Chapter 5

From a Sequential to a Continuous Approach for LVV-h7 Preparation during Enzymatic Proteolysis in a Microfluidic-Based Extraction Process

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Abstract

Intensification of process is increasingly interesting in the context of recovery of industrial wastes. Among these compounds, animal blood is underexploited although it is an important source of bioactive peptides. LVV-h7 (LVVYPWTQRF) is one of these bioactive peptides from bovine haemoglobin hydrolysate. Our innovative approach consists of a continuous process involving at microfluidic scale for enzymatic proteolysis of bovine haemoglobin by pepsin, selective extraction of LVV-h7 to an organic solvent during the enzymatic reaction, followed by a second extraction to an aqueous phase for organic solvent recycling. Thus, the obtainment of pure LVV-h7 peptide with an efficient methodology of extraction and solvent recycling was proved.

Keywords: haemoglobin, enzymatic hydrolysis, extraction, microfluidic, solvent recycling, bioactive peptides

1. Introduction

Intensification of process is considered as an indispensable part in the development of approach for obtaining product with high added value. The improvement of a process could concern the technology used, the safety or also the source of raw material employed. Among these sources, wastes of agricultural and food processing are considered as a cheap source of valuable components since the existent technologies allow the recovery of target compounds

and their recycling [1]. The process was developed at industrial scale to valorise these kinds of products [2]. In this work, animal blood was studied as a source of bioactive peptides [3–6]. Some of these peptides revealed their potential as antimicrobial [7], opioid [8], antihypertensive [8] or antioxidative activities [9]. These peptides are generally obtained by enzymatic degradation of haemoglobin alpha and beta chains [3]. Among these peptides, one opioid peptide was studied, known as LVV-haemorphin-7. LVV-h7 corresponds to the amino acid sequence LVVYPWTQRF (Leu-Val-Val-Tyr-Pro-Trp-Thr-Gln-Arg-Phe), obtained from β chain of haemoglobin (β 31–40) by pepsin hydrolysis and well known as bioactive peptides involved in the treatment of humans diseases [8, 10–12].

LVV-h7 presents also interesting physical properties, especially hydrophobic character which makes it able to transfer from aqueous to organic media in liquid/liquid extraction process [13]. Previous studies were interested to extract this peptide during haemoglobin hydrolysis using water/butan-2-ol-octan-1-ol liquid/liquid biphasic system [14–16]. Even if this process has shown the ability to extract selectively LVV-h7, its implementation was very complex and laborious, due to the long time of the process carried out (more than 10 h) to obtain low extraction yield of peptide (about 5%), the control of the immobilised enzyme stability during the process to avoid its inhibition by solvents and a high quantity of solvent.

Keeping in mind the economic and environmental impacts of a process development which requires the use of organic solvent, the microfluidic domain could bring solutions to reduce these disadvantages. First, reduction of scale allows to improve the surface/volume ratio and thus the molecular transfer capacity while reducing the volume of solvent used and energy [17, 18]. Second, the implementation for continuous flow platforms brings advantages of liquid-liquid transfer favoured by laminar flow [19–21], in the case of non-miscible liquids, for molecular transfer. Several studies revealed the real advantage of using LLE in microfluidic system [19, 22–23].

Previous study realised on the enzymatic hydrolysis of haemoglobin by pepsin in a microfluidic reactor has shown the influence of the microfluidic scale to the kinetics acceleration of bioactive peptides appearance, such as LVV-h7, comparing to bench scale [24]. This study shows also the potential of combining enzymatic microreactor with liquid/liquid biphasic system for LVV-h7 extraction. Therefore, the approach that we propose is based on continuous aqueous haemoglobin hydrolysis by pepsin, LVV-h7 extraction towards an intermediate organic phase and LVV-h7 DES extraction in a receiving aqueous phase, allowing the solvent recycling. This integrated process with solvent recycling is presented in **Figure 1**. Before fully designing the entire microfluidic process, where all the reactions are carried out simultaneously, we separately investigated each reaction to notably determine their respective optimal ranges of conditions before combination as follows: (1) haemoglobin hydrolysis by pepsin occurs in a primary aqueous feed phase, generating the LVV-h7 in a very complex peptide mixture with more than one hundreds of peptides with different primary sequences; (2) the as-formed LVV-h7 is then selectively extracted into an organic solvent (octan-1-ol); (3) eventually, the LVV-h7 is des extracted in a second aqueous phase (called receiving aqueous phase), which also allows the octan-1-ol recycling in the extraction step. Our approach is not only focused on the compatibility issues of enzymatic catalysis and opioid peptide extraction, but also pays particular attention to integrating all the steps to minimise separation and recycling burdens, which can be detrimental for the overall economics and efficiency of the process. The methodology envisioned to move from a

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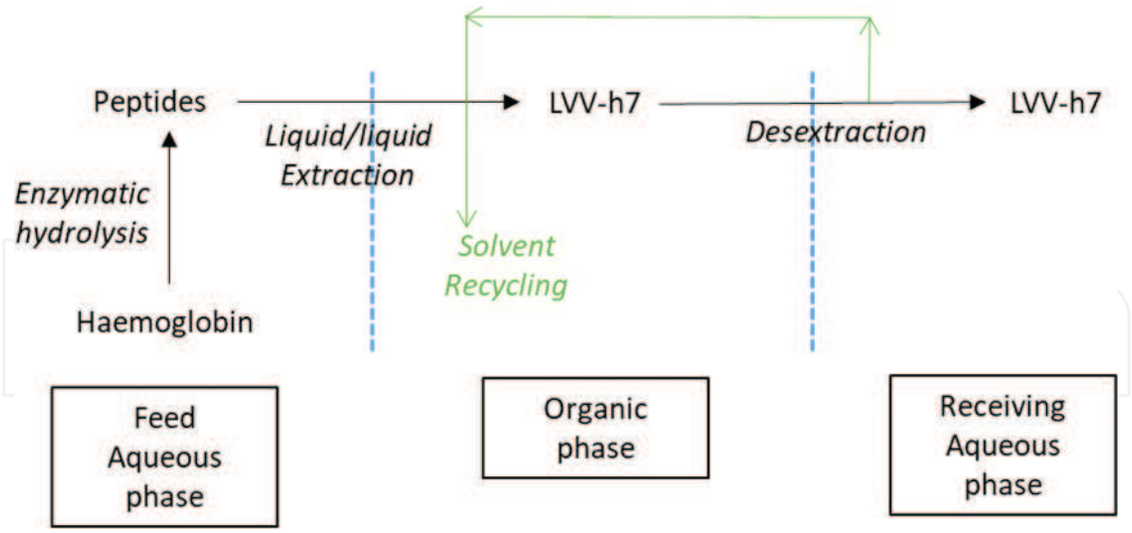

Figure 1. Simultaneous process applied to the haemoglobin hydrolysis by pepsin, the liquid/liquid extraction of LVV-h7 and the solvent recycling, to produce pure opioid peptide.

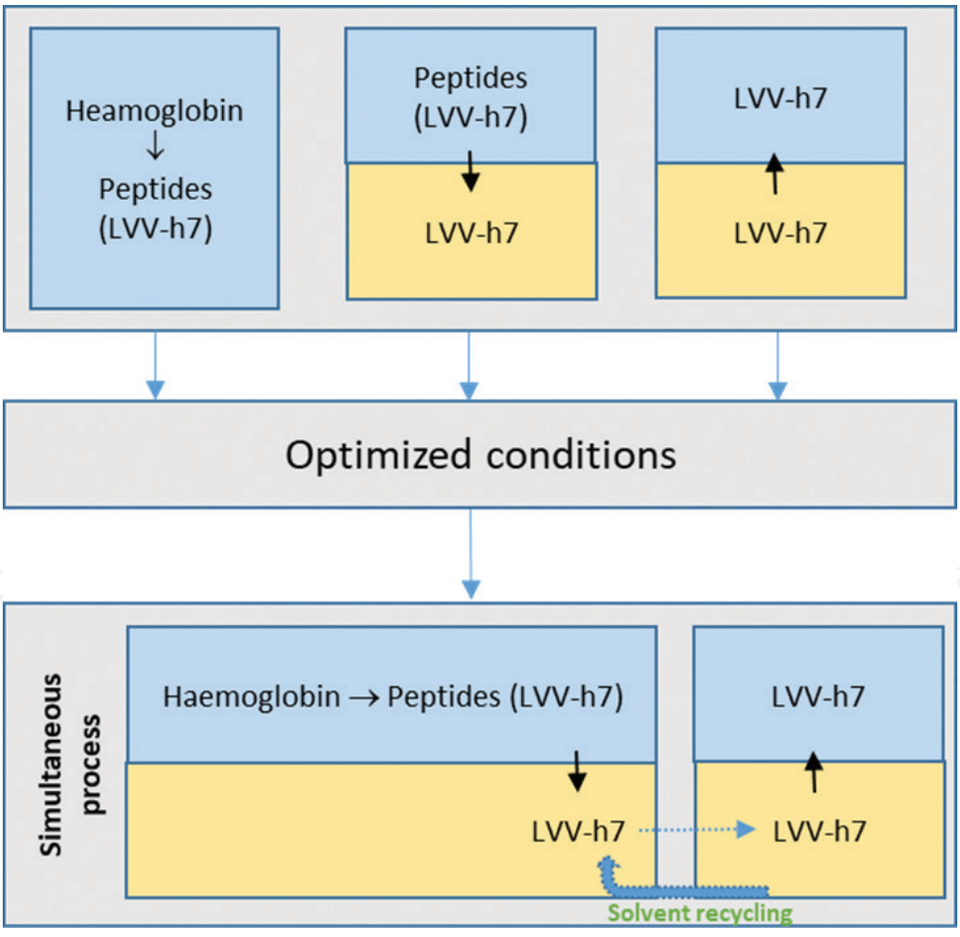

Figure 2. Methodology from a sequential approach towards an integrated continuous process.

sequential approach towards an integrated continuous process is schematised in **Figure 2**. Each step was optimised to obtain the best conditions concerning LVV-h7 concentration and purity, through residence time (i.e. flow rate) of both aqueous and organic phases inside the system.

After optimisation, enzymatic hydrolysis and extractions steps were combined for a continuous approach to obtain final aqueous phase containing the bioactive peptide. Quantification and purity of LVV-h7 were determined to evaluate the efficiency of integrated process.

2. Experimental procedure

2.1. Haemoglobin hydrolysis by pepsin

Bovine haemoglobin (64.5 kDa, Sigma Chemicals Co.) was hydrolysed by porcine pepsin (E.C. 3.4.23.1, 3.440 U mg^{-1}, 35 kDa, Sigma Chemicals Co.), protease from the family of aspartic acid proteases which preferentially catalyses the cleavage of peptide bonds at the carboxyl side of aromatic and hydrophobic amino acids. This proteolytic reaction leads to the appearance of product molecules called peptides. Haemoglobin was prepared under denaturing conditions at pH 3.0 by adding 2 M HCl for a 20 mg mL^{-1} final concentration. All aqueous solutions were prepared in 18.2 MΩ Milli-Q water (Millipore).

In order to obtain a maximum concentration of LVV-h7 peptides for the extraction step studies, kinetic of reaction was implemented in microfluidic system (75 μm inner diameter, 2 m length). Denatured bovine haemoglobin (1% w/v) was hydrolysed by porcine pepsin at different flow rates (9, 4.5, 1, 0.4 and 0.2 μL min^{-1}), which corresponded to a residence time of 15 s, 30 s, 2 min, 5 min and 10 min, respectively. Samples of peptidic solution were collected during the reaction and mixed with sodium hydroxide 1 M in order to inhibit the pepsin. Samples were conserved at 4°C for RP-HPLC analysis to determine the progress of the reaction.

2.2. Extraction of LVV-h7 from feed aqueous phase containing peptidic hydrolysate to octan-1-ol phase

Previous studies have shown the choice of octan-1-ol as the better extraction solvent for hydrophobic peptides and particularly in the case of LVV-h7 [16, 25]. The haemoglobin hydrolysate was pumped using peristaltic pump (Minipulls 3, Gilson Inc., Middleton, WI, USA) and sent in co-flow with octan-1-ol solution in the same capillary (75 μm inner diameter, 10 cm length) for liquid-liquid extraction (LLE) of peptides from haemoglobin phase to the organic phase. The liquid-liquid extraction was performed with different flow rates to appreciate the impact of contact time on the opioid peptide extraction. The two phases were collected out of the capillary in a 2 mL Eppendorf, immediately separated and analysed by RP-HPLC.

2.3. DES extraction of LVV-h7 from organic phase to receiving aqueous phase

Octan-1-ol phase obtained after the extraction procedure was pumped with peristaltic pump and fed in another capillary where acidic water (pH 3 obtained with acetic acid adjustment) was injected at different flow rates to perform a second liquid-liquid extraction from the octan-1-ol to the aqueous phase, called "DES extraction step". In these conditions, the acidic water extracts peptides and favours the extraction without mixing of the two phases [26]. Phases were collected out of the capillary in a 2 mL Eppendorf, immediately separated and analysed by RP-HPLC.

2.4. Coupling of extraction and DES extraction procedure

A coupling between both extractions optimised method was implemented, and its efficiency on LVV-h7 peptide extraction selectivity was measured. The entire system was represented on scheme **Figure 3** where the volume containing the peptidic hydrolysate and the octan-1-ol was pumped in the same capillary for extraction, followed by a pumping of octan-1-ol phase recovered in a second capillary, connected with acidic water for DES extraction of LVV-h7. For each part of the process, samples were collected for identification and quantification of species.

2.5. Reversed-phase-HPLC analysis

The liquid chromatographic system is consisted of a Waters 600E automated gradient controller pump module, a Waters Wisp 717 automatic sampling device and a Waters 996 photodiode array detector. Spectral and chromatographic data were stored in a NECImage 466 computer. Detection of the produced peptides was carried out at 215 nm by reverse phase HPLC (RP-HPLC) on a C4-column (Vydak 0.46 × 25 cm, 3 mm I.D.). The mobile phase was water/trifluoroacetic acid (100, 0.1, v/v) and acetonitrile/water/trifluoroacetic acid (60, 40, 0.1, by vol.) at 0.4 mL min^{-1} flow rate. All common chemicals and reagents were of analytical grade and were purchased from Sigma Chemicals Co. and Flandres Chimie. Identification and quantification of LVV-h7 were performed using peptide standard (purity: 91.63% M.W. 1308.56 Da) purchased from GeneCust Society (Luxembourg).

2.6. MALDI-TOF mass spectrometry analysis

The sample was loaded on a ground steel MALDI target (Bruker Daltonics, Bremen, Germany) following the dried droplet method. The MS (positive reflectron mode) and MS/MS (lift mode) measurements were performed in an automatic mode on an AUTOFLEXTM Speed

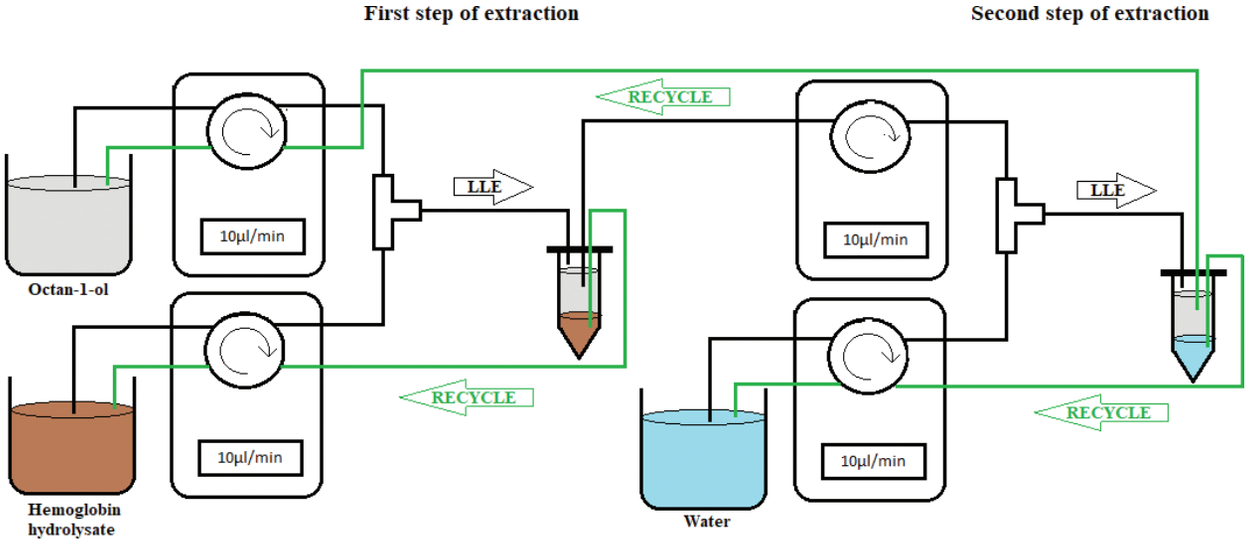

Figure 3. Implementation of microfluidic extraction and DES extraction steps of LVV-h7 from hydrolysate and recycling phases (black: employed method for extraction; green: recycling possibilities of solution).

TOF/TOF mass spectrometer (Bruker Daltonics) running FlexControlTM3.0 software (Bruker Daltonics). Peptide fragmentation was performed by automatic method of the manufacturer. MS and MS/MS spectra were processed using FlexanalysisTM3.3 and BioTools 3.4 software packages (Bruker Daltonics). Fragmentation pattern of peptides was deduced from matching of amino acid sequences (Uniprot accession numbers: P02070 & P01966) of each chain of bovine haemoglobin to the MS/MS spectra using BioTools 3.4.

3. Results and discussion

3.1. Haemoglobin hydrolysis by pepsin in feed aqueous phase

Haemoglobin 1% (w/v) was hydrolysed by porcine pepsin at room temperature, at pH 3 and at different residence time of substrate and enzyme in the microchannel. At the outlet of the capillary, samples were sent to an Eppendorf containing a disodium tetraborate buffer solution (0.32 M, pH 9.0), that caused enzyme denaturation and thus reaction stopping, and analysed by RP-HPLC.

Figure 4 shows the progressive decrease of alpha and beta chains of haemoglobin (between 40 and 60 min) to generate intermediate peptides (between 25 and 40 min) after 15 s of reaction. Next, after 30 s of reaction, there is an increase of peptide population between 25 and 30 min and an emergence of peptides between 15 and 25 min. For the last samples (5 and 10 min), there is a disappearance of peptides between 30 and 40 min to the profit of peptides between 5 and 25 min. This phenomenon was already described in the literature and was explained by an enzymatic hydrolysis of alpha and beta chains to produce a population of hydrophobic peptides with high molecular weight, observed between 30 and 35 min in **Figure 3** [3, 5]. Next, this population is hydrolysed to produce other peptides with intermediate molecular weight, observed between 20 and 30 min. Finally, these intermediate peptides are hydrolysed to generate small peptides with hydrophilic character (retention time between 5 and 20 min). This mechanism is called "zipper" mechanism, which is characterised by a denatured state of the initial haemoglobin structure. Moreover, this mechanism is more suitable for obtaining intermediate bioactive peptides, such as LVV-h7 (retention time of about 29 min in **Figure 4**), compared to a "one by one" mechanism where initial haemoglobin structure is in a native state [3, 5]. LVV-h7 was used as a standard to evaluate the efficiency of extraction process by octan-1-ol. Thus, for studying of the extraction step in microfluidic system, we decided to stop the reaction after 30 s of hydrolysis by sodium hydroxide 1 M in order to obtain the maximum quantity of LVV-h7 (**Figure 3c**).

3.2. Study of LVV-h7 extraction in octan-1-ol using microfluidic system

Haemoglobin hydrolysate whose concentration of LVV-h7 was the more important (1%, 30 s of hydrolysis with pepsin) was injected in co-flow (using a T connector) with octan-1-ol at different flow rates (5, 10, 20 and 50 μL min^{-1}). Samples were collected in Eppendorf, and octan-1-ol phase was analysed by RP-HPLC to highlight the extracted peptides. Results are shown in **Figure 5**.

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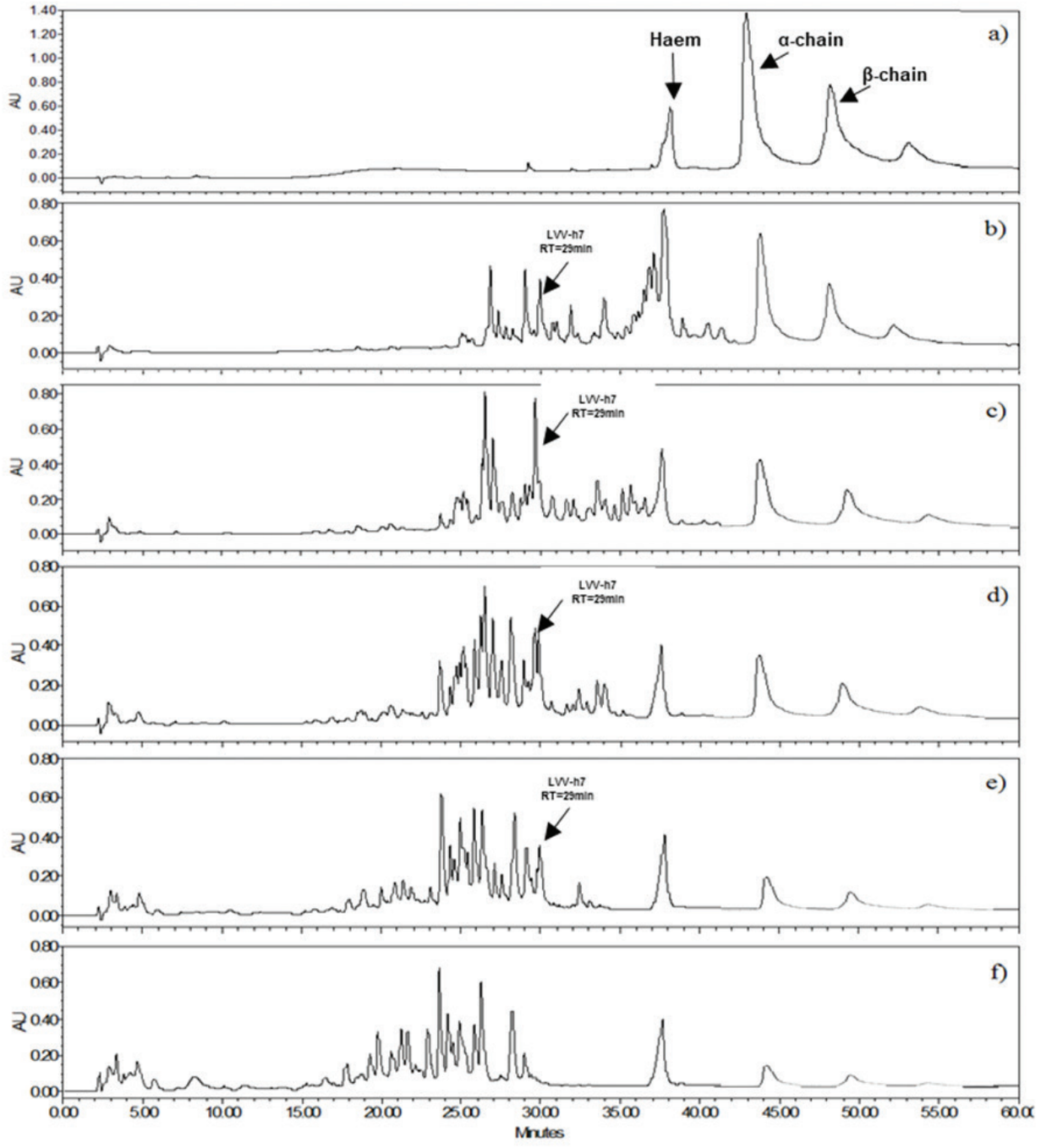

Figure 4. RP-HPLC chromatograms of haemoglobin 1% hydrolysed by porcine pepsin for different residence times of both haemoglobin and pepsin solutions. (a) Denatured haemoglobin without enzyme, (b) 15 s, (c) 30 s, (d) 2 min, (e) 5 min and (f) 10 min. Samples were analysed on C4 column.

The flow rates used for our analysis correspond to the additive flow rates of both solutions during the extraction step (i.e. initial flow rate multiplied by 2). Chromatograms show a good selectivity of the LLE method and octan-1-ol using microfluidic system. Indeed, we observe only three major peaks from the initial complex peptidic hydrolysate. Moreover, a predominance of LVV-h7 peptide is observed (RT of 29 min), which represents more than 55% of the peptides extracted by octan-1-ol. The selectivity of octan-1-ol for hydrophobic

peptides such as LVV-h7 was previously confirmed by our team in batch and continuous reactors from enzymatic haemoglobin hydrolysates, but not in microfluidic system [14–16]. Two other peaks (RT of 27 and 31 min) are observed and supposed having a hydrophobic character.

The three peaks identified in the octan-1-ol phase were analysed by MALDI mass spectroscopy to appreciate their composition (**Figure 6**).

Concerning the fraction eluted at a 29 min (**Figure 6b**), result from mass spectroscopy shows unique peak at m/z = 1308.930 Da corresponding to molecular weight for LVV-h7 (with one hydrogen more from mass analysis). No other components were observed, which shows that LVV-h7 peptide is pure. The other fractions collected were also identified. At retention time

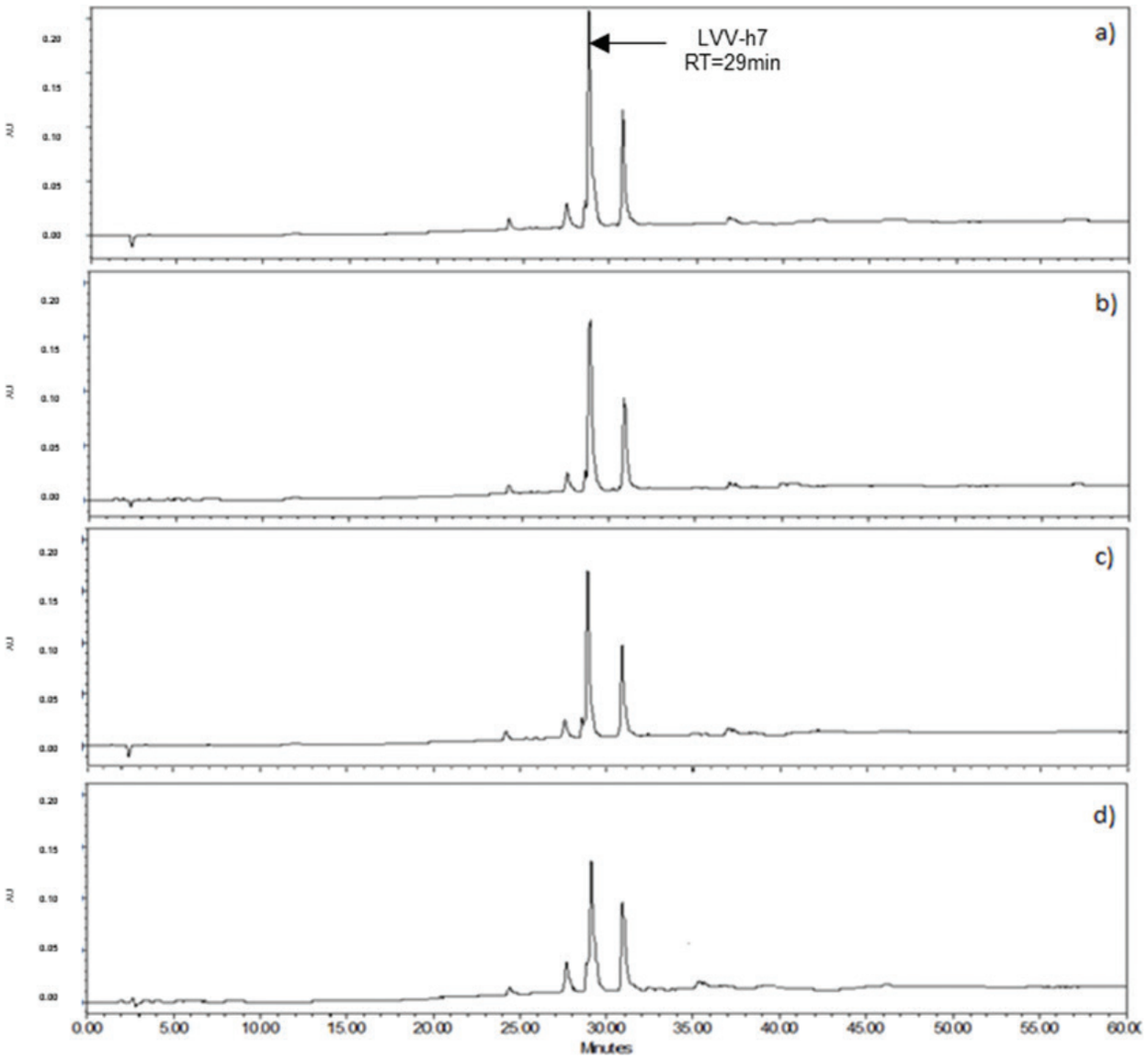

Figure 5. HPLC chromatograms of octan-1-ol phase after extraction applied on haemoglobin hydrolysate in microfluidic system at different flow rates ((a) 10 μL min^{-1}, (b) 20 μL min^{-1}, (c) 40 μL min^{-1} and (d) 100 μL min^{-1}).

of 27 min, another pure peptide with 1195.933 Da for molecular weight was identified. It corresponds to the VV-h7 peptide, also obtained by haemoglobin hydrolysis with pepsin and characterised as a hydrophobic bioactive peptide [7]. The fraction eluted at 31 min was composed of two major compounds (1422.138 and 1733.23 Da). These peptides correspond to other peptides without known biological activity.

Finally, using standard concentration curve (R^2 = 0.98) prepared with a pure standard of LVV-h7 (GeneCust, 91%), the quantity of pure LVV-h7 extracted from bovine haemoglobin hydrolysate was 6.12 ± 0.34 μg mL^{-1}. With an initial concentration of LVV-h7 calculated at 16.12 ± 0.85 μg mL^{-1} in the hydrolysate, performance of our system is around 38% of peptide extracted with only one cycle of extraction.

Table 1 resumes the total area of peptides extracted and particularly for LVV-h7 depending on the flow rate and thus on the residence time of hydrolysate in the microsystem. The area of peptides extracted from peptidic hydrolysate increases with the decreasing of flow rate used, confirming the influence of the time of contact between both phases in the capillary. A relative high time of contact between peptidic hydrolysate and octan-1-ol favours the diffusion of peptides to the solvent phase. However, even if the quantity of LVV-h7 obtained between 10

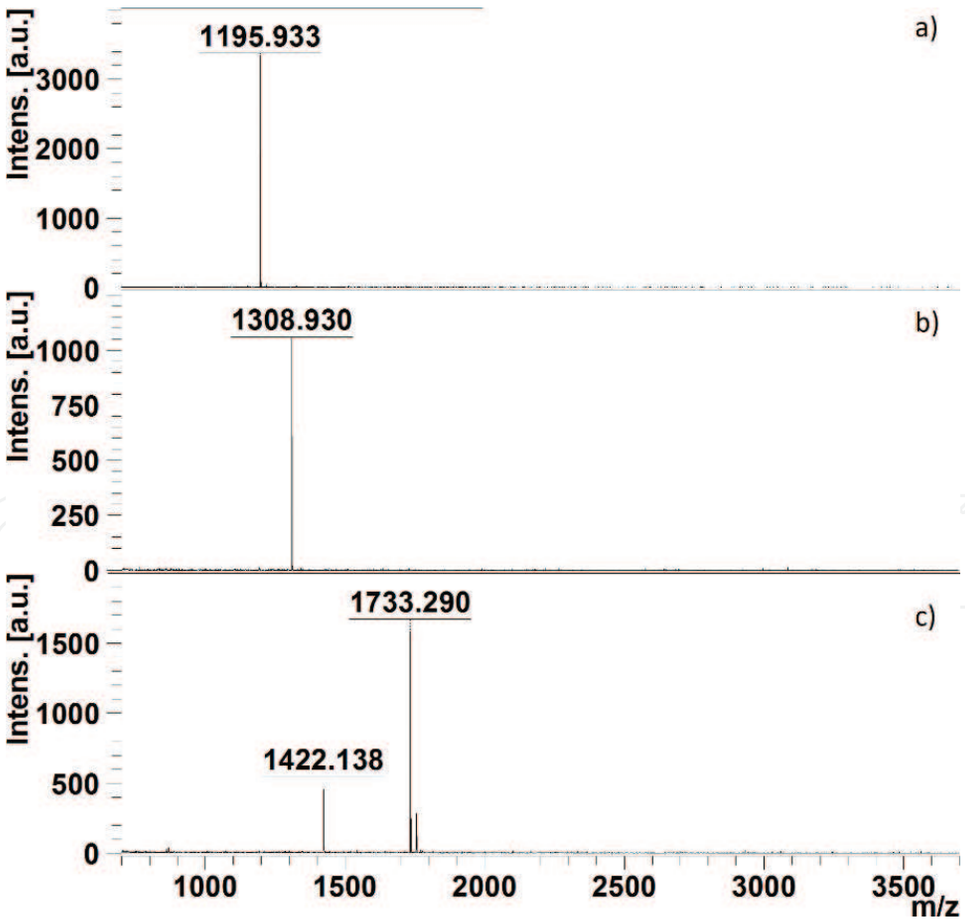

Figure 6. MALDI-TOF spectra obtained for each peak (elution time (a) 27 min, (b) 29 min and (c) 31 min) collected after octan-1-ol extraction and separated by RP-HPLC.

and 20 μL min^{-1} was relatively proportional (×1.5), this difference was less marked for flow rates used up to 20 μL min^{-1}.

3.3. Study of LVV-h7 DES extraction from octan-1-ol to receiving aqueous phase

Using the same process than in the first extraction (from peptidic hydrolysate to octan-1-ol), the octan-1-ol phase containing peptides was injected in co-flow with acidic aqueous phase in order to transfer these peptides from organic to receiving aqueous phase. Different flow rates were used to evaluate the impact of contact time between the two phases during the DES extraction process.

Chromatograms (**Figure 7**) show clearly a transfer of peptides from the organic phase to the aqueous phase by liquid-liquid DES extraction in the microfluidic conditions. The three major fractions corresponding to the peptides previously described and mainly present in the initial octan-1-ol phase were found in the receiving aqueous phase. Moreover, a small proportion of these peptides were still present in octan-1-ol after the DES extraction step. **Figure 8** illustrates the concentration of LVV-h7 in octan-1-ol and the receiving aqueous phase after the DES extraction for different flow rates.

The LVV-h7 concentration in the aqueous phase increases with the decrease of flow rate, indicating the influence of contact time between the two phases on the peptide diffusion. For 10 μL min^{-1}, more than 82% of the initial concentration of LVV-h7 phase was transferred in the aqueous phase. The calculation of LVV-h7 proportion in the aqueous phase compared to the total quantity of peptides extracted was 50 ± 1%. LVV-h7 fraction was analysed by MALDI mass spectroscopy to verify its purity (result not shown). Mass analysis reveals the purity of the LVV-h7 fraction to recover from organic phase to the receiving aqueous phase using microfluidic system. Both fractions (27 and 31 min) were also analysed by mass spectroscopy. It confirmed the same composition than obtained during the extraction step that is, a pure VV-h7 peptide for 27 min of elution time and other peptides for 31 min. Thus, the DES extraction step of peptides from octan-1-ol phase to an acidic aqueous phase using microfluidic system was validated with a good efficiency (more than 82% of transfer yield).

Flow rate (μL min^{-1})	Time of contact (s)	Total area of peptides (μV/*s)	LVV-h7 area (μV/*s)	Total % of LVV-h7[1]
10	2.65	10,793,556	6,292,478	58
20	1.32	6,976,911	4,064,156	58
40	0.66	6,316,966	3,599,507	57
100	0.26	5,921,488	3,181,856	54

[1]Total % LVV-h7 = (area of LVV-h7/total area) × 100)

Table 1. Proportion of LVV-h7 in total area of peptides after extraction process.

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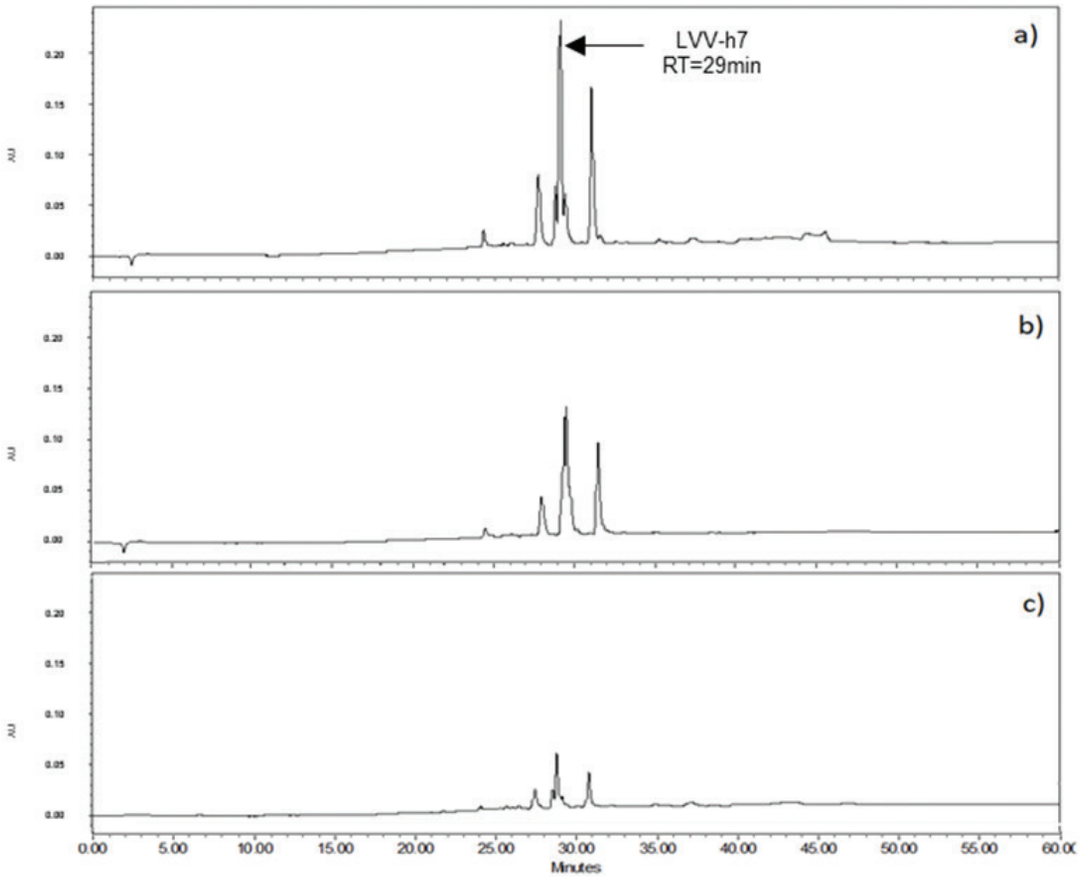

Figure 7. RP-HPLC chromatograms of octan-1-ol phase before DES extraction of peptides (a) water phase, (b) and octan-1-ol phase (c) obtained after DES extraction in microfluidic system for a flow rate of 20 μL min^{-1}.

3.4. Simultaneous haemoglobin hydrolysis by pepsin in feed aqueous phase, LVV-h7 extraction in octan-1-ol and DES extraction in receiving aqueous phase

In the optimal conditions previously determined, a continuous integrated process was tested. The microfluidic system, phases and matter flows are presented in **Figure 1**. The experiment was conducted following the same approach than those in **Figure 3** but with a feed aqueous phase formed by a haemoglobin solution, previously prepared under denaturing conditions at pH 3.0 (see part 3.1, haemoglobin final concentration of 1%, p/v), and a solution of pepsin with a E/S ratio of 1/11 (mol/mol). The introduction of these solutions in the microreactor (75 μm I.D. × 150 μm O.D.) was achieved by a syringe pump with a flow rate for both pepsin and haemoglobin solutions at about 4.5 μL min^{-1} for a capillary length of 2 m. The flow rates used correspond to the additive flow rates of both solutions, that is, initial flow rate multiplied by 2. The outlet fused silica capillary was in contact with a disodium tetraborate buffer solution (0.32 M, pH 9.0), thanks to a T-connection and a capillary (75 μm inner diameter, 10 cm length, flow rate of 5 μL min^{-1}), that caused enzyme denaturation and thus reaction stopping. Consequently to the enzymatic reaction, the resulting peptidic hydrolysate was sent in co-flow in a T-connector with

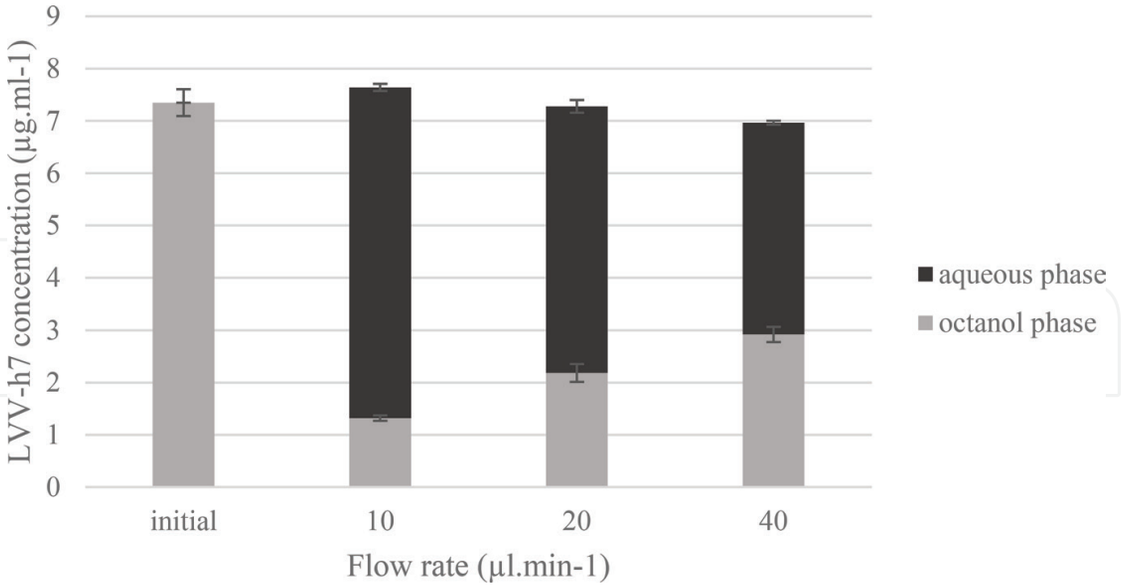

Figure 8. Proportion of LVV-h7 (μg mL^{-1}) in each phase after the DES extraction step in the microfluidic system for different flow rates.

octan-1-ol solution in a new capillary (75 μm inner diameter, 10 cm length) for LVV-h7 extraction at 10 μL min^{-1}. The two phases were collected out of the capillary in an Eppendorf. Octan-1-ol was pumped at 10 μL min^{-1} in a third capillary connected with acidic water for DES extraction of LVV-h7 thanks to a T-connector. Finally, the octan-1-ol phase remaining was pumped (10 μL min^{-1}) and re-injected in the initial octan-1-ol phase for a new cycle of extraction-DES extraction. To avoid the pumping of the bad phase, an offset of 10 min was done between each extraction step to have sufficient volume of phases. For each step of the continuous process, samples were collected for identification and quantification of species (**Figure 9** and **Table 2**).

First, the peptidic profile obtained during the enzymatic hydrolysis step confirms the results previously obtained by Elagli et al. [24] and the presence of LVV-h7 in the reaction medium (**Figure 9a**). Then, extraction of the opioid peptide to octan-1-ol phase is also observed in **Figure 9b**, showing the good selectivity of the organic phase for the same hydrophobic peptides detected before, whose LVV-h7 (**Figure 7**). Finally, we observe the haemorphin in the acidic receiving aqueous phase, confirming the DES extraction step efficiency. A proportion of the peptidic fractions is also found in the octan-1-ol phase after the DES extraction, translating an incomplete transfer in the receiving aqueous phase. However, results validate the process of simultaneous enzymatic hydrolysis of haemoglobin with LVV-h7 extraction and DES extraction at the microscale level.

The calculation of the different concentrations of LVV-h7 in each phase (**Table 2**) shows the transfer of 78% of the initial concentration of LVV-h7 from octan-1-ol to water. This result confirms the efficiency of the coupling approach of the extraction-DES extraction steps in microfluidic system after the enzymatic reaction. Moreover, after the DES extraction step, a low concentration of LVV-h7 remained in octan-1-ol phase (1.64 μg mL^{-1}). Thus, the reuse of octan-1-ol for a new cycle of extraction allows to obtain an efficient method of peptide recovery with a minimal of organic solvent quantity. An optimisation of the process, particularly the time of contact

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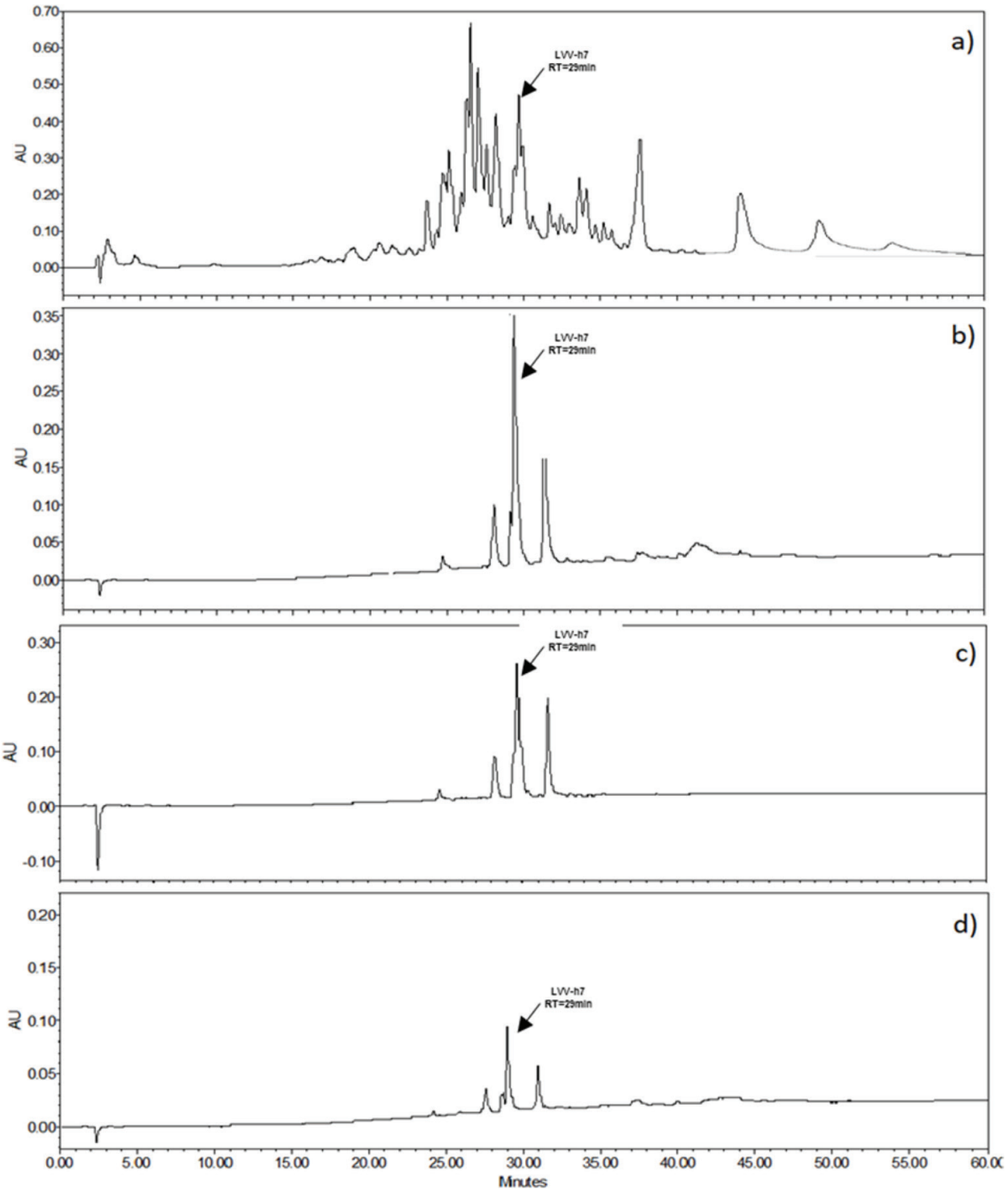

Figure 9. RP-HPLC chromatograms obtained from each step of the continuous process in the microfluidic system. (a) Peptidic hydrolysate after 30 s of haemoglobin hydrolysis by pepsin, (b) octan-1-ol phase after the extraction, (c) water phase after the DES extraction and (d) octan-1-ol phase after the DES extraction.

between feed aqueous/organic phases during the extraction and receiving aqueous/organic phases during the DES extraction could certainly improve the final concentration of LVV-h7 recovered and consequently decrease peptide concentration remained in the organic phase.

Phases collected	LVV-h7 area (μV/*s)	LVV-h7 concentration (μg mL^{-1})
Octan-1-ol (extraction step)	6,354,896	6.18
Octan-1-ol (DES extraction step)	1,687,646	1.64
Water (DES extraction step)	4,982,647	4.85

Table 2. Concentrations of LVV-h7 in each phase during the complete process.

4. Conclusion

In this work, we have first provided the conditions of the key parameters in microfluidic systems (residence times, flow rates, and concentrations) applied for a sequential process from liquid/liquid extraction of LVV-h7, present in a very complex peptidic hydrolysate, in octan-1-ol to its DES extraction in a second acidic aqueous phase. The optimised conditions have been then applied to an unprecedented integrated process in a specifically microfluidic approach. Therein, enzymatic hydrolysis of denatured haemoglobin by pepsin in a first microfluidic system was coupled with LVV-h7 extraction in octan-1-ol. This organic phase was then put in contact with a second aqueous phase for LVV-h7 DES extraction. The microfluidic scale allowed to increase the ratio surface/volume in order to favour the transfer of hydrophobic peptide to the organic solvent. A very good selectivity of extraction of the opioid peptide is obtained, from a very complex peptidic population generated during the enzymatic hydrolysis of haemoglobin by pepsin, with more than 38% of LVV-h7 initial concentration transferred to the organic phase. The DES extraction reveals also a very good transfer of LVV-h7 from octan-1-ol to the acidic aqueous phase, with more than 80%. Thus, the simultaneous process allowed to recover until more than 6 μg mL^{-1} of LVV-h7 with an excellent purity measured by mass spectroscopy with only one cycle of process (2.65 s of contact for each LLE).

The coupling of both extractions confirmed the feasibility of this process with a recycling of each phase to obtain a continuous process of extraction at microfluidic scale. An optimisation of the time of contact during the extraction is the key of peptide transfer between each phase and particularly for the recycling step. Currently, a study of a complete continuous process is in progress, where pepsin is immobilised in the microchannel and the outlet microcapillary is directly in contact with a solution of octan-1-ol, to avoid stopping the enzymatic reaction before extraction.

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