Immunosorbents Extracting Various Toxins

Subjects: Others

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The evolution of instrumentation in terms of separation and detection allowed a real improvement of the sensitivity and analysis time. However, the analysis of ultra-traces of toxins in complex samples requires often a step of purification and even preconcentration before their chromatographic analysis. Therefore, immunoaffinity sorbents based on specific antibodies thus providing a molecular recognition mechanism appear as powerful tools for the selective extraction of a target molecule and its structural analogs to obtain more reliable and sensitive quantitative analysis in environmental, food or biological matrices.

Keywords: toxins ; immunoaffinity ; immunosorbent ; molecularly imprinted polymers ; aptamers ; oligosorbents ; trace analysis ; complex samples ; matrix effects

1. Introduction

Given their presence at the trace level in food, biological or environmental samples, the analysis of toxins requires very sensitive and specific tools for their monitoring in very complex samples. For many years, liquid chromatography (LC) coupled with fluorescence detection (Fluo) has been used to monitor toxins with or without derivation steps depending on the physico-chemical properties of the targeted molecules—these toxins being mainly mycotoxins monitored in food matrices. Then, LC coupled with mass spectrometry (MS) has gradually become the preferred method to confirm the presence of these mycotoxins but also of other toxins at ultra-trace level in complex extracts. It also became the method of choice to eliminate the derivation step and allow the simultaneous monitoring of toxins of different classes that may be present in the same sample. However, when applied to the analysis of very complex extracts, LC–MS suffers from matrix effects during the ionization step that can lead to erroneous quantification. Thus, although initially developed to improve the reliability of less specific analytical methods such as LC-Fluo by allowing selective cleaning of sample extracts, immunoaffinity sorbents are still developed and used in combination with LC/MS to reduce or even eliminate matrix effects.

These immunoaffinity supports, also named immunosorbents (ISs), are based on the use of antibodies specific to the molecule(s) of interest. The high specificity and affinity of the antigen-antibody interactions allow the selective and efficient extraction of the target analyte(s) from complex samples, thus facilitating its final identification and quantification [1][2][3][4][5]. As a result, ISs are marketed as single-use cartridges and widely used for the monitoring of mycotoxins in foodstuffs. ISs are also still under development to propose new extraction formats such as magnetic beads to perform selective solid phase extraction (SPE) in dispersive mode (dSPE). ISs are also still under development for other types of toxins such as marine or plant toxins that need to be detected at trace levels in environmental matrices but also in biological fluids.

A molecular recognition mechanism can also be implemented using molecularly imprinted polymers (MIPs), whereby synthesis leads to the formation of specific cavities mimicking the recognition site of antibodies [6][7]. Another selective support, called oligosorbent (OS), has been also recently developed using aptamers immobilized onto a solid support. Aptamers are oligonucleotides with a specific sequence able to bind a given molecule with the same affinity as antibodies. OSs were recently successfully applied to the selective extraction of different target analytes from biological fluids and food samples [8][9]. Once the sequence is available, the development of an OS is less expensive than for an IS. MIPs and OSs present the advantage to be synthesized in a few days. In return, their application to real samples necessitates a careful optimization of the extraction procedure to reach the expected affinity and selectivity, while this selective procedure is very easy to develop when using ISs.

2. Immunoaffinity Sorbents

2.1. Antibody Production and Development of Immunosorbents

ISs were first described in the biological field because of the availability of antibodies specific to large molecules such as proteins. Indeed, small molecules (<1000 Da) are unable to evoke an immune response and make the production of antibodies more difficult. They have to be bound to a larger carrier molecule, usually a protein, to immunize the animal. After a few weeks or months of immunization by this immunoconjugate, the serum is collected and the antibodies, i.e., the immunoglobulin G (IgG) fraction, are purified. This purification generally results in polyclonal antibodies (pAbs) made of a heterogeneous mixture of antibodies [10][11]. These pAbs can bind with the antigen with different affinities, because they are directed against various antigenic determinants (epitopes) on the antigen/immunoconjugate. Therefore, when a small

molecule is targeted, it is commonly found in the literature that the mix of pAbs contains only about 15% active antibodies [10]. In return, techniques of hybridoma allow the production of only one type of IgG known as monoclonal antibodies (mAbs). Polyclonal antibodies are cheaper to obtain, but their production suffers from a lack of reproducibility in terms of time of response of an animal, of quantity and even of specificity and ethical issues. In contrast, the production of mAbs is costly but guarantees a long-term production of reproducible antibodies that do not require animals for further large-scale production. Once the antibodies are obtained, they are immobilized on a solid sorbent, called IS.

The first ISs that were developed for toxin analysis targeted mycotoxins, despite the difficulty to produce specific Abs for such small molecules. Indeed, their quantification at low concentration levels, mainly in foodstuff, represents an analytical challenge. Therefore, a considerable effort has led to the development of mycotoxin-specific ISs, which are currently marketed by many companies such as Vicam and R-Biopharm and to a lesser extent RomerLabs, Aokin, and Neogen, as shown in Table 1. They have been mostly developed for the four aflatoxins (AFs) (B_1, B_2, G_1, and G_2), for Ochratoxin A (OTA), for trichothecene toxins such as deoxynivalenol (DON), zearalenone (ZON), H-2 and HT-2 toxins, fuminosins (FUM B_1, B_2, B_3) and for sterigmatocystin (SMC). As illustrated in this table, while some of these ISs have been developed to trap a mycotoxin and possibly its structural analogs, some companies sell cartridges containing two or more antibodies to simultaneously trap multiple toxins and their structural analogs. As an example, the AlfaOchra Test cartridge allows the trapping of the four main aflatoxins and OTA simultaneously or the Myco6in1 of the four AFs, OTA and several trichothecenes, including their metabolites such as nivalenol (NIV) and acetyl-deoxynivalenol (ADON).

Table 1. Commercially available immunosorbents (ISs) for the analysis of single and multi-toxins in off-line solid phase extraction (SPE) mode.

Toxin(s)	Matrix	Marketed ISs (Company)	Extraction Solvent; Factor and Solvent of Dilution	V_{sample} (eq. of Solid Sample)	Wash
Single analyte and analogs/metabolites					
	Olive, peanut and sesame oils	Aflatest WB (Vicam)	MeOH/water 45/55; -, water	-	-
AFs (B_1, B_2, G_1, G_2)	Nuts and based-nut products	Alfaprep (R-biopharm)	MeOH/water 7/3, NaCl; ×3, water	15 mL (eq. 1 g)	Water
	Baby food and feed	AlfaOchra HPLC^{TM} (Vicam)	ACN/water 78/22 (solid), ACN (milk); dried extract dil. in water	10 mL	Water
	Sidestream cigarette smoke	Aflatest P aflatoxin (Vicam)	-; ×4, water	20 mL	
AF B_1					Water
	Organic spices and herbs	RIDA Aflatoxin column (R-Biopharm)	MeOH/water 7/3; ×4, water	1 mL (eq. 0.25 g)	

AF B_1 and AF M_1	Pig liver	Afla^{TM} wide bore for M1 Aflatest-P for AFB1 (Vicam)	MeOH/water, NaCl; ×5, PBS, Tween-20 2%	20 mL (eq. 1 g)	PBS,
DON	Wheat	DON-Test HPLC (Vicam)	Water; -, -	1 mL (eq. 0.25 g)	-
DON, NIV	Rice, bran	DON NIV WB (Vicam)	Water, NaCl; ×5, PBS	10 mL (eq. 0.4 g)	PBS +
FUMs (B_1, B_2)	Cornflakes	Fumoni Test™ (Vicam)	ACN/MeOH/water 25/25/50; ×5, PBS	10 mL (eq. 0.4 g)	PBS

	Cereals	Easi-extract (Biocode)	MeOH/water 1/1; ×3, PBS	50 ml	Water
	Wine	Ochraprep (OP, Rhone Diagnostic Technologies) et Ochratest (OT,Vicam)	pH adjusted	10 mL + 10 mL PBS (OT) or 4 mL + 10 mL PBS (OP)	PBS +
	Beer	Ochratest (Vicam)	Degassed; ×2, PEG - $NaHCO_3$	10 mL	NaCl 2 0.5% ·
	Urine		×2 (human) or ×3.4 (rat), $NaHCO_3$ + filtration		
OTA	Milk	Ochraprep (R-Biopharm) et Ochratest (Vicam)	-	50 mL	Water
	Grapes, dried wine fruit, winery products		-, ACN/water or ACN/MeOH/water	-	Water NaHC 1% +
	Ready-to-drink coffee	Ochratest (Vicam)	-; ×8, PB	5 mL	NaCl · 0.5% · NH_4Cl
	Wine		-; ×2 PEG 8000 (1%), $NaHCO_3$ (5%)	10 mL	NaCl, water
	Cereals, spices		MeOH/water 7/3; ×1.8, water	40 mL	Water 20
OTA, OTB, α-OTA	Milk	Ochraprep (R-Biopharm)	LLE with $CHCl_3$; back extraction with PBS	PBS extract	Drying
SMC	Cereal, cheese, beer	Easi-extract SMC (R-Biopharm)	ACN/water 8/2, NaCl; ×15, PBS	10–30 mL (eq. 0.25–0.5 g)	PBS +

T-2 toxin		T2 TAG (Vicam)	MeOH/water 8/2; ×5, water	10 mL (eq. 1 g)	
	Cereals	T-2 test (Vicam)	MeOH/water 9/1, NaCl; ×5, water	-	Water
		Easi-extract T2 (R-Biopharm), T-2 Test HPLC (Vicam)	MeOH/water 9/1; -, NaCl 4%	-	
		Easi-extract T2 (R-Biopharm)	MeOH/water 9/1 + 2% NaCl (oats)	-	-
T-2 & HT-2 toxins	Chinese herbal medicines and related products	HT-2 HPLC (Vicam)	MeOH/water 9/1, NaCl; ×5, water	10 mL (eq. 0.5 g)	Water
	Oats	Easi-extract T-2 and HT-2 (R-Biopharm)	MeOH/water 9/1, NaCl; ×5, NaCl 4%	5–25 mL (eq. 1.5–0.3 g)	Tweer water
	Food, Feed		MeOH/water 9/1, NaCl; ×5, water	25 mL (eq. 1 g)	Water
	Corn		ACN/water 9/1; ×10, water	10 mL	
ZON	Botanical root products, soybeans, grains, grain products extracted	ZearalaTest (Vicam)	MeOH/water 75/25; ×10, PBS, Tween 20 (0.5%)	50 mL (eq. 1 g)	MeOH Tweer water
ZON and metabolites (5)	Maize		ACN/water 9/1, NaCl; ×5, PBS, Tween 20 (0.1%)	10 mL	Water
Multi-analytes					

	Ginseng, ginger	AflaOchraTest (Vicam)	MeOH/water 7/3, 0.5%$NaHCO_3$; ×5, PBS, Tween 20 (1%)	-	PBS + water/
	Cereals	Aflatest and Ochratest (Vicam)	ACN/water 6/4 (OTA); MeOH/water 8/2 (AFs); ×5, PBS	50 mL (eq. 0.5 g)	Water
	Sicilian sweet wines	Ochraprep, Easi-extract for AFs (R-Biopharm)	-; ×2 PEG 6000 (1%), $NaHCO_3$ (5%)	20 mL	NaCl 2 NaHC water
AFs (B_1, B_2, G_1, G_2), OTA					
	Meat products	Aflatest and Ochratest (Vicam)	MeOH/water 6/4, NaCl; × 2, water (AF) MeOH/water, $NaHCO_3$ 1% 7/3; ×5, water (OTA);	10 mL (eq. 1 g)	Water 20/PB
	Spices and spices mixtures		MeOH/water 8/2, NaCl; ×10, PBS, Tween 20	20 mL	Tweer PBS +
	Ginger	AflaOchra HPLC (Vicam)	MeOH/water 7/3, $NaHCO_3$ 0.5%; ×4, PBS, Tween 20 (1%)	25 mL (eq. 0.3 g)	PBS +
AFs (B_1, B_2, G_1, G_2), OTA, FUMs (B_1, B_2), DON, ZON, T-2 and HT-2	Maize	AOFZDT2^{TM} (Vicam)	Water (A) and then water /MeOH 3/7; PBS	Percolation of B and then of A	PBS (
AFs (B_1, B_2, G_1, G_2), OTA, ZON	Airborne from poultry house	AOZ (Vicam)	aqueous extract + NaCl; -, -	-	-
AFs (B_1, B_2, G_1, G_2), OTA, ZON, FUM (B_1, B_2, B_3), T-2 and HT-2	Cereals	AOF-MS-Prep and DZT-MS-Prep used in tandem	MeOH/water 7/3, NaCl; -, -	-	-

AFs (B_1, B_2, G_1, G_2, M_1), OTA	Turkish dairy food	3 ISs (no supplier mentioned)	MeOH/water 8/2, NaCl (AF B and G), 7/3 (OTA) and $CHCl_3$, NaCl (AF M1); ×7, PBS (AF B/G, OTA), dried residue diluted in MeOH/PBS 2/98 (AF M1)	-	PBS (
AFs (B_1, B_2, G_1, G_2, M_1), OTA, DON, ZON, FUM (2), T-2 and HT-2	Food	AflaOchra Prep (R-Biopharm)	QuEChERSs method including LLE (hexane) to purify ACN extract; ×12.5, PBS	-	Water
AFs (B_1, B_2, G_1, G_2, M_1), OTA, DON, ZON, NIV, FUS-X, VCG; T-2 and HT-2; CTN, 3-ADON,15-ADON, SMC	Food and feed extracts (84% ACN)	Mycosep 226 Aflazon + (COCMY2226, Romer labs)	-; ×2, ACN	-	-
AFs (B_1, B_2, G_1, G_2), OTA, DON, ZON, FUM (3), T-2 and HT-2, NIV, 3-ADON, 5-ADON	Cereals	Myco6in 1 (Vicam)	Water + MeOH; ×3.5, PBS after partial evaporation	7 mL (eq. 0.5 g)	Water
AFs (B_1, B_2, G_1, G_2), DON, ZON, NIV, FUS-X, T-2 and HT-2, 3-ADON, 15-ADON, DAS	Corn, wheat, biscuit, cornflakes	Multisep 226 (Romer Labs)	ACN/water 85/15; none	10 mL	-

AFs (B_1, B_2, G_1, G_2), OTA, DON, ZON, FUMs (B_1, B_2), T-2 and HT-2, NIV	Spices, infant formula, coffee, nuts	AflaOchra Prep (R-Biopharm)	Water/ACN/AA 10/89.75/0.25 + salt ($MgSO_4$/NaCl) + LLE (Hexane); ×25, PBS	50 mL	Water
	Corn and corn-derived products		MeOH/water 7/3; ×10, PBS	20 mL (eq. 0.5 g)	
AFs (B_1, B_2, G_1, G_2), OTA, DON, ZON, FUMs (B_1, $B_{2,}$), T-2 and HT-2, NIV, 3-ADON	Cereal grains	Myco6in1 (Vicam)	ACN/water/AA 79.5/20/0.5; ×16, PBS	-	PBS +
AFs (B_1, B_2, G_1, G_2), OTA, DON, ZON, FUM (B_1, B_2, B_3), T-2	Herbs		MeOH/PBS 7/3 + LLE (hexane); ×26, PBS	-	NH_4H
AFs (B_1, B_2, G_1, G_2), OTA, DON, ZON, FUMs (B_1, B_2, B_3), T-2 and HT-2	Cereals, nuts		ACN/water/AA 79.5/20/0.5 + evaporation; -, PBS	10 mL (eq. 2.5 g)	
AFs (B_1, B_2, G_1, G_2), OTA, DON, ZON, FUM, T-2 and HT-2	Cereals	AOF MS PREP, DZT MS-PREP (R-Biopharm)	MeOH/water 7/3, NaCl; ×13, PBS	20 mL (eq. 0.38 g)	Water
AF M_1, OTA, DON, DON analog, ZON (α,β), FUM B_1	Urine	Myco6in1 (Vicam)	Oasis HLB SPE cartridge connected to the top of the IS; ×2, water	12 mL	
DON, ZON and 5 derivatives, 3-ADON, 15-ADON	Flour	Multi-IACs (Magnech Bio-Tech)	ACN/water 8/2; ×8, PBS	20 mL (eq. 0.25 g)	Tweer Water

DON, ZON (+conjugated and metabolites)	Calf serum	DON Prep and DZT MS-Prep (R-Biopharm), NeoColumns for DON and for ZEN (Neogen), AokinImmunoClean C for DON and for ZEN (Aokin), Easi-extract ZEA (R-Biopharm)	Protein precipitation + drying; PBS, 5% MeOH	10 mL		Water
DON, ZON, HT-2 andT-2	Wheat, biscuit	DZT MS-PREP (R-Biopharm); MultiSep 226 (Romer Labs)	MeOH/water, 75/25; ×4, PBS, MeOH (15%)	5 mL (eq. 0.25 g)		
DON, ZON, NIV, FUS-X, 3-ADON, T-2 and HT-2	Maize		ACN/water, 84/16; -	8 mL (eq. 2 g)	-	
DON, ZON, T-2 and HT-2	Cereal and cereal-based samples	Mycosep 226 and 227 (Coring systems Diagnostix)	ACN/water, 85/15; -	5 mL (eq. 1 g)	-	
DON, ZON, T-2 and HT-2	Wheat, Maize	DZT MS-PREP (R-Biopharm)	ACN/water 8/2; ×40, PBS	8 mL		Water

AA: acetic acid; CNT: citrinin; DAS: diacetoxyscirpenol; FA: formic acid; FUS-X: Fusarenon-X; LLE, liquid–liquid extraction; PEG: polyethylene glycol; PB: phosphate buffer (PBS: PB saline); PEG: polyethyleneglycol; VCG: verruculogen; ZER: zeranol. -: no data.

Table 2. Home-made ISs for the analysis of toxins.

Target toxin(s)	**Matrix**	**Extraction Solvent; Dilution Factor and Solvent**	**Sorbent, Amount of Abs**	**Grafting Yield or Density; Capacity**	**Extraction Mode**	**V_{sample} (eq. Sample Amount)/ Amount of Sorbent**	**Washing**	**Elu**
Toxins with MW < 1500								
Bacterial toxin								
TTX	Marine organisms	MeOH, 1% AA; PBS (20% MeOH)	CNBr-Sepharose (0.5 g); mAbs (6 mg)	1106 ng/mL; -	Off-line SPE	25 mL (eq. 1 g)/0.5 g	Water	Me (1%

Phycotoxins–Cyanotoxins								
Anatoxin-a	Pure water	-	NHS-Sepharose beads (27 µm, 10 µL); mAbs (100 µg)	-; 20 ng	dSPE	20 mL/ 10 µL	-	2-p
	Algae extracts	-	Poly(APTES-co-TEOS) monolith; pAbs	-; 0.38 pmol (2.1 µg/g sorbent)	On-line SPE	150 nL/ 45 x 0.1 mm i.d. capillary	PBS	AC (LC pha
MC-LR	Urine	-	streptavidin-magnetic beads; Biotin-Abs		dSPE	100 µL	-	Wa 7/3 (0.5
	Pure water	-	Sol-gel entrapment (TEOS)	-; 4.28 µg		1 L (eq 2.5 g)/ 0.5 g	-	AC 7/3
MC-LR, MC-RR, MC-YR	Real waters	-	Glutaraldehyde-silica; purified pAbs	-; 1.8 µg/g IS		20 mL (0.5% MeOH)/ 0.25 g	Water + water/MeOH 8/2	Me 8/2
	Cyanobacteria, real waters	MeOH/water, 75/25; x0.75, PBS	pAbs	-	Off-line SPE	100 µL	PBS + water + MeOH/water 25/75	Me
MC-LR, MC-RR, MC-YR, MC-LA	Algae and fish extracts, real waters	-; <15% MeOH	CNBr-Sepharose and silica; -	-		5–15 mL/ 0.1–0.2 g	PBS + water + MeOH/water 25/75	Me 8/2
	Real waters and blue green algae extracts	-	Sepharose CL-4B; pAbs (1 mg/mg sorbent)	-; 0.2 µg		10 mL/ 2 mg	PBS + water + water/MeOH 85/15	Me 80/ (2%

MC-RR, MC-YR, MC-LR, MC-AR, MC-FR, MC-WR, MC-LA, MC-LF, MCYST-LW and other MC variants	Real waters	Concentrated, filtered	CNBr-Sepharose and silica; pAbs	-; 200 ng/IS (Sepharose); 135 ng/IS (silica)		-	Water/MeOH 75/25	Me (+A
	Urine	-	Streptavidin-beads (2.5 µL); biotinylated Abs (0.5 µg)	-	dSPE	500 µL/ 2.5 µL	-	Wa 7/3 (0.5
Phycotoxins—Diarrheic shellfish poisoning (DSP) toxins								
OA	Shellfish	MeOH, NaOH; dried extract in PBS	Protein G-magnetic beads; mAbs (1 mg/mg sorbent)	-	dSPE	1 mL/ 1 mg	PBS	Me
	Algae extract	-; PBS/ACN 8/2	Silica; pAbs	-		-/125 mg	MeOH/water 3/7	Me 8/2
OA and derived form	Shellfish (hepatopancreas)	LLE; water/ACN 8/2	Glutaraldehyde-silica; pAbs	-; 16 µg/g IS	Off-line SPE	2 mL/ 125 mg	MeOH/water 7/3	PBS 7/3
OA, DTX-1 and DTX 2	Shellfish		-; Anti-AO mAbs	-		-	-	-
Mycotoxins								

AFs (B_1, B_2, G_1, G_2), OTA, ZON, SMC, T-2	Feed samples	ACN/water/AA 80/18/2; x3, PBS	CNBr-Crystarose; 4 mAbs (5 mg each/g sorbent)	-; 0.13 µg/mg Abs (sum of toxin)		10 mL (eq 0.6 g)/ 0.3 ml	PBS	
AFs (B_1, B_2, G_1, G_2), OTA, ZON, T-2	Peanuts, corn, wheat	ACN/water/AA 80/19/1; x3, PBS (≤ 20% ACN)	CNBr-Sepharose (1.3 g); mAbs (20 mg each)	-; 9 µg/mL IS (sum of toxin)		10 mL/ 0.1 mL		Me
AF B_1	Cereals, peanuts, vegetable oils, Chinese traditional food	MeOH/water 6/4; x6, water (<10% MeOH)	CNBr-Sepharose (1 g, 3.5 mL); mAbs (9.92 g)	99.8%; 260 ng/mL		30 mL (eq. 1 g)/ 1 mL	Water	
A- and β-Amanitins	Urine	Filtration; x1.8, PB	CNBr-Sepharose 2 mL; pAbs (6.4 mg)			9 mL/ 2 mL	PB + water + acetone/water 95/5	Ace Me
DON	Cereals	MeOH/water 8/2; x2, PBS (<10% MeOH)	CNBr-Sepharose (1 g, 3.5 mL); mAbs (30 mg)	95%; 9.67 nmol/mL	Off-line SPE	10 mL (eq. 0.5 g)/ 1 mL	Water + MeOH/water 1/9	Me
DON, 3-ADON, 15-ADON, deepoxy-DON	Foods, feeds (aqueous extracts)	-	Abs entrapped in silica gel (TMOS); mAbs	-; 1 µg/mg immob. Abs		/1 g	1% MeOH	AC 4/6
FUMs (B_1, B_2) B_3, OH-B_1)	Dried feed samples	-; 100 µL for 10 mg, buffer	Protein A/PS-DVB (POROS); serum/mL sorbent	-		100 µL/ 30 x 2.1 mm column	-	Wa 7/3
FUMs (B_1, B_2, B_3)	Cereals	-	CNBr-Sepharose 4B (0.5 g); pAbs (1.27 mg/mL, 400 µL)	-		-	-	-

OTA	Beer	Degassed; x2, PBS, 1% PEG 6000	anti-IgG + CNBr-Sepharose (non-covalent bonding)	-		-	PBS, 0.05%Tween	OTA con
	pure media	None	polyGMA-co-EGDMA monolith in a capillary; -	260 ng Ab/cm; 1.2 pmol OTA/cm	In-line SPE	10 µL/ 8.5 cm x 75 µm i.d	PBS+ borate buffer	Me
T-2, HT-2	Maize, cherry	MeOH/water 6/4; x6, water, ≤ 10% MeOH	CNBr-Sepharose (1 g); mAbs (30 mg)	-; 3 µg/mL IS (for each toxin)		30 mL (eq 0.5 g)/1 mL, 10 x 0.8 mm column	-	Me
ZER + 3 analogs	Bovine muscle	MeOH; x5, PBS	CNBr-Sepharose 2 g; mAbs (50 mg)	96.3%; 2.7 µg/mL gel		25 mL (eq 2.5 g) /1 mL, 10 x 0.8 mm column	PBS + water + water/MeOH 7/3	Me
ZON, DON, T-2, HT-2	Grain products	-	CNBr-Sepharose (0.2 g); DON Abs (1.25 mg), H-2/HT-2 Abs (0.2 mg), ZON Abs (0.3 mg)	100%; 198–281 ng (for each compound)	Off-line SPE	-	Water or PBS	Me
	Flour	-	Activated poly(GMA-co-DVB) µSpheres (0.3 g, 1 mL); DON Abs (1.25 mg), H-2/HT-2 Abs (0.2 mg), ZON Abs (0.3 mg)	-; 210–294 ng (for each compound)		/300 mg, 1 mL	-	-
ZON, T-2, HT-2	Feed samples	ACN/H_2O, 8/2; x3, PBS	Anti-IgG-Sepharose (0.5 g, 1.8 mL); mAbs (ZON) and pAbs (T-2)	-		/0.2 g	-	-
Phycotoxins—Paralytic shellfish p*oisoning* toxins								

STX	Human urine	-	Protein G-magnetic beads (30 mg/mL); mAbs, (1 mg/mL)	15 µg/mg (theory); -	dSPE	100 µL/1.5 mg	PBS + water	AC 1/1 (2.5
STX, NEO	Shellfish	-	NH_2-coated hollow glass	5.8 mg/g; -		1 mL/25–100 mg	PBS	Gly buf
PSP toxins	Algae culture	PBS	magnetic µSpheres; mAbs	5.5 mg/g; -				
Protein toxins								

		-					
Abrin	Milk		Tosyl-activated magnetic beads (14.8 mg); mAbs against 4 epitopes (140 µg)		dSPE	500 µL/0.2 mg	PBS + Tween 0.05% + PBS + water	Try dig
Androctonus australis Hector	Venom		CNBr-Sepharose 2 g; purified pAbs (0.15 µmole)		Off-line SPE	/20 x1 cm column, 2 g	Tris HCl, NaCl	FA Na
BoNT type A	Crude culture supernatant, food, environmental samples			-		500 µL/10–100 µL	HEPES	Try dig
ETX	Milk, serum							
	Pure media (buffer + BSA)		Protein G-magnetic beads (3 µm), pAbs (BoNT A) and mAbs (ricin)			500µL/100 µL	Ammonium acetate (pH 4)	RN inc
	Milk					500µL/5 µL	Buffer	5% 0.1 wat
Ricin	Milk, apple juice, human serum, saliva				dSPE	500µL/20 µL	PBS, Tween + water	AC
	Serum		Streptavidin-magnetic beads; biotinylated mAbs	55 µg/mg; 16.5 µg/mg		500 µL/20 µL	PBS + water	TFA

Ricin, SEB, BoTN A and B	Milk, orange and apple juices	M-280 tosyl-paramagnetic beads (250 µL); mAbs			200 µL/8 µL		
Ricin, SEB, ETX	Milk, human urine, plasma				1 mL/20 µL	PBS	Try dig
Shigatoxin (protein) + analogs	Cell culture	CH-Sepharose 4B (2 g); purified pAbs (4 mg)	-	Off-line SPE	/2 g	Tris HCl + NaCl	Gly 2.7 0.5
Staphilococcal enterotoxins A and E (proteins)	Dialyzed cell culture supernatant	Affigel 10 (agarose) 1 mL; mAbs (5 mg)			25 mL/1 mL	PB	AA,

AA: acetic acid; APTES: aminopropyltriethoxysilane; BoTN: botulinium neurotoxin; CE: capillary electrophoresis; CNBr: *cyanogen bromide;* ETX: epsilon toxin; FA: formic acid, HRP: horseradish peroxidase; LLE, liquid–liquid extraction; NEO: neosaxitoxin; NHS: N-hydroxysuccinimide; PB: phosphate buffer (PBS: PB saline); PEG: polyethylene glycol; SEB: staphylococcal enterotoxin B; TEOS: tetraethoxysilane; TFA: trifluoroacetic acid; TTX: tetrodotoxin; ZER: zeranol. -: no data.

While the nature of the solid phase used to immobilize antibodies is rarely described for commercially available ISs, the content of Table 2, dedicated to ISs developed in research laboratories, illustrates the wide variability in the nature of the possible sorbent and the final format of the immunoextraction device. Indeed, if agarose gel such as Sepharose is one of the most widely used sorbents for developing commercially available ISs and is still used to develop new ISs (as shown by 40% of the work reported in Table 2), other sorbents can be used, such as activated silica or polymers. These supports, available as beads, can be packed between two frits in a conventional SPE cartridge such as the commercially available ISs or can be directly introduced into the sample to perform the extraction in dispersive mode as discussed later. To immobilize Abs on a solid sorbent, the most common approach is with regard to their covalent bonding, which is often achieved by coupling an accessible amino group of the Abs with a support that contains reactive groups such as epoxy or aldehyde or groups that can be activated using glutardialdehyde, carbonyldiimidazole, cyanogen bromide (CNBr) or N-hydrosuccinimide (NHS). Some activated supports are commercially available such as NHS- or CNBr- activated Sepharose or glutardialdehyde activated silica. Non-covalent binding can also be used to couple Abs to the sorbent. For this purpose, proteins A- or G- based sorbents or sorbents grafted with anti-IgG can be used as these proteins bind a part of the constant region of Abs, allowing the orientation of the Abs with the antigen binding sites away from the surface and towards the solution. The same orientation effect can be obtained using streptavidin activated sorbent that can react with biotinylated Abs . However, the resulting non-covalent binding is quite strong under physiological conditions but can be easily disrupted by decreasing the pH of the surrounding solution. The sol–gel method can also be used to entrap Abs . In this case, Abs are then immobilized in the pores of a hydrophilic glass matrix that reduces the non-specific adsorption of apolar analytes. Moreover, narrow pores prevent the diffusion of large size molecules such as bacteria or proteolytic enzymes. Therefore, no bacteriostatic agent must be added in the phosphate buffer saline (PBS) solution for IS storage.

2.2. Immunoextraction Procedure on IS Cartridges

Numerous studies reported the use of commercially available ISs for toxin analysis in numerous samples, and in some cases more than 100 samples were analyzed. In most of the cases, the immunoextraction procedure provided by the vendor was directly applied by the user in terms of washing and elution conditions. For laboratory-made ISs, such as those reported in Table 2, both steps must be optimized but are very similar to those applied to commercially available ISs, with a washing step using water or a buffer and elution mainly with methanol. In addition to aqueous conditions, a low amount of solvent (or surfactant for proteins) can be added in the washing solution to improve the selectivity by removing the interfering compounds retained by non-specific interactions mainly caused by the solid-phase selected for the grafting. To evaluate the contribution of non-specific interactions in the retention of the target toxins, we proposed to compare their retention on their IS with their retention on a sorbent bonded with non-specific antibodies or on a non-bonded sorbent. The study of the retention on the IS of compounds having a polarity similar to one of the target toxins, but which should not be retained as they are not recognized by antibodies, was also proposed.

Concerning the elution step, the nature of the elution solvent can be optimized to improve its efficiency and thus reduce the elution volume (which improves the enrichment factor) or make it compatible with the analytical device used for toxin quantification. As an example, despite its efficiency, a glycine buffer was no longer used for the elution step due to its lack of compatibility with LC/MS–MS analysis.

As for conventional solid-phase extraction, the retention of an analyte on an IS during the percolation step depends on the volume of samples that is passed through the IS and the content of this sample. Therefore, the nature of the solvent used to extract the toxins from the samples (cereals, food, etc.) may vary according to the sample matrix to ensure a good extraction yield, but it must also be compatible with the percolation conditions on the ISs since antibodies have a high affinity in aqueous media. For polar toxins such as saxitoxins, the possibility to extract them from samples using phosphate buffer saline (PBS) solution constitutes a real advantage [114]. When solvents or hydro-organic mixtures are required, as often reported in Tables 1 and 2, they can be either evaporated and the toxins next dissolved in water or PBS, or directly diluted with these aqueous solutions to decrease the solvent proportion that affects the retention on IS. However, with this second approach, the dilution rate affects the final sensitivity of the method and has to be carefully optimized. Reported or calculated dilution factor values are mainly between 2 and 10. It is worthwhile noticing that a residual amount of solvent in the extract can sometimes be necessary to ensure the complete solubilization of the toxins. Moreover, some authors suggested to optimize the extraction conditions of the toxins from the sample not only regarding the final extraction recovery of the toxins but also by studying the effect of the nature of the extraction solvent on the final selectivity measured by the removal of the interfering peak in the final chromatogram. As an example, for mycotoxin analysis, it was often mentioned that the addition of NaCl in the extraction solvent strongly contributes to the improvement in selectivity because it induces the precipitation of the proteins that are thus removed from the extracts. The addition of a surfactant in the extract to be percolated was also reported to improve the clean-up effect as it contributes to limit nonspecific interactions of sample components with the IS. Among the parameters affecting extraction recoveries, the pH of the sample was sometimes also studied.

As previously mentioned, an IS that contains several antibodies allows the simultaneous extraction of toxins from different chemical groups, thus decreasing both the global analytical time and the cost of the method as only one cartridge is required. Nevertheless, if the targeted toxins have different physico-chemical properties, it improves the difficulties to find the extraction conditions leading to high recoveries for all the toxins without affecting the stability of some of them. This may explain why some authors preferred to run the samples over several ISs even if it means assembling the ISs in series for the elution step.

The volume of sample that can be percolated through an IS is limited by the affinity of the antibodies towards the antigen, as previously mentioned, but also by the number of antibodies immobilized, which defines the IS capacity that should not be overloaded. The capacity corresponds to the maximal amount of a target molecule that can be retained by the IS during the percolation step. It depends on the nature of the antibodies (mAbs or pAbs), of the grafting yield, and of the antibody accessibility for the antigen. This value can be provided by manufacturers, and values of about 1.4–1.6 µg of toxins were depicted for T2 or zearalenone affinity columns from Vicam for example.

To avoid the IS overloading, as can be seen from the data reported in Table 1, while the volume of sample/extract percolated is variable, the equivalent amount of sample contained in the percolation fraction never exceeds 1.5 g and is generally equal to or less than 0.5 g. This suggests that all commercial cartridges contain similar amounts of IS and therefore similar amounts of immobilized antibodies. Nevertheless, an easy way to determine the real capacity of an IS consists of measuring the amount of analyte retained as a function of the analyte amount present in the percolated sample. The amount of analyte retained by the IS can be determined by analyzing the elution fraction to measure the analyte amount that was fixed during the percolation and next desorbed applying the immunoextraction procedure. It can also be estimated by measuring the residual analyte amount in the percolating fraction after the percolation of a huge amount of toxin causing the overloading of the IS. Some data are presented in Table 2 for laboratory-made ISs. These values are difficult to compare because they are given in different units: per g or ml of sorbent, per number of antibodies, etc., but they are always in the range from the hundreds of ng to a few µg per gram or ml of sorbent. These capacity values partly result from the grafting yields, some values being listed in Table 2 and being close to 100% for most of the reported studies. However, lower grafting yields may be obtained if steric hindrances occur during the grafting. This is why it can be interesting to optimize the number of antibodies for a given amount of sorbent as reported. However, only a theoretical capacity can be calculated based on the grafting yield because the real capacity depends on the number of specific and active antibodies, which is unknown when using pAbs, and steric hindrances that could prevent the analyte from accessing the antibody recognition sites. The proportion of active antibodies can be deduced from the experimentally determined capacity value. As an example, values of 39% or 65% of active antibodies were reported for ISs prepared by the grafting of a poly(GMA-co-EGDMA) monolith or sol–gel entrapment, respectively. Concerning laboratory-made ISs, there are only a few studies that give data about the repeatability of the preparation of ISs. For an IS prepared by immobilizing antibodies on Sepharose, recoveries were found similar for the extraction of α- and β-amanitins on two independently prepared cartridges. The column-to-column reproducibility was also determined by preparing nine sol–gel immunoaffinity columns on nine different days and mean recovery for DON was found to be 97.8% with a relative standard deviation (RSD) value of 1.4% thus indicating the high repeatability of this preparation method based on the entrapment of Abs in sol–gel. For an IS prepared in a 100 µm i.d. capillary to be coupled on-line with nanoLC, the repeatability of the synthesis of monoliths estimated by the evaluation of their permeability was first studied and an RSD

value of only 6.2% was obtained for three independent synthesis. After their grafting with antibodies, a mean extraction recovery of 73% was obtained for microcystin-LR with an RSD of 5.4% showing the similarity of the results obtained with these three ISs.

At last, commercially available ISs are not reused, thus explaining the use of pure methanol or sometimes acetonitrile as eluting solvent with the possible addition of up to 2% of acetic acid to increase the elution strength. Regarding the ISs prepared in laboratories, softer elution conditions are indicated in Table 2, such as the use of a water–acetonitrile or water–methanol mixture, to favor the reuse of the ISs. However, it is worthwhile to notice that the use of pure methanol does not prevent the reuse of ISs [86,88,94,95]. The reusability of ISs was not so much studied even for laboratory-made ISs, but some works demonstrated that ISs can be reused 5, 6, 8 or even more than 60 times without observing a decrease in the extraction recoveries. For a saxitoxin IS, the elution of this polar toxin was achieved by a glycine/HCl buffer that was selected because those mild conditions offered the possibility to reuse the IS up to 50 times the IS. It was also reported that an IS prepared by Abs encapsulation in sol–gel can be reused 25 times and be stored at room temperature over 19 days in water or 20 weeks in PBS. So, if leaching of antibodies can be a problem in sol–gel techniques because of the high porosity of the sol–gel matrix and the fact that Abs are not covalently bound, no, or negligible, leaching seems to be observed. Other studies carried out with commercially available ISs or laboratory-made ISs prepared by covalent bonding reported the possibility to store the ISs during either 360 days at 4°C or 30 days at room temperature. Storage conditions are also given by manufacturers, such as, for example, the possibility to store ZearalaTest WB column 18 months at 4°C or 12 months at room temperature.

2.3. Immunoextraction Using Other Formats

In recent years, much research has been devoted to the development of miniaturized extraction devices with the aim of limiting reagent consumption and reducing sample volume. Thus, for toxin analysis, as an alternative to conventional cartridges used in off-line mode and containing from 2 to 500 mg and sometimes up to 2 g of IS, as shown in Table 2, other formats have been proposed. Among them, microparticles and nanospheres were prepared and used for solid-phase extraction in dispersive mode (dSPE), also named immunocapture, mainly in the field of protein extraction. This dSPE mode was reported for 34% of the studies cited in Table 2. The particles were prepared by the covalent immobilization of antibodies on NHS-activated Sepharose beads, or tosyl-activated magnetic beads or by non-covalent immobilization on protein G- or streptavidin- activated magnetic beads or on amino-coated hollow glass magnetic microspheres [100,101].

In dSPE, the extraction is carried out by introducing the sorbent directly in the sample instead of percolating the sample through a cartridge containing the sorbent. After a sufficient extraction time under stirring, the particles are recovered mainly by centrifugation or by a magnetic field (when using particles with a magnetic core) to be further introduced into a suitable desorption solvent. As for IS used in SPE cartridge, the nature of the extract put in contact with the IS particles, as well as the nature of the washing and elution solutions, rather called desorption solutions in dSPE, affects the extraction yields. In addition, it is necessary in this mode to optimize the extraction, the desorption times and the vortex speed. It appears that the extraction step takes from 1–10 min [70,72,79,80,100,101,104,105] to 1–2 h [99,102,107,108,110,111], the desorption step being carried out with a similar or shorter time. Most of the procedures include a washing step before desorption to ensure an optimal selectivity, but the duration of this step was never mentioned. The duration of the overall extraction procedure is therefore quite long, but only a small amount of phase is used, which reduces the costs of the device. Indeed, the polypropylene reservoir and frits, which can be clogged during the percolation of certain samples and thus requiring prior filtration, are no longer used. This certainly explains why most of the applications of ISs in dSPE mode concern protein toxins as illustrated in Table 2 that were often monitored in milk or plasma samples that contain huge amounts of other proteins that can clog frits. Indeed, dSPE was applied in 75% of the cases to these protein toxins in reduced sample volume by adding no more than 20 µL of beads in 500 µL of sample. Concerning the desorption of proteins, the addition of trypsin in the desorption solution was proposed to carry out simultaneously both desorption and digestion steps. This allows us to reduce the overall duration of the analysis but hinders the reuse of the IS, as the antibodies are also digested by the protease, thus leading also to peptides that will make the analysis of the target proteins more complex. At last, similar to SPE cartridge that may contain several antibodies to trap, simultaneously, toxins from different classes, multiplex-immunoextraction of three different toxins was described by Dupré et al. who mixed three batches of beads, each batch being prepared with antibodies specific of one toxin.

In addition to the dSPE mode, IS particles can be packed in a small size precolumn (5–20 mm length and 1 to 4.6 mm internal diameter (i.d.)) connected to switching valves and an LC column. Different types of set-up exist for this coupling and they allow the automation of the whole analytical procedure. This set-up at the conventional format was not described for toxin analysis but the integration of ISs on-line with the separation step was proposed under a miniaturized format thus requiring us to modify the way to prepare ISs. Indeed, in order to integrate the immunoextraction sorbents into miniaturized analytical methods, such as capillary electrophoresis (CE) and nanoLC, new approaches have been proposed that consist mainly in the in-situ synthesis of a monolith that are grafted in a second step with antibodies. This monolith must be hydrophilic to limit the contribution of nonspecific hydrophobic interactions during the extraction of the target analytes and must have an accessible function for antibody grafting. Such type of miniaturized ISs was recently reviewed showing the growing interest for the miniaturization of ISs, but the development of monolithic ISs for toxin

analysis is still reduced. One of the two reported works consisted of the in-situ synthesis of a 5 cm organic monolith on one end of a long silica capillary of 75 µm i.d. by radical polymerization using glycidyl methacrylate (GMA) as monomer and ethylene glycol dimethacrylate (EGDMA) as crosslinking agent. The hydrophilicity of GMA, which possesses an epoxy group allowing antibody grafting, has often been advanced to justify its use in order to reduce the risk of non-specific interactions by limiting the hydrophobic effect. This device was applied to the extraction of OTA from pure spiked water samples before its elution by a solvent plug and its detection by laser-induced fluorescence detection (LIF) through the capillary. The second development concerns the preparation of a 5 cm hybrid monolith that was synthesized in a 100 µm i.d. capillary by hydrolysis and condensation of organosilanes and alkylethoxysilanes (by the sol–gel process) and used for the covalent grafting of anti-microcystin-LR antibodies. The resulting monolithic IS was coupled on-line to nanoLC/UV via a nano-switching valve and applied to the analysis of microcystin LR in an algae extract. In this last case, a reduced sample volume of 150 nl was enough to determine microcystin-LR in the algae extract. For the poly-(GMA-EGDMA) monolithic-based IS, the amount of grafted mAbs was 18 mg/g thus allowing the retention of 1.2 pg/cm (3 fmol/cm) of OTA, which means that 39% of the randomly immobilized mAbs were active. A higher capacity of 40 mg/cm (40.2 nmol/cm, 2.11 nmol/g) of MC-LR was reported for the hybrid monolithic-based IS. This difference is mainly due to the higher specific surface area of hybrid than organic-based monoliths. This 40 mg/cm capacity of MC-LR corresponds to a binding density of 0.543 pmol/mL of active mAbs. This monolithic approach has also been used for the integration of ISs in chips but not yet applied for toxin analysis.

2.4. Potential of Immunosorbents for the Reliable Quantification in Real Samples

ISs constitute a good mean to concentrate the targeted toxin(s) while removing matrix effect thus allowing the analysis of the extract with simple and fast analytical methods adapted to numerous samples such as bioassays achieved in 96-well plates (ELISA or enzymatic inhibition assay) as it was performed mainly for microcystin analysis. In addition, it was shown by Chiavaro et al. that the high selectivity of the IS allows the direct determination of AFs B_1 and M_1 at 1 µg/kg in pig liver extracts using only fluorescence detection. Fluorescence was also directly applied to the analysis of OTA in wine, but it necessitates an additional step of purification of the IS eluate on amino silica. However, in the majority of cases, as shown in Tables 1 and 2, the ISs were applied upstream of liquid chromatography coupled initially mainly to fluorescence (LC/Fluo) for native fluorescent compounds or after post-column derivatization. The reliability of methods combining IS and LC/Fluo was proven by interlaboratory studies or applications to certified reference materials, as it was carried out for T2 and HT-2 toxin analysis. Comparison with the Association of Official Analytical Chemists (AOAC) method analysis was also performed showing the performance of the IS associated to LC/Fluo in terms of clean-up efficiency, but also by limiting solvent consumption, liquid–liquid extraction step being no more necessary as purification step as mentioned for ZON analysis.

Initially applied as a confirmatory method in the case of mycotoxins, the coupling of IS extraction with LC–MS is now unavoidable. This coupling has the advantage of being both more specific and applicable to a wide range of compounds. This constitutes a serious advantage when using multi-analyte ISs, but also adapted to new toxins such as protein toxins. It is well known that matrix effects can affect the sensitivity and accuracy of LC–MS/MS method. As such, it has been demonstrated by many authors that an IS clean-up can solve this problem by removing most of the interfering compounds from the final extract. Indeed, it was shown by Yue et al. that in contrast to conventional sorbents, ISs suppressed matrix effects for LC–MS/MS analysis of STX in bivalve extracts [114] thus allowing an external calibration. This simple calibration method was also applied to the quantification of T2 and HT-2 toxins in 20 different samples of food or a mix of mycotoxins in different samples as no matrix effects were observed using the IS. IS cleanup also helps the reliability of the LC–MS/MS analysis. As an example, Senyuva et al. reported that, in addition to the improvement in terms of sensitivity, peaks observed in LC–MS had Gaussian shapes and were essentially indistinguishable from standards. There was also much closer agreement of ion ratios with standards when samples received cleanup.

Nevertheless, despite the use of ISs, other authors mentioned that there are still some matrix effects that may affect the sensitivity and accuracy of LC–MS/MS. To circumvent the risk of false quantification in LC/MS caused by these matrix effects, a possibility is the use of matrix match calibration. It consists of using a blank extract of the studied matrix passed through the IS and spiked at different concentration levels to construct a calibration curve. This approach was also proposed by different groups to evaluate the clean-up effect of ISs. Indeed, it was considered as necessary for the simultaneous quantification of HT-2 and T-2 toxins in maize and cherry samples, of several toxins in cereals, feed samples, or urine. In return, matrix match calibration was studied and considered as not necessary for the analysis of a mix of mycotoxins in cereals or SMC in various samples thus allowing the use of the much simpler external calibration method. A similar conclusion was obtained for the extraction of OA from shellfish extracts in dSPE . For a study related to the simultaneous extraction of DON, ZON, T-2 and HT-2 toxins, matrix match calibration was only applied to the quantification of DON as the quantification of the other toxins was not affected by matrix effects. The use of a “IS calibration standards”, as named by Vaclavikova et al., and prepared by spiking the elution solvent of the IS was also considered as efficient and less time consuming than matrix match calibration to correct the signal suppression or enhancement that occurs for some mycotoxins.

Thus, it appears that the conclusions differ between studies as to the potential of ISs to suppress matrix effects in LC–MS analysis. Indeed, this potential may also depend on the level of optimization of the extraction procedure, and in particular on the washing step which can efficiently remove residual interferents when perfectly optimized. It may also depend on

the ionization capacities of the molecules in the source of the MS and on the level of sensitivity expected, the later point was illustrated by a study of Solfrizzo et al., showing that the choice of the calibration mode may depend on the contamination level. Indeed, for very low contamination levels they proposed to use labeled toxins (C13) to correct the quantification of some mycotoxins in cereals, while this was not necessary for higher levels of contamination. The use of isotopic dilution using C13-labeled mycotoxins is a good alternative to matrix match calibration that is quite tedious approach. It was also systematically used for the quantification of AF M_1, and only to quantify AFs and OTA at low levels of concentration. It was also applied to the quantification of SMC in various samples to correct matrix effects[115]. However, labeled toxins are very expensive and they are not available for all the studied toxins. By this fact, for the quantification of ZON, an analog of this compound was used as internal standard.

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