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Immunoaffinity solid-phase extraction for pharmaceutical and biomedical trace-analysis—coupling with HPLC and CE—perspectives

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Abstract

Immunoaffinity solid-phase extraction (SPE) technique is based upon a molecular recognition mechanism. The high affinity and the high selectivity of the antigen–antibody interactions allow the specific extraction and the concentration of the analytes of interest in one step. In pharmaceutical and biological fields, where most often matrices are complex and analytes at trace-levels, this approach constitutes a unique tool for fast and solvent-free sample preparation. This review presents a general description of this extraction technique and gives numerous examples of its applications in pharmaceutical and biomedical fields. It emphasizes the on-line coupling with chromatographic and electrophoretic separation techniques and introduces new developments. The future directions, especially with regards to the current development of analytical microsystems, are discussed.

Keywords: Review; Immunoaffinity extraction; Immunoextraction; Sample preparation

1. Introduction

Pharmaceutical and biomedical analysis concern a wide variety of analytes and matrices, as they cover a wide range of applications such as proteomic, diagnosis, drug discovery, investigation of intoxications, detection of potential drug abusers, control of drug addicts following withdrawal therapy, and risk assessment in occupational and environmental health. All these analysis have in common the complexity of the matrices and the low-concentration levels of the analytes. Consequently, time and solvent consuming sample preparation steps are often necessary prior to analytical determination of the target analytes.

For the last decade, conventional liquid–liquid extraction (LLE) has dropped dramatically following the obligatory reduction of chlorinated solvent usage. In parallel, intensive research in the area of solid-phase extraction (SPE) has promoted the development of new formats and new sorbents [1]. However, in most cases, analyte retention is based on hydrophobic

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interactions and co-extraction of analytes and matrice interferences occurs, which may become a major problem, especially when analytes are at trace-levels and interfering compounds at higher concentrations. Therefore, there is a considerable interest in having highly selective sorbents allowing extraction, concentration, and clean-up in a single step. Highly selective SPE sorbents involving antigen–antibody interactions have been thus developed and they are called immunosorbents (ISs).

In pharmaceutical and biological analysis, which constitute the focus of this paper and where immunological assays are commonly used, the approach involving antigen–antibody interactions is well-known. Nevertheless, the principles of immunoassays and immunosorbents are quite different. The strength of immunoassays is linked to their ability to detect highly sensitivity compounds in complex matrices without extensive sample preparation. This allows to carry out simple and rapid screens for identification of a small number of positive samples that may be present in the thousands of samples normally tested. However, immunoassays have some inherent drawbacks. First, a lack of homogeneity and activity in antibodies from varying commercial sources is drawback for this analytical method. Second, an analyte is often a member of a class of compounds and antibodies are not able to distinguish between structural analogues (with similar conformational shapes and electronic distributions), which induces wrong estimations of the levels of the target analytes, false-positives, and false-negatives. On the contrary, in immunoaffinity extraction, the cross-reactivity of antibody is advantageous because all compounds within a given class are extracted from the surrounding matrice and then separated and quantified individually by coupling with chromatographic or electrophoretic separation techniques. This is especially true for low-molar-mass analytes, which constitute the scope of this review.

The first extensive studies demonstrating the advantages of coupling immunoextraction to a separation technique, HPLC in the present case, have been made by Farjam et al. for the determination of estrogens and nortestosterone in plasma and urine, and aflatoxins in milk [2–7]. Nowadays, numerous studies have shown the great interest of immunoaffinity extraction in the pharmaceutical and biomedical fields. Applications which have illustrated the more clearly the high potential of ISs for class-selective extraction belong to environmental analysis: up to 12 triazines, 15 phenylureas, or 16 polyaromatic hydrocarbons (PAH) have been simultaneously extracted, separated by HPLC, and quantified by UV or fluorescence detection [8–33].

In this review, we present a general description of the immunoaffinity extraction technique and numerous examples of analysis of low-molar-mass compounds in pharmaceutical and biomedical areas are given. Thus, we emphasize the on-line coupling with chromatographic and electrophoretic separation techniques and introduce new developments. The future directions, especially with regards to the current development of analytical microsystems, are also discussed.

2. Immunoaffinity solid-phase extraction description

2.1. Immunosorbent

An immunosorbent is constituted of antibodies specific to the target analytes, immobilized on a solid-support. Thus, the first step in making an IS is to produce antibodies with the ability to recognize either one or a group of analytes. As compounds of low-molar-mass (<1.000 Da) are unable to evoke an immune response, they must be modified in a hapten, via the introduction of a functional group into the selected molecule, which can be linked to a carrier protein. To obtain antibodies with an appropriate specificity, the hapten design is fundamental.

Hapten design has often been based on trial-and-error assays: the conclusions are drawn after having produced and characterized the antibodies [34–36]. However, we have recently proposed a new tool for a better design of the hapten according to the desired specificity of antibodies for a group of structurally related compounds [37]. This approach involves molecular modeling followed by principal component analysis. In this study, we have characterized the specificity of three immunosorbents based on different monoclonal anti-triazine antibodies by extraction recoveries studies and with step-elution experiments. Both indicated that the anti-dichloroatrazine IS was specific of terbutylazine and cyanazine. The anti-atrazine IS was specific of the chlorotriazines, whereas the anti-ametryn IS could trap all the triazines. This

confirms the great influence of the hapten design on the specificity of the resulting antibodies, even if the target molecules are small. Thus, the steric and functional parameters of triazines, metabolites, and immunoconjugates were calculated using molecular modeling and principal components analysis was used to convert the data into distribution maps with the relative position of each immunoconjugate and all the target analytes. In the three cases, conclusions on specificity made with the analysis of the maps fitted well with the experimental results. Thus, this study demonstrated that it was possible to optimize the hapten design for good trapping of only one analyte or a group of structurally related analytes. This tool may be useful in the pharmaceutical and biomedical fields, even if numerous antibodies have already been produced for immunoassays, and consequently a wide variety of antibodies are already commercially available. Indeed, it is important to keep in mind that, for immunoassays, one is looking for antibodies with a specificity rather limited to only one compound, whereas in immunoaffinity extraction, one would rather preferred class-specific antibodies.

Both polyclonal and monoclonal antibodies have been immobilized on ISs, as shown in Table 1, which presents examples of pharmaceutical and biomedical applications of immunoaffinity extractions. An increase in the use of monoclonal ones has been observed during the last few years. Although monoclonal antibody development is more costly, it guarantees a long-term production of reproducible antibodies without the need for further animals.

The selected antibodies are immobilized on an appropriate solid-support. This solid-support has to be chemically and biologically inert, easily activated, and hydrophobic in order to limit the non-specific interactions. The most common approach involves immobilization of the antibodies onto an agarose gel (Sepharose in general) or silica beads (cf. Table 1). Agarose-based ISs have a low back pressure resistance, thus requiring application of samples under gravity flow or very low flow rate. Therefore, they are appropriate for use in off-line coupling with separation techniques. On the contrary, silica-based ISs are pressure resistant and allow a simple on-line coupling with separation techniques. Other natures of solid-support are sometimes encountered in the literature, such as glass, alumina, or polystyrene-divinylbenzene, but they have not shown advantages compared with silica.

The immobilization conditions should keep the biospecific activity of antibodies. Suitable bonding conditions should be in aqueous media, at a pH of 0.5–2 units from the isoelectric point of the antibodies, with an ionic strength of 0.01–0.05, at a temperature of 4–25 °C, and with a short reaction time (less than 16 h). The most common approach consists in a covalent bonding; a random or oriented immobilization is obtained depending on the activation of the solid-support and of the antibodies. However, a non-covalent bonding can also be used to couple antibodies to a sorbent, such as streptavidin, protein A, and protein G-based sorbents. These aspects have been discussed with more details in our previous reviews [38–40].

2.2. Immunoextraction procedure

The immunoextraction procedure is constituted of distinct steps: (i) conditioning; (ii) percolation of the sample; (iii) washing; (iv) elution of the target analytes; and (v) regeneration.

(i) The conditioning step allows to remove the storage solution, most often a phosphate buffer saline solution (PBS, 0.01 M sodium phosphate buffer with 0.15 M sodium chloride, pH 7.4) containing a small percentage of azide. It has to be done with an appropriate solution to create a favorable environment for specific interactions between the target analyte(s) and the solid sorbent. Thus, aqueous solutions, pure or containing a small organic solvent percentage, are generally used. This step can be constituted of different solutions with transitory properties from the ones of the storage solution to the ones of the sample.

(ii) The second step is the percolation of the sample. In pharmaceutical and biomedical fields, where matrices are complex, the percolated samples on the IS result most often of previous pretreatment steps, such as centrifugation, filtration, dilution, and modification of the pH. As an example, Schedl et al. diluted 1:1 urine aliquots with 0.1 M sodium acetate buffer (pH 5) [92], and Zhao et al. centrifuged the collected blood samples and then diluted 1:25 the supernatant with 0.01 M PBS [54] prior to the immunoextraction step. These samples are then percolated onto the IS.

Table 1
Examples of pharmaceutical and biomedical applications of off and on-line immunoextraction

Analyte(s) used for antibody production	Single or class-selective	Matrice	Lod	Elution solution	Analytical method	Coupling	Antibody type	Solid-support (activation)	Reference
Aflatoxins	C	Urine	50 ng/l	MeCN	HPLC–EC	Off	N.A.[a]	*Aflaprep (Rhônediagn. Tech.)*	[41]
Aflatoxins	C	Urine	7–18 pg/ml	MeCN	HPLC–Fluo	Off	Mono	*Aflaprep (Rhônediagn. Tech.)*	[42]
Aflatoxins	C	Urine	1 pg/ml	MeCN	HPLC–Fluo	Off	Mono	*Aflaprep (Rhônediagn. Tech.)*	[43]
Aflatoxin B1-N^7-guanine	S	Urine	2 μg/ml	DMSO–PBS (7/3)	ELISA	Off	Poly	Sepharose 4B (CNBr)	[44]
β-Amanitin	C	Plasma, urine	2.5 ng/l	MeOH–acetone (1/1)	HPLC–MS	Off	Poly	Sepharose 4B (CNBr)	[45]
Ampicillin, cloxacillin	C	PBS	N.A.	MeOH or glycine buffer-HCl 0.1 M (pH 2.5)	ELISA	Off	Mono	Sepharose 4B (CNBr)	[46]
Avermectin B1	S	Plasma, meat, pear	2 μg/l	MeOH	HPLC–UV	Off	Poly	Sepharose 4B (CNBr)	[47]
Avermectin B1	S	Liver	2 μg/kg	MeOH	HPLC–UV	Off	Poly	Sepharose 4B (CDI)	[48]
Avermectin B1	C	Liver	5 μg/kg	MeOH	HPLC–MS	Off	Poly	Sepharose 4B (CDI)	[49]
Benzimidazole (2 haptens)	C	Hepatocytes, liver	50 ng/ml	EtOH–water (7/3)	LSC	Off	Mono	Agarose (aldehyde)	[50]
Benzodiazepin	C	PBS	N.A.	PBS–acetic acid (98/2)	RAM–HPLC–MS	On	Mono, poly	Support (protein G)	[51]
Benzodiazepin	C	Urine	2 ng/ml	MeOH–water (9/1)	HPLC–UV	On via C_{18}	Poly	Support (glutardialdehyde)	[52]
Benzo(a)pyrene	C	Urine	0.7 pg/ml	MeOH	HPLC–Fluo	Off	Mono	Affinica kit (oriented immobilization)	[53]
Bisphenol A	S	Serum	N.A.	MeOH–water (4/1)	HPLC–UV	Off	Poly	Sepharose 4B (CNBr)	[54]
(*R*) and (*S*) bufuralol	C	Plasma	50 ng/ml	MeOH–acetic acid 0.01 M (pH 5) (95/5)	HPLC–UV	Off	Poly	Agarose (NHS)	[55]
Chloramphenicol	S	Muscle	10 μg/kg	MeOH	HPLC–UV	Off	Mono	Sepharose 4B (CNBr)	[56]
Chloramphenicol	S	Milk, egg	20 ng/kg	Glycine 0.2 M-NaCl 0.5 M (pH 2.8)	HPLC–UV	Off	Mono	Silica (CDI)	[57]
Chloramphenicol	C	Muscle, liver, kidney, urine	0.2–2 μg/kg	EtOH–water (7/3) or glycine 0.2 M-NaCl 0.5 M (pH 3)	GC–ECD	Off	Poly	Agarose	[58]
Chlortoluron	S	river and tap water, plasma, urine	0.1 μg/kg	EtOH–PBS (1/1)	HPLC–UV	Off	Poly	Silica (aldehyde)	[18]
Clenbuterol	S	Meat, liver	0.02–0.04 ng/g	EtOH–water (8/2)	HPLC–MS-MS	Off	N.A.	*RIDA Clenbuterol (R-Biopharm)*	[59]
Clenbuterol	C	Urine	50 ng/l	Acetic acid 2%	HPLC–MS–MS	On via C_{18}	N.A.	Sepharose (protein G)	[60]

Clenbuterol	S	Kidney, muscle	0.3 ng/g	EtOH–water (8/2)	HPLC–UV	Off	Poly	*RidaScreen (Bioman Products)*	[61]
Cortisol	S	Urine, serum	1 ng/μl	MeOH–water (6/4)	HPLC–UV, LSC	On	Poly	Silica	[62]
Cytokine	C	Plasma, blood, urine, saliva	N.A.	Citric acid 0.1 M (pH 2)	HPLC–Fluo	On	Mono, Fab	Silica beads (streptavidin coating)	[63]
Cytokine	C	Microdialysis of cell	fg/μl	0.1 M phosphate buffer pH 1.5	CE–LIF	On	Mono, Fab	Fused-silica capillary	[64]
10-Deacetylbaccatin III	C	*Taxus* needles, cell culture	N.A.	1 M acetic acid–MeOH (1/4)	HPLC–UV	Off	Poly	Sepharose 4B (CNBr)	[65]
Dexamethasone	C	Urine	0.1 ng/ml	MeOH–water (1/1)	HPLC–UV, GC–MS	On via C_{18}, off	N.A.	*Chromsep (Randox Laboratories Ltd.)*	[66]
Dexamethasone	C	Urine	3 ng/ml	MeOH–propionic acid 1 M (1/1)	HPLC–MS	On	Poly	Poly(hydroxyethylmethacrylate)	[67]
Dexamethasone and methylprednisolone (mixture)	C	Milk, liver, urine, faeces	0.25 μg/l	MeOH–water (8/2)	GC–MS	Off	N.A.	Tresyl gel	[68]
Diethylstilbestrol	C	Urine, plasma	10 ng/l	Acetone–water (95/5)	GC–MS	Off	Poly	Sepharose 4B (CNBr)	[69]
Diethylstilbestrol, zeranol, clostebol, nortestosterone (NT), fluoxymesterone, trenbolone, methylNT, ethynylestradiol (mixture)	C	Urine, faeces	2 ng/ml	MeOH–water (8/2)	GC–MS	Off	Poly	Sepharose 4B (CNBr)	[70]
Digoxin	S	Serum	0.3 ng/ml	Hydrochloric acid 1%	HPLC–Fluo	On	Poly	Silica	[71]
1α,25-Dihydroxyvitamin D_3	C	Plasma	N.A.	MeOH-water (95/5)	RRA	Off	Poly	Sepharose 4B (CNBr)	[72]
1-Amino-3,7,8-trichlorodibenzo-*p*-dioxin	C	Serum	N.A.	Tween-20 1%	GC–MS	Off	Poly	Sepharose 4B (CNBr)	[73]
2,3,7,8-Substituted-dioxin	C	Serum	N.A.	Acetone–water (1/1)	GC–MS	Off	Mono	Sepharose (CNBr)	[74]
Forskolin	C	Roots and cultures of Coleus forskohlii	1 ng/ml	PBS–MeOH (55/45)	ELISA	Off	Mono	Sepharose 4B (CNBr)	[75]
Ginsenoside Rb1	S	Roots	N.A.	MeOH–acetic acid (0.1 M), 0.5 M KSCN (1/4) pH 4	ELISA	Off	Mono	Agarose (hydrazide)	[76]
Gonadotropin-releasing hormone	S	Serum, urine	1 ng/ml	0.3 M Glycine–HCl buffer pH 2.5	CE–MS	On	Poly, Fab	Glass	[77]

Table 1 (*Continued*)

Analyte(s) used for antibody production	Single or class-selective	Matrice	Lod	Elution solution	Analytical method	Coupling	Antibody type	Solid-support (activation)	Reference
Imidazolinone	C	Urine, kidney, seed, fodder, wheat	N.A.	Water–MeOH (7/3)	HPLC–UV, LSC	Off	Mono, poly	Polymer (hydrazide)	[78]
LSD	C	Urine	2.5 ng/l	Acetic acid 2%	HPLC–MS-MS	On via C_{18}	Mono	Silica (protein G)	[79]
LSD	S	Blood, urine	39 pg/ml	EtOH or NEt_3 0.1 M	LSC	Off	Poly	Agarose (protein A)	[80]
LSD	S	Serum, hair	N.A.	MeOH	HPLC–Fluo	Off	Mono	*ImmunElute (Microgenics)*	[81]
Methamphetamine	S	Urine	N.A.	0.05 M Sodium acetate buffer pH 3.5	CE–UV	Off	Mono	Glass (DITC)	[82]
Morphine (Mo), Mo-3-β-D-glucuronide (G), and Mo-6-β-D-G (mixture)	S	Blood	3 ng/g	MeOH–glycine buffer 0.2 M, NaCl 0.05 M, HCl (pH 2) (9/1)	HPLC–Fluo	Off	Poly	Tris-acryl polymer (CDI)	[83]
Neuropeptides	C	Tear	N.A.	0.1 M Phosphate/0.2% Nonidet 40 buffer, 25 mm glycine pH 1.5	CE–LIF	On	Mono Fab	Fused-silica capillary	[84]
Normorphine	C	Urine	50 ng/ml	PBS (pH 4)–EtOH (6/4)	HPLC–EC	Off	Poly	Silica (aldehyde)	[85]
17β-19-Nortestosterone	C	Urine	50 ng/l	Aqueous solution of norgestrel (190 μg/l)–MeCN (95/5)	HPLC–UV	On via C_{18}	Poly	Sepharose 4B (CNBr)	[2]
17β-19-Nortestosterone	C	Urine, bile, meat, liver, kidney, tissues	0.05 μg/kg	Aqueous solution of norgestrel (250 μg/l)–MeCN (95/5)	HPLC–UV, GC–MS	On via C_{18}	Poly	Sepharose 4B (CNBr)	[3]
Ochratoxin	C	Blood, serum, milk, food	5–10 pg/g	MeOH	HPLC–Fluo	Off	Mono	*EASY-EXTRACT (Rhônediagn. Tech.)*	[86]
Ochratoxin	S	Urine	5 pg/g	MeOH	HPLC–Fluo	Off	N.A.	*OchraTest (Vicam)*	[87]
17β-Estradiol	S	Plasma, milk	1–10 pg/ml	Water–acetone (5/95)	LSC	Off	Poly	Sepharose 6B (CNBr)	[88]
β-Estradiol	S	Serum	2 ng/ml	MeOH–water (8/2)	HPLC–UV	On	N.A.	Alumina coating with an hydrophilic polymer (hydrazide or aldehyde)	[89]

Oestrogen	C	Urine	200 ng/l	Water–MeCN (95/5) with 17-β-estradiol and 17-β-estradiol-17-acetate (260 μg/l each)	HPLC–UV	On via C_{18}	Poly	Sepharose 4B (CNBr)	[4]
17β-Estradiol and estrone	C	Wastewater	0.1 ng/l	MeOH–water (7/3)	HPLC–MS	Off	Mono	Glass (hydrazide)	[90]
Phenitoin	S	Plasma	41 mg/l	Phosphate buffer 0.01 M (pH 7,4)–EtOH (6/4)	HPLC–UV	On	Poly	Silica (ester)	[91]
Pyrene	C	Urine	1–16 ng/l	MeCN–water (1/1)	HPLC–UV-vis, GC–MS	Off	Poly	Silica sol-gel	[92]
S-8921[b]	S	Urine	0.1 ng/ml	MeCN–trifluoroacetic acid (99.9/0.1)	RIA	Off	Poly	Tresyl gel	[93]
Salbutamol	C	Urine	4 ng/ml	EtOH–acetate buffer 0.03 M (pH 4) (8/2)	HPLC–EC	Off	N.A.	*Fractoprep*	[94]
Salbutamol	C	Liver, kidney, muscle	N.A.	EtOH–acetic acid (8/2)	ELISA	Off	Mono	Sepharose (divinylsulfone)	[95]
Salbutamol	C	Liver, kidney, muscle, hair	N.A.	EtOH–acetic acid (8/2)	ELISA, GC–MS	Off	Mono	Sepharose (divinylsulfone)	[96]
Sarafloxacin	C	Serum	5 ng/ml	Acetic acid 2% + HCl (pH 2.2)	HPLC–Fluo	On	Mono	Polystyrene–divinylbenzene	[97]
Sarafloxacin	C	Liver	4–8 ng/g	Acetic acid 2% pH 2.2	HPLC–Fluo	On	Mono	Polystyrene–divinylbenzene	[98]
Sarafloxacin	C	Serum	1 ng/ml	Acetic acid 2% + 0.1 M sodium phosphate pH 6	Fluo	On	Mono	Polystyrene–divinylbenzene	[99]
Solanidine	C	Serum	1 ng/ml	MeOH	MS	Off	Poly	Silica (carboxylate), Sepharose (CNBr)	[100]

Table 1 (*Continued*)

Analyte(s) used for antibody production	Single or class-selective	Matrice	Lod	Elution solution	Analytical method	Coupling	Antibody type	Solid-support (activation)	Reference
3β-Hemiglutaramide-testosterone (3-He-T), 5-He-T	C	Urine	N.A.	EtOH–water (8/2)	GC–MS	Off	Poly	Sepharose (CNBr)	[101]
Tetracycline	S	Milk	19 μg/l	MeOH	Fluo	Off	Mono	Agarose (hydrazide)	[102]
D^9-Tetrahydrocannabinol	C	Saliva	20 ng/ml	MeOH–NaCl 0.15 M (82/18)	HPLC–UV	On	Poly	Silica (epoxy)	[103]
Tetrodotoxin	S	Urine	2 ng/ml	Acetic acid 1%	HPLC–Fluo	Off	Mono	Sepharose (NHS)	[104]
Trenbolone	C	Urine	2 ng/ml	MeOH–water (7/3)	HPLC–UV	Off	N.A.	*(Randox Labs)*	[105
Troponin I	S	Serum	2 nmol/l	35 mm Tris-acetate + 7 mm β-mercaptoethanol + 5 mm EDTA + 2 M urea pH 3.5	CE–UV	On	Mono	Silica (glutardialdehyde)	[106]
Vitamin B12	S	Fermentation broth	N.A.	HCl + 0.15 M NaCl-MeOH (7/3)	HPLC–UV-vis	On	N.A.	Alumina coating with an hydrophilic polymer (hydrazide or aldehyde)	[89]

In italic: commercial ISs. Abbreviations: C, class-selective; CDI, carbonyl diimidazole; DITC, 1,4-phenylene diisothiocyanate; EC, electrochemical detection; ECD, electron capture detection; ELISA, enzyme linked immunosorbent assay; LIF, laser-induced fluorescence; LSC, liquid scintillation counter; NHS, N-hydroxy-succinimide; RRA, radioreceptor assay; S, single selective.

[a] N.A.: not available.

[b] S-8921: methyl-1-(3,4-dimethoxyphenyl)-3-(3-ethylvaleryl)-4-hydroxy-6,7,8-trimethoxy-2-naphtoate.

During the percolation step, it is important to prevent the loss in recovery of the target analyte(s), which can result from an overloading of the IS capacity and/or a low affinity of antibodies towards compounds. The capacity of an IS is defined as the maximal amount of analyte–antigen that can be bound onto the sorbent during the percolation of the sample. It is directly linked to the total number of specific, active, and accessible antibodies immobilized on the solid-support. This parameter can be easily measured experimentally [38–40]. Depending on the affinity of the antibodies, the maximum sample volume that can be percolated without loss in recovery, so-called breakthrough volume, varies. With biological matrices, for which the available volumes of samples are in general small, the percolated volume varies from 25 μl [62,63,91] up to 66 ml [56] in the examples of Table 1, with an average range of 1–10 ml. It is less than in the environmental field where the average range is rather 5–25 ml, and thus where the breakthrough volume value may be more easily reached. Nevertheless, even in pharmaceutical and biomedical fields this parameter has to be taken into account, especially because the presence of salts in the sample can reduce it [107].

An other parameter is the percolation flow rate, which can affect recoveries of analyte(s), especially with low-performance sorbent because of their slow mass transfer properties. During the sample percolation step, high flow rates may prevent analytes from binding to the immobilized antibodies [28,47,56]. As an example, an increase in recovery of an analyte from 25 to 95% was observed as the flow rate decreased from 2 to 0.2 ml/min [28].

During percolation, non-specific interactions can occur between analyte(s) and interferences present in the sample and the IS (solid-support and/or immobilized antibodies). This may be especially relevant in the biological field where matrices are complex. Most commonly, hydrophobic and ionic forces are responsible of undesirable adsorption, which results in a decrease of selectivity and detection limits. Agarose and silica-based ISs have been shown to minimize the interactions generated by the sorbent itself, especially when the bonding procedure is followed by the neutralization of the remaining free activated groups. However, in order to evaluate if the binding of the analyte(s) is due to specific or non-specific interactions, one can apply the same extraction protocol to a control sorbent, it means a sorbent treated in a similar manner as the immunoaffinity sorbent, but where immobilized antibodies have no affinity for the target analyte(s). Theodoridis et al. used this approach to demonstrate that the extraction of paclitaxel, a potent anticancer drug, from a plant extract with the devoted IS was mainly governed by non-specific interactions [65]. The retention of target analyte(s) and interferences by non-specific interactions can be limited when a small percentage of organic solvent or detergent, such as Tween-20 or Triton-X is added to the initial sample prior to percolation [24,29,31,32,71,98]. As an example, Holtzapple et al., in order to limit the non-specific adsorption of interferences affecting the chromatographic profile corresponding to the analysis of fluoroquinolones in chicken liver after immunoextraction, tested the addition of non-specific antibodies, 0.1% Tween-20, and up to 10% methanol [98]. Effective extraction and analysis of fluoroquinolones were finally achieved by the addition of 5% methanol in the sample. An other approach consists in placing a sorbent bonded with non-specific antibodies prior to the immunoaffinity sorbent to effectively remove matrice components that otherwise would bind non-specifically to the IS and interfere with the sample analysis [99].

(iii) The third step of an immunoextraction protocol is the washing step, which aims the removal of the potential interferences without eluting the target analyte(s). The percolation of some ml of a solution with properties close to the sample ones is in general sufficient.

(iv) The step is the elution of the target analyte(s). Different approaches are possible to induce the disruption of the analyte–antibody complexes, as it can be seen in Table 1: displacer agents, chaotropic agents, pH variations, water-organic modifier mixtures, or every associations of them. A displacer is a highly concentrated cross-reacting molecule able to induce a biospecific

desorption; in a large concentration excess, it competes with the target analytes to occupy the binding sites of the antibodies. This type of desorption has been barely used in immunoaffinity extraction [2–4]. Indeed, for optimum performance, the displacer has to meet severe and numerous criteria [38]. The use of chaotropic agents and/or pH variations is more extended, because it is usually done with target proteins. Nevertheless, it is not an efficient means to desorb small analytes: it requires often a large elution volume and induces a decrease of the enrichment factor [2,8]. We think that it is probably because the desorption of target proteins is partly based on changes in their own structure, in parallel with the changes in the structure of the immobilized antibodies; but small molecules are not sensitive to denaturation and need much more rigorous elution conditions. This is why in Table 1 only 25% of the elution solutions are purely aqueous; the 75% remaining ones involve an organic solvent. For commercial ISs (in italic in Table 1), the suppliers recommend always the partial or total use of an organic solvent (methanol, ethanol, and acetonitrile) for elution.

(v) Immunosorbent reusability requires a regeneration step. When antibodies are not covalently bound to the solid-support, the elution step may have washed away the antibodies. In such a case, a simple step involving antibodies dissolved in an appropriate solution can regenerate the IS surface state by simple percolation. With a covalent immobilization, the chemical bond between the antibody and the sorbent surface greatly increases the stability of antibodies, even in contact with harsh eluting conditions. Indeed, we have observed, via capacity measurements, which reflect the number of active antibodies, the complete regeneration of an anti-isoproturon IS after 2 days of storage in PBS at 4 °C after elution of the target analytes with 70% methanol [107]. Even if the kinetic is quite long, the full regeneration of an IS seems possible when organic solvents are used for elution. However, this may necessitate an organization that may not suit routine analysis laboratories and the reusability of ISs is not recommended by suppliers when complex samples are analyzed.

This described immunoextraction procedure allows an efficient extraction and concentration of target analyte(s) present in matrices, even complex. It is a practical alternative method for sample clean-up. It reduces the time and solvent consumption involved in traditional methods. As an example, Huwe et al. used in total, for a 25 ml serum sample, 3 ml acetone and 3 ml methylene chloride with the immunoaffinity extraction protocol compared 100 ml sulfuric acid and 1500 ml organic solvents with the conventional clean-up, and a sample can be processed in about 2 h instead of 24 h [74]. Moreover, immunoaffinity extraction can be coupled easily with a chromatographic or electrophoretic separation technique and give well-resolved and clean chromatograms.

3. Hyphenation with separation techniques

To take advantage of the cross-reactivity offered by the antibodies, many authors have analyzed the eluted solution from IS by chromatographic or electrophoretic techniques to separate and detect the extracted analytes (cf. Table 1). In that case, the high selectivity of the bioassays is combined with the high-resolution power of separation techniques. This can be applied either off or on-line. Table 2 presents the advantages and drawbacks of each approach.

3.1. Off-line immunoaffinity extraction

In the off-line method, the IS is often packed into a disposable cartridge. The previously described protocol is carried out and the resulting eluted extract is then partially injected into the desired separation system. This approach is very simple to carry out and does not require any adaptation of experimental set-ups or protocols. It is exactly like a conventional SPE process, but involving a much higher selectivity. Fig. 1 illustrates the relevant improvement in selectivity brought by an efficient IS compared to a classical SPE sorbent: a serum sample was percolated on a C_{18} sorbent or an anti-melatonin IS, and the resulting eluted extracts were analyzed by HPLC–MS [108].

This concept offers a high flexibility concerning the number of injections and of separation techniques that can follow the immunoextraction step. Indeed, Schedl et al. have analyzed urine immunoextracts containing

Table 2
Advantages and drawbacks of off and on-line coupling of immunoaffinity extraction with a chromatographic or electrophoretic separation method

	Advantages	Drawbacks
Off-line	Concentration post-extraction Use of complementary techniques for confirmation No limitation for the choice of the immunosorbent solid-support	Important sample handling Human exposure Analysis of only a fraction of the eluted solution from immunosorbent
On-line	Minimal sample handling Minimal solvent consumption Automatable Analysis of the total amount of the extracted analytes	Required compatibility of elution and analytical solutions Potentially reduced analyte resolution, broader peaks and substantial component tailing

PAH by HPLC–Fluo and, after a derivatization step, by GC–MS for confirmation [92]. It allows also to reach a higher enrichment factor. As an example, Ferguson et al. eluted the extracted analytes from 1 l wastewater with 4 ml of 70% methanol in water [90]. The IS eluent was thus evaporated to dryness and reconstituted in 200 μl of 25% acetonitrile in water. The enrichment factor raised from 250 to 5000. In the present case, the off-line approach allows also to add internal standards to the purified extract just prior to the analytical step.

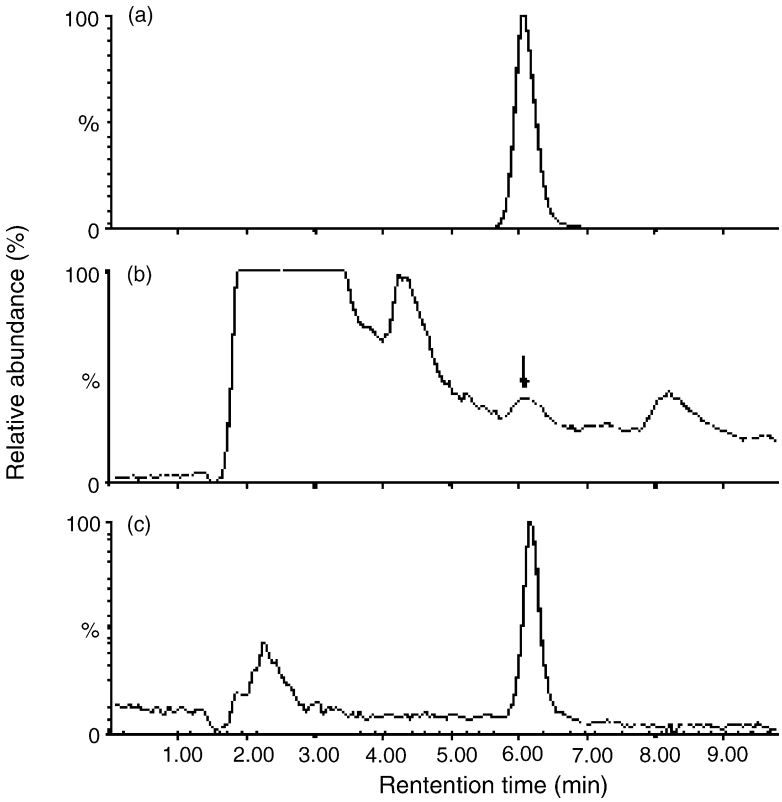

Fig. 1. Comparison of HPLC–MS analyses of (A) melatonin standard and a human serum sample processed (B) by SPE (C18 sorbent, the arrow indicates the melatonin peak) or (C) by an anti-melatonin IS. With permission, from [108].

Nevertheless, an increase of sample handling increases proportionally the risk of loss of analytes (walls of Eppendorf, pipette tips, evaporation, etc.). Especially true with biological matrices, excessive handling can also lead to denaturation, aggregation, or ageing of the sample, which yield poor recoveries. The off-line approach may result in a more important human exposure to previously unidentified materials of sometimes unknown toxicity too. For these considerations, the on-line system can be advantageous even if it is shown in Table 1 that more than 65% of the applications in pharmaceutical and biomedical fields have been carried out with an off-line approach.

3.2. Hyphenation with HPLC

For the on-line coupling of immunoextraction with HPLC, the IS is packed into a precolumn which is located in a six-port switching valve (Fig. 2A). In the load position, the immunoextraction sequence is performed and, in the inject position, the precolumn is connected to the analytical column, thus allowing desorption and transfer of the retained compounds by the mobile phase of the HPLC separation. With this configuration, the solid-support of the IS has to be pressure resistant.

Fig. 2B presents the on-line set-up used if the solid-support is not pressure resistant. It prevents a direct connection of the immuno-precolumn to the analytical column. This system has also been used when desorption is achieved with an aqueous solution of low pH that requires a large volume of eluent [28]. In this case, it is necessary to use a second precolumn, a classical one (C_{18} or polymer), to reconcentrate analytes between the immuno-precolumn and the analytical one.

These on-line experimental set-ups are quite simple, automatable, limit sample handling and solvent consumption, and the wholeness of the retained compounds by the antibodies is analyzed. Using a precolumn packed with either anti-isoproturon antibodies-based silica or a non-selective polymeric sorbent (PLRP–S), the excellent selectivity toward isoproturon and its metabolites has been demonstrated with direct extraction, analysis, and detection in spiked urine (cf. Fig. 3) [33]. In this case, after a washing with water, the immuno-precolumn was directly connected to the analytical column and an

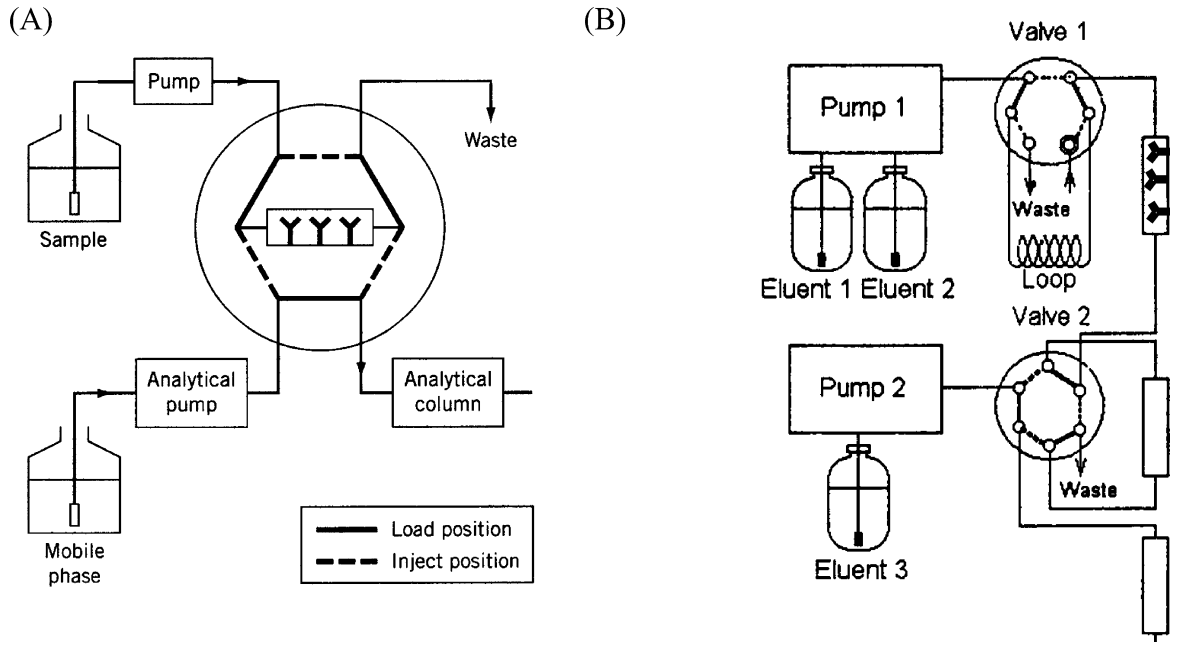

Fig. 2. Schematic of an on-line coupling of an immuno-precolumn with an HPLC analytical column (A) without and (B) with an intermediate trapping column. With permission, from [39].

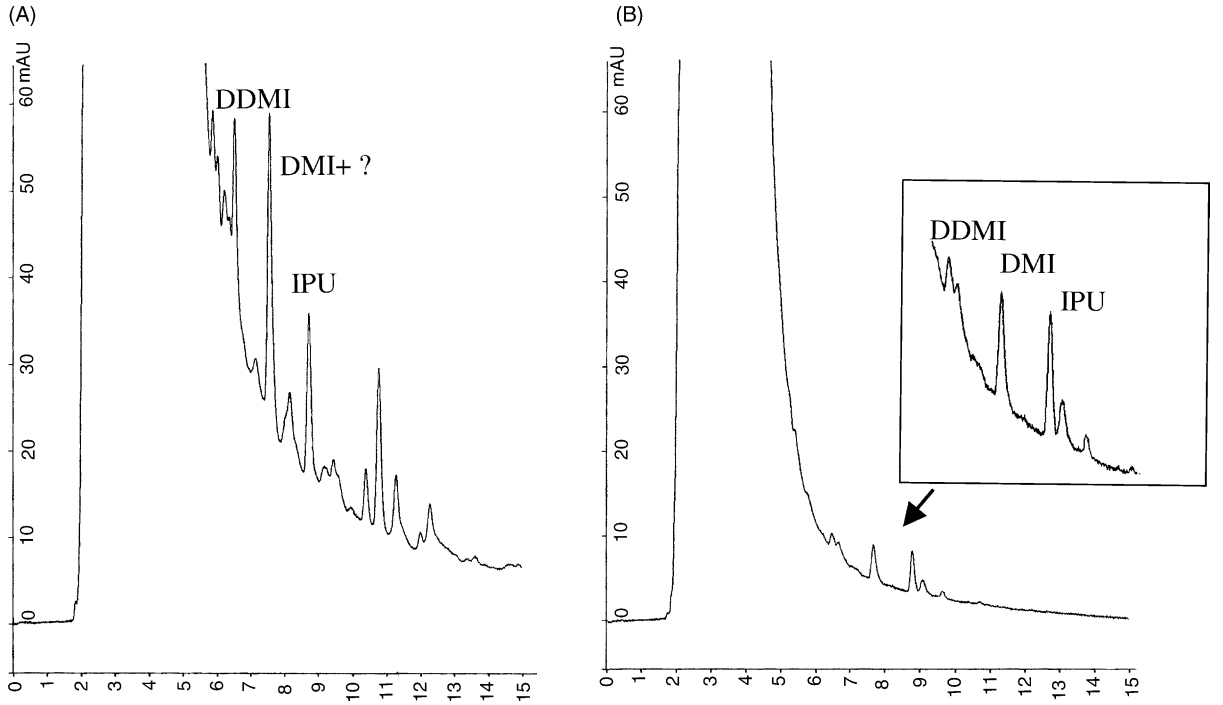

Fig. 3. Chromatograms obtained after percolation of 25 ml urine, spiked at 5 μg/l with isoproturon, demethylisoproturon (DMI), and di-demethylisoproturon (DDMI), and diluted 1:1 with PBS, through (A) PLRP-S and (B) anti-isoproturon IS, and analysis by HPLC–UV. With permission, from [33].

acetonitrile-phosphate buffer (pH 7.4) gradient allowing the analytical separation was percolated through the system. In order to prevent long contact of IS with high amount of acetonitrile, the connecting valve was switched off after 2 min. This system requires thus an efficient desorption of the retained analytes by the initial composition of the mobile phase at the beginning of the analytical gradient. This results often from a compromise between an elution of the fixed compounds from the IS in a small volume and a good separation of the analytes.

In Table 1, a few studies have involved a mass spectrometric detection after the immunoextraction and the HPLC separation. It is important to remind here that the presence of coextracted interferences from sample matrice can greatly suppress the formation of analyte ions in the electrospray process, leading to a dramatic drop of sensitivity. This effect is shown graphically in Fig. 4. The estrogens, their surrogate standards, and the internal standard are clearly distinguishable in the summed ion chromatogram from an effluent waste water extract that was purified using the IS (Fig. 4B). The chromatogram of the extract from the identical effluent sample that was not purified by immunoextraction reveals no peaks for any of the analyte, even for the equilin-d4 internal standard, which was added to the sample just prior to analysis (Fig. 4A). This indicates that ionization of the analytes and standards was suppressed at a level approaching 100% in this particular sample. Since this process occurs in the MS source during ion formation, the use of MS–MS would not solve the problem. Thus the IS purification allows the removal of interfering sample matrice compounds that would cause severe ionization suppression and also substantially reduce the baseline noise, which increase the signal-to-noise ratio.

3.3. Hyphenation with CE

Capillary electrophoresis separation techniques are still in a growth stage, but they present already some

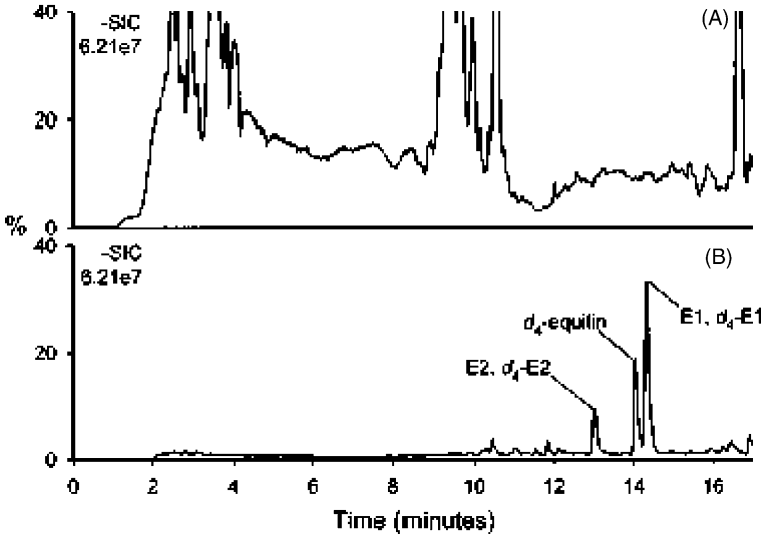

Fig. 4. Summed ion chromatograms of (A) raw and (B) IS-extracted sewage effluent, analyzed by negative polarity HPLC-ESI-MS. Both chromatograms are normalized to the same absolute intensity scale. E2: β-estradiol. d4-E2: β-estradiol-d4. E1: estrone. d4-E1: estrone-d4. With permission, from [90].

major advantages, such as an enormous resolution power and minimal sample volume requirements (typically, 1–20 nl), which can be useful for pharmaceutical and biomedical applications. The retention mechanism of CE is based on charge and size of the analytes whereas, in HPLC, it is mainly based on hydrophobic interactions with the stationary phase. Thus CE and HPLC can offer orthogonal, complementary analysis. Besides the sample pretreatment point of view, the hyphenation of immunoaffinity extraction with CE may counteract the low-sensitivity of CE, one of its usually major drawback, by the substantial concentration of analytes.

An hyphenated IS–CE device usually consists of an adsorptive phase at the inlet of the CE capillary and serves to enrich trace-levels of analytes, as well as allows on-line sample clean-up prior to component separation. Different technical solutions have been proposed to immobilize antibodies at the inlet of the CE system. The first and simplest concept consists in immobilizing antibodies on the walls of the capillary, either by adsorption on coated walls or by covalent binding to the modified capillary. In principle, a limited length of the capillary walls should be occupied by antibodies, so that an adequate length remains for CE separation.

The adsorptive binding is in general easier to achieve and opens up possibilities for the regeneration of the IS part. Ensing and Paulus showed that the use of a C_8-modified capillary was preferable over C_{18} and mercaptodimethylsilane-modified capillary [109]. They covered the first part of the capillary with anti-atrazine antibodies and the remainder was covered by adsorbed bovine serum albumin to eliminate non-specific interactions. The retained labeled-analytes were eluted with methanol-20 mm sodium tetraborate buffer pH 8 (25/75) without inactivation or mobilization of the antibodies. Moreover, the methanol caused stacking of fluorescein-labeled atrazine in the sample plug by a factor of 30, which was very advantageous for the quantification part. This protocol was carried out by an other team and they obtained a 1000-fold increase in detection level normally attainable using CE alone [110].

The covalent binding of antibodies to capillary walls has also been carried out [64,84]. Fig. 5 presents a diagrammatic representation of the immunoaffinity extraction hyphenated with CE, where only Fab fragments were covalently immobilized on one-third of the capillary. One hundred nanoliters of micro-dissection samples were introduced into the capillary via vacuum-injection and allowed to come

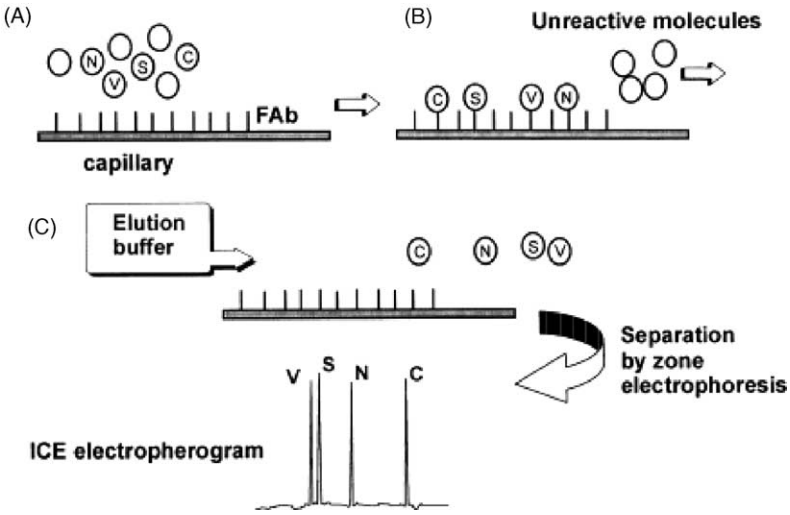

Fig. 5. Diagrammatic representation of the immunoaffinity extraction coupled to CE technique. (A) Analyte percolation and capture phase. (B) Washing of non-retained compounds. (C) Acid elution of the analytes and separation by CE. V: vasoactive intestinal peptide. S: substance P. N: neuropeptide Y. C: calcitonin gene-related peptide. With permission, from [84].

into direct contact with the immobilized antibodies for 5 min (Fig. 5A). The capillary was then purged with 200 μl of 100 mM phosphate buffer pH 7.4 containing 0.2% of the non-ionic detergent Nonidet-40. This step removed all extraneous unbound compounds (Fig. 5B). The electro-elution was performed with 100 mm phosphate/0.2% Nonidet-40 buffer, adjusted to pH 1.5 by the addition of 25 mm glycine. During this phase, the acidic buffer plus the electrical current disrupted the Ag–Ab complexes. Once free, the neuropeptides have been analyzed by CE–LIF. This approach allows the detection of regulatory molecules in sub-microliter quantities. One difficulty of this kind of extraction-separation is linked to the potential adsorption on capillary walls of proteins and peptides present in the samples. Manipulations such as adjustments of pH and chemical modifications of the capillary walls can circumvent it.

As the number of immobilized antibodies on capillary walls is restricted, inducing a low capacity, a second approach consists in a short packed bed of porous beads (silica or glass), containing covalently immobilized antibodies, directly integrated within the capillary [77,82,106]. The presence of solid-support into a capillary often requires frits to maintain the particles. Their preparation can be laborious and induce degradation of antibodies located next the frits, during their fabrication by heating. Frits can also generate irregular electro-osmotic flow and irreproducible migration times. Rashkovetsky et al. have immobilized antibodies on magnetic beads (supermagnetic polystyrene microspheres of 2.8 μm uniform size), kept in place in the capillary by a magnet [111]. This judicious approach does not require frits and provides an automatable way for the replacement of the solid-phase. This system has been successfully evaluated with immunoextraction of monoclonal mouse antibodies against human growth hormone with immobilized sheep anti-mouse immunoglobulins G. A priori, it could be similarly used for low-molar-mass analytes.

Thus, even if the hyphenation of immunoextraction with CE has not been commonly used yet, some possibilities exist. Nevertheless, one can not deny the higher degree of technical and experimental complexity compared to the hyphenation with HPLC, explaining that IS–CE hyphenation is still in its infancy whereas IS–HPLC is routinely used. However, the potential of this approach for microscale operation is huge (low sample and reagent consumption, automation, short analysis times, and enormous resolution power) and will contribute to the further dissemination of the use of Ag–Ab interactions in conjunction with CE.

4. New developments

4.1. Hyphenation with MS

Immunoextraction can be directly hyphenated with MS, without an intermediate chromatographic or electrophoretic technique. This could be carried out by a direct introduction of the eluting solution from the IS to the mass spectrometer, as it has been done by Holtzapple et al. with a UV detector [99]. In this case, the two targeted fluoroquinolones were separated from each other by the IS, during the elution step, on the basis of the differences in their relative affinities for the covalently attached antibodies. A similar approach could be tested with an MS detector, even if the eluted analytes are not well-resolved after elution from IS, because MS can be considered as a separation technique.

Drieger and Sporns have explored the feasibility of using MALDI–TOF–MS after an immunoaffinity extraction step, to detect individuals potato glycoalkaloids in serum [100]. They have evaluated several sample purification strategies with antibody-coated silica and agarose beads used in batch after a classical SPE step. The optimized conditions allowed the detection of solanine and chaconine down to 1 ng/ml of serum. The advantage of this approach is the speed, the possibility of unambiguous analyte identification based on molecular mass, and the opportunity to identify metabolites. In principle, the same approach can be used to detect sub-parts per million drugs and other compounds in different kinds of matrices.

4.2. Enantioselective immunoaffinity extraction

There is an increasing need for efficient enantioselective extraction and separation of chiral drugs and it has been shown that immunosorbents are perfectly adapted for that purpose [55,112,113]. As an example, Ikegawa et al. produced two batches of anti-bufuralol antibodies (anti-1S) and (anti-1R) and the relative affinity of both resulting antisera was determined with an enzymatic immunoassay [55]. Both antisera were characteristic of discriminating the corresponding antipodes with a cross-reactivity of a few percent or less. The resulting ISs allowed enantioselective immunoaffinity extraction of bufuralol and its metabolites in human plasma, followed by HPLC–UV analysis. Similar results have been obtained with an IS constituted of anti-D-methamphetamine monoclonal antibodies to directly extract D-methamphetamine from urine samples [113]. Recently, recombinant antibody fragments have been generated for the drug diarylalkyltriazole that contains two chiral centres [112]. The immobilized antibody fragments have been used successfully for an efficient separation of two enantiomers of the drug.

4.3. Immunoaffinity solid-phase microextraction

As the chemistry of antibody immobilization onto silica supports is now well-established, Pawliszyn et al. tried to combine the advantages of the immunosorbents with the solid-phase microextraction technique (SPME): a theophylline antiserum was covalently immobilized on the surface of a fused-silica fiber and used as a selective and sensitive extraction medium for the immunoaffinity solid-phase microextraction determination of theophylline in serum samples [114]. The theophylline immunoaffinity SPME fiber was positioned in a 1 ml vial containing a 100-fold diluted solution of serum with PBS and incubated for 3 h. After the incubation, the fiber was recovered from the solution, rinsed with PBS twice and placed in a scintillation vial for counting. An excellent agreement between the serum analysis and calibration curve results was observed.

This approach is simple, requires no extraction or desorption solvent, and presents a high potential of hyphenation, automation, and miniaturization. Improvement of the antibody immobilization procedure to increase the density of active antibodies and hyphenation with separation techniques are under way. This concept, once fully developed, could extend the existing immunoaffinity extraction techniques and their applications.

5. Future directions

Despite the current rapid progress in antibody engineering, the use of antibody fragments in immunoaffinity extraction has followed quite slowly. In Table 1, only four examples out of 71 have involved antibody fragments. However, in immunoaffinity extraction, only the antigen-binding domains are needed

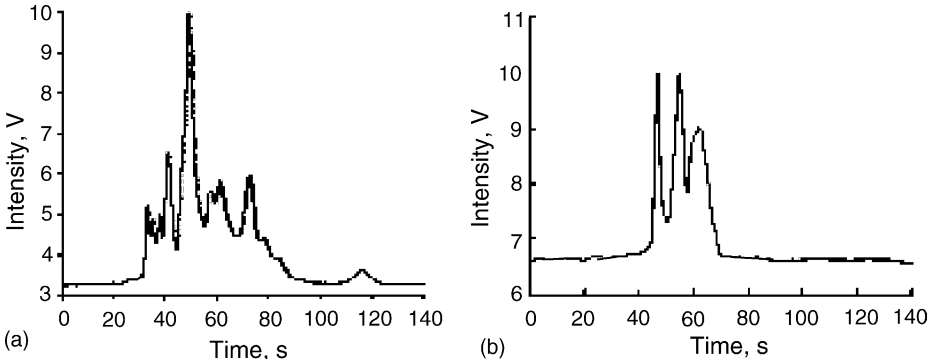

Fig. 6. On-chip separation of fluorescein isothiocyanate-labeled bovine serum albumin digest (A) before and (B) after affinity extraction. With permission, from [115].

for selective recognition. Indeed, antibodies subjected to partial enzymatic digestion to generate $F(ab)'_2$ fragments, subsequently reduced to the monovalent Fab antibody fragments, are still able to selectively extract the target analytes [63,64,77,84].

The use of antibody fragments allows to increase the number of binding sites per surface without causing steric hindrances. Consequently, the capacity, the breakthrough volume, and the linearity range of the resulting IS are increased, which means that higher enrichment factor and so better detection limit can be reached and quantitation in a more extended range can be done. There is so a great interest in immobilizing antibody fragments. In addition, the affinity, the specificity, and the stability of the antibody fragments can be improved by genetic engineering or using combinatorial mutagenesis coupled with the phage display technology [112]. Indeed, antibody affinity and specificity can be fine-tuned to recognize even minor differences in small organic molecules. The production of antibody fragments can be carried out in a relative large scale using microbial production systems, and various tags can be added to the engineered antibody fragments for efficient, pre-oriented immobilization on desired solid-supports.

An other future direction concerns the development of analytical microsystems, which is currently one of the major challenge in analytical chemistry and may play a role in the future of life science oriented research and development. The main incentives in miniaturization include a reduction of reagents and samples consumption, increased analytical performance, shorter analysis time, and high-throughput. The overall goal is progression towards a μ-total-analysis system (μTAS), whereby chemical information is periodically transformed into an electronic or optical signal, where analysis is carried out on a micrometer scale using centimeter-sized glass or plastic chips. However, samples from biological extracts will always be complex and target analytes at trace-levels. There is thus a great interest in adapting the immunoaffinity extraction technique to a micrometer scale.

A paper has recently reported affinity extraction in a chip format [115]. The study involved trypsin digestion, affinity extraction of histidine-containing peptides, and reversed-phase capillary electrochromatography of the selected peptides in a single polydimethylsiloxane chip. Copper (II)-immobilized metal affinity chromatography 5 μm-particles have been introduced into the chip. Frits have been fabricated in order to maintain the beads, with collocated monolithic support structures (COMOSS). They were able to trap particulate contaminants ranging down to 2 μm in size. Fig. 6 presents the on-chip separation of fluorescein isothiocyanate-labeled bovine serum albumin digest (A) before and (B) after affinity extraction. These results are very encouraging and offer challenging perspectives for the pharmaceutical and biomedical analysis.

6. Conclusions

The large number of examples presented in this review has demonstrated that immunoaffinity extraction

is a powerful and efficient tool in pharmaceutical and biomedical fields. The off or on-line coupling of immunoextraction with a chromatographic or electrophoretic separation allows to detect and quantify with a high sensitivity, selectivity, reproducibility, speed, and convenience a single analyte or a class of compounds, via the cross-reactivity of antibodies, contained initially in a complex matrice at trace-levels. New developments and future directions have shown the high potential of this technique and foresee the increasing place of this technique in analytical chemistry.

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