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Review

Immunoaffinity column clean-up techniques in food analysis: A review☆

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ABSTRACT

This review provides a critical assessment of the applications of immunoaffinity columns for sample clean-up in the field of food safety. The performance of immunoaffinity columns are compared in terms of specificity, binding capacity and recovery, and commercial disposable columns are contrasted with home-made columns. Areas covered include multiple-use of columns, multiple-analyte columns, use with automated systems and validation of IAC methods. Publications illustrating the many varied applications of IAC for sample clean-up in the areas of mycotoxins, veterinary drug residues, pesticide residues, environmental contaminants and vitamins have been compiled, comparing extraction methods, achievable recovery, and illustrating the variety of end-detection methods that have been employed.

Contents

1. Introduction

Immunoassays were first developed more than 50 years ago and the exploitation of antibodies in various formats has continued making a significant impact in the testing and diagnostic fields [1]. Antibodies in user-friendly formats have even been exploited in the home-testing arena, e.g. pregnancy tests. In the clinical field, urine or blood plasma, being relatively uncomplicated liquid samples, readily lend themselves to direct analysis [2–6], whereas the complexity of food matrixes means that direct measurement, e.g. by immunoassay can sometimes be problematic. Thus, although a number of immunoassays are being routinely and successfully used for screening purposes in food analysis [7–10], in general positive results or those above a regulatory limit require confirmation by

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instrumental analysis [11]. Although immunoassays have changed the way some food testing is conducted, arguably the biggest impact of antibody technology has probably been in the development and application of immunoaffinity columns (IAC) to trace analysis of foods.

Although in this review we use the terminology immunoaffinity throughout, it should be noted that a number of other terms are also used in the literature to describe the same process of sample extraction and clean-up. Thus, the process of using an IAC is sometimes described as immunoextraction, immunoaffinity-based solid-phase extraction or sol–gel immunoaffinity chromatography. Immunofiltration or immuno-ultrafiltration (IUF) are similar to IAC except with IUF the antibodies are not bound to a solid support material but are used in free form [12].

The principle of the IAC is relatively simple in that an antibody (polyclonal or monoclonal) raised against the analyte is immobilised on a gel, and generally about 0.2–0.5 ml of gel is packed into a small plastic column. The column is initially conditioned with phosphate buffered saline (PBS) and then the crude sample extract is applied slowly to the column at around 1–2 ml/min. The sample can be applied under gravity flow or under positive pressure from a syringe or can be sucked through the column under vacuum, but maintaining a uniform flow-rate in line with manufacturers recommendation is important.

In general the sample extract must be in aqueous solution because organic solvents can damage the antibody and can interfere with the antibody–antigen interaction and this may be a limitation when wishing to use this approach to analyse non-polar target analytes. During application of the sample extract the analyte becomes bound to the antibody and thereby bound to the IAC gel. The specificity of the antibody is important, in terms of the extent of recognition of the analyte (antigen) compared to structurally similar co-extractives which should not become bound to the antibody. The strength of the binding to the antigen (avidity) is also important as binding strength will influence recovery during this extraction stage. The capacity of the column in terms of the total number of antibody sites (quantity of antibody) available for binding will also be important as overloading the column will lead to poor recovery. After loading the extract onto the IAC, the gel is washed with PBS to completely remove any co-extractives. Finally, the analyte is eluted from the IAC by breaking the antibody–antigen bond. For small molecules this can be achieved with a small volume of methanol or acetonitrile, which is generally the procedure used with commercial columns [13,14]. Alternatively the analyte might be eluted for example with buffered glycine–NaCl [15,16] which is less damaging to the antibody and is the procedure employed with home-made columns which can then be re-generated and re-used many times [17]. Acidic conditions are required for the elution of macromolecules. Despite the specificity of the antibody it is also possible to get some non-specific binding to the gel itself, which can mean for some very dirty matrices, the eluted final extract from the column is not as clean as might otherwise be anticipated [18].

The only limitations as to the volume of sample extract that can be applied to the column are the breakthrough volume of the column (i.e. the affinity of the antibodies for the target compound) and the practicality of the time involved in passing a large volume through the column. For liquid samples such as milk [19–21], beer [22,23], wine [24–27], vinegar [28] and soy sauce [29], the sample can be passed directly through the IAC (or diluted with PBS and applied) without any need for prior extraction. To test the robustness of the column format, as much as 400 ml of milk was passed through a commercial IAC [30] demonstrating a capability of achieving ng/l (ppt) sensitivity, by concentration of trace amounts of analyte from a large volume of milk onto the column.

IACs have been used for many years as a method of sample purification and many of the early papers involve use of antibodies in columns prepared in the authors own laboratories [31]. These columns have tended to have a high capacity and to be sufficiently robust for multiple-use with suitable selection of eluting solvent so as not to denature the antibody. Although a range of monoclonal antibodies are commercially available from a number of suppliers, preparing columns does limit applicability to those with the skills and experience to couple the antibody to a gel and be able to prepare a robust column. Nevertheless, many recent publications still involve the use of IACs prepared in the authors own laboratory [32–34], and although full experimental details of preparation are given, most analytical chemists will err towards purchasing commercial columns if available. A comparison of commercial and in-house columns [35] clearly showed that even some commercial columns can fail to adequately entrap and then to elute the analyte. Poor antigen specificity, insufficient antibody concentration on the column, antibodies of poor avidity or poorly developed protocols for elution were all thought to be factors contributing to poor column performance.

IACs can be applied in many different formats in different analytical protocols, although in general the same principle of trapping on the column, washing off extraneous compounds and eluting the analyte is employed. As the analyte is eluted in a relatively pure form, some protocols have been developed for field-testing whereby the eluate is measured directly in solution either by fluorimetry [36–38] or visually under UV light. These are essentially screening tests and a number of commercial kits are available for screening mycotoxins in food and feed samples. Some enzyme linked immunosorbant assays (ELISA) for food and biological materials also require prior clean-up [39–43] and IACs are thus included as part of an ELISA test kit (e.g. products sold for screening for various mycotoxins).

Of the various potential applications in food safety the biggest impact has probably been made in the development of commercial IACs for analysis of mycotoxins. IACs are now commercially available for aflatoxins B_1, B_2, G_1 and G_2, aflatoxin M_1, ochratoxin A (OTA), deoxynivalenol (DON), zearalenone (ZON), T-2 and HT-2 toxins, fumonisins B_1, B_2 and B_3, citrinin and for various combination of toxins using mixed antibodies on the same column. Generally, these columns are used to provide a rapid and highly effective clean-up of extracts from food samples or direct extraction from beverages prior to HPLC analysis with fluorescence detection. However, the fact that the extracts are essentially free of interfering components also means these columns can be used to provide a clean extract for subsequent analysis by non-chromatographic measurement or for example by TLC analysis [44–47].

In this review we have focussed on food safety applications of IACs and have collected information on the relative performance of IACs and on wide range of applications. We have not attempted to make a critical assessment of the place of IACs compared to alternative approaches to clean-up, but there are a number of excellent recent reviews on advances in analysis of mycotoxins [48–55], veterinary drugs [56,57] and environmental contaminants [58] which provide such an insight. There are few reviews dealing specifically with IACs, although one has previously been published covering a range of small molecules [59], one on mycotoxins [48] and another on shellfish toxins [8].

1.1. Characterisation of IAC performance

The performance of an IAC is to a large extent determined by the quality of the antibody in terms of its specificity (and cross-reactivity), binding ability (avidity) together with the column capacity (the total amount of antibody which can be bound to the column gel). These three characteristics are critical in determining overall performance of an IAC in enabling effective clean-up of the analyte from co-extractives from the sample. In practical terms for

Table 1
Comparison of performance of commercially available affinity columns.

Analyte	Brand name	Manufacturer	Gel bead vol (ml)	Column capacity (ng)	Spike level for recovery (ng)	Spike conditions for recovery	Recovery (%)
Mycotoxins							
B1, B2, G1, G2, M1, M2[a]	AFLASCAN®	R-Biopharm Rhone Ltd	–	ca. 200	4 of each	10 ml, 30% methanol	Typical recoveries: >85% B1, B2, B3 and >70% G2
B1, B2, G1, G2, M1, M2[a]	AFLA-RHONE®	R-Biopharm Rhone Ltd	–	ca. 150	4 of each	10 ml, 10% methanol	Typical recoveries: >85% B1, B2, G1 and >80% G2
B1, B2, G1, G2, M1, M2[a]	EASI-EXTRACT®	R-Biopharm Rhone Ltd	–	ca 900	4 of each	16 ml, 10% methanol	Typical recoveries: >95% B1, B2, G1, >85% G2 and >95% M1 and M2
B1, B2, G1, G2, M1, M2	AflaTest®	VICAM®	0.250	300	100 total	Spiked sample extract	B1, G1, ≥90%; B2, ≥85%; G2, ≥80%
B1, B2, G1, G2, M1, M2	AflaTest® WB	VICAM®	0.300	400	200 total	Spiked sample extract	B1, G1, ≥90%; B2, ≥85%; G2, ≥80%
B1, B2, G1, G2, M1, M2	AflaTest®WB SR	VICAM®	0.375	500	400 total	Spiked sample extract	B1, G1, ≥90%; B2, ≥85%; G2, ≥80%
B1, B2, G1, G2	NeoColumn™	Neogen corp	0.216 or 0.500	>100	not given	Not given	>90%
B1, B2, G1, G2	AflaStar™	Romer Labs®	0.300	1,875	5 ng/g of each	Corn extracts in 20% MeOH	B1, G2, 90%; B2, 100%; G1, 85%
B1, B2, G1, G2	AflaStar™Fit	Romer Labs®	0.200	300	2.5 ppb of each	Corn extracts in 20% MeOH	B1, 97%; G1, 93%; B2, G2, 100%
B1, B2, G1, G2, M1, M2[a]	AFLAPREP®	R-Biopharm Rhone Ltd	–	ca. 200	4 of each	10 ml, 10% MeOH	Typical recoveries >90% B1, B2, G1 and >80%G2
B1, B2, G1, G2, M1, M2[a]	AFLAPREP® M	R-Biopharm Rhone Ltd	–	ca. 500	20	25 ml of 10% (w/v) milk in water	Typical recoveries >90% M1
M1, M2	AFLA M1™ HPLC	VICAM®	1.000	150	2.5	Spiked skim milk	M1>85%
M1	AflaStar™ M1	Romer Labs®	0.300	200	0.05 ng/g 0.5 ng/g	Matrix extract	Milk 90%; cheese 96%
OTA, OTB and OTC[b]	OCHRAPREP®	R-Biopharm Rhone Ltd	–	ca. 2,000	5	48 ml, 5% AcCN in PBS	Typical recoveries >95% OTA
OTA, OTB and OTC[b]	OCHRASCAN®	R-Biopharm Rhone Ltd	–	ca. 2,000	5	20 ml, 1% (w/v) aq. bicarbonate	Typical recoveries >95% OTA
OTA, OTB and OTC[b]	OCHRA-RHONE®	R-Biopharm Rhone Ltd	–	ca. 400	5	10 ml, 10% MeOH in PBS	Typical recoveries >80% OTA
OTA	OchraTest™	VICAM®	0.200	100	20	Spiked extract	≥85%
OTA	OchraTest™ WB	VICAM®	0.250	300	20	Spiked extract	≥85%
OTA	NeoColumn™	Neogen	0.500	>500			>95%
OTA	OchraStar™	Romer Labs®	0.300	1,500	10 ng/g	Wheat extracts in 20% MeOH	95%
OTA	OchraStar™Fit	Romer Labs®	0.250	250	10 ng/g	Wheat extracts in 20% MeOH	90%
ZON	EASI-EXTRACT®	R-Biopharm Rhone Ltd	–	ca. 1,500	75	15% AcCN in PBS	Typical recoveries >95% ZON
ZON	ZeralaTest™	VICAM®	0.350	1,500	1500	Spiked extract	≥85%
ZON	ZERALATEST™ WB	VICAM®	0.350	1,500	1500	Spiked extract	≥85%
ZON	NeoColumn™	Neogen corp	0.500	2,000	–	–	>90%
ZON	ZeraStar™	Romer Labs®	0.300	2,700	200ppb	Corn extracts in 10% MeOH	100%
DON, 3-AcDON, 15-Ac-DON	DONPREP®	R-Biopharm Rhone Ltd	–	ca. 2,000	500	2 ml water	Typical recoveries >90% DON
DON	DONTest™	VICAM®	0.400	1,250	25	Spiked water	≥90%
DON	DONTest™ WB	VICAM®	0.450	1,250	25	Spiked water	>90%
DON	NeoColumn™	Neogen corp	0.525	1,000	–	–	≥85%
DON	DonStar™	Romer Labs®	0.500	2,500	2000 ng/g	Corn Extract	90%

Table 1 (*Continued*)

Analyte	Brand name	Manufacturer	Gel bead vol (ml)	Column capacity (ng)	Spike level for recovery (ng)	Spike conditions for recovery	Recovery (%)
T-2[c] HT-2[c]	EASI-EXTRACT® T2 and HT2	R-Biopharm Rhone Ltd	–	1,500	100 total	18% Aqueous methanol	Typical recoveries >95% T-2 >95% HT-2
T2, HT2	T-2Test ™ HPLC	VICAM®	0.300	1,500 T-2	1500 T-2	Spiked PBS	≥85% T-2
T2, HT2	T2/HT2™ HPLC	VICAM®	0.600	2,000 total	1400 total	Spiked PBS	≥85% of each
FB1[d] FB2[d]	FUMONIPREP®	R-Biopharm Rhone Ltd	–	10,000 B1 + B2	1334 μg B1 + 666 μg B2	8 ml PBS + 1.5 ml MeCN/MeOH/water	Typical recoveries >95% FB1 >95% FB2
FB1, FB2 and FB3	FumoniTest™	VICAM®	0.300	10,000	7000	Spiked PBS	≥85%
FB1, FB2 and FB3	FumoniTest™ WB	VICAM®	0.350	10,000	7000	Spiked PBS	≥85%
FB1, FB2 and FB3	FumoniStar™	Romer Labs®	0.500	5,700	1000ppb	Corn extracts in 10% MeOH	B1, B3, 90%; B2, 93%
B1, B2, G1 and G2	AFLAOCHRAPREP®	R-Biopharm Rhone Ltd	–	200	1 of each	10 ml, 10% MeOH in PBS	Typical recoveries >85% B1, B2, G1 and >80% G2
OTA				400	1		>90% OTA
DON	DZT®	R-Biopharm Rhone Ltd	–	n/a	1000	15% MeCN	Typical recoveries >85% DON
ZON			–	n/a	40 ppb	Water	>90% ZON
T-2			–	n/a	25 ppb	18% MeOH	>90% T-2
HT-2			–	n/a	25 ppb	18% MeOH	>90% HT-2
B1, B2, G1 and G2	AFLAOCHRA HPLC™	VICAM®	0.500	100	100	Spiked sample extract	>85%
OTA				100			>85%
B1, B2, G1, G2	AOZ HPLC™	VICAM®	0.700	100	100	Spiked sample extract	>85%
OTA				100	100		>85%
ZON				1,000	50		>85%
Citrinin	CITRITEST® HPLC	VICAM®	0.350	20	20	Spiked phosphoric acid solution	>80%
Vitamins							
Vitamin B_9	EASI-EXTRACT® Folic acid	R-Biopharm Rhone Ltd	–	n/a	100 ng	10 ml PBS	Typical recoveries >90%
Vitamin B_{12}	EASI-EXTRACT® B12	R-Biopharm Rhone Ltd	–	n/a	500 ng	25 ml Water	Typical recoveries >90%
Vitamin B_{12}	EASI-EXTRACT® B12 LGE	R-Biopharm Rhone Ltd	–	n/a	–	Not given	Typical recoveries >90%
Veterinary drugs							
β-Agonists (10 compounds)	Clenbuterol	Randox Labs	1.0	50 each	≤Binding capacity	Not given	70–130%
Stilbenes (3 compounds)	Hexestrol Diethylstilbestrol Dienestrol	Randox Labs	1.0	50 each	≤Binding capacity	Not given	70–130%
Zeranol	Zeranol	Randox Labs	1.0	100 each	≤Binding capacity	Not given	70–130%
Cortiosteroids	Dexamethasone Flumethasone Betamethasone	Randox Labs	1.0	250 each	≤Binding capacity	Not given	70–130%

[a] Cross-reactivity with other aflatoxins metabolites.
[b] Cross-reactivity with OTB and OTC although performance reported for OTA alone.
[c] Cross-reactivity with diacetoxyscirpenol (DAS).
[d] Cross-reactivity with FB3.

commercial columns, the performance tends to be measured based on the recovery which can be achieved with standards, and the total amount of extract which can be loaded onto the column before there is seen to be any analyte breakthrough (giving an indication of IAC capacity). The breakthrough volume of the IAC depends on the total number of binding sites accessible to the analyte and how strongly the analyte is bound to the antibody. The selection of suitable solid support materials used for covalent bonding is thus of prime importance since a good choice should provide a high capacity and avoid any kind of non-specific binding [59]. Commercial IACs intended for the same application will differ in practical terms because different antibodies are being employed and are being used on a different solid supports. A good solid support should be chemically and biologically inert, easily activated, mechanically stable, uniform in particle size and have large sample pores because antibodies are large molecules [59]. Manufacturers of IACs provide datasheets indicating some aspects of performance such as cross-reactivity particularly to analytes, which are structurally similar to the target, but for commercial reasons do not disclose the nature of the solid supports. For home-made columns, of necessity the laboratory making the column must establish its specificity through practical tests. The necessary column capacity will vary depending on the likely concentrations of the analyte of interest. As can be seen from Table 1 the capacity of commercial IACs for DON, ZON and fumonisins is significantly higher (2500–5700 ng) than for aflatoxins (200–250 ng) as the former commonly occur at mg/kg levels in foods, whereas the latter only occur naturally at μg/kg in most circumstances. When highly contaminated samples are found and it is suspected that the amounts loaded may have exceeded the column capacity, these samples must be re-analysed after suitable dilution of the extract.

The analyte recovery from an IAC is a function of antibody specificity, concentrations of antibody on the column and the accessibility of analyte to antibody when the sample extract is passed through the column. The accessibility is dependant on the characteristics of the gel, and thus sample flow-rate through the column is important. High flow-rates may prevent analytes from binding and generally there is an optimum flow through the column during sample application to ensure good recovery. Flow of sample can be by gravity, under pressure or using a vacuum manifold, but whichever method is employed a flow-rate of about 1 drop/s equivalent to about 3 ml/min is thought to be about optimum. There is no agreed protocol as to how to determine recovery and as can be seen in Table 1 all the major manufacturers choose to determine recovery in different ways making comparison difficult. Some manufacturers use a spiked blank sample extract whereas other choose to employ standards in pure solvent. Applying sample extracts to the column is probably the more realistic approach for establishing recovery, although as explained below official methods frequently set performance based on applying analytes in solvent. In comparing column performance one would additionally expect concentrations to be in the typical range one might anticipate with naturally contaminated samples. Recent work by Trebstein et al. [60] compared the performance of two commercial IAC columns sold for the purposes of simultaneous determination of T-2 and HT-2 toxins. With one of the two columns (EASI-EXTRACT®) recoveries of better than 80% were achieved at spiking levels from 10 to 200 ng/g for both T-2 and HT-2 toxins whereas for the other column (T-2Test™ HPLC) recoveries remained constant at around 60% independent of spiking levels. It was concluded that the second column had both a lower capacity and also was affected by matrix compounds [60].

The selectivity of an IAC depends on the specificity of the immobilised antibodies that have been generated. These antibodies may be either polyclonal or more likely and certainly the case for commercial IACs they will be monoclonal. Polyclonal antibodies are prepared by injection, generally into a rabbit, of the low-molecular weight analyte coupled to a functional protein. After an immunization period (maybe several weeks) the serum is collected and the IgG fraction is isolated and purified. Polyclonal antibodies are thus a heterogeneous mixture of antibodies and available only in limited amounts. In contrast monoclonal antibodies are produced from cloned long-lived cell lines, and are well-defined in terms of being a single antibody rather than a mixture. Monoclonal antibodies can be continuously generated to a consistent standard and can be made available in the large quantities necessary for large-scale manufacture of commercial disposable columns. If antibodies with a very high specificity are used, cross-reactive and structurally analogous components can be removed during clean-up on the column. In most cases this is the goal of the IAC clean-up and the IAC is very specific to the target analyte. However, in some instances, e.g. in the case of aflatoxins, high specificity to the common four ring-system of aflatoxins is essential, but as most analysts want to determine all four structurally similar aflatoxins B_1, B_2, G_1 and G_2, antibodies have been generated against B_1 but have a high cross-reactivity to aflatoxins B_2, G_1 and G_2. Some theoretical background concerning the affinity of antibodies when incorporated into column format can be found elsewhere [17].

When IAC-based methods have been collaboratively studied, it is important that the IAC, which is at the heart of the method, is described in generic terms rather than based on a proprietary product. Although there are inevitably differences in column performance, the methods have in most cases stipulated the minimum performance that the IAC itself should meet. Thus, for example, for AOAC Official Method 999.07 [61] the requirement is that the column should have a maximum capacity of not less than 100 ng for aflatoxins B_1, B_2 and G_1 and not less than 60% for aflatoxins G_2 when applied as an aqueous standard solution (10% methanol) containing 5 ng of each toxin. Similar IAC performance criteria have been stipulated for aflatoxin B_1 in baby food [62], aflatoxin M_1 in milk [63], OTA in cereals [64], wine and beer [26] and coffee [18], DON in cereals [65], ZON in cereals [66] and fumonisins in corn and corn products [67]. Similar IAC performance criteria have been prescribed in Official Methods published by other organisations like CEN.

The only area where it is difficult to objectively assess column performance is related to non-specific binding to the gel to which the antibody is bound in the column. There has been evidence of false positive results for fumonisins in wheat using FumoniTest™ IAC when compared with SAX clean-up, although no LC/MS was carried out [68]. For two commercial T-2 toxin columns one appeared to show non-specific binding [60], and after comparing a number of supports HEMA-Afc-Bio which is a macroporous hydrophilic copolymer of 2-hydroxyethyl methacrylate and ethylene dimethacrylate exhibited non-specific binding of the aflatoxins to the column, and was used on-line for clean-up without any coupling of antibodies [69]. Clearly testing for non-specific binding needs to be carried out whenever a new matrix is used, and when problems are experienced, e.g. with interferences it is worthwhile looking to see whether columns from a different manufacturer before differently in this respect.

1.2. IACs manufactured in authors own laboratory (home-made)

In publications where IACs have been made in the author's own laboratory rather than being obtained commercially, the IACs have invariably been intended for multiple-use, and antibodies (polyclonal or monoclonal) have either been generated by the authors or purchased from commercial suppliers. The preparation of the columns involves antibody purification of the antiserum (if not purchased) and then antibody immobilisation onto a support. Typically the purified antibody in coupling buffer is passed though a column containing gel material, allowed to stand and then washed with coupling buffer. The non-coupled active groups are then capped

Table 2
Comparison of performance of IACs in different application areas.

Analyte	Matrix	Column capacity	Recovery	Analysis	Repeat use	Reference
Process contaminants						
Heterocyclic amines	Meat	Not given	Not given	HPLC	–	[6]
Phycotoxins						
DSP toxins (OA)	Mussels	300 ng	55–95%	HPLC	–	[137]
Okadaic acid	Mussels	16 μg/g	87–100%	LC/MS and LC-FLD	X50	[59]
Tetrodotoxin	Urine	Not given	58–88%	HPLC	X1	[5]
Pesticides						
Thifluzamide	Peanuts	4600 μg	100%	HPLC	X10	[128]
Bioallethrin	Fruit, veg	>5000 μg	87%	ELISA	–	[40]
Imazalil	Citrus fruit	2.0 μg	85–98%	HPLC	X30	[129]
s-Triazines	Water	1.5 μg	84–98%	GC	–	[130]
Phenylurea herbicides	Water	Not given	16–97%	LC/MS and LC-DAD	–	[94]
Carbofuran	Potato	28 ng	Not given	On-line LC/MS	–	[160]
Environmental						
1-Nitropyrene	Herbs	68 ng	96%	HPLC	X30	[161]
1,3,7,8-TCDD	Milk	Not given	>90%	GC/MS	–	[142]
Veterinary drugs						
Tetracyclines	Milk	Not given	94–100%	Fluorescence	–	[38]
Hormones		30 mg	88–94%	Electrophoresis	–	[34]
DES, dienestrol, HES	Biological samples			NICI GC/MS	–	[162]
Methamphetamine	Urine			LC/MS		[163]
Ractopamine	Muscle, kidney	5000 ng	89%	HPLC (FL)	X20	[16]
Fluoroquinolones	Liver	Not given	86–94%	HPLC	–	[105]
Fluoroquinolones	Milk	Not given			–	[104]
Chloramphenicol	Muscle	70 ng	54–95%	GC	X100	[17]
Chloramphenicol	Milk, muscle	1 mg	64–70%	HPLC	–	[70]
Penicillins	Buffer	5.4–6.6 μg	67–100%	ELISA	–	[15]
Anabolic steroids	Urine faeces	>900 ng	50%	GC/MS	–	[164]
Streptomycin	Honey	4.47 μg	100%	EIA/HPLC	–	[7]
Sulfathiazole	Honey	3.0 μg	105%	EIA/HPLC	–	[7]
Packaging						
Bis-phenol A	Canned food	280 ng	53–75%	HPLC	X15	[165]
Bis-phenol A	Wine	Not given	74–81%	HPLC	–	[166]
Vitamins						
Vitamin B_9 (folic acid)	Milk, cereals	Not relevant	88–101%	Biosensor	–	[167]
Vitamin B_{12}	Foods	–	>85%	HPLC	X1	[143]
Vitamin B_{12}	Foods	–	>85%	HPLC	X1	[144]
Vitamin B_{12}	Foods	–	>85%	HPLC	–	[145]
Biotin	Fluids		94–100%	Fluorescence	X30	[148]

by alternate cycles of ethanolamine and acetate buffer [32]. The antibodies are thus covalently bound to the activated support (column gel), such as agarose, trisacyl or CNBr-activated Sepharose 4B [70]. Columns containing the gel in ready-to-use form can be purchased, e.g. *N*-hydroxysuccinimide (NHS)-activated Sepharose columns (Hi-Trap R HHS-activated from Pharmacia Biotech), or glass columns can be packed after preparing the gel in a similar manner. It should be noted that these supports are unsuitable for automated procedures as after column switching the gel would collapse as a result of the high pressure. In automated systems a pre-concentration column (C_{18}) is therefore used for desorption of the analyte, to avoid direct exposure of the IAC to high pressure.

In Table 2 the performance of a number of home-made IACs are compared from different application areas with the exception of mycotoxins which are covered in more depth later in this review. In Table 2 the column capacities are compared and range from the relatively low (300 ng) to high capacity (hundreds of μg) depending on the application area. As explained above, the required capacity is driven by the anticipated levels of contamination. Recoveries in most instances are reported as quantitative, although in some instances recoveries around 50–60% are given which is probably not acceptable for other than a research method. Table 2 shows that a wide range of analytical procedures have been combined with IAC clean-up and HPLC, GC, GC/MS and LC/MS have been utilised.

1.3. Use of IAC in screening methods

IACs have been incorporated into many test kits, primarily intended for screening purposes, which can be used by relatively unskilled personnel or for field use when access to laboratory facilities is not possible. A simple system for aflatoxin testing involves extraction of the foodstuff, passing a portion through the IAC, and then washing the column. Instead of eluting and collecting the eluate in a tube, a small plastic 'tip' containing silica gel is connected to the column exit and the eluate is passed through the 'tip'. The aflatoxins are concentrated on the few milligrams of silica gel and when viewed under UV light can be seen as a fluorescent band. Visual comparison with known levels of aflatoxins, which have been similarly processed enables a semi-quantitative assessment of the original concentration in the food or feed. Alternatively, by eluting into a tube rather than passing through the tip, after addition of a few drops of reagent the concentration can be directly read using a simple fluorimeter, as exemplified by analysis of aflatoxins in tahini paste [71].

A number of commercial manufacturers of ELISA kits also provide an IAC for a preliminary clean-up prior to conducting the assay. IACs have been used to purify extracts prior to ELISA for the analysis of aflatoxin B_1 in chilli pepper [42]. A specific screening technique was developed for detecting and quantifying the antibiotic, monensin, present as a residue in chicken tissues. Monensin was extracted from chicken tissues by enzymic hydrolysis, followed

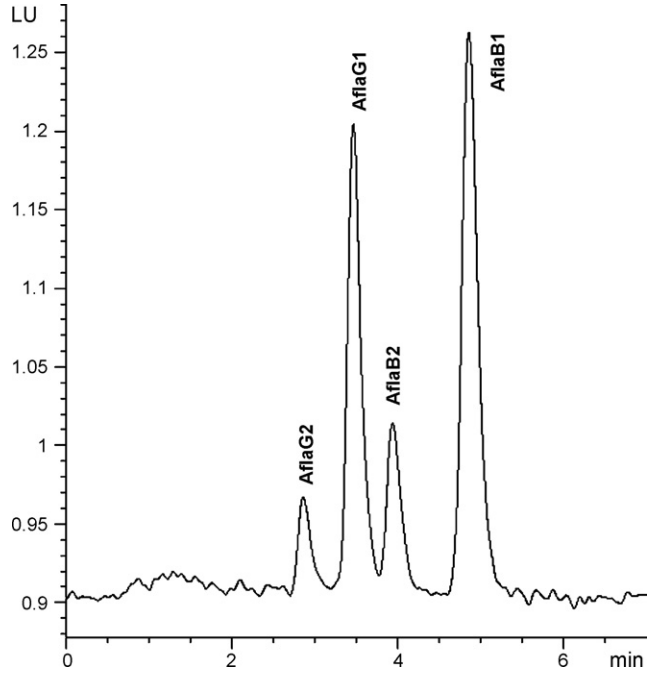

Fig. 1. HPLC chromatogram of aflatoxins in a sample of naturally contaminated hazelnuts. Sample extracted with methanol wafer and clean-up with IAC column. Fluorescence detection with post-column derivatisation with Kobra cell; aflatoxin G_2 (AflG2): 0.8 μg/kg, aflatoxin G_1 (AflG1): 3.7 μg/kg, aflatoxin B_2 (AflB2): 0.7 μg/kg, aflatoxin B_1 (AflB1): 3.1 μg/kg, total aflatoxins = 8.2 μg/kg.

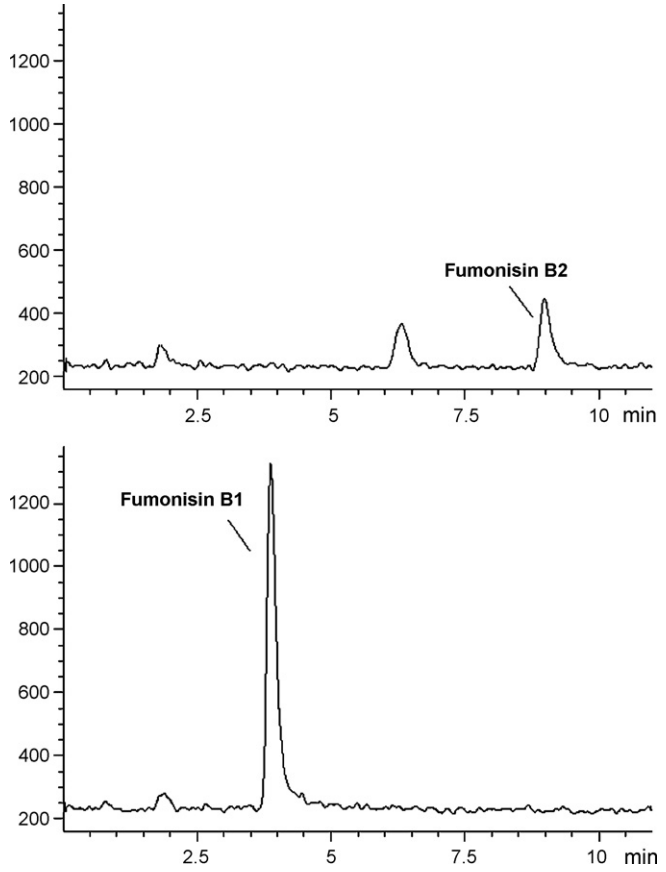

Fig. 2. Selected ion monitoring LC/MS chromatograms showing fumonisins in naturally contaminated corn with methanol/water extraction and IAC clean-up. Fumonisin B_2 (m/z = 706): 162 μg/kg; fumonisin B_1 (m/z = 722): 790 μg/kg by LC/MS.

by IAC clean-up and quantitative assessment by chemiluminescent ELISA [43]. Haloanisoles responsible for musty taint problems in wines have been determined by dilution of the wine sample with PBS (3 ml diluted to 30 ml) extraction on an IAC and then direct ELISA determination of the eluate [32].

1.4. Comparison of IAC with other clean-up techniques

The approach of using an IAC is very attractive as a clean-up method for determining a single or a limited number of analytes particularly in complex food or feed extracts containing potential interferences. Despite the self-evident attractions of IAC clean-up there have been a number of publications comparing IAC with alternative clean-up methods. In early work [72] comparing IACs with a phenyl-bonded phase SPE column for aflatoxin analysis of sorghum and maize, the latter was preferred in terms of accuracy and precision. The explanation was that acetone as an extractant, which was compatible with the SPE column, gave better recoveries than acetonitrile–water which was used with IACs. Notwithstanding the recovery issue, the extracts were as in many comparisons significantly 'cleaner' by IAC than by SPE [72]. This is illustrated in Fig. 1, which shows an HPLC chromatogram for the four aflatoxins in a sample of naturally contaminated hazelnuts using fluorescence detection [13]. This chromatogram is typical of many where IAC clean-up has been employed and the chromatograms are essentially indistinguishable from those where the corresponding standards have been analysed. When an IAC method was compared with a multi-functional column for the analysis of OTA in cereals, raisins and green coffee beans [73], the HPLC chromatograms using the multi-functional column contained multiple peaks eluting on the tail of a large co-extracted peak. In comparison the IAC chromatograms were very clean showing only the OTA peak, enabling measurement at levels of a few ng/g in all three matrices. The speed and simplicity of the multi-functional column was recognised, but its lack of specificity was a severe disadvantage [73]. However, when the original AOAC method for OTA in green coffee (chloroform extraction and back-extraction into bicarbonate solution) was compared with a newer IAC method some researchers have indicated a preference for the original method [74]. Testing was at a high contamination level and for roasted coffee and lower levels the benefits of IAC were recognised. When two different commercial IACs (OCHRAPREP® and OchraTest™) were compared for the determination of OTA by analysing the wine directly and after a liquid/liquid extraction, recovery and precision were comparable for both IACs, with and without extraction and similar quantitative results were obtained for naturally contaminated red and white wine samples [75]. It has been proposed that solid-phase microextraction (SPME) is a simpler and more cost-effective clean-up than IAC [76] for the analysis of OTA in wine, although using SPME the LOD was seven times higher than IAC and chromatograms appeared to be significantly dirtier than those achievable by IAC.

For the analysis of fumonisins the use of a strong ion exchange column (SAX) has been the standard approach to clean-up [77] until the recent introduction of fumonisin IACs from two suppliers. A comparison of SAX and IAC clearly demonstrated (for fumonisins in infant formula naturally contaminated at 1500 ng/g) that although the HPLC chromatogram showed FB2 free of interferences for both clean-up methods, FB1 eluted on a tailing front and contained a shoulder using SAX clean-up but was a single sharp peak using IAC [78]. LC/MS [14] and LC/MS/MS [79] have also been used as detection systems for fumonisin analysis in corn-based foods after IAC clean-up avoiding derivatization required for HPLC with fluorescence detection. An example of the analysis of fumonisins in naturally contaminated corn is shown in Fig. 2 using a commercial FUMONIPREP® column [14]. In this example recoveries from 90 to

101% were achieved, and using LC/MS avoided the need for derivatization.

For the analysis of DON in cereals and cereal-based foods, DON was extracted with water or acetonitrile/water and clean-up was carried out comparing two different IACs (DONtest™ and DONPREP®) with a charcoal/alumina column (MycoSep). HPLC analysis was carried out with diode array detection and fluorescence detection. For a cereal bar sample with the MycoSep clean-up the DON peak was only partially resolved from an interference peak, whereas with the IAC clean-up an essentially clean chromatogram was obtained [80]. Sol–gel IACs have been also used for clean-up of DON in food and feed being claimed as superior to commercial columns with regard to production costs, storage stability and re-usability [81]. Immuno-ultrafiltration where antibodies are not bound to a column during clean-up has also been employed for clean-up in DON analysis [12]. With this technique the clean-up was carried out in a special device containing an excess of antibodies. The extraction by centrifuge used a cut-off membrane to retain antibody–antigen complex but allowed smaller molecules to pass through, enabling the complex to be initially washed and then the analyte released by breaking the complex. HPLC chromatograms with UV detection were very similar for DON in wheat by either immuno-ultrafiltration or IAC clean-up [12].

1.5. Multiple-analyte affinity columns

In some situations it is highly desirable to be able to monitor a number of co-occurring residues or contaminants in foods, which requires an IAC with the capability of extraction and purification of multiple analytes. Where the target analytes are structurally very similar, such as aflatoxins B_1, B_2, G_1 and G_2 there is good cross-reactivity using a single antibody, although as can be seen from Table 1 recoveries for commercial IACs tend to be only around 80% for G_2 compared to 90+% for aflatoxin B_1. Recently developed IACs for the trichothecenes T2-toxin and HT-2 toxin are presumably also based on selection of a single antibody which exhibits cross-reactivity with recoveries of 85–95% indicated by the two manufacturers. Interestingly as can be seen from Table 1 one manufacturer gives much more detail about cross-reactivity, indicating that the T-2/HT-2 column also cross-reacts to diacetoxyscirpinol. This is probably also the case for other commercial IAC although not specifically indicated. An IAC for T-2/HT-2 toxins has been applied to the analysis of naturally contaminated wheat, maize and barley [82] with derivatization and fluorescence HPLC detection. The IAC had a recovery ranging from 70 to 103% for the two toxins at spiking levels from 25 to 500 ng/g. In the phycotoxin area a monoclonal antibody against saxitoxin cross-reacted against all other PSP toxins and was used in an IAC with a column capacity of 3 μg and was reusable up to 25 times when eluted with 0.1 M glycine–NaCl buffer [83].

To meet a requirement to determine co-occurring but structurally different analytes IACs have been prepared containing mixed antibodies on the same column. Columns for simultaneous determination of both aflatoxins and OTA have been the most widely tested showing recoveries from 72 to 101% for all five toxins in matrices such as maize cereals, whole maize, maize snacks, cornflour, polenta, and peanut butter [84]. These columns have also been successfully applied to the analysis of aflatoxins and OTA in ginseng and ginger [85] as well as other botanical roots such as ginger, kavakava, licorice and echinacea [86]. Using IAC clean-up for ginseng at 4–16 ng/g recoveries for aflatoxins and OTA were about 70% and were disappointingly from 55 to 60% for the other botanicals [86]. Although not really a foodstuff the same multi-analyte column was used to analyse 20 samples of bee pollen [87] producing very clean chromatograms with recoveries from 72 to 93% for both toxins at spike levels from 0.6 to 2.5 ng/g. The same authors subsequently applied methanol/water extraction to red paprika [88] to simultaneously determine aflatoxins and OTA with recoveries somewhat lower than would be expected with the single analyte columns. For the analysis of penicillins a combination of two antibodies on one column gave recoveries of amoxicillin, ampicillin, cloxacillin, dicloxacillin, penicillin G and oxacillin (in buffer solutions) in the range from 67 to 100% [15].

The approach of multi-analyte columns has been further extended to the development of an IAC containing antibodies to aflatoxins, OTA and ZON. Simultaneous determination in cereals such as rye and rice was performed by extraction into a mixture of acetonitrile–water (60 + 40, v/v), IAC clean-up, derivatization with trifluoroacetic acid, and simultaneous determination by HPLC with fluorescence detection [89]. Recoveries from rice spiked at 5 ng/g for aflatoxins and OTA and 100 ng/g for ZON were 81–96% with the exception of aflatoxin G_2 which averaged 55% recovery [89]. The development of a multi-toxin column has been extended further with a multi-antibody column being developed for simultaneous extraction of aflatoxins, OTA, FB1, FB2, DON, ZON, T-2, and HT-2 toxins [90]. Given the chemical diversity of these toxins the extraction step needed to be optimised and a double extraction was proposed initially with methanol/PBS followed by methanol giving recoveries from 79 to 104% with hydrolysed T-2 and HT-2 being determined together. This method relied on using LC/MS/MS for quantification the tandem MS providing a capability for detection of structurally diverse compounds [90].

1.6. Proficiency testing as an indicator of performance

A good source of intelligence concerning the extent of penetration of IACs into the market-place is to examine proficiency testing reports such as those from FAPAS®. These reports provide a detailed breakdown of the methods used by those taking part, who are normally laboratories undertaking routine analysis. For the determination of aflatoxins in dried figs in a 2009 report, of the 54 participants around 80% used IAC clean-up, which shows little change from a 2002 FAPAS® report on the same matrix where 90% similarly used IAC clean-up. For the determination of aflatoxins in Brazil nuts, maize and almonds 76, 87 and 86% of participants respectively used IAC in 2009 FAPAS® rounds. This compares with only 59% of 97 participants in a 1995 FAPAS® round who used IAC clean-up for the determination of aflatoxins in nut powder. The predominance of laboratories using IAC clean-up, but varying other method parameters makes it difficult to draw any conclusions concerning overall method performance of IAC methods.

1.7. Automation of IAC

Automation of IAC clean-up can take the form of a fully automated system using commercial IACs, with clean-up using the IAC being carried out off-line [91,92]. Using the ASPEC™ automated system marketed by Gilson, the loading of sample extracts, washing and elution is carried out off-line, although the automated system subsequently makes an injection of the extract onto the HPLC system, so the whole system can be run unattended. Such an automated analysis has been successfully employed for aflatoxins in a range of commodities [91], and ochratoxin A and ZON in wheat, barley, oats and rye [92,93], and phenylurea herbicides in water using an ASPEC™ system coupled to both diode array detection and LC/MS [94]. The ASPEC™ system is highly flexible and has proved itself not only for use in large-scale mycotoxin surveys [95] but also has been employed in collaborative studies demonstrating the equivalence of manual and automated methods [61,63]. An alternative approach to automation has been to use a home-made robotic system which has been demonstrated for aflatoxin M_1 in milk [96], to use the commercial Zymark robotic system for aflatoxins in a

Table 3
Summary of some key features of IAC methods in food analysis.

Analyte	Matrix	Extraction	Brand name	Analytical technique	Detection	Reference
Mycotoxins						
Aflatoxins	Paprika, peanut, pistachio	MeOH/water (8 + 2, v/v)	Rhone-Poulenc	TLC	Densitometry	[47]
OTA	Beer	4% Aqueous sodium bicarbonate + PBS	OchraTest™	HPLC	Fluorescence	[168]
OTA	Wine	PH 7.8 PBS adjusted 2 M NaOH	Ochraprep® and OchraTest™	HPLC	Fluorescence	[75]
OTA	Olive oil	MeOH/water (8 + 2, v/v)	Ochraprep® and OchraTest™	HPLC	Fluorescence	[169]
OTA	Green coffee	MeOH + 3% aqueous sodium bicarbonate	OchraTest™	TLC	Densitometry	[44]
OTA	Milk	No extraction necessary	Ochraprep®	HPLC	Fluorescence	[21,170]
FB1	Corn	MeOH/water (8 + 2, v/v)	FumoniTest™	TLC	Densitometry	[45]
FB1, FB2	Cornflakes	AcCN + MeOH + water (25 + 25 + 50)	FumoniTest™	HPLC	Fluorescence	[67]
FB1, FB2	Corn products	MeOH + water (8 + 2, v/v)	FumoniTest™	LC–MS/MS	SIM	[79]
Afla, OTA, ZON	Grains	AcCN + water (60 + 40, v/v)	AflaOchraZea™	HPLC	Fluorescence	[89]
11 Mycotoxins	Corn	PBS + methanol	AOFZDT2	LC–MS/MS	SIM	[90]
Pesticides						
Thifluzamide	Peanut	AcCN + water (50 + 50)	Silica-based IAC	HPLC	UV–vis	[128]
Bioallethrin	Fruit and vegetable	Acetone	Sol–gel IAC	GC/MS	SIM	[40]
Imazalil	Citrus fruit	MeOH + PBS	Home-made	HPLC	UV–vis	[129]
s-Triazines	Water, orange juice	Direct after filtration	Home-made	GC	FID, NPD	[130]
Phenylurea herbicides	Potatoes, carrot	Methanol extraction—centrifuged dilute PBS	Home-made	HPLC	UV	[131]
Triazine herbicides	Apple, carrot, celery, peas corn, potato	Methanol extraction—centrifuged dilute PBS	Home-made	HPLC	UV	[132]
Vitamins						
Vitamin B_{12}	Liver, chicken, egg, beef, fish, milk	Acetate + pepsin	easi-extract® B12	HPLC	Fluorescence	[143]
Vitamin B_{12}	Milk-based infant formula, cereals, pre-mixes	Acetate buffer + NaCN + amylase incubated 42° for 30 min	easi-extract® B12	HPLC	UV–vis	[144]
Vitamin B_{12}	Cereals, infant formula, pre-mixes	Acetate buffer + NaCN + amylase incubated 42° for 30 min	easi-extract® B12	HPLC	UV–vis	[145]
Veterinary drugs						
Tetracyclines	Milk	Diluted with PBS	Home-made	Fluorescence spectrophotometer	Fluorescence	[38]
Hormones		Methanol + water	Home-made	Capillary electrophoresis	Electrophoresis	[34]
DES, dienestrol, HES	Biological samples	Phosphate buffer	Home-made	GC/MS	NICI	[162]
Methamphetamine	Urine	No information	Home-made	LC/MS	Scan	[163]
Ractopamine	Muscle, kidney, liver	Diluted with PBS	Home-made	HPLC	Fluorescence	[16]
Fluoroquinolones	Liver	0.1 M NaOH + 1 M phosphoric and diluted with 5.5 ml of PBS containing 10% methanol	Home-made	HPLC	Fluorescence	[105]
Fluoroquinolones	Milk	Centrifuged to defat—direct analysis	Home-made	On-line HPLC	Fluorescence	[104]
Chloramphenicol	Muscle	Acetonitrile + 4% aqueous NaCl (1:1)	Not given	GC	Electron-capture	[17]
Chloramphenicol	Milk, muscle	15% TCA	Home-made	HPLC	Variable-wavelength detector	[70]
Penicillins	Buffer	Diluted with PBS	Home-made	ELISA		[15]
Anabolic steroids	Urine faeces	Acetate buffer	Home-made	GC/MS	Scan	[164]
Streptomycin	Honey	Sodium acetate buffer		HPLC	EIA	[7]
Sulfathiazole	Honey	Sodium acetate buffer		HPLC	EIA	[7]

wide range of food matrices [97] or a Millilab work station for the automated analysis of aflatoxicol in human urine [98].

The alternative approach to automation involves establishing a system where the IAC is used on-line and is directly coupled through a system of switching values to the HPLC or LC/MS. This system necessitates that the same column is used repeatedly imposing conditions on elution of the analyte. Urano et al. [69] manufactured IACs using four types of epoxy-activated affinity supports for potential use in an on-line system for aflatoxin analysis. They found that Durasphere-Epoxy and Affi-Prep 10 were unable to withstand the 500 psi pressure of the system, but the two other supports were suitable. Interestingly with HEMA-Afc-Bio which is a macroporous hydrophilic copolymer of 2-hydroxyethyl methacrylate and ethylene dimethacrylate there was non-specific binding of the aflatoxins to the column, and in fact the authors advocated use of the columns on-line for clean-up without any coupling of antibodies [69]. Whilst in this specific situation this might offer a viable analytical system more generally non-specific binding must be avoided as it can reduce analyte specificity of the IAC. On-line IAC coupled with HPLC with column switching and electrospray ionization mass spectrometry, has been used for automated determination of fumonisins. The analytes were captured on 100 μl home-made IAC, eluted to a reverse-phase pre-column, switched to a reverse phase analytical column and finally analysed by ESI LC/MS [99]. Other on-line columns switching systems have been developed for chloramphenicol in milk and muscle tissue [70], natural hormones in calf urine [100], calf tissue [101], clenbuterol in urine [102], trenbolones in calf and cattle urine [101], and fluoroquinolones in serum [103], milk [104] and chicken liver [105].

2. Application areas for IAC clean-up

As is evident from the above discussion, IACs are currently being used for clean-up for a wide range of different analytes and for a range of different matrices. Some examples illustrating this diversity of matrices and analytes are shown in Table 3.

Liquid matrices such as milk, fruit juice, wine or beer are frequently de-fatted and/or filtered and perhaps diluted with PBS before being passed directly through the IAC. In these cases the IAC is simultaneously performing an extraction and clean-up function. With solid matrices, solvent extraction is required and this is frequently carried out in a blender or by shaking. The common feature of the extraction solvent mixtures used in Table 3 is that they need to be aqueous-based to be compatible with the IAC and thus methanol/water and acetonitrile/water combinations are frequently used, with differing amounts of water. It is necessary to have a suitable pH in some cases, e.g. ochratoxin A, although some debate as to whether alkaline conditions can adversely affect recovery [106]. It can also be seen from Table 3 that IACs have been successfully used by laboratories, where only TLC is available [44,45,47], and the improved clean-up means that the lack of separation on the TLC plate becomes less disadvantageous. At the other end of the spectrum IAC clean-up has been used with sophisticated chromatographic end-determinations being used in LC/MS and LC/MS/MS [107,108].

2.1. Mycotoxins

Some of the earliest publications using home-made IACs were in the analysis of aflatoxins adducts in human blood plasma samples [31] looking at human exposure to aflatoxins through the diet. This area of application has continued to be exploited in particular with ochratoxin A being monitored in human blood plasma [3,109–111], and human milk [111]. Antibodies against mycotoxins began to be exploited in food analysis initially in the form of ELISA systems then in the form of IACs utilised for various screening formats and coupled with conventional HPLC. Applications to mycotoxins has been exploited to the greatest extent in terms of uptake by end-users of commercial single-use IACs. From Table 1 it can be seen that there are four major manufacturers of disposable IACs all providing broadly similar products covering the aflatoxins, OTA, DON, ZON, fumonisins and T-2 + HT-2 toxins. For application to aflatoxin analysis, monoclonal antibodies have been raised against aflatoxin B_1 but cross-reactivity is observed against B_2, G_1 and G_2. One manufacturer indicates that the column has cross-reactivity to aflatoxin M_1 and all other similar metabolites that can occur, and this is probably the case with columns from other manufacturers although not specifically stated. A similar situation applies to the OTA columns where cross-reactivity to ochratoxins B and C is to be expected, and for the IAC for DON where cross-reactivity to 3-AcDON, 15-Ac-DON is reported by one manufacturer and is probably also the case for the other products. In addition to IACs marketed for the determination of individual mycotoxins such as OTA, it has been recognised that in some circumstances there is a need to determine co-occurring toxins. Thus two companies market IACs, which are applicable for isolating both aflatoxins and OTA simultaneously from food extracts. A fuller discussion of this area is covered in the section above on multi-analyte columns.

For regulatory purposes where analytical methods are required for food control or as referee methods for disputes, there has been a move away from prescribed 'official methods' to a criteria-based approach. This more flexible approach is based upon a published minimum set of performance criteria that methods must meet to be accepted for use for official purposes. Thus the European Commission has published performance criteria for veterinary drugs [11] and for mycotoxins methods [112] and CEN has published its own criteria, which are used to make a preliminary selection of methods [113]. To establish these method performance parameters it is necessary to conduct a full inter-laboratory method validation (collaborative study). The method performance of mycotoxins methods using IAC clean-up are summarised in Table 4.

The minimum performance criteria that need to be met include precision data such as intra-laboratory relative standard deviation (% RSD_r), inter-laboratory relative standard deviation (% RSD_R) and recovery. The detection limits of the methods to be used are not stipulated as the precision values are given at the concentrations of interest. Thus, for example for ochratoxin A with a regulatory limit in the range 1–10 $\mu g\,kg^{-1}$ the method must have a recovery from 70 to 110%, and an RSD_r of <20% and an RSD_R of <30%. Similar performance criteria are stipulated for aflatoxin B_1 and total aflatoxins, aflatoxins M_1, DON, ZON, fumonisins FB_1 and FB_2 (measured as total) and T-2 and HT-2 toxins (measured separately). From Table 4 it can be seen that methods have been validated using IAC clean-up for total aflatoxins, aflatoxin M_1, OTA, DON, ZON and fumonisins, but no IAC method has yet been validated for T-2 and HT-2 toxins where the IACs are relatively new. In most cases the validated methods meet European Commission method requirements [112], although in a few cases whilst the recoveries are acceptable, the precision characteristics fall just outside the minimum stipulated. It should be noted that whilst in most cases IAC methods can be readily transferred from one matrix to another, there have been instances of non-specific binding causing interferences and as a minimum single-laboratory validation is required for each new matrix.

Although in Table 4 it can be seen that methods for the majority of the important combinations of matrices and mycotoxin analytes have been validated by inter-laboratory collaborative study, a far wider range of matrices have been analysed using IAC-based methods. In Table 5 we have compiled a listing of the mycotoxins and matrices that have been the subject of food surveillance programmes indicating the commercial brand name of the IAC, which

Table 4
Method performance characteristics for inter-laboratory validated IAC methods for mycotoxins.

Analyte	Matrix	Level (ng/g)	RSDr (%)	RSD_R (%)	Recovery (%)	Brand name or manufacturer	Reference
Aflatoxin B1 Total aflatoxins	Peanut, pistachio, fig, paprika	0.9–3.6 0.8–7.9	3.1–20 4.6–23.3	9.1–32.2 14.1–34.2	82–109 71–92	Rhone-Poulenc	[61]
Aflatoxin B1 Total aflatoxins	Hazelnut	1.36–3.82 4.17–12.08	2.2–3.2 2.3–3.4	7.3–7.8 6.1–7.0	85.9–88.8 86.9–89.2	R-Biopharm Rhone Ltd	[13]
Aflatoxin B1	Baby food	<0.02, 0.07, 0.09, 0.17	3.5–14	9–23	92–101	Performance specified[a]	[62]
Aflatoxin B1	Corn	2.20–17.60	11.03–28.71	24.42–36.83	82–84	R-Biopharm Rhone Ltd	[171]
Aflatoxin B1	Senna pods Ginger root Devil's claw	0.8–14.9 0.9–2.1 0.8–0.9	5.1–22.0 4.2–10.0 5.5–7.8	18.1–35.2 5.8–30.2 7.6–10.5	78 91–92 83–92	EASI-EXTRACT® EASI-EXTRACT® EASI-EXTRACT®	[172]
Aflatoxin B1 Aflatoxin M1	Cattle feed Milk	0.87–4.19	5.9–8.7	17.5–19.6	74–157	VICAM® and R-Biopharm Rhone Ltd AFLAPREP® M	[173] [46]
Aflatoxin M1	Milk	0.02–0.103	8–18	21–31	74–107	VICAM®	[63]
OTA	Barley	1.3–4.5	15–33	12–17	93	R-Biopharm Rhone Ltd	[64]
OTA	Green coffee	<0.12–13.5	7.4–20.9	16.3–29.2	92.8	OchraTest™	[174]
OTA	Roasted coffee	1.2–5.4	2–22	14–26	65–97	VICAM® and R-Biopharm Rhone Ltd	[18]
OTA	Baby food	0.05–0.22	18–36	29–63	108	VICAM® and R-Biopharm Rhone Ltd	[175]
OTA	White wine Red wine Beer	0.105–1.76 0.19–1.69 0.07–1.4	6.6–10.8 6.5–10.9 4.7–16.5	13.1–15.9 11.9–13.6 15.2–26.1	88.2–105 84.3–93.1 87–95	OchraTest™	[26]
OTA	cocoa beans	0.4–12.0	1–4	n/a	89	OchraTest™	[176]
OTA	Cocoa powder	0.18–0.95	15–31	29–40	80	OchraPrep®	[177]
OTA	Currants Sultanas Raisins Mixed fruits Figs	4.5 11.4 7.5 1.1 2.5	5.7 5.6 4.9 8.6 8.7	28 14 14 14 18	73.6 74.5 72.2 69.2 72.9	Performance specified[a]	[178]
DON	Cereals	85–1768	3.1–14.1	11.5–26.3	78–87	Performance specified[a]	[179]
ZON	Baby food Animal feed	9.1–44 <20–307	2.8–9.0 5.7–9.5	8.3–13.3 15.5–21.4	78–119 51–122	Performance specified[a]	[180]
ZON	Baby food Barley Corn Polenta Wheat	10.9 143.0 87.2 66.5 226.6	35.8 6.9 14.2 8.9 8.3	38.2 17.9 20.6 16.4 17.0	100 92 91 91 95	EASI-EXTRACT® EASI-EXTRACT® EASI-EXTRACT® EASI-EXTRACT® EASI-EXTRACT®	[181]
ZON	Dairy feed Distillers Grain Wheat	134 250 189	9.8 5.8 9.7	16.6 13.4 12.5	– – 107.3	Performance specified[a]	[182]
FB1 and FB2	Corn	200–500	2.8–7.1	n/a	90–101	FUMONIPREP®	[14]
FB1 and FB2	Corn Cornflakes	650–1410 130–1050	18.5–26.8 9.2–21.7	22.1–28.2 26.1–34.8	72–76 97–110	FumoniTest™	[67]

[a] Performance specified—specific commercial columns were not indicated but the minimum performance that must be achieved in terms of recovery and column capacity was specified in the validation study.

Table 5
Applications of IAC methods in mycotoxin surveys.

Analyte	Matrix	Recovery	Brand name	No. samples in survey	Country	Years of survey	Reference
Aflatoxins	Dried figs	92%	AFLAPREP®	2,643	Turkey	2007	[115]
	Pistachio	80%		80			
	Hazelnut	98%		28			
	Peanuts	85%		10			
	Paprika	85%		23			
Aflatoxins	Paprika	64–76%	AflaOchra®	21	Spain	2004–2007	[88]
Aflatoxins	Ginseng roots	83–92%	AflaTest® P	23	USA	2002–2003	[183]
Aflatoxins	Dried figs	88–94%	AFLAPREP®	4,917	Turkey	2007	[184]
Aflatoxins	Dried figs	>85%	AFLAPREP®	10,396	Turkey	2003–20066	[114]
Aflatoxins	Dried fruits	68–96%	AflaTest®	62	Brazil	2002–2003	[185]
Aflatoxins	Beer	87–109%	AflaTest®	304	Canada	1998–2002	[22]
Aflatoxins	Breakfast infant cereals	69–90%	AflaTest®	349	Canada	2002–2005	[186]
Aflatoxins	Ginseng, ginger	60–70%	AflaTest®P, AflaOchraTest®	35	USA	2006–2007	[85,86,187]
Aflatoxins	Spices	83–87%	EASI-EXTRACT®	75	Turkey		[188]
B1	Tiger-nut drinks	88%	EASI-EXTRACT®	22	Spain, Belgium	2004	[189]
M1	Retail milk	91–114%	AFLAPREP®M	241	Colombia	2004–2005	[170]
M1	Milk and cheese	67–98%	AFLAPREP®M	69	Libya	2002	[190]
M1	Domestic milk	91%	AflaTest®P	208	Japan	2001–2002	[191]
B1	Pig liver	76–84%	AflaTest®P	50	Italy	2004	[36]
M1		74–77%	AflaTest®M	50			
Aflatoxicol	Milk	90%	EASI-	580	Mexico	1996–1998	[192]
B1		98%	EXTRACT®				
OTA	Spices	78–83%	OchraTest™	91	Hungary	2004	[193]
B1		81%	AflaTest®				
OTA	Rice	83%	OchraTest™	20	Morocco	2005	[194]
OTA	Dried fruits	72–94%	OchraTest™	100			
OTA	Sultanas	–	OchraTest™	1,885	Turkey	1999–2003	[195]
OTA	Sultanas	87–93%	OchraTest™	264	Turkey	1998–2004	[196]
OTA	Dried vine fruit	77–92%	OchraTest™	151	Canada	1998–2000	[197]
OTA	Wine, grape juice	88%	OchraTest™	251	Canada	1999–2002	[198]
OTA	Wine, grape juice	82–91%	OchraTest™	64	Brazil	–	[199]
OTA	Beer	97%	OchraTest™	82	Belgium	1998–2001	[168]
OTA	Beer	96%	OchraTest™	61	Italy	–	[200]
OTA	Sweet wine	87–91%	OCHRAPREP® and OchraTest™	290	Spain	2001–2005	[201]
OTA	Wine	88–93%	OchraTest™	56	Italy	–	[25]
OTA	Wine	93–100%	OchraTest™	30	S. Africa	200–2001	[27]
OTA	Cocoa beans	78–89%	OchraTest™	22	–	2003–2004	[202]
OTA	Cocoa and chocolate	80–84%	OCHRAPREP®	181	Different origin	–	[203]
OTA	Milk	89%	OCHRAPREP®	12	Spain	–	[21]
OTA	Liquorice	91%	RIDA®-OTA	–	Spain	–	[204]
OTA	Liquorice	91%	RIDA®-OTA	30	Spain	–	[205]
OTA	Virgin olive oils	108%	OCHRAPREP®	50	Greece	1998–2001	[169]
OTA	Ham	84%	OchraTest™	42	–	–	[37]
OTA	Grapes	94%	OCHRAPREP®	50	Greece	2005	[206]
Aflatoxins	Ginseng,	60–101%	AflaOchraTest®	36	USA	2006	[85]
OTA	ginger	58–89%					
Aflatoxins	Ginseng and botanical roots	59–73%	AflachroTest®	5	USA	2005–2006	[86]
OTA		52–67%					
M1	Duplicate diets	68–74%	AflaOchra HPLC™	123	Netherlands	1994	[207]
B1		95–97%					
OTA		75–83%					
OTA	Beer	91%	OchraTest™	80	Belgium	2003–2004	[23]
DON		93%	DONPREP®	80			
FB1, FB2	Corn, corn products	79–99%	FumoniTest™	67	Portugal	2005	[208]
FB1	Corn	85%	FumoniTest™	214	Brazil	1998	[45]
FB1	Corn	–	FumoniTest™	259	China	2001–2002	[209]

has been used, the size of the survey, country conducted and year undertaken.

In fact it is relatively uncommon to publish survey results so this is really only a very small insight into the extent of mycotoxin monitoring that is undertaken world-wide. The surveys undertaken in Turkey [114,115] indicate that several thousand aflatoxin determinations are undertaken each year and this is only for one food product and for analyses undertaken by one country for export. The EU Rapid Alert System for Food and Food (RASFF) gives some indications of the extent of monitoring of imports into the EU for aflatoxins [116], yet apart from notification of consignments above EU limits there is no compilation of this data. In addition to applying validated IAC methods in surveys Table 5 indicates that IAC clean-up has been applied to other matrices where there has been no formal validation such as for aflatoxins in breakfast cereals, beer, tiger-nut drinks, ginger, ginseng spices and pig liver, aflatoxin M_1 in cheese, ochratoxin A in rice, cocoa beans, chocolate, liquorice, olive oil, cured meat (ham) and duplicate diets. This table illustrates

the diversity of matrix types where IAC methods have been applied and the extent to which they have received global acceptance as the preferred approach to clean-up in this area irrespective of the method chosen for end-determination whether low-technology TLC or high-technology LC/MS/MS instrumentation.

2.2. Veterinary drugs

Despite the potential benefits of using IAC for clean-up of animal and seafood tissue samples for routine veterinary residue testing, and notwithstanding the availability of commercial columns (see Table 1) the uptake by users compared with mycotoxins has been surprising low. This may in part be due to the fact that, with the exception of drugs such as chloramphenicol, most veterinary residue testing is nowadays carried out for classes of compounds such as hormones, antibiotics etc rather than for single analytes. LC/MS/MS methods are also now being developed to screen and cover classes of compounds and therefore generic rather than specific clean-up approaches are being exploited. An exhaustive comparison has been carried out of fifteen IAC types from five different manufacturers [35] comparing their ability to bind different drugs in fortified bovine urine samples.

A number of papers have reported systems using on-line coupling of IAC with column switching [70,104,117–119] for veterinary drugs analysis. For example for the analysis of chloramphenicol in milk and muscle tissue, elution from the column was with 20 ml of glycine–NaCl buffer (0.2 M glycine and 0.5 M NaCl at pH 2.8) with an analysis time of 60 min for milk and 50 min for muscle tissue extract. The system was used for 3 months in which time 150 samples were analysed with recoveries from 64 to 70% [70]. Chloramphenicol in muscle, liver, kidney and urine of pigs has also been analysed using IAC followed by GC/MS determination. Elution was with 15 ml ethanol or 40 ml of glycine–NaCl giving a recovery of 95% [17]. A rapid and sensitive gas chromatography (GC) method was developed for chloramphenicol in chicken tissues. Extracted samples were passed through a home-made IAC (chloramphenicol capacity of 3265 ng/ml gel) prepared by coupling anti-chloramphenicol monoclonal antibody with cyanogen bromide-activated Sepharose 4B [120].

β-19-Nortestosterone and its metabolites were determined in urine, bile and tissue samples with IAC clean-up and analysis by HPLC with UV detection and confirmation by GC/MS. The IAC was on-line and the analytes transferred to a C-18 pre-column prior to the determinative step [117]. Oestrogen steroids have also been determined in spiked urine samples using automated column-switching method with a total analysis time of 45 min and capacity to analyse 30 samples per day [118]. Other use of IACs have included natural hormones in calf urine [100], and tissues [101], clenbuterol in urine [102] and trenbolones in calf and cattle urine [121]. A GC/MS method with IAC clean-up was developed for the determination of derivatised zeranol and related compounds, taleranol, zearalanone, and α-zearalenol in bovine muscle, using methanol extraction and IAC columns containing monoclonal antibodies raised against zeranol coupled to CNBr-activated Sepharose 4B [122]

On-line coupling of IAC with automated with column switching has been used for the determination of four fluoroquinolone antibiotics in chicken liver [105]. Monoclonal antibodies were raised to sarafloxacin, which exhibited cross-reactivity to enrofloxacin, ciprofloxacin and difloxacin with recoveries ranging from 85.9 to 93.5% from fortified liver samples. The fluoroquinolones were found to elute from the IAC on the basis of their relative affinities for the antibodies [105]. Seven fluoroquinolones were captured on monoclonal antibody raised to sarafloxacin IAC, then eluted onto a hydrophobic interaction chromatography column (HIC) by switching on- and off-line and finally eluted and fluorescence detected [123]. The separation and quantification of two fluoroquinolones in serum was carried out using two columns in series, one containing gel but no antibody, to remove any non-specific binding interferences, followed by the HPIAC where the two fluoroquinolones are bound, then eluted separately and determined by fluorescence detection with HPLC [103,119]. Four fluoroquinolones were determined in milk [104] with IAC on-line with HPLC. No significant interferences were observed from the sample matrix, indicating good selectivity with the IAC. Recoveries from fortified raw milk samples (5–50 ppb of each fluoroquinolone) ranged from 72 to 90%, with standard deviations of <8% [104]. More recently a method was reported for the analysis of 10 quinolones from chicken muscle [33]. This off-line HPLC method used a norfloxacin monoclonal antibody in a home-made reusable IAC with cross-reactivity to the other fluoroquinolones [33].

A column was prepared taking two different antibodies both raised against ampicillin but with different characteristics in terms of cross-reactivity. The column gave recoveries from 67 to 100% for ampicillin, amoxicillin, cloxacillin, dicloxacillin, penicillin G and oxacillin when tested at a concentration of 100 μg/ml [15]. The β-agonist ractopamine and its glucuronides was determined in cattle urine, muscle, liver and kidney samples with HPLC and fluorescence detection. The column could be used up to 20 times with 50 mM glycine buffer elution [16].

An IAC method was developed for nine sulfonamides (sulfamethazine, sulfadimethoxine, sulfamerazine, sulfathiazole, sulfamethoxazole, suifamethizole, sulfadiazine, sulfamonomethoxine, and sulfapyridine) from chicken tissue (muscle and liver) samples. Two monoclonal antibodies (antisulfamethazine and antisulfamethoxazole) were simultaneously covalently coupled to CNBr-activated Sepharose 4B for the preparation of a re-usable IAC. After extraction with methanol–water and IAC clean-up, the sulfonamides were determined by reversed-phase liquid chromatography and UV detection at 270 nm. Recoveries at fortification levels of 10–50 ng/g ranged from 74.1 to 108.9% with relative standard deviations from 1.9 to 11.5% with limits of detection of 2 ng/g [124]. In swine meat tissue, after extraction with methanol–water (8 + 2), sulfamonomethoxine, sulfadimethoxine, and sulfaquinoxaline were cleaned-up by IAC, and determined by reversed-phase liquid chromatography with UV detection at 270 nm [125].

A specific screening technique was developed for detecting and quantifying the antibiotic, monensin, present at residue levels in chicken tissues. Monensin was extracted from chicken tissues by enzymic hydrolysis, followed by IAC clean-up and quantitative assessment by chemiluminescent ELISA. [43] A simple and sensitive method was reported for determining ivermectin in sheep serum. The sample was cleaned up by IAC and determined by HPLC with detection at 245 nm. Recoveries ranged from 90.3 to 98.8% [126].

2.3. Pesticides

From a food safety perspective pesticide residue monitoring is generally based on targeted analysis, but requires screening to be undertaken for a large number of possible residues. Thus, multi-residue methods have evolved with generic clean-up such as the QuEChers approach with GC/MS or LC/MS/MS as the end-determination [127]. Such methods have a capability in principle of screening for several hundred residues covering different structural classes of compound. Thus, although the market for pesticide residue testing is huge, and a generic IAC for a class of pesticides would be useful, the need for very specific clean-up for a single residue is somewhat limited. However, in the case of pesticide registration a significant dataset is often required covering a range of matrices such as soil, water and the crops for the intended use but only for a single analyte (plus metabolites) and thus the development of an IAC may be justified. Such an approach was adopted to determine thifluzamide in peanuts [128] where the IAC clean-up

replaced five successive SPE applications and significantly reduced solvent usage, producing HPLC chromatograms (UV detection) free of background interference.

An IAC clean-up has been applied to imazalil [129] in lemon, orange and grapefruit, replacing multiple clean-up steps and showing substantially cleaner HPLC chromatograms using UV detection. A particularly elegant approach to using IAC clean-up has been online coupling of the IAC to a GC system (FID/NPD) using switching values to achieve a fully automated system [130]. This approach has been employed to determine triazines in liquids including orange juice, which is a complex matrix, clearly demonstrating the potential of IACs in an automated system. A polyclonal antibody raised against the urea herbicide isoproturon showed substantial cross-reactivity to six other urea herbicides [131]. This home-made IAC was used as a single-step clean-up for in potatoes and carrots, but an additional clean-up step using a strong anion-exchange solid-phase extraction column was required when analysing grapes, onion, celery, corn and strawberries [131]. This group extended their work to the determination of seven trazine herbicides [132], in matrices such as apple, celery, carrot, corn, potato and peas with detection limits from 2 to 10 ng/g and with recoveries ranging from 65 to 94% using HPLC with UV detection. In general the number of applications of IACs in pesticide residue analysis is rather limited and other examples use the IAC as a preliminary clean-up prior to ELISA determination for atracine herbicides [133], and the pyrethroid bioallethrin [40].

2.4. Phycotoxins (seafood toxins)

Phycotoxins are widely occurring toxins in shellfish and a number of different classes of compound are known which are responsible for paralytic shellfish poisoning (PSP), diarrhetic shellfish poisoning (DSP) and amnesic shellfish poisoning (ASP). As groups of compounds rather than individual toxins are responsible for poisoning and as not all are well characterised, a mouse bioassay is still used as a screening test (and official method), although being progressively replaced by instrumental methods such as LC/MS/MS. ELISA methods have proved to be attractive for monitoring these toxins [8,134–136], but even though 10 years ago an IAC was reported for analysis of PSP toxins [83] there has been no uptake commercially and applications in the area are limited. An IAC has also been reported for the clean-up of DSP toxins (okadaic acid and dinophysistoxins) using home-made columns but commercially available anti-okadaic monoclonal antibody [137]. Others have reported a similar approach to analysis of okadaic acid in shellfish and algae using the IAC for clean-up after derivatization prior to HPLC analysis with fluorescence detection, or prior to LC/MS [138]. Tetrodotoxin is a powerful neurotoxin found in exotic fish like puffer fish which can result in human poisoning incidents. An IAC for tetrodotoxin has proved effective in analysing urine samples [5] from patients, and as a clean-up tool for isolating microcystins from tap water [139].

2.5. Process and environmental contaminants

Cooking particularly on a barbeque can produce a range of heterocyclic aromatic amines such as 2-amino-3-methylimidazo[4,5-f]quinoxaline (IQ) and 2-amino-3,8-dimethylimidazo[4,5-f]quinoxaline (MeIQx). Studies have been carried out monitoring these mutagens in beef broth and also in the urine of exposed humans using IACs which provided an effective sample clean-up prior to HPLC analysis monitoring by UV and radioactivity [6,140,141]. These IACs have been very much developed as research tools and although important these mutagens are important there has been little demand for routine testing in terms of food surveillance.

In contrast dioxins and furans (PCDDs and PCDFs) are environmental contaminants of considerable importance in food safety terms, accumulating in fatty foods and being of significance in animal and seafood products. The well-established conventional analysis involves extraction, extensive elaborate clean-up and GC separation with determination by high resolution MS using selected ion monitoring (10,000 resolution). Although there are individually 75 PCDDs, 135 PCDFs and 209 PCB cogeners, only those few containing the 2,3,7,8-chlorine substitution demonstrate toxicity and are monitored to estimate total equivalence for toxicity. In principle an antibody with generic specificity which could recognise only 2,3,7,8-substitution in the dibenzodioxin or dibenzofuran structures could have enormous benefit in sample clean-up, replacing a lengthy alumina–silica gel–carbon column procedure. In practice although antibodies have been successfully raised they have tended to be rather too specific to 2,3,7,8-TCDD itself which has limited the usefulness of both IAC for clean-up or the development of ELISA for screening.

An IAC generated by cross-linking a polyclonal chicken antibody with CNBr-activated Sepharose 4B was used for isolation of 1,3,7,8-tetrachlorodibenzo-p-dioxin (1,3,7,8-TCDD) from bovine milk. However, direct application to a milk sample, even if diluted 1:50 with 0.05% Triton X-100, interfered with the binding of 1,3,7,8-TCDD to the column. However, pre-IAC clean-up using liquid–liquid extraction followed by solid-phase extraction produced excellent retention of 1,3,7,8-TCDD by the column. The TCDD was eluted by increasing the Triton X-100 concentration to 0.5%. Although analysis of milk samples requires more stringent clean up than serum samples, the immunoaffinity chromatography column procedure did provide specific clean-up for these samples [142].

2.6. Vitamins

The vitamins as a class of compounds have some similarities to mycotoxins in that single analytes (or small groups of analytes) are monitored and there is also a sufficiently large market in testing by the food industry to justify commercial exploitation. Additionally traditional methods such as microbiological assays can be time-consuming and lack specificity, meaning that there can be substantial technical benefit in introducing a robust clean-up stage into the assay, followed by instrumental determination. In Table 1 it can be seen that there are now two commercially available IACs for vitamins B_9 and B_{12}. A method was first reported [143] to determine free and total vitamin B_{12} in various foods such as pig liver, eggs, beef, salmon mackerel and milk by extraction (hydrolysis for total vitamin B_{12}) purification using IAC and pre-column derivatisation prior to HPLC with fluorescence detection. A very similar IAC method [144] similarly enables determination of vitamin B_{12} as total and/or free vitamin B_{12} in food products and premixes by extraction (and enzyme treatment to determine total B_{12}) followed by IAC clean-up prior to HPLC determination in this case with UV detection. Fig. 3 shows a typical HPLC chromatogram for the analysis of vitamin B_{12} in Fig. 3. This method has been validated albeit by single-laboratory rather than inter-laboratory study [145] with repeatability of 2.1% and reproducibility of 4.3%. In a recent proficiency testing round of a total of 38 participants only one used an IAC method, but did achieve a satisfactory *z*-score indicating equivalence in performance to conventional methods [146].

For the analysis of vitamin B_9 (folic acid) using an IAC clean-up, the sample is extracted, diluted with buffer, centrifuged and the supernatant filtered before being passed slowly through the EASI-EXTRACT® Folic acid column where binding takes place between the antibody and the vitamin. The column is then washed to remove any extraneous unbound pigments and folic acid is released from the column using 1 ml of elution solution (30% acetonitrile, 70% water containing 0.2% trifloroacetic acid) and collected, and injected onto

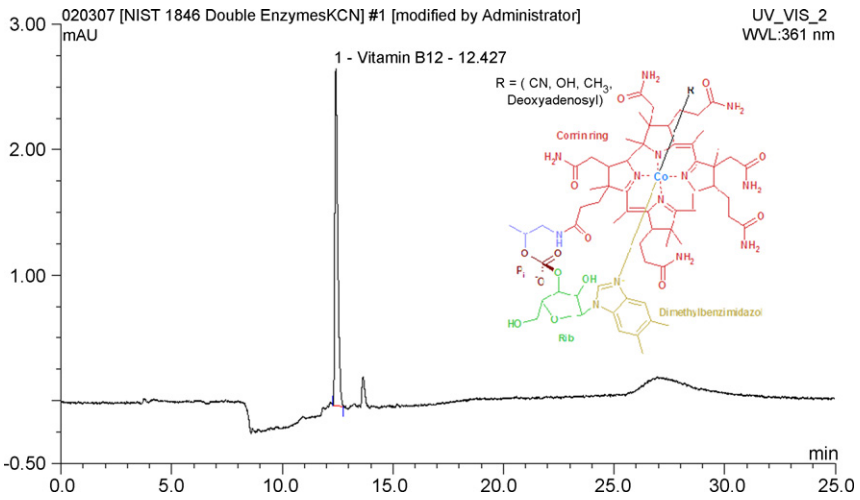

Fig. 3. HPLC chromatogram illustrating the analysis of vitamin B_{12} in NIST infant formula certified reference material (NIST CRM 1846). The vitamin B_{12} was released from the matrix by enzyme treatment and KCN used to convert hydroxy, methyl and adenosyl forms to the cyanocobalamin form. The filtered sample was cleaned-up on and Easi-Extract® vitamin B_{12} column and analysed by HPLC with UV detection at 361 nm. The CRM was found to contain 3.84 μg/100 g of vitamin B_{12}.

the HPLC. Methods have been developed for infant formula powder, ready-to-use infant formula milk, flour, dietetic milk powder, soya milk, cereals, vitamin premix powders and certified reference materials [147].

An IAC produced in the authors own laboratory has been demonstrated in the form of a biosensing system to determine vitamin B_7 (biotin). The IAC is initially used to bind the biotin from the sample, free antibody sites on the column are then bound by a biotin-tagged fluorescent dye and finally the dye is released and measured by fluorimetry to indirectly quantify the biotin level in the original sample [148].

3. Future prospects

For the future, alternatives to the use of antibodies to manufacture high specificity clean-up columns are being explored and molecular imprinted polymers (MIPs) and aptamers offer exciting possibilities.

Molecular imprinting involves creating a synthetic 'receptor' to the analyte in question. This is achieved by forming a complex between a functional monomer and the template (analyte) molecule. A cross-linking monomer is used to impart a degree of rigidity to the created receptor. There have been hopes that MIPs might begin to replace IACs as these columns are more robust and certainly reusable, thereby reducing consumable costs. MIPs are nevertheless still in their infancy in terms of development and exploitation although highly promising publications have shown their application to the analysis of stilbene-type synthetic hormones (hexestrol, diethylstilbestrol and dienestrol) [149,150], and mycotoxins such as moniliformin [151], fumonisins [152], deoxynivalenol and zearalenone [153].

Aptamers are single stranded DNA or RNA ligands which can be selected for different targets starting from a huge number of molecules containing randomly created sequences [154]. These nucleic acids are able to fold into a well-defined three-dimensional structures, which can show high affinity and specificity for target molecules. From this very large number of molecules, the ones of interest are selected by a process called 'Systematic Evolution of Ligands by Exponential Enrichment (SELEX)'. SELEX involves iterative cycles of selection and amplification starting from as many as 10^{15} different structures, and gradually by selective amplification creating a new mixture enriched with nucleic acid molecules having a high affinity for the target. After several cycles of this selection process the pool is enriched with high affinity molecules at the expense of the low affinity ones. Once this process is complete, the aptamer sequence can be established and consequently through chemical synthesis unlimited amounts of the aptamer can be prepared. On overview of aptamers has recently been published [155], and analytical applications have been reviewed [154,156]. Aptamers are claimed to offer many advantages over traditional antibodies such as they can be generated against any target compared with antibodies where an immune response in an animal is an essential pre-requisite. As aptamers are produced by chemical synthesis they can be purified to a high degree, and modifications to the structure can be introduced enhancing stability, affinity and specificity [156]. They also have the advantages compared to antibodies they do not involve use of animals for their production, can be made in weeks rather than months, in terms of solvent tolerance they are not limited in use to physiological fluids and have an unlimited shelf-life.

Although aptamers have been known for 20 years they have mostly been exploited in the field of biomolecules [155] rather than small molecules. The potential of aptamers has been demonstrated with respect to capturing some small molecules such vitamins [157] and antibiotics such as streptomycin [158], although little has been done to date to exploit aptamers in the form of clean-up columns. However, in a recent publication [159] an aptamer was reported which bound with a high affinity and specificity to ochratoxin A, but 100 times less affinity for the close structurally related ochratoxin B. The aptamer was prepared in the form of a resin column and its potential for clean-up demonstrated by passing a methanol wheat extract through the column and determining OTA directly by fluorescence without any subsequent chromatographic analysis. The performance of this aptamer column was not really established in terms of recovery, capacity etc but its potential was demonstrated, clearing indicating that the field of aptamers is one where with appropriate commercial exploitation clean-up columns rivaling IACs could very easily be produced.

4. Conclusions

This review clearly demonstrates by the numbers of publications and the extent of applications of IAC in food analysis, that the approach of using an IAC for sample clean-up, particularly for mycotoxins, has now become the method of choice. There are four main manufacturers of commercial IACs for mycotoxins and these columns differ in analyte capacity, although recoveries based on manufacturer's specifications tend to be broadly comparable. It is

however evident that although antibodies potentially offer high specificity in clean-up, non-specific binding to the column matrix can occur and instances of both interferences and low recoveries caused by such binding have been reported. Although in many instances an existing protocol can be transferred to a previously untested matrix, it is nevertheless essential that for any new application of an IAC, method validation is carried out to demonstrate transferability.

The main drawback for users of commercial IACs tends to be the cost, which is particularly the case for those in poorer countries who have the greatest need for a reliable clean-up technique. Although re-use of commercial IACs has been explored, it has not been widely adopted and for obvious reasons is not actively encouraged by commercial manufacturers. This is somewhat surprising as home-produced IACs have been demonstrated with suitable elution conditions to be re-usable as many as 100 times before recovery begins to decline.

Manufacturers of commercial IACs have started to develop columns containing more than one antibody meeting a demand to be able to simultaneously determine co-occurring analytes. For mycotoxins, IACs containing antibodies against aflatoxins and ochratoxin A, and containing antibodies against zearalenone together with deoxynivalenol and other *Fusarium* toxins are being marketed. Providing the costs of IACs for multi-analytes do not increase in proportion to the number of antibodies contained in the column, this is a welcome development, and should lead to a further expansion in applications of IACs. In the veterinary drug reside field, two different IACs are marketed which can extract as many as 10 β-agonists and ten growth promoters respectively, although it is not clear the extent to which these are based around multiple antibodies or generic antibodies with cross-reactivity within the class of drugs.

The main reason why IACs for veterinary drugs have not found such widespread application as those for mycotoxins is the demand in the former case for multi-residue methods covering several classes of compound. To an extent in the mycotoxin field there has been a similar move from single toxin to multi-toxin methods. There are a number of publications advocating sample extraction with minimal or no clean-up followed by LC/MS/MS analysis of the crude extract thereby making IAC clean-up superfluous. Whilst arguably there may be situations where aflatoxins and *Fusarium* toxins should be simultaneously monitored the use of LC/MS/MS significantly increases analytical costs and still remains a sophisticated technology outside the reach of many laboratories.

The recent introduction of commercial IACs for vitamins although presently limited to only columns for vitamin B_{12} and vitamin B_9 is a welcome development, as this is a difficult area of analysis and improved sample extraction and clean-up could radically improve this field where large-scale monitoring is essential for the food industry. An expansion in the numbers of different vitamin columns and suitable combinations of different vitamin antibodies on the same columns will further encourage development in this area.

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