

## **Extraction of Peptides from Body Fluids Using Supported Liquid Membranes**

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Sample pre-treatment is a very important step in many analytical procedures, especially when the analyte is presented in low concentration in complex sample matrices. In this paper, potential using of the supported liquid membrane (SLM) technique as a sample preparation step in order to isolate, pre-concentrate and separate small peptides and phosphono dipeptides from aqueous solutions and body fluids is discussed. An influence of various parameters including carrier type, donor and acceptor phase compositions, presence of salts and proteins in analysed samples on extraction efficiency and selectivity is presented. Additionally, comparison of SLM extraction efficiency from aqueous samples and body fluids is presented. Finally, a fully automated system consisted of SLM extraction coupled on-line with HPLC-UV for the analysis of selected peptides from blood plasma is shown.

**K e y w o r d s:** peptides, phosphono dipeptides, supported liquid membranes, extraction

### **1. Introduction**

Determination of peptides in biological samples is a very important aspect in the diagnosis and treatment of many diseases [1, 2]. Information about peptide profiles in biological samples on one side, and monitoring of metabolism of peptide pharmaceuticals from the other can effectively help in treatment of patients, e.g., with heart failures, Alzheimer's and Parkinson's diseases. For this reason, the determination of peptides has met a great interest in recent years. Peptides usually exist in the biological samples (i.e. blood plasma, urine, cerebrospinal fluid) in low concentration together with large amounts of other endogenous compounds (proteins and salts). As a consequence, development of sensitive, simple and fast analytical methods for peptides analysis is still a challenge for many researchers. Till now, reversed-phase

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high performance liquid chromatography (RP-HPLC) is considered as the most widely used instrumental method for the separation of relatively simple samples of peptides [3, 4]. Capillary zone electrophoresis (CZE) has also been successfully used, especially in the case of analysis of some polar peptides [5].

## 2. Sample Preparation Methods in Peptide Analysis

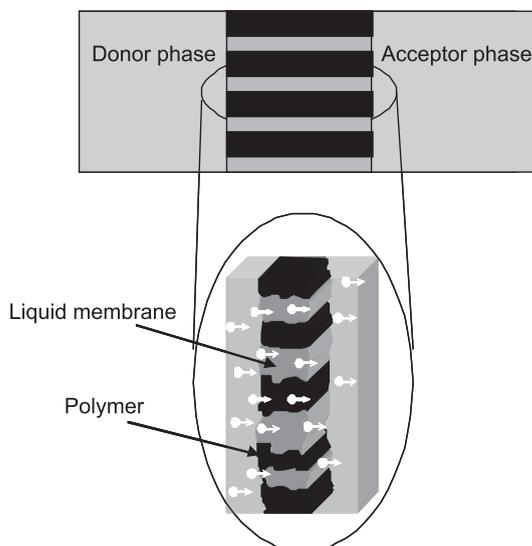
Unfortunately, most of the analyzed peptide-containing samples are very complex. Therefore, even when the peptide(s) of interest might tolerate separation conditions, the sample matrix components very often produce a significant decrease in resolution and might also cause the equipment fouling. To avoid those problems, it is necessary to carry out appropriate sample preparation steps to isolate, pre-concentrate and separate the analyte before final analysis. Nowadays, the importance of the sample pre-treatment is recognized by most analytical chemists as the bottle-neck of the almost every analytical method. It was addressed to high number of review papers and books on this subject published in last 20 years [6–9].

In the case of peptide analysis, a wide range of techniques and methods (e.g. extraction and membrane techniques) are available for determination of peptides in biological samples such as blood plasma, serum or urine. The most often used techniques are: extraction methods including liquid-liquid extraction (LLE) [4, 10] and solid-phase extraction (SPE) [11, 12] as well as some membrane-based systems namely ultrafiltration (UF) [13] and dialysis (D) [14]. Each of those techniques has its specific advantages and limitations. For example, liquid-liquid extraction is a technique less attractive (although widely used). The reason for this is that it is tedious and time consuming and not easy to automate. It also causes formation of emulsion and is also environmental unfriendly due to large volumes of used organic solvents. The main disadvantage of the other extraction technique – the solid-phase extraction is the use of substantial amounts of often hazardous organic solvents. Additionally, a necessity of carrying out many laborious operations such as washing, elution and solvent evaporation, significantly extends the time of analysis. In case of the membrane techniques (ultrafiltration and dialysis) low selectivity and problems with membrane clogging as well as large sample dilution, especially in the case of the dialysis limit their application. Thus, a great interest has recently arisen, in exploring new and faster sample pre-treatment methods, especially in the automated mode.

## 3. Supported Liquid Membrane Extraction

The ideal chemical analytical procedure is the one that can be characterised by minimal and simple number of steps but still retains its selectivity towards the analyte. In this mode, the supported liquid membrane (SLM) technique has recently shown

interesting potential considering peptide analysis [15, 16, 17]. The strength of the technique is based on the selective enrichment of various classes of compounds from the complex sample matrices such as blood plasma and urine. The main advantages of SLMs over the traditional separation methods are small amounts of organic phase and extractant (carrier), the occurrence of the mass transfer in one step, possibility of achieving high separating factors and concentration. Furthermore, simplicity of the SLM preparation, as well as full coupling with chromatographic system is also advantageous. The main problem of SLM extraction is its stability, caused by leakage and/or losses of membrane phase components during extraction process. Nevertheless, by proper choice of membrane phase this instability can be significantly reduced.



**Fig 1.** Schematic scheme of supported liquid membranes

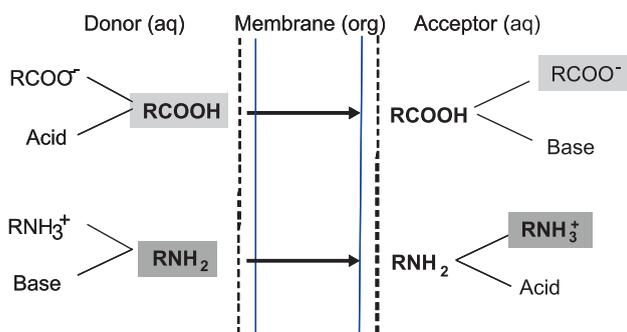
The principles and application of supported liquid membrane extraction have been described several times [18–20]. During SLM extraction, an organic solvent is immobilised in the pores of a porous polymer support material by capillary forces, separating two aqueous solutions: the donor and the acceptor phase (Fig. 1). The analytes are partitioned from the aqueous sample donor phase into an organic solvent immobilised in polymeric support, and then they are extracted back to the other side of the membrane – into the acceptor phase. The driving force of the process is the difference of the analyte concentration between aqueous phases. In order to reach the effective enrichment level, the solute – analyte of interest must exist in two forms. Firstly, in the non-ionic form on the donor side to be able to dissolve in the organic membrane phase. Secondly, in the ionic form in the acceptor phase to prevent its back diffusion into the membrane. By appropriate selection of the donor and acceptor

composition, the high extraction efficiency of different monocharged compounds as organic acids and amines could be simple achieved. In case of various permanently charged species, e.g. metal ions, amino acids or peptides, which are nonextractable, a high mass transfer can be obtained by adding an chelating and/or ion-pairing reagents as well as various carriers to the donor or membrane phase, respectively.

The transport mechanisms in SLM can be divided into two main groups: simple permeation and carrier mediated transport. In the simple permeation, the analyte dissolves into the membrane and diffuses to the acceptor side due to the concentration gradient across the membrane. Selectivity is mainly governed by proper choice of the donor and acceptor solutions so that the compound(s) are transformed in non-ionic or ionic form respectively. Selectivity is also further enhanced by solubility differences between the analytes and the matrix components into the membrane (Fig. 2a).

Sometimes, when the solubility of the compounds is too low showing poor extraction efficiency, a powerful approach is to incorporate a mobile carrier into the membrane that selectively binds the analytes. The carrier in the membrane forms a complex with the analyte in the donor that diffuses across the membrane to the acceptor. Once it reaches the acceptor, the analyte is converted to a non-extractable

a) Diffusion transport:



b) Carrier mediated transport:

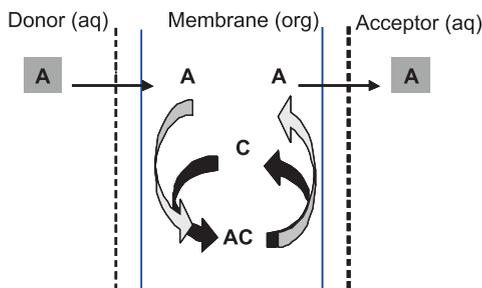


Fig. 2. SLM transport mechanism

form (Fig. 2b). It is important to emphasize that the carrier and its complex form with the analyte must be soluble only in the membrane but not in the adjacent aqueous phases. Introducing of a carrier allows supported liquid membranes to be applicable to a variety of the compounds, including very polar molecules.

Three different SLM units have been reported, i.e. the flat, spiral and tubular modules, which differ by the ratio between membrane surface area and volumes of aqueous phases [20]. The ratio should be high to obtain large enrichment factors. It is the highest for the tubular, medium the spiral, and the lowest for the flat modules. When the access of sample is limited as in the case of plasma, blood or cerebrospinal fluid, the SLM units with small volume of acceptor channel (~1 up to 20  $\mu\text{l}$ ) should be used. In the case of urine samples due to its large volume availability and low content of proteins the modules with larger volume of acceptor phase can be applied. Nevertheless clean-up and pre-concentration of those types of samples enables the determination only peptides metabolites. While, for monitoring a real correlation between the drug concentration (peptide drug) and therapeutic effect the analysis of plasma or serum are indispensable and essential.

#### 4. SLM Extraction of Peptides from Aqueous Samples

In recent years, the supported liquid membrane (SLM) extraction has been demonstrated as a useful tool for the selective extraction and enrichment of various classes of compounds from very complex samples [21–24]. Successful determination of amine [25] and drug compounds [22, 26] in biological samples by combining the SLM with gas chromatography, high-performance liquid chromatography or capillary electrophoresis have been reported. The consequence of this as well as the immense importance of peptides, brought about the interested in SLM application for peptide transport and also peptide extraction for analytical purposes [17, 27].

The first studies, concerning peptides extraction through supported liquid membranes, only dealt with the aspects of the transport mechanism and factors influencing mass transfer effectiveness. For example, the SLM system was used for the extraction of diastereomers of dipeptide and phosphono dipeptide hydrochlorides through 1-decanol membranes containing macrocyclic carriers namely Kryptofix 5 and Kryptofix 222 immobilised in pores of polyethylene hollow fibre support [28]. The mass transfer was easily accomplished either by passive or by carrier-facilitated transport. The flux of the peptides in carrier-mediated transport depended on the donor phase pH. Unfortunately the low solubility of macrocyclic carriers in the solvent of the membrane phase significantly limited the effectiveness of the mass transfer the practical application of this system is very limited.

The other example describes the use of the ionic surfactant, sodium di-2-ethyl-hexyl sulfosuccinate (Aerosol OT/AOT), as a carrier dissolved in an oleyl alcohol for the separation and recovery of short chain peptides from a mixture of amino acids

and peptides [29]. The carrier concentration and pH of the donor phase significantly influence the effectiveness of the analyte permeation. It was observed that during the transport of a mixture of tryptophan (Trp as an amino acid) and tryptophan-leucine (TrpLeu as a peptide) the transport rate of the peptide was much higher than amino acid alone. However, the increased of the flow rate did not affect the extraction efficiency. Nevertheless due to the problems with stability of liquid membrane (only few hours) the developed method has not found wider application.

The more successful, in the practical relevance, application of supported liquid membrane extraction for isolation, pre-concentration and separation of peptides was achieved when di-2-ethylhexylphosphoric acid (D<sub>2</sub>EHPA) and trioctylmethylammonium chloride (Aliquat 336) were used as a carriers incorporated in the membrane phase [17, 27]. The presented in the literature studies concerns different aspects of the peptide transport parameters for procedure optimization to obtain the high efficiency of the extraction. Membrane unit with 1 ml volume of the donor and acceptor phase was applied for all experiments. In both cases the transport of peptides across the organic liquid membrane was carrier-mediated. In the case of SLM system with anionic carrier (Aliquat 336) the gradient of chloride ions was a driving force and determined the effectiveness of the analyte permeation [15], whereas for the system with cationic carrier (D<sub>2</sub>EHPA), the proton gradient of hydrochloric acid was the driving force of the process [16]. In both systems, the concentration of counter-ion in the acceptor phase was crucial for extraction efficiency. If it was not high enough, the peptide-carrier complex could not be broken at the membrane-acceptor interface, and the peptides were able to be carried back across the membrane, what resulted in extraction efficiency decrease. It was demonstrated that the extraction of the analyzed short chain peptides was influenced by molecular structure of the transported compounds. It has been found that the hydrophobicity of the investigated peptides was not the major factor, influenced their SLM permeation. It has been observed, in studied conditions, that the transport of peptide-carrier complex depended on the acidic-basic properties of the analyte. Additionally, the extraction efficiency strongly depended on the donor and acceptor phase pH, carrier concentration and type of organic solvent used as membrane phase. High selectivity and efficiency were achieved for extraction of peptides from aqueous solutions, in most cases the values reached more than 80%.

## 5. SLM Extraction of Peptides from Biological Samples

Described above modelling and process optimization studies led to the application of SLM for the pre-concentration of peptides from body fluid spiked samples. Because of limited volume of the body fluids the smaller membrane unit (20  $\mu$ l) was used. In the first stage the system with D<sub>2</sub>EHPA was applied [27]. The application of acidic acceptor phase allowed for using the capillary electrophoresis analysis in

off-line mode for the determination of the analytes concentration in the samples. The extraction efficiency from plasma samples was lower of about 20–40% compared to aqueous samples. In the case of the urine samples, the extraction efficiency was higher compared to plasma samples and it ranged from 20 to 70% depending on the peptide structure. The minimum concentration of the investigated peptides was at the ppb level.

Finally, the application of full automated system consisted of supported liquid membranes coupled on-line with high performance liquid chromatography was applied for enrichment of selected peptides from blood plasma [17]. Taking into consideration specificity of the chromatographic system the module with anionic carrier – Aliquat 336 was used. Compared to analysis of aqueous samples markedly lower extraction efficiency was obtained in the case of plasma samples. Extraction efficiency of peptides from blood plasma samples using Aliquat 336 as a membrane carrier was also lower comparing with supported liquid membrane containing D<sub>2</sub>EHPA as a carrier. The limiting factor was the presence of salts and proteins in blood plasma samples. Therefore, in this experiment it was necessary to perform two more steps: ultrafiltration and dialysis prior to SLM extraction procedure. The application of those two additional techniques overcame the protein interaction with liquid membrane surface. As a result it helps to decrease the concentration of inorganic ions that strongly interfered in efficient extraction of the investigated peptides. Even the ultrafiltration and dialysis procedures were performed before the SLM extraction; the total time of analysis was twice shorter in comparison with the methods based on other sample preparation techniques [5]. Moreover, compared to SLM extraction methods used for extraction and pre-concentration of basic and antidepressant drugs from blood plasma samples, the achieved values of peptide extraction efficiencies were comparable, taking into account the fact that peptides are the compounds strong bounded with plasma proteins [30]. The obtained detection limits 90-130 ng/ml was similar to other analytical methods. Additionally the developed SLM extraction system offered the possibility of automation of the analysis.

Recently, the supported liquid membrane technique with described above carriers has been used also for extraction and pre-concentration of phosphono dipeptides [31]. Those compounds have recently received considerable attention in bioorganic chemistry, medicine and agriculture due to their unique biological activity. They mostly show antibiotic properties (for example phosalacine or alaphosphalin), but some of them as well as synthetic phosphono peptides have also shown the herbicidal (bialaphos), neuroregulatory (phosphonoic analogues of enkephalins) and even anticancer (inhibitor of ras-farnesyl protein transferase ) activity [32]. The transport of phosphono dipeptides was also strongly influenced by their structure and concentration. The extraction efficiency using Aliquat 336 was higher compared to peptides with carboxylic groups. Contrary to those results, the experiments with D<sub>2</sub>EHPA application showed that the presence of phosphonic group in phosphono peptide

chain was a factor significantly limiting high values of extraction efficiency. In this model transport of analytes proceed only until the system reaches the equilibrium.

## 6. Conclusions

Summarized, SLM extraction turns out to be an efficient method for isolation and pre-concentration of short peptides from complex samples matrices. High selectivity and efficiency were achieved for extraction of peptides from aqueous samples when both Aliquat 336 and D<sub>2</sub>EHPA were used as a carrier in the membrane. The transport of peptides through supported liquid membrane was in all cases strongly depended on donor, membrane and acceptor compositions. The mass transfer was significantly influenced by the analyte structure in particular its hydrophobicity and acidic/basic properties. The lowering of analyte concentration in donor phase resulted in its higher extraction efficiency. Compared to peptides extraction from aqueous solutions, the extraction efficiencies, in the case of body fluids samples analysis, were considerably lower. However from the point of view of trace analysis the obtained detection limits were satisfied. Therefore the developed methods of supported liquid membrane extraction of peptides could be seen as a complementary to other available sample pre-treatment techniques.

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