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Nanoparticle-based capillary electrochromatography

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Abbreviations

CE	Capillary electrophoresis
CEC	Capillary electro chromatography
EOF	Electro-osmotic flow
MEKC	Micellar electrokinetic chromatography
NPs	Nanoparticles
PSP	Pseudostationary phase
MS	Mass spectrometry
SDS	Sodium dodecyl sulfate
UV	Ultra violet
MALDI-TOF	Matrix assisted laser desorption/ionization-time of flight
SERS	Surface Enhanced Raman Spectroscopy
MOPS	3-(N-morpholino) propanesulfonic acid
LC	Liquid chromatography
Old	It means that nanoparticles were stored for about a year
Fresh	Nanoparticles were freshly prepared within a week

Contents

Abstract

In this work, lipid nanoparticles were used as pseudostationary phase, PSP, for protein separation in capillary electrochromatography, CEC. The aim of the work was to use a new analytical separation technique for future analysis of protein drugs by using lipid nanoparticles as a pseudo-stationary phase with conventional UV-detection.

PSP-CEC was combined with UV-detection to study proteins elution. The tested proteins BSA, cytochrome *c* and insulin have been selected according to different hydrophobicities, sizes and isoelectric points (pI). A zwitter ionic buffer, MOPS, was used as an electrolyte at pH 7.4. The nanoparticles were tested for their compatibility with MALDI-TOF/MS (Matrix assisted laser desorption/ionization-time of flight). MADI-TOF/MS works with a suspension of lipid nanoparticles in MOPS that contains insulin and it can be a future possible detection method for PSP-CEC. All three proteins were successfully eluted with PSP-CEC combined with UV-detection. Nanoparticles coated with silver might be studied with "surface enhanced Raman spectroscopy"-detection.

Key words: PSP-CEC, UV and MALDI-TOF/MS

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1. Introduction

1.1 Aim of project

This work was done with the purpose of developing a new analytical separation technique for future analysis of protein drugs by using lipid nanoparticles as pseudo-stationary phase, ”PSP”, with conventional UV-detection.

1.2 Nanoparticle-based CEC

Lipid nanoparticles “NPs” were used as pseudo-stationary phase “PSP” for CEC separation [2, 3, 4]. NPs have a relatively high surface/volume ratio due to their small size that can improve the separation efficiency [5]. The advantage of using NPs as PSP, that it can be used once each experiment, so there is no need for column regeneration [5].

1.3 PSPs historical development in PSP-CEC

The separation in capillary electrophoresis is based on the difference in electrophoretic mobility of the analytes and to improve the selectivity, different additives have been used, for example by Terabe *et al*. He managed to separate neutral analytes that cannot be separated in normal CE by using micelles as PSP in a new technique that was named MEKC (Micellar electrokinetic chromatography) [6, 7]. The surfactants as SDS “sodium dodecyl sulfate” were added to the target electrolytes above a certain concentration “critical micelle concentration” and the detergent can be “seen” in free and in micellar form. In MEKC, the negatively charged SDS “go against” the EOF “electro osmotic flow” [6, 7].

1.4 Capillary electrophoresis

The separation in CE is based generally on the differential migration velocities of the analytes through a very thin "narrow diameter" with our capillary "inner diameter of 25-100 µm" across a high voltage is applied. As the capillary has a high surface/volume ratio, the generated Joule heating is dissipated "through an internal large surface area" and there is a minimum consumption of analytes and electrolyte.

Fig. 1 below shows the flow profile in CE compared to liquid chromatography "LC" [8]. The generated flow in CE gives more sharp peaks "because of a lower band broadening" than in LC.

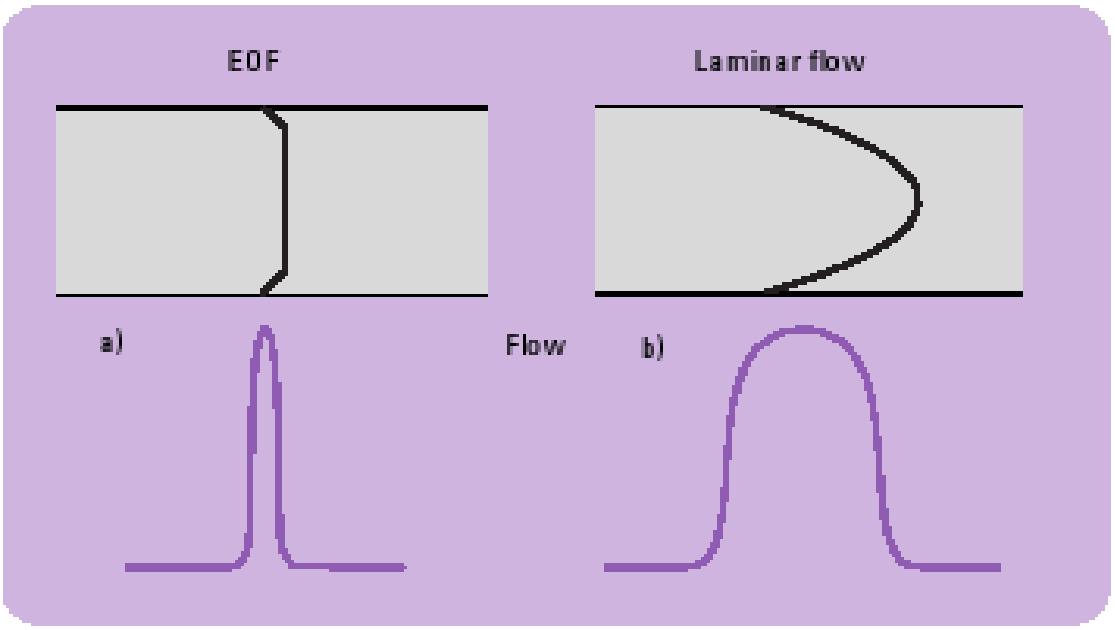

Fig. 1 shows the flow behaviour in a) EOF in CE, b) pressure based flow in LC [9].

1.5. Modes of CEC

The fundamental difference between CE and CEC is that the separation is not based on the electrophoretic mobility difference only but on the interaction with the stationary phase as well.

The modes of CEC can be divided into four groups:

1.5.A. Packed column CEC

The stationary phase is packed in the capillary and retained frits are fabricated in the capillary. One of the earliest packed column was performed by Pretorious and it was as a glass capillary, which was packed with silica particles [10]. In a packed column, smaller particles are used compared to LC because of the absence of back pressure [10]. The disadvantage with packed column CEC preparation is because it is complicated due to the technical preparation of packing. The adsorption of sample on retaining fritis can cause air bubbles that give zone broadening [11, 12].

1.5.B. Monolithic column CEC

Monoliths are defined as continuous pieces of cross-linked macroporous material that are fixed inside the capillary from the monomer mixtures. The monolithic column is an alternative to a packed column to avoid problems of column packing and retained frits [13, 14]. The advantage of monolithic columns is that they can easily to be prepared and they benefit from its large surface area. Pioneering monolithic column research was done by Hjertén based on poly-acrylamide for applications in CEC [15, 16]. Monolithic columns such as poly(styrene-co-vinylbenzyl chloride-co-divinylbenzen) and poly(4-methylstyrene-co-vinylbenzyl chloride-co-divinylbenzene) have been used early to achieve CEC separation by Svec *et al* [17].

1.5.C. Open-tubular column CEC

The open-tubular column was illustrated by Tsuda using a C_{18} coated capillary in 1982 [18, 19]. The mentioned mode has an advantage of an ease of preparation and a disadvantage of a relatively low sample capacity and the long diffusion distance of the target analytes to the surface interaction phase "mobile phase". The problem of a long diffusion distance can be solved by using a rather small inner diameter capillary ~25 µm, but the detection sensitivity will decrease.

1.5.D. PSP-CEC

There are some limitations such as "carry over effects" of previous modes such as adsorption of sample, sample matrix components to the stationary phase (SP) as the column regeneration is extremely important to give the aimed reproducibility, so an alternative mode was used i.e. PSP. PSP is used only once and to be added to the buffer/electrolyte, and it is an alternative to immobilized SPs [20].

1.6 Amount ratio of Proteins to NPs

As we did not see any separation in PSP-CEC combined with UV-detection in a mixture of two proteins that differ in size and pI "BSA and cytochrome C" were mixed, however they have been eluted in one peak, some mathematical calculations of the outer surface area of the nanoparticles and proteins have been done, and how many protein molecules have been done to get a proper protein separation, e.g., if two proteins are mixed and they have a bigger surface area than the outer surface area of the nanoparticles, in this case the separation will not be performed , then the outer surface area of one nanoparticle should be calculated to see if differences can be noticed in the separation when the proteins differ in sizes for example in case of using a small size such as for insulin and cytochrome c or a big size such as for BSA and ɣ-globulin. The affinity chromatography can also affect the separation if more than one protein molecules are mixed and they separate as one signal. The calculations depend on some variables such as the protein size for example the outer surface area will be less in case of insulin "6000 Da" and cytochrome C "12000 Da" than the outer surface area of BSA "66000 Da" and ɣ-globulin "112000 Da". It also depends on the ratio of pore size of nanoparticles/proteins and if the nanoparticles pore size are bigger than the protein molecule or smaller, while smaller protein molecules can penetrate the nanoparticles pores. PSP-CEC with UV-detection cannot, so far, separate a mixture of proteins; they elute in one peak, due to too few nanoparticles, i.e too little a surface area of the NPs

The mathematical expression can be introduced as below,

The area of 1 NP "spherical" is $\underline{4 \pi r^2} = 1.5*10^{-14}\ m^2$ (1)

The outer diameter of that particle is 70 nm.

The volume of 1 NP is $4/3\ \pi\ r^3 = 1.8*10^{-22}\ m^3$ (2)

The total volume of the cylindrical capillary is $\pi\ r^2\ l = 5.9*10^{-10}\ m^3$ (liter), that is roughly 0.6 µl.

Where the inner diameter of the used capillary is 50 nm, and l is the length of the capillary and it is 30 cm.

The total number of particles in the capillary can be calculated to $6.5*10^{10}$ particles.

So, the area of all NPs = area of 1 NP * total number of NPs (3)

$= (1.5*10^{-14}) * (6.5*10^{10}) = 10^{-3}\ m^2$.

To calculate how many (amount of) protein molecules should be used, the concentration of protein "cytochrome C" is 1 mg/mL, that can be represented as 1 ng/nL, and the protein mass is 12 KDa, where Da = g/mole and 1 ng = 10^{-9} g.

Then the number of moles of protein = $(1/14000) * 10^{-9} = 7.14*10^{-14}$.

So, the actual total number of (cytochrome C) molecules =

$(7.14*10^{-14}) * N_A$ (Avogadro number) = $6.2*10^7$ in case of concentration of 1 mg/mL/ (1ng/1nL) of protein molecule.

The nanoparticle pore is around 100 A^O, so small size proteins should be used as cytochrome C "12 KDa" and insulin "6 KDa".

1.6. UV-detection

UV-methodology was connected to CE/CEC to see the protein separation via pseudo-stationary phase. Nanoparticles with and without silver were used as pseudo-stationary phase.

1.7. Mass spectrometry "MALDI-TOF/MS"

Nanoparticles as pseudo-stationary phase "PSP" were combined to MALDI-TOF/MS. The principle of mass spectrometry is to separate and detect the gas phase ions. MALDI-TOF/MS is a mass spectrometric technique used in the identification of large biomolecules such as proteins, peptides, DNA, etc…, MALDI expanded widely and attracted much interest during the last two decades in identification either for biological analysis or large organic molecules such as polymers [18]. MALDI-TOF technique was introduced as a principle by Karas and Hillenkamp [18].

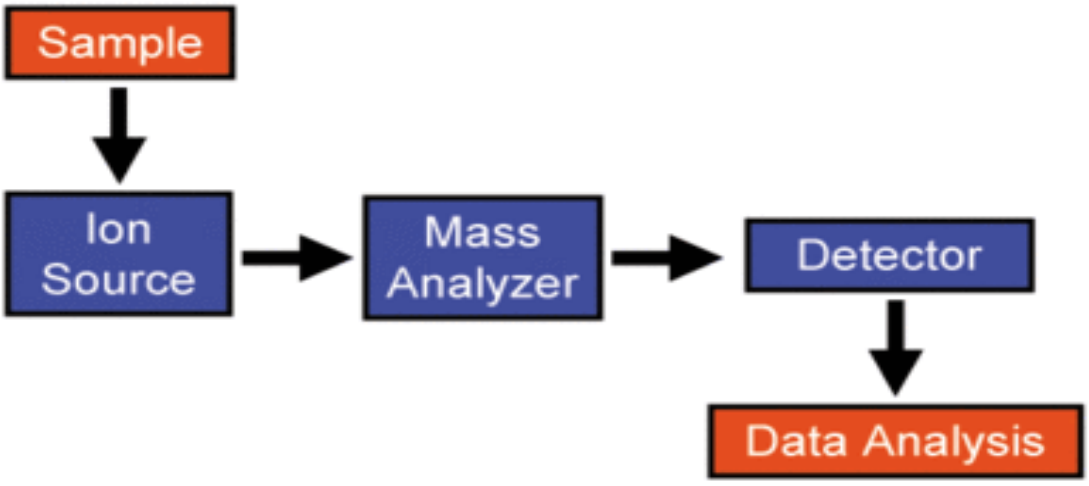

Fig. 2/. A simplified schematic diagram of MALDI-TOF/MS "is taken from www.google.wekipedia, MALDI-TOF/manual/instrument.en.com".

In MALDI-TOF/MS, the protein sample is embedded of within a crystal structure of organic compounds that is called matrix. The most common matrices are, α-cyano-4-hydroxy cinnamic acid (CHCA) and 2,5-dihydroxybenzoic acid (DHB). It is deposited on a highly conductive sample support "MALDI-plate" [(19)]. The cocrystals are normally irradiated by a UV-laser beam with wave length of 266 or 337 nm. The resulting energy from the UV-laser causes a decomposition of the irradiated cocrystals, consequently, a particle cloud is generated called a plume, in which ions are extracted by an electric field [(19)].

The mechanism behind MALDI is divided to two steps, desorption of the matrix by the UV-laser beam and decomposition of the plume by an electric field or by the energy resulting from the UV-laser beam. The UV-laser beam hits the cocrystalized matrix and the resulting energy transfers to the target sample and vaporizes the sample, then directs the plume to the ion source see figure 2. The plume is sent to the mass analyzer that includes reflector mirror and the fragmented ions are separated depending on their masses/velocities, where the lighter (mass to charge) goes first then the heavier ones to the detector. Most ions can be found as a single positive charge that makes it consequently easier in reading the spectra, MALDI is a preferred instrumental technique in proteomics analysis.

2. Experimental method

2.1. Sample preparation

2.1.A. Old NPs preparation

2.1.B. Freshly prepared NPs preparation

1.6 g of MOPS was added to 50 mL of M.Q water. 100 mM MOPS, pH 7.4 was diluted with M.Q water to ~15 mM. 1 mg of protein cytochrome *c* was added to 2% of NPs with and without silver to give a concentration of 1 mg/mL. Two NPs "buffer samples" were prepared as the following; A 20% of NPs was diluted to 2% with 15 mM MOPS and 150 µl of the diluted NPs was added to each buffer sample. The third vial sample was the injected NPs containing 1 mg/mL cytochrome c. The same preparation methodology was repeated different times but for different concentrations (0.5 and 0.25 mg/mL).

2.2. UV-detection

The capillary was of 30 cm a length and of 50 µm an inner diameter and was fixed in a Beckman cassette. The Beckman instrument was set up for UV-detection as follows:

Set detector at 5 Hz. Rinse for 3 min, using forward pressure for the outlet samples. Zero detector, then Separate for 10 min at 10 KV, the maximum current should not exceed 100 µA, the outlet samples are located in positions "11&1" on the rotating plate. Inject at High pressure for 3 sec, the inlet sample is located in position "12". Then separate again after injection at 10 KV and the maximum current should not exceed 100 µA.

2.3. Mass spectrometry “MALDI-TOF”

2.3.A. Insulin identification

Pure insulin was identified as the following: 0.05 mg of insulin was dissolved in 1 mL of 15 mM MOPS. 0.5 µl of 0.05 mg/mL insulin sample was spotted on MALDI-plate, then another 0.5 µl of matrix, CHCA, 10 mg/mL, was added to the sample. The same procedure in pure insulin preparation was used however, the insulin was dissolved in 2% NPs with and without silver instead of MOPS buffer.

3. Results and discussion

The work is divided to two parts. The first part shows the behavior and the activity of old nanoparticles that act as a pseudo stationary phase and the second part shows freshly prepared nanoparticles as PSP.

3.1. Old prepared NPs

Old NPs show good stability as a pseudostationaryphase.

3.1.A. NPs with silver

Two different proteins were tested, cytochrome c and BSA. Cytochrome c has a pI 9 above the buffer pH so, the protein has a negative charge. BSA has a pI 4.6 below the buffer pH so, the protein has a positive charge. Cytochrome c has a size of 12 KDa and BSA has a size of 64 KDa.

Cytochrome c was injected in 20% of NPs with silver. Figure 1 shows a sharp peak of cytochrome c in a case of high pressure injection. The run was repeated at the same conditions but in a case of electro-kinetic injection at 2 KV and 3 KV, see figures 2 and 3.

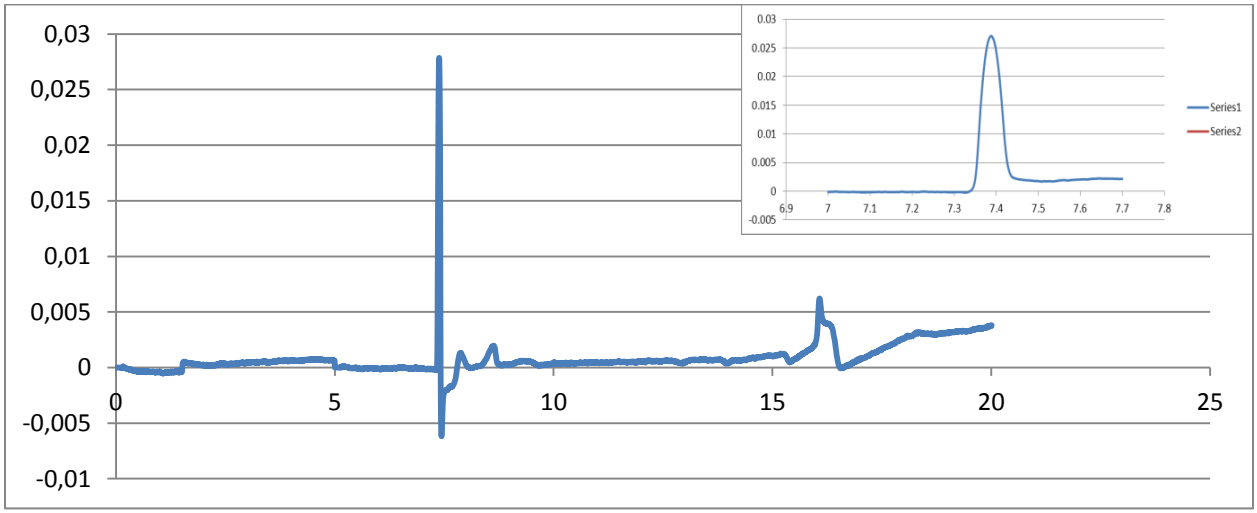

Fig. 1, shows the behavior of old NPs with silver in case of cytochrome c injection at High pressure for 3 s. The injection was after 5 min_and the voltage all over the run was 10 KV. Cytochrome c concentration is 1.6 mg/mL. The current all over the run was 8 µA. The buffer concentration is 9 mM

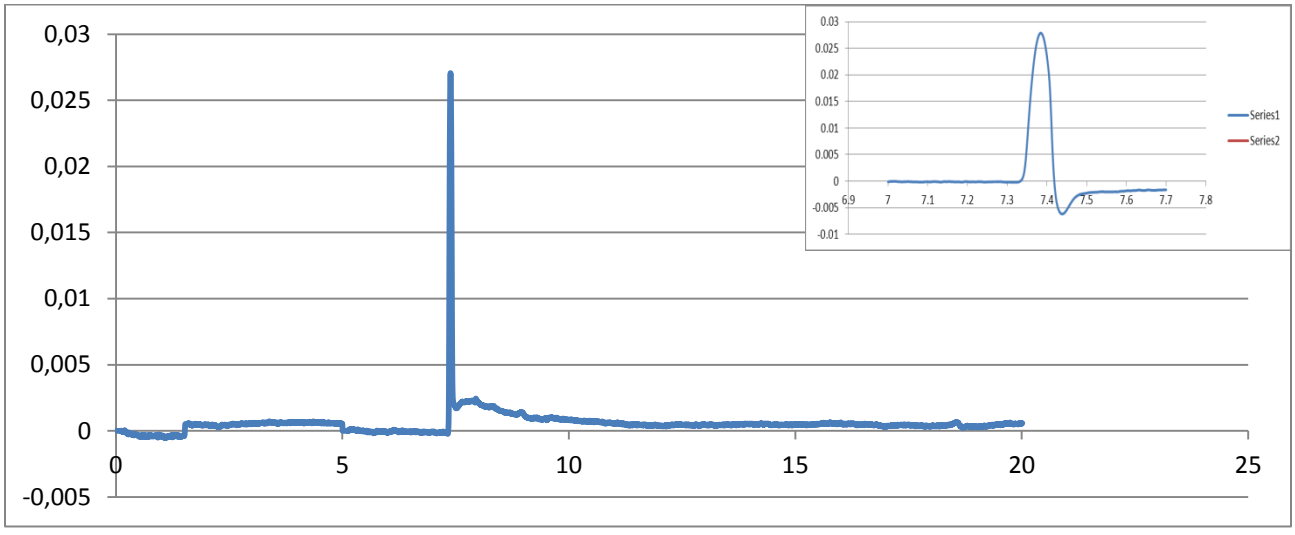

Fig. 2, shows the behavior of old NPs with silver in a case of cytochrome c injection at 3 KV for 3 s. The injection was after 5 min and the voltage all over the run was 10 KV. BSA concentration is 1 mg/mL. The current all over the run was 8 µA. The buffer concentration is 9 mM

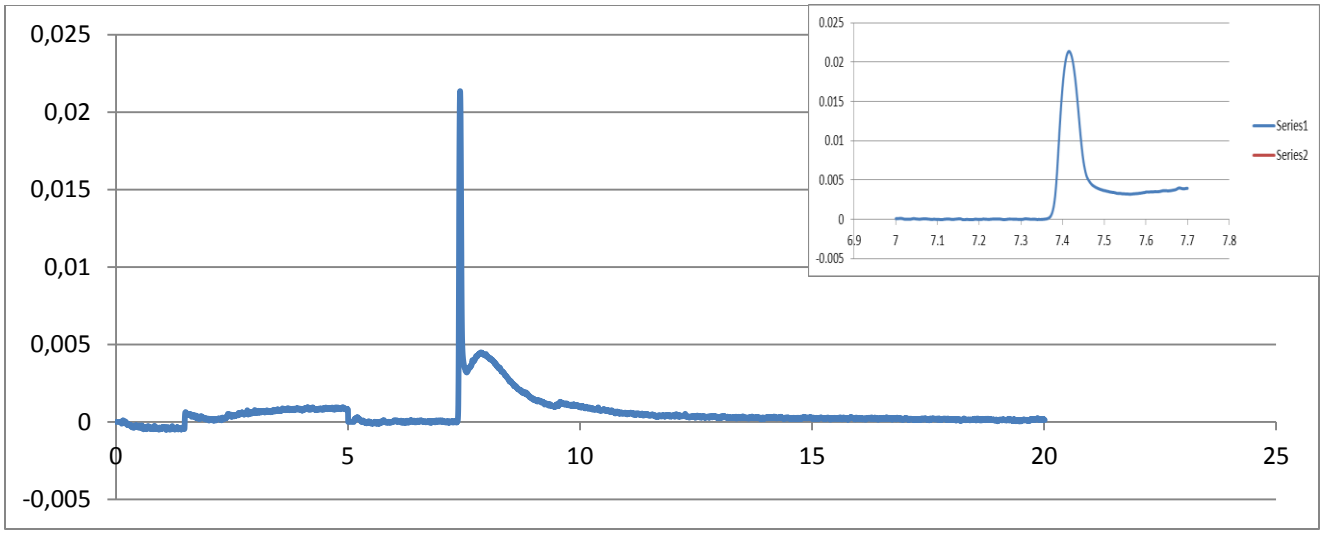

Fig. 3 shows the behavior of old NPs with silver in a case of cytochrome c injection at 2 KV for 3 s. The injection was after 5 min and the voltage all over the run was 10 KV. Cytochrome c concentration is 1 mg/mL. The current all over the run was 8 µA. The buffer concentration is 9 mM.

The magnifications of figures 1, 2 and 3 at retention time between 7.3 and 7.5 minutes show that all sharp peaks have the same retention time and similar height. These results show the stability of NPs with silver and that they can be used as a PSP.

BSA “bovine serum albumin” shows two peaks, see figures 4, 5 and 6. The two peaks could be a dimer. Because BSA has a low pI and big size, the interaction between cytochrome c and NPs is stronger than BSA and NPs and consequently the peak comes at a longer retention time in case of cytochrome c.

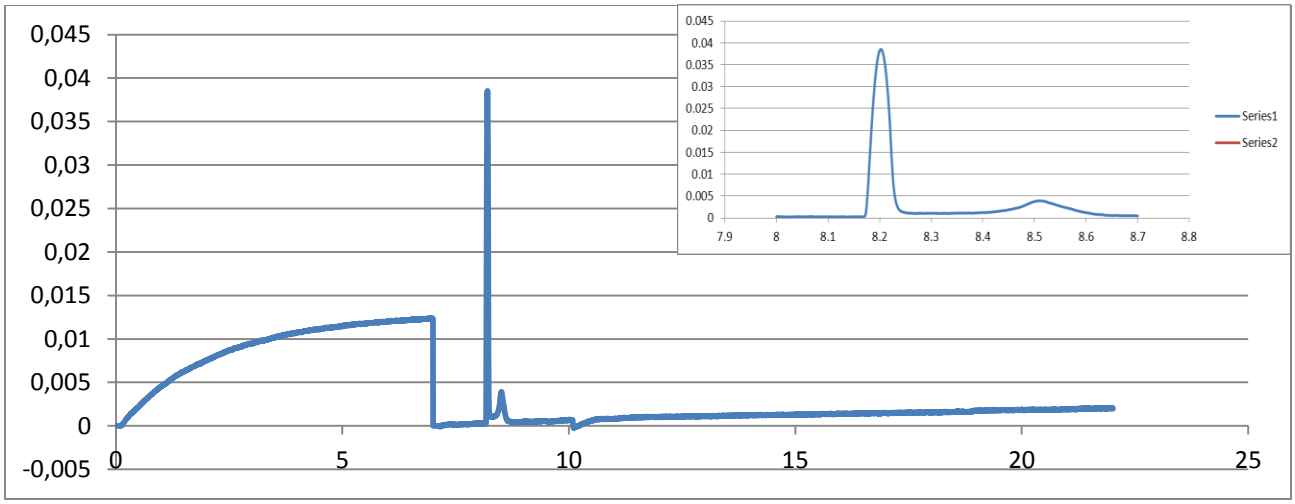

Fig. 4 shows the behavior of old NPs with silver in a case of BSA injection at 3 KV for 3 s. The injection was after 7 min and the voltage all over the run was 10 KV. BSA concentration is 1 mg/mL. The current all over the run was 10 µA. The buffer concentration is 10 mM.

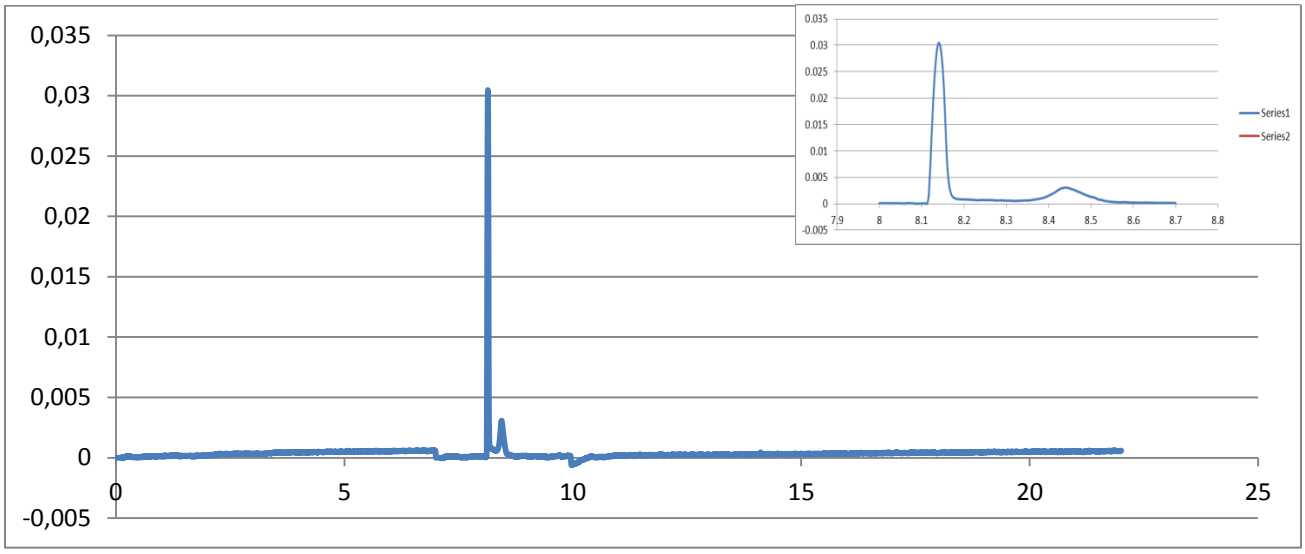

Fig. 5 shows the behavior of old NPs with silver in a case of BSA injection at 2 KV for 3 s. The injection was after 7 min and the voltage all over the run was 10 KV. BSA concentration is 1 mg/mL. The current all over the run was 10 µA. The MOPS concentration is 10 mM.

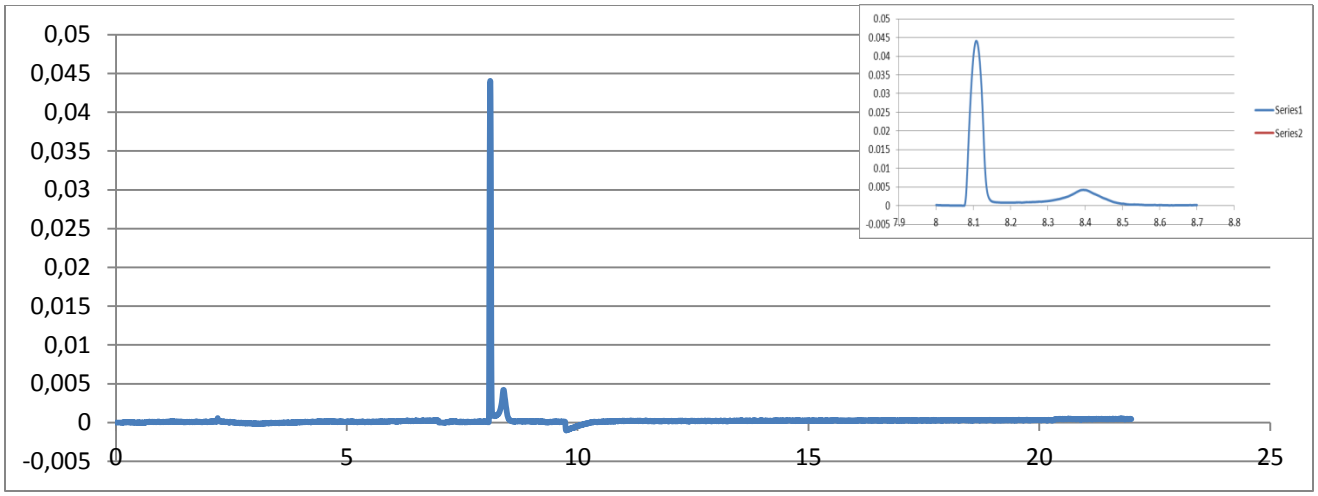

Fig. 6 shows the behavior of old NPs with silver in case of BSA injection at high pressure for 3 s. The injection was after 7 min and the voltage all over the run was 10 KV. BSA concentration is 1 mg/mL. The current all over the run was 10 µA. The buffer concentration is 10 mM.

3.2. Freshly prepared NPs

3.2.A. NPs with silver, cytochrome c as injected protein

According to the previous mathematical expression of the outer surface area of nanoparticles and the small pore size of NPs which is 100 A^{o}, it was suggested that rather small size molecules should be used. The two used proteins are cytochrome c and insulin. Insulin size is 6 KDa. The 20% NPs was diluted to 2% with 15 mM of MOPS. Cytochrome c was injected at high pressure, see figure 7.

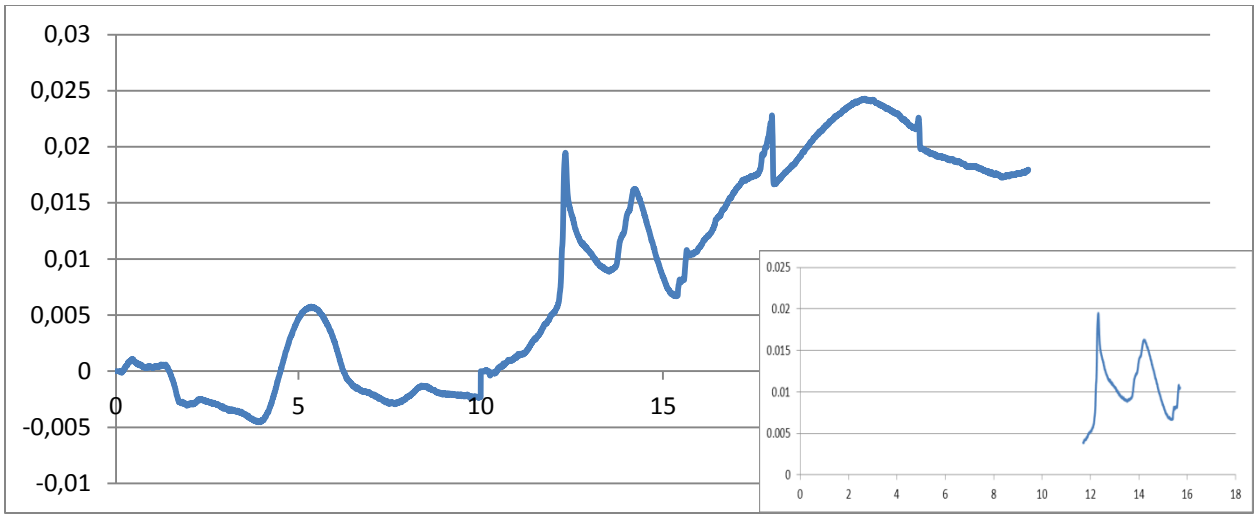

Fig. 7 shows the behavior of NPs with silver in case of cytochrome c injection at high pressure for 3 s. The injection was after 10 min and the voltage all over the run was 10 KV. Cytochrome c concentration is 1 mg/mL. The current all over the run was 20 µA. The buffer concentration is 15 mM.

Figure 7 shows that the sample was overloaded. Thus the run was repeated at the same conditions but the concentration was diluted from 1 to 0.5 mg/mL to see if it was a real spectra, see figures 8, 9. The magnification of figures 8 and 9 at a retention time between 13.1 and 14.2 minutes show that the runs are reproducible.

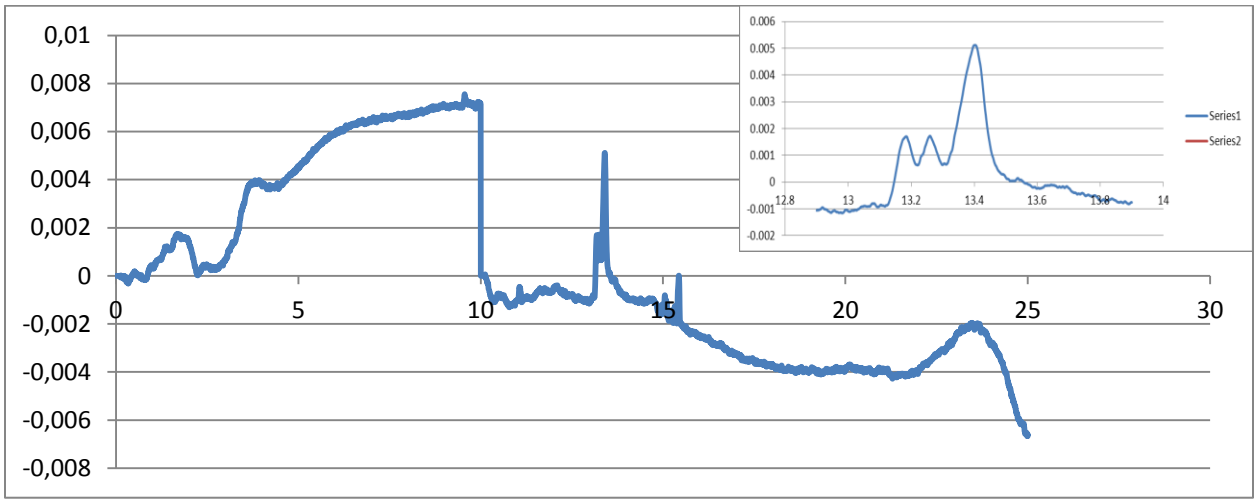

Fig. 8 shows the behavior of NPs with silver in case of cytochrome c injection at high pressure for 3 s. The injection was after 10 min and the voltage all over the run was 10 KV. Cytochrome c concentration is 0.5 mg/mL. The current all over the run was 20 µA. The MOPS buffer concentration is 15 mM.

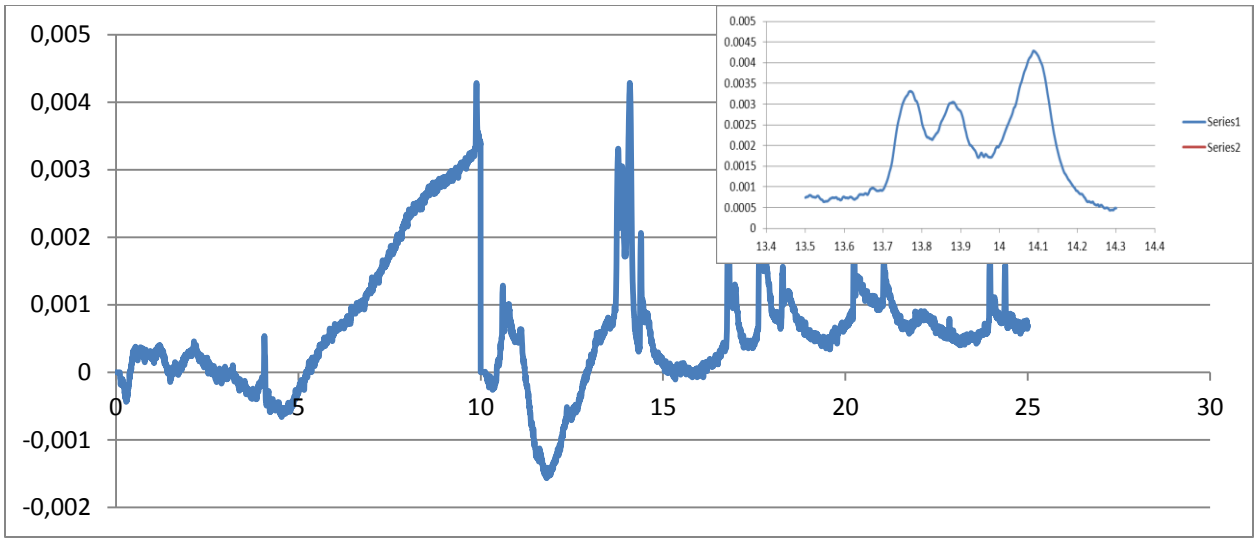

Fig. 9, shows the reproducibility of Fig. 8 with a protein concentration of 0.5 mg/mL. The current all over the run was 20 µ Amp.

The concentration of 0.5 mg/mL was diluted to 0.25 mg/mL to see if UV-detection can be obtained even for a very small concentration, see figure 10.

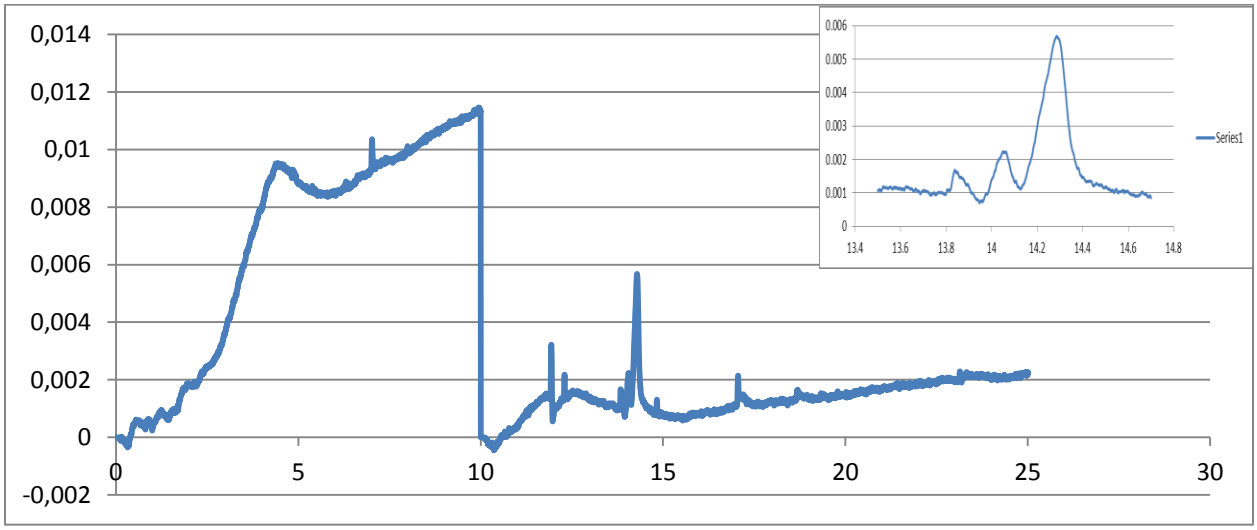

Fig. 10 shows the behavior of NPs with silver in case of cytochrome C injection at high pressure for 3 s. The injection was after 10 min and the voltage all over the run was 10 KV. Cytochrome c concentration is 0.25 mg/mL. The current all over the run was 20 µA. The buffer concentration is 15 mM.

The magnified spectra in figure 10 shows the same signals that have been shown in case of using a concentration of 0.5 mg/mL as shown before in figures 8 and 9. The peak height should be half of the peak height in figure 9 as the concentration is a half which is not seen. The explanation that the area in figure 9 is more broadening than in figure 10. The run was applied once more and it was reproducible, see figure 11.

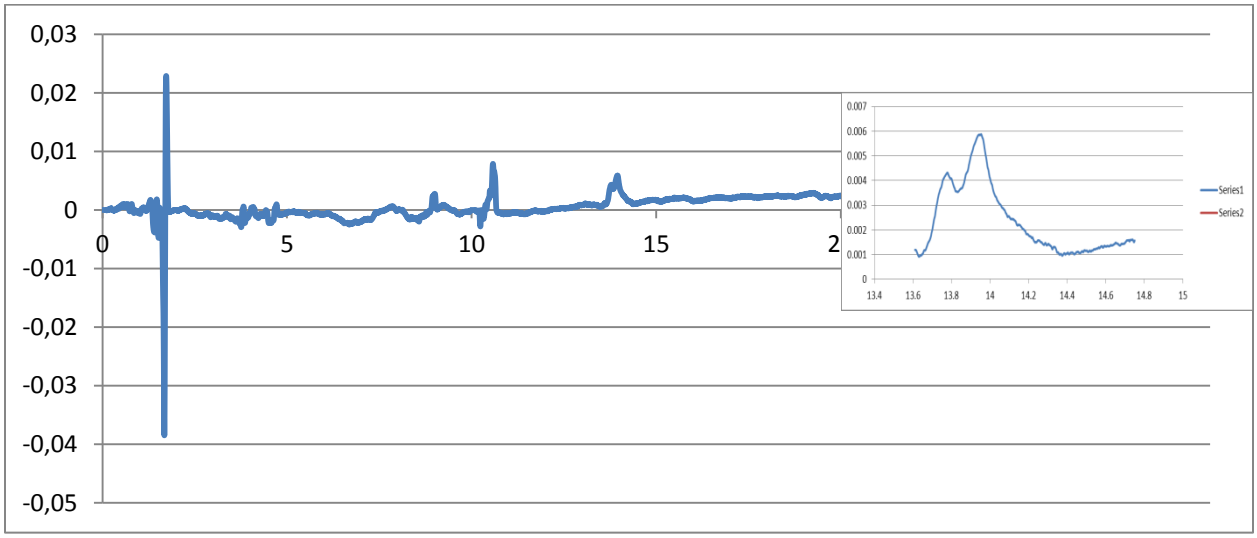

Fig. 11, shows the reproducibility of Fig. 4 with a protein concentration of 0.25 mg/mL. The current all over the run was 20 µA, see Fig. 12.

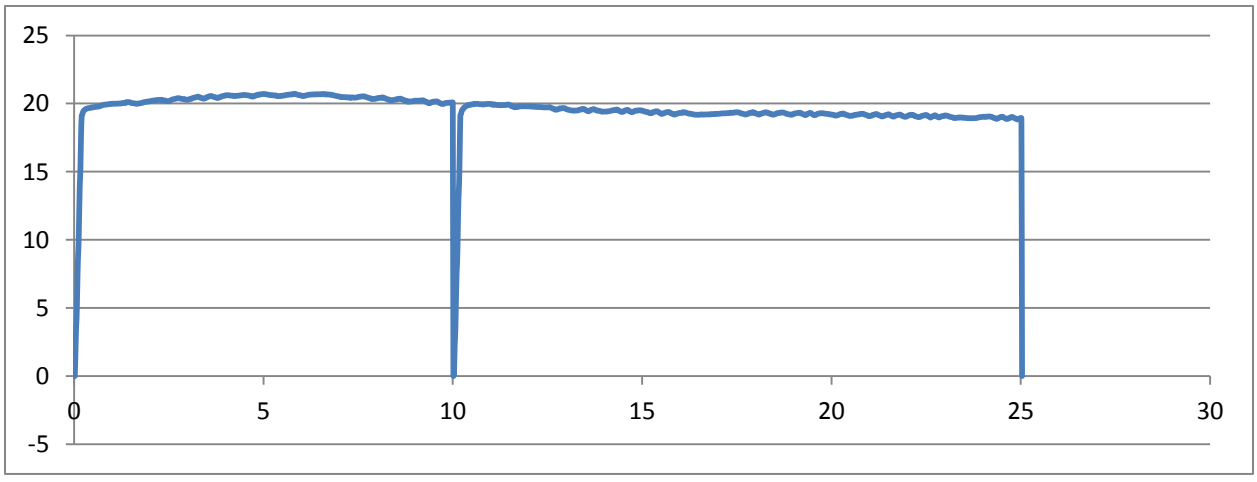

Fig. 12 shows that the current was high and stable all over the previous runs.

Two samples of 2% NPs with silver were used as buffer samples and the run was applied at the same conditions as before but with zero injection (without cytochrome c), see figure 13.

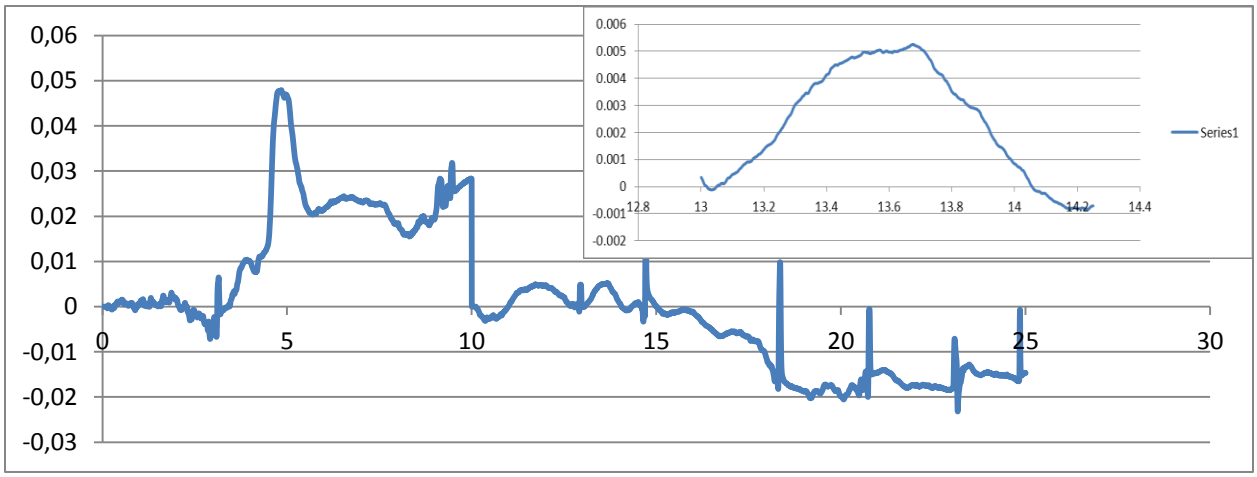

Fig. 13 shows NPs with silver in case of zero injection.

The magnification of figure 13 gives a noisy base line without any signals as it is expected, and this indicates that the previous peaks results in case of cytochrome c injection were real peaks. It gives highly detailed information that NPs can be used as a PSP in CEC separation combined with UV-detection.

3.2.B. NPs without silver, cytochrome C as injected protein

The same procedures were performed but in case of NPs without silver. Different concentrations were used (1 mg/mL, 0.5 mg/mL and 0.35 mg/mL).

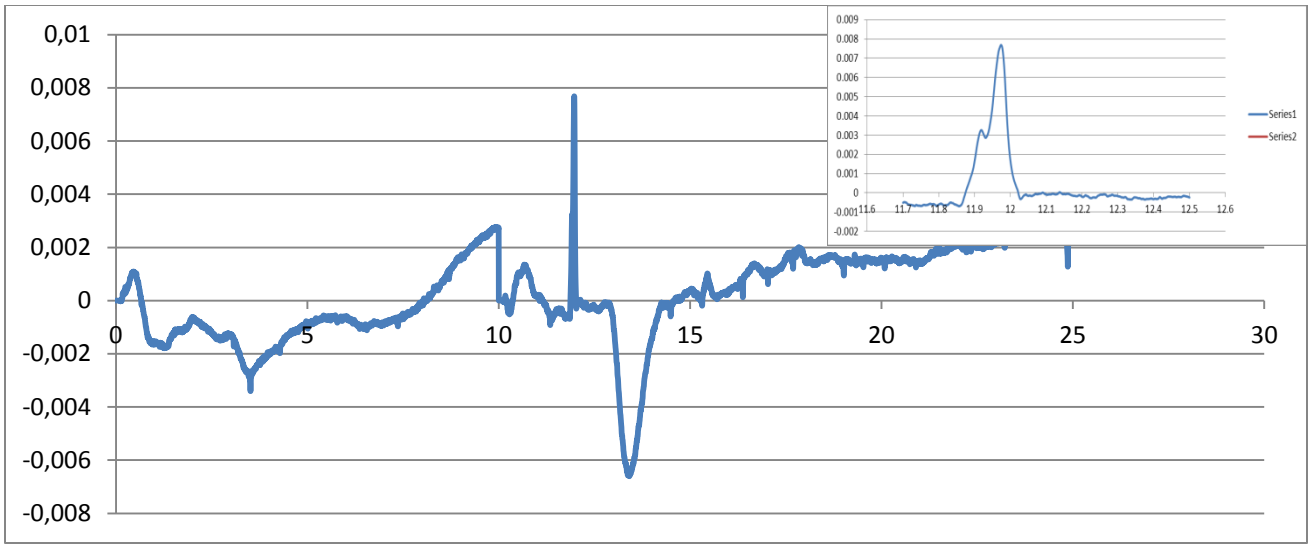

Fig. 14 shows the behavior of NPs without silver in case of cytochrome c injection at High pressure for 3 s. The injection was after 10 min and the voltage all over the run was 10 KV. Cytochrome c concentration is 0.35 mg/mL. The current all over the run was 20 µA. The buffer concentration is 15 mM.

The magnification of figure 14 at retention time between 11.7 and 12.5 minutes shows a signal that has a height of 0.009 with a shoulder but at this step, it is not known yet if it is a protein peak or a nanoparticle peak. The run was repeated at the same conditions, see figure 15, which shows that it is reproducible.

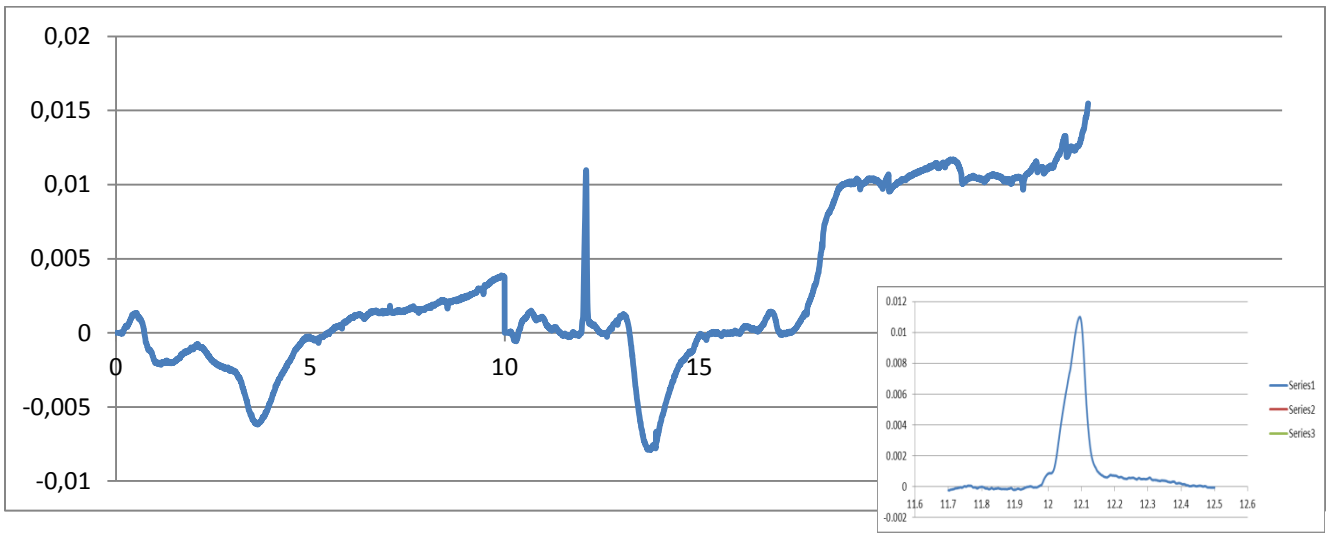

Fig. 15 shows the reproducibility of Fig. 8 at protein concentration of 0.35 mg/mL. The current all over the run was 20 µA.

The magnification of figure 15 shows that the peak height is 0.010 which is the same as shown in figure 14 and at the same retention time at 12 min. In this instance the shoulder is gone. Cytochrome c peak has a shoulder that could be due to a part of protein 3D-structure has been hydrolyzed.

The run was applied once more at the same conditions but the voltage all over the run was increased to 15 KV instead of 10 KV, see figure 16.

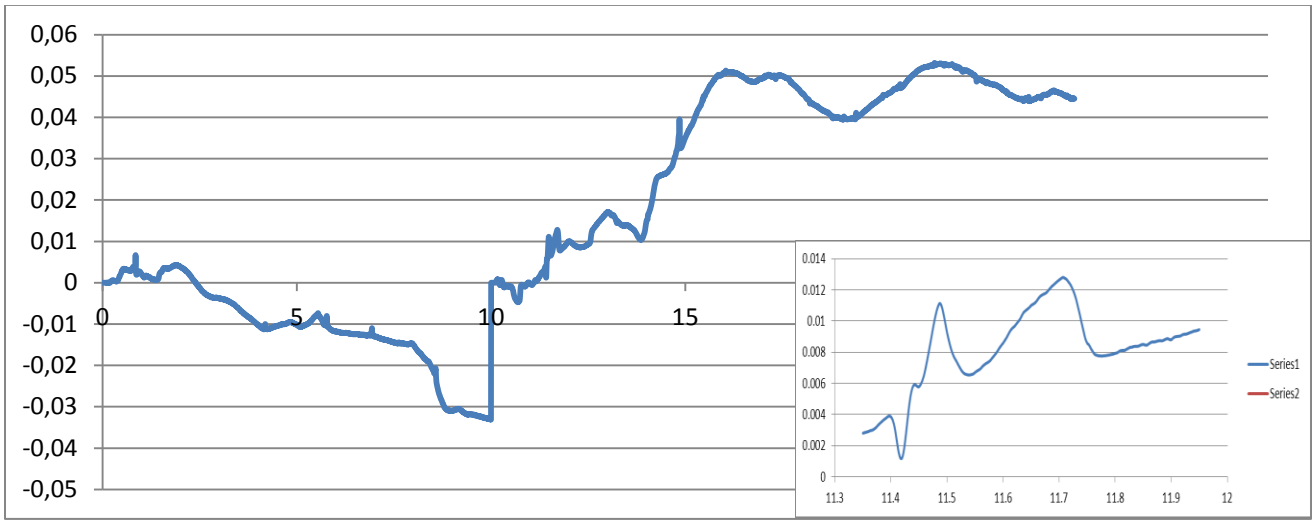

Fig. 16 shows the behavior of NPs without silver in case of cytochrome C injection at High pressure for 3 s. The injection was after 10 min and the voltage all over the run was 15 KV. Protein concentration is 0.35 mg/mL. The current all over the run was 44 µA. The buffer concentration is 15 mM

The magnification of figure 16 shows a peak at a similar time as in the case of 10 KV. It comes to a similar height but it is broadening. This is due to CEC allowing more interactions to happen.

The run was applied and it was reproducible, see Fig 17. The peak height of 0.005 is similar to the peak in figure 16 and the retention time is the same as well.

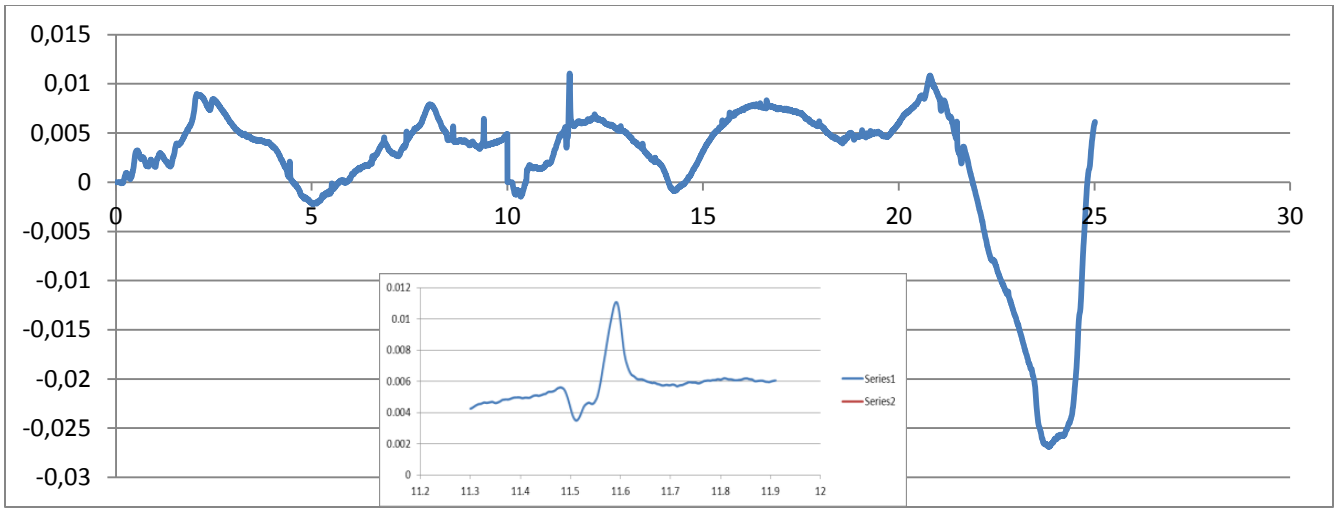

Fig. 17, shows the reproducibility of Fig. 21 at protein concentration of 0.35 mg/mL. The current all over the run was 44 µA. The buffer concentration is 15 mM.

Two samples of 2% NPs with silver were used as buffer samples and the run was applied at the same conditions as before but with zero injection (without cytochrome c), see figures 18 and 19.

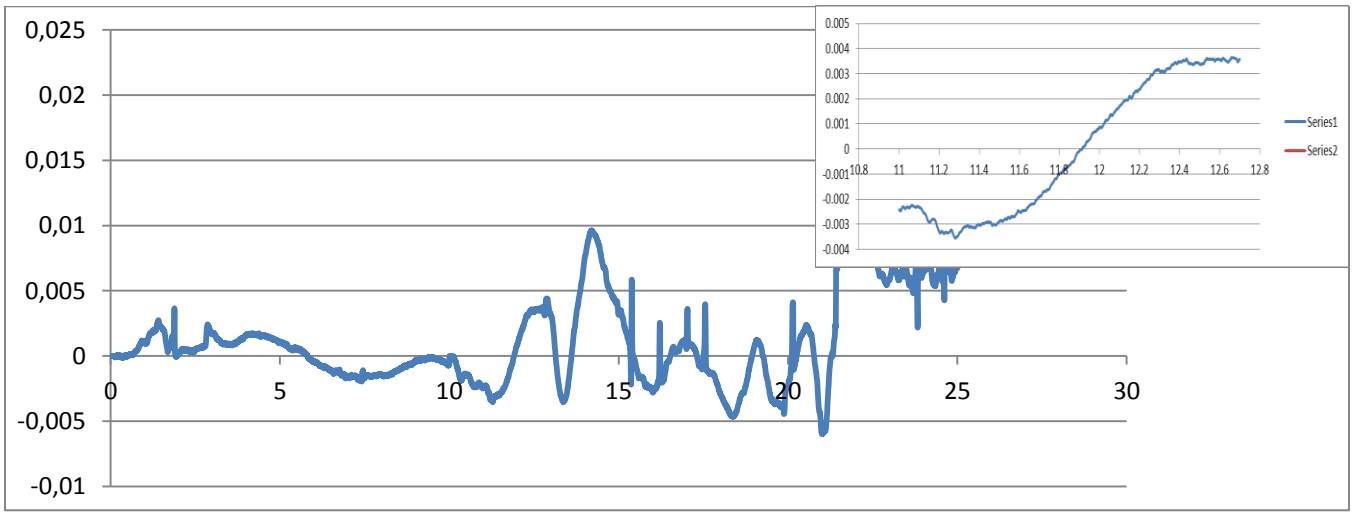

Fig. 18 shows the run was performed without cytochrome c injection.

Fig. 18, shows that there is just a noisy base line without any real peak and this is an indication that the shown signal in case of 10 KV or 15 KV is a real peak that comes from cytochrome c.

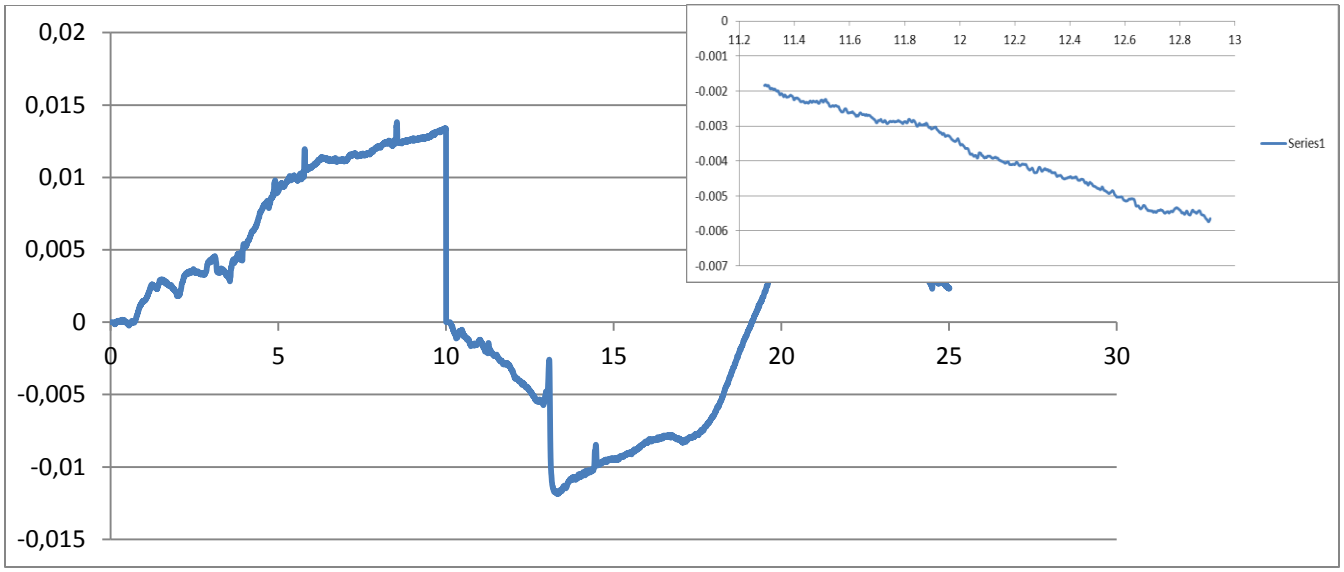

Fig. 19 shows the run was applied without cytochrome c injection.

3.2.C. NPs with silver, insulin as injected protein

Insulin has a half size of cytochrome c. Insulin was injected instead of cytochrome c at the same conditions. Different concentrations were used (1 and 0.5 mg/mL). Figures 19 and 20 show a successful reproducibility of the runs giving the same real insulin peak at similar retention time with the same height of 0.01 in case of insulin concentration of 1 mg/mL.

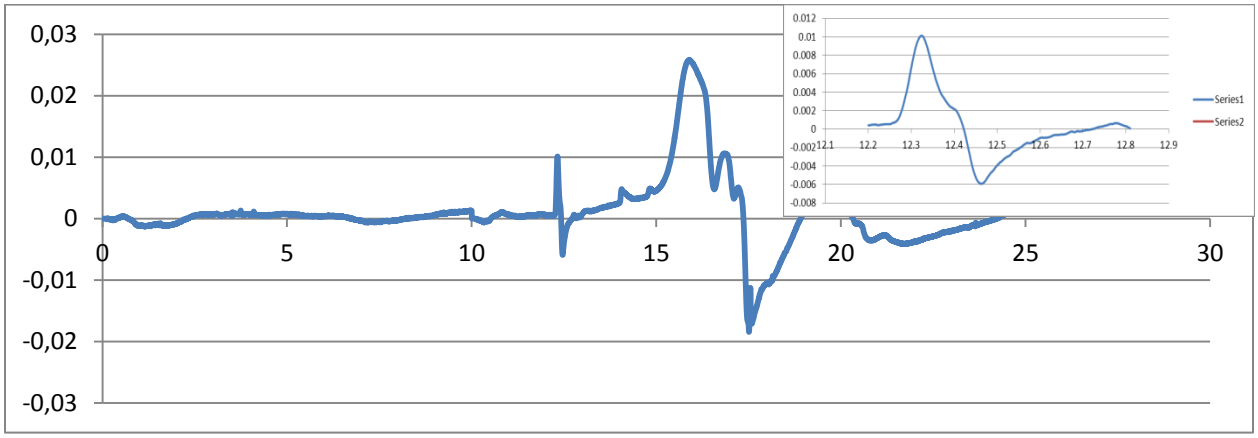

Fig. 19 shows the behavior of NPs with silver in case of insulin injection at high pressure for 3 s. The injection was after 10 min and the voltage all over the run was 10_KV. Insulin concentration is 1 mg/mL. The current all over the run was 15µA. The buffer concentration is 13 mM.

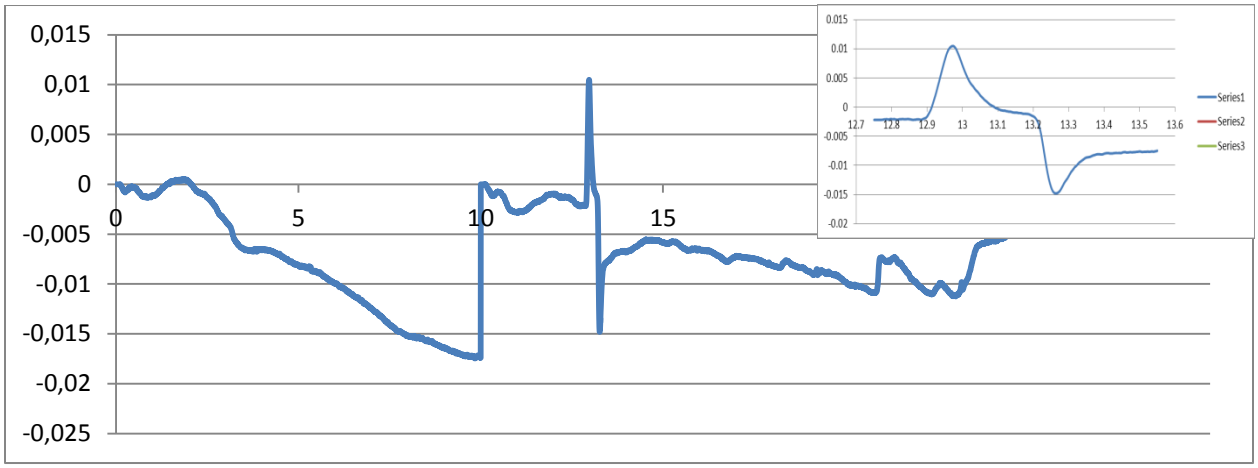

Fig. 20 shows the behavior of NPs with silver in case of insulin injection at igh pressure for 3 s. The injection was after 10 min and the voltage all over the run was 10 KV. Insulin concentration is 1 mg/mL. The current all over the run was 15 µA. The buffer concentration is 13 mM.

The run in figure 21 was applied at the same conditions as in figure 20 however the insulin concentration was diluted to 0.5 mg/mL.

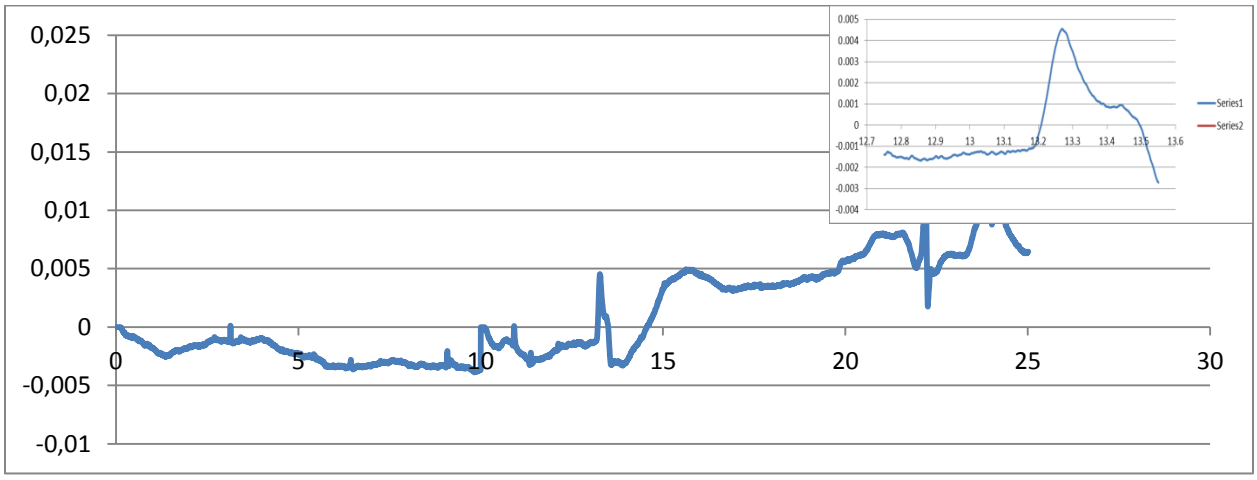

Fig. 21 shows the behavior of NPs with silver in case of insulin injection. The same conditions as in figures 19 and 20. Insulin concentration is 0.5 mg/mL. The current all over the run was 15µA. The buffer concentration is 13 mM.

The magnification of figure 21 shows a peak at similar time with half the height of that was shown in case of 1mg/mL in figures 19 and 20.

Two samples of 2% NPs with silver were used as buffer samples and the run was applied at the same conditions as before but with zero injection (without insulin), see figures 22 and 23.

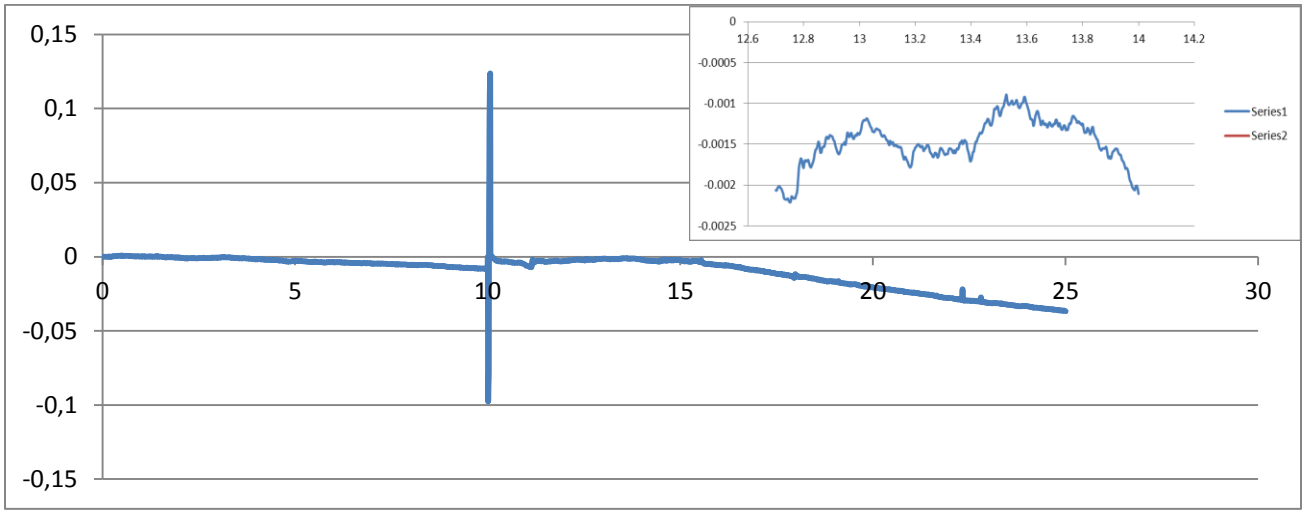

Fig. 22, shows that the run was performed without insulin injection. The same conditions as in figures 19 and 20. The current all over the run was 15 µA. The buffer concentration is 13 mM.

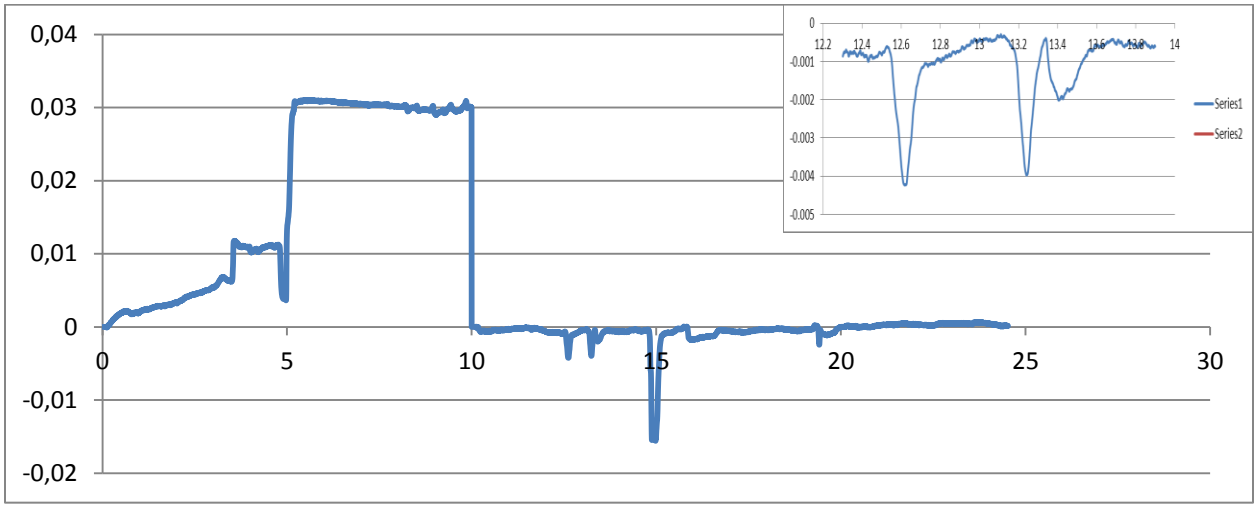

Fig. 23 shows that the run was performed without insulin. The same conditions as in figures 19 and 20. The current all over the run was 15µA. The buffer concentration is 13 mM.

There is a deep negative shift in figure 23 and it could be due to the presence of acetic acid as the insulin was in a stock solution of 10 µM of acetic acid. It was diluted 10 times with 15 mM of

MOPS to give a weight of 6 mg/L then diluted once more with MOPS and NPs with silver to give a final concentration of 1mg of insulin/1mL of NPs with silver. This got a suitable pH as the acetic acid is highly acidic with low pH.

The results mentioned above give an indication of the presence and good separation of insulin peak, thus NPs with silver can be used as PSP.

3.2.D. NPs without silver, insulin as injected protein

The runs were applied at the same conditions as injected insulin in case of using NPs with silver. Different concentrations were used (1 and 0.5 mg/mL). The magnifications of figures 24 and 25 show a high reproducibility giving the same insulin peak at the similar retention time at (12.5 minutes in figure 24 and at 13 minutes in figure 25) with the similar height (0.0035 in figure 24 and 0.004 in figure 25) in case of using a concentration of 1 mg/mL.

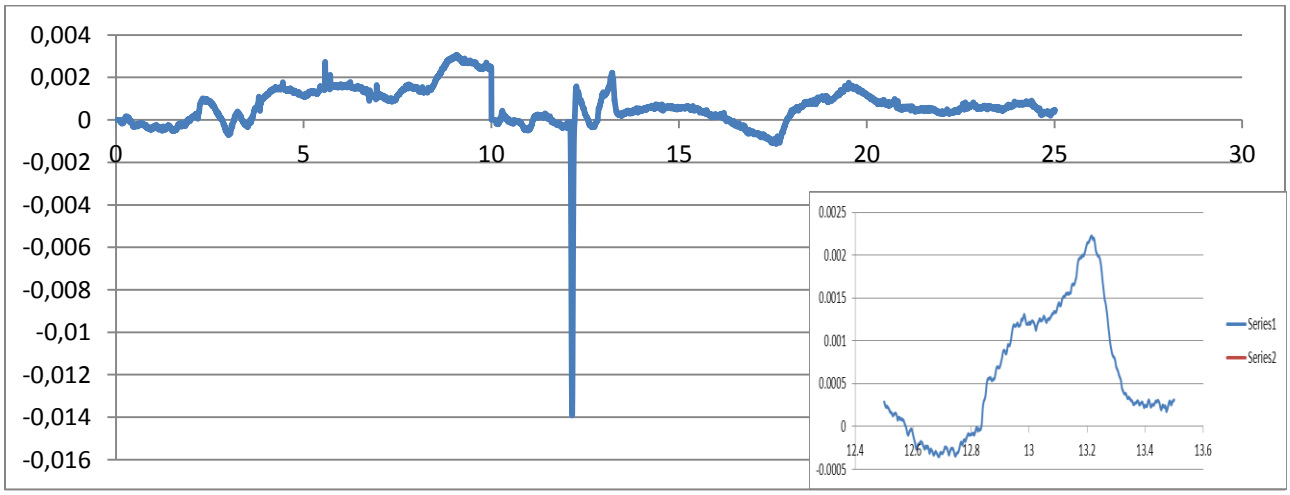

Fig. 24 shows the behavior of NPs without silver in case of insulin injection at high pressure for 3 Sec. The injection was after 10 min and the voltage all over the run was 10 KV. Protein concentration is 1 mg/mL. The current all over the run was 15µA. The buffer concentration is 13 mM.

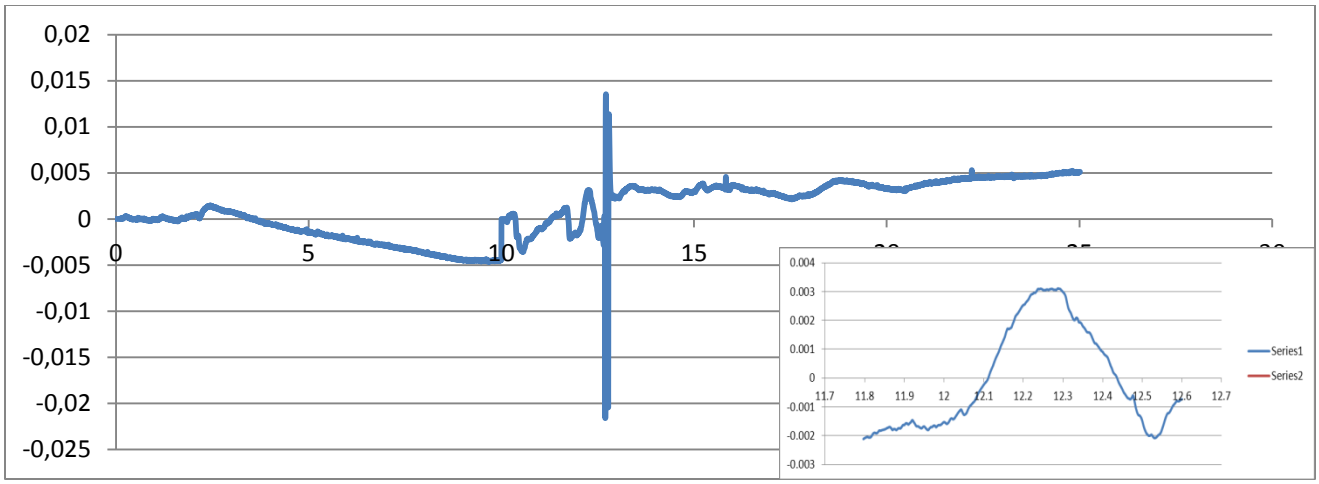

Fig. 25 shows the behavior of NPs without silver in case of insulin injection at high pressure for 3 s. The injection was after 10 min and the voltage all over the run was 10 KV. Protein concentration is 1 mg/mL. The current all over the run was 15µA. The buffer concentration is 13 mM.

The same run was applied using a concentration of 0.5 mg/mL, see figure 26. The magnification of figure 26 shows a peak at similar retention time which is 12.9 min and with half the height of the peak that was shown in figures 24 and 25 in case of a concentration of 1mg/mL.

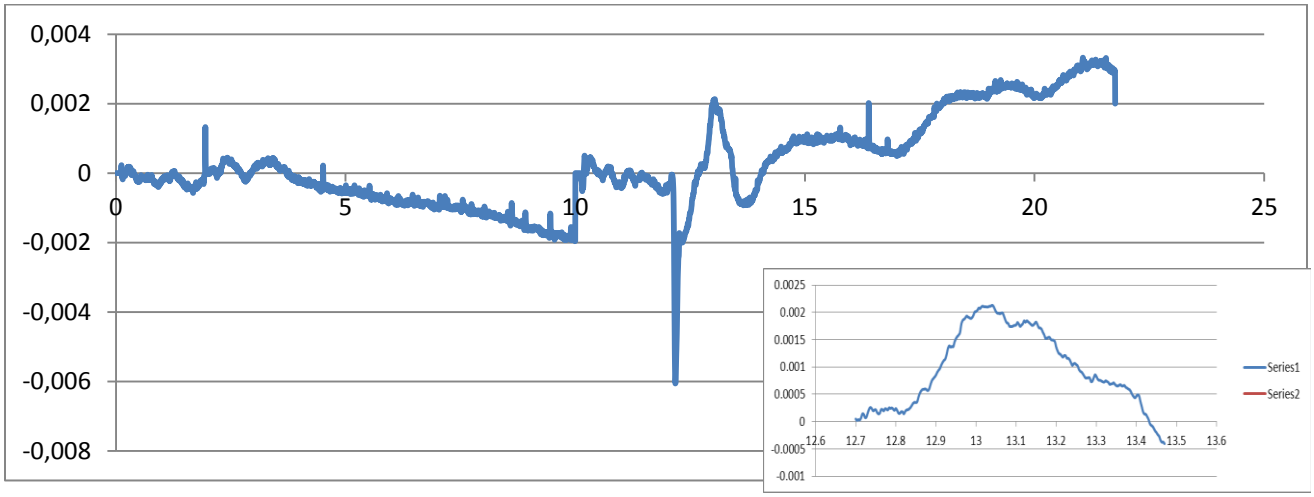

Fig. 26 shows the behavior of NPs with silver in case of insulin injection. The same conditions as in figures 30 and 31 Protein concentration is 0.5 mg/mL. The current all over the run was 15 µA. The buffer concentration is 13 mM.

Two samples of 2% NPs with silver were used as buffer samples and the run was applied at the same conditions as before but with zero injection (without insulin), see figures 27 and 28.

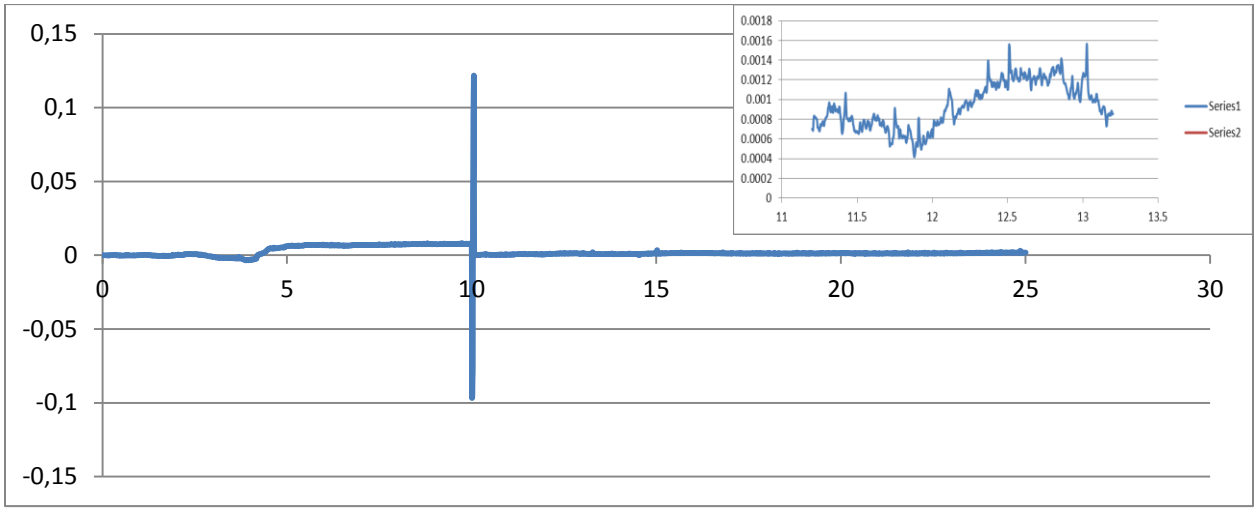

Fig. 27 shows that the run was performed without protein "insulin" injection. The same conditions as in figures 25 and 26. The current all over the run was 15 µA. The buffer concentration is 13 mM.

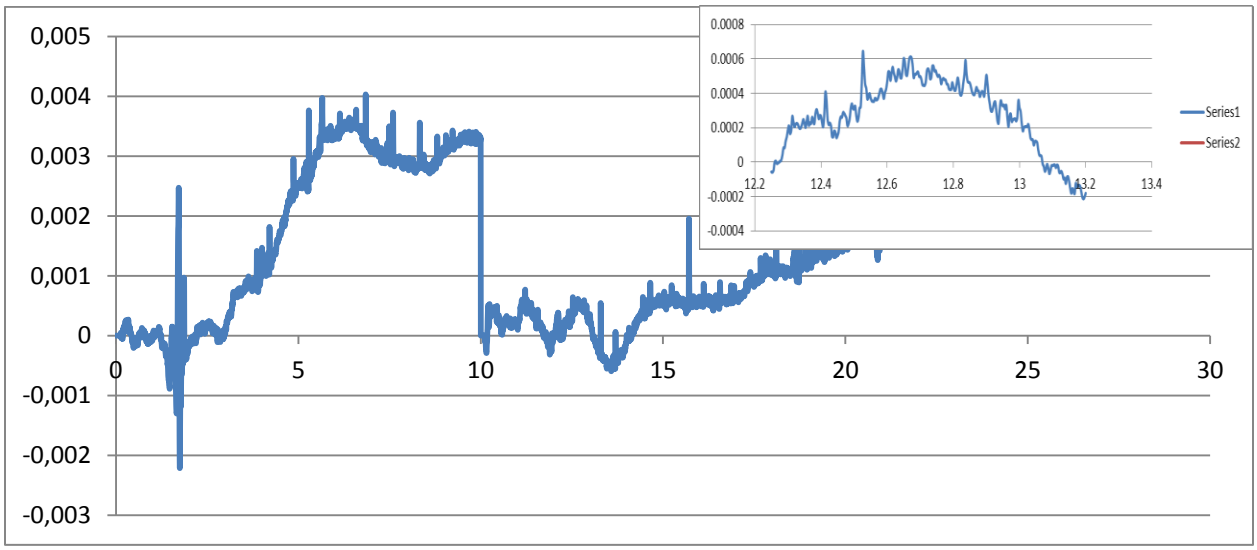

Fig. 28 shows that the run was performed without p insulin injection. The same conditions as in figures 25 and 26. The current all over the run was 15 µA. The buffer concentration is 13 mM.

The results mentioned above give an indication of the presence and good separation of insulin peak, thus NPs without silver can be used as PSP.

3.3 Mass spectrometry "MALDI-TOF" of Insulin

MALDI-TOF-MS was tested to investigate its compatibility with lipid nanoparticles. Insulin was successfully identified in a pure sample (insulin + electrolyte solution) with a mass of 5808 Da, see figure 29.

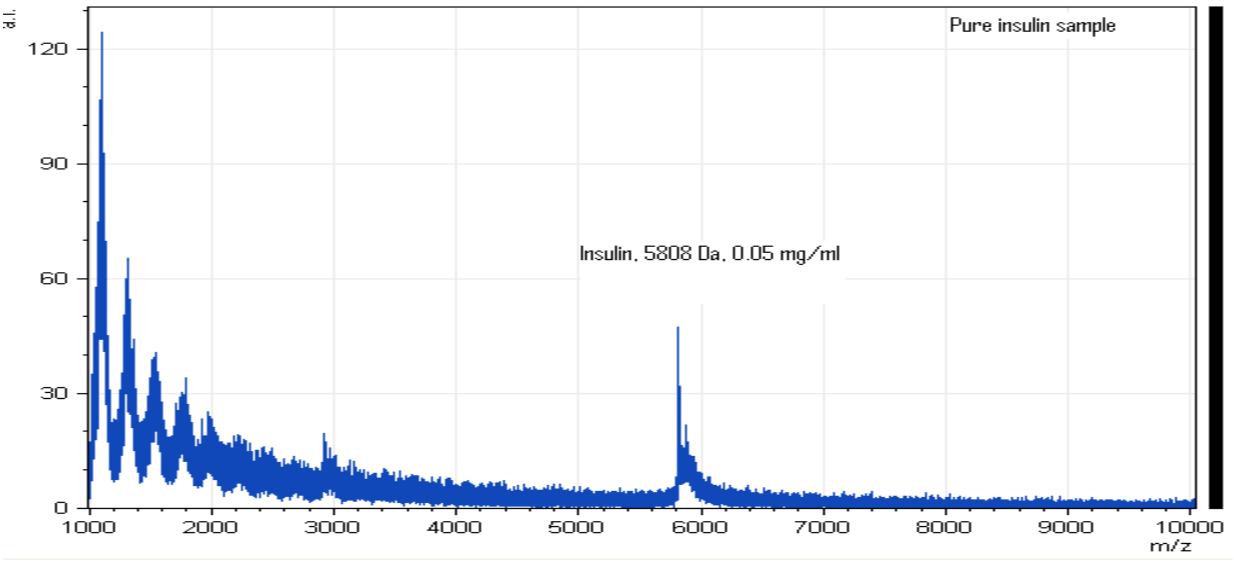

Fig. 29 shows the insulin peak at 5808 Da.

Insulin was also identified in case of using nanoparticles with silver (insulin + nanoparticles) instead of using MOPS, see figure 30.

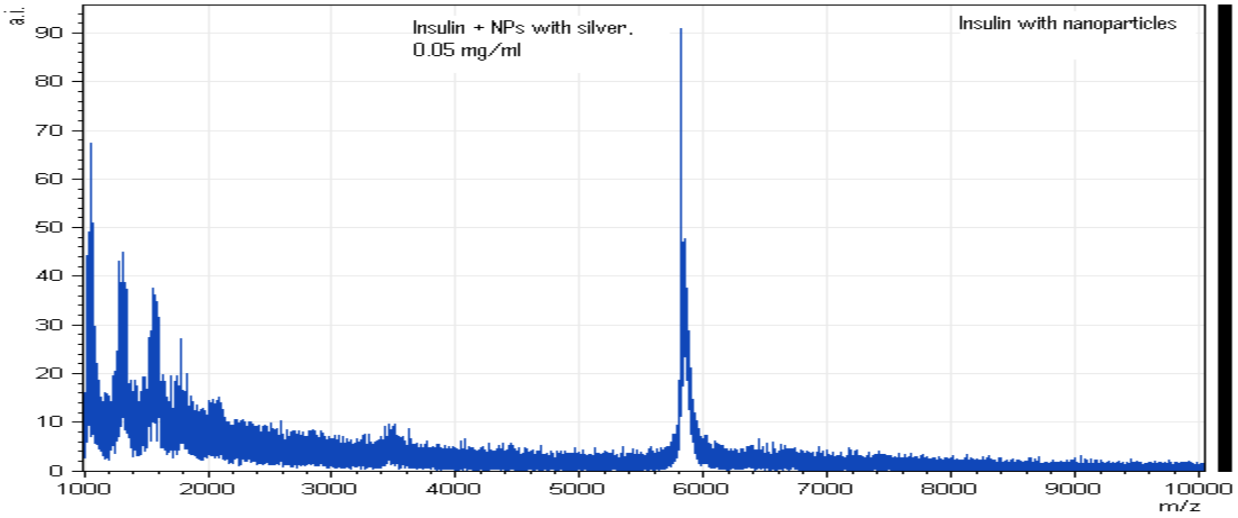

Fig. 30 shows the insulin peak at 5808 Da.

Insulin was identified in case of using nanoparticles without silver instead of using MOPS, see figure 31.

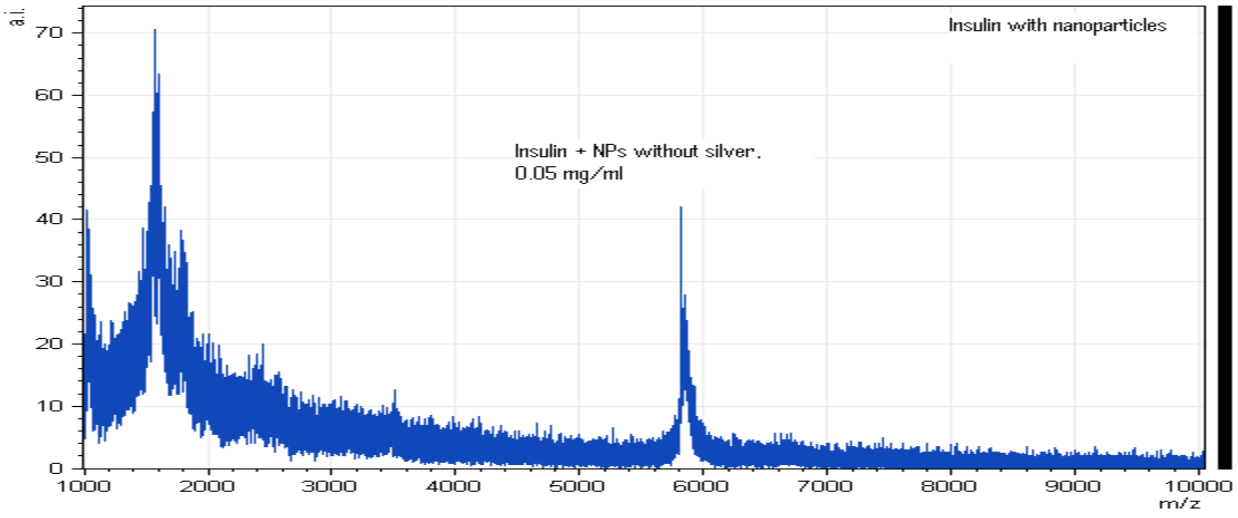

Fig. 31 shows the insulin peak at 5808 Da.

Figures 30 and 31 show that nanoparticles with and without silver can be used as a PSP due to the insulin identification in both cases. MALDI-TOF/MS works well with lipid nanoparticles.

Conclusion

Lipid nanoparticles with or without silver can be used as a pseudostationary phase. Different proteins with different hydrophobicities and sizes have been separated such as insulin, cytochrome c and BSA. PSP-CEC in combination with UV-detection is a good method to elute insulin. MADI-TOF/MS works well with lipid nanoparticles and it can be used as a future possible detection method for the outcome PSP-CEC. PSP-CEC with UV-detection cannot separate a mixture of proteins; they have been eluted in one peak due to too few nanoparticles, i.e. too little surface area of NPs.

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