REVIEW

Approaches in Sample Handling before HPLC Analysis of Complex Matrices

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A short review on trends in sample handling before HPLC analysis is presented. The paper deals with the use of solid-phase extraction (SPE) for sample preparation. Attention is focused on new polymer hydrophilic-lipophilic balanced sorbents Oasis, immunosorbents, and molecularly imprinted polymers and their application in SPE technique.

Despite advances in the sensitivity of analytical instrumentation for the end-point determination of analytes in environmental or biological samples, a pretreatment is usually required to extract and isolate the analytes of interest from complex matrices. Solid-phase extraction (SPE) is one such sample preparation technique that has rapidly developed in recent years. It provides the good possibility for sample clean-up and preconcentration of analytes.

For many years most of the off-line SPE procedures have been achieved using reversed-phase (RP) silicas. One limitation of RP silica is that it must be conditioned with a wetting solvent and remain wetted before sample application. New patented sorbents have been introduced in the market and they are capable to extract acidic, basic, and neutral compounds whether polar or nonpolar. That is hydrophilic-lipophilic balanced (HLB) sorbent Oasis HLB (Waters, USA) and it is a copolymer of *m*-divinylbenzene and *N*-vinylpyrrolidone [1]. Additionally, this HLB sorbent is fully wettable with water, therefore it is not necessary to include any sorbent-drying steps.

In the last few years there has been a lot of interest in immobilizing antibodies onto solid supports such as silica to provide highly selective SPE. This approach uses the specificity of antibodies along with the ability of HPLC and GC to separate structurally closely related analytes that might cross-react [2].

Materials based on antigen—antibody interactions allow selective extraction. Antibodies can be linked to an appropriate solid support to form an immunoaffinity sorbent (IAS), either by covalent bonding, adsorption or encapsulation. As a result of the high affinity and high selectivity of antigen—antibody interactions, immunoaffinity sorbents provide a high degree of molecular selectivity and have been increasingly used in sample preparation [3].

The high selectivity provided by immunoextraction has led to attempt to synthesize antibody mimics. One approach has been the development of molecularly imprinted polymers (MIPs). They are crosslinked polymers with specific binding (recognition) sites for certain molecules. These binding sites are tailor-made *in situ* by the copolymerization of crosslinking monomers and functional monomers in the presence of the print molecule, called the template. After polymerization, the template is removed and the resulting polymer has cavities, which are the "imprints". These cavities are the recognition sites allowing binding of the template molecule. Like with IASs, the recognition is due to shape and various hydrogen, hydrophobic and electronic interactions [1, 4].

The advantages of MIPs, *e.g.* physical robustness, high strength, resistance to elevated temperatures and pressures, and inertness towards acids, bases, metal ions, and organic solvents, have been well exploited in a large number of applications, *e.g.* in LC they were used as normal and chiral stationary phases, in areas where they can be substitutes of natural antibodies (immunoassays, sensors), and in SPE [1, 5].

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The review is based on the literature published in the last five years dealing with the use of HLB sorbents, IAS and MIP sorbents for sample preparation of complex matrices, such as plant, food, beverage, and clinical samples before HPLC analysis. Preseparation and chromatographic conditions are summarized in Tables 1—3.

OASIS SORBENTS

Oasis HLB is a macroporous copolymer consisting of two monomers, the lipophilic *m*-divinylbenzene and the hydrophilic *N*-vinylpyrrolidone (Formula 1 [6]). These two monomers construct a hydrophilic-lipophilic balance. The utilization of a method using the Oasis products instead of RP silicas needs less sorbents because of the up to five times higher capacity. In contradistinction to silica-based RP-resins the macroporous and wettable resin shows a less complicated adsorption mechanism that is not influenced by the metallic impurities or the changing concentration of silanol groups.
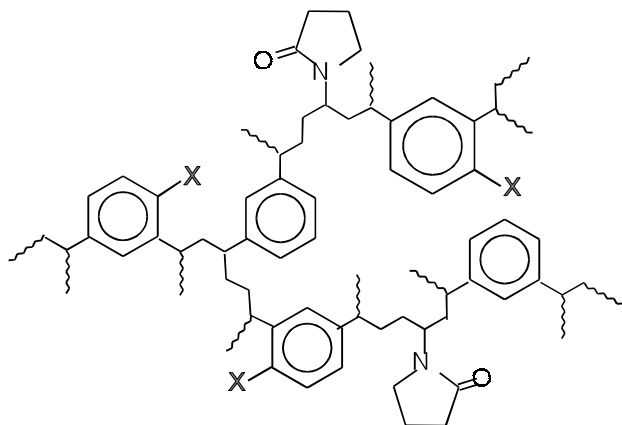
Formula 1. OASIS sorbents. *a*) HLB, X = H, *b*) MCX, X = SO₃H, *c*) MAX, X = CH₂N⁺R₃.

The material can be used for the determination of polar and apolar organic compounds from aqueous solutions. Acidic, neutral, and basic components can be isolated using a very simple method that shows high recoveries (greater than 85 %) and excellent reproducibility values (less than 5.5 % RSD). Because of the high pH-stability (pH = 1—14) and the high compatibility to organic solvents clean extracts can be achieved by simple pH-modifications or modifications in the concentration during the elution step. In this way analytes are retained in nonionic and strong lipophilic form. Interferences can be removed with higher concentrated organic solvents. Because of wetting ability, the retention and the high recoveries are kept constant for a wide spectrum of analytes even if air is flushed through the SPE cartridge.

Besides the classical Oasis HLB there also exists the Oasis MCX (mixed mode: cation-exchange and reversed-phase) sorbent that combines the properties of the HLB sorbents with the characteristics of a strong cation exchanger. The additionally introduced sulfonic acid groups (—SO₃H) cause an improved selectivity and sensitivity for basic additives in plasma, urine or whole blood. The combined RP and cation exchanger properties of the Oasis MCX material allow to separate samples into an acidic-neutral fraction and into a basic fraction [7]. High selectivity and sensitivity for acidic compounds and metabolites can be achieved using the Oasis MAX (mixed mode: anion-exchange and reversed-phase) sorbent.

Plant and Food Samples

Plant and food material contains various ballast compounds, *e.g.* waxes, oils, sterols, *etc.*, which can cause the damage of HPLC columns and may interfere with analytes. This is the main reason for purification of such samples before analysis. Liquid-liquid extraction (LLE) is the first preparation step of solid samples, as SPE procedure requires application of samples in liquid state. Simple filtration or centrifugation follows after LLE and then filtrate or supernatant is applied on SPE cartridge (see Table 1).

A fast, sensitive, and precise method for isolation, purification, and determination of isoflavones in plant material is described in paper [8] where classical sorbents were compared with new polymer sorbents. The material was milled, dispersed in water and then mixed with HCl and extracted with ethanol. Ethanolic extracts were used for SPE on C8, C18, Amide 2, RP105, ABN (Applied Separations, USA) and Oasis HLB cartridges. Eluates were analyzed on C18 column by means of gradient elution and DAD. Extraction efficiencies were 84—102 % for the first five sorbents and 91—99 % for Oasis HLB.

In the study [9] five different commercial sorbents C18, SCX, Certify (Varian, USA), CN (J. T. Baker, Holland), and Oasis HLB were compared for the SPE of potato glycoalkaloids. The recoveries were determined using α-solanine, α-chaconine, and α-tomanine. The dried leaves of *S. brevidens* were ground, extracted with 5 % acetic acid and then the suspension was filtered. Samples were applied to the SPE cartridges. The eluates were analyzed by RP-HPLC under gradient elution conditions using C18 column. The highest recovery (≈ 100 %) was achieved with Oasis HLB cartridges.

HPLC method for determining hydroxymethylfurfural, furfural, 2-furoic acid, 3-furoic acid, and methyl anthranilate in honey and honeydew samples has also been described [10]. To remove interferences, procedures based on LLE and SPE on Oasis HLB were assayed. Recoveries obtained for LLE ranged from 35 to 88 %. Before SPE, samples were dissolved with sulfu-

Table 1. The Preseparation and Separation Conditions for Oasis SPE Clean-Up

Compound	Matrix	Presep.	Column	Mobile phase	Detection	Ref.
			Plant and Food Samples			
Isoflavones	Plant material	milling, disper., acidif., extr., filtr., SPE on C8, C18, Amide 2, RP105, ABN, Oasis HLB	Zorbax SB C18, 75 mm × 4.6 mm, 3.5 μm	grad. ACN—phosph. buff. (or acetic acid or TFA)	DAD, $\lambda = 280$ nm	[8]
α-Solanine, α-chaconine, α-tomanine	*S. brevidens* leaves	milling, extr., filtr., SPE on C18, SCX, CN, Certify, Oasis HLB	Zorbax Rx-C18, 250 mm × 4.6 mm, 5 μm	grad. ACN—phosph. buff.	DAD, $\lambda = 205$ nm	[9]
Hydroxymethyl-furfural, furfural, 2-furoic acid, 3-furoic acid, methyl anthranilate	Honey	dissolv., LLE, SPE on Oasis HLB	Luna C18, 250 mm × 4.6 mm, 5 μm	grad. acetic acid—ACN—water	UV, $\lambda = 250$ nm	[10]
Tetracyclines	Bovine milk and tissues	homog., acidif., centr., SPE on Oasis HLB	Hypersil C8, 250 mm × 4.6 mm, 5 μm	oxalic acid—ACN—MeOH	DAD, $\lambda = 365$ nm	[11]
Quinolones (13 compounds)	Feeds	extr., evapor., filtr., SPE on Oasis HLB	C5 Luna, 150 mm × 4.6 mm, 5 μm	grad. ACN—THF— KH$_2$PO$_4$	DAD, $\lambda = 278$ nm FD, $\lambda_{ex} = 278$ nm $\lambda_{em} = 446$ nm	[12]
Phenolic acids	*Melissa officinalis* L.	LLE, filtr., SPE on Oasis HLB	Symmetry C18, 150 mm × 3.9 mm, 5 μm	grad. MeOH—acidic water	DAD, $\lambda = 260$ nm, 280 nm, 330 nm	[13]
			Clinical Samples			
Catecholamines	Human plasma	SPE on Isolute C8, BondElut C8, Bond Elut CH, Oasis HLB	Apex C8, 150 mm × 4.6 mm, 5 μm	MeOH—citric acid—EDTA—OSA	ED	[14]
Catecholamine + its metabolites	Plasma	deprotein., SPE on Oasis HLB and C18	Spherisorb ODS 2, 250 mm × 4.0 mm, 5 μm	THF—NaH$_2$PO$_4$· 2H$_2$O—sodium citrate—EDTA—DEA—OSA	ED	[15]
Moxifloxacin	Human plasma, lung tissue	milling, homog., centr., SPE on Oasis HLB	Supelcosil ABZ+, 150 mm × 4.0 mm, 5 μm	ACN—phosph. buff.	UV, $\lambda = 296$ nm	[16]
Rufinamide, ICL670, anticonvulsant agent AA1	Plasma, brain	homog., dil., precipit., filtr., SPE on C18 and Oasis HLB	Supelcosil LC-18, 150 mm × 4.6 mm, 5 μm Alltima C18, 50 mm × 4.6 mm, 3 μm	ACN—MeOH—phosph. buff.	UV, $\lambda = 230$ nm, 295 nm, 316 nm	[17]
Clarithromycin, roxithromycin	Plasma	precipit., centr., SPE on C8, C18 and Oasis HLB	Inertsil ODS-3, 250 mm × 4.0 mm, 5 μm	phosph. buff.—MeOH—ACN—iPrOH	ED	[18]
Rutin	Human plasma	dil., SPE on Oasis MAX	Luna C18, 150 mm × 2.1 mm, 5 μm	ACN—ammonium acetate, EDTA—acetic acid	UV, $\lambda = 370$ nm	[19]
Caffeine + 10 metabolites	Serum, urine	deprotein., centr., SPE on C18, Nexus and Oasis HLB	MZ Kromasil C4, 250 mm × 4.0 mm, 5 μm	acetate buff.—MeOH	UV, $\lambda = 275$ nm	[20]
Caffeine + its 14 metabolites	Urine	filtr., acidif., SPE on Oasis C18	Eclipse XDB-C18, 250 mm × 4.6 mm, 5 μm	grad. water—acetic acid—THF—ACN	UV, $\lambda = 270$ nm, 290 nm	[21]
Hyperforin	Human plasma	precipit., centr., SPE on Oasis HLB	Luna C18, 150 mm × 4.6 mm, 3 μm	MeOH—ACN—water—formic acid—TEA	UV, $\lambda = 287$ nm	[22]

Table 1. (Continued)

Compound	Matrix	Presep.	Column	Mobile phase	Detection	Ref.
Zidovudine, lamivudine, nevirapine	Plasma	SPE on Oasis HLB	Nova-Pack C8, 150 mm × 3.9 mm, 5 μm	phosph. buff.+ OSA—ACN	UV, $\lambda = 265$ nm	[23]
Uric acid	Saliva	alkalin., SPE on Oasis MAX	Capcell Pack C18 UG120, 150 mm × 2.0 mm, 5 μm	phosph. buff.	UV, $\lambda = 284$ nm, ED	[24]
Morphine, M3G, M6G	Human plasma	homog., centr., SPE on Oasis HLB	Symmetry Shield C8, 150 mm × 4.6 mm, 5 μm	ACN—phosph. buff.	UV, $\lambda = 210$ nm	[25]
5-Hydroxyoxindole	Serum, tissue	acidif., homog., centr., SPE on Oasis HLB	Symmetry Shield RP18, 250 mm × 4.6 mm, 5 μm	phosphate buffer— ACN—SDS— nitrilotriacetic acid	ED	[26]
3-Amino-2-oxazolidone	Liver	acidif., neutr., extr., centr., evapor., dissolv., SPE on Oasis HLB	Luna C18, 250 mm × 4.6 mm, 5 μm	MeOH—ACN— KH_2PO_4	UV, $\lambda = 275$ nm	[27]
Benzimidazoles	Liver	homog., SFE, SPE on Oasis MCX, C18 and SCX	XTerra C18, 150 mm × 3.0 mm, 3.5 μm	grad. ammonium buffer—MeOH— ACN	DAD, $\lambda = 298$ nm	[28]
Oxcarbazepine + metabolites	Plasma	SPE on Oasis HLB	Microsorb MV Rainin, 150 mm × 4.6 mm, 5 μm	phosphate buffer— ACN—MeOH— TEA	UV, $\lambda = 237$ nm	[29]

ric acid and recoveries of this procedure ranged from 67.9 to 99.7 %. Samples were analyzed on C18 column by means of gradient elution. LOD of the method was 10—80 μg dm^{-3}.

HPLC-DAD was optimized and validated for the determination of tetracyclines in bovine milk and tissues by authors [11]. Milk and muscle were homogenized and then TCA and McIlvaine buffer were added. Centrifuged sample was applied to SPE Oasis HLB cartridge and tetracyclines were eluted with methanol. The analyses were carried out using the mobile phase of oxalic acid—ACN—methanol on C8 column. Recoveries of tetracyclines from spiked samples were higher than 81.1 % in milk and 83.2 % in muscle. The decision limit was in the range 113.2—127.2 μg kg^{-1} and 107.7—129.9 μg kg^{-1} for all compounds in milk and muscle, respectively.

Authors [12] describe simple multiresidue method for assaying 13 quinolones in feeds. The samples were extracted by a metaphosphoric acid in water—ACN mixture and purified on Oasis HLB cartridges. The analytes were eluted with methanol and the determination was achieved by gradient elution using C5 analytical column either with DAD or FD. Average recoveries ranged from 51 to 103 % at the content of 5 mg kg^{-1}. LOD of the method was calculated at 0.4— 1.5 mg kg^{-1}.

Authors [13] tested four different preparation techniques, including off-line SPE on Oasis HLB columns, for the isolation of some phenolic antioxidants from *Melissa officinalis* L. Plant samples were ground to powder and extracted with acidified water or with mixture of methanol—water. Extracts were loaded onto cartridge and phenolic compounds were eluted with mixture of methanol—water. The separation of phenolics was achieved on C18 column by means of gradient elution. Recoveries of phenolics ranged from 88.4 to 99.9 %.

Clinical Samples

Clinical samples (blood, serum, plasma, urine, *etc.*) are another type of complex matrices, which contain many interferences. Preparation of such samples usually includes dilution, precipitation, filtration or centrifugation. LLE is necessary only in the case of solid samples, like tissues.

Isolation and determination of catecholamines [14] and catecholamines with their metabolite [15] in plasma samples are described. Authors of the first article carried out several experiments using different kinds of cartridges, *e.g.* Isolute C8 (International Sorbent Technology, UK), BondElut C8, BondElut CH (Varian, USA), and Oasis HLB. Extracts were analyzed on C8 column with the mobile phase consisting of a mixture of methanol—citric acid solution— EDTA—OSA and with ED. The efficiency yield for all catecholamines was from 92 to 98 %. Authors [15] loaded deproteinized plasma onto Oasis HLB cartridge and analytes were eluted with methanol. Eluate was analyzed on C18 column, the mobile phase was mixture of THF, $NaH_2PO_4 \cdot 2H_2O$, sodium citrate, EDTA, DEA, and OSA and detection was done with ED. The recovery of the compounds averaged 61—99 % and

limits of detection for all analytes were 10—50 ng dm^{-3}.

The aim of the study [16] was to develop HPLC-UV assay for the determination of moxifloxacin, new methoxyquinolone, in human plasma and lung tissue. The extraction procedure was characterized by a fully automated SPE using Oasis HLB column as the solid phase. Blood samples were centrifuged and lung samples were ground in PBS and the tissue suspension obtained was homogenized and centrifuged. Thus prepared samples were loaded onto cartridges and analyte was eluted with methanol—TFA. Supelcosil ABZ+ column was used for the separation and mobile phase was a mixture of ACN—phosphate buffer. Recovery from plasma samples was 94.2—100.6 % and from lung 90—98.4 %. LODs of this method were 6.52 ng cm^{-3} and 50 ng g^{-1}, for plasma and lung tissue, respectively.

C18 Empore 96-well extraction disc plates have been employed in [17] for the analysis of three drugs with different polarities (anticonvulsant agents: rufinamide and AA1, iron chelator ICL670) in plasma in conjunction with HPLC-UV. The method developed for the assay of AA1 in plasma was applied to its determination in brain using an Oasis HLB plate following homogenization and protein precipitation. Recovery of analyte from Oasis column was up to 60 %. RP chromatography was applied using short (5 cm) column for the determination of ICL670 and AA1 and two parallel columns (15 cm) for the determination of rufinamide.

The macrolide antibiotics clarithromycin and roxithromycin in plasma samples were determined by HPLC-ED in [18]. Samples were prepared by mixing plasma with 80 % methanol and ACN. After mixing, the samples were centrifuged and transferred to the extraction column (C8 from Alltech, C18 from Supelco, USA, Baker, Holland, Rigas Labs, and Oasis HLB). The drugs were eluted with methanol and HPLC analyses were performed by means of C18 column. When Oasis columns were used, the yields of extraction were 87.7—93.4 % at a concentration 1 μg cm^{-3} for both compounds.

Study [19] describes the HPLC-UV method for determining a flavonol glycoside, rutin, in human plasma. Sample was diluted with water and applied to Oasis MAX cartridge. Analyte was eluted with a mixture of phosphoric acid—ACN. HPLC analyses were performed on C18 column with mobile phase consisting of ACN—ammonium acetate solution containing EDTA—glacial acetic acid. The recoveries were 79.7 % (for concentration level of 157.09 ng cm^{-3}) and 80.3 % (for 1042.29 ng cm^{-3}) and LOD was approximately 0.75 ng cm^{-3}.

HPLC-UV determination of caffeine and its metabolites is described in papers [20, 21]. Authors [20] treated samples (blood serum, urine) with ACN to precipitate proteins, then centrifuged and transferred them to cartridge (Oasis HLB, C18 from Supelco, USA and Nexus from Varian, USA). Caffeine and metabolites were eluted using methanol—acetate buffer and analyzed on C4 column with mobile phase consisting of acetate buffer—methanol. Recovery of caffeine and metabolites after SPE on Oasis columns ranged from 84.4 to 100.8 %. LOD of the assay was 0.3 ng. Authors [21] describe extraction procedure of these compounds from urine. Samples were filtered, spiked and then acidified. The solution was loaded onto SPE column Oasis C18 and analytes were eluted with sodium acetate in methanol—ACN. Analytical separation was performed on C18 column by means of gradient elution. Recoveries ranged between 83 % and 99 % and LOD was evaluated at 2—3 ng.

SPE and isocratic HPLC-UV were developed by authors [22] to determine active component of St. John's wort (*Hypericum perforatum*) hyperforin in human plasma. Sample was mixed with acetonitrile and centrifuged. Supernatant was mixed with water, loaded onto Oasis HLB cartridge and hyperforin was eluted with methanol. Extracts were analyzed on C18 column with mobile phase consisting of methanol—ACN—water. Absolute recovery of hyperforin was 88.9—91.5 % for concentration levels from 25 to 500 ng cm^{-3}. LOD of the method was 4 ng cm^{-3}.

Authors [23] developed a new HPLC-UV assay for the simultaneous determination of therapeutical agents for the treatment of HIV infection (zidovudine AZT, lamivudine 3TC, and nevirapine) in human plasma. Samples were spiked and then treated using SPE procedure on Oasis HLB. Water—ACN was used to elute the analytes. The compounds were separated using mobile phase containing phosphate buffer—OSA—ACN on C8 analytical column. Extraction recoveries of the analytes were higher than 92 % and the LODs were 13.3—28.8 ng cm^{-3}.

The aim of the study [24] was to establish a highly sensitive method for the determination of uric acid (UA) in human saliva. KOH was added to the samples and alkalinized saliva was applied onto SPE cartridge Oasis MAX. Elution was performed with acidified water and RP-HPLC was used for the analytical separation of compounds with phosphate buffer as mobile phase. Authors compared efficiencies of UV detection with amperometric detection with a single electrode and coulometric detection with a multiple electrode array. The recoveries of UA were above 95 %.

Paper [25] describes simple SPE assay for HPLC-UV analysis of analgetic morphine and its two metabolites (morphine-3-glucuronide, M3G, morphine-6-glucuronide, M6G) in serum samples. Samples were homogenized, centrifuged and then loaded onto Oasis HLB cartridge. Elution was performed with methanol. C8 analytical column and phosphate buffer or mixture of acetonitrile and phosphate buffer was used for the chromatographic separation. High extraction recoveries have been obtained for simultaneous isolation of all three analytes: 90 % for morphine,

and 96 % and 99 % for M3G and M6G, respectively.

Authors [26] designed assay to determine 5-hydroxyoxindole, metabolite of triptophan, in serum and tissue using SPE and HPLC-ED. Serum sample was acidified and then applied on Oasis HLB column. Sample of tissue was homogenized in $HClO_4$, centrifuged and supernatant was transferred to Oasis HLB cartridge. Elution was performed with ethanol in both cases. Eluates were analyzed on C18 column with mobile phase consisting of phosphate buffer—ACN—SDS—nitrilotriacetic acid. Extraction recoveries were 93—103 % and LOD was 0.5 nmol dm^{-3}.

Paper [27] describes SPE clean-up procedure for 3-amino-2-oxazolidone (metabolite of furazolidone) from liver tissue. Samples were acidified with HCl and after derivatization with nitrobenzaldehyde neutralized and extracted with ethyl acetate. Extracts were passed through the Oasis MAX column and eluted with methanol. Eluates were applied to Oasis HLB cartridge and eluted with ammonia—methanol. Recovery of the method was 84—90 %. Analyses were performed by means of HPLC-UV on C18 column with methanol—ACN—KH_2PO_4 as mobile phase.

Authors [28] used SPE clean-up of SFE extracts for determination of anthelmintics benzimidazoles in liver. Sample was homogenized and, after SFE, extract was applied to SPE column. A number of different procedures were evaluated on C18, SCX (Varian, USA), and Oasis MCX. Mixture of ACN—ammonia was used for elution and extract was injected onto C18 analytical column and separated by means of gradient elution. Mean recovery of analytes was between 51 % and 115 % and LOD was 50 μg kg^{-1}.

Method based on HPLC-UV in combination with SPE was developed [29] for analysis of antiepileptic drug oxcarbazepine and its metabolites in plasma. The extraction of analytes was carried out by means of Oasis HLB cartridges from which analyte was eluted with methanol. The analytical separation was obtained on C18 column using phosphate buffer—ACN—methanol—TEA mixture as the mobile phase. Recovery for all analytes was up to 95 % and LOQs were 15 ng cm^{-3}.

IMMUNOAFFINITY SORBENTS

The first step in making immunosorbent is to develop antibodies with the ability to recognize either one or a group of analytes. Antibody is protein produced by mammalian immune system in response to the presence of a foreign substance (antigen), which is capable of producing an immune response. Compounds of low relative molecular mass (< 1000) are unable to evoke an immune response; therefore they must be coupled to a carrier molecule (usually a protein).

After immunization, either polyclonal or mono-

clonal antibodies can be obtained. Antibodies present in the antiserum taken directly from the animal are known as polyclonal antibodies, because antiserum contains a mixture of different antibodies produced by different cells. The procedure for obtaining monoclonal antibodies involves removing mammalian spleen antibody-producing cells. Each cell is fused with an immortal line of myeloma (tumour) cells in culture. The resulting hybrid cells are screened in order to select one cell that will produce a desired antibody indefinitely. As the resulting antibodies are homogeneous, they are referred to as monoclonal [2, 3].

Sorbent that is used for immobilizing of antibodies must be chemically and biologically inert, easily activated, and hydrophilic to avoid any nonspecific interactions. The most common approach involves covalent bonding of the antibodies onto activated silica or agarose gel but noncovalent binding can also be used to couple antibodies to the sorbent. Another method, the sol-gel method, consists in encapsulating antibodies in the pores of a hydrophilic glass matrix [3]. Separation conditions on IAS are given in Table 2.

Plant, Food, and Beverage Samples

The following text deals with the applications of IAS-SPE to treatment of plant, food, and beverage samples.

Ochratoxin A (OA) was determined in coffee [30], in wine [31—33], and in beer [34]. Authors [30] used LLE with methanol—aqueous $NaHCO_3$, then the extract was applied to NH_2 (Baker, Holland) SPE cartridge and then it was cleaned up by means of IA column from which OA was eluted with methanol. C18 analytical column and FD were used for the analysis. In paper [31], wine was diluted and then applied to IA column. OA was eluted with methanol and quantified by RP-HPLC C18 column with FD. The direct wine clean-up with IA columns or IA clean-up following LLE with chloroform is described in [32]. Elution of OA was performed with methanol and the extract was analyzed by HPLC on C18 column with FD. Authors [33] compare different analytical methods for the determination of OA in wine. Sample clean-up was based on SPE with RP-18 (Phenomenex, USA) or IAS materials. In the first method, sample was spiked with OA, applied to RP-18 SPE column and eluate was injected into the LC-MS system. In the second and the third method, wine was diluted, applied to the IA column and analyses were performed in the LC-MS and LC-FD system. The fourth method involved mixing of the sample with phosphoric acid and NaCl, extraction with chloroform, and IA clean-up. HPLC analysis was performed with FD. In the short communication [34], authors quantified OA in beers. Beer was degassed, diluted and then applied to an IA column. OA was eluted with methanol

Table 2. The Preseparation and Separation Conditions for IAS Clean-Up

Compound	Matrix	Presep.	Column	Mobile phase	Detection	Ref.
			Plant, Food, and Beverage Samples			
Ochratoxin A	Roasted coffee	extr., dil., SPE-NH$_2$ column, IAC	Discovery C18, 250 mm × 4.6 mm, 5 μm	ACN—water—acetic acid	FD, $\lambda_{ex} = 333$ nm $\lambda_{em} = 460$ nm	[30]
Ochratoxin A	Wine	dil., IAC	Discovery C18, 150 mm × 4.6 mm, 5 μm	ACN—water—acetic acid	FD, $\lambda_{ex} = 333$ nm $\lambda_{em} = 460$ nm	[31]
Ochratoxin A	Wine	extr., evapor., dissolv., IAC	Inertsil ODS-2, 250 mm × 4.0 mm, 5 μm	ACN—water—acetic acid	FD, $\lambda_{ex} = 333$ nm $\lambda_{em} = 460$ nm	[32]
Ochratoxin A	Wine	dil., extr., evapor., dissolv., IAC	Superspher 100RP-18e, 125 mm × 3 mm, Hypersil C18, 250 mm × 4.6 mm	MeOH—water—acetic acid ACN—water—acetic acid	MS FD, $\lambda_{ex} = 333$ nm $\lambda_{em} = 460$ nm	[33]
Ochratoxin A	Beer	degass., dil., IAC	Discovery C18, 150 mm × 4.6 mm, 5 μm	ACN—water—acetic acid	FD, $\lambda_{ex} = 333$ nm $\lambda_{em} = 460$ nm	[34]
Deoxynivalenol (mycotoxin)	Wheat	etxr., IAC	Nova-Pack C18, 150 mm × 3.9 mm, 4.0 μm	ACN—water	UV, $\lambda = 218$ nm	[35]
Thifluzamide (pesticide)	Peanut	extr., defatting (hexane), IAC	Supelcosil LC-18, 150 mm × 4.6 mm, 4 μm	grad. ACN—water	UV, $\lambda = 280$ nm	[36]
10-Deacetylbaccatin	Plant, cell extract	extr., IAC	Taxsil-3, 250 mm × 4.6 mm, 5 μm, Altima C18, 250 mm × 4.6 mm, 5 μm	ACN—ammonium acetate MeOH—ACN—ammonium acetate	UV, $\lambda = 280$ nm	[37]
T-2 toxin	Wheat, corn, barley, oats, rice, sorghum	milling, extr., centr., filtr., dil., IAC	Symmetry C18, 150 mm × 4.6 mm, 5 μm	ACN—water	FD, $\lambda_{ex} = 381$ nm $\lambda_{em} = 470$ nm	[38]
			Clinical Samples			
Morphine, morphine-3-glucuronide, morphine-6-glucuronide	Human blood	dil., centr., IAC	LiChrospher60-RP-Select B, 250 mm × 4 mm, 5 μm	ACN—phosph. buff.	FD, $\lambda_{ex} = 235$ nm $\lambda_{em} = 345$ nm	[39]
epi-Trenbolone, trenbolone, epi-nortestosterone, nortestosterone	Bovine urine	adjusted pH 7—9, IAC	Nova-Pack, 150 mm × 3.9 mm, 4 μm	ACN—MeOH—water	UV, $\lambda = 340$ nm, 241 nm	[40]
Bisphenol A	Serum	dil., IAC	Zorbax Eclipse XDB C8	ACN—water	DAD, $\lambda = 340$ nm, 241 nm FD, $\lambda_{ex} = 231$ nm $\lambda_{em} = 420$ nm	[41]

and quantified by HPLC with FD on C18 analytical column. Recoveries of all five studies were up to 72 %.

A simple and accurate method to quantify mycotoxin deoxynivalenol (DON) in wheat is described in [35]. Samples were extracted into water and IA chromatography was used for isolation of analyte. DON was eluted with methanol and injected into HPLC-UV system. Analytical separation was performed on C18 column by means of mobile phase consisting of ACN—water. LOD was at least 0.10 μg g^{-1}. Fraction recovery for the entire assay range averages 90 %.

Authors [36] prepared specific columns to selectively extract pesticide thifluzamide from peanut samples. Two types of cartridges were assayed based on two different support materials: silica-based (Baker, Holland) and Sepharose 4B CL (Pharmacia) cartridges. Peanuts were extracted with solution consisting of ACN—water and defatted by means of hex-

ane. Aqueous ACN layer was spiked, diluted and then passed through the IA column. Elution was performed with mixture of methanol—water and HPLC analyses were carried out on the C18 column with gradient elution and UV detection.

The application of IAC for the purification of *Taxus* plant and cell extracts, prior to HPLC analysis is described in paper [37]. Polyclonal antibodies raised against 10-deacetylbaccatin (10-DAB III) were characterized by ELISA. Plant samples were extracted with methanol and the extract was passed through the IA column. The recovery after IA chromatography was approximately 80 %. Chromatographic analyses were performed on two columns with UV detection.

A method for the determination of T-2 toxin in cereal grains is described in paper [38]. The samples were ground, extracted with methanol—water, the extracts were applied to IA column and the toxin was eluted with methanol. Extracts were quantified by RP-HPLC-FD with mobile phase consisting of ACN—water. The recoveries from the different cereals were from 80 to 99 % and LOD was 5 ng g^{-1}.

Clinical Samples

Another application of IAS-SPE is to use it for preparation of clinical samples in the analysis of drugs, toxins, *etc.*

The development of an IA-based extraction method for the determination of morphine and its glucuronides M3G, M6G in human blood is described in [39]. The blood was mixed with PBS, centrifuged and loaded onto IA column. The resulting extracts were analyzed by RP-HPLC-FD with mobile phase consisting of phosphate buffer—ACN. The mean recoveries from spiked samples were 71 %, 76 %, and 88 % for morphine, M3G, and M6G, respectively and LOD was 3 ng g^{-1} blood.

The procedure [40] uses commercially available IA clean-up cartridges followed by determination of residues of hormones *epi*-trenbolone, trenbolone, *epi*-nortestosterone and nortestosterone in bovine urine. Samples were adjusted to pH 7—9 and loaded onto IA column. A mixture of methanol and water was used for elution of analytes. Extracts were analyzed by HPLC-UV on RP column by means of mobile phase consisting of ACN—methanol—water. Recoveries were on an average 97 %.

The development of IA column for selective extraction of bisphenol A from serum samples is described by authors [41]. Samples were diluted with PBS and applied to IA column. BPA was recovered by mixture of methanol—water with average recovery of 91.8 %. Analyses of extracts were performed on C8 analytical column with ACN—water as mobile phase and with DAD and FD.

MOLECULARLY IMPRINTED POLYMERS

Molecular imprinting (MI) of synthetic polymers with a target molecule can be achieved if the target is present as a template during the polymerization process. Monomers carrying certain functional groups are arranged around the template through covalent or noncovalent interactions and are "frozen" into position by polymerization with a high degree of crosslinking. In the first approach, polymerizable derivatives of the template molecule are copolymerized with a crosslinking monomer. These derivatives are obtained by forming covalent bonds between the template and suitable polymerizable monomers. To remove the template from the polymer, these covalent bonds have to be chemically cleaved. The second approach is based on the formation of a prepolymerization complex between monomers carrying suitable functional groups and the template through noncovalent bonds such as ionic interactions or hydrogen bonding. The template can be removed after the polymerization simply by solvent extraction. This "self-assembly" approach is more similar to the natural process, in the sense that most biomolecular interactions are noncovalent in nature (Fig. 1 [42]).

In analytical applications, problems may be encountered owing to small amounts of template remaining in the polymer even after very thorough washing. A possible solution of this problem is to use a molecule with a structure very closely related to the target analyte as the template [42]. Separation conditions on MIP are given in Table 3.

Plant, Food, and Beverage Samples

The application of MIP as SPE sorbent is the most common in the analytical separation field. The following chapters include the applications of MIP in preparation of plant, food, and beverage samples and clinical samples.

Authors [43] prepared a selective and affinitive MIP for flavonoid quercetin using a noncovalent imprinting approach. Application of this polymer for extracting compounds of the flavonoids from gingko leaves was investigated. Leaves were extracted by methanol, hydrolyzed with HCl and after removing of acid, redissolved in methanol. Hydrolyzate was applied onto MIP column and, after washing, eluted with methanol—acetic acid. Fraction was analyzed by HPLC-UV on C8 column. Recovery from MIP cartridge was 90 %.

A MIP in [44] was prepared with caffeine as the template molecule and produced polymer was evaluated as selective SPE cartridges for trapping and preconcentration of caffeine from beverage and plasma sample. Sample of cola beverage was diluted with water and then applied to the MIP-SPE. The spiked human plasma was extracted with acetonitrile, diluted
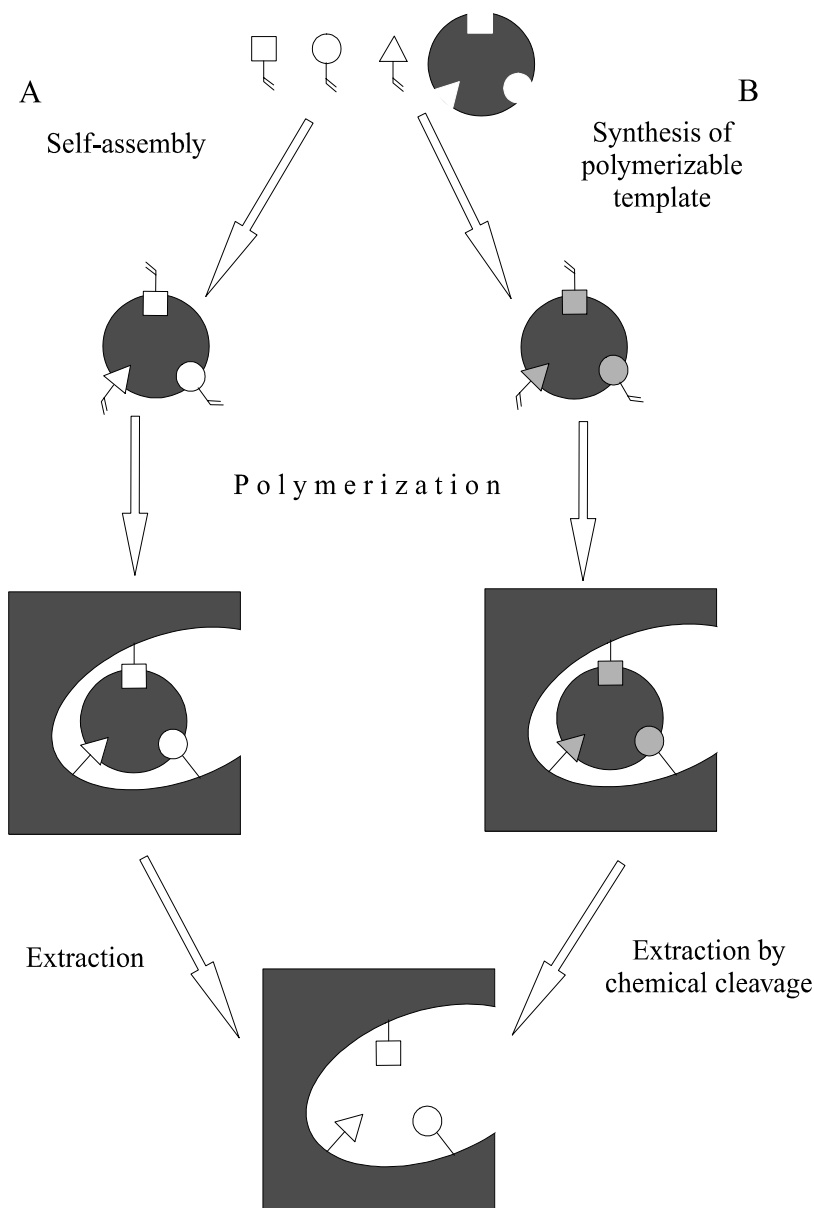
Fig. 1. Schematic representation of the molecular-imprinting principle: A: self-assembly approach, B: covalent approach.

with water and the mixture was applied to the MIP-SPE cartridge. Extracts were analyzed on C18 column and detected with UV detector. Extraction recoveries from both matrixes were up to 81 %.

Study [45] describes using of MIP as SPE material for separation of inhibitors of epidermal growth-factor receptor (E)-piceatannol and butein from roots of *Caragana Jubata*. MIP was prepared with quercetin as template. Sample was extracted with ethanol, extract was evaporated and redissolved and then loaded onto MIP-SPE column. Analytes were eluted with methanol—acetic acid and separated on C18 analytical column by means of gradient elution and UV detector. Recoveries of compounds were 76 % for butein and 80 % for (E)-piceatannol.

MIP-SPE method for the enrichment and clean-up of herbicide fenuron (used as template) from plant extracts was developed by authors [46]. Sample was extracted with ACN, centrifuged and loaded on MIP cartridge. Analyte was eluted with ACN—acetic acid, analyzed on C18 column with mobile phase consisting of water and ACN and detected with UV detector. Recoveries from different kinds of samples were 98—115 %.

Clinical Samples

The use of MIP as sorbent material in SPE for clean-up of β-agonist clenbuterol from urine [47, 48] and from milk, urine and liver [49] is described. The MIP was produced using clenbuterol [47, 49] or brombuterol [48] as a template. Liver samples were ex-

Table 3. The Preseparation and Separation Conditions for MIP-SPE Clean-Up

Compound	Matrix	Presep.	Column	Mobile phase	Detection	Ref.
			Plant, Food, and Beverage Samples			
Quercetin	Gingko leaves	extr., hydrol., evapor., dissolv., MIP-SPE	Zorbax Eclipse XDB-C8, 150 mm × 4.6 mm	grad. MeOH—phosph. acid	UV, $\lambda = 365$ nm	[43]
Caffeine	Cola beverage, human plasma	extr., dil., MIP-SPE	Discovery C18, 150 mm × 4.6 mm, 5 μm	MeOH—ammonium acetate	UV, $\lambda = 272$ nm	[44]
(E)-Piceatannol, butein	Roots of *Caragana Jubata*	extr., evapor., dissolv., MIP-SPE	Zorbax Extend C18, 150 mm × 2.1 mm	grad. water—MeOH	UV, $\lambda = 254$ nm MS	[45]
Fenuron (pesticide)	Wheat, barley, potato, carrot	extr., centr., filtr., evapor., dissolv., MIP-SPE	Hypersil 5 ODS, 150 mm × 4.6 mm	water—ACN	UV, $\lambda = 244$ nm	[46]
			Clinical Samples			
Clenbuterol	Calf urine	MIP-SPE	LiChroCART 250-4, 250 mm × 4 mm, 5 μm	ACN—phosph. buff.	ED	[47]
Clenbuterol	Calf urine	centr., dil., MIP-SPE	BetaBasic C18, 150 mm × 2.1 mm, 5 μm	MeOH—phosph. buff.	UV, $\lambda = 210$ nm	[48]
Clenbuterol + other β-adrenergic agents	Bovine urine, liver, milk	extr., concentr., dissolv., MIP-SPE	LiChrospher RP 18, 250 mm × 4.0 mm, 5 μm	grad. ACN—water	DAD, $\lambda = 245$ nm, 275 nm, 360 nm	[49]
Tramadol	Serum	on-line RAM SPE, MIP-SPE	LiChrospher CN, 125 mm × 4 mm, 5 μm	ACN—water	FD, $\lambda_{ex} = 235$ nm $\lambda_{em} = 296$ nm, UV, $\lambda = 220$ nm	[50]
Scopolamine	Urine, serum	extr., centr., dil., acidif., MIP-SPE	Discovery C18, 150 mm × 4.6 mm, 5 μm	ACN—CH_3COONH_4	UV, $\lambda = 210$ nm	[51]

tracted and concentrated on Extrelut 20 columns before MIP-SPE. Extracts from MIP cartridge were injected into RP-HPLC system in all cases and analytes were detected by means of ED or UV detection ([47] and [48, 49], respectively). Recoveries of presented works were 75 % in [47, 48] and > 91 % in [49].

A multidimensional SPE sample-processing platform for complex fluids, which relies on the combination of small LC columns packed with RAM and MIPs is described in [50]. Method was applied for analysis of analgesic drug Tramadol in serum sample. After injection, the sample was directed to the RAM column with mobile phase consisting of water—ACN. Transfer to the MIP column was performed by use of ACN and Tramadol was eluted from the MIP with ACN—water, transferred to the CN analytical and detected with FD or UV detector.

MIPs selective for alkaloid scopolamine were produced using hyoscyamine as template molecule in [51]. The produced polymers were used as media for SPE of analyte from urine and serum samples. Serum was extracted with ACN, centrifuged and applied to MIP column. Urine sample was adjusted to pH 5 with HCl and loaded to cartridge. HPLC-UV determination was carried out on C18 column with the mixture of ACN—CH_3COONH_4 as mobile phase. Recoveries ranged from 60 to 79 % depending on concentration.

CONCLUSION

Presented work is focused on the use of SPE as method for the sample preparation and three types of new sorbents were discussed.

The first, HLB solid phase sorbents (Oasis) and their applications in SPE of clinical, plant, and food samples were described. From the reviewed literature it is obvious that properties of this new sorbent enable to develop simple procedures for simultaneous extraction of compounds with different chemical behaviour. Most of the papers describe the use of only methanol for the simple elution of analytes. Some authors recommend to use LLE before SPE.

Further, the presented work deals with the application of immunosorbents in SPE. Thanks to the high affinity and high selectivity of the antigen—antibody interaction, they allow high degree of molecular selectivity and became a unique tool in the sample preparation. This technique can be used, likewise Oasis sor-

bents, for the preparation of clinical, food, and plant samples and also some applications in beverage analysis were described. Some examples illustrate the use of immunosorbents in on-line mode of SPE.

Finally, molecularly imprinted polymers and their applications were described. Reviewed studies demonstrate that MIP-SPE is a promising technique for cleaning up and enrichment of analytes from complex samples such as mentioned plant, food, and clinical samples.

ABBREVIATIONS

ACN	acetonitrile
BPA	bisphenol A
DAD	diode-array detector
DEA	diethylamine
DON	deoxynivalenol
ED	electrochemical detection
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
FD	fluorescence detection
HLB	hydrophilic-lipophilic balance
IA	immunoaffinity
IAC	immunoaffinity chromatography
IAS	immunoaffinity sorbent
LLE	liquid-liquid extraction
LOD	limit of detection
LOQ	limit of quantification
MAX	mixed mode: anion-exchange
MCX	mixed mode: cation-exchange
MI	molecular imprinting
MIP	molecularly imprinted polymer
M3G	morphine-3-glucuronide
M6G	morphine-6-glucuronide
OA	ochratoxin A
OSA	octanesulfonic acid
PBS	phosphate-buffered saline
RAM	restricted access media
RP	reversed phase
RSD	relative standard deviation
SFE	supercritical fluid extraction
SPE	solid-phase extraction
TCA	trichloroacetic acid
TEA	triethylamine
TFA	trifluoroacetic acid
THF	tetrahydrofuran
UA	uric acid

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