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Review

Restricted access materials and large particle supports for on-line sample preparation: an attractive approach for biological fluids analysis

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Abstract

An analytical process generally involves four main steps: (1) sample preparation; (2) analytical separation; (3) detection; and (4) data handling. In the bioanalytical field, sample preparation is often considered as the time-limiting step. Indeed, the extraction techniques commonly used for biological matrices such as liquid–liquid extraction (LLE) and solid-phase extraction (SPE) are achieved in the off-line mode. In order to perform a high throughput analysis, efforts have been engaged in developing a faster sample purification process. Among different strategies, the introduction of special extraction sorbents, such as the restricted access media (RAM) and large particle supports (LPS), allowing the direct and repetitive injection of complex biological matrices, represents a very attractive approach. Integrated in a liquid chromatography (LC) system, these extraction supports lead to the automation, simplification and speeding up of the sample preparation process. In this paper, RAM and LPS are reviewed and particular attention is given to commercially available supports. Applications of these extraction supports, are presented in single column and column-switching configurations, for the direct analysis of compounds in various biological fluids.

Keywords: Reviews; Restricted access materials; Large particle supports; Direct injection

Contents

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1. Introduction

In the pharmaceutical field, continued efforts are engaged in the development of faster analytical methods with concomitant higher sensitivity and selectivity. Liquid chromatography (LC) can be considered as the technique of choice currently used for drug and metabolites analysis in biological fluids. However, biological matrices, such as serum and plasma, are complex mixtures incompatible with a direct injection into conventional LC supports. Indeed, when proteineous matrices are directly injected on reversed stationary phases, protein denaturation occurs with an irreversible adsorption on particles, which causes a rapid deterioration of chromatographic performances and clogging of the column. To overcome this problem, a sample preparation step is necessary. The extraction techniques commonly used, such as solid-phase extraction (SPE), liquid–liquid extraction (LLE) and membrane extraction (i.e. ultrafiltration, dialysis), are generally achieved in off-line mode and are, consequently, recognised as being tedious and labour-intensive and are considered as the time-limiting step in the analytical process [1–4]. Traditional protein precipitation (PP) procedures are considered as the fastest and simplest extraction techniques [5–7]. PP can be used as a sample preparation process as well as a pre-treatment in other extraction techniques. However, its low selectivity can induce analyte co-precipitation or mass spectrometry (MS) signal suppression [8–12].

The development of special and selective extraction supports, allowing the direct and multiple injections of biological matrices, is an attractive means to reduce the sample preparation time. Among different supports, restricted access media (RAM) [13–16] and large particles supports (LPS) [17–20] are considered, nowadays, as the most popular extraction materials (Fig. 1). These different extraction supports possess the common property of excluding macromolecules while analytes are generally retained by hydrophobic or electrostatic interactions. Connected to a detector in a single column configuration or to an analytical column in a column-switching configuration, these special extraction supports allow automating, simplifying and speeding up of the sample preparation step. During

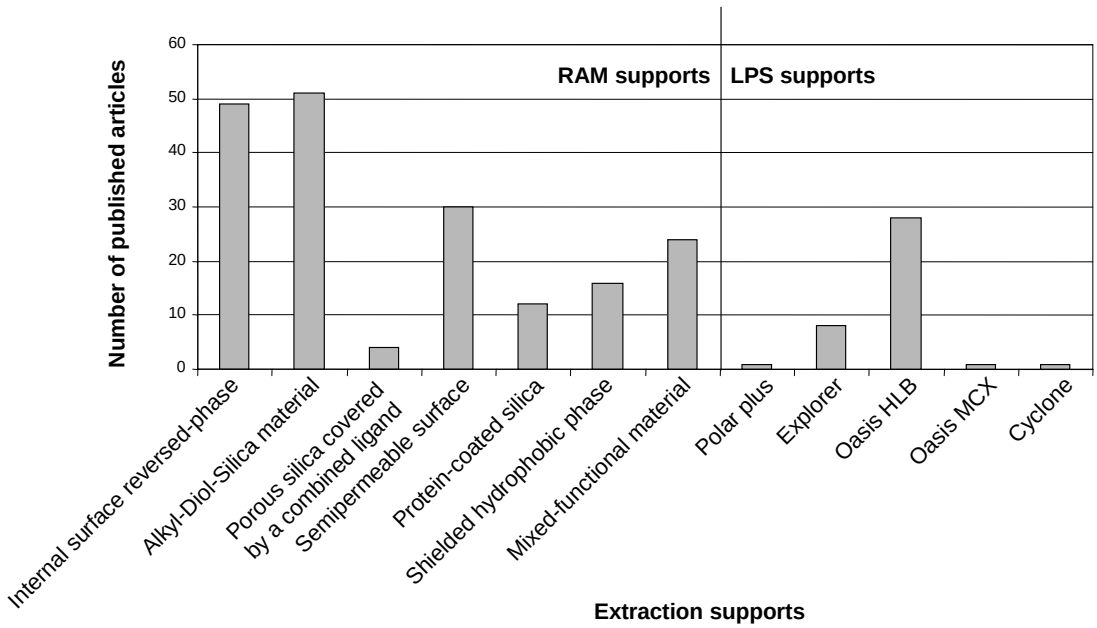

Fig. 1. Articles published on the use of RAM and LPS supports for the on-line sample preparation of biological matrices.

recent years, this on-line sample extraction process has been widely used for the fast analysis of a large variety of compounds in various biological matrices.

This paper describes the use of restricted access media and large particle supports. In the first part, the fundamental characteristics of RAM and LPS sorbents are reviewed and particular attention is given to commercially available supports. In the second part, these extraction supports, in single column and column-switching configurations are discussed and their application to the analysis of compounds in various biological fluids is presented.

2. Special extraction supports for direct and repetitive injection of biological fluids

2.1. Restricted access media

The restricted access media term was introduced by Desilets et al. in 1991 [21]. It designates a support family which allows direct injection of biological fluids by limiting the accessibility of interaction sites within the pores to small molecules only. Macromolecules are excluded and interact only with the outer surface of the particle support coated with hydrophilic groups, which minimises the adsorption of matrix proteins. RAM can be classified according to the protein exclusion mechanism. Macromolecules can be excluded by a physical barrier due to the pore diameter or by a chemical diffusion barrier created by a protein (or polymer) network at the outer surface of the particle. Boos and Rudolphi [13] completed this classification by subdividing RAM sorbents with respect to their surface chemistry. Indeed, these authors discerned phases with different types of bonding in external and internal surfaces (bimodal phases) and phases with a unique bonding to both surfaces (unimodal phases). In the present paper, the RAM classification was based on the exclusion mechanism only to review the characteristics of seven commercially available RAM sorbents. The latter were applied to the direct analysis of numerous compounds in biological matrices as, reported in Table 1.

2.1.1. RAM with a physical barrier

2.1.1.1. Internal surface reversed-phase. The first support for the direct injection of biological matrices was introduced, in 1985, by Hagestam and Pinkerton [22] under the trade name of internal surface reversed-phase (ISRP). This new extraction sorbent was commercialised in 1986. It is constituted of porous silica particles with the outer surface covered by a hydrophilic moiety limiting the adsorption of protein (diol-glycine groups) with a hydrophobic tripeptide partitioning phase (glycine-L-phenylalanine-L-phenylalanine or GFF) only on the internal surface. A schematic drawing of GFF material is presented in Fig. 2. When serum or plasma is directly injected onto this sorbent, macromolecules are excluded from the internal surface region by a size exclusion mechanism (physical barrier). Through enzymatic synthesis, GFF supports are characterized by a particle pore diameter of approximately 8 nm, which enables to exclude proteins larger than about 20,000 Da [22,23]. Therefore, blood protein such as albumin with a molecular weight of 65,600 can be directly eluted from the support while small molecules of interest may be retained within the pores by the tripeptide phase [23,24]. The retention mechanism is mainly due to π-electron interactions. An additional weak-cation exchanger functionality was demonstrated for this support due to the free carboxyl group of the terminal phenylalanine [24]. A second generation of GFF support (GFF II) was developed by Perry et al. [25] to improve performances in terms of efficiency, retentivity and reproducibility. ISRP supports allowed to withstand several hundred plasma or serum injections (total volume of 6–7 ml) without losing performance [26,27]. GFF material has been recognized as a suitable approach for the direct injection of drugs and metabolites in biological matrices [26–43]. This support also shows a real potential for the direct analysis of endogenous substances in serum [44,45] and peptides from complex extracts [46,47].

2.1.1.2. Alkyl-diol-silica material. Alkyl-diol-silica (ADS) support is certainly, with GFF sorbent, the most popular RAMs material. Produced at the beginning of the 1990's, the structure of ADS material is closed to GFF particles [48–50]. Indeed, a physical barrier (pore size 6 nm) excludes macromolecules, which are not adsorbed onto the support thanks to the hydrophilic groups (glycerylpropyl, i.e. diol moieties) bounded at the outer surface of particles (Fig. 2). The RAM phase is characterised by the different available reversed phases (C4, C8 or C18) on the internal surface. Recently, a new ADS support was developed with sulphonic acid groups bonded at the inner surface of the particles. This restricted access cation exchanger, namely XDS (exchange diol silica), demonstrated promising performances for the direct analysis of endogenous compounds [51,52] and pharmaceuticals [51] in biological fluids. The ADS column can tolerate up to 80–100 ml of plasma [50,53,54]. The LiChrospher ADS-RP was able to achieve the direct analysis of pharmaceutical compounds in several biological matrices such as plasma [49,50,53,55–73], serum [42,43,74–79], urine [60,75,76,78,80], microdialysate [81,82], saliva [64], liver homogenate [49], intestinal aspirates [76], cell cultures [68,83], bronchial secretions [74], milk [84] and tissue [67].

2.1.1.3. Porous silica covered by a combined ligand. ChromSpher BioMatrix was introduced as a new extraction sorbent for the direct injection of biological matrices [85,86]. This sorbent consists of bonded porous silica (pore size 13 nm) with a ligand possessing hydrophobic and hydrophilic properties. Alkanolic groups (polyglycidol) of the ligand prevent protein adsorption onto the particle while

Table 1
Compounds analysed by direct injection of biological matrices onto RAM

Compounds (matrix)	RAM sorbent	References	Configuration[a]
AINS (plasma)	LiChrospher RP-18 ADS	[53,71]	CS
AINS (serum)	BioTrap C18	[109]	CS
AINS (serum)	SPS C18, GFF, Hisep	[38]	S
AINS[b] (aqueous humor)	Capcell Pak MF Ph-1	[131]	CS
AINS[b] (cell culture media)	BioTrap 500 MS	[113]	CS
AINS[b] (plasma)	Capcell Pak MF Ph-1	[117]	CS
AINS[b] (plasma)	GFF	[35]	S
AINS[b] (serum)	SPS C18	[103]	S
Aloesin (plasma)	Capcell Pak MF Ph-1	[124]	CS
Amoxicillin (serum)	SPS C18	[102]	CS
Anti-epileptic (serum)	GFF	[37]	S
Anti-epileptic (serum)	GFF II	[31]	S
Antipyrine (serum)	SPS C8	[99]	S
Anxiolytic agent CP-93 393 (plasma)	BioTrap C18	[108]	CS
Arachidonic acid (urine)	LiChrospher RP-18 ADS	[60]	CS
Artemisinin (plasma, saliva)	LiChrospher RP-18 ADS	[64]	CS
Asiaticoside (plasma, bile)	Capcell Pak MF Ph-1	[120]	CS
Atropine (plasma)	LiChrospher XDS	[51]	CS
Azole pesticides (urine)	GFF II	[39]	CS
Barbiturates (plasma)	LiChrospher RP-18 ADS	[63]	CS
Barbiturates (serum)	SPS C8, GFF, GFF II	[40]	S
Barbiturates (serum)	Capcell Pak MF Ph-1	[127]	CS
Benzodiazepines (plasma, serum)	BioTrap MS	[110]	CS
Benzodiazepines (plasma, urine)	LiChrospher RP-18 ADS	[78]	CS
Beta-adrenoceptor ligand (plasma)	ChromSpher BioMatrix	[87,89]	S
Beta-blockers (plasma)	LiChrospher RP-8 ADS	[65]	CS
Beta-blockers (plasma)	BioTrap C18	[109]	CS
Beta-blockers (plasma)	LiChrospher XDS	[51]	CS
Beta-blockers (plasma, microdialysate)	LiChrospher RP-8 ADS	[81]	CS
Beta-blockers (microdialysate)	LiChrospher RP-18 ADS	[82]	CS
Beta-blockers (serum)	BioTrap C18	[109]	CS
Beta-blockers (serum)	GFF II, SPS C18, LiChrospher RP-18 ADS	[43]	CS
Beta-blockers (urine)	LiChrospher RP-18 ADS	[80]	CS
Bilirubins (serum)	GFF	[45]	S
Biphenyldimethyl dicarboxylate (plasma)	Capcell Pak MF Ph-1	[115]	CS
10-Hydroxycampthotecine (serum)	Hisep	[138]	S
Carbamazepine (serum)	Hisep	[135]	S
Carbamazepine (serum)	GFF	[37]	S
Carbamazepine (serum)	SPS C8, GFF, GFF II	[40]	S
Carbamazepine[b] (plasma)	SPS C18	[93]	S
Carbamazepine[b] (plasma)	LiChrospher RP-18 ADS	[72]	CS
Cardiovascular drugs (serum)	GFF II	[32]	CS
Catecholamines (urine)	Hisep	[137]	S
Cefpiramide (plasma)	GFF	[34]	S
Ceftazidime (bronchial secretions, serum)	LiChrospher RP-8 ADS	[74]	CS
Chlozoxazone[b] (serum)	SPS C18	[100]	S
Cisapride (serum)	Capcell Pak MF Ph-1	[116]	CS
Citalopram[b] (plasma)	LiChrospher RP-4 ADS	[55]	CS
Clenbuterol (serum)	GFF, SPS C18, LiChrospher RP-18 ADS	[43]	CS
Clomipramine (plasma)	Capcell Pak MF Ph-1	[121]	CS
Cocaine[b] (plasma)	LiChrospher RP-18 ADS	[69]	CS
Creatinine (serum)	GFF	[41]	CS
Digoxin (serum)	LiChrospher RP-4 ADS	[79]	CS
Dopamine agonist (plasma)	GFF	[27]	CS
Epirubicin[b] (plasma, liver homogenate, liver tumour homogenate)	LiChrospher RP-4 ADS	[49]	CS
Entacapone glucuronide (plasma)	LiChrospher RP-18 ADS	[56]	CS
Felodipine (plasma, tissue)	LiChrospher RP-18 ADS	[67]	CS

Table 1 (*Continued*)

Compounds (matrix)	RAM sorbent	References	Configuration[a]
Fenoterol (plasma)	LiChrospher XDS	[51]	CS
Fleroxazin (serum)	Hisep	[142]	S
Flunitrazepam[b] (plasma)	BioTrap MS LiChrospher RP-18 ADS	[57]	CS
Furosemide (plasma)	GFF	[35]	S
Granisetron[b] (plasma)	GFF II	[26]	CS
Heroin[b] (urine)	Capcell Pak MF SCX	[134]	CS
4-Hydroxyanisol (serum)	GFF	[30]	S
Indoxyl sulfate (serum)	GFF	[44]	S
Ipratropium (plasma)	LiChrospher XDS	[51]	CS
Lamotrigine (plasma, serum)	GFF	[28]	S
Linezolid (serum, urine)	LiChrospher RP-8 ADS	[75]	CS
Local anaesthetics (plasma)	SPS C8	[92]	CS
Local anaesthetics (plasma)	LiChrospher RP-18 ADS	[59]	CS
Matrix metalloprotease inhibitors (plasma)	SPS C18	[95]	CS
Meropenem (bronchial secretions, serum)	LiChrospher RP-18 ADS	[74]	CS
Methadone[b] (serum)	GFF II, LiChrospher RP-4 ADS	[42]	S, CS
Methotrexate[b] (plasma)	LiChrospher RP-8 ADS	[61,62]	CS
8-Methoxysporalen (plasma)	LiChrospher RP-8 ADS	[50]	CS
Mitomycin C (plasma)	Capcell Pak MF Ph-1	[118]	S
Montelukast sodium (plasma)	Capcell Pak MF Ph-1	[119]	CS
Mycophenolic acid (serum)	Capcell Pak MF Ph-1	[133]	CS
Neuropeptide Y (plasma)	LiChrospher XDS	[52]	CS
Nitrendipine (plasma, tissue)	LiChrospher RP-18 ADS	[67]	CS
NTBC[c] (plasma)	BioTrap C18	[107]	CS
Omeprazole[b] (plasma)	Capcell Pak MF Ph-1	[130]	CS
Oxytetracycline (serum)	Hisep	[139]	S
Paclitaxel (plasma)	LiChrospher RP-4 ADS	[58]	CS
Paraquat (plasma)	LiChrospher RP-18 ADS	[73]	CS
Pentoxifylline (plasma)	Hisep	[141]	S
Pirlindol (plasma)	LiChrospher RP-4 ADS	[66]	CS
Procaine (plasma)	LiChrospher XDS	[51]	CS
Propafenone[b] (serum)	LiChrospher RP-18 ADS	[77]	CS
Propentofylline (serum)	Hisep	[140]	CS
Propofol (plasma)	GFF	[36]	S
Proton pump inhibitor (KR60436)[b] (plasma)	Capcell Pak MF Ph-1	[129]	CS
Rifapentine[b] (plasma)	Hisep	[143]	S
Ropivacaine and bupivacaine (plasma)	SPS C18	[94]	CS
Salbutamol (serum)	GFF, SPS C18, LiChrospher RP-18 ADS	[43]	CS
Sildenafil[b] (plasma)	Capcell Pak MF Ph-1	[125]	CS
Steroid compounds (liver microsomes)	BioTrap C18	[111]	CS
Steroid compounds (plasma)	LiChrospher RP-18 ADS	[60]	CS
Steroid compounds (cell culture)	LiChrospher RP-4 ADS	[83]	CS
Steroid compounds (hepatocytes)	BioTrap 500 MS	[112]	CS
Sulfamonomethoxine, miloxacin, oxolinic acid (serum, muscle)	Hisep	[144]	S
Sulfamonomethoxine[b] (serum)	Hisep	[136]	S
Tamoxifen[b] (plasma)	SPS CN	[97]	CS
Terbutaline (plasma)	LiChrospher XDS	[51]	CS
Tetracyclines (urine)	ChromSpher BioMatrix	[88]	CS
Thiacetazone (plasma)	Capcell Pak MF Ph-1	[126]	S
Tofisopam (serum)	Capcell Pak MF Ph-1	[132]	CS
Trimethoprim (milk)	LiChrospher RP-18 ADS	[84]	CS
Vancomycin (serum)	SPS C8	[104]	S
Verapamil[b] (serum)	Hisep	[101]	S
Verapamil[b] (serum)	SPS C8	[101]	CS
Verapamil[b] (cell culture, plasma)	LiChrospher RP-8 ADS	[68]	CS
YM087, YM440[b] (plasma)	LiChrospher RP-18 ADS	[70]	CS

[a] S: single column configuration, CS: column-switching configuration.
[b] Metabolites.
[c] 2-(2-Nitro-4-trifluoromethylbenzoyl)-1,3-cyclohexanedione.

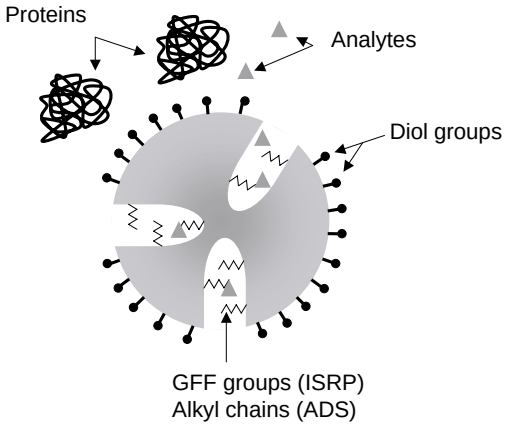

Fig. 2. Internal surface reversed-phase (ISRP) with GFF groups and alkyl-diol-silica (ADS) with alkyl chains.

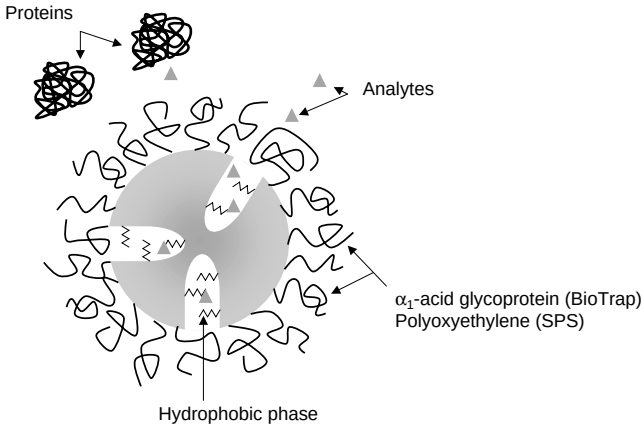

Fig. 4. Semi-permeable surface and protein-coated silica supports.

phenyl groups provide hydrophobic interaction with small analytes (Fig. 3). ChromSpher Biomatrix supports have been applied to the direct analysis of small compounds from several complex matrices [85–89].

2.1.2. RAM with a chemical barrier

2.1.2.1. Semi-permeable surface. Desilets et al. [21] demonstrated that polyoxyethylene polymer bonded to the surface of a reversed-phase chromatographic packing (i.e. C8 or C18 phase) formed a semi-permeable hydrophilic layer which can restrict protein access to the underlying hydrophobic stationary phase. On this basis, another RAM sorbent was developed under the trade name of semi-permeable surface phase (SPS; Fig. 4) [40,90]. SPS material has outer and inner moieties independently synthesized and covalently grafted at the surface of the silica particle. The outer surface repels large molecules such as proteins, while the inner surface can be any of several common hydrophobic-reversed phases. Small analytes, which can penetrate through the polymer layer, interact with the hydrophobic sites. SPS materials with different inner surfaces (nitrile, phenyl, C8 and C18) are commercially available. The life span of SPS material has been found equivalent to ADS material [91]. Indeed, up to 50 ml of plasma can be loaded on this extraction sorbent [92]. SPS material has been successfully applied to the analysis of small molecules in biological fluids [38,43,92–104].

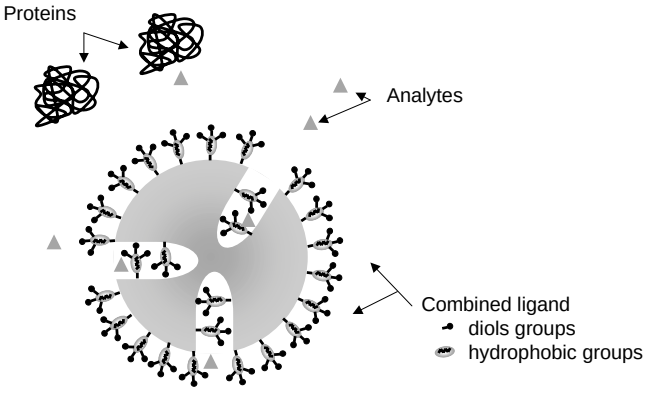

Fig. 3. Porous silica covered by a combined ligand.

2.1.2.2. Protein-coated silica. Protein-coated silica material is based on macromolecule exclusion by a chemical diffusion barrier, as previously described. However, this sorbent uses a protein network at the outer surface instead of a polymer (Fig. 4). This extraction support developed for the direct injection of biological fluids, was introduced by Hermansson and Grahn in 1994 [105] and was commercialized under the trade name of BioTrap [106]. This material is constituted of porous silica particles with the outer surface covered with the human plasma protein; α_1-acid glycoprotein (AGP). This makes the external surface of the particles compatible with a proteineous sample which cannot penetrate into small pores (10 nm). Hydrophobic groups (C8 or C18) at the inner surface are responsible for interaction with small analytes. Protein-coated silica material has exhibited good performances for the analysis of pharmaceutical compounds in biological matrices [31,107,108]. BioTrap can tolerate up to 30 ml of biological fluids without notable performance deterioration [109]. Nowadays, the BioTrap support is available with a hydrophobic polymer (BioTrap MS) and presents the advantage of being used in a wider pH range (from 2 to 10) than a silica material (pH range from 2.5 to 7.5). This new support generation was successfully used for the analysis of drugs in biological matrices [57,110–113].

2.1.2.3. Mixed-functional material. In 1994, a silicone polymer-coated mixed-functional silica material was introduced by Kanda et al. [114] for the direct injection of biological fluids. This concept of mixed-function extraction phase was commercialised under the Capcell Pak MF trade name. Both internal and external surfaces consist of

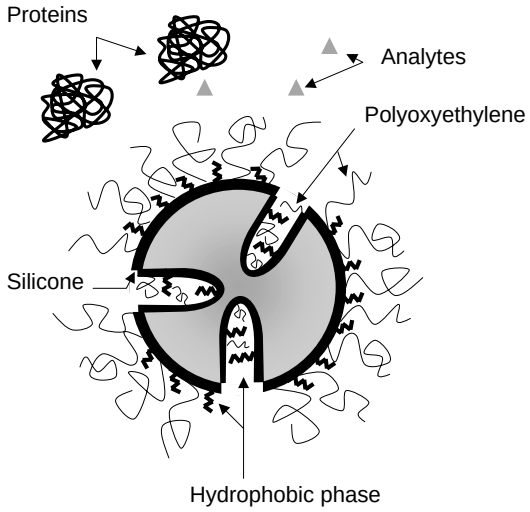

Fig. 5. Mixed-functional material.

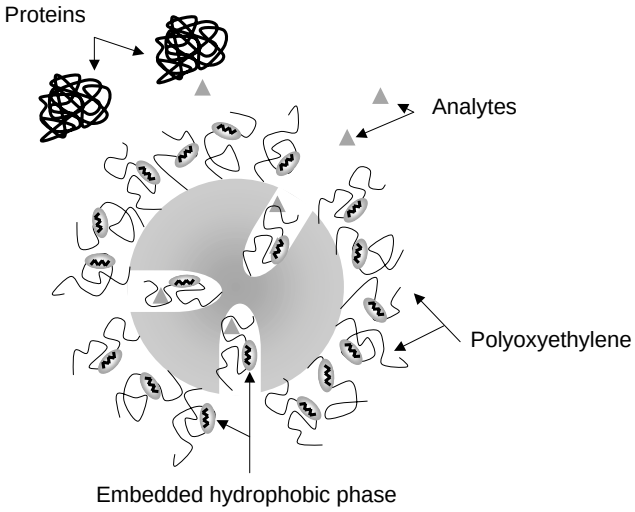

Fig. 6. Shielded hydrophobic phase.

a mixture of hydrophilic polyoxyethylene and hydrophobic styrene groups grafted on silicone polymer-coated silica (8 nm). The long polyoxyethylene chains limit the access to macromolecules. Therefore, matrix components interact only with the hydrophilic, non-adsorptive polymer network and elute in the void volume. Several extraction materials with phenyl, C8 and strong-cation exchanger (SCX) moieties instead of styrene groups are available. Small analytes are retained on the bonded hydrophobic or ion exchanger groups. This extraction sorbent is represented in Fig. 5. Several ml of biological fluids can be directly injected on this extraction material without loss of performances [115,116]. Capcell Pak MF [115–133] and Capcell Pak SCX [134] materials were suitable tools for the direct determination of drug-containing biofluids.

2.1.2.4. Shielded hydrophobic phase. In 1988, a new design for direct injection analysis was developed by Gisch et al. [135] and termed shielded hydrophobic phase (SHP). This extraction support was introduced on the market under the name of Hisep. This sorbent is a silica-based material covered with a hydrophilic network of polyethylene oxide with embedded hydrophobic phenyl groups (Fig. 6). Macromolecules are prevented from contact with the surface by hydrophilic shielding. Small molecules can penetrate through the polymer layer and interact with hydrophobic groups. Hisep column can tolerate 16 ml of serum without significant loss of performance [136]. Hisep material was used for the direct analysis of endogenous compounds [137] and pharmaceuticals in biological matrices [101,135,136,138–144].

2.2. Large particle supports

2.2.1. Concept of large particle supports

Another approach for the direct injection of biological fluids is the use of large particle supports. These extraction supports are packed with particles whose diameter is 30–50 μm permitting to apply a high mobile phase flow rate without generating high backpressure in the system. Under these conditions, the rapid percolation of proteins and other hydrophilic endogenous material through the extraction support can be performed while analytes are retained by means of hydrophobic interactions. This extraction technique was patented in 1997 by Quinn and Takarewski, under the name of turbulent flow chromatography [145]. In fact, this concept had already appeared in the 1960's as a new approach for performing fast analysis in open tubular column [146] and in the 1980's in packed columns [147].

The choice of the flow rate can be considered as the key parameter of the extraction procedure. Indeed, Wu et al. [148] demonstrated that under high (turbulent) flow rate, LPS tolerated several hundred plasma injections of 100 μl without any deterioration of the system, whereas, under conventional (laminar) flow rate, extraction supports were rapidly damaged. According to the authors, eddy strengths, generated by a high flow rate, allow to extricate large molecules blocked in a small channel (Fig. 7). Then, macromolecules can be directed towards another larger pathway and pass though the extraction support without plugging the system. A schematic representation of the improved mass transfer produced by eddies, under high flow rate conditions, is reported in Fig. 8.

2.2.2. Available LPS

The description of LPS is very simple. Indeed, LPS supports are packed with commonly used chromatographic stationary phases. The main differences between conventional LC material and LPS are particle and column diameters. Given the high mobile phase flow rate, the extraction column is packed with large particles and is usually commercialized in microbore column dimension to ensure

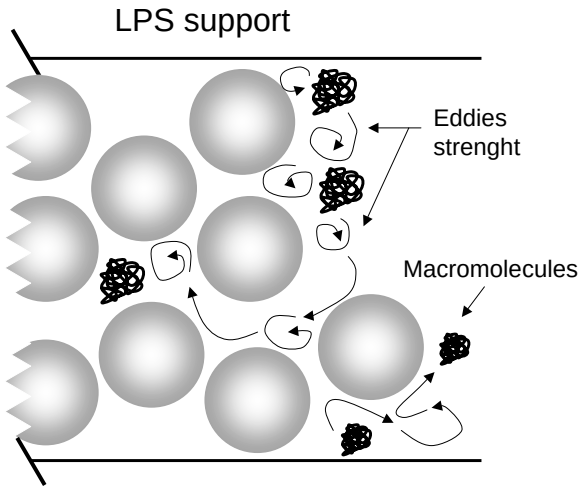

Fig. 7. Schematic drawing of LPS with eddies strengths implied to the protein exclusion mechanism.

compatibility with conventional LC systems. Generally, a flow rate of 3–5 ml min^{-1} is applied onto a LPS column of 1 mm i.d. [149–152].

Different stationary phases have been introduced on the market such as micro and capillary columns packed with 50–60 μm particles of various sorbents. Two types of stationary phases can be distinguished: (1) silica particles coated by classical alkyl chains (C2, C8 or C18), phenyl groups or mixed apolar/polar phase and (2) polymeric particles (styren-divinylbenzene cross-linked copolymer). An extraction column packed with a polymeric sorbent (divinylbenzene-*N*-vinylpyrrolidone copolymer) is available under the name of Oasis HLB. A Oasis cation exchanger (MCX) is also commercialized.

Table 2 offers a survey of the applications of these extraction supports.

2.3. Monolithic phases

Recently introduced to achieve fast chromatographic separation [153,154], monolithic phases are also suitable for the direct injection of biological fluids. Thanks to their high permeability [153,154], the extraction of biological samples can also be performed with a high flow rate without generating high backpressure. However, in this case, the flow rate remains laminar and is 5–10 times higher than generally used with conventional supports. It is noteworthy that, contrary to RAM and LPS, monolithic phases produced by the sol–gel polymerisation technology do not require frits at column extremities, which often remain the main source of endogenous material adsorption. Plumb et al. [155] have demonstrated that monolithic supports can tolerate several ml of plasma without significant performance degradation. Commercialized monolithic columns also exhibited good performances for the analysis by direct injection of a drug cocktail in human plasma [156]. However, the number of commercially available monolithic columns is limited and their dimensions (i.e. 4.6 mm i.d.) are not really adapted to extraction procedures with conventional instrumentation. Therefore, monolithic supports cladded in miniaturized column dimension are necessary to implement this material in clinical and pharmaceutical laboratories.

3. On-line sample preparation set-up

The supports reviewed in this paper have been developed to tolerate the direct and repetitive injection of biological fluids with different designs and functionalities. These supports can be integrated in an analytical system to automate the sample purification step. Two configurations have been described: in the first device, called single column or direct configuration, the extraction support is directly connected to a detector. In this case, the support is used for the extraction and separation steps. The second configuration is based on an extraction support and an analytical column coupled with a switching-valve. This configuration, termed column-switching, only uses the support as extraction pre-column.

3.1. Direct mode configuration

In the direct mode configuration, the analytical procedure involves three steps: (1) sample extraction; (2) analyte elution; and (3) re-equilibration of the extraction support. The single column configuration is represented in Fig. 9.

Firstly, the biological fluid is injected onto the extraction support with an appropriate mobile phase. During this extraction step, analytes are retained while endogenous components are eluted within the void volume. Afterwards, analytes are eluted from the support to the detector. Finally, the extraction support is re-conditioned with the loading mobile phase and is ready for the next injection.

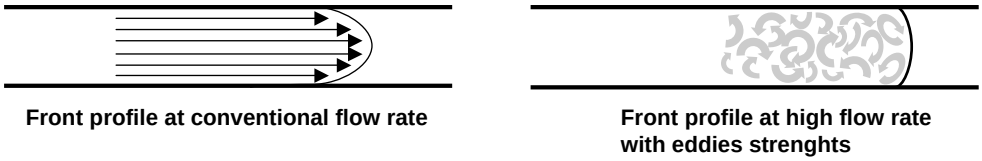

Fig. 8. Conventional and high flow rate in packed column.

Table 2
Compounds analysed by direct injection of biological matrices onto LPS

Compounds (matrix)	LPS sorbent	References	Configuration[a]
Adatenserine (microsomal incubate)	HTLC C8	[191]	CS
Aminopterin (plasma)	Oasis HLB	[192]	S, CS
Apomorphine (plasma)	Oasis HLB	[192]	S, CS
Benzoylecgonine (plasma)	Oasis HLB	[192]	S, CS
Benzoylecgonine (plasma)	Oasis HLB	[193]	CS
Beta-lactam[b] (plasma)	Oasis HLB	[177]	CS
Catechins (plasma)	HTLC C18	[194]	CS
Citalopram, fluvoxamine, paroxetine (plasma)	Oasis HLB	[195]	CS
Clemastine, diflunisal (plasma)	Oasis HLB	[190]	S, CS
Clozapine, olanzepine[b] (plasma)	Oasis HLB	[183]	CS
Dofetilide (plasma)	HTLC C18	[187]	CS
Doxazosin (plasma)	HTLC C18	[187]	CS
Fenfluramine (plasma)	Oasis HLB	[184]	CS
Guanidine-containing drug (plasma)	Oasis HLB	[189]	CS
Haloperidol (microsomal incubate)	HTLC C8	[191]	CS
Haloperidol (plasma)	Oasis HLB	[193]	CS
Isoquinoline compound (plasma)	Oasis HLB	[160,161,163]	S
Isoquinoline compound (plasma)	Explorer C18[c]	[160]	S
Ketoconazole (plasma)	HTLC C18	[188]	CS
Melatonin (serum)	Oasis HLB	[196]	S
Methotrexate (plasma)	Oasis HLB	[197]	CS
Narcotic (plasma)	Oasis HLB	[17]	S
Oxazepam, alprozolam, carbamazepam, clobazam, estazolam, temazepam (plasma)	Oasis HLB	[184]	CS
Oxazepam, alprozolam, carbamazepam, clobazam, estazolam, temazepam (plasma)	Oasis HLB	[192]	S, CS
Oxazepam, alprozolam, carbamazepam, clobazam, estazolam, temazepam (plasma)	Oasis HLB	[193]	CS
Oxycodone (plasma)	Oasis HLB	[193]	CS
Phentolamine (plasma)	Oasis HLB	[193]	CS
Pravastatine[b] (serum)	Oasis HLB	[159]	S, CS
Propranolol (plasma, urine)	Oasis HLB, Oasis MCX	[198]	CS
Promethazine[b] (urine)	Oasis HLB	[152]	CS
Puromycin (plasma)	Oasis HLB	[193]	CS
Simvastatin[b] (plasma)	Oasis HLB	[151]	CS
Tamoxifen (plasma)	Oasis HLB	[184,193]	CS
Terbinafine (plasma)	HTLC C18	[149]	CS
Tetracyclines (kidney)	Oasis HLB	[164]	S
Triprolidine (plasma)	Oasis HLB	[193]	CS
Vancomycin (serum, urine)	Oasis HLB	[186]	CS
Venlafaxine (microsomal incubate)	HTLC C8	[191]	CS
Tricyclic antidepressants (plasma)	Oasis HLB	[17]	S
Tricyclic antidepressants (plasma)	Oasis HLB	[199]	CS
Others[d]			
Anticanceral drug (plasma)	Polar Plus	[200]	CS
Drugs A, B (plasma)	Oasis HLB	[165]	S
Drugs A, B (plasma)	HTLC C18	[165]	S
Compounds I, II (plasma)	Oasis HLB	[150]	CS
Compound A (plasma)	Cyclone	[201]	CS

[a] S: single column configuration, CS: column-switching configuration.
[b] Metabolites.
[c] Old version of HTLC C18 from Cohesive Technologies.
[d] Not revealed.

According to the nature of the extraction supports and detection system, two different approaches can be discerned. Indeed, RAM and LPS belong to two different generations of extraction supports and their development were mainly influenced by the properties of the detection system. As previously mentioned, RAM columns appeared in the middle of the 1980's when the UV spectrophotometer was the current detection system. Consequently, given the relatively low selectivity of UV detection, special focus was given to the chromatographic efficiency of these extraction sup-

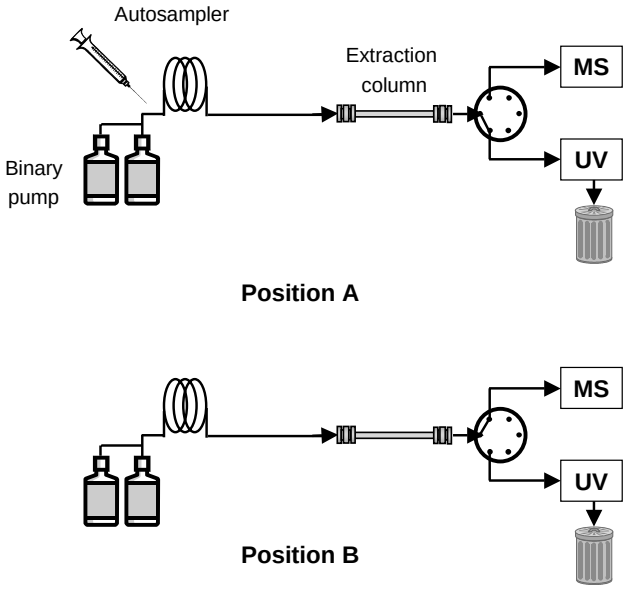

Fig. 9. Single column configuration.

ports commercialized in conventional column dimensions. Concerning LPS, the latter were introduced 10 years later and were generally coupled directly to a mass spectrometer which appears to be the system of choice thanks to its high selectivity and sensitivity. In this context, LPS in short column dimensions were developed with a special emphasis on high throughput analysis.

3.1.1. Single column mode with UV detection

In order to evaluate their potential, first commercialized RAM supports (ISRP and SHP) for the direct analysis of compounds in biological fluids, were integrated in direct mode with UV detection [30,34,36,37,40,44,135,141,143]. These extraction supports packed in columns with a conventional dimension (i.e. 150–250 mm length, 4.6 mm i.d.) not only allowed extraction but also chromatographic separation. In this context, Riva et al. [143] developed a direct method for the simultaneous determination of rifapentine and its metabolite in plasma, based on the extraction and complete analyte separation onto a Hisep column (150 mm × 4.6 mm i.d.) [143]. An ISRP column (250 mm × 4.6 mm i.d.) also allowed extraction from serum and separation of three anti-epileptic drugs with a sufficient resolution [37]. Due to good method performances in terms of precision, trueness and robustness, many methods based on single column configuration and UV detection have recently been published. In 1999, Haque and Stewart [38] developed and validated a method for the analysis of several non-steroidal anti-inflammatory drugs (NSAIDS) in serum with ISRP and SPS columns coupled to a UV detector. Recently, a quantitative analysis of vancomycin in serum was performed onto SPS supports connected to a UV detection [104]. Generally, total analysis time (TAT), including sample preparation and analyte separation steps is superior to 15 min.

However, due to the small injection volume (i.e. 5–50 μl) and the low sensitivity of UV detection, these methods suffer from low sensitivity. For example, with a direct injection of 10 μl of plasma onto a GFF support (150 mm × 4.6 mm i.d.) coupled to a UV detector, Pullen et al. [36] obtained a limit of detection of 1 μg ml^{-1} for propofol. In order to improve the sensitivity of single column methods using conventional UV detection, extraction supports packed in smaller diameter columns are recommended [157,158]. However, RAM supports packed in semi-micro (2 mm i.d.) or in micro (1 mm i.d.) columns are not yet commercially available.

LPS in a single column configuration using UV detection are not recommended since they present low chromatographic performances [159].

3.1.2. Single column mode with MS detection

In the beginning of the 1990's, the introduction of mass spectrometry equipped with atmospheric pressure ionisation (API) techniques such as electrospray ionisation (ESI) and atmospheric pressure chemical ionisation (APCI) compatible with liquid chromatography contributed to the development of LPS supports for performing high-throughput analysis. Fast and sensitive analytical methods based on the use of an extraction support coupled to MS were developed for the determination of drugs and metabolites in biofluids [17,42,159–165].

Thanks to its high selectivity, LC–MS methods were developed with short extraction columns packed with LPS supports. Consequently, incomplete analyte separation and high flow rates contributed to the speeding up of the process. Indeed, a TAT of less than 3 min was achieved [17,160,161,165–167]. Ding and Neue [17] developed a fast method for the analysis of drugs in biological fluids. The procedure was based on an Oasis HLB support coupled to MS and was achieved in 1.3 min. A similar configuration was successfully applied to determine a novel isoquinoline drug in biological fluids with an analysis time of 1.2 min [161].

In order to increase the sample throughput, multiple analyses can be achieved [5,168]. Bayliss et al. [163] reported a system with four LPS coupled in parallel to the MS with a multi-sprayer interface which allows the analysis of 120 plasma samples per hour.

Given the high MS sensitivity, compounds can be extracted and analyzed at the ng ml^{-1} level in complex matrices [159,161,165]. Jemal et al. [159,167] developed a single column method with an Oasis HLB support coupled to a triple quadrupole mass spectrometer for the on-line extraction of a pharmaceutical compound in plasma. Method sensitivity can be improved with smaller i.d. columns [157,158]. Ayrton et al. [162] developed a method with a LPS column on the capillary scale (180 μm i.d.) for the direct analysis of isoquinoline drug in plasma at sub ng ml^{-1} concentrations.

To our knowledge, only few papers describe RAM supports directly coupled to MS detection. Ortelli et al. [42] developed a method with a GFF column connected to ESI-MS detector for the analysis of methadone and its primary metabolite in serum. Mixed-functional material was coupled to a triple quadrupole mass spectrometer for the analysis of drug cocktails in plasma [122,128].

3.1.2.1. MS requirements. With MS detection in a single column configuration, some points have to be considered:

- An additional switching-valve is required to avoid contamination of the MS source with the biological material. A schema of the single column mode with MS detection is shown in Fig. 9. During the extraction step, the valve is in position A and the effluent containing endogenous components is directed to waste. After elution of the biomaterial, the valve is switched to position B and analytes are eluted from the extraction support to the MS detector.
- With regard to LC–MS techniques, a selection of mobile phase constituents has to be performed. Indeed, non-volatile mobile phase constituents such as phosphate buffers and ion-pairing agents have to be avoided. Mobile phases must be suitable for sample loading, analyte elution and compatible with MS detection. Guidelines for LC–MS operating conditions are described elsewhere [169,170].
- Compatibility between the mobile phase flow rate and the MS source is of utmost importance. Flow rates of 5–10 $\mu l\,min^{-1}$ and 50–200 $\mu l\,min^{-1}$ were generally applied with ESI and pneumatically-assisted ESI techniques, respectively [171]. Despite several efforts engaged to develop MS interfaces compatible with high flow rates [172], a flow splitter is necessary when extraction supports operate at a high flow rate. An elegant solution is to use extraction columns with a small i.d. operating at a low flow rate and which allows sensitivity improvement.
- The absence of selectivity, which speeds up the analytical process, can become an important limitation. Indeed, the co-elution of analytes and interfering substances in biological matrices can modify the MS response of the target molecules [8,173]. This undesirable phenomenon, termed matrix effect, can considerably affect the analytical performances of the method [174,175]. During method development, special attention has to be paid to the absence of matrix effect. Biotransformation products can also hamper the quantitative determination of compounds in biological matrices by LC–MS [176,177]. Analysis of isomeric compounds can also become a real problem [159]. Therefore, despite high MS selectivity, chromatographic separation remains essential. In this context, the coupling of an analytical column to the extraction support (i.e. column-switching configuration) appears as a suitable alternative.

3.2. Column-switching configuration

In the last decades, the column-switching configuration has proved to be a useful approach for the determination of compounds in biological matrices [54,178–181]. The extraction support, used for the extraction and/or pre-concentration of the sample, is coupled to an analytical column allowing the separation of analytes before detection. For this purpose, an additional pump and a switching-valve are required. A schematic representation of a column-switching configuration working in backflush mode is reported in Fig. 10.

During the extraction step, the switching-valve is in position A. The sample is injected into the extraction pre-column with the loading mobile phase. Simultaneously, the analytical column is conditioned with an elution mobile phase. After the extraction, the valve is switched to position B. Analytes are eluted in backflush mode from the extraction support with the mobile phase and transferred to the analytical column. Afterwards, the valve is switched to its initial position (position A). Analytes are separated onto the analytical column and directed to the detector. Simultaneously, the extraction pre-column is re-equilibrated with the loading mobile phase. Finally, the system is ready for the next sample injection.

In recent years, RAM and LPS have been widely used in the column-switching device. Independently of the extrac-

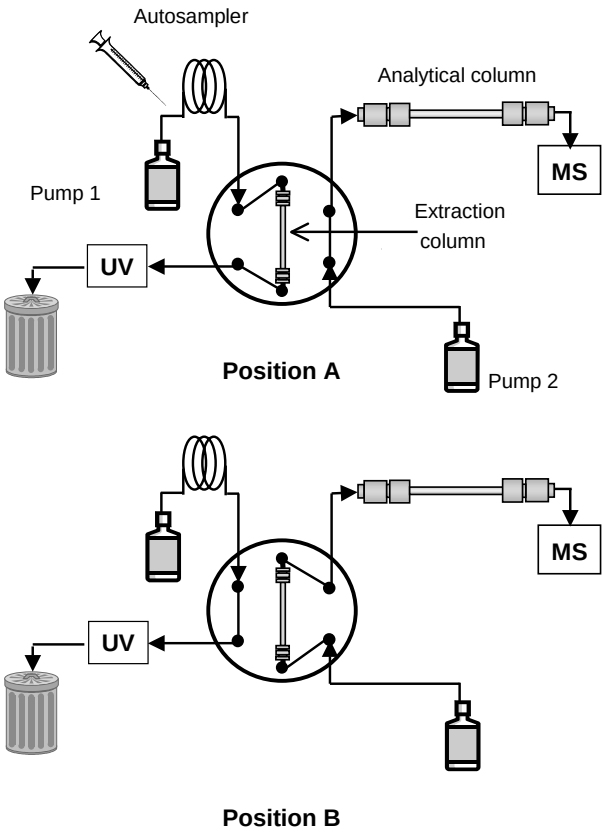

Fig. 10. Column-switching configuration.

tion support, this configuration offers great selectivity and sensitivity.

3.2.1. Selectivity

In the column-switching configuration, short extraction columns, termed pre-column, are generally used. A pre-column allows to extract analytes from endogenous material while chromatographic separation between analytes is performed in the analytical column. The column-switching device affords significant flexibility to the system because various extraction and analytical columns with different selectivities can be used. The conventional RP-18 stationary phase has been the most popular analytical column integrated in a column-switching system [39,42,50,88,95,111, 119,151,182,183]. An example is the method described for the determination of five benzodiazepines in serum and urine [78]. After sample extraction onto a LiChrospher RP-18 ADS, separation was performed on a C_{18} analytical column. An octadecyl analytical column coupled to a pre-column packed with large particles was also found suitable for the simultaneous analysis of methadone and its primary metabolite in plasma [182]. Octyl [26,49], phenyl [55,108], cyano [97,152], mixed-mode [69] and carbonaceous [94] analytical columns coupled to different extraction pre-columns were also suitable for the analysis of several compounds in biological matrices. Recently, Zeng et al. [184] developed a column-switching method using an Oasis HLB coupled to a monolithic column for the analysis of a drug cocktail in plasma. This configuration allowed a fast analyte transfer from the pre-column to the analytical column because the high flow rate on the monolithic phase can be applied on the LPS supports for analyte desorption without high back-pressure. Under these conditions, the TAT was of 2 min.

Concerning the analyte time transfer, it is recommended for an efficient separation, to use an extraction sorbent less retentive than the chromatographic support. Indeed, analyte elution from the extraction support must be performed in a small mobile phase volume to avoid peak broadening which could negatively affect the separation [54].

Using a chiral stationary phase (CSP) in column-switching configuration is a powerful technique for the stereoselective analysis of drugs in biological fluids, as already demonstrated [185]. Various CSP with different chiral selectors, such as cyclodextrins [98], cellulose derivatives [63,66], proteins [53] and antibiotics [65,82], have been successfully coupled to extraction pre-columns. A typical application is a CSP packed with human serum albumin as selector coupled to a LiChrospher RP-18 ADS for the determination of ketoprofen enantiomers in plasma [53]. Biological samples are injected onto the extraction support with a phosphate buffer and, after the extraction step, analytes are backflushed to the chiral column, with a loading mobile phase constituted of a mixture of phosphate buffer, propanol and octanoic acid.

3.2.2. Sensitivity

On-line sample extraction based on a column-switching device is characterized by high sensitivity. Indeed, a pre-concentration step based on a large injection volume of the biological sample onto the extraction support can be performed. Gordi et al. [64] developed a sensitive method for the quantification of artemisinin in saliva and plasma. With the injection of 1 ml of saliva on a LiChrospher ADS coupled to an octadecyl column and a UV detection, concentrations down to 2 ng ml^{-1} were quantified. The limit of detection was of 30 ng ml^{-1} for several local anaesthetics injected with a volume of 500 μl of plasma onto a SPS pre-column integrated in a column-switching device with UV detection [92]. Song and Putcha [152] described a method for the simultaeous analysis of promethazine and three metabolites in urine with an injection volume of 1–2 ml onto an Oasis HLB extraction support. With UV detection, analyte concentrations were between 10 and 20 ng ml^{-1}. Sensitive column-switching methods using a derivatisation procedure were described [61,97]. Yu et al. [61] induced a post-column photochemical reaction in a column-switching device for the determination of methotrexate and its metabolite in plasma. The system consisted of a LiChrospher ADS RP-8 coupled to an octadecyl analytical column, followed by a photoreactor and fluorimetric detection. With an injected plasma volume of 100 μl, analyte concentrations were detected up to 2 ng ml^{-1}.

Method sensitivity was improved with mass spectrometry. A highly sensitive column-switching method based on a GFF II pre-column and a triple quadrupole mass spectrometer was described for the quantitation of granisetron and its metabolite in plasma [26]. With an injection volume of 80 μl, the concentration at sub-ng ml^{-1} level for each analyte was quantified. Similar performances were obtained for the analysis of pravastatine and its positional isomer biotransformation product in serum using an LPS [159]. The method allowed to quantify of analytes in serum at 0.5 ng ml^{-1}.

3.2.3. Analysis time

The TAT depends on the pre-column and the analytical column. With LPS, which allow a rapid extraction step due to the high mobile phase flow rate applied, chromatography is often the limiting step. In order to reduce the latter, short analytical columns packed with small particles operated at high flow rates are coupled to the extraction supports. Under this configuration, TAT are less than 5 min [151,177,182,186–189]. In the case of RAM sorbents, TAT depends not only on the separation time but also on the extraction time. Indeed, conventional flow rates are used for sample extraction on RAM supports and total analysis time are usually longer to 5 min [42,43,67,78,88,95,108,123,140]. Nevertheless, alkyl-diol-silica material have, recently, been found suitable for a fast and efficient sample extraction at a high flow rate [16,167]. In this context, a mobile phase at 4 ml min^{-1}, applied to a 25 mm × 2 mm i.d. LiChrospher

ADS, allows a plasma sample cleanup in less than 1 min. This material with particle diameter of 25 μm belongs also to LPS and can operate at high flow rate.

As described previously for the single column mode, the sample throughput can be increased by combining several supports in parallel via 10-port or 6-port switching-valves [5,168]. A typical system connects an analytical column to two alternating extraction pre-columns. With this device, one of the extraction supports was re-equilibrated while chromatographic separation was performed on the analytical column coupled on-line with the second extraction column. Therefore, analyses were performed in parallel and no re-equilibration time of the extraction support was added to the overall analytical process. This approach was applied for the determination of granisetron and its metabolite in plasma [26]. Alternate injections of biological sample were performed onto two GFF II pre-columns in a parallel configuration coupled to an octyl silica analytical column. With this set-up, a TAT was of 6 min. A similar configuration based on RAM sorbents such as LiChrospher ADS [76] or BioTrap [108] coupled to a conventional analytical column were described for the analysis of drugs and metabolites in various biological samples. Xia et al. [189] developed a high-throughput method which allowed the analysis of guanidine drugs in plasma injected in two LPS extraction supports coupled to an octadecyl analytical column in less than 2 min. A similar process was applied for the determination of acidic and basic drugs in plasma [190].

4. Conclusion

Dedicated extraction supports such as RAM and LPS which allow direct and repetitive injection of biological fluids have revolutioned the field of bioanalysis. Compared to conventional extraction techniques in off-line mode such as LLE, SPE, these extraction supports, integrated in a LC system, allow the automation, speed-up and simplification of the extraction step. Nowadays, various classes of extraction supports with different functionalities and properties are commercially available.

RAM and LPS in single column mode were applied for the analysis of different compounds in various biological matrices by direct injection. Despite satisfactory analytical performances, this configuration presents some limitations in terms of selectivity and sensitivity. To overcome these drawbacks, column-switching emerged as an attractive alternative. Indeed, this configuration offers a great opportunity to minimize interferences and enhance the system selectivity and sensitivity. Thanks to their high versatility, column-switching methods including RAM or LPS exhibited excellent performances for the analysis of a wide range of substances in different matrices.

Because of the bioanalytical potential of RAM and LPS sorbents and their increasing popularity, the development of new supports based on various column dimensions and chemical properties and compatible with LC–UV and LC–MS methods is certainly the next step in on-line extraction research.

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