Rev. Toxicol (2018) 35: 2 - 6

Dietary exposure to mycotoxins through fruits juice consumption

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Abstract: A study on fruit juice products (apple, pineapple, apricot, orange and pear) was carried out to determine the natural occurrence of fifteen mycotoxins by gas chromatography coupled to tandem mass spectrometry (MS/MS). A developed multi-mycotoxin procedure was carried out by dispersive liquid-liquid microextraction (DLLME). 36% of the analyzed samples presented mycotoxin contamination. PAT was detected in orange, apple, mixed fruits and pineapple juices with prevalence of 86%, 60%, 29%, 14% at mean concentrations of 34.57 µg/L, 33.41 µg/L, 8.59 µg/L, 8.02 µg/L, respectively. One orange juice sample, exceeded the maximum level (ML) established by EU for PAT (50 µg/L). HT-2 toxin was found in mixed juice (43%) at mean level of 22.38 µg/L. Overall no toxicological concern was associated to mycotoxins exposure for children and adult population and the results obtained highlight the necessity for rigorous monitoring studies on HT-2 in fruit juice.

Keywords: mycotoxins, fruit juice, daily intake

Resumen: Exposición a micotoxinas a través del consumo de zumo de frutas.

Se presenta un estudio sobre zumos de frutas a base de manzana, piña, albaricoque, naranja y pera para determinar la presencia natural de quince micotoxinas mediante cromatografía de gases acoplada a espectrometría de masas en tándem (EM/EM). El procedimiento desarrollado de multi-micotoxinas se llevó a cabo mediante micro-extracción líquida-líquida dispersiva (DLLME). El 36% de las muestras analizadas presentaron contaminación con micotoxinas y una muestra de jugo de naranja, superó el nivel máximo (ML) establecido por la UE para PAT (50 µg/L). Se detectó PAT en naranja, manzana, frutas mezcladas y jugos de piña con una prevalencia de 86%, 60%, 29%, 14% a concentraciones promedio de 34.57 µg/L, 33.41 µg/L, 8.59 µg/L, 8.02 µg/L, respectivamente. La toxina HT-2 estaba presente en el jugo mixto (43%) a un nivel medio de 22.38 µg/L. En general, ninguna preocupación toxicológica se asoció con la exposición a micotoxinas en la población de niños y adultos, los resultados ponen de relieve la necesidad de estudios rigurosos de monitoreo de HT-2 en el zumo de fruta.

Palabras claves: micotoxinas, zumos de frutas, ingesta diaria

Introduction

Mycotoxins are secondary metabolites produced by filamentous fungi as *Aspergillus, Penicillium, Fusarium and Claviceps.* More than 400 mycotoxins are known and only some of them represent a real threat to food security. The most relevant are aflatoxins (AFs), ochratoxin A (OTA), Patulin (PAT) fumonisins (FBs), zearalenone (ZEA) and trichothecenes (TCs) [1]. Chronic exposition to some mycotoxins can produce carcinogenic, mutagenic, teratogenic, cytotoxic, neurotoxic, nephrotoxic, immunosuppressive and estrogenic effect. Their seriousness effects depend largely on the ingested amounts and duration of exposure that may result from simultaneous ingestion of various mycotoxins [2]. Mycotoxins can be present along the entire process of food production; in field, before and after the harvest, during processing, storage and also in a finished product [3].

Various factors affect the levels of contamination of mycotoxins in fruit and fruit products such as type and variety of fruit, climate conditions, geographical location, year production treatments before and after the harvest, use and pesticides, damage to the surface of the fruit, and storage conditions [4]. European Food Safety Authority (EFSA) have established maximum permitted levels for certain mycotoxins as aflatoxins B_1, B_2, G_1, G_2 y M_1, OTA, PAT, ZEA, DON and fumonisins B_1 and B_2, T-2 and HT-2 toxins [5]. In fruits and fruit juices, only PAT and OTA are legislated. A maximum up to 50 µg/kg is set for Patulin in reconstituted concentrated fruit juices and fruit nectar (Table1)

Table 1. *Summary of maximum levels specified for patulin in fruit and fruit juice*

Food products	Maximum level µg/kg
Fruit juices, concentrated fruit juices as reconstituted and fruit, nectars, spirit drinks, cider and other fermented drinks derived from apples or containing apple juice	50
Apple juice and solid apple products	10

While for Ochratoxin A a maximum level of 2 µg/kg is set up in grape juices, reconstituted concentrated grape juice, grape nectar, grape must and reconstituted concentrated grape must, intended for direct human consumption [5].

The presence of mycotoxins has been highly investigated in different fruit juices such as apple juice [6], orange juice [7], pear juice [8] apricot and peach juice [9] and berry juice [10]. The most of the method used for extraction mycotoxins in fruit juice are QuEChERS extraction [11], liquid-liquid extraction [12], and dispersive liquid-liquid microextraction [13]. DLLME offers some advantages over traditional technique being simple, fast and low cost technique extraction [14].

Regarding analytical method for mycotoxins determination, liquid chromatography mass spectrometry in tandem (LC-MS/MS) and gas chromatography coupled to mass spectrometry detector (GC-MS/MS) have become the most extensively technique used for determination of mycotoxins in biological and food samples [15, 16]. The gas chromatographic techniques offers some advantages as lower detection limits and greater selectivity [17].

In this sense the aim of the present study was to evaluate the presence of fifteen mycotoxins DON, 3-AcDON, 15-AcDON, NEO, DAS, NIV, ZON, α-ZOL, β-ZOL,α-ZAL, β-ZAL, FUS X, T-2, HT-2 and PAT and to carry a risk exposure of the population to these mycotoxins through the fruit juice consumption.

Material and methods

Chemicals and reagents

Solvents (acetonitrile, hexane, chloroform and methanol) were supplied by Merck (Darmstadt, Germany). Deionized water (<18.2 MΩ cm resistivity) was obtained in the laboratory using a Milli-QSP® Reagent Water System (Millipore, Beadford, MA, USA).

Ammonium formate (99%) and formic acid (≥98%), sodium chloride were supplied by Sigma Aldrich (Madrid, Spain). Syringe nylon filter (13mm diameter 0.22 µm pore size) were obtained from Analysis Vinicos S.L. (Tomelloso Spain). The derivatization reagent composed of BSA (N,O-bis(trimethylsily) + TMCS (trimthylcholorosilane) + TMSI (N-trimethylsilyimidazole) (3:2:3) was obtained from Supelco (Bellefonte, PA). Sodium dihydrogen phosphate and disodium phosphate, used to prepare phosphate buffer, were acquired from Panreac Quimica S.L.U. (Barcelona, Spain).

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Standards and solutions

The standards of DON, 3-ADON, 15-ADON, DAS, NIV, FUS-X, NEO ZON, α-ZAL, β-ZAL, α-ZOL, β-ZOL, T-2 and HT-2 toxins were purchased from Sigma Aldrich. Individual stock of all analytes were prepared to obtain 20 mg/L in methanol and multianalyte working solutions of 2 mg/L were also used by diluting the individual stock solutions. The multianalyte working standard solution was used for standard calibration curves, matrix-matched calibration curves and recovery assays. All standards were stored in darkness and kept at-20°C.

Procedures

Commercial samples

A total of 42 samples were purchased from different supermarkets in Valencia, Spain. The samples were classified in as 10 samples of apple juice, 7 samples of each of pineapple, apricot, mixed fruits and orange juices and 4 samples of pear juice.

Dispersive liquid-liquid microextraction

Samples extraction was performed according to the previously validated method by Pallarés et al. [18]. Briefly, 5 mL aliquot of fruit juice sample were placed in a 10 mL conical tube, a mixture of dispersion solvent (950 µL of ACN) and the extraction solvent (620 µL of EtOAc) were added; the resulting mixture was shaken for 1 min, forming a cloudy solution of the three components. The mixture was centrifuged 4000 rpm for 5 min, and the organic phase at the top of the tube was recovered and placed in second conical tube. Next, a mixture of dispersion solvent (950 µL of MeOH) and extraction solvent (620 µL of CHCl3) was added to the remaining residue, and after agitation and centrifugation, the separated organic phase was recovered and added to the first organic phase. The solvent in the conical tube containing the two recovered phases was evaporated to near dryness under a nitrogen stream using turvovap LV Evaporator (Zimark, Hopkinton, MA). The residue was reconstituted with 1 mL of 20 mM ammonium formate (MeOH/ACN) (50/50 v/v) and filtrated through a 13-mm/0.22 µm nylon filter (Membrane Solutions, Plano, TX). Finally, reconstituted extract was evaporated to dryness under a gentle nitrogen flow.

Derivatization

Before GC-MS/MS analysis, 50 µL of BSA + TMCS + TMSI (3:2:3) was added to the dry extract and left 30 min at room temperature. Then 200 µL of hexane was added, mixed thoroughly on vortex for 30 s, washed with 1 ml of phosphate buffer (60 mM, pH7) and mixed until the upper layer was clear. Finally, the hexane layer was transferred to an auto sampler vial.

GC-MS/MS analysis

Gas chromatographic determination was carried out using a GC system Agilent 7890A coupled with an Agilent 7000A triple quadruple mass spectrometer with inter electron-impact ion source (EI, 70Ev) and Agilent 7693 auto sampler (Agilent Technologies, Palo Alto, USA). Quantitation data were acquired at selection reaction monitoring (SRM). The transfer line and source temperatures were 280° and 230°, respectively. The collision gas for MS/MS experiments was nitrogen, and the helium was used as quenching gas, both at 99.999% purity supplied by Carburos Metálicos S.L. (Barcelona, Spain). Analytes were separated on a HP-5MS 30m x 0.25mm x 0.25µm capillary column. A total one microliter of the final clean extract of mycotoxins was injected in splitless mode in programmable temperature vaporization (PTV) inlet at 250°C employing helium as the carried gas at fixed pressure of 20.3 psi. The oven temperature started at 80°C, and increased to 245°C at 60 ºC/min, hold their time for 3 min and increased to 260 ºC progressively at to 3 ºC/min and finally to 270 ºC at 10 ºC/min and then held for 10 min. Data were acquired and processed using Agilent Masshunter version B.04.00 software.

The criteria established in Document No. SANCO 11813/2017 [19] was achieved for quantification purposes. For each analyte, two transitions of SRM were required for each compound and compliance of the SRM ratio, defined as the relative intensities of ions between the area of both the quantitation (Q) and the confirmation transition (q). The most intense SRM transition was selected for quantification purposes. The specific parameters of MS/MS for each mycotoxin are detailed in table 2.

Table 2. *MS/MS parameters of the selected mycotoxins*

Mycotoxin	Retention time (min)	Quantitative Transition (Q) Q1	Q3	CE, eV (Dt, ms)	Qualitative transition (q) Q1	Q3	CE, eV (Dt, ms)
DON	8.4	392	259	10 (25)	407	197	10 (25)
3-ADON	9.68	392	287	5 (35)	467	147	10 (25)
15-ADON	9.65	392	217	20(35)	392	184	20(35)
NIV	10.15	289	73	15 (35)	379	73	15 (35)
NEO	11.68	252	195	10 (25)	252	167	15 (35)
DAS	9.85	350	229	15 (35)	378	124	10 (25)
HT-2	14.39	347	157	10 (25)	347	185	10 (25)
T-2	14.8	350	259	10 (25)	350	229	15 (35)
FUS-X	9.73	450	260	10 (35)	450	245	20 (35)
PAT	4.3	226	73	10 (50)	183	75	15 (50)
ZON	15.95	462	151	10 (25)	462	333	10 (25)
α-ZAL	15.45	433	309	20 (35)	433	295	20 (35)
ß-ZAL	15.68	433	295	15 (35)	307	73	10 (35)
α-ZOL	16.45	305	73	15 (25)	305	289	15 (20)
ß-ZOL	16.82	536	333	10 (35)	536	446	15 (20)

Exposure assessment

The probable daily intake (PDI) is considered one of the most important aspect for the measure and characterization of risk assessment of contaminants in food. With the aim of estimating exposure to the substance in the population [20], the probable daily intakes (PDIs) was calculated and expressed in µg/L bw day as indicated by the following equation:

PDI= (C*K)/bw

Where "C" is the mean concentration of mycotoxin detected in food expressed as µg/L. "K" represents fruit juice consumption expressed in L per day and "bw" is the average weight of the age groups studied. Fruit juice consumption data were available in the statistical data base of the Spanish Ministry of Agricultural and Environment (MAPAMA), considering a consumption of 10 L/annual [21]. As this fruit juice by general population including children, two different body weights were considered (25 kg and 70 kg for children and adults, respectively).

The health risk characterization of mycotoxin (%) percentage of relevant TDI) was performed by dividing the calculated PDI by the tolerable daily intake (TDI) (µg/kg bw day) of the respective mycotoxins.

%TDI= (PDI/TDI)*100

The established provisional maximum tolerable daily intake (PMTDI) for PAT of 400 ng/kg bw was established [22] and 60 ng/kg bw for the sum T-2 and HT-2 toxins [23].

Results and discussion

Method validation and analytical parameters

The analytical method was validated for fruit juice samples (Table 2). Matrix effects were corrected by matrix-assisted calibration curve a mycotoxin-free sample. For the evaluation of the linearity, calibration curves were constructed at eight concentration levels (250 to 1.97 µg/L for all studied analytes).

The results showed good correlation coefficients (r^2 >0.9986). Limits of detection (LODs) were calculated using a signal-to-noise of 3 (from 0.58 to 2.34 µg/L). Limit of quantification (LOQ) were calculated using a signal-to-noise of 10 (from 1.95 to 7.81 µg/L). The accuracy was evaluated through essays recoveries at three concentrations (50, 100, 200 µg/L). Intraday and interday precision of the method were carried out by spiking fruit juice at the three levels previously indicated was assessed based on three determination on the same day, and interday precision was assessed based on three determinations on consecutive. Method precision was estimated by calculating the relative standard deviation (RSD) using the results obtained during the same day (intraday) and on three different days (interday) by the repeated analysis three times at the three spiked levels. Recoveries values at three spiked levels were ranged from 61 to 114 %.

Natural occurrence of mycotoxin in fruit juice

A total de 42 different samples were evaluated. The results of the natural occurrence are shown in the table 3.

Table 3. *Analytical parameters for the used method: limits of detection (LOD) and quantification (LOQ), recovery, interday relative standard deviation (RSD_R) (n=9), absolute matrix effect, and linearity expressed as correlation coefficient (r^2)*

			Recovery (RSD_R) (%)							
Analyte	LOD µg/L	LOQ µg/L	50 µg/L		100 µg/L		200 µg/L		Matrix effect (%)	Linearity
DON	0.58	1.95	68	(3)	73	(4)	62	(3)	103	0.996
3ADON	1.17	3.90	103	(2)	103	(7)	105	(11)	88	0.991
15ADON	0.58	1.95	95	(7)	98	(1)	96	(7)	114	0.993
DAS	1.17	3.90	103	(1)	91	(7)	84	(9)	96	0.991
FUS-X	2.34	7.81	84	(9)	106	(9)	98	(4)	106	0.995
T-2	2.34	7.81	98	(3)	96	(12)	78	(12)	71	0.991
HT-2	0.58	1.95	106	(11)	107	(5)	97	(5)	78	0.991
NIV	1.17	3.90	61	(3)	88	(9)	97	(7)	87	0.995
NEO	0.58	1.95	105	(6)	114	(1)	90	(6)	95	0.991
PAT	2.34	7.81	61	(9)	96	(6)	92	(13)	78	0.998
ZON	2.34	7.81	98	(2)	113	(9)	103	(9)	115	0.991
AZAL	0.58	1.95	101	(10)	97	(4)	99	(3)	88	0.992
BZAL	2.34	7.81	97	(5)	1055	(8)	104	(4)	91	0.994
AZOL	0.58	1.95	80	(1)	113	(1)	95	(11)	62	0.992
BZOL	1.17	3.90	98	(4)	93	(3)	102	(6)	69	0.992

Occurrence of Patulin

Six apple juice samples (60%) presented patulin contamination at mean levels of 33.41 µg/L, which are above 10 µg/kg of ML established for apple juice. Orange juice samples also resulted contaminated by PAT in 86% of the sample with mean content of 31.29 µg/L, 29% of mixed juiced sample resulted contaminated by PAT, but only one sample of pineapple juice contained PAT at mean level of 8.05 µg/L.

Figure 1**.** MRM chromatogram of a) standard matrix matched with fruit juice at 125 µg/L of PAT and HT-2 toxin. b) positive orange juice sample containing 14.78 µg/L and positive mixed juice containing HT-2 toxin 21.38 µg/L.

Several studies reported presence of PAT in different types of fruit juice at highest concentrations to those found in this survey. In a recent study Li et al. [6] reported high concentration of PAT until 1234 µg/L in apple juice brand. The presence of PAT in orange juice also was reported in other study conducted by Cho et al. [24] who reported natural occurrence of PAT in orange juice in 8% of sample ranged between 9.9 to 30.9 µg/L. Regarding pineapple juice, similar incidence to those found in this study were reported by Lee et al. [25] who confirmed the presence of PAT in only one sample at concentration of 33.7 µg/L. Drusch and Ragab [26] confirmed in a published review presence of PAT in fruit juice sample that contain pineapple with a maximum concentration of 60 µg/L.

Occurrence of HT-2 toxins

HT-2 toxins were present in 43% of the mixed juice (apple, mango, pineapple) in ranging from 21.38 to 24.15 µg/L. The mycotoxins of the trichothecene group are generally present in grains and grain-based products and, as far as we know, very rarely in juices. However, López et al. [27] reported presence of T-2 toxins in apple juice composite at 14 µg/L and HT-2 toxins only was detected below LOQ (<20 µg/L). Those authors mentioned that cross contamination during manufacturing the preparation of compound may have caused the presence of T-2 in apple juice composite samples.

In this study mycotoxins were not found in apricot and pear juice samples. However Poapolathep et al. [28] has investigated PAT in 40 apricot juice sample, 10% of samples contained PAT at mean levels of 4.51 µg/L. Similar content were found by Sparado et al. [9] who quantified PAT in 29% of sample at mean concentration of 3.6 µg/L. Moukas et al., et al. [29] also reported PAT in apricot juice samples at mean level of 13.70 µg/L.

Concerning presence of mycotoxin in pear juice, Rahimi et al. [30]

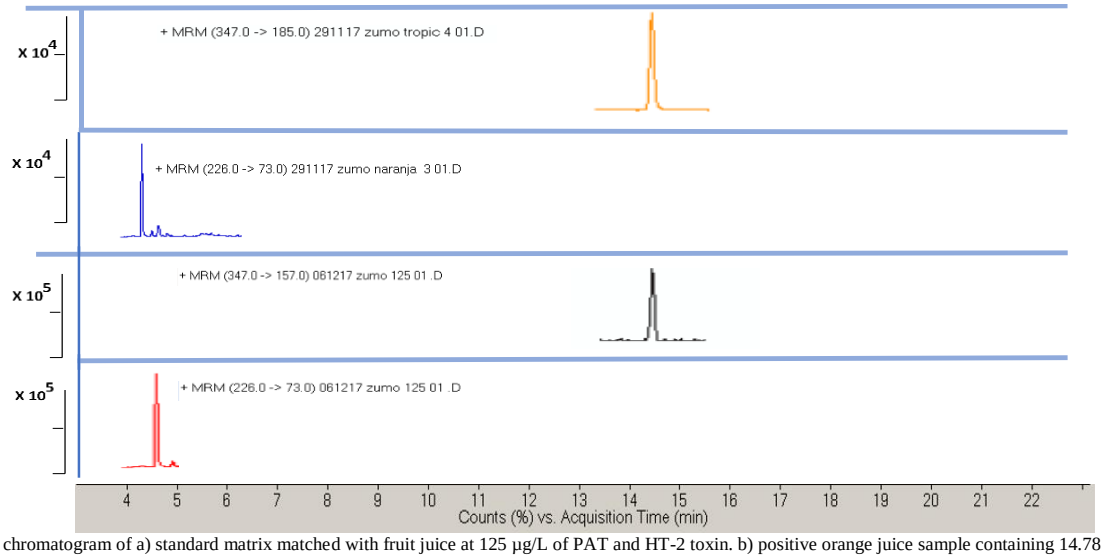

Figure 1: MRM chromatogram of a) standard matrix matched with fruit juice at 125 µg/L of PAT and HT-2 toxin. b) positive orange juice sample containing 14.78 µg/L and positive mixed juice containing HT-2 toxin 21.38 µg/L.

Table 4. *Occurrence of patulin and HT-2 in fruit juice analyzed*

Mycotoxin	Parameters	Apple	Pineapple	Orange	Mixed fruits	Apricot	Pear
PAT	Incidence	6/10	1/7	6/7	2/7	0/7	0/4
	Mean (µg/L)	33.41	8.05	34.57	8.59	-	-
	Range (µg/L)	28.07-47.82	8.05	14.78-50.95	6.3-10.89	n.d	n.d.
HT-2	Incidence	-	-	-	(3/7)	-	-
	Mean (µg/L)	n.d.	n.d.	n.d.	22.38	n.d	n.d
	Range (µg/L)	n.d.	n.d.	n.d.	21.38-24.15	n.d	n.d

analyzed 15 pear juice samples, but only two samples resulted contaminated by PAT at mean concentrations of 22.9 µg/L. Similar results were detected by Drush and Ragab [26] who also reported presence of PAT at maximal concentration of 20 µg/L. Highest content were reported by Zouaoui et al. [8] who detected PAT in in 47% of pear juice samples at average concentration of 62.5 µg/L. This value exceed ML established of PAT in fruit juice which it is 50 µg/kg.

Calculation PAT and HT-2 toxins intake

The PDI calculated values and comparison with the PMTDI for the risk assessment in children and adults population are reported in the Table 5. The PDI obtained for patulin in apple juice was 26.72 and 9.54 ng/kg bw day. The PDI calculated in orange juice were 27.65 and 8.94 ng/kg bw day. In pineapple juice was obtained PDI of 2.44 and 0.87 ng/kg bw day. PDI ranging from were 6.87 and 2.45 ng/kg bw day through the consumption mixed juice. The results indicated a low exposure to PAT through fruit juice consumption.

In others studies, estimate daily intake value calculated for PAT through the consumption of apple juice consumed in Spain ranged between 155 and 55 ng kg/bw day for children and adult, respectively [12]. EDI of PAT obtained by Poapolathep et al. [28] for the children Thailand population through the consumption of apple juice were 0.96 µg/kg bw day.

Table 5 shows the exposure estimates for HT-2 toxins. The PDI calculated were between 17.90 and 6.39 ng/kg bw day. The %PMTDI were 29.84% and 10.65% for children and adults population.

Table 5. *Patulin exposure calculated for children and adults through fruit juice consumption*

Commodity	Population group	PDI ng/kg bw	PMTDI (ng/kg bw)	%PMTDI
Apple juice	Children	26.72	400	6.68
	Adults	9.54	400	2.38
Orange juice	Children	27.67	400	6.91
	Adults	8.94	400	2.35
Pineapple juice	Children	6.44	400	1.61
	Adults	2.3	400	0.57
Mixed juice	Children	6.87	400	1.71
	Adults	2.45	400	0.61

Table 6. *HT-2 toxin exposure calculated for children and adults through fruit juice consumption*

Commodity	Population group	PDI ng/kg bw	PMTDI (ng/kg bw)	%PMTDI
Mixed juice	Children	17.90	60	29.84
	Adults	6.39	60	10.65

Conclusions

The herein used analytical procedure was suitable to quantify fifteen mycotoxins in fruit juice products. 36% of the analyzed samples were contaminated by PAT and 7% resulted positive for HT-2 toxins. One orange juice sample exceeded the maximum limit of PAT (50 µg/L).

The risk assessment shows that the intake of patulin through the consumption of fruit juice does not represent a risk for the population. Nevertheless, a risk dietary exposure for HT-2 toxins by mixed juice samples reached 29.84% of PMTDI. The results highlight the necessity for rigorous monitoring studies.

Acknowledgements

This research was supported by the Spanish Ministry of Economy and Competitiveness (AGL2016-77610-R) and Government Scholarship program "Carlos Antonio López – Paraguay"

Conflict of interest

The authors declare no conflict of interest and informed consent was obtained from all individual participants included in the study.

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