majority of target analytes were similar with d-SPE, recovery of **3** was seriously affected, resulting in omission of the d-SPE step.

EE values varied from 69%-80%, 72%-86% and 59%-75% in tomato juice, sauce and concentrate, respectively. Very strong signal suppression (<10% of signal recovered) was observed for **7** in all three tomato product matrices and for **2** in tomato concentrate, while only very limited signal suppression could be observed for **10** in tomato juice and concentrate (77% and 83% of signal recovered, respectively). Concerning all other target analytes in all three tomato product matrices, *SSE* values ranged from 10%-44%.

Additionally, screening experiments with several commercially available tomato products pointed out that representative blank matrices suitable for future fortification and validation experiments, could not be identified yet. Therefore, in a next phase, representative blank tomato juice, sauce and concentrate matrices were obtained through processing of fresh tomatoes based upon several in-house developed protocols. Dry weight percentages of these products proved to be similar to those of commercially available tomato products, thereby rendering them suitable for the intended use.

Lyophilisation enables a more profound homogenization of the sample matrix and facilitates accurate weighing of the analytical sample. Furthermore, long-term storage of samples is improved. However, it is deemed a prerequisite to assess whether the lyophilisation process still allows accurate quantitation of all target analytes in an unknown sample. Quantitation of pre-lyophilisation fortified samples using a calibration curve in representative blank lyophilised matrix indicated that 95% of the apparent recoveries (10 target analytes, 18 individual samples), taking into account the expanded measurement uncertainty (*U*) on the analytical result, ranged between 80 and 120%, thereby confirming sufficiently accurate

quantitation after lyophilisation. Ultimately, satisfactory pre-validation quantitation experiments (cf. fruit and vegetable juices) were performed.

Method validation

The analytical methodologies for the simultaneous determination of (modified) *Alternaria* toxins **1**-**10** in fruit and vegetable juices and tomato products were successfully validated. Regarding **6**, validation was only performed for the methods in fruit and vegetable juices and tomato juice due to depletion of the standard stock solution.

No interfering peaks (S/N ≥ 3) were detected in the 2.5% margin of the relative retention time (RRT) for all target analytes in blank samples per investigated matrix, confirming the specificity of the analytical methodologies.[35] Additionally, adequate linearity in the applied concentration ranges was demonstrated. Furthermore, homoscedasticity was assessed as previously described.[31] Weighted least squares linear regression (WLSLR) with an optimal weighting factor ($w_i = 1/x^2$) was used to counteract the observed heteroscedasticity. Indeed, it has been shown that an heteroscedastic situation, which has not been corrected for through WLSLR, will result in an impaired accuracy in the lower end of the calibration range.[38]

The developed methods allowed for the detection of all target analytes at low parts per billion (µg/kg) levels. LOQ values in fruit and vegetable juices ranged from 1.1-5.7 µg/kg, while LOD and LOQ values in lyophilised tomato products ranged from 3.0-18.3 µg/kg and from 9.8-61.5 µg/kg, respectively. The latter values appear to be quite elevated, but it must be taken into consideration that these LOD and LOQ values were determined on lyophilised matrix. To obtain the corresponding µg/kg values for fresh (wet) weight of the different

types of tomato products, these values need to be multiplied by a conversion factor based on the dry weight percentage of the corresponding sample.

The apparent recovery, ranging from 87.0%-109.8% and from 89.3%-110.6% for all analytes in fruit and vegetable juices and lyophilised tomato products, respectively, was in good agreement with the imposed guideline ranges (80-110%).[35] RSD_r and RSD_R ranged from 0.8%-15.7% and 1.2%-15.7%, and from 1.1%-15.6% and 2.4%- 17.9% for all analytes in fruit and vegetable juices and lyophilised tomato products, respectively. Acceptance limits for the imprecision of quantitative methods (RSD_r and RSD_R) are concentration dependent and are calculated by the Horwitz Equation[35], or set at 20% and 25%, respectively, for concentrations lower than 100 µg/kg according to an in-house developed standard operating procedure on analytical method validation. For all the analytes, the expanded measurement uncertainty *U* ranged from 9.1%-54.3%, and from 14.3%-60.0% in fruit and vegetable juices and lyophilised tomato products, respectively. It is confirmed that uncertainty and vice versa accuracy is best for **2** and **3**, analytes for which corresponding isotope-labelled standards are available.

Alternaria toxins in commercially available foodstuffs

The prevalence (% of positive samples), mean upper bound (UB) concentration, concentration range and median values (µg/kg) of (modified) *Alternaria* toxins found in each type of commercially available food products (n_{total}=129) in Belgium are represented in Table 1 (data of fruit and vegetable juices not shown). Regarding fruit and vegetable juices, only **3** was detected in 79% (11/14) of grape juice samples and 8% (2/24) of apple juice samples in rather low concentration ranges (grape juice: <LOQ to 19.4 µg/kg; apple juice: <LOQ to 7.9 µg/kg), whereas no **3** or other target analytes were detected in any of the carrot juice samples analyzed. Likewise, despite the widespread occurrence of *A. alternata* on organic

carrots, and the reported ability of its isolates to produce mycotoxins when grown on carrot culture discs, the analysis of 266 carrot samples from various carrot cultivars and 87 carrot based commercial products revealed a total absence of **1**, **2**, **3** and **6**[41]. On the contrary, natural occurrence of **1**, **2** and **3** has been reported in decayed apples and apple products such as juices, sauces and concentrates, albeit at trace level (**2**) or in rather low concentration ranges (**1** and **3**, <10 µg/kg).[3,5,7] Regarding grape juice, our results are largely in agreement with the previous reports. Whereas several studies only sporadically reported trace levels of **1** and **2**, Asam et al.[7] detected low levels of **3** (≤7 µg/kg) in all four red grape juice samples analyzed due to the highly sensitive stable isotope dilution assay (SIDA) applied.

Regarding tomato products, **3** proved to be ubiquitously present in all tomato juice, sauce and concentrate samples in concentrations up to 333 µg/kg, while **1**, **2**, **5** and to a lesser extent **4** were also frequently detected, albeit in much lower concentrations. Whereas the prevalence of **1** (71-86%), **2** (54-78%), **4** (21-64%) and **5** (32-56%) is comparable in the different types of tomato products, significantly (p<0.05) higher mean concentrations for these toxins could be observed in tomato concentrate samples as compared to tomato juice and sauce samples due to the concentration procedure during processing. Co-occurrence of four *Alternaria* toxins (**1**, **2**, **3** and **4** or **5**) was observed in 8% of all tomato product samples (7/83), while **1**, **2** and **3** were observed to co-occur in 12% of all samples (10/83). Contamination with **3** and either **1** or **2** was observed in 29% of all samples (24/83).

Whereas in this study **5** was detected in concentrations up to 6.1, 12.1 and 62.0 µg/kg in 50%, 32% and 56% of tomato juice, sauce and concentrate samples respectively, no **5** was found in other surveys.[6] Regarding **4**, Noser et al.[6] reported similar prevalences and concentrations ranges in tomato sauce and concentrate samples, while López et al.[42]

reported all tomato product samples (*n*=8) to be negative for **4**. In contrast to the other *Alternaria* toxins investigated here, **6** could not be detected in any of the samples under study, which confirmed the results of a recent study based on SIDA.[43] Similar prevalences, median concentrations and concentration ranges for **1**, **2** and **3** in tomato products have previously been reported[3-6,33] and also by EFSA[14] and very recently by López et al.[42] in the Netherlands. On the contrary, Van de Perre et al.[44] reported much lower prevalences for **1** (18%) and **2** (12%) in tomato concentrates and purees (*n*=33), as well as a complete absence of these mycotoxins in tomato juice, sauce and ketchup samples (*n*=50) from the Belgian market. This discrepancy could be attributed to the lower sensitivity (LOD_1 = 12.2 µg/kg; LOD_2 = 13.5 µg/kg) of the semi-quantitative LC-TOF-MS analytical method used in the latter study.[44] Furthermore, da Motta and Soares[2] could not detect **1** or **2** in 80 tomato derived products from Brazil, while comparable concentrations of **3** (29-111 µg/kg) were only found in tomato sauces and concentrates, albeit in a minor fraction of the samples (25%, 11/44). Terminiello et al.[11], however, reported concentrations up to 8.8, 1.7 and 4.0 mg/kg for **1**, **2** and **3**, respectively, in a fraction of 80 tomato puree samples from Argentina, using the same analytical methodology.[2] For clarification, the authors hinted at the likelihood of mouldy tomatoes being included during tomato processing. Indeed, recently the stability of **1** and **2** throughout the production of derived tomato products was reported[13], leading to the conclusion that the presence of *Alternaria* toxins in commercial end products might be indicative of a lack of quality control, e.g. the use of mouldy raw material in tomato processing plants.

This study reports the novel detection of modified *Alternaria* toxins (specifically, sulfates of **1** and **2**) occurring in tomato products. Particularly in tomato concentrate, **8** and **10** were detected in 26% and 78% of all samples, in concentrations up to 8.7 and 9.9 µg/kg,

respectively. This study meets the recommendations to identify modified, and as-yet uncharacterized mycotoxins, as well as to gather occurrence data using properly validated analytical methods listed in EFSA's scientific opinion on modified mycotoxins.[19] Figure 2 depicts the chromatogram of a tomato sauce sample showing co-occurrence of **1**, **2**, **3**, **5** as well as **8** and **10**. Additionally, for **10**, the residual plot and calibration curve, the chromatogram of a calibration standard (with comparable area under the curve) and finally four identification criteria (Commission Decision (EC) N° 2002/657: identification points for LC-MS/MS ≥ 4, S/N ratio for both fragment ions ≥ 3 and both ion ratio and relative retention time (RRT) within maximum permitted tolerances[35]) to unambiguously confirm the presence of this target analyte are also depicted. Whether these conjugates originate from fungal metabolism or from the plant detoxification system, remains to be elucidated. Usually, phase II conjugation reactions for detoxification *in planta* lead to glucose, malonic acid or glutathione conjugates.[25] However, regarding the occurrence of sulfated conjugates of other mycotoxins, ZEN was found to be partially converted to ZEN-14-sulfate (ZEN14S) both during fungal and plant metabolism.[45,46] Subsequently, Vendl et al.[27] reported the natural occurrence of ZEN14S in various cereal-based foodstuffs, also in rather low concentrations (<LOQ - 6.1 µg/kg). Very recently, the potential of wheat to form sulfate conjugates of DON (DON-3-sulfate and DON-15-sulfate) was reported, supporting the theory that sulfation can indeed be regarded as a detoxification reaction *in planta*.[47]

Dietary exposure assessment

This survey demonstrated a high contamination frequency of different types of tomato products, mostly with **1**, **2** and **3**. Moreover, EFSA considered it appropriate to use the threshold of toxicological concern (TTC) approach to assess the relative level of concern of

these mycotoxins for human health. Based on the genotoxic potential of **1** and **2** (these mycotoxins displayed *in vitro* genotoxicity in bacterial and mammalian cell lines[48,49]), a TTC value of 2.5 ng/kg b.w./day was assigned. Since there is no evidence for genotoxicity of **3** in bacteria, or clear structural alerts, a TTC value of 1500 ng/kg b.w./day was assigned.[14] A deterministic dietary exposure assessment was carried out to evaluate the risk associated with the exposure to **1**, **2**and **3** due to consumption of tomato products. Table 2 gives an overview of the minimum (min), mean, median, P75, P90, P95, P99 and maximum (max) dietary exposure to **1**, **2** and **3** for the adult Belgian population both for LB and UB concentration scenarios. For **1** both the estimated mean chronic (3.49 - 12.6 ng/kg b.w./day) and 95th percentile dietary exposures (25.0 - 90.4 ng/kg b.w./day) regarding all tomato products (LB and UB scenario) exceeded the TTC value of 2.5 ng/kg b.w./day. For **2**, mean dietary exposure (LB and UB) regarding tomato juice and sauce (0.50 - 0.96 ng/kg b.w./day), and LB mean dietary exposure regarding tomato concentrate (1.93 ng/kg b.w./day) were lower than the TTC value, while UB mean dietary exposure regarding tomato concentrate (5.27 ng/kg b.w./day) exceeded the TTC value. Furthermore, 95th percentile dietary exposures (LB and UB) regarding all tomato products (3.55 - 37.7 ng/kg b.w./day) largely exceeded the imposed TTC value. In general, both for **1** and **2**, mean and high dietary exposure values (LB and UB) regarding tomato concentrate were more than 3-fold higher than corresponding values regarding tomato juice and sauce. For **3**, both the estimated mean chronic (104.2 - 104.3 ng/kg b.w./day) and 95th percentile dietary exposures (745.5 - 746.1 ng/kg b.w./day) regarding all tomato products (LB and UB scenario) were well below the imposed TTC value of 1500 ng/kg b.w./day. These results are in good agreement with an indicative exposure assessment conducted by EFSA on the European level.[14] Recently, Van de Perre et al.[13,44] also conducted a dietary exposure assessment for **3** from derived tomato

products. Both mean and high (2900 - 7430 ng/kg b.w./day) exposure estimates using a conservative approach largely exceeded the imposed TTC value. However, exposure assessment was carried out using concentration data expressed on lyophilised samples, without application of the conversion factor considering the dry to fresh weight ratio of the corresponding tomato products.[13,44]

In conclusion, for **1** and **2**, the outcomes of this study confirm the need for additional toxicity data to assess their potential health risk, whereas the intake of **3** via fruit juices and tomato products is unlikely to be of human health concern. However, it is worth mentioning that the risk assessment conducted by EFSA not only covers tomato products, but also other products containing *Alternaria* toxins. Other food groups that significantly contribute to the chronic dietary exposure to **1**, **2** and **3** are grain and grain-based products, vegetable oils, oilseeds and alcoholic beverages.[14]

Moreover, since dietary exposure in this study was only calculated for the adult population, it is likely that dietary exposure in children (higher food consumption per kg body weight) or in population groups exhibiting a different consumption pattern (e.g. vegetarians with higher intake of plant-based foodstuffs), is even higher. This has only recently been shown for millet-based infant cereals containing high amounts of **3**.[50] Furthermore, overall dietary exposure to **1** and **2** might even be more elevated if foodstuffs exhibiting higher concentrations of these mycotoxins, such as oilseeds and vegetable oils (unpublished results), would also be taken into consideration. Additionally, synergistic effects of *Alternaria* toxins, the presence of their modified forms, provided they are equally toxic and bioavailable, and the possibility of conversion of these modified forms into their native forms during their passage in the gastric tract might also lead to an underestimation of the overall effect of *Alternaria*-infested foodstuffs on human health. Finally, it should also be

taken into account that, in case of limited oral bioavailability, (modified) *Alternaria* toxins may exert their effects locally rather than exhibiting a systemic toxicity.

Acknowledgements

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Supporting information

The supporting information is available free of charge via the Internet at http://pubs.acs.org: Visualization of parallelism assessment of MMC curves in juices matrices and different tomato products (Figure 1); High resolution mass spectrometric data of spiked versus naturally contaminated tomato product sample (Figure 2); Performance characteristics of validation parameters for all analytes in each investigated matrix (Table 1-5).

References

1. Ostry, V. *Alternaria* mycotoxins: an overview of chemical characterization, producers, toxicity, analysis and occurrence in foodstuffs. *World Mycotoxin J.* **2008**, *1*, 175-188.

2. da Motta, S.; Soares, L.M.V. Survey of Brazilian tomato products for alternariol, alternariol monomethyl ether, tenuazonic acid and cyclopiazonic acid. *Food Addit. Contam.* **2001**, *18*, 630-634.

3. Asam, S.; Konitzer, K.; Schieberle, P.; Rychlik, M. Stable isotope dilution assays of alternariol and alternariol monomethyl ether in beverages. *J. Agric. Food Chem.* **2009**, *57*, 5152-5160.

4. Asam, S.; Konitzer, K.; Rychlik, M. Precise determination of the *Alternaria* mycotoxins alternariol and alternariol methyl ether in cereal, fruit and vegetable products using stable isotope dilution assays. *Mycotoxin Res.* **2011**, *27*, 23-28.

5. Ackermann, Y.; Curtui, V.; Dietrich, R.; Gross, M.; Latif, H.; Martlbauer, E.; Usleber, E. Widespread occurrence of low levels of alternariol in apple and tomato products, as determined by comparative immunochemical assessment using monoclonal and polyclonal antibodies. *J. Agric. Food Chem.* **2011**, *59*, 6360-6368.

6. Noser, J.; Schneider, P.; Rother, M.; Schmutz, H. Determination of six *Alternaria* toxins with UPLC-MS/MS and their occurrence in tomatoes and tomato products from the Swiss market. *Mycotoxin Res.* **2011**, *27*, 265-271.

7. Asam, S.; Lichtenegger, M.; Yang, L.; Richlyk, M. Content of the *Alternaria* mycotoxin tenuazonic acid in food commodities determined by a stable isotope dilution assay. *Mycotoxin Res.* **2012**, *28*, 9-15.

8. Logrieco, A.; Moretti, A.; Solfrizzo, M. *Alternaria* toxins and plant diseases: an overview of origin, occurrence and risks. *World Mycotoxin J.* **2009**, *2*, 129-140.

9. Stinson, E.; Osman, S.; Heisler, E.; Siciliano, J.; Bills, D. Mycotoxin production in whole tomatoes, apples, oranges, and lemons. *J. Agric. Food Chem.* **1981**, *29*, 790-792.

10. Harwig, J.; Scott, P.M.; Stoltz, D.R.; Blanchfield, B.J. Toxins of molds from decaying tomato fruit. *Appl. Environ. Microbiol.* **1979**, *38*, 267-274.

11. Terminiello, L.; Patriarca, A.; Pose, G.; Fernández Pinto, V. Occurrence of alternariol, alternariol monomethyl ether and tenuazonic acid in Argentinean tomato puree. *Mycotoxin Res.* **2006**, *22*, 236-240.

12. Scott, P.M.; Kanhere, S.R. Stability of *Alternaria* toxins in fruit juices and wine. *Mycotoxin Res.* **2001**, *17*, 9-14.

13. Van de Perre, E. Farm to fork risk assessment of emerging mycotoxins in fresh produce: the case of tomato considering climate change. *Doctoral thesis, Ghent University, Belgium.* **2014**.

14. EFSA on Contaminants in the Food Chain (CONTAM); Scientific Opinion on the risks for animal and public health related to the presence of *Alternaria* toxins in feed and food. EFSA Journal 2011;9(10):2407. [97 pp.] doi:10.2903/j.efsa.2011.2407. Available online: www.efsa.europe.eu/efsajournal

15. Berthiller, F.; Dall'Asta, C.; Schuhmacher, R.; Lemmens, M.; Adam, G.; Krska, R. Masked mycotoxins: determination of a deoxynivalenol glucoside in artificially and naturally contaminated wheat by liquid chromatography-tandem mass spectrometry. *J. Agric. Food Chem.* **2005**, *53*, 3421-3425.

16. Engelhardt, G.; Zill, G.; Wohner, B.; Wallnofer, P.R. Transformation of the *Fusarium* mycotoxin zearalenone in maize cell-suspension cultures. *Naturwissenschaften* **1988**, *75*, 309-310.

17. Gareis, M.; Bauer, J.; Thiem, J.; Plank, G.; Grabley, S.; Gedek, B. Cleavage of zearalenone-glycoside, a masked mycotoxin, during digestion in swine. *J. Vet. Med. , Ser. B* **1990**, *37*, 236-240.

18. Rychlik, M.; Humpf, H.U.; Marko, D.; Danicke, S.; Mally, A.; Berthiller, F.; Klaffke, H.; Lorenz, N. Proposal of a comprehensive definition of modified and other forms of mycotoxins including "masked" mycotoxins. *Mycotoxin Res.* **2014**, *30*, 197-205.

19. EFSA CONTAM Panel (EFSA Panel on Contaminants in the Food Chain), 2014. Scientific Opinion on the risks for human and animal health related to the presence of modified forms of certain mycotoxins in food and feed. EFSA Journal 2014;12(12):3916. [107 pp.] doi:10.2903/j.efsa.2014.3916. Available online: www.efsa.europe.eu/efsajournal

20. Berthiller, F.; Schuhmacher, R.; Buttinger, G.; Freudenschuss, M.; Adam, G.; Krska, R. Synthesis of deoxynivalenol-glucosides and their characterization using a QTrap LC-MS/MS. *Mycotoxin Res.* **2003**, *19*, 47-50.

21. Berthiller, F.; Hametner, C.; Krenn, P.; Schweiger, W.; Ludwig, R.; Adam, G.; Krska, R.; Schuhmacher, R. Preparation and characterization of the conjugated Fusarium mycotoxins zearalenone-4*O*-β-D-glucopyranoside, α-zearalenol-4*O*-β-D-glucopyranoside and β-zearalenol-4*O*-β-D-glucopyranoside by MS/MS and two-dimensional NMR. *Food Addit. Contam. , part A* **2009**, *26*, 207-213.

22. Fruhmann, P.; Skrinjar, P.; Weber, J.; Mikula, H.; Warth, B.; Sulyok, M.; Krska, R.; Adam, G.; Rosenberg, E.; Hametner, C.; Frohlich, J. Sulfation of deoxynivalenol, its acetylated derivatives, and T2-toxin. *Tetrahedron* **2014**, *70* (34), 5260-5266.

23. Mikula, H.; Weber, J.; Svatunek, D.; Skrinjar, P.; Adam, G.; Krska, R.; Hametner, C.; Frohlich, J. Synthesis of zearalenone-16-β-D-glucoside and zearalenone-16-sulfate: A tale of protecting resorcylic acid lactones for regiocontrolled conjugation. *Beilstein J. Org. Chem.* **2014**, *10*, 1129-1134.

24. Mikula, H.; Skrinjar, P.; Sohr, B.; Ellmer, D.; Hametner, C.; Frohlich, J. Total synthesis of masked *Alternaria* mycotoxins-sulfates and glucosides of alternariol (AOH) and alternariol-9-methyl ether (AME). *Tetrahedron* **2013**, *69*, 10322-10330.

25. Berthiller, F.; Crews, C.; Dall'Asta, C.; De Saeger, S.; Haesaert, G.; Karlovsky, P.; Oswald, I.P.; Seefelder, W.; Speijers, G.; Stroka, J. Masked mycotoxins: A review. *Mol. Nutr. Food Res.* **2013**, *57*, 165-186.

26. Kostelanska, M.; Hajslova, J.; Zachariasova, M.; Malachova, A.; Kalachova, K.; Poustka, J.; Fiala, J.; Scott, P.M.; Berthiller, F.; Krska, R. Occurrence of deoxynivalenol and its major conjugate, deoxynivalenol-3-glucoside, in beer and some brewing intermediates. *J. Agric. Food Chem.* **2009**, *57*, 3187-3194.

27. Vendl, O.; Crews, C.; MacDonald, S.; Krska, R.; Berthiller, F. Occurrence of free and conjugated *Fusarium* mycotoxins in cereal-based food. *Food Addit. Contam. , part A* **2010**, *27*, 1148-1152.

28. De Boevre, M.; Diana Di Mavungu, J.; Landschoot, S.; Audenaert, K.; Eeckhout, M.; Maene, P.; Haesaert, G.; De Saeger, S. Natural occurrence of mycotoxins and their masked forms in food and feed products. *World Mycotoxin J.* **2012**, *5*, 207-219.

29. Yoshinari, T.; Sakuda, S.; Furihata, K.; Furusawa, H.; Ohnishi, T.; Sugita-Konish, Y.; Ishizaki, N.; Terajima, J. Structural determination of a nivalenol glucoside and development of an analytical method for the simultaneous determination of nivalenol and deoxynivalenol, and their glucosides, in wheat. *J. Agric. Food Chem.* **2014**, *62*, 1174-1180.

30. Lattanzio, V.M.T.; Visconti, A.; Haidukowski, M.; Pascale, M. Identification and characterization of new *Fusarium* masked mycotoxins, T-2 and HT-2 glycosyl derivatives, in naturally contaminated wheat and oats by liquid chromatography high-resolution mass spectrometry. *J. Mass Spectrom.* **2012**, *47*, 466-475.

31. Walravens, J.; Mikula, H.; Asam, S.; Rychlik, M.; Njumbe Ediage, E.; Diana Di Mavungu, J.; Van Landschoot, A.; Vanhaecke, L.; De Saeger, S. Development and validation of an ultra-high-performance liquid chromatography tandem mass spectrometric method for the simultaneous determination of free and conjugated Alternaria toxins in cereal-based foodstuffs. *J. Chromatogr. A* **2014**, *1372*, 91-101.

32. De Vriese, S.; De Backer, G.; De Henauw, S.; Huybrechts, I.; Kornitzer, K.; Leveque, A.; Moreau, M.; Oyen, H. The Belgian food consumption survey: aims, design and methods. *Belg. Arch.* **2005**, *63*, 1-16.

33. Asam, S.; Liu, Y.; Konitzer, K.; Rychlik, M. Development of a stable isotope dilution assay for tenuazonic acid. *J. Agric. Food Chem.* **2011**, *59*, 2980-2987.

34. Commission Regulation (EC) No 401/2006 of 23 February 2006 laying down the methods of sampling and analysis for the official control of the levels of mycotoxins in foodstuffs. *Off.J.Eur.Commun.*, **2006**, *L70*, 12-34.

35. Commission Decision (EC) No 2002/657 implementing Council Directive 96/23/EC concerning the performance of analytical methods and the interpretation of results. *Off.J.Eur.Commun.*, **2002**, *L221*, 8-36.

36. ICH, Harmonised Tripartite Guideline. Validation of analytical procedures: text and methodology Q2(R1). International Conference on Harmonisation of technical requirements for registration of pharmaceuticals for human use. **2005**.

37. Sulyok, M.; Berthiller, F.; Krska, R.; Schuhmacher, R. Development and validation of a liquid chromatography/tandem mass spectrometric method for the determination of 39 mycotoxins in wheat and maize. *Rapid Commun. Mass Spectrom.* **2006**, *20*, 2649-2659.

38. Almeida, A.M.; Castel-Branco, M.M.; Falcao, A.C. Linear regression for calibration lines revisited: weighting schemes for bioanalytical methods. *J. Chromatogr. B* **2002**, *774*, 215-222.

39. Van Loco, J. Method Validation for Food Analysis: Concepts and Use of Statistical Techniques. In *The Determination of Chemical Elements in Food: Applications for Atomic and Mass Spectrometry;* Caroli, S., Eds.; John Wiley & Sons, Inc.: Hoboken, NJ, USA, 2007; pp. 135-163.

40. Yogendrarajah, P.; Jacxsens, L.; Lachat, C.; Walpita, C.N.; Kolsteren, P.; De Saeger, S.; De Meulenaer, B. Public health risk associated with the co-occurrence of mycotoxins in spices consumed in Sri Lanka. *Food Chem. Toxicol.* **2014**, *74*, 240-248.

41. Solfrizzo, M.; De Girolamo, A.; Vitti, C.; Tylkowska, K.; Grabarkiewicz-Szczesna, J.; Szopinska, D.; Dorna, H. Toxigenic profile of *Alternaria alternata* and *Alternaria radicina* occurring on umbelliferous plants. *Food Addit. Contam. , part A* **2005**, *22*, 302-308.

42. Lopez, P.; Venema, D.; de Rijk, T.; de Kok, A.; Scholten, J.M.; Mol, H.G.J.; De Nijs, M. Occurrence of *Alternaria* toxins in food products in the Netherlands. *Food Control* **2016**, *60*, 196-204.

43. Liu, Y.; Rychlik, M. Biosynthesis of seven carbon-13 labeled *Alternaria* toxins including altertoxins, alternariol, and alternariol methyl ether, and their application to a multiple stable isotope dilution assay. *Anal. Bioanal. Chem.* **2015**, *407* (5), 1357-1369.

44. Van de Perre, E.; Deschuyffeleer, N.; Jacxsens, L.; Vekeman, F.; Van Der Hauwaert, W.; Asam, S.; Rychlik, M.; Devlieghere, F.; De Meulenaer, B. Screening of moulds and mycotoxins in tomatoes, bell peppers, onions, soft red fruits and derived tomato products. *Food Control* **2014**, *37*, 165-170.

45. Plasencia, J.; Mirocha, C. Isolation and characterization of zearalenone sulfate produced by *Fusarium spp*. *Appl. Environ. Microbiol.* **1991**, *57*, 146-150.

46. Berthiller, F.; Werner, U.; Sulyok, M.; Krska, R.; Hauser, M.T.; Schuhmacher, R. Liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) determination of phase II metabolites of the mycotoxin zearalenone in the model plant *Arabidopsis thaliana*. *Food Addit. Contam.* **2006**, *23*, 1194-1200.

47. Warth, B.; Fruhmann, P.; Wiesenberger, G.; Kluger, B.; Sarkanj, B.; Lemmens, M.; Hametner, C.; Frohlich, J.; Adam, G.; Krska, R.; Schuhmacher, R. Deoxynivalenol-sulfates: identification and quantification of novel conjugated (masked) mycotoxins in wheat. *Anal. Bioanal. Chem.* **2015**, *407*, 1033-1039.

48. Schrader, T.J.; Cherry, W.; Soper, K.; Langlois, I. Further examination of the effects of nitrosylation on Alternaria alternata mycotoxin mutagenicity in vitro. *Mutat. Res. , Genet. Toxicol. Environ. Mutagen.* **2006**, *606*, 61-71.

49. Pfeiffer, E.; Eschbach, S.; Metzler, M. *Alternaria* toxins: DNA strand-breaking activity in mammalian cells *in vitro*. *Mycotoxin Res.* **2007**, *23*, 152-157.

50. Asam, S.; Rychlik, M. Potential health hazards due to the occurrence of the mycotoxin tenuazonic acid in infant food. *Eur. Food Res. Technol.* **2013**, *236*, 491-497.

Figure captions

Figure 1. Structures of (modified) *Alternaria* toxins: alternariol **1**, alternariol monomethyl ether **2**, tenuazonic acid **3**, tentoxin **4**, altenuene **5**, altertoxin-I **6**, alternariol-3-glucoside **7**, alternariol-3-sulfate **8**, alternariol monomethyl ether-3-glucoside **9** and alternariol monomethyl ether-3-sulfate **10**.

Figure 2. Chromatogram (both transitions) of a tomato sauce sample showing co-occurrence of [**A**, **B**] **5**, [**C**, **D**] **3**, [**E**, **F**] **1**, [**G**, **H**] **2**, [**I**, **J**] **8** and [**K**, **L**] **10**; [**M**] Residual plot and calibration curve for **10**; [**N**, **O**] Chromatogram of a calibration standard (50 µg/kg) and [**P**] 4 identification criteria for **10**.[35]

Tables

Table 1. Prevalence, mean UB concentration and concentration range (µg/kg) of (modified) *Alternaria* toxins in each type of commercially available tomato product in Belgium.

***Alternaria* toxins** [a]	**Tomato juice (*n*=28)**			**Tomato sauce (*n*=28)**			**Tomato concentrate (*n*=27)**		
	$\%_{pos}$ [b]	Mean $_{UB}$ [c]	Range (median) [d]	$\%_{pos}$	Mean $_{UB}$	Range (median)	$\%_{pos}$	Mean $_{UB}$	Range (median)
1	71	2.1	<LOQ to 27.0 (0.5)	86	2.7	<LOQ to 41.6 (0.8)	85	7.6	<LOQ to 31.0 (2.1)
2	54	0.6	<LOQ to 3.3 (0.6)	78	0.6	<LOQ to 3.8 (0.5)	67	3.2	<LOQ to 6.10 (3.6)
3	100	53.1	3.7 to 333.1 (28.6)	100	84.3	7.7 to 330.6 (64.1)	100	49.6	<LOQ to 174.3 (36.1)
4	64	0.4	<LOQ (0.5)	21	0.6	<LOQ (0.5)	37	2.5	<LOQ to 8.9 (1.5)
5	50	2.2	<LOQ to 6.1 (0.7)	32	2.2	<LOQ to 12.1 (1.1)	56	20.4	18.7 to 62.0 (20.5)
6	nd [e]	-	-	nd [e]	-	-	nd [e]	-	-
8	21	0.9	<LOQ (0.7)	11	0.6	<LOQ to 2.6 (0.5)	26	3.0	4.5 to 8.7 (5.1)
10	50	0.7	<LOQ to 1.7 (0.3)	32	0.5	<LOQ to 2.3 (0.3)	78	3.6	<LOQ to 9.9 (1.3)
7	nd	-	-	nd	-	-	nd	-	-
9	nd	-	-	nd	-	-	nd	-	-

[a] **1**: alternariol - **2**: alternariol monomethyl ether - **3**: tenuazonic acid - **4**: tentoxin - **5**: altenuene - **6**: altertoxin-I - **7**: alternariol-3-glucoside - **8**: alternariol-3-sulfate - **9**: alternariol monomethyl ether-3-glucoside **- 10**: alternariol monomethyl ether-3-sulfate.
[b] Percentage of samples with a concentration above the limit of detection (LOD)
[c] Mean upper bound concentration (µg/kg); "-" : not applicable (no positive samples)
[d] Concentration range of positives (with median values); "<LOQ" : below limit of quantitation, detected but not quantifiable; "-" : not applicable (no positive samples)
[e] nd: not detected

Table 2. Deterministic dietary exposure assessment for adult population (ng/kg b.w./day) associated with the consumption of tomato products contaminated with *Alternaria* toxins **1**, **2** and **3** using the lower bound - upper bound scenarios in Belgium.

Tomato product consumption (usual intake [a], g/kg b.w./day)	**1 [b] (juice and sauce) [c]**		**1 [b] (concentrate) [c]**		**2 [b] (juice and sauce) [c]**		**2 [b] (concentrate) [c]**		**3 [b] (tomato products)**	
	LB [d]	UB [d]	LB	UB	LB	UB	LB	UB	LB	UB
Min (0.000)	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Mean (1.668)	**3.49** [e]	**3.99**	**11.4**	**12.6**	0.50	0.96	1.93	**5.27**	104.2	104.3
Median (0.224)	0.47	0.54	1.53	1.70	0.07	0.13	0.26	0.71	14.0	14.0
P75 (1.418)	**2.96**	**3.39**	**9.68**	**10.7**	0.42	0.82	1.64	**4.48**	88.5	88.6
P90 (7.313)	**15.3**	**17.5**	**49.9**	**55.4**	2.18	**4.22**	**8.46**	**23.1**	456.6	457.0
P95 (11.940)	**25.0**	**28.5**	**81.5**	**90.4**	**3.55**	**6.89**	**13.8**	**37.7**	745.5	746.1
P99 (11.940)	**25.0**	**28.5**	**81.5**	**90.4**	**3.55**	**6.89**	**13.8**	**37.7**	745.5	746.1
Max (11.940)	**25.0**	**28.5**	**81.5**	**90.4**	**3.55**	**6.89**	**13.8**	**37.7**	745.5	746.1

[a] Consumption data of derived tomato products from an adult population obtained from a Belgian food consumption survey conducted in 2004[32], and converted to the usual food intake (expressed as g/kg body weight per day) using the Multiple Source Method (MSM) program.

[b] **1**: alternariol - **2**: alternariol monomethyl ether - **3**: tenuazonic acid

[c] **1** and **2**: Data pooling to tomato products not allowed since mean concentrations in juice, sauce and concentrate differed significantly (one-way ANOVA, $p<0.05$).

[d] LB: lower bound scenario; UB: upper bound scenario (both based on mean toxin concentration values).

[e] Values exceeding the TTC value for **1** and **2** (2.5 ng/kg b.w./day) and **3** (1500 ng/kg b.w./day) are shown in bold.

Figure graphics

Figure 1

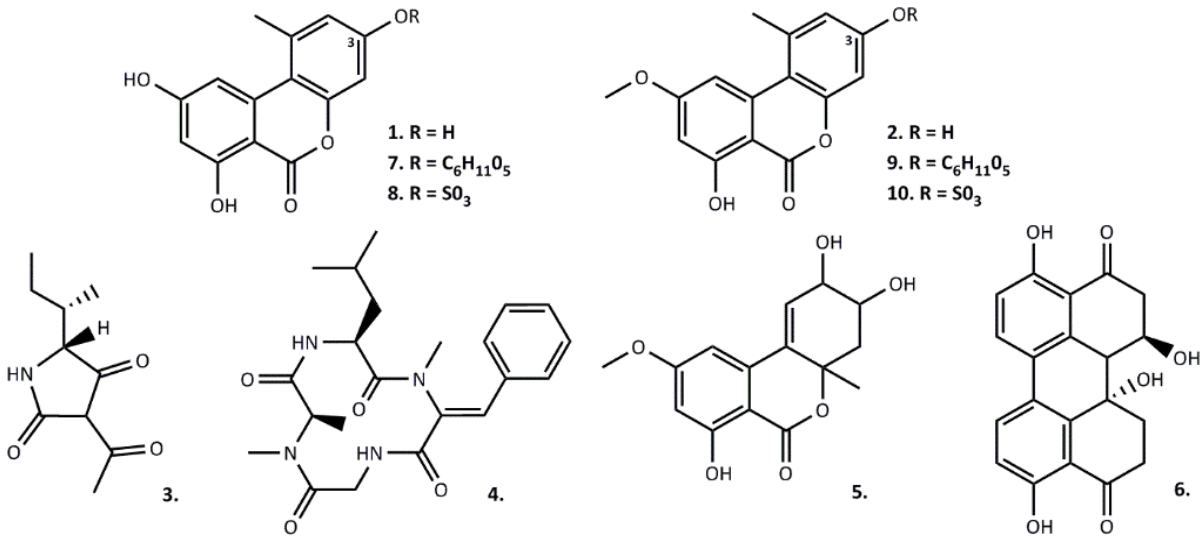
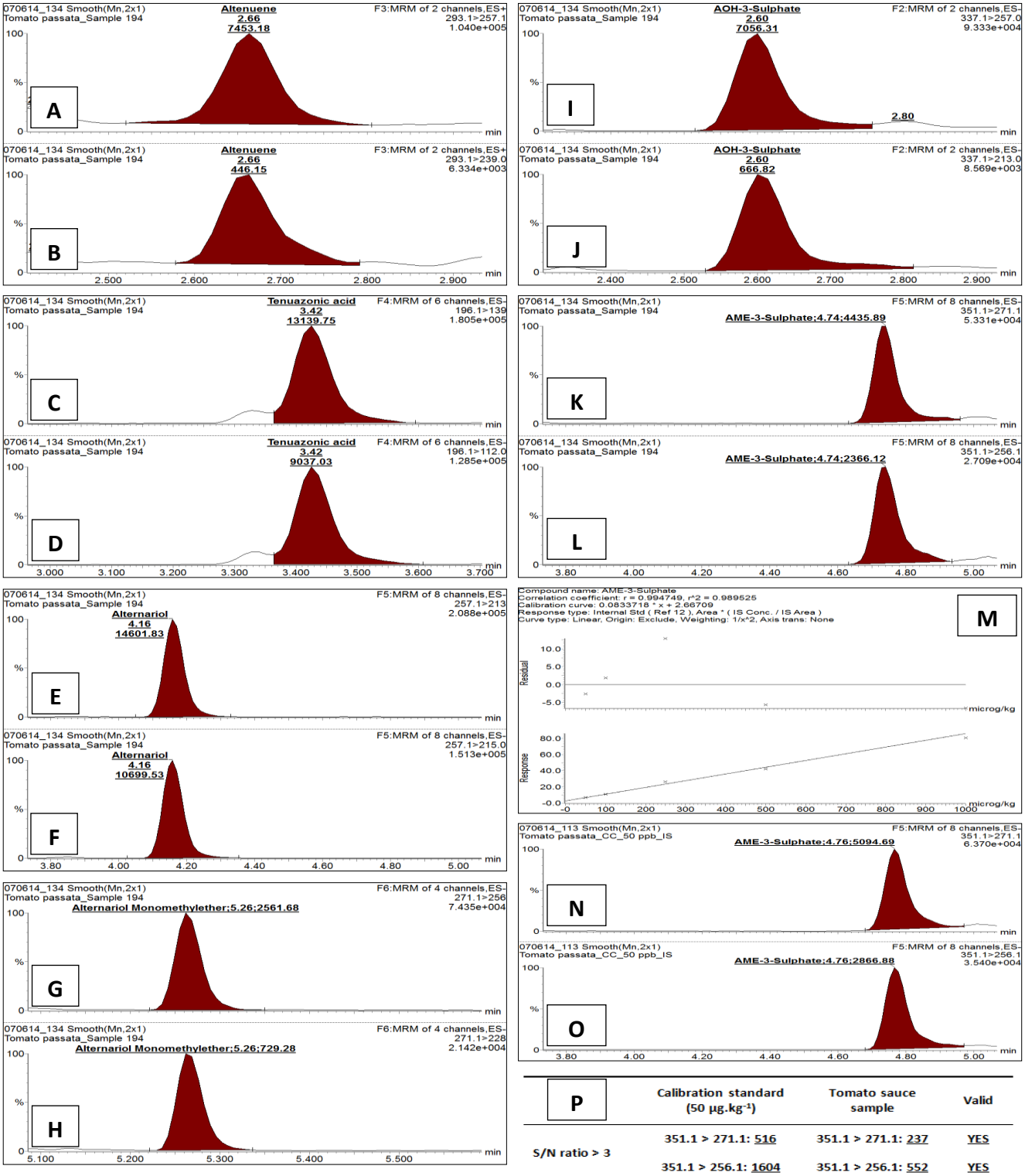
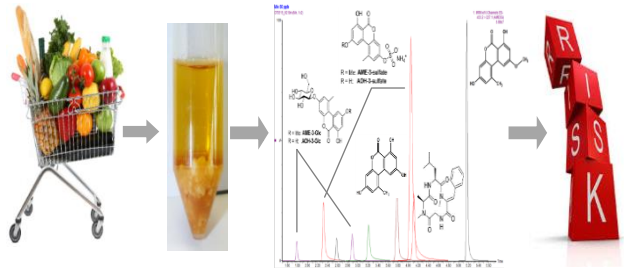


Figure 2



P	Calibration standard (50 µg.kg^{-1})	Tomato sauce sample	Valid
S/N ratio > 3	351.1 > 271.1: 516	351.1 > 271.1: 237	YES
	351.1 > 256.1: 1604	351.1 > 256.1: 552	YES
RRT	Range: 1.370 - 1.420	1.390	YES
Ion ratio	Range: 0.450 - 0.675	0.533	YES

Table of Contents Graphic



Supporting information

Results and discussion

Optimization of the sample preparation and extraction methodology

Visual comparison of the slopes (curve in standard mixture vs MMC curve with identical concentration range) confirmed the presence of signal suppression due to matrix effects and necessitated further use of MMC curves for all three juice matrices and the different types of tomato products. Upon construction of MMC curves, significantly different slopes (non-parallelism of the curves, confirmed by *t*-test[1]) were observed for the majority of target analytes. This revealed the necessity to use matrix specific MMC curves for quantitation purposes (Figure 1).

Method validation

In Tables 1 and 2, regression coefficients (R^2) and experimental *p*-values from lack-of-fit tests for every analyte in each investigated matrix are summarized in Tables 1-2. Additionally, LOD and LOQ values are represented.

Homoscedasticity was assessed as previously described.[2] Briefly, homoscedasticity is evaluated by applying an *F*-test. If the experimental *F*-value is higher than the tabled *F*-value,

this is indicative of an heteroscedastic situation, which can be counteracted through weighted least squares linear regression (WLSLR). The optimal weighting factor, w_i, is chosen according to a percentage relative error %RE:

$\%RE=([C_{experimental}-C_{assigned}]/C_{assigned})*100$

The effectiveness of a weighting factor is evaluated by calculating $\sum$%RE (the sum of absolute %RE values). In Table 3, $\sum$%RE and accuracy (in terms of bias, %) at three concentration levels obtained by using unweighted (w_i = 1) and weighted ($w_i = 1/x^2$) linear regression for all target analytes in tomato juice and tomato sauce are displayed. The weighting factor $1/x^2$ not only produced the least $\sum$%RE for these data sets, but also considerably improved the accuracy for the majority of analytes, particularly at the lowest concentration level of the calibration curve.

Apparent recovery, RSD_r, RSD_R and *U* values for every analyte in each investigated matrix are displayed in Tables 4-5.

Alternaria toxins in commercially available foodstuffs

This study reports the novel detection of modified *Alternaria* toxins (specifically, sulfates of (**1**) alternariol and (**2**) alternariol monomethyl ether) occurring in tomato products. Particularly in tomato concentrate, alternariol-3-sulfate (**8**) and alternariol monomethyl ether (**10**) were detected in 26% and 78% of all samples, in concentrations up to 8.7 and 9.9 µg/kg, respectively.

A Synapt G2-Si mass spectrometer, operated in high resolution MS^E continuum mode (ESI^-), was used to analyse tomato product samples from the survey in which sulfates of **1** and **2** were reported by low resolution tandem mass spectrometry. Accurate masses of both sulfates with an acceptable mass deviation (< 2 mDa) were detected in low energy as well as

high energy mode. Component identification was performed by comparing the retention time under identical chromatographic conditions and by matching the high energy fragmentation spectra of the precursor ion from spiked samples to that of naturally contaminated samples (Figure 2). Chromatographic separation was performed using a Waters Acquity UPLC system (Waters, Milford, MA) equipped with a FTN autosampler. A sample volume of 5 µl was injected into an HSS T3 column (1.8 µm, 2.1 x 100 mm) held at 35 °C with a flow rate of 400 µl/min. A gradient elution program with solvent A (ultra-pure water, 1% acetic acid) and solvent B (acetonitrile, 1% acetic acid) was applied as follows: 95% A and 5% B for 0.5 min followed by an increase to 95% B from 0.5 to 16.0 min, 95% B maintained from 16.0 to 17.0 min, ramping back to 95% A from 17.0 to 17.1 min, and maintaining starting conditions from 17.1 to 20 min. Mass spectrometric detection was performed using a SYNAPT G2-Si (Waters, Milford, MA) equipped with an electrospray ionization source operating in negative mode with a capillary voltage of 2.5 kV and a sampling cone voltage of 30 V. The full-scan data were acquired in MS^E continuum high resolution mode within a 50 to 1200 Da mass range with a 0.1 s survey scan time over a 17.5 min run time. In high energy mode, the trap MS collision energy was ramped from 30.0 to 50.0 eV. Desolvation temperature was 500 °C, source temperature 150 °C, cone gas flow 150 L/h and desolvation gas flow 1000 L/h. During acquisition, accurate masses were generated through correction using an external reference (Lock Spray, a 1 ng/µL solution of leucine encephalin infused at a flow rate of 10 µL/min) via a lock spray interface, generating a reference ion of m/z 554.2615 ($[M-H]^-$) in negative ionization mode.

References

1. Van Loco, J. Method Validation for Food Analysis: Concepts and Use of Statistical Techniques. In *The Determination of Chemical Elements in Food: Applications for Atomic and Mass Spectrometry;* Caroli, S., Eds.; John Wiley & Sons, Inc.: Hoboken, NJ, USA, 2007; pp. 135-163.

2. Walravens, J.; Mikula, H.; Asam, S.; Rychlik, M.; Njumbe Ediage, E.; Diana Di Mavungu, J.; Van Landschoot, A.; Vanhaecke, L.; De Saeger, S. Development and validation of an ultra-high-performance liquid chromatography tandem mass spectrometric method for the simultaneous determination of free and conjugated Alternaria toxins in cereal-based foodstuffs. *J. Chromatogr. A* **2014**, *1372*, 91-101.

Figure captions

Figure 1. Non-parallelism (confirmed by *t*-test)[1] of the matrix matched calibration (MMC) curves of both [A] **7** and [B] **4** in tomato juice versus tomato concentrate, [C] **1** in apple juice versus grape juice and [D] **8** in grape juice versus carrot juice. Parallelism[1] of the MMC curves of [E] **2** in tomato juice versus tomato concentrate and [F] **3** in tomato juice versus tomato paste due to the application of the corresponding isotope-labelled internal standards [$^{2}H_{4}$]-**2** and [$^{13}C_{6}$,^{15}N]-**3**.

Figure 2. Extracted ion chromatogram of **10** (m/z 351.0175) in [A] spiked tomato product sample (MS^{E} high energy mode), [B] spiked tomato product sample (MS^{E} low energy mode), [C] naturally contaminated tomato concentrate sample (MS^{E} high energy mode) and [D] naturally contaminated tomato concentrate sample (MS^{E} low energy mode). Comparison of fragmentation spectra (MS^{E} high energy mode) of **10** (m/z 351.0175) in [E] spiked tomato product sample and [F] naturally contaminated tomato concentrate sample.

Tables

Table 1. R^2 values and *p*-values (lack-of-fit test, SPSS) of the matrix-matched calibration curves (range 5-100 µg/kg) in fruit and vegetable juices (apple, carrot and grape juice), supplemented with limits of detection (LOD) and limits of quantitation (LOQ) for all the analytes (µg/kg).

Alternaria toxins [a]	Apple juice				Carrot juice				Grape juice			
	R^2	*p*	LOD	LOQ	R^2	*p*	LOD	LOQ	R^2	*p*	LOD	LOQ
7	0.992	0.981	0.7	2.2	0.997	0.064	1.5	5.0	0.997	0.387	1.2	4.0
8	0.997	0.749	0.4	1.4	0.999	0.098	1.5	4.8	0.996	0.375	1.4	4.5
5	0.995	0.759	1.1	3.6	0.998	0.914	1.5	5.0	0.997	0.770	1.5	5.0
9	0.994	0.833	1.6	5.2	0.998	0.643	1.7	5.6	0.998	0.545	1.6	5.2
3	0.998	0.718	1.3	4.4	0.997	0.986	1.2	4.1	0.998	0.924	1.5	5.0
6	0.993	0.612	1.5	5.0	0.993	0.410	1.7	5.7	0.996	0.157	1.2	4.0
1	0.992	0.647	1.3	4.3	0.997	0.065	1.4	4.8	0.997	0.088	1.4	4.7
4	0.996	0.925	1.0	3.4	0.998	0.141	1.4	4.6	0.997	0.314	1.5	4.9
10	0.994	0.614	1.5	4.8	0.997	0.075	1.2	4.1	0.989	0.163	1.6	5.4
2	0.998	0.945	0.3	1.1	0.998	0.501	0.7	2.2	0.999	0.299	0.8	2.8

[a] **1**: alternariol - **2**: alternariol monomethyl ether - **3**: tenuazonic acid - **4**: tentoxin - **5**: altenuene - **6**: altertoxin-I - **7**: alternariol-3-glucoside - **8**: alternariol-3-sulfate - **9**: alternariol monomethyl ether-3-glucoside **- 10**: alternariol monomethyl ether-3-sulfate.

Table 2. R^2 values and *p*-values (lack-of-fit test, SPSS) of the matrix-matched calibration curves (range 50-1000 µg/kg) in lyophilised tomato products (juice, sauce and concentrate), supplemented with limits of detection (LOD) and limits of quantitation (LOQ) for all the analytes (µg/kg, expressed on fresh weight of the tomato products applying the experimentally determined conversion factor [CF]).

***Alternaria* toxins** [a]	**Tomato juice (CF=0.052)**				**Tomato sauce (CF=0.077)**				**Tomato concentrate (CF=0.216)**			
	R²	***p***	**LOD**	**LOQ**	***R²***	***p***	**LOD**	**LOQ**	***R²***	***p***	**LOD**	**LOQ**
7	0.996	0.871	0.5	1.6	0.995	0.717	1.1	3.6	0.991	0.825	1.3	4.3
8	0.996	0.822	0.7	2.4	0.994	0.335	0.5	1.5	0.992	0.733	1.5	5.0
5	0.995	0.546	0.5	1.6	0.992	0.369	1.1	3.6	0.994	0.174	1.6	5.3
9	0.996	0.957	1.0	3.2	0.996	0.824	0.4	1.4	0.991	0.436	1.0	3.5
3	0.994	0.859	0.3	1.1	0.995	0.588	0.4	1.2	0.994	0.779	1.0	3.3
6	0.994	0.170	0.4	1.4	-	-	0.3	1.1	-	-	1.2	3.8
1	0.996	0.547	0.3	0.8	0.993	0.802	0.4	1.4	0.992	0.907	1.1	3.5
4	0.982	0.258	0.2	0.7	0.988	0.781	0.5	1.8	0.991	0.975	1.5	5.0
10	0.991	0.753	0.3	0.9	0.993	0.185	0.3	1.0	0.992	0.969	1.3	4.3
2	0.993	0.990	0.3	0.9	0.997	0.933	0.2	0.8	0.993	0.867	1.4	4.7

[a] **1**: alternariol - **2**: alternariol monomethyl ether - **3**: tenuazonic acid - **4**: tentoxin - **5**: altenuene - **6**: altertoxin-I - **7**: alternariol-3-glucoside - **8**: alternariol-3-sulfate - **9**: alternariol monomethyl ether-3-glucoside **- 10**: alternariol monomethyl ether-3-sulfate.

[b] Because of depletion of the stock solution of **6**, validation experiments for ATX-I in tomato sauce and concentrate were not performed

Table 3. Sum of the relative errors (∑%RE) and accuracy (Bias, %) at low (50 µg/kg), medium (250 µg/kg) and high (1000 µg/kg) concentration level obtained by using unweighted (w_i = 1) and weighted (w_i = 1/x^2) linear regression for all the target analytes in tomato juice and tomato sauce.

Alternaria toxins [a]	Tomato juice					Tomato sauce				
	w_i	∑%RE	Bias (%)			w_i	∑%RE	Bias (%)		
			low	medium	high			low	medium	high
7	**1**	630.5	35.5	8.8	0.6	**1**	603.0	6.6	5.5	2.9
	1/x^2	**296.8**	**8.2**	**8.6**	**5.1**	**1/x^2**	**309.0**	**9.1**	**4.6**	**1.3**
8	**1**	405.0	-6.5	4.3	4.7	**1**	428.4	33.6	-1.0	-6.6
	1/x^2	**261.2**	**4.6**	**4.5**	**3.0**	**1/x^2**	**277.5**	**-0.8**	**-0.5**	**0.5**
5	**1**	376.5	7.6	4.2	5.8	**1**	522.5	14.2	-4.5	-8.6
	1/x^2	**297.5**	**-6.8**	**4.9**	**9.4**	**1/x^2**	**382.4**	**1.7**	**-4.4**	**-6.0**
9	**1**	324.7	-2.5	4.4	6.0	**1**	430.4	8.0	-0.4	-8.1
	1/x^2	**271.9**	**-2.2**	**4.5**	**6.2**	**1/x^2**	**296.4**	**2.7**	**-0.5**	**-7.3**
3	**1**	461.7	-12.3	0.9	3.6	**1**	455.8	-15.7	0.3	-0.8
	1/x^2	**294.3**	**-3.3**	**1.6**	**2.5**	**1/x^2**	**292.5**	**2.5**	**0.4**	**-3.9**
6 [b]	**1**	604.6	-25.1	6.2	5.0	**1**	-	-	-	-
	1/x^2	**300.2**	**-1.8**	**5.8**	**0.4**	**1/x^2**	**-**	**-**	**-**	**-**
1	**1**	450.8	-17.6	8.4	8.4	**1**	454.2	-14.9	-1.1	-7.3
	1/x^2	**263.5**	**-1.8**	**8.3**	**5.5**	**1/x^2**	**335.5**	**-1.2**	**-1.3**	**-9.6**
4	**1**	1448.8	-40.6	14.8	32.3	**1**	677.0	-18.6	1.6	-8.3
	1/x^2	**497.8**	**-1.4**	**10.6**	**8.0**	**1/x^2**	**392.9**	**-5.0**	**1.3**	**-10.6**
10	**1**	600.7	-14.0	5.0	1.9	**1**	532.7	-16.2	-2.2	-5.1
	1/x^2	**351.7**	**0.1**	**3.6**	**-1.6**	**1/x^2**	**301.6**	**3.9**	**-2.7**	**-8.8**
2	**1**	631.1	1.5	5.8	0.7	**1**	317.6	15.2	5.7	0.0
	1/x^2	**278.0**	**4.9**	**5.6**	**-0.6**	**1/x^2**	**219.1**	**3.1**	**5.5**	**2.0**

[a] **1**: alternariol - **2**: alternariol monomethyl ether - **3**: tenuazonic acid - **4**: tentoxin - **5**: altenuene - **6**: altertoxin-I - **7**: alternariol-3-glucoside - **8**: alternariol-3-sulfate - **9**: alternariol monomethyl ether-3-glucoside **- 10**: alternariol monomethyl ether-3-sulfate.

[b] Because of depletion of the stock solution of **6**, validation experiments for ATX-I in tomato sauce and concentrate were not performed.

Table 4. Repeatability (RSD_r), intermediate precision (RSD_R), apparent recovery (R_A, %) and expanded measurement uncertainty (*U*, %) values for all the analytes at low, medium and high concentration level (μg/kg) in fruit and vegetable juices (apple, carrot and grape juice).

Type of juice	Concentration (μg/kg)	7 [a]				8 [a]				5 [a]				9 [a]				3 [a]			
		RSD_r [b]	RSD_R [b]	R_A [b]	*U*	RSD_r	RSD_R	R_A	*U*	RSD_r	RSD_R	R_A	*U*	RSD_r	RSD_R	R_A	*U*	RSD_r	RSD_R	R_A	*U*
Apple	**5**	12.5	12.5	96.5	48.4	13.7	13.7	99.5	49.4	7.0	13.8	99.3	49.3	15.7	15.7	91.2	54.3	9.1	9.7	104.5	29.1
	50	4.5	4.5	89.1	42.6	3.3	4.0	99.0	36.3	5.1	5.2	95.7	38.4	5.6	5.6	95.9	38.4	3.3	3.3	100.1	9.1
	100	5.6	5.6	93.3	40.0	3.8	4.8	100.0	37.0	6.4	6.4	95.9	38.9	9.5	9.5	93.3	44.0	0.8	3.6	98.8	9.6
Carrot	**5**	3.2	6.6	108.5	42.4	6.7	12.5	96.5	47.3	5.3	12.6	104.7	48.4	15.5	15.5	96.7	53.3	4.0	7.4	101.4	19.4
	50	1.5	2.9	107.2	38.3	2.1	2.4	101.0	35.3	4.7	9.5	105.0	43.7	7.7	7.7	104.7	41.8	3.8	4.2	104.5	15.1
	100	5.9	5.9	100.3	37.5	2.3	3.1	100.0	35.6	6.8	10.0	100.2	43.5	5.7	5.7	101.6	38.4	4.3	5.7	102.8	16.7
Grape	**5**	13.8	13.8	104.2	49.7	6.7	8.5	94.3	42.7	9.2	9.2	100.9	42.9	8.5	11.8	102.1	47.1	4.4	7.1	96.6	19.5
	50	5.0	5.0	105.4	38.4	1.5	3.8	105.6	37.7	5.7	5.7	108.0	41.2	5.6	5.6	103.0	37.8	4.4	4.4	100.6	11.4
	100	3.5	3.5	103.3	49.7	2.5	2.7	105.4	42.7	4.1	4.4	100.5	42.9	5.3	5.6	97.3	47.1	4.0	4.0	98.0	19.5

Type of juice	Concentration (μg/kg)	6 [a]				1 [a]				4 [a]				10 [a]				2 [a]			
		RSD_r [b]	RSD_R [b]	R_A [b]	*U*	RSD_r	RSD_R	R_A	*U*	RSD_r	RSD_R	R_A	*U*	RSD_r	RSD_R	R_A	*U*	RSD_r	RSD_R	R_A	*U*
Apple	**5**	15.0	15.0	97.3	52.7	12.5	13.6	96.4	37.8	12.8	14.7	98.9	40.2	7.9	7.9	99.7	43.7	11.2	11.2	93.0	31.2
	50	3.9	3.9	104.0	37.0	5.4	5.4	94.5	17.3	3.7	3.7	95.2	13.7	4.6	4.6	95.1	37.9	3.3	3.3	95.6	12.7
	100	1.7	6.0	98.8	37.7	10.1	10.1	94.5	26.7	6.8	6.8	96.0	18.8	2.8	5.0	99.0	37.0	4.2	5.9	95.6	17.8
Carrot	**5**	11.8	11.8	87.0	52.5	6.0	6.1	93.5	21.3	7.2	7.2	95.9	21.7	6.7	6.7	96.0	39.4	9.0	9.0	100.5	23.4
	50	6.6	7.3	104.2	41.2	3.2	3.2	105.9	14.5	3.8	3.8	105.1	15.1	2.6	2.6	100.7	35.4	3.5	4.1	97.9	12.0
	100	5.3	5.3	96.0	38.3	6.2	6.2	97.9	16.0	5.7	5.7	98.9	14.7	1.0	1.5	100.4	34.9	2.5	3.4	98.2	9.8
Grape	**5**	8.5	8.5	103.8	41.5	10.7	10.7	96.6	29.9	10.4	10.8	90.8	34.4	11.0	15.3	98.2	53.1	6.8	6.8	103.1	18.1
	50	5.4	5.4	107.2	40.0	4.6	4.6	109.8	23.0	5.7	5.7	108.4	22.3	0.8	1.2	99.7	34.8	3.7	3.7	100.9	10.3
	100	6.4	6.4	96.6	41.5	6.7	6.7	99.4	29.9	4.3	4.3	101.0	34.4	15.3	15.3	108.1	53.1	1.0	2.9	100.2	18.1

[a] **1**: alternariol - **2**: alternariol monomethyl ether - **3**: tenuazonic acid - **4**: tentoxin - **5**: altenuene - **6**: altertoxin-I - **7**: alternariol-3-glucoside - **8**: alternariol-3-sulfate - **9**: alternariol monomethyl ether-3-glucoside **- 10**: alternariol monomethyl ether-3-sulfate.

Table 5. Repeatability (RSD_r), intermediate precision (RSD_R), apparent recovery (R_A, %) and expanded measurement uncertainty (*U*, %) values for all the analytes at low, medium and high concentration level (µg/kg) in lyophilised tomato products (juice, sauce and concentrate).

Tomato product	Concentration (µg/kg)	7 [a]				8 [a]				5 [a]				9 [a]				3 [a]			
		RSD_r [b]	RSD_R [b]	R_A [b]	*U*	RSD_r	RSD_R	R_A	*U*	RSD_r	RSD_R	R_A	*U*	RSD_r	RSD_R	R_A	*U*	RSD_r	RSD_R	R_A	*U*
Juice	**50**	8.1	8.1	108.2	44.3	4.5	10.3	104.6	44.6	14.6	17.9	93.2	60.0	8.5	13.8	97.8	49.8	15.4	15.4	96.7	43.3
	250	6.7	6.7	108.6	42.3	5.0	5.0	104.5	38.0	4.5	4.5	104.9	38.1	6.4	6.4	104.5	39.4	6.0	6.4	101.6	18.5
	1000	2.3	5.6	105.1	38.9	2.0	3.1	103.0	36.1	2.4	2.4	109.4	40.0	3.4	5.2	106.2	39.4	6.4	6.4	102.5	17.6
Sauce	**50**	8.9	12.4	109.1	52.0	7.8	13.1	99.2	48.4	3.1	11.0	101.7	44.4	6.7	10.5	102.7	44.7	9.3	12.8	102.5	34.8
	250	4.3	6.0	104.6	39.4	5.5	8.5	99.5	41.2	8.9	8.9	95.6	43.2	6.6	6.6	99.5	38.6	5.1	5.5	100.4	15.6
	1000	4.6	6.3	101.3	38.6	6.4	8.1	100.5	40.9	3.4	12.3	94.0	47.1	5.8	6.3	92.7	41.2	7.9	7.9	96.1	22.2
Concentrate	**50**	8.5	8.5	93.6	43.2	3.6	9.9	105.5	44.4	6.5	6.5	96.2	38.9	9.5	11.4	100.5	46.5	7.7	12.5	106.1	35.6
	250	7.5	9.8	101.3	43.7	4.4	10.0	99.1	42.9	1.1	7.5	98.5	39.3	4.8	6.4	100.2	38.7	3.4	7.8	105.0	22.7
	1000	5.2	7.4	99.7	39.8	6.2	6.2	92.7	41.2	4.0	5.8	97.9	38.0	3.5	11.0	93.4	45.6	5.5	7.4	95.6	21.4

Tomato product	Concentration (µg/kg)	6 [a,c]				1 [a]				4 [a]				10 [a]				2 [a]			
		RSD_r [b]	RSD_R [b]	R_A [b]	*U*	RSD_r	RSD_R	R_A	*U*	RSD_r	RSD_R	R_A	*U*	RSD_r	RSD_R	R_A	*U*	RSD_r	RSD_R	R_A	*U*
Juice	**50**	8.2	11.2	98.2	45.6	5.8	5.8	98.2	16.7	11.1	11.1	98.6	29.1	9.9	10.1	100.1	44.9	6.6	6.6	104.9	20.5
	250	9.8	9.8	105.8	44.7	8.0	8.0	108.4	26.4	15.6	15.6	110.6	48.6	8.5	8.5	103.6	41.3	2.0	4.6	105.6	16.5
	1000	5.6	5.6	100.4	37.6	3.7	3.7	105.5	14.7	3.4	4.1	108.0	19.8	4.8	4.8	98.4	43.2	5.4	7.6	99.4	20.2
Sauce	**50**	-	-	-	-	5.8	5.8	98.8	25.8	8.5	12.2	95.0	33.2	9.4	9.4	103.9	43.5	5.7	11.0	103.1	29.3
	250	-	-	-	-	8.0	8.0	98.7	23.8	6.4	6.4	101.3	17.0	6.0	6.8	97.27	39.6	5.3	5.3	105.5	17.4
	1000	-	-	-	-	3.7	3.7	90.4	24.9	2.8	10.3	89.4	32.3	9.3	9.3	91.2	46.4	5.4	5.4	102.0	14.3
Concentrate	**50**	-	-	-	-	9.5	10.8	93.7	31.5	10.0	10.0	89.3	33.9	9.1	11.1	98.4	45.9	4.8	9.5	90.7	29.9
	250	-	-	-	-	4.7	10.0	98.9	25.5	5.0	9.9	95.8	26.2	5.1	9.1	96.0	42.3	5.6	5.6	93.8	18.6
	1000	-	-	-	-	6.6	6.6	92.3	23.2	7.2	8.4	91.3	28.3	7.2	8.6	93.5	43.4	3.7	10.1	95.3	26.6

[a] **1**: alternariol - **2**: alternariol monomethyl ether - **3**: tenuazonic acid - **4**: tentoxin - **5**: altenuene - **6**: altertoxin-I - **7**: alternariol-3-glucoside - **8**: alternariol-3-sulfate - **9**: alternariol monomethyl ether-3-glucoside - **10**: alternariol monomethyl ether-3-sulfate.

[b] **RSD_r** and **RSD_R** acceptance criteria: 20 and 25%, respectively; **R_A** imposed guideline ranges: 80-110%.

Figure graphics

Figure 1

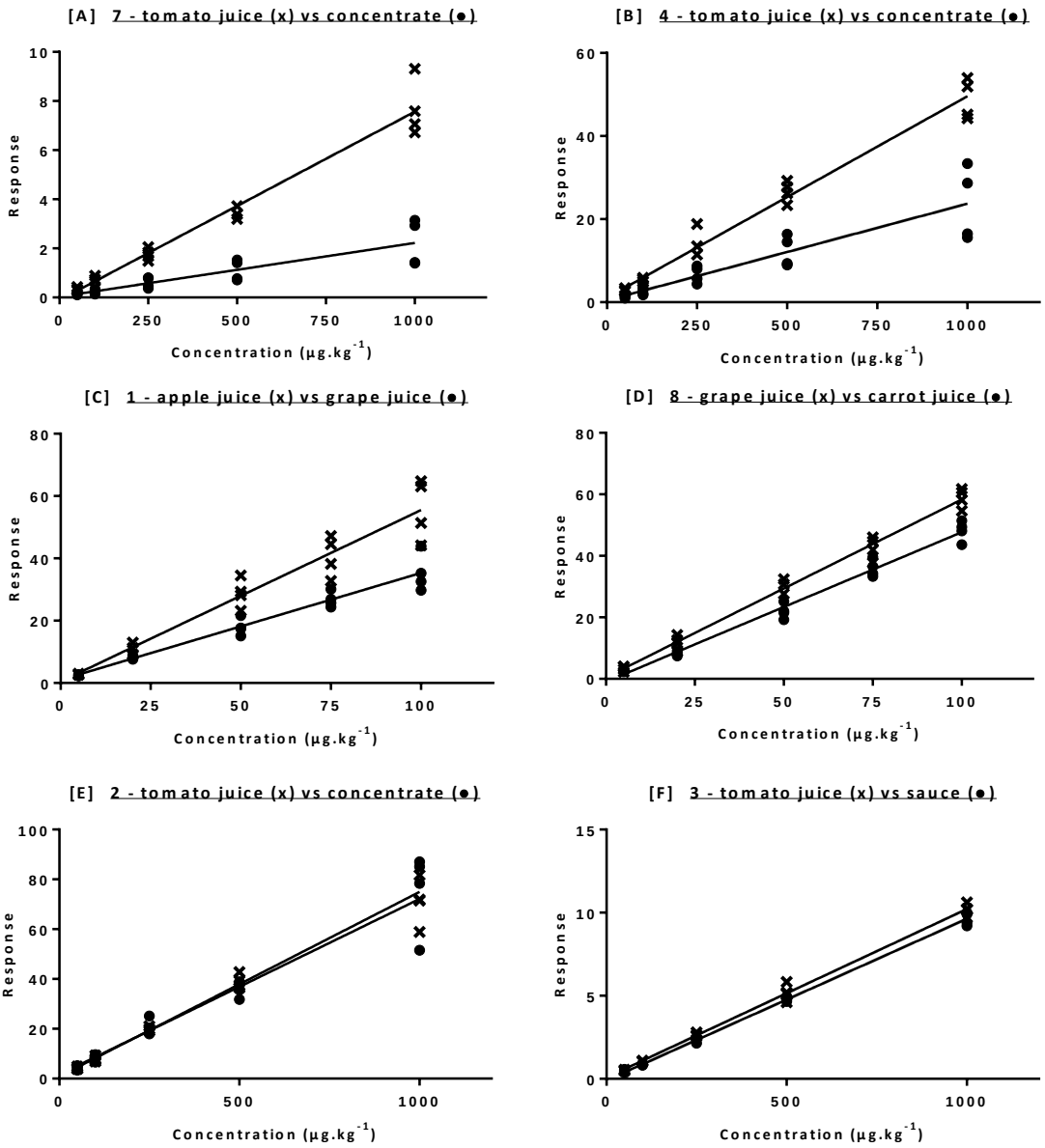
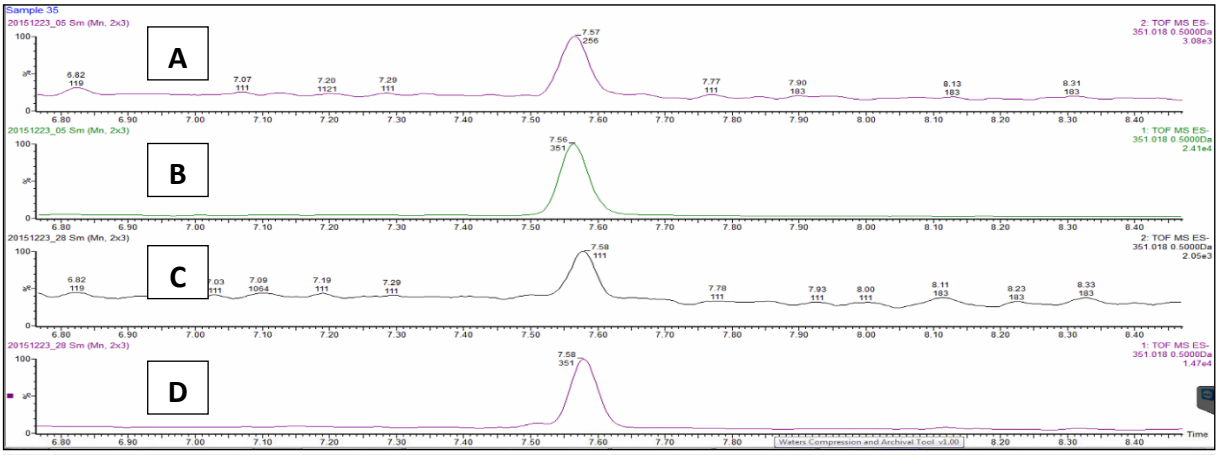
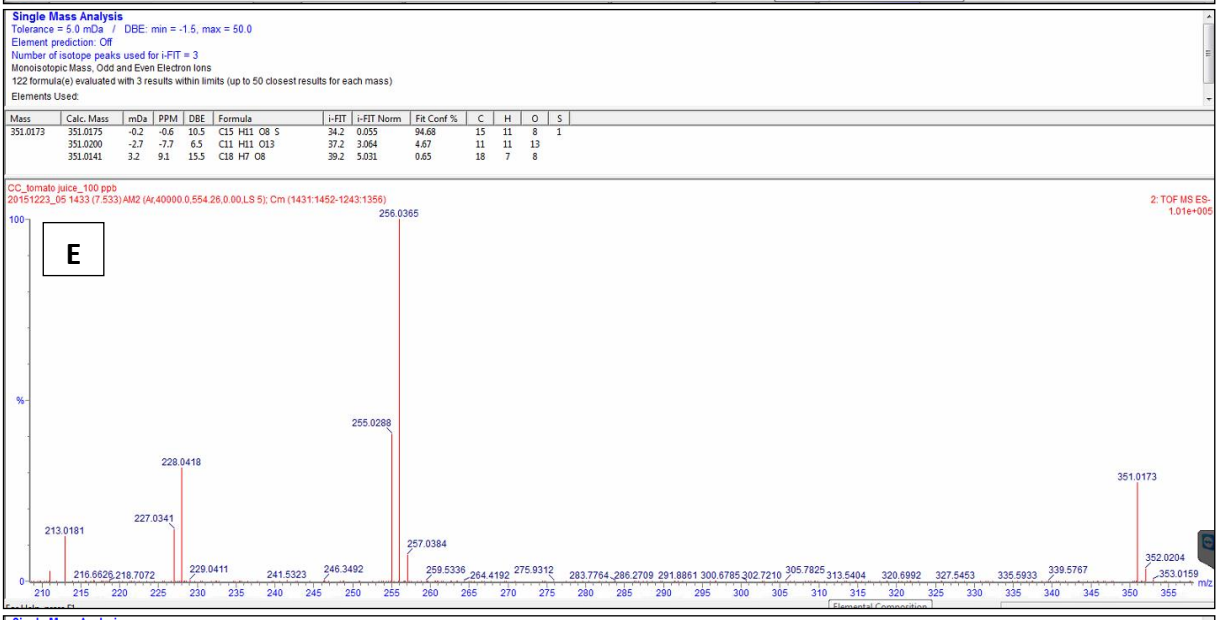
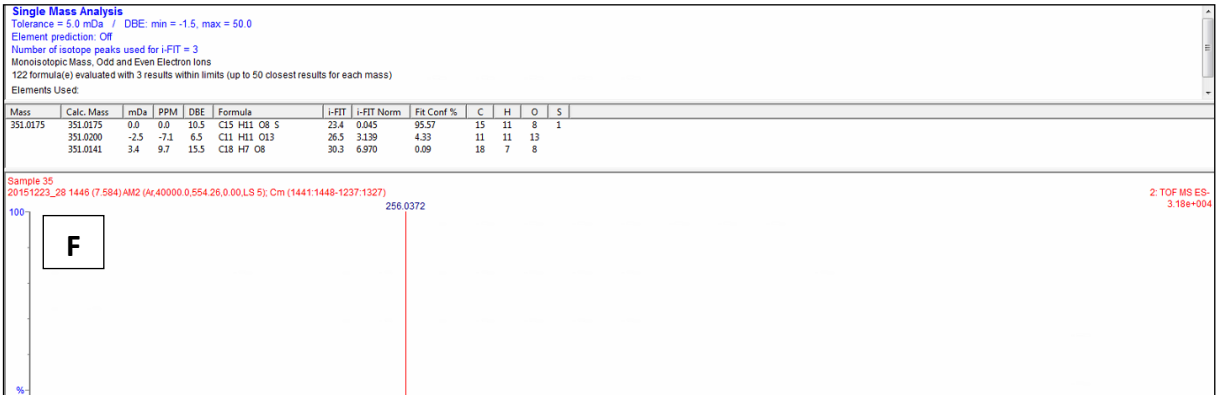


Figure 2


Sample 35
20151223_05 Sm (Mn, 2x3)
A
B
20151223_28 Sm (Mn, 2x3)
C
D
Time


Single Mass Analysis
Tolerance = 5.0 mDa / DBE: min = -1.5, max = 50.0
Element prediction: Off
Number of isotope peaks used for i-FIT = 3
Monoisotopic Mass, Odd and Even Electron Ions
122 formula(e) evaluated with 3 results within limits (up to 50 closest results for each mass)
Elements Used:
Mass | Calc. Mass | mDa | PPM | DBE | Formula | i-FIT | i-FIT Norm | Fit Conf % | C | H | O | S
351.0173 | 351.0175 | -0.2 | -0.6 | 10.5 | C15 H11 O8 S | 34.2 | 0.055 | 94.68 | 15 | 11 | 8 | 1
351.0200 | -2.7 | -7.7 | 6.5 | C11 H11 O13 | 37.2 | 3.064 | 4.67 | 11 | 11 | 13
351.0141 | 3.2 | 9.1 | 15.5 | C18 H7 O8 | 39.2 | 5.031 | 0.65 | 18 | 7 | 8

CC_tomato juice_100 ppb
20151223_05 1433 (7.533) AM2 (Ar,40000.0,554.26,0.00,LS 5); Cm (1431:1452-1243:1356)
E
256.0365
255.0288
228.0418
227.0341
213.0181
257.0384
351.0173
352.0204
353.0159
m/z


Single Mass Analysis
Tolerance = 5.0 mDa / DBE: min = -1.5, max = 50.0
Element prediction: Off
Number of isotope peaks used for i-FIT = 3
Monoisotopic Mass, Odd and Even Electron Ions
122 formula(e) evaluated with 3 results within limits (up to 50 closest results for each mass)
Elements Used:
Mass | Calc. Mass | mDa | PPM | DBE | Formula | i-FIT | i-FIT Norm | Fit Conf % | C | H | O | S
351.0175 | 351.0175 | 0.0 | 0.0 | 10.5 | C15 H11 O8 S | 23.4 | 0.045 | 95.57 | 15 | 11 | 8 | 1
351.0200 | -2.5 | -7.1 | 6.5 | C11 H11 O13 | 26.5 | 3.139 | 4.33 | 11 | 11 | 13
351.0141 | 3.4 | 9.7 | 15.5 | C18 H7 O8 | 30.3 | 6.970 | 0.09 | 18 | 7 | 8

Sample 35
20151223_28 1446 (7.584) AM2 (Ar,40000.0,554.26,0.00,LS 5); Cm (1441:1448-1237:1327)
F
256.0372
255.0289
228.0421
213.0186
227.0349
257.0391
351.0175
352.0197
m/z
Elemental Composition