Alternaria Toxins and Ergot Alkaloids in food by LC-MS/MS: results of four years monitoring in Umbria and Marche regions (central Italy).

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INTRODUCTION

Molds of the *Alternaria* and *Claviceps* families can contaminate many different food matrices through the production of secondary metabolites called mycotoxins. Alternaria toxins (ATs) (Altenuene ALT, Alternariol-monomethyl ether AME, Alternariol AOH, Tenuazonic acid TeA and Tentoxin TEN) often contaminate grains, high water vegetables and oilseeds while ergot alkaloids (EAs) (Ergocornine, α and β Ergocristinine, Ergocristinine, Ergocristinine Ergometrina, Ergosina, Ergotamina and their corresponding epimers -inines) are mainly found in cereals and baked goods. Maximum levels of mycotoxins in food are regulated by the EU Regulation (1881/2006 and its amendments) but the impact and toxicological effects of emerging mycotoxins such as ATs and EAs on public health are not yet clear, so there is currently no regulation available for these contaminants but from January 1st 2022 Regulation (EU) 1399/2021 will come into force amending regulation 1881/2006 with which the limits of AEs in cereals and cereal-based foods for babies will be regulated.
In our study, two LC-MS / MS analytical methods for the analysis of AT and EA in food were developed and validated. The activity was supported by the Ministry of Health (Research project RC008 / 2016 IZSUM)

MATERIALS AND METHODS

Alternaria Toxins

Weigh 2 g of sample

↓

Extraction (MeOH:H_2O:AcOH, 80:19:1)

↓

AcOH 1% diluition and SPE purification

↓

PTFE 0.22 µm filtration

↓

LC-MS/MS injection

Levels of ATs and EAs in food were determined by LC-MS/MS (UHPLC Shimadzu Nexera X2 coupled with Sciex API 3200 in MRM mode) operating in APCI- and ESI+ mode respectively. Chromatographic separation of five *Alternaria* toxins was carried out through reversed-phase C18 column (Ascentis Express – 100x2.1mm, 2.7 µm) using MeOH and Ammonium buffer 5mM pH 8.7 as mobile phases. ATs were quantified using ILIS (Isotopically Labeled Internal Standards) to compensate matrix effects. Twelve Ergot Alkaloids were determined using a reversed-phase C18 column (Acquity UPLC BEH C18 – 150x2.1mm, 1.7 µm), which also allows the separation of α and β Ergocryptine isomers, using acetonitrile and an aqueous solution of ammonium carbonate 200 mg/L as mobile phases. ATs validation was carried out at two mass fraction levels (LOQs and 10xLOQs) on the three different matrices (tomatoes, wheat, and sunflower seeds). The validation levels were the following: 25 and 250 µg/kg, for ALT, 10 and 100 µg/kg for AOH and TeA, and 2.5 and 25 µg/kg for AME and TEN. EAs validation was performed in wheat at 3 mass fraction levels, 2.5, 5 and 10 µg/kg (corresponding to LOQs, 2xLOQs and 4xLOQs respectively). RSDr and RSD_{WLR} for each molecule were evaluated according to UNI CEN/TR 16059:2010. All validation data fulfill acceptability requirements.

Ergot Alkaloids

Weigh 25 g of sample

↓

Extraction (ACN:NH_4CO_3, 84:16)

↓

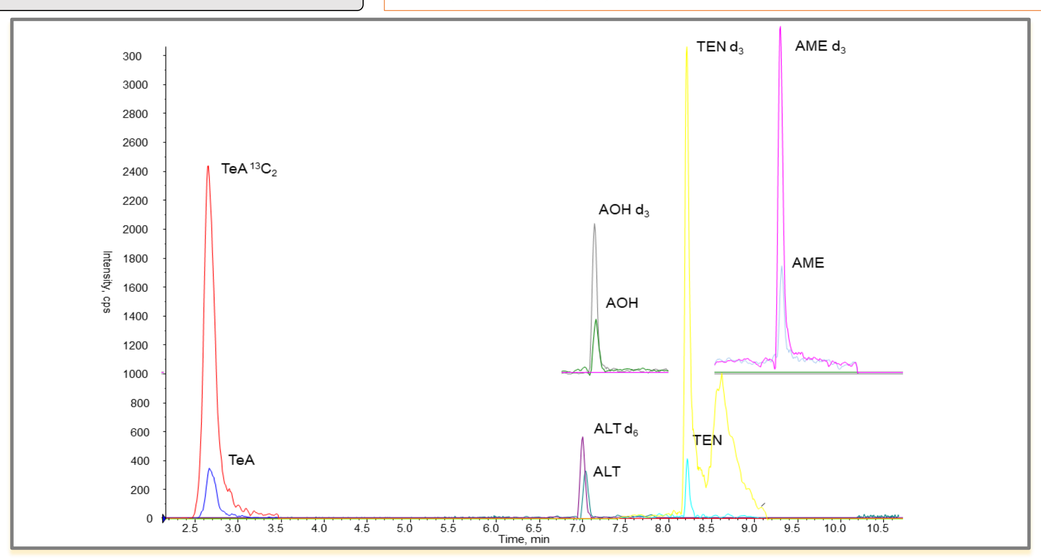
Mycosep 150 purification

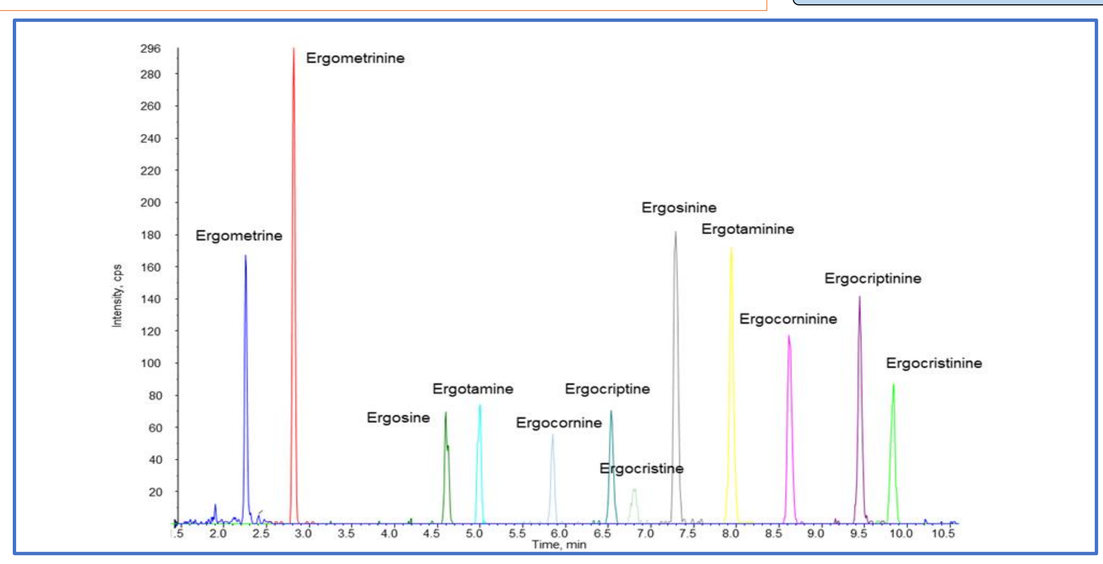
↓

PTFE 0.22 µm filtration

↓

LC-MS/MS injection





RESULTS AND DISCUSSION

Tomato (n= 36)

ITALIAN REGION	YEAR	SAMPLE	ALT	TeA	TEN	AME	AOH
			µg/Kg				
Marche	2017	TOMATO JUICE	N.D.	N.D.	N.D.	N.D.	N.D.
Umbria		TOMATO JUICE		52,9			
Umbria		TOMATO JUICE		26,9			
Marche		TOMATO SAUCE		99,3			
Umbria		TOMATO JUICE		43,7			
Umbria		TOMATO JUICE		484,9			
Umbria	2018	TOMATO JUICE		6,2			
Marche		TOMATO SAUCE		26,4			
Umbria		TOMATO JUICE		22,6			
Umbria		TOMATO JUICE		56,7			
Marche		TOMATO SAUCE		30,4			
Marche		TOMATO JUICE		5,3			
Umbria		TOMATO JUICE		87,9			
Umbria	2019	TOMATO SAUCE		26			
Umbria		TOMATO JUICE		N.D.			
Marche		TOMATO SAUCE		113			
Marche		TOMATO JUICE		15			
Marche		TOMATO JUICE		N.D.			
Marche		TOMATO JUICE		N.D.			
Marche		TOMATO JUICE		N.D.			
Marche		TOMATO JUICE		412			
Marche		TOMATO SAUCE		12			
Marche		TOMATO JUICE		414			
Marche		TOMATO JUICE		73			
Marche		TOMATO JUICE		25			
Umbria	2020	TOMATO SAUCE		55			
Umbria		TOMATO SAUCE		18			
Umbria		KETCHUP		26			
Umbria		TOMATO SAUCE		126			
Umbria		KETCHUP		N.D.			
Umbria		TOMATO SAUCE		112			
Umbria		KETCHUP		32			
Umbria		TOMATO SAUCE		18			
Umbria		TOMATO SAUCE		62			
Umbria		TOMATO SAUCE		81			
Umbria		KETCHUP		21			

Sunflower seeds (n = 27)

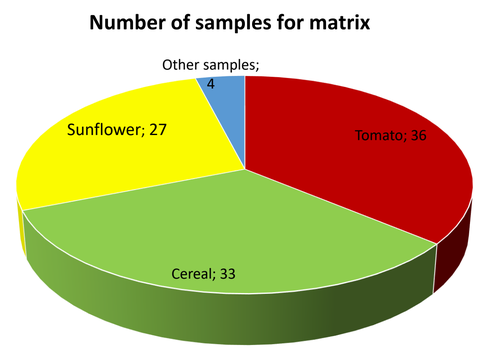
ITALIAN REGION	YEAR	SAMPLE	ALT	TeA	TEN	AME	AOH
			µg/Kg				
Umbria	2017	SUNFLOWER SEEDS	N.D.	166	N.D.	N.D.	N.D.
Marche				46			
Marche				68			
Marche				37			
Umbria				48			
Marche				62			
Umbria	2018			443			
Umbria				1021	225	7,5	46
Marche				182	N.D.	N.D.	N.D.
Marche				3180	34	8,6	33
Marche				**16572**	**570**	**6,1**	**25**
Marche				38	N.D.	N.D.	N.D.
Marche				403	332	5,4	16
Umbria	2019			206	8,3	N.D.	N.D.
Umbria				313	N.D.		
Marche				537	6,6		
Marche				222	N.D.		
Marche				290	3,7		
Marche				2020	32		
Marche				319	18		
Marche				189	N.D.		
Marche				208			
Marche				5079	214	110	
Marche				7075	261	2,6	
Umbria	2020			228	N.D.	N.D.	N.D.
Umbria				98	N.D.	N.D.	N.D.
Umbria				128	149	19	37

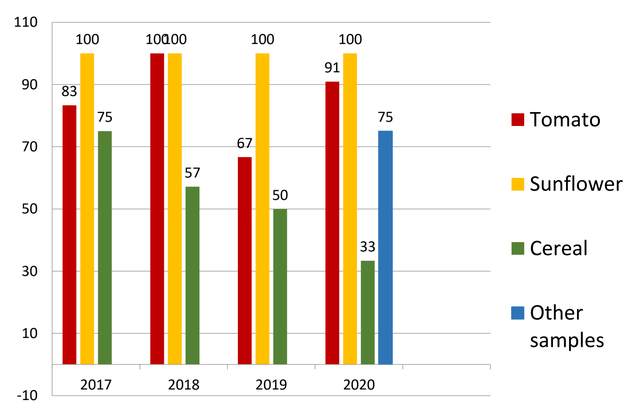
Other Samples (n = 4)

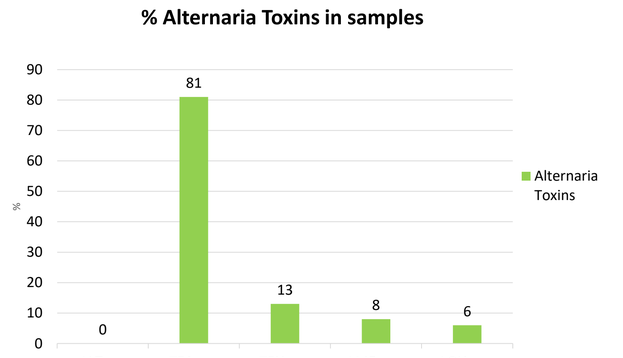
ITALIAN REGION	YEAR	SAMPLE	ALT	TeA	TEN	AME	AOH
			µg/Kg				
Umbria	2019	DRIED FIGS	N.D.	564	N.D.	N.D.	N.D.
Umbria	2019	DRIED FIGS	N.D.	519	N.D.	N.D.	N.D.
Marche	2020	APRICOT MARMELADE	N.D.	26	N.D.	N.D.	N.D.
Marche		APPLE	N.D.	N.D.	N.D.	N.D.	N.D.

Cereals (n = 33)

ITALIAN REGION	YEAR	SAMPLE	ALT	TeA	TEN	AME	AOH
			µg/Kg				
Marche	2017	PASTA	N.D.	11	N.D.	N.D.	N.D.
Umbria		WHEAT		100			
Umbria		WHEAT		14			
Marche		SPELT		65			
Umbria		SPELT		11			
Marche		CORN FLOUR		N.D.			
Marche		WHEAT FLOUR		N.D.			
Umbria		SPELT		41			
Marche	2018	BREAD		7,9			
Marche		CORN FLOUR		N.D.			
Marche		PASTA		8,1			
Marche		RICE		6,9			
Umbria		SPELT		5,8			
Umbria		OATS		N.D.			
Marche		WHEAT FLOUR		N.D.			
Umbria	2019	SPELT		31			
Umbria		BREAD		N.D.			
Marche		WHEAT FLOUR		54			
Marche		CORN		N.D.			
Umbria		BABY FOOD		N.D.			
Marche		BARLEY		N.D.			
Marche		SPELT		69			
Umbria		SPELT		N.D.			
Marche		SPELT		149			
Marche		BARLEY		N.D.			
Umbria		BABY FOOD		N.D.			
Marche		RICE		283			
Marche		WHEAT		175	3,9		
Marche		SPELT		46	N.D.		
Marche		**WHEAT**		**636**	**8,8**	**6,6**	**14**
Umbria		BABY FOOD		N.D.	N.D.	N.D.	N.D.
Marche	2020	BABY FOOD		N.D.	N.D.	N.D.	N.D.
Marche		BABY FOOD		22	N.D.	N.D.	N.D.



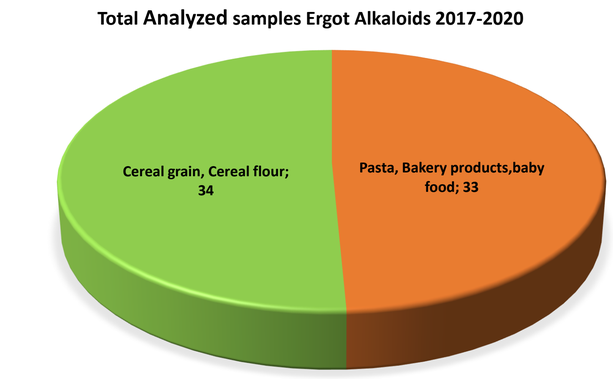


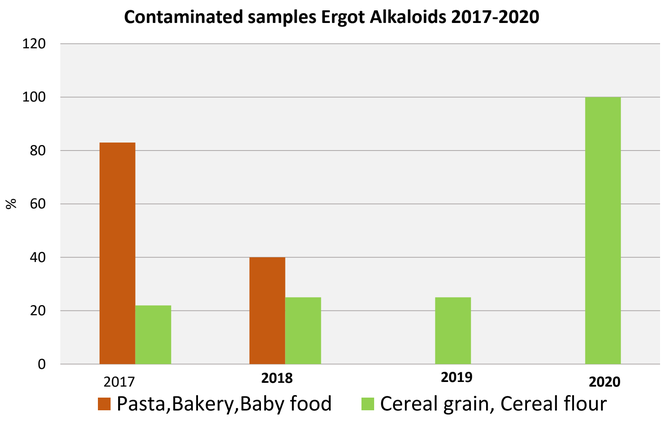


From 2017 to 2020, 100 samples belonging to three different groups were analyzed for Alternaria toxins: vegetables and plant products (tomato), cereals and cereal products (cereals), and oil seeds (sunflower seeds), furthermore other samples such as dried figs, apples and apricot jam were also analyzed.
Sunflower seeds showed the highest levels of TEA (16572 µg/kg) and TEN (570 µg/kg) being, on average, the most contaminated matrix (100%). ATs were also detected to a lesser extent in tomato-based products (up to 485 µg/kg) and cereals (up to 636 µg/kg). AOH and AME were only rarely detected at levels below 25 µg/kg while ALT was never found. Overall the samples were contaminated with TEA (81%), TEN (13%), AME (8%), AOH (6%) while ALT was never detected.

ITALIAN REGION	YEAR	SAMPLE	ERGOTAMININE	ERGOCRIPTYNE	ERGOCRIPTY NINE	ERGOMETRINA	ERGOMETRININE	ERGOSINE	ERGOSININE	ERGOCORNINE	ERGOCORNINININE	ERGOCRISTINE	ERGOCRISTININE	ERGOTAMINE	EAs Tot
			µg/kg												
Marche	2017	PASTA	N.D.	**3,6**	**2,6**	**5,8**	N.D.	N.D	N.D. .	N.D.	N.D.	**7,9**	**3,8**	**6,1**	**30**
Umbria		CEREAL FLAKES		N.D.	N.D.	**20**						N.D.	N.D.	N.D.	**20**
Umbria		PASTA				N.D.	N.D.	**4,1**							**4,1**
Umbria		BREAD				**5,5**		**3,8**							**9,3**
Umbria		CRACKERS		**5,6**		N.D.		N.D							**5,6**
Marche		WHEAT		N.D.		**9,3**	**7,9**	**5,7**							**23**
Umbria		WHEAT				**6,2**									**6,2**
Umbria	2018	CEREAL FLAKES				**7,6**	N.D.	N.D		**7,6**					**15**
Marche		BREAD				N.D.				N.R.		**2,6**		**3,9**	**6,5**
Umbria		WHEAT BRAN	**9,7**	**28**	**19**	**9,9**	**3,1**	**9,0**	**5,4**	**14**	**12**	**94**	**48**	**18**	**271**
Puglia		WHEAT	N.D.	**7,8**	N.D.	**25**	N.D.	**24**	**6,2**	**11**	N.D.	N.D.	**3,1**	N.D.	**77**
Puglia		WHEAT FLOUR		N.D.		N.D.		N.D	N.D.	N.D.		**2,7**	N.D.		**2,7**
Marche	2019	WHEAT FLOUR										**3,4**			**3,4**
Umbria		WHEAT FLOUR						**2,9**				**6,0**		**3,0**	**12**
Marche		WHEAT FLOUR						N.D				**11**	**4,1**	N.D.	**15**
Umbria	2020	WHEAT FLOUR	**2,5**	**2,5**	**2,5**	**2,5**	N.D.	N.D.	N.D.	N.D.	N.D	**2,5**	**2,5**	**2,5**	**18**
Umbria		WHEAT FLOUR	N.D.	N.D.	N.D.	N.D.	**2,5**	**2,5**	**2,5**	**2,5**	**2,5**	N.D	N.D	N.D	**13**

In the same period, 67 samples were analyzed for ergot alkaloids. The diagram on the side shows the high percentage of samples detected for each year. Only in one wheat bran sample, analyzed in 2018, all 12 molecules of EAs were detected, the most abundant ones were Ergocristine (94 µg/kg) and its epimer –inine (48 µg/Kg) with a total level of 271 µg/kg.





CONCLUSIONS

The determination of emerging mycotoxins plays a role of primary importance for the protection of public health and reliable and validated analytical methods are important for official control to provide data to EFSA for risk assessment. In our survey, 85% of the samples monitored for Alternaria toxins were tested positive (> LOQ) for at least one molecule, confirming that contamination of food by Alternaria species is widespread, with a prevalence of TeA and TEN and a lower presence of AOH and AME, while ALT was never detected in any sample analyzed. Ergot alkaloids were frequently found in raw materials and flours, while in pasta and baby bakery products, EAs levels were, on average, much lower, probably because the grinding and cooking processes may be responsible for the decrease of their concentrations.

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