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.

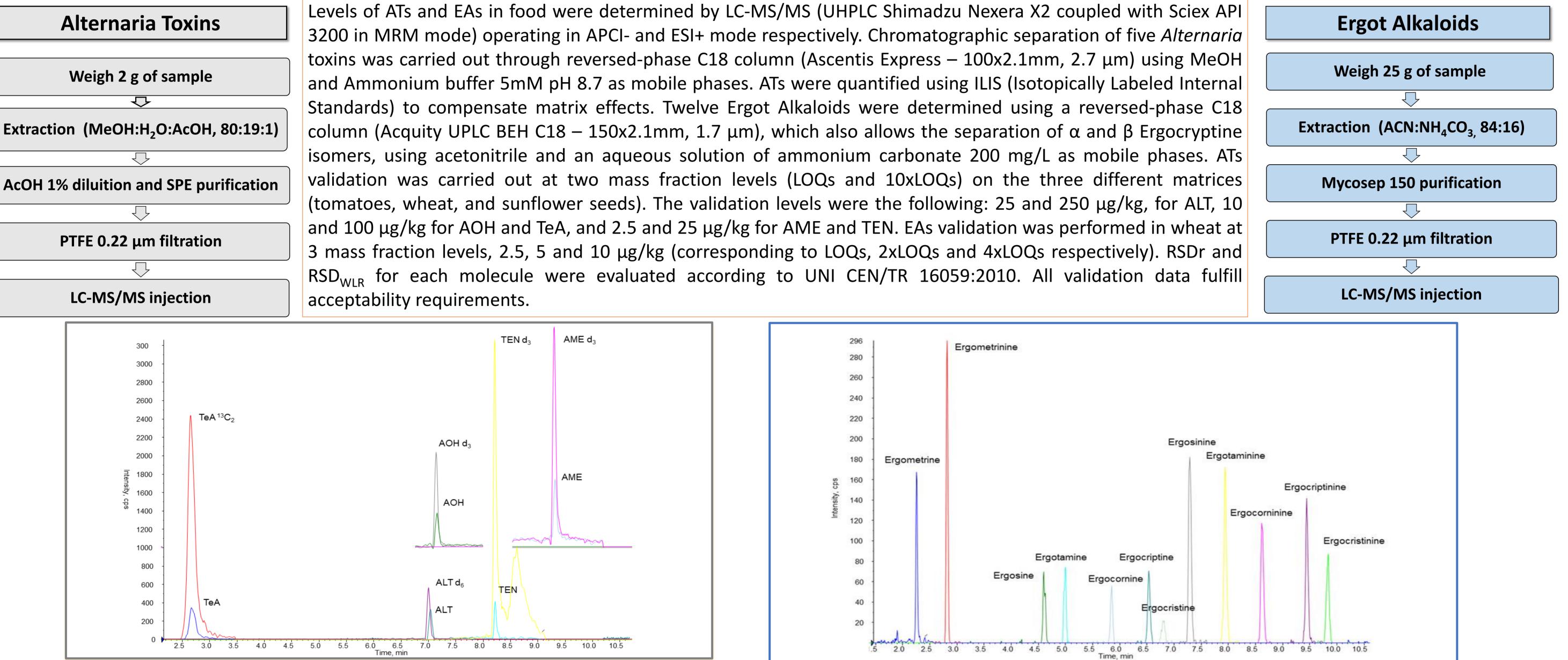
In our study, two LC-MS / MS analytical methods for the analysis of AT and EA in food were developed and validated. The activity was supported by the Ministry of Health (Research project RC008 / 2016 IZSUM)

MATERIALS AND METHODS

Alternaria Toxins
Weigh 2 g of sample
Extraction (MeOH:H ₂ O:AcOH, 80:19:1)
AcOH 1% diluition and SPE purification
\Box
DTEE 0.22 um filtration

MycoKey

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 μ m), 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





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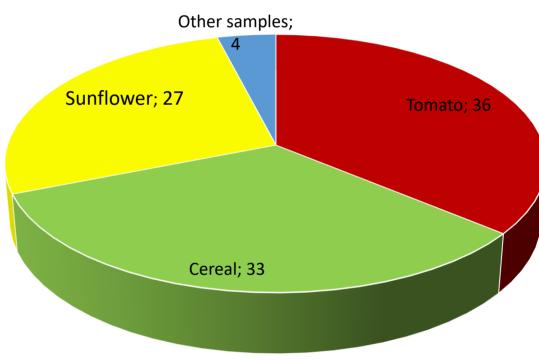
RESULTS AND DISCUSSION

							ITA	IAN YEAR	SAMPLE	ALT	TeA	TEN	AME	AOH												Other	samples;	
	То	mato	(n= 36)				REG		SAIVIPLE		1	μg/Kg					C	Cereals (n	= 33)								4	
						АОН	Un	bria			166	_							TeA	TEN	AME	AOH			Cumfley			
TALIAN REGION	SAMPLE	ALT	TeA	TEN	AME	АОП	Ma	rche			46						R SAMPLE			µg/Kg	1				Sunflov	/er; 27		
				µg/Kg			Ma	che 2017			68					Marche	PASTA		11									
Marche			N.D.	-			Ma	rche			37	N.D.	N.D.	N.D.		Umbria	WHEAT		100									
Umbria Umbria	TOMATO JUICE		52,9	-			Un	bria			48					Umbria	WHEAT		14									
Marche 2017	TOMATO JOICE		26,9 99,3	-				rche			62	-				Marche 2017	SPELT		65									
Umbria	TOMATO JUICE		43,7	-			Un		_		443	-				Umbria	SPELT		11									
Umbria	TOMATO JUICE		484,9				Un				1021	225	7,5	46		Marche	CORN FLOUR		N.D.							Cere	al; 33	
Umbria	TOMATO JUICE		6,2													Marche	WHEAT FLOUR		N.D.									
Marche	TOMATO SAUCE		26,4	-			Ma				182	N.D.	N.D.	N.D.	1 -	Umbria	SPELT		41									
Umbria	TOMATO JUICE		22,6	1				rche 2018			3180	34	8,6	33		Marche	BREAD	_	7,9									
Umbria 2018	TOMATO JUICE		56,7				Ma	rche			16572	570	6,1	25		Marche	CORN FLOUR	_	N.D.									
Marche	TOMATO SAUCE		30,4	_			Ma	rche			38	N.D.	N.D.	N.D.		Marche 2018	PASTA		8,1									
Marche	TOMATO JUICE		5,3	-			Ma	rche			403	332	5,4	16					6,9									
Umbria	TOMATO JUICE	N.D.	87,9	N.D.	N.D.	N.D.	Un	bria	SUNFLOWER SEEDS	N.D.	206	8,3				Umbria	SPELT OATS		5,8	N.D.					0/ .			.
Umbria	TOMATO SAUCE		26	-			Un	bria	SEEDS		313	N.D.				Umbria Marche	WHEAT FLOUR		N.D. N.D.		N.D.	N.D.			% p	ositive s	amples	for ma
Umbria Marche	TOMATO JUICE TOMATO SAUCE		N.D. 113	-			Ma				537	6,6				Umbria	SPELT		31									
Marche	TOMATO JUICE		115	-			Ma				222	N.D.				Umbria	BREAD		N.D.				110	100	10000	100	10	0
Varche	TOMATO JUICE		N.D.	-												Marche	WHEAT FLOUR		54					100	10000	100	_	
Marche	TOMATO JUICE		N.D.	1			Ma				290	3,7	N.D.			Marche	CORN		N.D.				00				91	
Marche 2019	TOMATO JUICE		N.D.]			Ma	rche 2019			2020	32		N.D.		Umbria	BABY FOOD		N.D.				90	83				
Marche	TOMATO JUICE		412				Ma	rche			319	18				Marche Marche	BARLEY SPELT	_	N.D. 69					75				75
Marche	TOMATO SAUCE		12	_			Ma	rche			189					Umbria	SPELT		N.D.				70			67		
Marche	TOMATO JUICE		414	-			Ma	rche			208	N.D.				Marche 2019		_	149				/0					
Marche	TOMATO JUICE		73	-			Ma	rche			5079	214	110			Marche	BARLEY		N.D.						57			
Marche	TOMATO JUICE		25					rche			7075	261	2,6			Umbria	BABY FOOD	_	N.D.				50				50	
Umbria Umbria	TOMATO SAUCE		55 18	-				bria	-		228	N.D.	N.D.	N.D.	1	Marche	RICE		283		-							
Umbria	KETCHUP		26	1												Marche	WHEAT		175	3,9								33
Umbria	TOMATO SAUCE		126	1				bria 2020			98	N.D.	N.D.	N.D.		Marche	SPELT		46	N.D.			30	╶╉╴╶╉╴				
Umbria	KETCHUP		N.D.				Un	bria			128	149	19	37		Marche	WHEAT		536	8,8	6,6	14						
Umbria 2020	TOMATO SAUCE	N.D.	112	N.D.	N.D.	N.D.										Umbria	BABY FOOD		N.D.	N.D.	N.D.	N.D.						
Umbria	KETCHUP		32							Other Sa	amples (n =	4)				Marche 2020	BABY FOOD		N.D. 22	N.D.	N.D.	N.D.	10 -					
Umbria	TOMATO SAUCE		18				ITAL	AN		ALT			AME	AOH		warche	BABY FOOD		22	N.D.	N.D.	N.D.						
Umbria	TOMATO SAUCE		62	-			REG		SAMPLE			μg/Kg												2017	2018	3 201	9 2	020
Umbria	TOMATO SAUCE		81	-			Umł		DRIED FIGS	N.D.	564		N.D.	N.D.									-10					
Umbria	KETCHUP		21				Uml		DRIED FIGS				N.D.	N.D.														

										Sunfle	owers	seeds (I	-27												t i	Numbei	r of sa	mple
											ALT	TeA		AME	АОН												Other sa	mnles
	_							alian Gion	YEAR	SAMPLE			μg/Kg		Aon									I.			4	npics,
	Το	mato	(n= 36					mbria				166	<u>80' 18</u>				1	C	ereals (r	i = 33)		1	_	1				
		ALT	TeA	TEN	AME	AOH		larche			-	46				ITALIAN	YEAR	SAMPLE	ALT	TeA	TEN	AME	AOH	4	Sur	nflower; 27	.7	
REGION YEAR	SAMPLE _			μg/Kg							-					REGION					µg/Kg			I				
Marche	TOMATO JUICE		N.D.					larche	2017		-	68			ND	Marche	-	PASTA		11	-							
Umbria	TOMATO JUICE		52,9					larche			-	37	N.D.	N.D.	N.D.	Umbria	-	WHEAT		100	_			1				
Umbria 2017	TOMATO JUICE		26,9					mbria			-	48				Umbria	-	WHEAT		14	_			1				
warche	TOMATO SAUCE		99,3				N	larche				62				Marche	2017	SPELT	-	65	-			1				
Umbria	TOMATO JUICE		43,7				U	mbria				443				Umbria	_	SPELT		11	_			1				
Umbria	TOMATO JUICE		484,9				U	mbria				1021	225	7,5	46	Marche Marche	-	CORN FLOUR WHEAT FLOUR		N.D. N.D.	-			1			Cereal;	33
Umbria	TOMATO JUICE		6,2	_			N	larche			F	182	N.D.	N.D.	N.D.	Umbria	-	SPELT	1 –	41	-			1				
Marche	TOMATO SAUCE		26,4	_				larche	2018		F	3180	34	8,6	33	Marche		BREAD	┨ ├─	7,9	-			1				
Umbria	TOMATO JUICE		22,6	_					2010							Marche	-	CORN FLOUR		N.D.	-			1				
Umbria 2018	TOMATO JUICE		56,7	_				larche				16572	570	6,1	25	Marche	-	PASTA	1	8,1	-			1				
Marche	TOMATO SAUCE		30,4	_				larche			-	38	N.D.	N.D.	N.D.	Marche	2018	RICE	1 –	6,9	-			1				
Marche Umbria	TOMATO JUICE TOMATO JUICE	N.D.	5,3 87,9	N.D.	N.D.	N.D.	N	larche				403	332	5,4	16	Umbria	-	SPELT	1 –	5,8	-			1				
Umbria	TOMATO SAUCE	11.0.	26		11.5.	11.2.	U	mbria		SUNFLOWER SEEDS	N.D.	206	8,3			Umbria	-	OATS		N.D.	N.D.			1		% positi	ivo sar	nnlo
Umbria	TOMATO JUICE		N.D.	_			U	mbria				313	N.D.			Marche		WHEAT FLOUR		N.D.		N.D.	N.D.	1		70 positi	ive sai	ihic
Marche	TOMATO SAUCE		113	_			N	larche				537	6,6			Umbria		SPELT		31				110				
Marche	TOMATO JUICE		15	_				larche			F	222	N.D.			Umbria		BREAD	N.D.	N.D.				110 100	0 100	100	100	
Marche	TOMATO JUICE		N.D.								F			N.D.		Marche		WHEAT FLOUR		54				1				
Marche	TOMATO JUICE		N.D.					larche			-	290	3,7	N.D.		Marche	_	CORN	-	N.D.	_			90 92				93
Marche 2019	TOMATO JUICE		N.D.					larche	2019		-	2020	32		N.D.	Umbria Marche	-	BABY FOOD BARLEY		N.D. N.D.	-			90 83				
Marche	TOMATO JUICE		412	_			N	larche				319	18			Marche	-	SPELT	1 -	69	-			1	75			
Marche	TOMATO SAUCE		12	_			N	larche				189				Umbria	-	SPELT		N.D.	-			70			67	
Marche	TOMATO JUICE		414	_			N	larche				208	N.D.			Marche	2019	SPELT	1	149	-							
Marche	TOMATO JUICE		73	_			N	larche				5079	214	110		Marche	-	BARLEY	1	N.D.	-			(57		
Marche	TOMATO JUICE		25					larche			F	7075	261	2,6		Umbria		BABY FOOD		N.D.				50 -	· · · · · · · · · · · · · · · · · · ·	<mark></mark> ′	50	
Umbria Umbria	TOMATO SAUCE		55	-						-	F	228	N.D.	N.D.	N.D.	Marche		RICE		283								
Umbria	KETCHUP		18 26	-				mbria			-					Marche		WHEAT		175	3,9			1				
Umbria	TOMATO SAUCE		126	-				mbria	2020		-	98	N.D.	N.D.	N.D.	Marche		SPELT		46	N.D.			30	· · · · · · · · · · · · · · · · · · ·	<mark></mark> ′		
Umbria	KETCHUP		N.D.	-			U	mbria				128	149	19	37	Marche		WHEAT		636	8,8	6,6	14					
Umbria 2020	TOMATO SAUCE	N.D.	112		N.D.	N.D.										Umbria		BABY FOOD		N.D.	N.D.	N.D.	N.D.	4				
Umbria	KETCHUP		32								Other Sar	mples (n = 4	1)			Marche	2020	BABY FOOD		N.D.	N.D.	N.D.	N.D.	10 -		 ′		
Umbria	TOMATO SAUCE		18					LIAN			ALT			AME	AOH	Marche		BABY FOOD		22	N.D.	N.D.	N.D.	i 📕 📙				
Umbria	TOMATO SAUCE		62						YEAR	SAMPLE		icA												20		2019	2010	
Umbria	TOMATO SAUCE		81						2019	DRIED FIGS	N.D.	564	μ g/Κ ε N.D.	5 N.D.	N.D.									-10 20	1/	2018	2019	
Umbria	KETCHUP		21																									
Umbria	KETCHUP		21						2019	DRIED FIGS	N.D.	519	N.D.	N.D.	N.D.													

								ITALIAN	YEAR	SAMPLE	ALT	TeA	TEN	AME	AOH											Ot	her samples
	Tom	nato (n= 36)				REGION	TLAN	SAIVIFLE			µg/Kg	1				C	ereals (n = 33)							4
ITALIAN REGION	SAMPLE	ALT	TeA	TEN	AME	AOH		Umbria Marche				166 46	-			ITALIA REGIO		SAMPLE	ALT	TeA	TEN μg/Kg	AME	AOH		Sunfl	ower; 27	
				µg/Kg	1			Marche	2017			68				March	e	PASTA		11							
Marche	TOMATO JUICE		N.D.	-				Marche	2017			37	N.D.	N.D.	N.D.	Umbri	a	WHEAT		100	-						
Umbria	TOMATO JUICE		52,9	-				Umbria				48	1			Umbri	a	WHEAT		14	-			, v			
Umbria 2017	TOMATO JUICE		26,9	-			-	Marche				62	-			March		SPELT	-	65	-						
Marche Umbria	TOMATO SAUCE TOMATO JUICE		99,3 43,7	-			-			-			4			Umbri	2017	SPELT		11	-			,			
Umbria	TOMATO JUICE		43,7				-	Umbria				443				March		CORN FLOUR		N.D.	_					<u> </u>	Cereal; 33
Umbria	TOMATO JUICE		6,2					Umbria				1021	225	7,5	46	March		WHEAT FLOUR		N.D.	-						
Marche	TOMATO SOICE		26,4	-				Marche				182	N.D.	N.D.	N.D.	Umbri	a	SPELT		41							
Umbria	TOMATO JUICE		20,4	-				Marche	2018			3180	34	8,6	33	March	e	BREAD		7,9							
Umbria 2018	TOMATO JUICE		56,7	-				Marche				16572	570	6,1	25	March	e	CORN FLOUR		N.D.							
Marche	TOMATO SAUCE		30,4	-				Marche				38	N.D.	N.D.	N.D.	March	e	PASTA		8,1							
Marche	TOMATO JUICE		5,3	-				Marche				403	332		16	March	e 2018	RICE		6,9							
Umbria		N.D.	87,9	N.D.	N.D.	N.D.	-			SUNFLOWER				5,4	10	Umbri	a	SPELT		5,8	N.D.						
Umbria	TOMATO SAUCE		26	1				Umbria		SEEDS	N.D.	206	8,3	_		Umbri	Э	OATS		N.D.		N.D.	N.D.		%	positive	e sample
Umbria	TOMATO JUICE		N.D.					Umbria				313	N.D.	_		March		WHEAT FLOUR		N.D.	_	N.D.	N.D.				
Marche	TOMATO SAUCE		113					Marche				537	6,6			Umbri		SPELT	N.D	31	_			110			
Marche	TOMATO JUICE		15					Marche				222	N.D.			Umbri		BREAD		N.D.	_			100	0 10000) 1	L00
Marche	TOMATO JUICE		N.D.					Marche				290	3,7	N.D.		March		WHEAT FLOUR		54	_						
Marche 2019	TOMATO JUICE		N.D.	_			-	Marche	2019			2020	32	-	N.D.	March Umbri		CORN BABY FOOD	-	N.D. N.D.	-			90 92			
iviarche	TOMATO JUICE		N.D.	_			-		2019					-	N.D.	March		BABITOOD	-	N.D.	-			65			
Marche	TOMATO JUICE		412	_				Marche				319	18	_		March		SPELT		69	-				75		
Marche	TOMATO SAUCE		12	_				Marche				189	N.D.			Umbri		SPELT		N.D.	-			70		67	
Marche			414	-				Marche				208				March	e 2019	SPELT		149						57	
Marche	TOMATO JUICE		73 25	-				Marche				5079	214	110		March	e	BARLEY		N.D.						57	50
Marche Umbria	TOMATO SAUCE		55					Marche				7075	261	2,6		Umbri		BABY FOOD	-	N.D.	_			50			50
Umbria	TOMATO SAUCE		18	-				Umbria		-		228	N.D.	N.D.	N.D.	March	e	RICE		283		-					
Umbria	KETCHUP		26	-			-		2020							March	e	WHEAT		175	3,9	_					
Umbria	TOMATO SAUCE		126	-			-	Umbria	2020			98	N.D.	N.D.	N.D.	March	e	SPELT		46	N.D.			30			
Umbria	KETCHUP		N.D.	-			L	Umbria				128	149	19	37	March	e	WHEAT		636	8,8	6,6	14				
Umbria 2020		N.D.	112	N.D.	N.D.	N.D.										Umbri		BABY FOOD		N.D.	N.D.	N.D.	N.D.				
Umbria	KETCHUP		32	1							Other Sa	mples (n =	4)			March	<u> </u>	BABY FOOD	_	N.D.	N.D.	N.D.	N.D.	10 -			
Umbria	TOMATO SAUCE		18					TALIAN			ALT			AME	AOH	March	e	BABY FOOD		22	N.D.	N.D.	N.D.				
Umbria	TOMATO SAUCE		62					REGION	YEAR	SAMPLE															.17	10	2010
Umbria	TOMATO SAUCE		81						2010	DRIED FIGS	N.D.	EC4			N.D.									-10 203	17 20	ΔΤΆ	2019
Umbria	KETCHUP		21						2019																		
								Umbria	2019	DRIED FIGS	N.D.	519	N.D.	N.D.	N.D.												





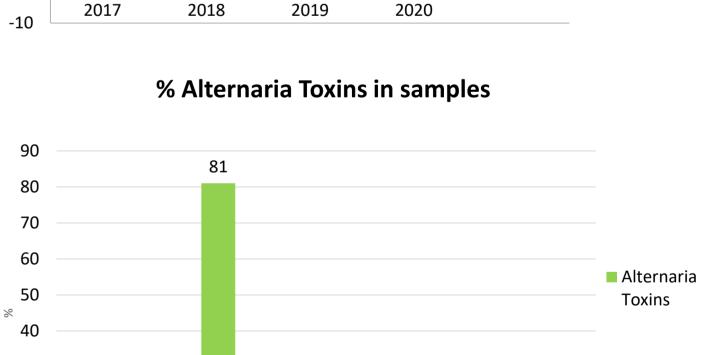
Tomato

Sunflower

Cereal

Other

samples



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.

N.D.

N.D.

26

N.D.

N.D.

N.D.

N.D.

N.D.

N.D.

N.D.

APRICOT

MARMELADE

APPLE

Marche

Marche

2020

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	ERGOCORNININE	ERGOCRISTINE	ERGOCRISTININE	ERGOTAMINE	EAs Tot
									μg/kg						
Marche		PASTA		3,6	2,6	5,8		ND				7,9	3,8	6,1	30
Umbria		CEREAL FLAKES				20		N.D							20
Umbria		PASTA		N.D.		N.D.	N.D.	4,1							4,1

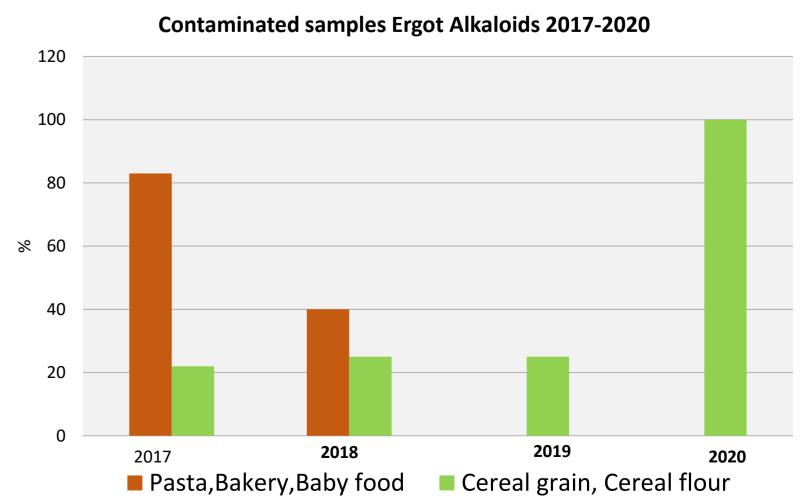
AL⁻

20

Total Analyzed samples Ergot Alkaloids 2017-2020



Umbria	2017	BREAD				5,5		3,8		N.D.					9,3
Umbria		CRACKERS	N.D.	5,6		N.D.		N.D	N.D.		N.D.	N.D.	N.D.	N.D.	5,6
Marche		WHEAT			N.D.	9,3	7,9	5,7					N.D.		23
Umbria		WHEAT		N.D.		6,2									6,2
Umbria		CEREAL FLAKES		N.D.		7,6	N.D.	N.D		7,6					15
Marche		BREAD				N.D.				N.R.		2,6		3,9	6,5
Umbria	2018	WHEAT BRAN	9,7	28	19	9,9	3,1	9,0	5,4	14	12	94	48	18	271
Puglia		WHEAT		7,8		25		24	6,2	11		N.D.	3,1		77
Puglia		WHEAT FLOUR						N.D				2,7		N.D.	2,7
Marche		WHEAT FLOUR	N.D.	N.D.	N.D.		N.D.	N.D	ND	ND	N.D.	3,4	N.D.		3,4
Umbria	2019	WHEAT FLOUR		N.D.		N.D.		2,9	N.D.	N.D.		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	2020	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 μ g/kg) and its epimer –inine (48 μ g/Kg) with a total level of 271 μ g/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.



CONFERENZA INTERNAZIONALE MycoTWIN-MycoKey

Bari, dal 9 al 12 novembre 2021

Palace Hotel

