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DATA ACQUISITION OF TRIPLE QUADRUPOLE LC/MS FOR THE CITRININ DETERMINATION

ABSTRACT: The analysis of citrinin is challenging because it needs to be detected in low concentrations in complex sample matrices. Before citrinin quantification, the data acquisition of LC-MS/MS must be performed, which includes the determination of ion monitoring reaction (SRM), finding fragmentation energies (Frag.) and collision cell energies (CE) for which the response of citrinin will be the highest for the given conditions. The best response of citrinin is obtained for Frag. of 66 V and CE of 17 and 29 V.

KEYWORDS: CIN, data acquisition, LC-MS/MS

INTRODUCTION

Mycotoxins are a group of natural contaminants in raw agricultural materials, foods, and feeds, mainly produced by filamentous fungi as a series of secondary metabolites (Ji *et al*. 2015). The most predominant mycotoxins are the aflatoxins (AFs – AFB1, AFG1, AFB2 and AFG2) produced by *Aspergillus* species, ochratoxin A (OTA) produced by both *Aspergillus* and *Penicillium* species, and toxins from *Fusarium* fungi, deoxynivalenol (DON), zearalenone (ZON), T-2 and HT-2 toxins, and fumonisins (FBs – FB1 and FB2) (Škrbić *et al*. 2011). To date, approximately 400 compounds have been identified as mycotoxins (Kim *et al.* 2017), one of which is citrinin (CIN).

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Citrinin is a polyketide mycotoxin with the molecular formula $C_{13}H_{14}O_5$, (IUPAC: (3*R*,4*S*)-4,6-dihydro-8-hydroxy-3,4,5-trimethyl-6-oxo-3*H*-2-benzopyran-7-carboxylic acid). Citrinin was first isolated as a pure compound from a culture of *Penicillium citrinum* by Hetherington and Raistrick in 1931 (Flajs and Peraica, 2009). Ostry *et al*. (2013) tabulated the mold that can produce this mycotoxin (Table 1), but Doughari (2015) indicated that out of main *Aspergillus (A. niger, A. ostianus, A. fumigates, A. niveus, A. awamori*, and *A. parasiticus) A. niger* is the most potent producer of citrinin. CIN is formed after harvest under storage conditions and it occurs in cereals and cereal products, rice, apples, fruit juices, black olive, almonds, peanuts, hazelnuts, pistachio nuts, sunflower seeds, spices (turmeric, coriander, fennel, black pepper, cardamom, and cumin) and food supplements based on rice fermented with red microfungus *Monascus purpureus* (Ostry *et al*. 2013). The strains of *Monascus* are traditionally used in China to produce red and yellow pigments for food. Western countries limit the use of synthetic food colorants due to their toxicity and mutagenicity. The natural food pigments obtained from *Monascus* were good candidates for their substitution because the reports on their toxic effects have been scarce for more than 1,000 years (Flajs and Peraica, 2009). Compared with other *Monascus* metabolites, CIN can be present in products in the range of concentrations from 0.1 to 500 mg/kg (Li *et al*. 2012). The European Food Safety Authority has also reported contamination of cheese by citrinin where toxigenic strains directly grow in the cheese mass.

Table 1. Penicilium and *Monascus* species as citrinin producers

Genera	Subgenus	Series	Species
Penicilium	Furcatum		*P. citrinum*
	Penicilium	*Expansa*	*P. expansum*
	Penicilium	*Carymbifera*	*P. radicicola*
	Penicilium	*Verrucosa*	*P. verrucosum*
Monascus	*M. purpureus*	Food supplement with fermented red rice	
	M. ruber	Soya bean, sorgum, rice, oat	

CIN is decomposed at 175 °C by dry heating, but decomposition temperature decreases to 140 °C in the presence of a small amount of water. The decomposition products obtained by heating CIN with water at 140 °C to 150 °C are CIN H1 and CIN H2 (Figure 1). CIN H2 shows no significant cytotoxicity, while CIN H1 shows increased cytotoxicity as compared to the parent compound (Doughari, 2015). Toxicity studies indicated that citrinin had cytotoxic, genotoxic, mutagenic and immunotoxic effects on humans and animals, and the most susceptible organ is kidney (Ji *et al.* 2015).

According to Commission Regulation (EC) 1881/2006, as amended by Regulation (EU) 212/2014, the maximum level of 2,000 μg/kg of citrinin in food supplements based on rice fermented with red yeast *Monascus purpureus* was to be reviewed before 1 January 2016 in the light of information on the exposure to citrinin from other foodstuffs and updated information on the toxicity of citrinin in particular as regards carcinogenicity and genotoxicity.

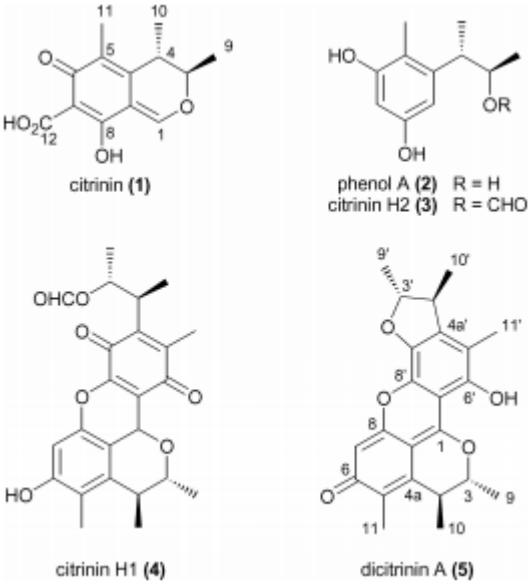


Figure 1. CIN decomposition products (Clark *et al*. 2006)

Thanks to the planar structure CIN has natural fluorescence, which indicates its detection by high-performance liquid chromatography with fluorescent detection (HPLC-FLD), but in recent years a rapid and sensitive liquid chromatography tandem mass spectrometry (LC-MS/MS) has been used (Li *et al*. 2011).

The analysis of CIN is challenging because it needs to be detected in low concentrations in complex sample matrices. Before CIN quantification, the data acquisition of LC-MS/MS must be performed.

MATERIAL AND METHODS

Analytical Standards. Citrinin of the highest possible purity was purchased from Biopure, Romer Labs as a solution of 100.4 μg/mL in acetonitrile. Working standard solutions were in the concentration of 1.0 and 10 μg/mL in acetonitrile.

Acquisition procedure. Before performing the calibration or quantification of CIN it is necessary to establish the acquisition parameters of mass spectrometry: to determine the reaction to ion monitoring (SRM), find fragmentation energies (Frag.) and collision cell energies (CE) for which the response of the studied mycotoxin will be the highest for set conditions. SRM was determined using MassHunter Optimizer Software Version B03.01 (Agilent Technologies, 2010), as well as the data from scientific papers. Then, experimentally, the needed optimum fragmentation energy and CE for each SRM were determined by introducing the solution standard of CIN (1.0 μg/mL) into ion source. In the

process, the chromatographic column from the system can, but not necessarily, be removed. In case that MassHunter Optimizer does not detect SRM, the precursor and product ion have to be detected gradually. CIN is recorded in SCAN mode where the molecular ion is detected from mass spectre. Then, based on the familiar molecular ion in Product Ion mode, by the application of various fragmentations energy, the molecular ion is fragmented and based on the obtained mass spectres the most intensive ions are found which are supposed to be formed during the fragmentation. For each formed ion in the mode Precursor Ion the source ion is confirmed. By combining these assumptions, at least two SRMs for the given analyte are confirmed.

Instrumentation and chromatographic conditions for LC-MS/MS. LC was performed with an Agilent 1200 HPLC system equipped with a G1379B degasser, a G1312B binary pump, a G1367D autosampler, and a G1316B column oven (Agilent Technologies, USA). Chromatography separation was achieved by Zorbax Ecllipse XDB C18 column (50 x 4.6 mm, 1.8 μm) (Agilent, USA) maintained at 30 °C. The analytical separation was performed using methanol as mobile phase A, and water as mobile phase B, both containing 0.1% formic acid with gradient mode (0 min – 40% B, 10 min – 5% B, 15 min – 5% B, stop time – 17 min, post time – 5 min). The flow rate was maintained at 0.5 mL/min. The mass analysis was carried out with an Agilent 6410B Triple Quadrupole mass spectrometer equipped with multi-mode ion source (MMI, Agilent Technologies, Palo Alto, CA, USA). The data acquisition and quantification were conducted using MassHunter Workstation software B.06.00 (Agilent Technologies, 2012). The following ionization conditions were used: electrospray ionization (+ESI) in positive ion mode, drying gas (nitrogen) at the temperature of 325 °C, vaporizer at 200 °C, drying gas flow rate 5 L/min, nebulizer pressure of 40 psi and capillary voltage of 2,500 V. The dwell time was 50 ms.

RESULTS AND DISCUSSION

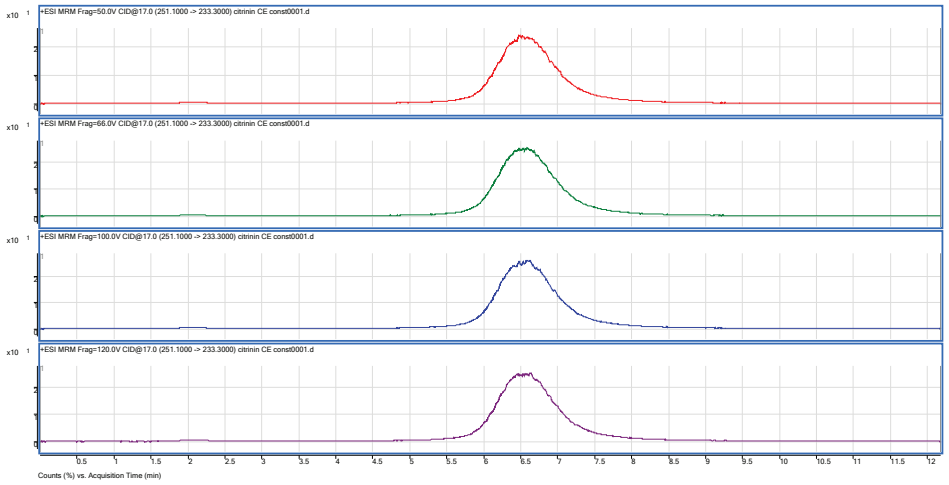
The first adjustments implied the constant collision cell energy, with various fragmentation energies (Table 2).

Table 2. Constant collision cell energy, with various fragmentation energies

Precursor Ion	MS1 Res	Product Ion	MS2 Res	Dwell	Frag (V)	CE (V)	Cell Acc. Voltage	Polarity
251.1	Wide	233.3	Unit	50	100	17	7	Positive
251.1	Wide	233.3	Unit	50	66	17	7	Positive
251.1	Wide	233.3	Unit	50	50	17	7	Positive
251.1	Wide	233.3	Unit	50	120	17	7	Positive

The effect of the fragmentation energy at the same collision energy values is shown in Figure 2.

a



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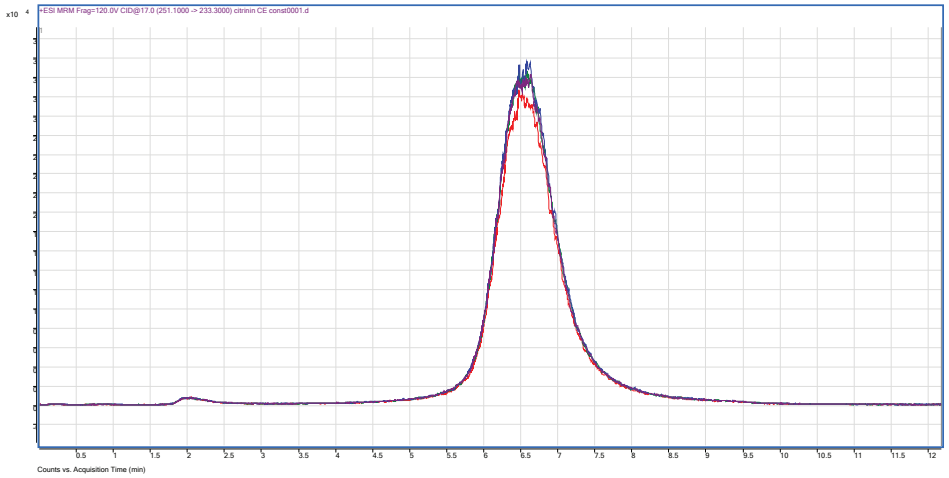


Figure 2. The effect of various fragmentation energy at constant CE (15 V)

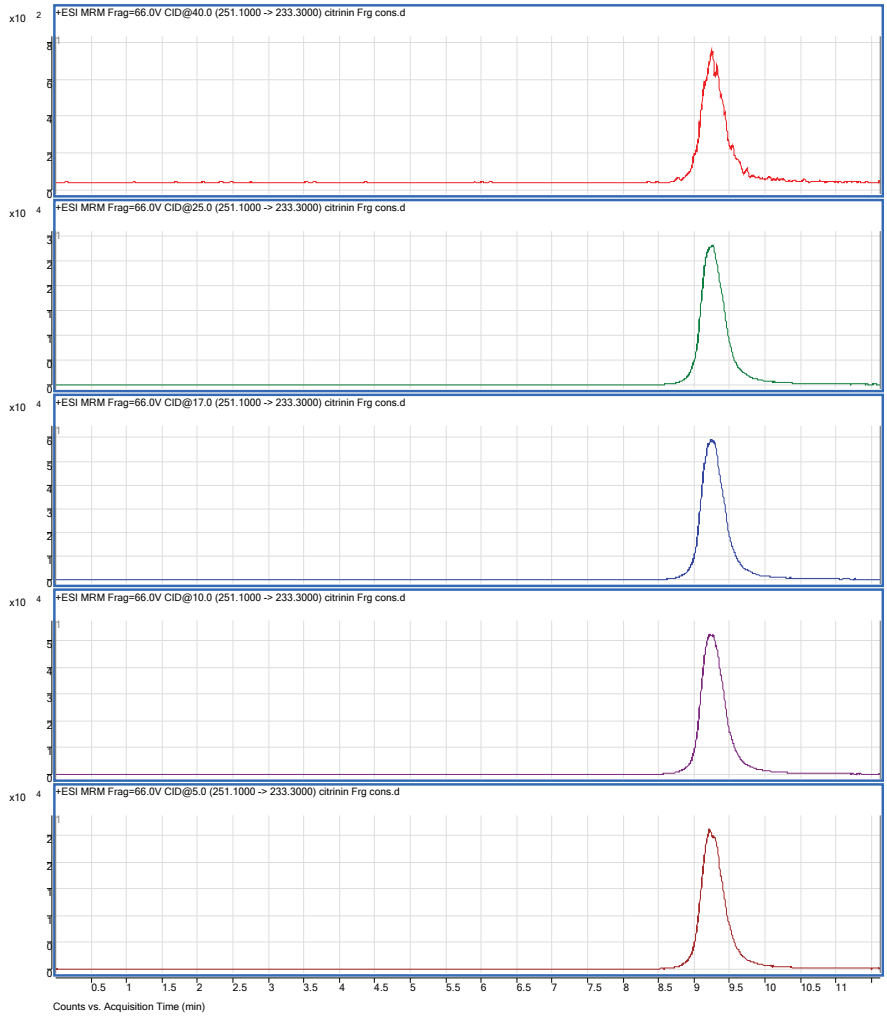
In the overlapped chromatogram (Figure 2b) it is shown that the change of collision cell energy does not significantly effect the citrinin response. Still, the best response is obtained at the collision cell energy of 66 V.

When the fragmentation energy is determined (in our case 66 V) for which the signal is the most intensive, then the fragmentation energy is kept constant and collision cell energy is changed (Table 3).

Table 3. Constant Frag. with the different CE

Precursor Ion	MS1 Res	Product Ion	MS2 Res	Dwell	Frag (V)	CE (V)	Cell Acc. Voltage	Polarity
251.1	Wide	233.3	Unit	50	66	10	7	Positive
251.1	Wide	233.3	Unit	50	66	17	7	Positive
251.1	Wide	233.3	Unit	50	66	25	7	Positive
251.1	Wide	233.3	Unit	50	66	40	7	Positive
251.1	Wide	233.3	Unit	50	66	5	7	Positive

At the constant Frag. energy of 66 V, by changing the collision energy (Figure 3) the response of the signal of studied mycotoxin in the given conditions was monitored.



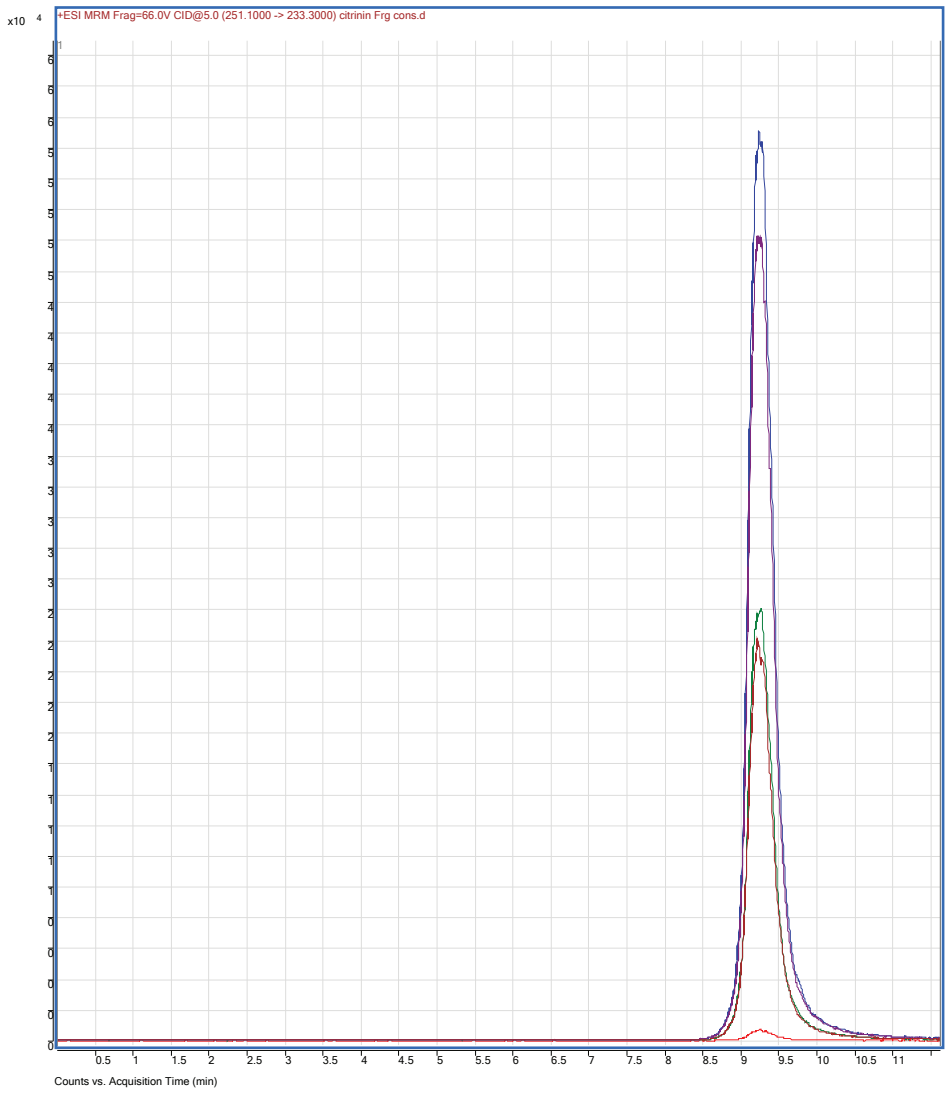


*Figure 3***.** The effect of energy change in collision cell at the constant fragmentation energy (Frag. of 66 V)

CE of 17 V gives the best CIN signal, followed by energy of 10 V. The collision cell energy of 40 V gives the weakest citrinin response.

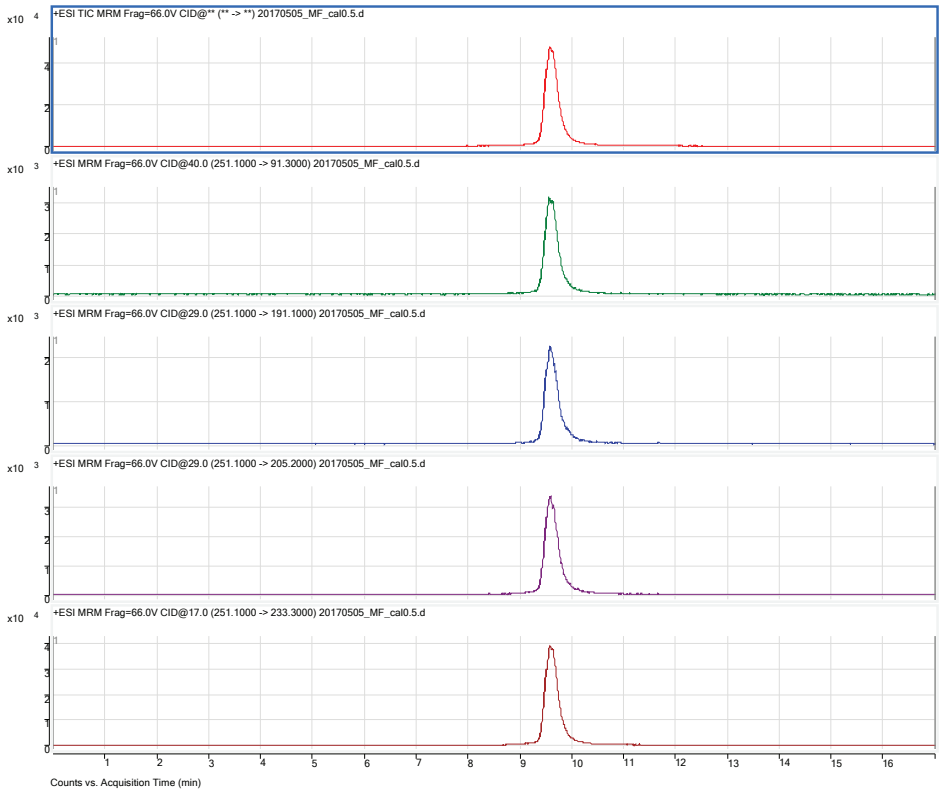
After SRM is established, it is necessary to find out the chromatographic conditions for the best separation of the studied analytes. It is necessary to emphasize that the total ion chromatogram (TIC) does not need to have a good separation resolution, because SRM chromatograms, which contained only one peak and are suitable for further quantitative analyses, are extracted from it.

Table 4. SRM transitions

Compound		Precursor Ion	MS1 Res	Product Ion	MS2 Res	Frag (V)	CE (V)	Cell Acc. Voltage	Polarity
Citrinin	q1	251.1	Wide	205.2	Unit	66	29	7	Positive
Citrinin	q2	251.1	Wide	191.1	Unit	66	29	7	Positive
Citrinin	q3	251.1	Wide	91.3	Unit	66	40	7	Positive
Citrinin	Q	251.1	Wide	233.3	Unit	66	17	7	Positive

The molecular weight of citrinin is 250.25 g/mol, the identification of the target mycotoxin through the selection of specific MRM transitions from 251.1 (used as qualification ion) to 233.3 (used as quantification ion) m/z was carried out at the constant Frag. (66 V) and CE of 40, 25, 17, 10, and 5 V.

As the response, i.e. the peak area, is the largest at 17 V, the other CE values are not taken into consideration (Figure 4).



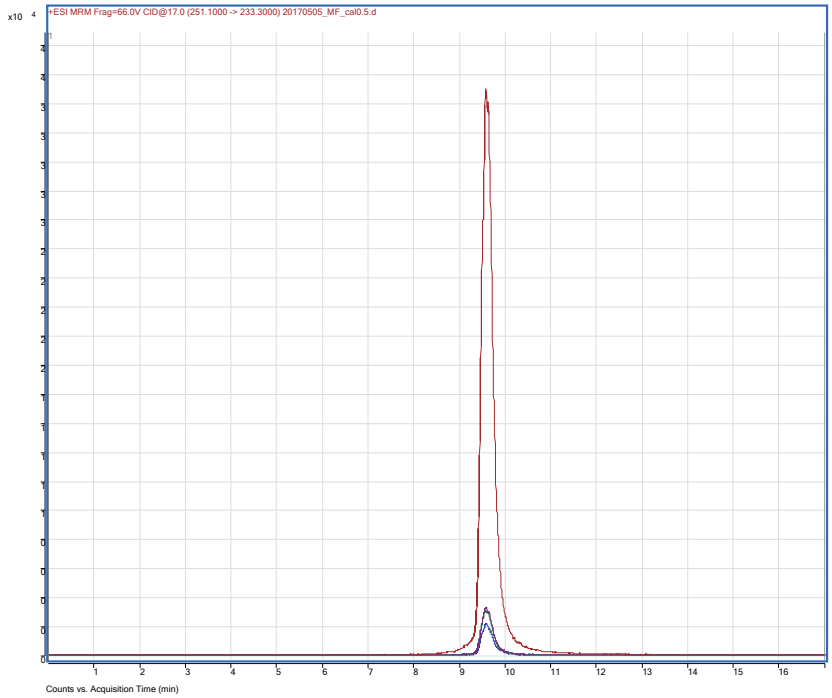


Figure 4. MRM citrinin transitions

The mechanism of citirinin fragmentation was given in Figure 5 (Shu and Lin, 2002).

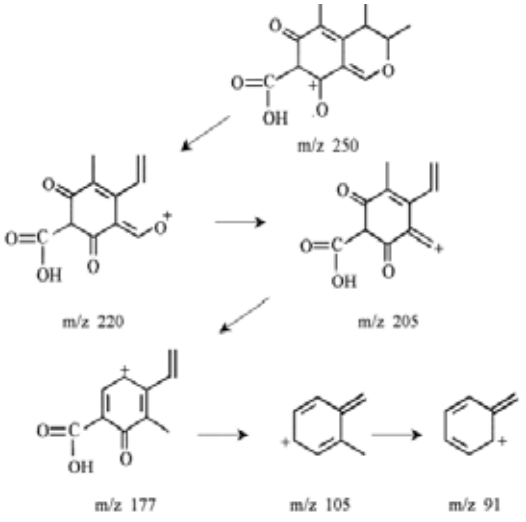


Figure 5. Fragmentation pattern of citrinin

CONCLUSION

Before doing the calibration or quantification of CIN it is necessary to establish the acquisition parameters of mass spectrometry: to determine the reaction to ion monitoring (SRM), find fragmentation energies (Frag.) and collision cell energies (CE) at which the response of the studied mycotoxin will be the highest for set conditions. The best response is obtained at the collision cell energy of 66 V. Collision cell energies of 17 V give the best CIN signal.

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АКВИЗИЦИОНИ ПАРАМЕТРИ ТРОСТРУКОГ КВАДРОПОЛА LC/MS ОДРЕЂИВАЊА ЦИТРИНИНА

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РЕЗИМЕ: Анализа ЦИН-а представља велики изазов из разлога што га је потребно детектовати у веома ниским концентрацијама у различитим узорцима. Пре квантификације ЦИН-а потребно је поставити аквизиционе параметре LC-MS/MS, који укључују одређивање реакције праћења јона (CPM), проналажење енергије фрагментације (Фраг.) и енергије колизионе ћелије (ЦЕ) при којој ће одговор ЦИН-а бити највећи за дате услове. Најбољи одговори ЦИН-а добијају се при Фраг. од 66 V и ЦЕ од 17 и 29 V.

КЉУЧНЕ РЕЧИ: ЦИН, аквизициони параметри, LC-MS/MS