Validation of analytical methods for determining mycotoxins in foodstuffs

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The European Union (EU) has established demanding regulatory limits for controlling aflatoxins B_1, B_2, G_1 and G_2, in cereals, nuts, nut products and dried fruit, aflatoxin M_1 in milk, and ochratoxin A in cereals. These limits are likely to be extended in the future to additional commodities and other mycotoxins. For enforcement purposes and in particular for resolving any disputes between parties, it is essential that validated methods are available, with performance characteristics that meet certain minimum criteria. As such methods were not available and had not previously been validated either for matrices of interest in Europe or at the low European limits compared to the USA, the EU funded a method-validation project to fulfil this requirement. Immunoaffinity column clean-up methods with HPLC determination were established for aflatoxins B_1, B_2, G_1 and G_2 in peanut butter, pistachios, fig paste and paprika, aflatoxin B_1 in baby food, aflatoxin M_1 in liquid milk, and ochratoxin A in roasted coffee and baby food. For patulin in apple juice and apple puree, solvent extraction and solid-phase clean-up HPLC methods were developed. To undertake collaborative studies, particular care was taken in preparation of naturally-contaminated test materials containing the toxins at levels close to regulatory limits and in demonstrating the homogeneity of batches of material. To ensure that participants in the validation exercise could follow the procedures to be tested, videos of the methods were prepared showing, in particular, any critical steps. Prior to undertaking the method validation, participants were invited to collaborative study workshops to ensure that they fully understood the methods and their role in the study. This care in planning and executing the collaborative studies led to impressive performance characteristics and adoption of six procedures by AOAC International as First Action Methods and seven methods by CEN as European standards. The valuable lessons learned in undertaking these validation exercises are now being put to further use in studies aimed at validating methods for mycotoxins in foodstuffs, which are appropriate for developing countries based on TLC as the end determination but use more modern sample clean-up techniques.

Keywords: Method validation; Mycotoxins; Performance characteristics; Regulatory limits

1. Introduction

Mycotoxins are toxic metabolites produced by various fungi growing in a wide range of food and animal feedstuffs. The main mycotoxins that occur frequently are aflatoxins, ochratoxin A, patulin fumonisins, trichothecenes (such as nivalenol, deoxynivalenol, T-2 toxin) and zearalenone. The pattern of occurrence is closely

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linked to specific food commodities, for example, aflatoxins in peanuts and maize, fumonisins in maize and patulin in apple juice. Monitoring mycotoxins depends on having available precise, reliable analytical methods. To date, several hundred mycotoxins have been identified, deriving from approximately 200 different fungi [1] However only a limited number (about 20) occur frequently at significant concentrations in food and feedstuffs. About 20% of food products, mainly of plant origin, are significantly contaminated with mycotoxins. Monitoring is important for not only consumer protection but also producers of the raw products prior to transport or processing.

Regulations have been set in more than 70 countries in order to restrict the intake of mycotoxins [2] However, the legal limits vary significantly both from country to country and by mycotoxin type and matrix. For example, limits for aflatoxins in foodstuffs range from 0 to 50 ng/g.

The frequent occurrence, particularly of aflatoxins, has already led to temporary bans of certain "high-risk" foods imported into Europe (for example, Egyptian peanuts and Iranian pistachios), where limits have been established at relatively low levels. As a result of the establishment of the EU and its aim of harmonisation of the internal market, the European Commission (EC) drafted regulations concerning certain contaminants. The current maximum levels for aflatoxins set by the EC are 2 ng/g for aflatoxin B_1 and 4 ng/g for total aflatoxins in groundnuts, nuts, dried fruits and cereals [3] These are to be extended to cover spices with limits of 5 ng/g and 10 ng/g for aflatoxin B_1 and total aflatoxins, respectively [4] These levels are about five times lower than those established in the USA. New limits are being established in the EU for aflatoxin B_1 in baby food (most probably 0.1 ng/g) and animal feeds (in discussion at 1 ng/g). The latter is regulated because aflatoxin B_1 can be metabolised in cattle to aflatoxin M_1, for which a limit of 0.05 ng/L has been set. Limits for other mycotoxins are being established in the EU.

Since the discovery of mycotoxins, several methodologies for their determination have been developed [5,6] Methods routinely used today for mycotoxins are mainly based on high-performance liquid chromatography (HPLC), thin-layer chromatography (TLC) or enzyme-linked immunosorbent assay (ELISA) although gas chromatography (GC) and GC/MS are employed for analysing trichothecenes. Analytical methods used by enforcement laboratories for the implementation of legislation must be subject to validation procedures, in order to show that the method produces reliable results [7] These methods need to provide accurate and reproducible results both within and between laboratories. This is especially important in view of legal actions and trade specifications, as well as monitoring and risk-assessment studies.

Several methods have been validated for the determination of various mycotoxins in a number of food commodities, and a summary of published methods for aflatoxins and for other mycotoxins are tabulated in this paper. These validated methods differ in many parameters for each matrix, the contamination levels vary significantly, and the validation does not always comply with international guidelines. For adoption as an official method, any proposed method should be validated not only in a collaborative trial study, but also in the matrices of concern and at levels close to the regulatory limits.

In 1995, the EC was aware that official methods at that time had been validated only at levels higher than those set as the maximum allowed limit [4] For this reason, the EC Standards, Measuring and Testing (SMT) Programme commissioned a project to develop and validate methods for the determination of various mycotoxins in a wide range of foodstuffs (aflatoxins in various food commodities including milk and baby food, and ochratoxin A in coffee, barley and baby food, as well as patulin in apple juice and apple puree) (see Table 1).

The project (CT96-2045) ran from 1996 to 2000 and involved six laboratories from five EU Member States plus the EC's Joint Research Centre (JRC) as the main partners responsible for method development, planning, implementation

and co-ordination of the validation studies. In total, some 57 participants from 16 European countries took part in the seven validation exercises, some participants taking part in more than one. The participants were mainly from government and food-control laboratories in the EU, although some industry and university laboratories participated, and laboratories from Switzerland and Norway were also involved.

Validation of analytical methods should be carried out at both national and international level, according to the IUPAC/AOAC/ISO international harmonised protocol for collaborative studies [8] Validated methods may subsequently be adopted as official international methods or as European Standards by bodies such as AOAC International or CEN, respectively. In the case of AOAC International, there is rigorous peer review by a number of committees, primarily of the way in which the collaborative study was conducted, before adoption as an AOAC First Action method. CEN is less rigorous in terms of assessing the conduct of a study but does go through an exhaustive consultation and international voting process before final adoption of a method as a European Standard.

For mycotoxins, a specific CEN Committee (CEN 275 WG5) uniquely established minimum requirements for the performance that the methods should meet, depending on the contamination level [9] In most cases, methods were required to have a recovery in the range from 70–110% and RSD_r and RSD_R values of $\leqslant 20\%$ and $\leqslant 30\%$, respectively. Any method that has been validated and adopted by AOAC International, CEN or ISO is recognised as being an official method for the purposes of enforcement or international trade.

This article has assessed the current position regarding validated mycotoxin methodology, taking into account the latest developments in legislation. The approach adopted for mycotoxin method validation to meet European food-control needs is described in detail. As this approach was very successful, it can provide a useful pointer for future similar projects. Recognising that European legislation on mycotoxins impacts frequently on developing countries that need to export commodities to Europe, the article also describes current activities relating to mycotoxin method validation that have been established to meet the requirements of developing countries.

Table 1
Performance characteristics of mycotoxin methods validated as part of a Standards, Measuring and Testing (SMT) project funded by the European Commission

Mycotoxin	Commodity	Contamination level (ng/g)	Performance characteristic average over range		
			RSD_r (%)	RSD_R (%)	Horrat ratio
Aflatoxin B_1	Peanut butter	0.8–3.6	3.1–9.9	14.3–32.2	0.4–0.7
Total aflatoxins	Peanut butter	1.9–7.9	6.2–13.4	14.1–34.2	0.4–0.8
Aflatoxin B_1	Pistachio paste	0.7–3.3	3.9–20.0	16.1–23.2	0.4–0.8
Total aflatoxins	Pistachio paste	2.0–7.8	11.9–23.3	17.8–24.3	0.4–0.7
Aflatoxin B_1	Fig paste	1.1–3.6	5.9–16.8	12.8–28.5	0.3–0.7
Total aflatoxins	Fig paste	2.2–7.8	8.8–17.6	16.5–32.4	0.5–0.8
Aflatoxin B_1	Paprika	0.9–3.4	5.3–14.0	9.1–18.8	0.2–0.4
Total aflatoxins	Paprika	1.7–7.1	6.4–17.0	14.2–33.5	0.4–0.7
Aflatoxin B_1	Baby food	0.07–0.18	3.5–14.0	9.0–23.0	0.1–0.4
Aflatoxin M_1	Liquid milk	0.04–0.10	8.0–18.0	21.0–31.0	0.3–0.4
Ochratoxin A	Barley	1.3–4.5	4.0–24.0	12.0–33.0	0.3–0.8
Ochratoxin A	Roasted coffee	1.2–5.4	2.0–22.0	13.0–26.0	0.4–0.6
Ochratoxin A	Baby food	0.05–0.22	18.0–47.0	29.0–63.0	0.4–0.9
Patulin	Clear apple juice	26–128	8.0–11.0	11.0–33.0	0.5–1.2
Patulin	Cloudy apple juice	26—106	6.0–35.0	12.0–35.0	0.5–1.2
Patulin	Apple puree	23—121	10.0–27.0	13.0–36.0	0.6–1.3

2. Assessment of published official methods for aflatoxins and other mycotoxins

Official analytical methods available for the analysis of mycotoxins in food and feedstuffs are listed in the AOAC International Official Methods of Analysis [10] More recently, European Standards have been developed (prEN 14123 — Aflatoxins in peanuts, pistachios, figs and paprika; prEN14132 — ochratoxin A in barley and coffee; prEN 14177 — patulin in apple juice; and, prEN 14133 — ochratoxin A in wine and beer).

A full compilation of official methods of analysis for aflatoxins is presented in Table 2. Table 3 covers other mycotoxins, arranged by toxin and matrices in alphabetical order. Where performance parameters were available, these have been included in the Tables 2 and 3, although it can be seen that a number of the methods were adopted, for example by AOAC, some 20–30 years ago. In these cases, no performance parameters are given or only recoveries for the methods (by spiking) and coefficients of variation (CVs) are reported. Many of these methods will not have been validated as rigorously as is currently required and will not have followed the International Harmonised Protocol [8]

Although it is difficult to draw direct comparisons between the performance characteristics of these published methods, in general terms, TLC methods have rather high CVs and have been tested only at aflatoxin contamination levels too high to be of relevance to current control limits. Even a more recent TLC study [31], conducted in 1994 using a densitometer, gave RSD_r and RSD_R values between 42% and 57%.

ELISA methods [15,29,33], although in some instances giving acceptable performance, have not been validated at sufficiently low levels and are limited in the range of matrices examined.

HPLC methods that have achieved official status use either column chromatography [32,37], multi-functional columns [11], solid-phase extraction [30,31,40] or immunoaffinity columns [16,22,23] Again, although in many instances, good performances were obtained, the levels were not sufficiently low to meet European needs and the range of matrices tested was not sufficient to cover the general classes of nuts, dried fruit and spices controlled by European regulations. In the case of aflatoxin M_1 in milk, there is an official HPLC method using conventional column clean-up with good performance parameters [48], and an affinity column HPLC method was validated [78], but not accepted by AOAC, as the study design did not include spiked samples to assess recovery. However, although the affinity column method [78] meets EU limits, the official method [48] goes down to only 0.08 ng/L. This assessment of aflatoxin methods concluded that available methods had not been validated at the low limits required and did not cover the range of matrices of interest.

Official methods for mycotoxins other than aflatoxins are summarised in Table 3. Although Table 3 includes fumonisins and zearalenone, at the time of the SMT project, only ochratoxin A and patulin were under consideration for regulatory control.

For ochratoxin A, official HPLC methods are available for barley [59], corn [58], wheat [63], kidney [58], and wine and beer [54] Although, for ochratoxin A, the range of matrices covers those of interest, and the methods perform well, only in the case of wine and beer [54] are the levels sufficiently low. There were no validated methods for ochratoxin A in roasted coffee, although a number of methods have been published, and many have been used in survey work.

For patulin, there are a number of good HPLC methods [65,66] for clear apple juice at the levels of interest, although cloudy juice and apple puree have not been examined.

The overall conclusion from the examination of the data in Table 3 was that there was a need for validated methods for ochratoxin A in barley and roasted coffee at low European limits and for patulin in cloudy apple juice and apple puree.

Table 2
Performance characteristics of validated and official methods for aflatoxins

Aflatoxin	Matrix	Applicability range (ng/g)	Official method	Date	Method principle	Clean-up	Method performance			Reference
							Recovery (%)	RSD_r (%)	RSD_R (%)	
Total	Almonds	5–30	AOAC 994.08	1994	HPLC	Multi-column	116	16	17–26	[11]
Total	Brazil nuts	5–30	AOAC 994.08	1994	HPLC	Multi-column	97–140	9	12–29	[11]
Total	Cocoa	8–35	AOAC 971.23	1971, 1978	TLC	CC	57–104	15–54	6–48	[12,13]
Total	Coconut, Copra	31–47	AOAC 971.24	1971, 1988	TLC	968.22 & 971.22	-	-	21–57	[14]
Total	Corn	$\geqslant$30	AOAC-IUPAC 990.32	1989	ELISA	None	Screening method only			[15]
Total	Corn	2–30	AOAC 991.31	1991	HPLC	IAC	106–124	17–20	14–62	[16]
Total	Corn	5–30	AOAC 994.08	1994	HPLC	Multi-column	98–102	6–20	15–22	[11]
Total + (B_1+B_2)	Cotton seed	30–97	None	1969	TLC	CC	85–90	12–18	-	[17]
Total (B_1+B_2)	Cotton seed	7–266	AOAC	1975	TLC Vis/Densitom	CC	-	9–26	30	[18]
Total	Cotton seed	20	AOAC-IUPAC	1989	ELISA	Solvent extraction		Qualitative		[15]
Total	Fig paste	2.2–5.2	AOAC and CEN adoption	2000	HPLC	IAC	81–92	9–18	17–32	[19]
Total	Paprika powder	2.4–7.9	AOAC and CEN adoption	2000	HPLC	IAC	74	6–17	14–34	[19]
Total	Peanut butter, peanut meal	14–192	None	1968	TLC	CC	-	37–129	-	[20]
Total	Peanut products	20	AOAC-IUPAC	1989	ELISA	None		Qualitative		[15]
Total	Peanut butter	90	AOAC 991.45	1992	ELISA	None	-	14–30	29–37	[21]
Total	Peanut butter	4–38		1991	HPLC	IAC	51–62	15–26	33–45	[22]
Total	Peanut butter	2–33	AOAC 991.31	1991	HPLC	IAC	107–131	12–31	11–37	[16]
Total	Raw peanuts	1–30	AOAC 991.31	1991	HPLC	IAC	97–115	13–87	13–108	[16]
Total	Peanut butter	7–48	None	1992	HPLC	IAC	-	17–45	30–57	[23]
Total	Peanuts	5–30	AOAC 994.08	1994	HPLC	Multi-column	74–91	7	22–70	[11]
Total	Peanut butter	0.8–9.6	AOAC and CEN adoption	2000	HPLC	IAC	81–83	11–23	18–24	[19]
Total	Pistachios	5–30	AOAC 994.08	1994	HPLC	Multi-column	91–92	23	20–33	[11]
Total	Pistachio paste	2.2–5.2	AOAC and CEN adoption	2000	HPLC	IAC	81–92	9–18	17–32	[19]
Total	Soybeans	15–58	AOAC 972.27	1972	TLC	968.22 & 972.26	-	-	41–65	[24]

(Table continued on next page)

Table 2 (*continued*)

Aflatoxin	Matrix	Applicability range (ng/g)	Official method	Date	Method principle	Clean-up	Method performance			Reference
							Recovery (%)	RSD_r (%)	RSD_R (%)	
Total	Animal feed	20	AOAC-IUPAC	1989	ELISA	Solvent extraction	Qualitative			[25]
B_1	Almonds	3–15	AOAC 994.08	1994	HPLC	Multi-column	91–95	9	10–20	[11]
B_1	Baby food	0.07–0.18	AOAC in adoption	2001	HPLC	IAC	92–101	4–14	9–23	[27]
B_1	Brazil nuts	3–15	AOAC 994.08	1994	HPLC	Multi-column	94–140	9	11–19	[11]
B_1	Corn	11–56	AOAC	1989	ELISA	None	-	19–39	53–61	[29]
B_1	Corn	5–50	AOAC 990.33	1990	HPLC	SPE	64–69	8–27	16–38	[30]
B_1	Corn	10–30	AOAC-IUPAC 991.31	1991	Fluorimetry	IAC	-	17–20	8–21	[16]
					HPLC	IAC	-	14–33	5–51	
B_1	Corn	5–50	AOAC 993.17	1994	TLC/Densitom	SPE	-	42–57	42–57	[31]
B_1	Corn	3–15	AOAC 994.08	1994	HPLC	Multi-column	97–103	4–21	9–27	[11]
B_1	Cotton seed	18–87	AOAC 980.20	1980	TLC	CC	-	33	-	[32]
					HPLC			26		
B_1	Cotton seed, mixed feed	16–50	AOAC 989.06	1989	ELISA	Solvent extraction	Screening method only			[33]
B_1	Eggs	>1	AOAC 978.15		TLC	968.22 & 980.20	>75	5–38	-	[34]
B_1	Fig paste	0.3–3.6	AOAC and CEN adoption	2000	HPLC	IAC	90–109	6–25	13–31	[19]
B_1	Paprika powder	0.8–3.4	AOAC and CEN adoption	2000	HPLC	IAC	85–86	4–14	9–19	[19]
B_1	Peanut butter, peanut meal	14–192	None	1968	TLC	CC	-	-	32–64	[20]
B_1	Peanut butter	8–24	None	1970	TLC	CC	-	-	22–56	[35]
B_1	Peanut butter	3–12	None	1973	TLC (2-dim)	Extraction with $CHCl_3$	-	-	35	[36]
B_1	Peanut butter	5–20	None	1984	HPLC (NP)	CC	79–103	20	34	[37]
					HPLC (RP)			19	32	
B_1	Peanut butter	4.5–84	None	1988	ELISA	None	-	-	18–45	[38]
B_1	Peanut products	11–56	AOAC	1989	ELISA	None	-	23–74	23–74	[29]
B_1	Peanut butter	12–35		1990	TLC or HPLC	IAC	72	-	36–45	[39]
B_1	Peanut butter, raw peanuts	5–25	AOAC 990.33	1990	HPLC	SPE	65–112	11–19	24–33	[30]
B_1	Peanut butter	2.6–26		1991	HPLC	IAC	72	16–29	37–45	[22]
B_1	Peanut butter	10–30	AOAC-IUPAC 991.31	1991	Fluorimetry	IAC	-	12	11–21	[16]
					HPLC		-	17	21–30	
B_1	Peanut butter	0.19–6.2	None	1992	HPLC	IAC	-	17–48	39–63	[23]
B_1	Raw peanuts	5–25	AOAC 993.17	1994	TLC/Densitom	SPE	-	21–37	26–37	[31]
B_1	Peanuts	3–15	AOAC 994.08	1994	HPLC	Multi-column	77–87	7	23–72	[11]

(Table continued on next page)

Table 2 (*continued*)

Aflatoxin	Matrix	Applicability range (ng/g)	Official method	Date	Method principle	Clean-up	Method performance			Reference
							Recovery (%)	RSD_r (%)	RSD_R (%)	
B_1	Peanut butter	0.8–3.7	AOAC and CEN adoption	2000	HPLC	IAC	87–91	3–10	14–32	[19]
B_1	Pistachios	3–15	AOAC 994.08	1994	HPLC	Multi-column	88–92	28	9–32	[11]
B_1	Pistachio paste	0.7–3.3	AOAC and CEN adoption	2000	HPLC	IAC	82	4–20	16–23	[19]
B_1	Animal feed	8–14	None	1991	HPLC	SPE		11	18	[40]
B_1	Animal feed	0.5–4.2	AOAC in adoption	2001	HPLC	IAC	75–157	6–9	18–20	[41]
B_1	Extracts from peanuts, cotton seed, ginger roots	<5	None	1985	Mass spectrometry	TLC or CC	Validation of identity confirmation only			[42]
B_2	Almonds	1–3	AOAC 994.08	1994	HPLC	Multi-column	88–92		15–19	[11]
B_2	Brazil nuts	1–3	AOAC 994.08	1994	HPLC	Multi-column	83–98	10	15–19	[11]
B_2	Corn	1.5–15	AOAC 990.33	1990	HPLC	SPE	65–85	9–20	17–39	[30]
B_2	Corn	3–15	AOAC 993.17	1994	TLC/Densitom	SPE	-	27–46	42–57	[31]
B_2	Corn	1–3	AOAC 994.08	1994	HPLC	Multi-column	91–98	10	12–22	[11]
B_2	Cotton seed	17–56	AOAC 980.20	1980	TLC HPLC	CC	- -	- -	35 18	[32]
B_2	Fig paste	0.1–1.3	AOAC and CEN adoption	2000	HPLC	IAC	95–123	5–23	9–22	[19]
B_2	Paprika powder	0.1–1.3	AOAC and CEN adoption	2000	HPLC	IAC	88–91	10–24	13–24	[19]
B_2	Peanut butter	3–12	None	1970	TLC	CC	-	-	33–39	[35]
B_2	Peanut butter	5–20	None	1984	HPLC (NP) HPLC (RP)	CC	92–104	33 22	33 46	[37]
B_2	Peanut butter, raw peanuts	1.5–7.5	AOAC 990.33	1990	HPLC	SPE	65–123	11–18	11–27	[30]
B_2	Peanut butter	0.6–4.8	None	1991	HPLC	IAC	-	14–62	34–66	[22]
B_2	Raw peanuts	1.5–7.5	AOAC 993.17	1994	TLC/Densitom	SPE	-	37–38	37–50	[31]
B_2	Peanuts	1–3	AOAC 994.08	1994	HPLC	Multi-column	89–93	8	11–32	[11]
B_2	Peanut butter	0.2–0.7	AOAC and CEN adoption	2000	HPLC	IAC	93	4–15	13–21	[19]
B_2	Pistachios	1–3	AOAC 994.08	1994.	HPLC	Multi-column	72–89	10	11–53	[11]
B_2	Pistachio paste	0.1–0.7	AOAC and CEN adoption	2000	HPLC	IAC	88–91	10–24	13–24	[19]
G_1	Almonds	2–9	AOAC 994.08	1994	HPLC	Multi-column	89–103	16	26–38	[11]
G_1	Brazil nuts	3–9	AOAC 994.08	1994	HPLC	Multi-column	111–53	13	14–71	[11]
G_1	Corn	5–15	AOAC 990.33	1990	HPLC	SPE	58–64	8–35	33–45	[30]

(Table continued on next page)

Table 2 (*continued*)

Aflatoxin	Matrix	Applicability range (ng/g)	Official method	Date	Method principle	Clean-up	Method performance			Reference
							Recovery (%)	RSD_r (%)	RSD_R (%)	
G_1	Corn	5–50	AOAC 993.17	1994	TLC/Densitom	SPE	-	36–44	43–61	[31]
G_1	Corn	0.9–9	AOAC 994.08	1994	HPLC	Multi-column	102–108	9–83	25–121	[11]
G_1	Fig paste	0.7–2.8	AOAC and CEN adoption	2000	HPLC	IAC	70–93	6–24	29–42	[19]
G_1	Paprika powder	0.1–2.5	AOAC and CEN adoption	2000	HPLC	IAC	58–62	10–54	31–55	[19]
G_1	Peanut butter, peanut meal	13–35	None	1968	TLC	CC	-	-	86–126	[20]
G_1	Peanut butter	9–16	None	1970	TLC	CC	-	-	41–65	[35]
G_1	Peanut butter	5–20	None	1984	HPLC (NP) HPLC (RP)	CC	74–89	28 38	39 51	[37]
G_1	Peanut butter, raw peanuts	5–25	AOAC 990.33	1990	HPLC	SPE	74–82	16–29	26–37	[30]
G_1	Peanut butter	0.5–2	None	1991	HPLC	IAC		14–39	55–69	[22]
G_1	Peanut butter	5.4–38	None	1992	HPLC	IAC		15–45	30–55	[23]
G_1	Peanuts	0.9–9	AOAC 994.08	1994	HPLC	Multi-column	71–96	15	30–73	[11]
G_1	Peanut butter	0.3–3.3	AOAC and CEN adoption	2000	HPLC	IAC	83–76	7–19	15–31	[19]
G_1	Pistachios	3–9	AOAC 994.08	1994	HPLC	Multi-column	96–100	21	32–40	[11]
G_1	Pistachio paste	0.7–3.0	AOAC and CEN adoption	2000	HPLC	IAC	71–76	16–22	24–36	[19]
G_2	Almonds	1–3	AOAC 994.08	1994	HPLC	Multi-column	98–116		16–34	[11]
G_2	Brazil nuts	1–3	AOAC 994.08	1994	HPLC	Multi-column	97–153	16	39–145	[11]
G_2	Corn	1.5–15	AOAC 990.33	1990	HPLC	SPE	56–83	35–70	35–96	[30]
G_2	Corn	3–15	AOAC 993.17	1994	TLC/Densitom	SPE		45–49	45–63	[31]
G_2	Corn	1–3	AOAC 994.08	1994	HPLC	Multi-column	108–115	18	28–42	[11]
G_2	Fig paste	0.2–0.6	AOAC and CEN adoption	2000	HPLC	IAC	72–104	10–31	31–63	[19]
G_2	Paprika powder	0.1–0.5	AOAC and CEN adoption	2000	HPLC	IAC	62–66	18–21	25–26	[19]
G_2	Peanut butter	5	None	1970	TLC	CC	-	-	69	[35]
G_2	Peanut butter, raw peanuts	1.5–7.5	AOAC 990.33	1990	HPLC	SPE	69–110	9–26	29–33	[30]
G_2	Peanut butter	5–20	None	1984	HPLC (NP) HPLC (RP)	CC	88–163	17 31	34 52	[37]
G_2	Peanut butter	0.3–0.9	None	1991	HPLC	IAC		47	55–85	[22]
G_2	Peanuts	1–3	AOAC 994.08	1994	HPLC	Multi-column	93–101	14	23–51	[11]

(Table continued on next page)

Table 2 (*continued*)

Aflatoxin	Matrix	Applicability range (ng/g)	Official method	Date	Method principle	Clean-up	Method performance			Reference
							Recovery (%)	RSD_r (%)	RSD_R (%)	
G_2	Peanut butter	0.1–0.6	AOAC and CEN adoption	2000	HPLC	IAC	76–80	10–18	13–32	[19]
G_2	Pistachios	1–3	AOAC 994.08	1994	HPLC	Multi-column	76–80	21–27	24–30	[11]
G_2	Pistachio paste	0.2–0.6	AOAC and CEN adoption	2000	HPLC	IAC	72–104	10–31	31–63	[19]
M_1	Milk, Milk powder	0.05–0.2 ng/ml, 1.5–3.8	Paragraph 35 LMBG	1977	TLC (2-dim)	-	-	-	47–74	[43]
			01.0014			-	-	-	20–35	
M_1	Cheese, milk powder, butter	0.3	AOAC 980.21, IUPAC	1980	TLC(2-dim)	Solvent extraction, CC		19–40	23–57	[44]
		1.0					-	19–36	29–37	
		1.0						8–68	24–82	
M_1	Liver	0.1–1.0	AOAC 982.26	1981,1982	TLC	CC	80–90	24–26	36–41	[45,46]
M_1	Milk powder	0.15–0.4	Paragraph 35 LMBG L01.00–34	1985	ELISA	None		36	36	[47]
M_1	Liquid milk	0.08–1.3 ng/ml	AOAC 986.16	1986	HPLC	CC	85–103	28	44	[48]
M_1	Milk powder	0.08–0.3	Paragraph 35 LMBG L01.00–15	1987	TLC	CC		35	35	[49]
M_1	Liquid milk	0.02–0.1 ng/ml	AOAC 2000.08 and CEN in adoption	2000	HPLC	IAC	74–103	8–18	21–31	[50]
M_2	Liquid milk	0.03–0.13 ng/ml	AOAC 986.16	1986	HPLC	CC	100–127	24	65	[48]

TLC, thin layer chromatography; HPLC, high-performance liquid chromatography; NP, normal phase; RP, reverse phase; ELISA, Enzyme-linked immunosorbent assay; Multi-column, Mycosep 224 Romer multi-functional column or equivalent; CC, column clean-up; IAC, immunoaffinity column; SPE, solid-phase extraction column; Densitom, TLC densitometer.

Table 3
Performance characteristics of validated methods for mycotoxins other than aflatoxins

Mycotoxin	Matrix	Applicability range (ng/g)	Official method	Date	Method principle	Clean-up	Methods Performance			Reference
							Recovery (%)	RSD_r (%)	RSD_R (%)	
Deoxynivalenol	Wheat	50–1000	AOAC 986.17	1986	TLC	CC	78–96	30–64	33–87	[51]
Deoxynivalenol	Wheat	⩾350	AOAC 986.18	1986	GC	CC	67–119	23–48	31–52	[26]
Fumonisin B_1	Corn	50–2000	IUPAC	1993	HPLC	AE		8–26	18–27	[52]
Fumonisin B_1	Corn	500–8000	AOAC-IUPAC	1996	HPLC	SPE	79–84	6–22	15–22	[53]
Fumonisin B_1	Corn	370–1400	AOAC, CEN in adoption	2001	HPLC	IAC	76	19–24	22–28	[54]
Fumonisin B_1	Cornflakes	320–1050	AOAC, CEN in adoption	2001	HPLC	IAC	110	9–21	27–32	[54]
Fumonisin B_2	Corn	50–750	IUPAC	1993	HPLC	AE	-	13–37	28–46	[52]
Fumonisin B_2	Corn	200–3200	AOAC-IUPAC	1995	HPLC	SPE	76–83	8–12	17–28	[55]
Fumonisin B_2	Corn	90–560	AOAC, CEN in adoption	2001	HPLC	IAC	72	19–27	022–30	[54]
Fumonisin B_2	Cornflakes	130–460	AOAC, CEN in adoption	2001	HPLC	IAC	97	8–22	26–35	[54]
Fumonisin B_3	Corn	100–1600	AOAC-IUPAC	1995	HPLC	SPE	75–87	8–25	19–26	[55]
Ochratoxin A	Baby food	70.1	CEN in adoption	2001	HPLC	IAC	108	18–47	29–63	[56]
Ochratoxin A	Barley	25–100	AOAC 973.37	1973	TLC	CC	81	12–56	-	[57]
Ochratoxin A	Barley	10–50	AOAC 991.44	1992	HPLC	CC	72–74	8	27–28	[58]
Ochratoxin A	Barley	2–9	AOAC-IUPAC-NMKL	1996	HPLC	CC	64–72	12–21	18–31	[59]
Ochratoxin A	Barley	1–5	AOAC 2000.03 CEN in adoption	2000	HPLC	IAC	93	4–24	12–33	[60]
Ochratoxin A	Beer	0.2–1.5 ng/ml	AOAC 2001.20	2001	HPLC	-	87–95	7–17	15–26	[54]
Ochratoxin A	Coffee, green	41–230	AOAC 975.38	1975	TLC	CC	60–86	21–32	16–25	[61]
Ochratoxin A	Coffee, roasted	1–5	AOAC 2000.09 CEN in adoption	2001	HPLC	SPE-IAC	85	2–22	13–26	[62]
Ochratoxin A	Corn	10–50	AOAC 991.44	1992	HPLC	CC	77–82	20	20–32	[58]
Ochratoxin A	Kidney	5–50	AOAC 991.44	1992	HPLC	CC	53–97	16	33–68	[58]
Ochratoxin A	Wheat meal	0.4–1.2	Paragraph 35 LMBG 15.00–1	1995	HPLP	SPE		35	35	[63]
Ochratoxin A	Wine, white	0.1–2 ng/ml	AOAC 2001.20	2001	HPLC	-	88–105	7–11	13–16	[54]
Ochratoxin A	Wine, red	0.2–3 ng/ml	AOAC 2001.20	2001	HPLC	-	84–93	7–11	12–14	[54]
Patulin	Apple juice	50–340 ng/ml	AOAC 974.18	1974	TLC	CC	70–99	CV: 36–51		[64]
Patulin	Apple juice	5–250 ng/ml	IUPAC	1988	HPLC	Solvent extraction	77–85	CV: 7–15		[65]
Patulin	Apple juice	20–200 ng/ml	AOAC-IUPAC-IFJU 995.10	1996	HPLC	Solvent extraction	91–108	11–20	15–29	[66]
Patulin	Apple juice	26–128	AOAC2000.02 CEN in adoption	2000	HPLC	Solvent extraction	80–89	6–35	11–35	[67]
Patulin	Apple puree	23–121	AOAC 2000.02 CEN in adoption	2000	HPLC	Solvent extraction	92	10–27	13–36	[67]
Zearalenone	Corn	100–2000	AOAC 976.22	1976	TLC	CC	88–129	-	27–53	[68]
Zearalenone	Corn	50–4000	AOAC 985.18	1985	HPLC	Solvent extraction	77–123	31	34–38	[69]
Zearalenone	Corn	⩾800	AOAC 994.01	1994	ELISA	Solvent extraction	-	3–11	16–26	[70]
Zearalenone	Wheat	⩾800	AOAC 994.01	1994	ELISA	Solvent extraction	-	10–15	16–31	[70]
Zearalenone	Feed	⩾800	AOAC 994.01	1994	ELISA	Solvent extraction	-	14–16	27–57	[70]
Zearalenone	Corn	35–700	None	2001	HPLC	Multi-column	81–107	1–27	-	[80]
Zearalenone	Corn	50–200	AOAC 985.18	1985	HPLC	Solvent extraction	81–100	23	26–47	[69]

TLC, thin layer chromatography; HPLC, high-performance liquid chromatography; GC, gas chromatography; ELISA, Enzyme-linked immunosorbent assay; Multi-column, Mycosep 224 Romer multi-functional column or equivalent; CC, column clean-up; AE, strong anion-exchange column; IAC, immunoaffinity column; SPE, solid-phase extraction column.

For baby food, validated methods were not available for aflatoxin B_1 or ochratoxin A, and certainly none was available for the very low limits under discussion by the EC.

3. Selection and development of methods for validation

The methods developed and validated in SMT project CT96–2045 were required to fulfil the following general criteria:

- use readily available analytical techniques accessible to enforcement laboratories;
- be validated according to international harmonised guidelines [8];
- perform according to CEN requirements at low regulatory limits set by the EC [9];
- take into consideration economical and ecological (environmentally friendly) factors, as well as efficiency in time and materials;
- adopt a uniform approach between methods (with application of the same principles for a range of mycotoxins and matrices); and,
- achieve wide acceptance by European laboratories.

These criteria had to be taken into account in developing methods for the determination of aflatoxins, ochratoxin A and patulin in the various food commodities, as prescribed in the project.

As HPLC is now the most commonly used chromatographic method for a wide variety of mycotoxins, especially for those mycotoxins on which the project focused (Table 1), all methods were developed with HPLC as the chromatographic stage.

Although there was debate as to the relative merits of the more traditional solid-phase clean-up technique, as opposed to immunoaffinity columns, the latter were selected for this project for a number of reasons. Immunoaffinity columns were thought to be more robust in terms of applicability to different matrices without the need for major adjustments to the method. Immunoaffinity columns offer the opportunity to concentrate large volumes of sample extract to achieve high sensitivity, which was the requirement for aflatoxin B_1 and ochratoxin A in baby foods. Immunoaffinity columns are also less demanding in terms of the skills and experience required. The disadvantage of cost was of less concern, but it was felt that official methods should not be tied tightly to particular manufacturers or suppliers. For this reason, two different suppliers of immunoaffinity columns were involved in the project, and the immunoaffinity columns to be used in the methods were ultimately described in the protocol in generic terms as to the performance that columns should achieve, rather than linking the methods to specific, proprietary products.

In several cases, the methods validated in the SMT project were based upon methods that had already been described in the scientific literature. For example, the method for aflatoxins was based on one that had been collaboratively tested for the determination of aflatoxins in peanut butter and corn in 1991 [16] It comprised extraction with an aqueous organic solvent (acetonitrile), purification of the extract using an immunoaffinity column, and identification and quantification by HPLC.

However, the method developed in the SMT project had to cover not only peanut butter but also pistachio paste, fig paste, paprika powder and baby food. For a universal approach, the same extraction solvent would need to be employed. Because of the international agreement to move away from using chlorinated solvents, their absence was essential. Several solvents were therefore investigated and, in the end, a mixture of methanol and water was found to be suitable for all matrices with some modifications for matrices with a high fat content [25] The selected methanol-water extractant had the advantage of being less toxic than others, such as acetonitrile-water.

Other aspects considered during method development were the kind of derivatisation technique to be used (for example, for aflatoxin

B_1 and ochratoxin A. In order to enhance fluorescence for better quantifications, several options were compared before finalising the methods. In the case of aflatoxin B_1, bromination was found to be the derivatisation of choice, as it offered the simplest, most readily available, reliable approach. Again simplicity and accessibility were seen as important factors, so an approach using pyridinium hydrobromide perbromide (PBPB) was evaluated, as it avoided the need to purchase additional pumps (for post-column iodination) or a Kobra cell for bromination.

4. Preparation of naturally-contaminated-mycotoxin test materials for validation studies

Particular care was taken in preparation of the test materials because of the relatively low levels of contamination in the naturally-contaminated test materials, coupled with the well-documented problems of the inhomogeneous distribution of mycotoxins in naturally-contaminated matrices. Surprisingly, international harmonised guidelines for validation studies [8] do not stipulate the need to demonstrate the homogeneity of the test samples. However, within this project, this aspect was given high importance, and we believe that inferior performance characteristics previously obtained in some mycotoxin validation studies may well have been attributable to lack of attention to this aspect.

Homogeneity testing in this project was carried out on the bulk material as well on the packed test samples. The sections below exemplify the procedures followed for sample preparation and homogeneity testing. Only those naturally-contaminated matrices containing aflatoxins are described, but similar procedures were followed for ochratoxin A in barley flour, roasted coffee and baby food, and for patulin in apple puree. For patulin in apple juice and aflatoxin M_1 in milk, although sample mixing of liquids was more straightforward, there were other difficulties in procuring naturally-contaminated samples containing the target mycotoxins at levels of interest.

4.1. Sample preparation – peanuts, pistachios, figs, paprika and baby food

Because of inhomogeneity of commercially-available peanut pastes, the blank sample was prepared from raw material containing shelled and blanched peanuts that were selected from a number of different materials by analysis of the aflatoxin contents. The selected raw material was roasted using a normal commercial process. The blank material was blended with contaminated, commercially-available peanut paste in the appropriate mass ratios in order to obtain test samples with the required contamination levels for the validation study. Lecithin was added to avoid separation of oil in the pastes. As it is also used in commercially-available peanut paste, this did not cause any problems for the method developed.

As blank pistachio material was unavailable commercially for a reasonable price (only hand-selected material containing no aflatoxins was available on the market), the blank sample was prepared from green, unripe pistachios. These were dried and prepared as a paste. However, this material still contained 0.2 ng/g total aflatoxins. The same procedure was carried out with two different commercial, ripe, shelled pistachio materials that naturally contained different levels of aflatoxins. The final pistachio pastes were stabilised with 0.4% lecithin.

Considerable effort was put into procuring non-contaminated figs. However, the best blank material obtained, after preparation of 10 different batches, still contained low amounts of aflatoxins (up to 0.4 ng/g). This material was blended with other higher naturally-contaminated materials and ground finely. Glycerin containing 0.3% potassium sorbate was added to obtain a paste.

In contrast to the other materials, blank samples and naturally-contaminated paprika powder were easily obtained from the market, whereas it was more laborious to obtain infant-formula materials (target value for validation 0.1 ng/g).

The infant formula was prepared by mixing rice flour (representing the blank) containing less than 0.02 ng/g aflatoxin B_1 with naturally-contaminated maize flour in order to obtain the desired contamination levels. Considerable emphasis was put on careful grinding and sieving of the materials. All test materials were analysed for particle size and particle-size distribution prior to homogeneity testing.

4.2. Homogeneity testing

All bulk samples were analysed for homogeneity using an accredited, in-house laboratory method for extraction and determination. The bulk homogeneity was investigated by means of "analysis of variance" (ANOVA). As required by the international harmonised protocol for proficiency testing of analytical laboratories [79], 10 samples were taken randomly and analysed in duplicate. In addition, the variance of 10 spiked blank samples was analysed. A systematic difference of the respective variances was investigated by calculating an F-statistic.

The homogeneous bulk materials were then put into appropriate containers from which every 10th sample was subjected to analysis. Data were analysed using ANOVA that allows the partitioning of the whole variance into the individual components of variability. In this study, 10 samples were analysed in duplicate; from each sample, two sub-samples were investigated. It was found that the material met the criteria that variances should not differ significantly from the variance of the repeatability of the method that had been determined prior to the validation study in an in-house performance study.

The equality of the variances was then checked using the F-test. The results of the ANOVA and of the test for equality of variances obtained for all materials revealed that the difference in the between-group-variance and the within-group-variance and the latter and the overall-variance (F-test) were not significant with respect to the variance of the repeatability of the in-house validated method. This demonstrated that all materials were sufficiently homogeneous to be used in the final validation studies.

A typical example showing the results of homogeneity testing for pistachio test materials containing levels of 1 to 4 ng/g of aflatoxins is shown in Fig. 1 and the statistical tests carried out to examine for analytical drift and for "equality of variances" are shown in Table 4.

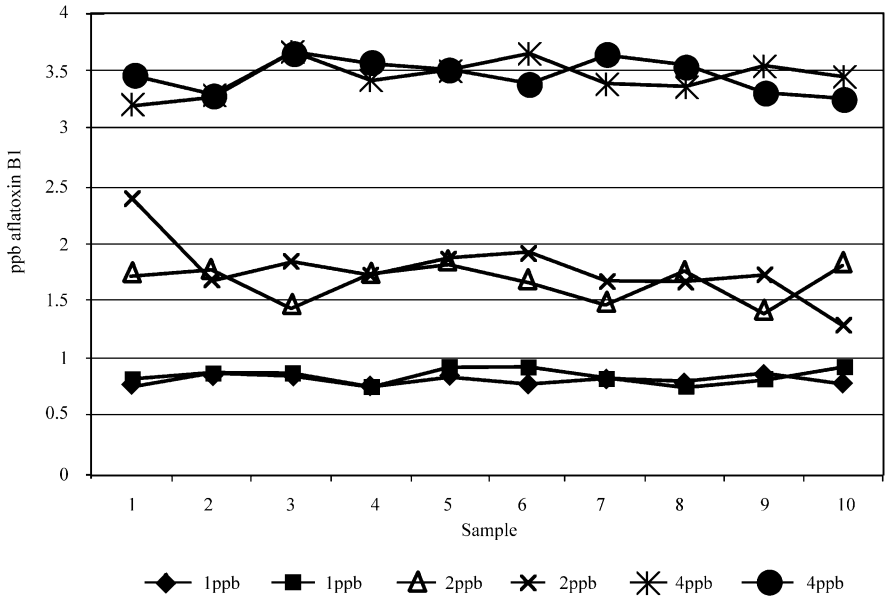

Fig. 1. Results for homogeneity testing of aflatoxins in pistachio paste contain 1–4 ng/g of total aflatoxins.

Table 4
Statistical tests for homogeneity of pistachio test material showing checks for (a) drift in analytical results and (b) test of "equality of variances"

(a)

Material	Content B_1 (μg/kg)	$\bar{x}_1$	$\bar{x}_2$	t_{calc}	$t_{crit,\ df=9}$
Pistachios	1	0.89	0.83	1.18	2.26
	2	1.56	1.51	0.43	2.26
	4	3.39	3.51	−0.55	2.26

(b)

Material	Content B_1 (μg/kg)	RSD (%) method	Within group variability			Overall variability		
			RSD (%)	F_{crit}	F_{calc}	RSD (%)	F_{crit}	F_{calc}
Pistachios	1	11	7	3.2	0.4	7	2.5	0.4
	2	11	14	3.2	1.5	13	2.5	1.5
	4	11	4	3.2	0.1	4	2.5	0.1

All data for all test materials were used to carry out the ANOVA without any outlier exclusion. Although a small number of samples showed a considerable difference in the content of the corresponding sub-samples, it was taken into account for this study that sub-samples (2 × 25 g from the 50 g) were used. In contrast to this, the total content of a sample sachet was subsequently analysed for each of the matrices in the validation study. In some cases, the calculated within-standard deviation, reflecting the analytical error, hinted at a considerable improvement in the achievable precision (for example, for figs containing 1 ng/g of aflatoxin B_1, the within-standard deviation was 3% and the method repeatability was 8%). However, the F-test indicated that the observed differences in the corresponding variances were not significant ($p=0.95$). Statistical analysis of the data confirmed that all materials can be considered as homogeneous and they fulfilled the criteria for homogeneity established at the outset of the project.

5. Videos demonstrating mycotoxin methods

In planning the SMT project, the decision was taken that the analytical method should not only be well described, as required for a CEN standard, but also visually demonstrated using a video format. This was envisaged as benefiting not only validation-study participants but also the wider analytical community, particularly those not familiar with analysis of mycotoxins. The video format enables a laboratory to demonstrate, visually, any manipulatively-difficult steps that are sometimes impossible to describe adequately in writing.

The availability of low-cost video equipment and computer editing of video and graphics significantly reduced the costs of the exercise, making it an attractive medium for demonstrating how to undertake a method of analysis. In the SMT project, a 45- minute video was filmed covering the practical steps in the clean-up by using an immunoaffinity column as applied to aflatoxins and ochratoxin A, with variations being shown for the slightly different approaches to handling different matrices. The analysis of patulin in apple juice and apple puree was also demonstrated. One project partner co-ordinated the production of the video and two other partners were directly involved in setting up and undertaking the work that was filmed. The video was distributed to all validation trial participants in advance of attending the Study Workshop.

Subsequent to the project, the video was translated into French, German and Spanish,

and sufficient copies were made in various formats, such as PAL, SECAM, and NTSC, to make the video useful internationally. The video is still available and copies can be obtained free of charge, on request from the authors of this paper. A CD-ROM version has now been made, enabling the video to be easily viewed on most PCs.

6. Planning methods validation (collaborative studies)

In principle, the results of a validation exercise should reflect those obtainable by any competent laboratory, and there should no "pre-selection" of participants. Whilst these rules need to be observed in planning a collaborative study, equally the organiser must guard against participants who are using participation as a "learning exercise" and who may therefore compromise the performance characteristics ultimately obtained. This is particularly critical for methods being validated close to analytical limits of detection, such as those for mycotoxins in this case, and for methods where unusual precautions need to be observed, such as avoiding exposure of extracts to sunlight and minimising the effects of active sites on glass surfaces. In practice, only laboratories with some experience in analysis of mycotoxins were included as participants, but there were no restrictions in terms of particular experience with the type of methods being validated.

7. Workshops for collaborative-study participants

All participants were invited to take part in workshops for each of the validation exercises. It should be emphasised that these workshops were not intended to provide training, which is against the spirit of objective method evaluation, as the methods should be sufficiently well described to be able to be undertaken by any experienced analyst without specific training. The main thrust of the workshops was to explain the rationale for the methods, so that, knowing why certain decisions had been taken, the participants did not feel they could improve on the methods by making changes to the way any part of the method was undertaken. Second, the workshops were used to explain the study design of the validation exercise and again, as far as possible, to ensure that participants complied with all instructions, for example, with respect to analysing samples in batches and timing of analyses.

8. Performance characteristics of methods for mycotoxins

The performance characteristics of the seven methods [19,27,50,56,60,62,67] that were validated in the SMT project are shown in Table 1. All of the methods with the exception of the ochratoxin A in baby food [56] achieved AOAC International First Action status. All seven methods met the criteria established by CEN [9] as being the minimum performance necessary for mycotoxin methods to be used in support of EC Directives [2–4] These methods have all been tabled at CEN TC 275 and are currently at various stages of scrutiny and voting before adoption as European Standards.

The Horwitz curve [49], based on collaborative trial data collected over many years, has been used to predict the most likely achievable performance of methods as a function of their limit of quantification. For six of the methods that were validated at ng/g and sub-ng/g limits, the performance characteristics achieved were better than would have been predicted by Horwitz [49] This was partly because the Horwitz curve was based, to a large extent, on historical collaborative trial data that tended to skew it away from 'best achievable' performance, and partly because the function used to predict became more uncertain approaching low ng/g levels. Taking account of the six methods validated in this project, as well as other recent collaborative trial data obtained at ng/g levels, Thompson [28] made a modification to the Horwitz function that is now accepted as a

better predictor of best achievable performance at low limits.

9. Validation of TLC methods for mycotoxins for developing countries

As described above, mycotoxin analysis in developed countries has in recent years become almost invariably based on HPLC, because it offers good sensitivity and precision, coupled with ease of automation. However, HPLC is expensive in initial capital investment and requires skilled and experienced staff to operate and maintain equipment. By contrast, TLC is a simple, robust technique [71,72], which is relatively inexpensive to establish in a testing laboratory, and most laboratories in developing countries have considerable expertise and experience with it. TLC offers the possibility of separating all mycotoxins from each other (for example, the various aflatoxins), provided that they can be concurrently extracted, suitably cleaned up from co-extractives and visualised on the TLC plate. One disadvantage of TLC is its lack of separating power, and thus an inability to discriminate any possible co-extracted interferences from the toxins of interest. However, with much-improved modern clean-up techniques, such as immunoaffinity, solid-phase or multi-functional columns, interfering matrix residues can be much more easily removed in the clean-up stage prior to TLC. As such extracts will frequently contain only the toxins of interest, the separating power of the chromatography becomes less critical and TLC then becomes viable, provided detection limits are not impaired. The use of an immunoaffinity column prior to TLC determination has been described for the determination of aflatoxins at levels of 10–50 ng/g [73], for aflatoxin B_1 in corn [74], fumonisin B_1 in corn [81] and ochratoxin A in green coffee [83]

TLC is unquestionably the method of choice in many parts of the world, especially in developing countries that are the main exporters to Europe of food and food products, which have the potential for aflatoxin contamination (for example, figs, peanuts, pistachios, and spices). TLC methods are potentially comparable alternatives to HPLC methods, although, to date, there has been little effort put into validating modern TLC procedures.

Many of the TLC methods for aflatoxins shown in Table 2 were validated more than 20 years ago [12–14,17,18,20,24,28] and require the use of chlorinated solvents for extraction or sample clean-up or use chlorinated solvents in the TLC mobile phase. Since these solvents are considered to be ecologically undesirable or of toxicological concern, they are systematically being replaced in routine laboratory work, where possible. For many long-standing TLC methods, performance has not been established by collaborative study, and where, for example, there has been a more recent validation of a TLC method [31], the performance of the methods has only been established at levels of 5–50 ng/g, which are too high to make the methods of any use for testing compliance of samples against European regulatory limits. There are some recent examples of where TLC methods with modern clean-up have been employed for surveillance work for aflatoxins, zearalenone and fumonisins [81,82] Although these methods have not been subjected to full collaborative studies, there is good, published, in-house validation data. For example, in-house performance (RSDs) of methods were obtained for: aflatoxins using florisil clean-up of 11–14% (recoveries 85–97%) [81]; fumonisin B_1 using immunoaffinity column clean-up of 19–20% (recovery 85) [81]; and, zearalenone using a Romer Mycosep column of 16–18% (recoveries 81–107%) [80,81]

To address the need for validated TLC methods that could be used to test samples for compliance with European regulatory limits, simple, user-friendly TLC methods have been developed as spin-off of the SMT project for the determination of aflatoxins in various food matrices at the same low levels as for the HPLC methods [19,50] These TLC methods use essentially similar affinity column clean-up, but employ TLC instead of HPLC, although they do not use chlorinated organic solvents and require

only normal silica-gel plates. These validation exercises for aflatoxin B_1 in corn and peanuts and for aflatoxin M_1 in liquid milk have been undertaken as IUPAC Food Chemistry Commission and Joint IUPAC/IDF projects, respectively.

In-house validation of these new TLC methods has shown that they are a simple, robust and efficient alternative to modern HPLC methods, and can quantify aflatoxin B_1 and total aflatoxins at 2 ng/g and 4 ng/g, respectively, and aflatoxin M_1 at 0.05 ng/L. The methods are currently under interlaboratory validation, involving mainly laboratories from developing countries, taking into account the experience and lessons learnt from the SMT project described above.

Although visual quantification of TLC plates has been employed in the initial validation exercises, attention has also been directed at ways in which densitometry might be made more accessible at minimal cost for developing countries. Recently, two simplified densitometers have been developed, one based on a miniaturized, low-power-consuming detector cell (battery, fully semiconductor-based) [75], and the other on a modified computer office scanner [76] Both detection approaches have shown excellent results during in-house validation studies. Further developments are being explored to exploit low-cost, but high-technology, equipment (such as Game Boy), which might be used as an alternative to a PC as an aid for the application of TLC methods [77]

It is essential that TLC is not always seen as being inferior to HPLC and that, when combined with modern clean-up techniques and low-cost plate scanning, methods are validated. There is no reason why TLC methods should not have comparable performance to HPLC methods and be available as robust, low-cost alternatives to HPLC for official monitoring and control of mycotoxins in foods.

10. Conclusions

In this article, we have attempted to demonstrate that, with considerable care and attention to detail, it is possible to obtain impressive performance characteristics for mycotoxin methods at low limits. In the past, when collaborative studies were carried out at mycotoxin concentrations of 20–50 ng/g, little attention was paid to demonstrating the homogeneity of test samples. At low ng/g and sub-ng/g levels, grinding of samples to fine particulate material and demonstration of homogeneity is expensive and time-consuming, but absolutely essential.

It has also proved necessary to minimise other possible variables, such as differences in concentrations of calibrants that might adversely affect method performance, and thereby ensure that performance characteristics truly reflect only the performance of methods and are not influenced by extraneous factors. We were fortunate that the EC funded the mycotoxin method-validation project described here and that the work was shared amongst a number of partners. To be undertaken properly and to obtain results that accurately reflect method performance, it must be recognised that validation is an expensive, time-consuming process. This EC-funded project presents a good model for method validation that should be emulated in other areas.

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