

Fabrication of a molecularly imprinted monolithic column via the epitope approach for the selective capillary microextraction of neuropeptides in human plasma

LEI, Xiaoyun; Huang, Ting; Wu, Xiaoping; Mangelings, Debby; Van Eeckhaut, Ann; Bongaerts, Jana; Terryn, Herman; Vander Heyden, Yvan

Published in:
Talanta

DOI:
[10.1016/j.talanta.2022.123397](https://doi.org/10.1016/j.talanta.2022.123397)

Publication date:
2022

Document Version:
Accepted author manuscript

[Link to publication](#)

Citation for published version (APA):

LEI, X., Huang, T., Wu, X., Mangelings, D., Van Eeckhaut, A., Bongaerts, J., Terryn, H., & Vander Heyden, Y. (2022). Fabrication of a molecularly imprinted monolithic column via the epitope approach for the selective capillary microextraction of neuropeptides in human plasma. *Talanta*, 243, [123397].
<https://doi.org/10.1016/j.talanta.2022.123397>

Copyright

No part of this publication may be reproduced or transmitted in any form, without the prior written permission of the author(s) or other rights holders to whom publication rights have been transferred, unless permitted by a license attached to the publication (a Creative Commons license or other), or unless exceptions to copyright law apply.

Take down policy

If you believe that this document infringes your copyright or other rights, please contact openaccess@vub.be, with details of the nature of the infringement. We will investigate the claim and if justified, we will take the appropriate steps.

Fabrication of a molecularly imprinted monolithic column via the epitope approach for the selective capillary microextraction of neuropeptides in human plasma

Xiaoyun Lei ^{a, b}, Ting Huang ^a, Xiaoping Wu ^{a,*}, Debby Mangelings ^b,
Ann Van Eeckhaut ^c, Jana Bongaerts ^{b,c}, Herman Terryn ^d, Yvan Vander
Heyden ^{b,*}

*^a Key Laboratory for Analytical Science of Food Safety and Biology; College of Chemistry,
Fuzhou University, Fuzhou 350116, China*

*^b Department of Analytical Chemistry, Applied Chemometrics and Molecular Modelling, Vrije
Universiteit Brussel – VUB, Brussels, Belgium*

*^c Department of Pharmaceutical Chemistry, Drug Analysis and Drug Information, Center for
Neurosciences, Vrije Universiteit Brussel – VUB, Brussels, Belgium*

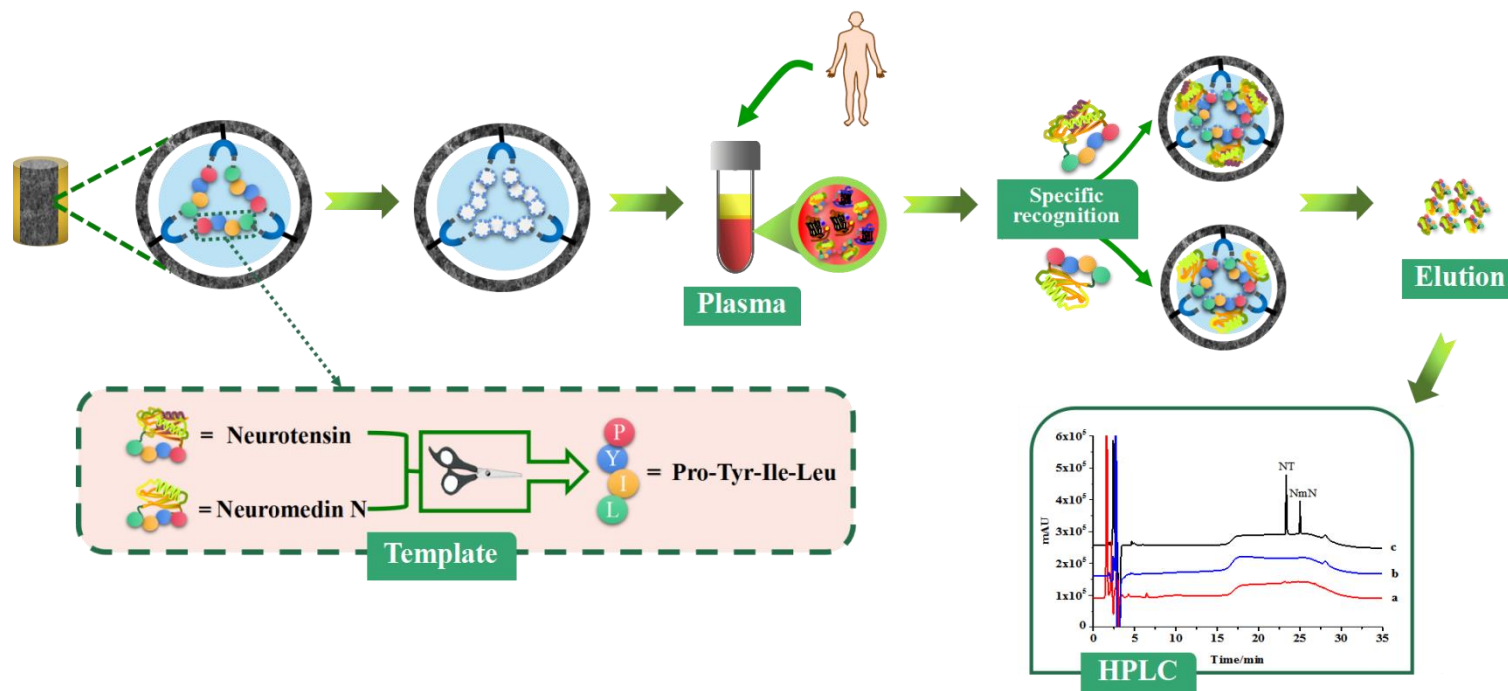
*^d Department Materials and Chemistry, Research group Electrochemical and Surface Engineering,
Vrije Universiteit Brussel – VUB, Brussels, Belgium*

***Corresponding authors**

X. Wu: wapple@fzu.edu.cn; Tel&Fax: +86-591-22866131

Y. Vander Heyden: Yvan.Vander.Heyden@vub.be; Tel: +32-2-477-47-34

Graphical Abstracts



Highlights

- Molecularly imprinted monolith (MIM) synthesized via epitope approach with dummy template.
- Neurotensin (NT) and neuromedin N (NmN) are targeted.
- A selective capillary microextraction (CME) technique for NT and NmN is developed.
- The optimal synthesized monolithic column showed high affinity and selectivity for the neuropeptides.
- A MIM-CME/HPLC-UV method for neuropeptides analysis in human plasma.

Abstract

A novel molecularly imprinted monolithic (MIM) column was designed and fabricated using the epitope approach, and was used for the selective capillary microextraction (CME) of the neuropeptides neurotensin (NT) and neuromedin N (NmN). The MIMs were synthesized in a capillary by thermally initiated polymerization of the functional monomer (methacrylic acid (MAA)), in the presence of a dummy template (Pro-Tyr-Ile-Leu (PYIL)), a crosslinker and porogens. The resulting monoliths were characterized by scanning electron microscopy, pore size distribution measurement, and Fourier transform infrared spectroscopy. Different synthesis conditions of the MIM column were investigated. The parameters affecting the MIM-CME performance, including loading, washing and elution protocols, were optimized as well. The MIMs were used to enrich NT and NmN from human plasma prior to HPLC-UV analysis. The imprinted monolith showed an excellent maximum adsorption capacity of 245-711 mg mL⁻¹ and selectivity (imprinting factor of 5.7–13.4) towards its target peptides. Low detection limits of 0.62 and 1.20 nM, and satisfactory recoveries (82.5-98.8%) were obtained for NT and NmN, respectively. The proposed MIM-CME/HPLC-UV method was found suitable to be used as an effective tool for the highly efficient and specific analysis of NT and NmN in human plasma samples.

Keywords:

Neuropeptides; Molecularly imprinted monolithic column; Capillary microextraction; Epitope approach

1. Introduction

Neuropeptides belong to one of the most widespread signaling molecules, which are distributed in the central and peripheral nervous system, and play a significant role in neurotransmitter modulation and hormone regulation [1]. Neurotensin (NT) behaves as a neurotransmitter or neuromodulator in the central nervous system (CNS) and as a hormone in the gastrointestinal tract, thus it is expressed both in the peripheral and central nervous system [2]. Over the years, different roles have already been described for neurotensin in body temperature regulation, feeding, analgesia, ethanol sensitivity, psychosis, substance use, and pain [3]. Due to its association with such a wide variety of neurotransmitters, NT has been implicated in the pathophysiology of major mental disorders such as schizophrenia, drug abuse, and autism [4]. NT and the NT-related peptide neuromedin N (NmN) are synthesized from a common precursor peptide and contain the same four amino acid sequences in the C-terminal [5]. High concentrations from immunoreactive NT and NmN expressions were also found to be associated with many types of solid tumors, for example, gastric cancers, chondrosarcoma and human neuroendocrine pancreatic tumor [6]. Therefore, the simultaneous monitoring of NT and NmN concentrations in body fluids is important

for clinical diagnostics and the treatment of both central and peripheral nervous system disorders [7,8].

Because of their low content and the complexity of the matrices, quantification of neuropeptides in biological samples is still challenging. NT-related neuropeptides have been detected frequently by immuno-based techniques, such as the radioimmunoassay (RIA). Although the technique provides high sensitivity, it suffers from the obvious disadvantages of the need for high-cost antibodies, high cross-reactivity and the difficulty of simultaneous monitoring [9-11]. In recent years, several separation techniques have been utilized for the simultaneous determination of NT-related neuropeptides in biological matrices, including capillary zone electrophoresis (CZE) [12], liquid chromatography - mass spectrometry (LC-MS) [13-15], and electrospray ionization multistage mass spectrometry (ESI-MSⁿ) [16-19]. However, direct injections of bio-fluids in these systems, without any sample pretreatment, are usually impossible due to the presence of proteins in biofluids that can block the chromatographic column. In addition, the very low concentration levels, possible matrix effects and the presence of structurally-related endogenous substances further complicate the analysis of these neuropeptides. Therefore, a suitable sample pretreatment procedure that allows to selectively capture and preconcentrate the target neuropeptides prior to chromatographic or MS analysis would be highly valuable.

Molecularly imprinted polymers (MIPs) are widely recognized as artificial receptors since they possess a typical recognition specificity and structure

predictability, reminiscent of basic functions of natural receptors. As a result, MIPs have gained popularity for versatile applications in solid phase extraction (SPE) and chemical sensing, displaying high affinity, selectivity and loading ability to target small molecules in complex matrices [20, 21]. Traditional MIPs are synthesized by tedious processes which consist of bulk polymerization, followed by grinding and sieving. As an efficient alternative to MIPs, molecularly imprinted monolithic (MIM) columns, which can easily be prepared in-situ in micro-columns or capillaries, have gained wide interest in recent years [22]. Owing to their continuous unitary porous structure and low back pressure, MIMs offer fast mass transport, regular and adjustable bulk shapes and low consumption of template and solvents for sample pretreatment [23,24]. Although the MIMs have become a convenient tool for the enrichment of small molecules, the molecular imprinting of larger molecules, such as proteins and peptides, is difficult because of the lack of stable and conformational matching templates, and an inefficient mass transfer [25-28]. The epitope approach proposed by Rachkov and Minoura [29] emerged as a promising strategy to overcome these limits, and was adopted to capture peptides, including angiotensin II, enkephalins, phosphopeptides, cardiac troponin I, tyrosine-phosphorylated peptides and acetylated lysine [27,30]. In this approach, an accessible short peptide fragment of the target macromolecule is employed as template. The resulting imprinted polymer is able to recognize the amino acid sequence of both the template and the homologous polypeptides, owing to the specific cavities for the epitope [31].

This work aims to develop a MIM-based capillary microextraction (CME) method for the highly selective recognition of NT and NmN in complex biological samples. A novel MIM column to extract NT-related neuropeptides was designed and prepared in a capillary through the epitope approach. A tetrapeptide Pro-Tyr-Ile-Leu (PYIL) was employed as dummy template for the in-situ copolymerization of a MIM, as shown in Fig.1. The recognition experiment was conducted with targets and its analogues, neuromedin U (NmU) and neuromedin B (NmB), to study the specific selectivity of the MIM column. Subsequently, the obtained MIM column was used in CME to enrich NT and NmN from human plasma prior to HPLC-UV analysis. The parameters affecting the monolithic preparation, extraction and separation were optimized. The specific enriching performance, including binding equilibrium and selectivity, are discussed.

2. Experimental

2.1 Chemicals and Materials

Porcine neurotensin (NT), neuromedin N (NmN) and neuromedin U (NmU) were obtained from the Polypeptide Group (Strasbourg, France). Neuromedin B (NmB) was purchased from Bachem (Bubendorf, Switzerland). Template peptide PYIL (Pro-Tyr-Ile-Leu) was purchased from GL Biochem (Shanghai, China). Methacrylic acid (MAA) and 2,2'-azobisisobutyronitrile (AIBN) were supplied by Acros Organics (Geel, Belgium). Ethylene glycol dimethacrylate (EDMA), formic acid, 3-(trimethoxysilyl) propyl methacrylate (γ -MAPS), acrylamide (AM) and sodium

acetate trihydrate were purchased from Merck (Overijse, Belgium). Acrylic acid (AA) and 4-vinylpyridine (4-VP) were obtained from Alfa Aesar (Kandel, Germany). Glacial acetic acid (HAc), isooctane, HPLC-grade acetonitrile (ACN) and methanol (MeOH) were purchased from Fisher Chemicals (Leicestershire, United Kingdom). All solutions were prepared with ultrapure water from an in-house Sartorius Stedim Biotech Atrium® system (Göttingen, Germany). Bare fused-silica capillaries (530 μm i.d. \times 690 μm o.d.) were purchased from Yongnian Optic Fiber Plant (Hebei, China).

The neuropeptides stock solutions of NmN, NT and NmB were prepared by dissolving 1.0 mg lyophilized peptide in 1000 μL water/acetonitrile/formic acid (45:50:5 v/v/v), and further a consecutive 1:1 dilution in ultrapure water. For NmU, 1.0 mg of lyophilized peptide was dissolved in 1261 μL water/acetonitrile (80:20 v/v) to obtain the stock solution. Stock solutions were aliquoted in 500 μL Eppendorf tubes and stored at -20°C . Prior to use, peptide stock solutions were thawed at room temperature and vortexed [1]. Working solutions of standard peptides were prepared by diluting the stock solutions to different concentrations with 50 mM acetate loading solvent.

2.2 Materials characterization

The surface morphology and structure of the monolithic columns were examined by a JEOL JSM-IT300 scanning electron microscope (SEM) (JEOL, Tokyo, Japan) and a Thermo Nicolet 6700 Fourier transform infrared spectrometer (FT-IR) (Thermo Fisher, Waltham, Massachusetts, USA). The Brunauer–Emmett–Teller (BET) was

applied to measure the specific surface area, while the pore-size distribution of the polymers was delineated using the Barrett-Joyner-Halenda (BJH) model, which are determined by a Micromeritics ASAP 2460 nitrogen adsorption apparatus (Micromeritics, Atlanta, Georgia, USA).

2.3 Preparation of molecularly imprinted capillary monolithic column

The inner surface of the bare fused-silica capillary was first pretreated using previously described procedures [32]. In brief, the inner walls of the fused silica capillaries were silane-activated by rinsing with 1.0 mol L⁻¹ HCl, water, 1.0 mol L⁻¹ NaOH for 30 min each. Then the capillary is pretreated with γ -MAPS/methanol (1:1, v/v) solution at 60°C for 16 h to immobilize a layer of vinyl groups for anchoring the monolithic column matrix. The resulting capillary was then rinsed with methanol and dried under a stream of nitrogen at 70°C for 3 h before use. Subsequently, a homogenized pre-polymerization mixture, containing 12.0 mg functional monomer and dummy template, in which the weight ratio between PYIL and MAA is 1 to 2, was dissolved in a centrifuge tube in 414.0 mg ternary porogenic solvent. The latter consisted of 250.0 mg methanol, 150.0 mg acetonitrile and 14.0 mg isooctane. After 20 min of ultrasonication, 74.0 mg cross-linker EDMA and 1 wt% initiator AIBN were added to the above mixture. Subsequently, the mixture was degassed by ultrasonication for 15 min and introduced into a silanized capillary with 10 cm length. Both capillary ends were sealed with a rubber septum before polymerization. The polymerization reaction was performed in a water bath at 60°C for 16 h. Finally, the

resulting molecularly imprinted monolithic column (C5) was rinsed with methanol for 30 min and MeOH/H₂O (50/50, v/v) for 2 h to remove the unreacted reagents and template. The non-imprinted monolith (NIM) column was also synthesized under the same conditions as the PYIL one, but in the absence of the template.

2.4 MIM-CME procedure

All solutions were loaded into a syringe and delivered by a syringe infusion pump (KD Scientific, Holliston, Massachusetts, USA). Prior to each CME run, a 2 cm length of the monolithic column was cut off and they were preconditioned with 1.0 mL methanol and 1.0 mL water, both containing 0.1% acetic acid, at a flow rate of 70.0 $\mu\text{L min}^{-1}$. Then 3 mL sample solution, prepared in 50 mmol L⁻¹ acetate solution (pH 6.5), was loaded on the monolith at a constant flow rate of 50.0 $\mu\text{L min}^{-1}$, followed with a rinse for 30 min with 50% (v/v) of methanol/water to elute interferences. Finally, the target neuropeptides were eluted at 70.0 $\mu\text{L min}^{-1}$ with 20 μL MeOH/H₂O (80:20, v/v), containing 0.1% acetic acid, and the eluent was collected for further HPLC-UV analysis.

2.5 HPLC analysis

The chromatographic analysis was performed on a Shimadzu SPD-M20A HPLC system (Shimadzu, Kyoto, Japan), which was equipped with two LC-20AD pumps, a SIL-20A autosampler, an SPD-M20A diode array detector and a CTO-20AC column oven. A CortecsTM C18+ column (150 \times 2.1 mm i.d., 2.7 μm) obtained from Waters (Zellik, Belgium) was utilized at 30°C to separate the neuropeptides. For sample

injection, 5.0 μL peptides solution was injected into the HPLC system and eluted at a flow rate of 130 $\mu\text{L}/\text{min}$. A wavelength of 210 nm was set for UV detection. Data were collected and processed by the LC solution chromatographic workstation system (Shimadzu).

Mobile phase A, used for HPLC gradient elution, consisted of water/methanol (90:10, v/v) with 0.1% acetic acid (v/v). Mobile phase B was a mixture of methanol/water (90:10, v/v) containing 0.1% acetic acid (HAc). Peptides were separated with the following gradient: first, a 2 min step is performed with 100 % mobile phase A, followed by a gradient up to 80% B in 13 min. Then a return to 0 % B was performed in 2 min, followed by a re-equilibration for 13 min at 100% A. The total run time was 30 min.

2.6 Evaluation of the enrichment performance of the MIM and NIM columns

The enrichment factor (EF), to assess the enrichment abilities of the MIM columns, was calculated according to equation (1). In Eq. (1), C_t (μM) is the final concentration of neuropeptides in the eluted solution, estimated with the calibration curve, and C_o (μM) the initial analyte concentration in the loading solution.

$$EF = C_t/C_o \quad (1)$$

The dynamic binding capacities (Q) of the MIMs and NIMs were determined using frontal elution. 5.0 mM NT and NmN were prepared with 50 mmol L^{-1} acetate solution (pH 6.5) and pumped through the monolithic extraction column at a flow rate

of 10.0 $\mu\text{L min}^{-1}$. The eluting solution was detected in the HPLC system at 210 nm. The Q values ($\mu\text{g mL}^{-1}$) of the NIM and MIM columns, for the neuropeptides were calculated using equation (2) [33]. The imprinting factor (IF) to evaluate the specificity of the MIM column was defined and calculated according to equation (3).

$$Q = ct_v/V \quad (2)$$

$$IF = Q_{MIM}/Q_{NIM} \quad (3)$$

Here, c ($\mu\text{g mL}^{-1}$) is the neuropeptide concentration, t is the break time pointed to the middle of the steep slope (min), v is the flow rate (mL min^{-1}) and V is the volume of the monolithic column (mL). Q_{MIM} and Q_{NIM} are the dynamic binding capacities of neuropeptides extracted on the MIM and NIM columns, respectively.

2.7 Sample preparation

Human plasma samples were provided by The Red Cross (Brussels, Belgium) and stored at -20°C . A series of spiked samples was prepared by adding neuropeptides working solutions to the plasma, and the obtained final concentrations of NT and NmN in plasma samples were 0.05, 0.50, and 5.00 μM , respectively. After filtration with a 0.22 μm Nylon membrane filter, the sample solutions were loaded onto the MIM columns, then eluted and analyzed by the MIM-CME procedure as described in section 2.4.

3. Results and discussion

3.1 Fabrication of MIM column for the neuropeptides

A self-designed tetrapeptide PYIL, comprising the four-amino-acid C-terminal sequence of NT and NmN, was selected as the dummy template for the epitope imprinting (Fig.1; Table 1 for AA sequences of NT and NmN). PYIL was interacting by electrostatic forces and hydrogen bondings with the functional monomers at the polymer matrix, which is synthesized via in-situ polymerization at 60 °C. The template molecules were removed using a washing solvent (50/50 (v/v) methanol/water) from the specific recognition sites, which were generated during the polymerization. The prepared MIM column should exhibit affinity and specificity for the neuropeptides with the imprinted amino acid sequences, owing to the presence of multiple interaction sites and the complementary space to the targets [34].

A suitable functional monomer plays an essential role in forming a specific donor-receptor complex between the MIM and the template. Four typical functional monomers, i.e., MAA, 4-VP, AM and AA, were selected to prepare different MIM columns in the same way as described in section 2.3. The enrichment factors (EFs) of NT and NmN on the corresponding MIMs are presented in Table 2. The highest EF was obtained on the M1 column, suggesting that MAA, as a versatile functional monomer, enhances the imprinting effect by dimerization in the systems [35,36] as well as the various interactions with the template [37]. In addition, MAA has been proved to have bonded with relatively few rotational degrees of freedom and the ability to interact with templates in various ways, for instance, as H-bond donors, H-bond acceptors, through formal ion-pair formation, and weaker dipole-dipole

interactions [38]. The pore volume of the NIM polymer was 0.13 cm³g and it had a specific surface area of 56.45 m²g⁻¹. However, a larger pore volume, 0.16 cm³g, and a higher surface area, 76.15 m² g⁻¹, were obtained in the MIM polymer material (shown in Fig. S1) when using MAA as functional monomer. This may provide more accessible cavities and binding sites for target analytes on the MIM column [39].

The ratio of the template to the number of monomers was optimized to acquire a MIM column with high selectivity and stability. As shown in Table 3, the EFs of NT and NmN increased when the molar ratio of PYIL to MAA was changed from 1:23 (C2) to 1:12 (C5), while a decrease is obtained when further increasing the molar ratio to 1:8 (C8) or 1:6 (C9). This phenomenon indicates that abundant imprinted sites on the column are formed by increasing the fraction of template molecules, but a too high template content results in a low yield of specific binding sites due to inadequate complexation. Therefore, a template to monomer ratio of 1:12 was selected (C5, same composition as M1) for further investigations.

In order to investigate the influence of cross-linker on the polymers, three different molar ratios of MAA to EDMA from 1:3.5 to 1:4.5 were tested. The EFs results from C3, C5 and C7 in Table 3 indicate that the number of specific binding sites increased with an intermediate amount of cross-linker. A higher content of crosslinker EDMA (C7), probably inhibiting the access of the template, resulted in a low permeability (hard to rinse the column) and a decreased enrichment factor for NT.

The formulation of porogens is crucial for controlling pore size, permeability and

stability in preparing MIM columns. In this work, methanol was selected as the polymerization solvent because of the good solubility of the template, while acetonitrile and isooctane were used as the less polar organic solvents to improve the imprinting efficiency. Various weight ratios of MeOH/ACN (5/3, w/w) to isooctane were investigated. The results revealed that C5 column with a weight ratio of 1.67 to 3.4 showed a higher enrichment factor for NT and NmN than that obtained from C4 (1.35/3.4) and C6 (2.33/3.4). Taking into account all results, the C5 column was finally selected for the subsequent experiments.

3.2 Characterization of MIMs

The scanning electron microscopy (SEM) images of the optimized MIM column (C5) are shown in Fig. 2. As illustrated in Figs 2A and 2B, the MIM column possesses a uniform porous morphological structure and a polymer matrix that is well anchored to the inner wall of the capillary. The pore diameter of the synthesized MIM was around 5 μm (Figs 2C and 2D), which accounts for a high permeability and a decreased mass-transfer resistance during extraction.

The FT-IR spectra of leached MIM and of NIM were recorded (Fig. 3) to identify the functional groups present at the surface of the polymers. As shown in Figs 3a and 3b, the MIM has similar absorption bands to the NIM, indicating that the PYIL template was completely removed from the polymer during the extraction process. The strong characteristic peak at 1148 cm^{-1} was assigned to the C–O stretching vibration in the ester of EDMA, while peaks at 1248 cm^{-1} or 1249 cm^{-1} were from the

stretching vibration of C–O in a carboxylic acid of MAA. The stretching vibrations of saturated alkyl group were observed around 2958 cm^{-1} . Fig. 3c shows a sharp intensity band of stretching vibrations of $\text{C}=\text{C}$ (1626 cm^{-1}), while it almost completely disappeared in Figs 3a and 3b. All results indicated that both MIM and NIM columns were successfully synthesized.

The pore-size distribution and specific surface area of monoliths were characterized by BJH and BET analysis. As shown in Fig. S1, the MIM and NIM have a similar type of IV isotherm of nitrogen physisorption, indicating that the presence of the template did not exert influence on the skeleton structure of the monolithic polymers. The surface area of the MIM was $76.15\text{ m}^2\text{ g}^{-1}$, which is somewhat larger than that of the NIM ($56.45\text{ m}^2\text{ g}^{-1}$). Moreover, the average pore diameters from the MIM and NIM were 9.0 nm and 8.4 nm, respectively. These parameters demonstrated that more mesopores (between 2 and 50 nm) could be obtained after removal of the template on the MIM, leaving more rough cavities and binding sites for target analytes. As a result, the specific interaction between targets and imprinted absorbent can be further increased during CME.

3.3 Evaluation of the adsorption capacity and selectivity of the MIM

The adsorption capacity is one of the most important properties that represents the loadability of the column. The MIM and NIM columns presented satisfying dynamic binding capacity (Q) to the neuropeptides, as shown in Fig. 4. The loading of both analytes on the monoliths increased rapidly at a certain range and then reached an

adsorption equilibrium. Meanwhile, the breakthrough times of NT and NmN could also be clearly observed. According to Eq. (2), the values of Q for NT and NmN on the MIM column were 711.3 and 245.3 mg mL⁻¹, and their corresponding values on the NIM column were 284.5 and 84.6 mg mL⁻¹, respectively. The higher adsorption capability of the neuropeptides on the MIM suggested that the binding sites on this column facilitated analyte diffusion into the imprinting cavities. The weak adsorption on the NIM column results from non-specific interactions with the polymer matrix. Moreover, the adsorption amounts of targets on the NIM column, over the whole concentration range, were always lower than those on the MIM column, which further confirmed the existence of high-amount and specific-recognition binding sites on the MIM column.

To validate the selectivity of the MIM to target neuropeptides, a mixture of four structural analogues, i.e., NT, NmN, NmU and NmB, was prepared at a concentration of 1.0 μ M. The EFs and IFs of the MIM for these peptides are shown in Table 1, and the EF values of NIM were determined for comparison. The IFs for targets NT and NmN were much higher than those for NmU and NmB, because the MIM column possesses specific cavities which matched better the spatial arrangement of NT and NmN. Fig. 5 showed the chromatographic behavior of the four peptides before and after extraction using the NIM and MIM columns. The peak heights of NT and NmN on MIM are higher than that on NIM, indicating the existence of specific three-dimensional cavities on the MIM that results in extraction and enrichment of the

neuropeptides. Due to the non-specific adsorption that dominates on the NIM column, similar EFs and peak heights for the four neuropeptides were obtained (Fig. 5b). These results indicate that the MIM column possesses specific imprinted sites and may act as an artificial receptor to enrich targets from complex sample matrices.

3.4 Optimization of MIM-CME conditions

As previously mentioned, the C5 column was selected as the optimal to investigate the CME conditions for NT and NmN prior to HPLC analysis. Several extraction parameters, i.e., pH of loading solvent, type of washing solvent and of elution solvent were studied.

3.4.1 Effect of loading solvent

Generally, the loading solvent is one of the primary factors to provide a suitable environment for the recognition of molecularly imprinted polymers towards analytes at low concentrations. In this work, polar solvents, such as ACN and MeOH, were first considered as loading solvents, but the results showed a low response due to their disruption of the recognition ability of the MIM [40]. To avoid this problem, a 50 mmol L⁻¹ acetate solution was applied as loading solvent to extract peptide through the MIM and NIM columns.

The pH of the loading solvent plays a vital role in the MIM-CME procedure since it has a large influence on the specific interactions between adsorbents and targeted molecules [41]. As shown in Fig. 6a, a relatively lower extraction efficiency

(represented by peak area) was obtained at pH values below 6.5 (Fig. 6A). This is probably because the deprotonation of carboxyl group on the MIM column increases when raising the pH, while the targets (pI NT = 7.09 and pI NmN = 9.06) are positively charged [42]. A relatively strong electrostatic interaction could contribute to increase the extraction efficiency of NT and NmN. Moreover, when pH is higher than 6.5, the carboxyl group is completely anionic, but NmN and NT are close to their zwitter-ionic form, thus the electrostatic interaction could again decrease and lead to a lower extraction efficiency. Therefore, pH 6.5 was selected as optimal, where a stronger electrostatic interaction between the analytes and the polymer is present and a higher extraction efficiency obtained.

3.4.2 Effect of washing solvent

The washing process is a crucial step to elute interferences and to reduce the nonspecific interaction with binding sites at the molecularly imprinted polymer. The effect of the washing solvent was monitored by using different solvents or their mixtures. Fig. 6b shows that the enrichment performance for both targets decreased when using ACN instead of MeOH or MeOH/H₂O (1/1). In addition, using acidified acetonitrile, containing 0.1% acetic acid, leads to the lowest extraction efficiency. This is probably caused by an increase of the peptide solubility in the washing solvent due the presence of the acid, which leads to competition with the selective binding retained by hydrogen bonding in the imprinted sites on the polymer. Thus, 1/1 of methanol/water (v/v) was selected as the washing solvent to maximally maintain

the interaction between the binding sites and target analytes.

3.4.3 Effect of elution solvent

Fig.6c shows the effect of different elution solvents on the extraction efficiency (represented by the peak area) of MIM. The use of a mixture of methanol/water (8/2, v/v) resulted in much higher peak areas than acetonitrile/water (8/2, v/v). Comparison to methanol/water (1/1, v/v) and methanol/water (1/1, v/v) + 0.1% acetic acid showed that increasing the fraction of MeOH in the mixture resulted in a further enhancement of the extraction efficiency, which may be due to the more efficient elution of analytes via a higher solvent strength of the elution solvent. The addition of 0.1% acetic acid to the mixture methanol/water (8/2, v/v) offered the highest elution efficiency, indicating that acetic acid affects the strong hydrogen bonding between the imprinted polymer and the targets, and thus further facilitated the elution of the neuropeptides. Finally, a mixture methanol/water (8/2, v/v) containing 0.1% acetic acid was chosen as the elution solvent for subsequent tests.

3.5 Method validation

The MIM column was utilized for the CME of NT and NmN prior to HPLC-UV analysis. Under the optimized extraction and chromatographic conditions, the calibration curves showed a good linear relationship in the ranges 0.01-10 μM for NT and 0.02-15 μM for NmN, respectively (Table 4). The limits of detection (LODs, S/N=3) for NT and NmN were 0.62 nM and 1.20 nM, while the limits of quantification (LOQs, S/N=10) reached 2.06 nM and 4.00 nM, respectively. The

RSDs of intra-day precision analysis were 3.7% and 3.5% for 0.5 μ M NT and NmN, while the RSDs for inter-day precision on the same concentration were 4.6% and 5.4%, respectively. Fig. S2 shows the chromatograms of NT and NmN standard solution without enrichment (trace a), and after enrichment using the MIM column (trace c). The results obviously indicate increases in the peak areas of both targets using the proposed MIM-CME-HPLC method, demonstrating an excellent imprinting capability towards NT and NmN.

3.6 Analysis of neuropeptides in human plasma by MIM-CME/HPLC method

It is still a challenge to accurately quantify the neuropeptides at low concentration in plasma. For this purpose, the MIM column was employed to extract NT and NmN from human plasma before HPLC-UV analysis. Fig. 7 shows the chromatograms of a standard solution, a blank plasma sample and a plasma sample spiked with 1.0 μ M NT and 3.0 μ M NmN), all three enriched by the MIM column. It can be observed that the peak areas of the neuropeptides were highly enlarged after enrichment via MIM-CME, indicating specific recognition of NT and NmN on the MIM polymer. Intra-day and inter-day recoveries of three concentration levels of NT and NmN in spiked plasma samples were in the ranges 89.2-99.8% and 82.5-97.3%, respectively, as shown in Table 5. The repeatability of the method was expressed as RSD (n=3), and values below 5.8% and 5.9% for NT and NmN, respectively were obtained. The results imply that the MIM-based CME method can successfully capture the target peptides and eliminate other interfering species from human plasma sample.

4. Conclusion

This work presents the preparation and application of a new molecularly imprinted monolithic column for the specific enrichment of neuropeptides. The MIM column was fabricated in a capillary by the epitope approach and allowed to be directly connected to a syringe to perform the CME procedure. The morphological characterizations indicated the presence of a high number of mesopores and macropores on the MIM column, which contributed to a higher adsorption capacity. Meanwhile, the generation of three-dimensional imprinted sites that are complementary to the template provide target selectivity to the MIM column. At the optimized conditions, the MIM-CME method was employed to couple with HPLC-UV analysis for quantifying NT and NmN in human plasma samples. Satisfying recoveries, repeatability and low LODs were achieved, demonstrating the feasibility of the MIM-CME method for application in complex biological sample analysis.

Author contributions

Xiaoyun Lei: Methodology, Formal analysis and Investigation, Writing - original draft; Ting Huang: Methodology, Validation; Xiaoping Wu: Supervision, Project administration, Resources, Conceptualization, Funding acquisition, Writing - review & editing; Debby Mangelings: Project administration, Supervision, Writing - review & editing; Ann Van Eeckhaut: Supervision, Writing - review & editing; Jana Bongaerts: Validation, Writing - review & editing; Herman Terryn: Software, Test;

Yvan Vander Heyden: Supervision, Project administration, Resources, Writing - review & editing.

Conflicts of interest

There are no conflicts of interest to declare.

Acknowledgements

The authors are grateful for financial support from the National Natural Science Foundation (Grant No. 22076024), and the Major Project of Science and Technology of Fujian Province (Grant No. 2018YZ0002-1) of China.

References

- [1] Y. Van Wanseele, J. Viaene, L. Van Den Borre, K. Dewachter, Y. Vander Heyden, I. Smolders, A. Van Eeckhaut, LC-method development for the quantification of neuromedin-like peptides. Emphasis on column choice and mobile phase composition, J. Pharm. Biomed. Anal. 137 (2017) 104-112, <https://doi.org/10.1016/j.jpba.2017.01.014>.
- [2] Z. Wu, D. Martinez-Fong, J. Trédaniel, P. Forgez, Neurotensin and its high affinity receptor 1 as a potential pharmacological target in cancer therapy, Front. Endocrinol. 3 (2012) 184, 10.3389/fendo.2012.00184.
- [3] M.L. Torruella-Suárez, Z.A. Mcelligott, Neurotensin in reward processes. Neuropharmacology 167 (2020) 108005, <https://doi.org/10.1016/j.neuropharm.2020.108005>.
- [4] M. Boules, P. Fredrickson, A. Muehlmann, E. Richelson, Behav. Sci. 4 (2014) 125-153, <https://doi.org/10.3390/bs4020125>.
- [5] P. Kitabgi, Neurotensin and neuromedin N are differentially processed from a common precursor by prohormone convertases in tissues and cell lines, Results Probl. Cell. Differ. 50 (2010) 85-96, https://doi.org/10.1007/400_2009_27.
- [6] K. Galoian, P. Patel, Epigenetic control of cancer by neuropeptides, Biomed Rep. 6 (2017) 3-7, <https://doi.org/10.3892/br.2016.804>.
- [7] D. Hoyer, T. Bartfai, Neuropeptides and neuropeptide receptors: drug targets, and

peptide and non-peptide ligands: a tribute to Prof. Dieter Seebach, *Chem. Biodivers.* 9 (2012) 2367-2387, <https://doi.org/10.1002/cbdv.201200288>.

[8] G. Heine, H.D. Zucht, M.U. Schuhmann, K. Bürger, H. Jurgens, M. Zumkeller, C.G. Schneekloth, H. Hampel, P. Schulz-Knappe, H. Selle, High-resolution peptide mapping of cerebrospinal fluid: a novel concept for diagnosis and research in central nervous system diseases, *J. Chromatogr. B.* 782 (2002) 353-361, [https://doi.org/10.1016/S1570-0232\(02\)00571-8](https://doi.org/10.1016/S1570-0232(02)00571-8).

[9] A. Van Eeckhaut, K. Maes, N. Aourz, I. Smolders, Y. Michotte, The absolute quantification of endogenous levels of brain neuropeptides in vivo using LC–MS/MS, *Bioanalysis*, 11 (2011) 1271-1285, <https://doi.org/10.4155/bio.11.91>.

[10] Z.R. Liu, M. Welin, B. Bragee, F. Nyberg, A high-recovery extraction procedure for quantitative analysis of Substance P and opioid peptides in human cerebrospinal fluid, *Peptides*, 21 (2000) 853-860, [https://doi.org/10.1016/S0196-9781\(00\)00219-9](https://doi.org/10.1016/S0196-9781(00)00219-9).

[11] M. Jaszberenyi, Z. Bagosi, K. Csabafi, M. Palotai, G. Telegdy, The actions of neuropeptide SF on the hypothalamic-pituitary-adrenal axis and behavior in rats. *Regul. Peptides*, 188 (2014) 46-51, <https://doi.org/10.1016/j.regpep.2013.11.004>.

[12] A.L. Freed, J.D. Cooper, M.I. Davies, S.M. Lunte, Investigation of the metabolism of Substance P in rat striatum by microdialysis sampling and capillary electrophoresis with laser-induced fluorescence detection, *J. Neurosci. Methods.* 109 (2001) 23-29, [https://doi.org/10.1016/S0165-0270\(01\)00397-1](https://doi.org/10.1016/S0165-0270(01)00397-1).

- [13] P. Song, O.S. Mabrouk, N.D. Hershey, R.T. Kennedy, In vivo neurochemical monitoring using benzoyl chloride derivatization and liquid chromatography–mass spectrometry, *Anal. Chem.* 84 (2012) 412–419, <https://doi.org/10.1021/ac202794q>.
- [14] Y. Zhou, J.M. Wong, O.S. Mabrouk, R.T. Kennedy, Reducing adsorption to improve recovery and in vivo detection of neuropeptides by microdialysis with LC–MS, *Anal. Chem.* 87 (2015) 9802–9809, <https://doi.org/10.1021/acs.analchem.5b02086>.
- [15] B.A. Sinnaeve, M.L. Storme, J.F. Van Bocxlaer, Capillary liquid chromatography and tandem mass spectrometry for the quantification of enkephalins in cerebrospinal fluid, *J. Sep. Sci.* 28 (2015) 1779–1784, <https://doi.org/10.1002/jssc.200500114>.
- [16] W.E. Haskins, Z.Q. Wang, C.J. Watson, R.R. Rostand, S.R. Witowski, D.H. Powell, R.T. Kennedy, Capillary LC-MS² at the attomole level for monitoring and discovering endogenous peptides in microdialysis samples collected in vivo, *Anal. Chem.* 73 (2001) 5005–5014, <https://doi.org/10.1021/ac010774d>.
- [17] K. Maes, G. Bechade, J. Van Schoors, Y. Van Wanseele, J. Van Liefferinge, Y. Michotte, S.N. Harden, E.E. Chambers, J. Claereboudt, I. Smolders, A. Van Eeckhaut, An ultrasensitive nano UHPLC–ESI–MS/MS method for the quantification of three neuromedin-like peptides in microdialysates, *Bioanalysis*, 7 (2015) 605–619, <https://doi.org/10.4155/bio.14.269>.
- [18] K. Lanckmans, B. Stragier, S. Sarre, I. Smolders, Y. Michotte, Nano-LC-MS/MS

for the monitoring of angiotensin IV in rat brain microdialysates: limitations and possibilities, J. Sep. Sci. 30 (2007) 2217-2224, <https://doi.org/10.1002/jssc.200700159>.

[19] N. Xu, C. Qiu, W. Wang, Y. Wang, C. Chai, Y. Yan, D. Zhu, HPLC/MS/MS for quantification of two types of neurotransmitters in rat brain and application: myocardial ischemia and protection of Sheng-Mai-San, J. Pharm. Biomed. Anal. 55 (2011) 101-108, <https://doi.org/10.1016/j.jpba.2010.12.015>.

[20] L. Chen, X. Wang, W. Lu, X. Wu, J. Li, Molecular imprinting: perspectives and applications, Chem. Soc. Rev. 45 (2016) 2137-2211, <https://doi.org/10.1039/C6CS00061D>.

[21] G. Becerra, F. Baez, S. Lucangioli, S. Flor, V. Tripodi, Miniaturized imprinted solid phase extraction to the selective analysis of Coenzyme Q10 in urine, J. Chromatogr. B. 1116 (2019) 24-29, <https://doi.org/10.1016/j.jchromb.2019.03.029>.

[22] A.G. Mayes, M.J. Whitcombe, Synthetic strategies for the generation of molecularly imprinted organic polymers, Adv. Drug Deliv. Rev. 57 (2005) 1742-1778, <https://doi.org/10.1016/j.addr.2005.07.011>.

[23] W. Zhang, Z. Chen, Preparation of micropipette tip-based molecularly imprinted monolith for selective micro-solid phase extraction of berberine in plasma and urine samples, Talanta, 103 (2013) 103-109, <https://doi.org/10.1016/j.talanta.2012.10.014>.

[24] T. Nema, E.C.Y. Chan, P.C. Ho, Applications of monolithic materials for sample

preparation, J. Pharm. Biomed. Anal. 87 (2014) 130-141,
<https://doi.org/10.1016/j.jpba.2013.05.036>.

[25] L. Liu, T. Zhong, Q. Xu, Y. Chen, Efficient molecular imprinting strategy for quantitative targeted proteomics of human transferrin receptor in depleted human serum, Anal. Chem. 87 (2015) 10910–10919,
<https://doi.org/10.1021/acs.analchem.5b02633>.

[26] C. Rossetti, A.A. Qader, T.G. Halvorsen, B. Sellergren, L. Reubsaet, Antibody-free biomarker determination: exploring molecularly imprinted polymers for pro-gastrin releasing peptide, Anal. Chem. 86 (2014) 12291-12298,
<https://doi.org/10.1021/ac503559c>.

[27] H. Li, D. Li, Preparation of a pipette tip-based molecularly imprinted solid-phase microextraction monolith by epitope approach and its application for determination of enkephalins in human cerebrospinal fluid, J. Pharm. Biomed. Anal. 115 (2015) 330-338, <https://doi.org/10.1016/j.jpba.2015.07.033>.

[28] L. Cenci, A. Anesi, M. Busato, G. Guella, A.M. Bossi, Molecularly imprinted polymers coupled to matrix assisted laser desorption ionization mass spectrometry for femtomoles detection of cardiac troponin I peptides, J. Mol. Recognit. 29 (2016) 41-50 (2016), <https://doi.org/10.1002/jmr.2494>.

[29] A. Rachkov, N. Minoura, Recognition of oxytocin and oxytocin-related peptides in aqueous media using a molecularly imprinted polymer synthesized by the epitope

approach, J. Chromatogr. A. 889 (2000) 111–118,
[https://doi.org/10.1016/S0021-9673\(00\)00568-9](https://doi.org/10.1016/S0021-9673(00)00568-9).

[30] X.D. Wang, G. Chen, P. Zhang, Q. Jia, Advances in epitope molecularly imprinted polymers for protein detection: a review, Anal. Methods, 13 (2021) 1660-1671, <https://doi.org/10.1039/D1AY00067E>.

[31] E. Moczko, A. Guerreiro, C. Caceres, E. Piletska, B. Sellergren, S.A. Piletsky, Epitope approach in molecular imprinting of antibodies, J. Chromatogr. B. 1124 (2019) 1-6, <https://doi.org/10.1016/j.jchromb.2019.05.024>.

[32] B.Y. Zhang, X.Y. Lei, L.J. Deng, M.S. Li, S.C. Yao, X.P. Wu, Ultrafast preparation of a polyhedral oligomeric silsesquioxane-based ionic liquid hybrid monolith via photoinitiated polymerization, and its application to capillary electrochromatography of aromatic compounds, Microchim. Acta. 185 (2018) 318–327, <https://doi.org/10.1007/s00604-018-2847-x>.

[33] F. Wu, C. Xu, N. Jiang, J. Wang, C.F. Ding, Poly (methacrylic acid-co-diethenyl-benzene) monolithic microextraction column and its application to simultaneous enrichment and analysis of mycotoxins, Talanta, 178 (2018) 1-8, <https://doi.org/10.1016/j.talanta.2017.08.030>.

[34] Y. Zhang, D. Song, L.M. Lanni, K.D. Shimizu, Importance of functional monomer dimerization in the molecular imprinting process, Macromolecules, 43 (2010) 6284–6294, <https://doi.org/10.1021/ma101013c>.

[35] L. Malosse, P. Palmas, P. Buvat, D. Ades, A. Siove, Novel Stoichiometric, Noncovalent Pinacolyl Methylphosphonate Imprinted Polymers: A Rational Design by NMR Spectroscopy, *Macromolecules*, 41 (2008) 7834-7842, <https://doi.org/10.1021/ma801171g>.

[36] Y. Zhang, D. Song, L.M. Lanni, K.D. Shimizu, Importance of functional monomer dimerization in the molecular imprinting process, *Macromolecules*, 43 (2010) 6284 – 6294, <https://doi.org/10.1021/ma101013c>.

[37] K. Golker, B.R.C. Karlsson, G.D. Olsson, A.M. Rosengren, I.A. Nicholls, Influence of composition and morphology on template recognition in molecularly imprinted polymers, *Macromolecules*, 46 (2013) 1408-1414, <https://doi.org/10.1021/ma3024238>.

[38] A.G. Mayes, M.J. Whitcombe, Synthetic strategies for the generation of molecularly imprinted organic polymers, *Adv. Drug Deliv. Rev.* 57 (2005) 1742–1778. <https://doi.org/10.1016/j.addr.2005.07.011>.

[39] N.H. Omran, H.A. Wagdy, M. Abdel-Halim, R.M.E. Nashar, Validation and application of molecularly imprinted polymers for SPE/ UPLC–MS/MS detection of gemifoxacin mesylate, *Chromatographia*, 82 (2019) 1617-1631, <https://doi.org/10.1007/s10337-019-03782-1>.

[40] G.J. Maranata, N.O. Surya, A.N. Hasanah, Optimising factors affecting solid phase extraction performances of molecular imprinted polymer as recent sample

preparation technique, Heliyon, 7 (2021) e05934,

<https://doi.org/10.1016/j.heliyon.2021.e05934>.

[41] D. Djozan, M.A. Farajzadeh, S.M. Sorouraddin, T. Baheri, Molecularly imprinted-solid phase extraction combined with simultaneous derivatization and dispersive liquid-liquid microextraction for selective extraction and preconcentration of methamphetamine and ecstasy from urine samples followed by gas chromatography, J. Chromatogr. A. 1248 (2012) 24-31, <https://doi.org/10.1016/j.chroma.2012.05.085>.

[42] Y.Van. Wanseele, K. Maes, K. Lanckmans, J.Van. Schoors, I. Smolders, A.Van. Eeckhaut, Surface and solvent dependent adsorption of three neuromedin-like peptides in glass and plastic syringes, Chromatographia. 81 (2018) 65-72, <https://doi.org/10.1007/s10337-017-3397-9>.

Table 1 Enrichment factor (EF) and imprinting factor (IF) of the MIM and the NIM column for four neuropeptides.

Peptide	Amino acid sequence	EF _{MIM}	EF _{NIM}	IF
NT	pGlu-Leu-Tyr-Glu-Asn-Lys-Pro-Arg-Arg-Pro-Tyr-Ile-Leu	17.4	1.30	13.4
NmN	Lys-Ile-Pro-Tyr-Ile-Leu	6.30	1.10	5.72
NmB	Gly-Asn-Leu-Trp-Ala-Thr-Gly-His-Phe-Met-NH ₂	2.40	1.01	2.37
NmU	Tyr-Lys-Val-Asn-Glu-Tyr-Gln-Gly-Pro-Val-Ala-Pro-Ser-Gly-Gly-Phe-Phe-Leu-Phe-Arg-Pro-Arg-Asn-NH ₂	1.06	1.04	1.02

Table 2 Influence of different functional monomers on the enrichment factor (EF) of the resulting MIM column.

MIMs	Monomers	Template/monomer ratio (% , w/w)	EF	
			NT	NmN
M1	MAA	1/2	17.4	6.3
M2	4-VP	1/2	11.5	4.9
M3	AM	1/2	10.8	4.3
M4	AA	1/2	17.2	5.9

Table 3 Chemical composition of the NIM, MIMs and corresponding enrichment factor (EF).

Column	Molar ratio	Molar ratio	Porogens mixture (% w/w)		EF	
	n _{PYIL} : n _{MAA}	n _{MAA} : n _{EDMA}	MeOH/ACN	Isooctane ^b	NT	NmN
C1 ^a	/	1:2.6	1.67	3.4	1.3	1.1
C2	1:23	1:3.3	1.67	3.4	12.5	3.9
C3	1:12	1:3.5	1.67	3.4	16.8	4.1
C4	1:12	1:4.0	1.35	3.4	12.1	3.5
C5	1:12	1:4.0	1.67	3.4	17.4	6.3
C6	1:12	1:4.0	2.33	3.4	15.9	5.8
C7	1:12	1:4.5	1.67	3.4	13.2	6.5
C8	1:8	1:4.6	1.67	3.4	12.2	4.4
C9	1:6	1:5.4	1.67	3.4	5.4	2.6

a: NIM column

b: Percentage of isooctane in the porogens mixture

Table 4 Linear range, regression equation, limits of detection and quantitation of NT and NmN.

Analyte	Linear range (μM)	Regression Equation ^a	R^2	LOD (nM)	LOQ (nM)	RSD% (n=3)	
						Intra-day precision	Inter-day precision
NT	0.01-10	$y = 1000000x + 15378$	0.997	0.62	2.06	3.7%	4.6%
NmN	0.02-15	$y = 164786x + 33823$	0.992	1.20	4.00	3.5%	5.4%

a: x: concentration of analyte, (μM), y: peak area of the analyte.

Table 5 Intra-day and inter-day recovery and precision (RSD) of the MIM-CME-HPLC analysis for NT and NmN spiked in plasma.

Analyte	Spiked	Intra-day (n = 3)		Inter-day (n = 3)	
	Level	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)
	(μ M)				
NT	0.05	89.8	5.8	89.2	5.2
	0.50	92.5	3.3	90.8	2.9
	5.00	99.8	1.9	98.8	2.0
NmN	0.05	87.6	5.9	82.5	5.8
	0.50	88.4	2.4	86.9	2.5
	5.00	97.3	1.3	93.8	1.7

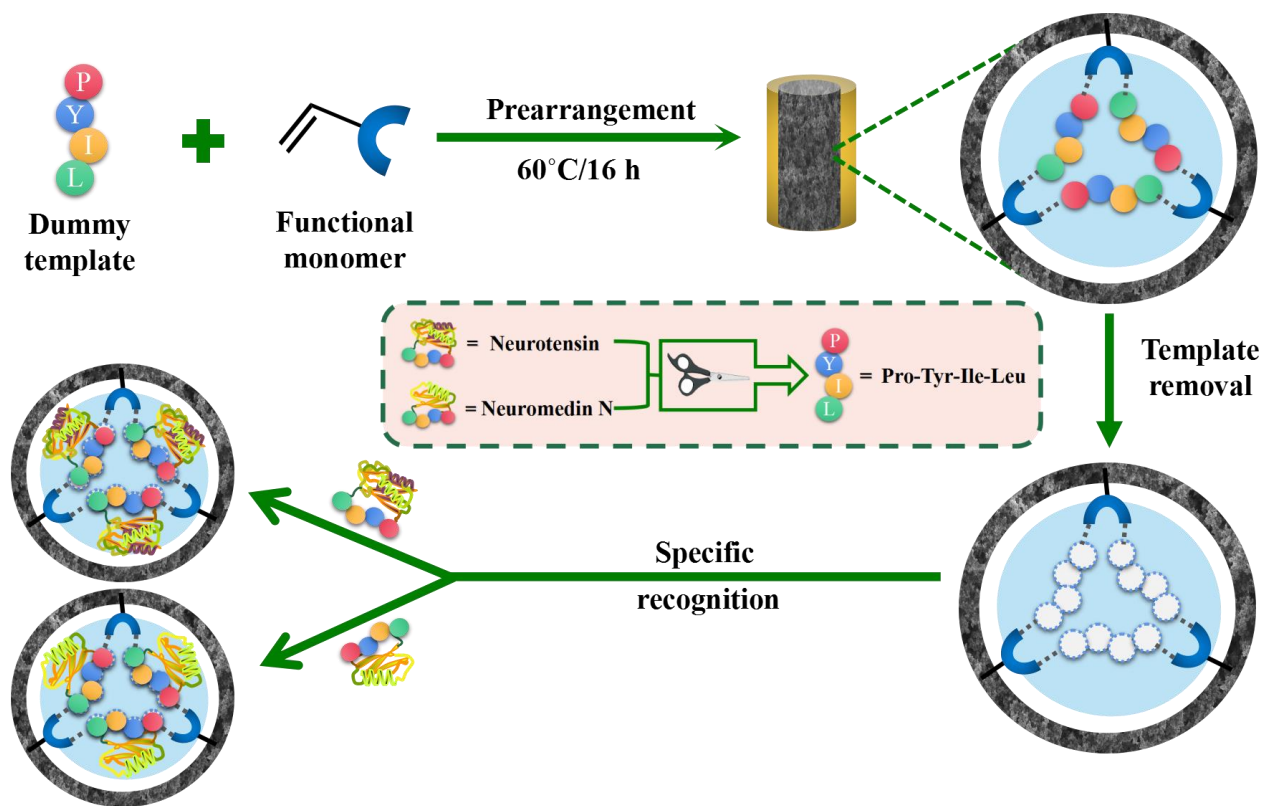


Fig. 1. Schematic illustration of the preparation and recognition process of a MIM column for neuropeptides.

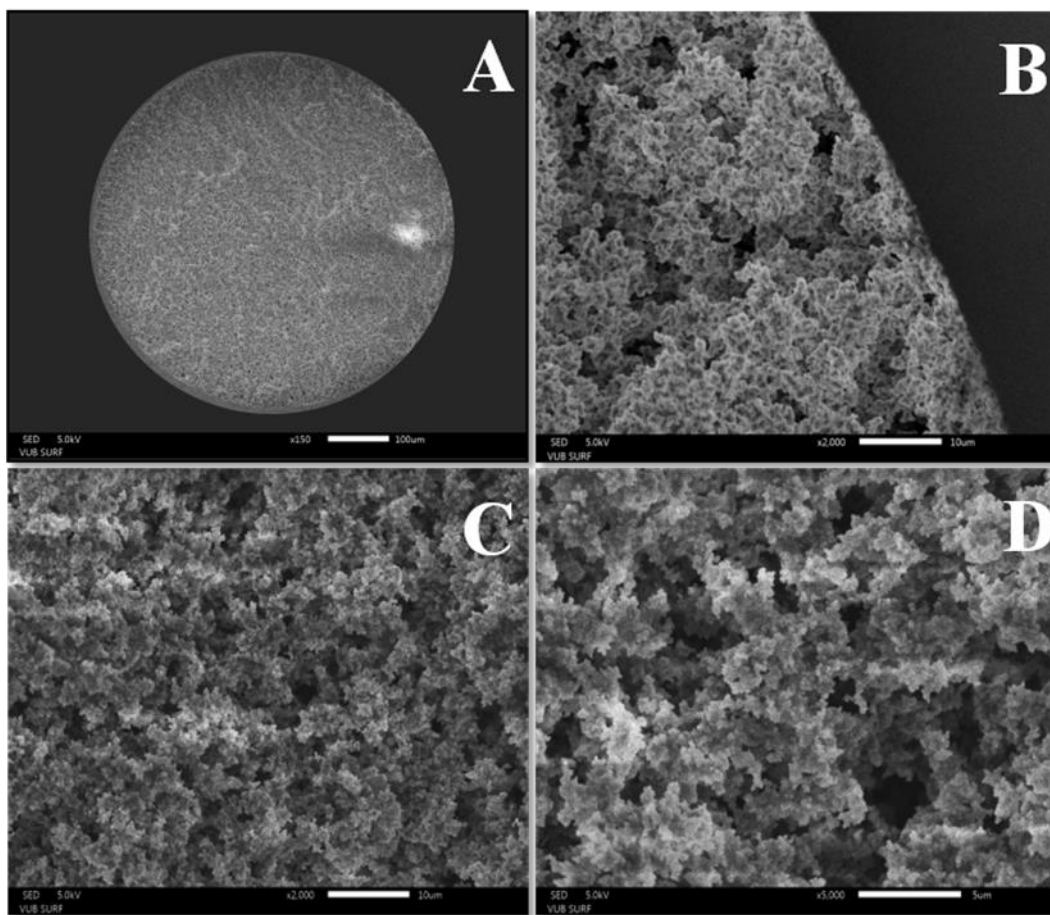


Fig. 2. SEM micrographs of MIM column C5. Magnification: A: 150×; B and C: 2,000×; D: 5,000×.

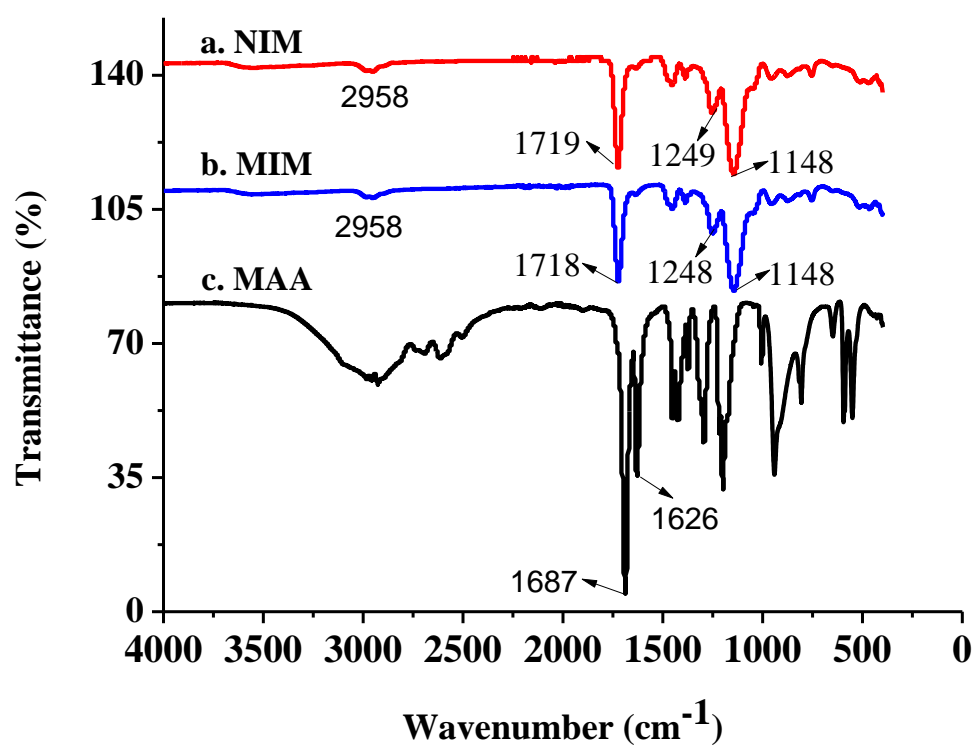


Fig. 3. FT-IR spectrum of (a) NIM, (b) leached MIM, and (c) MAA.

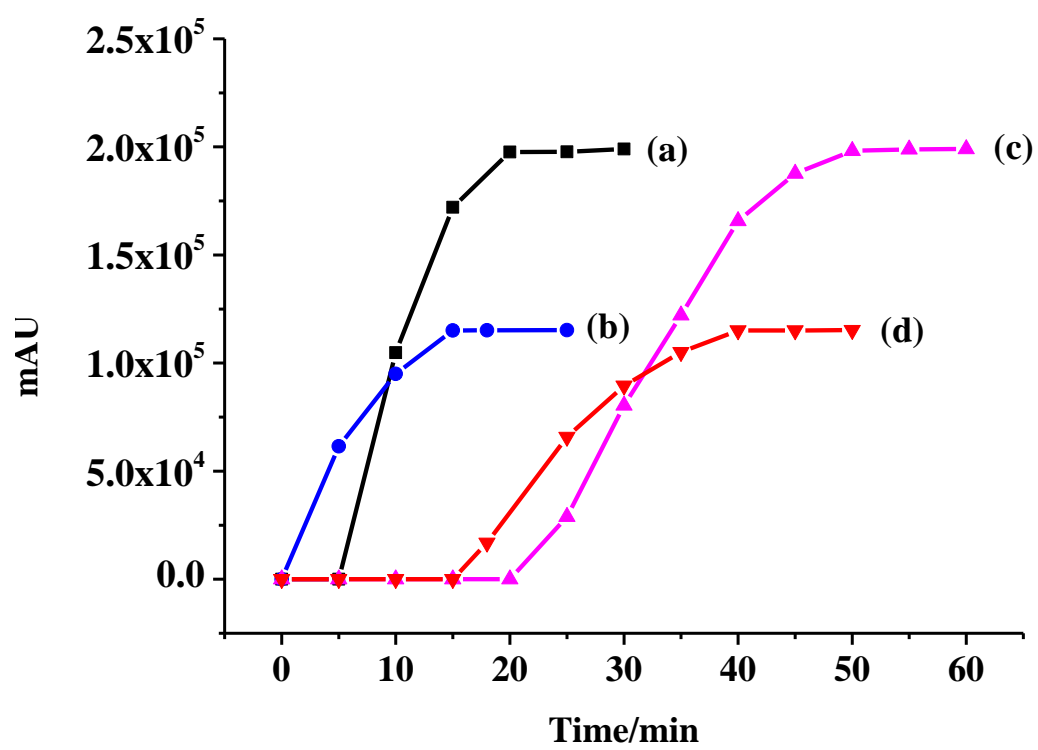


Fig. 4. The breakthrough curves of (a) NT and (b) NmN onto the NIM column; (c) NT and (d) NmN onto the MIM column.

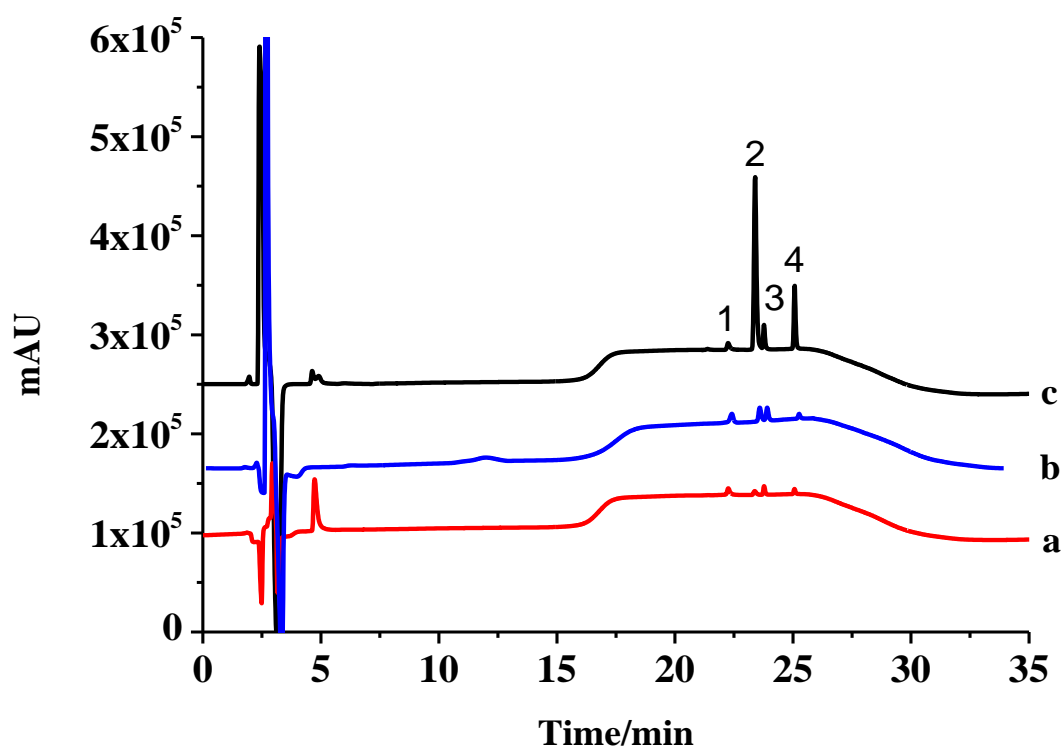


Fig. 5. Chromatograms of four neuropeptides standard mixture at concentration 1.0 μM , obtained by (a) direct HPLC analysis, (b) NIM-CME/HPLC and (c) MIM-CME/HPLC analysis on a CortecsTM C18+ column (2.7 μm , 150 \times 2.1 mm i.d.). Peaks: (1) NmU (2) NT (3) NmB (4) NmN. HPLC conditions (see also text): gradient elution with water/methanol containing 0.1% HAc; flow rate, 130 $\mu\text{L}/\text{min}$; UV detection at 210 nm.

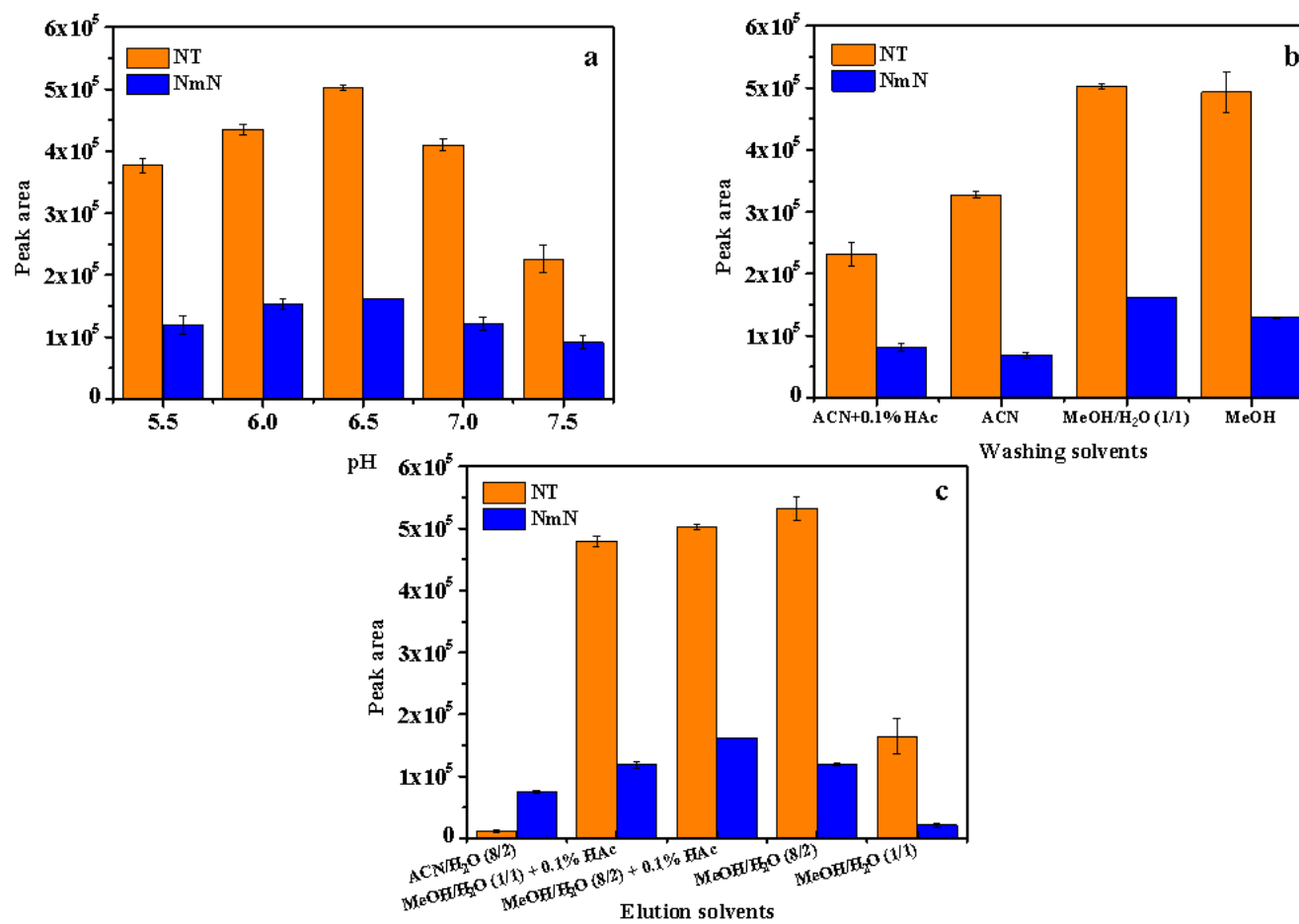


Fig. 6. Effects of different conditions on the extraction performance of the MIM. (a) pH of loading solvent; (b) type of washing solvent; (c) type of elution solvent. Response shown is the peak area after elution of the peptide (n=3).

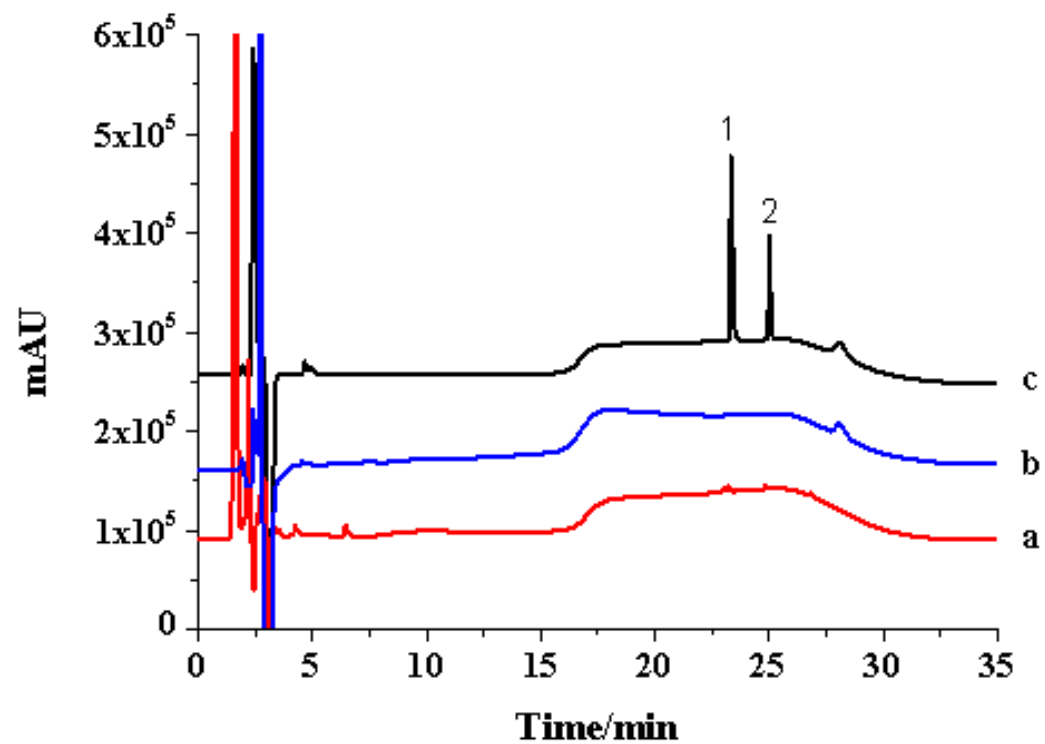


Fig.7. Chromatograms obtained by (a) direct HPLC analysis of a standard solution containing 1.0 μM NT and 3.0 μM NmN , (b) MIM-CME/HPLC analysis of a blank human plasma sample, (c) MIM-CME/HPLC analysis of a human plasma sample spiked with 1.0 μM NT and 3.0 μM NmN. Peaks: (1) NT (2) NmN. Conditions as in Fig. 5

Fabrication of a molecularly imprinted monolithic column via epitope approach for selective capillary microextraction of neuropeptides in human plasma

Xiaoyun Lei ^{a,b}, Ting Huang ^a, Xiaoping Wu ^{a,*}, Debby Mangelings ^b, Ann Van Eeckhaut ^c, Jana Bongaerts ^{b,c}, Herman Terryn ^d, Yvan Vander Heyden ^{b,*}

^a *Key Laboratory for Analytical Science of Food Safety and Biology; College of Chemistry, Fuzhou University, Fuzhou 350116, China*

^b *Department of Analytical Chemistry, Applied Chemometrics and Molecular Modelling, Vrije Universiteit Brussel – VUB, Brussels, Belgium*

^c *Department of Pharmaceutical Chemistry, Drug Analysis and Drug Information, Center for Neurosciences, Vrije Universiteit Brussel – VUB, Brussels, Belgium*

^d *Department Materials and Chemistry, Research group Electrochemical and Surface Engineering, Vrije Universiteit Brussel – VUB, Brussels, Belgium*

***Corresponding authors**

X. Wu: wapple@fzu.edu.cn; **Tel&Fax:** +86-591-22866131

Y. Vander Heyden: Yvan.Vander.Heyden@vub.be; **Tel:** +32-2-477-47-34

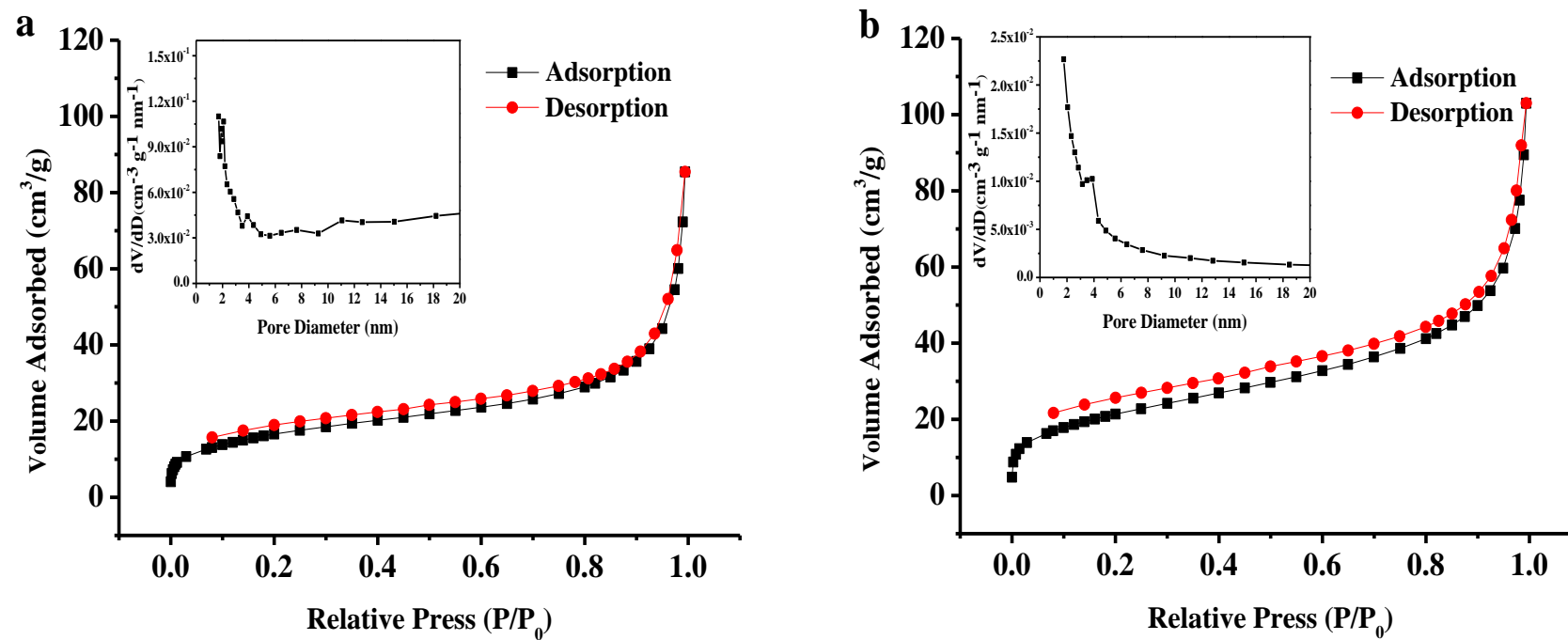


Fig. S1. N_2 adsorption/desorption isotherms and pore-size distributions (insert) of (a) NIM and (b) MIM columns.

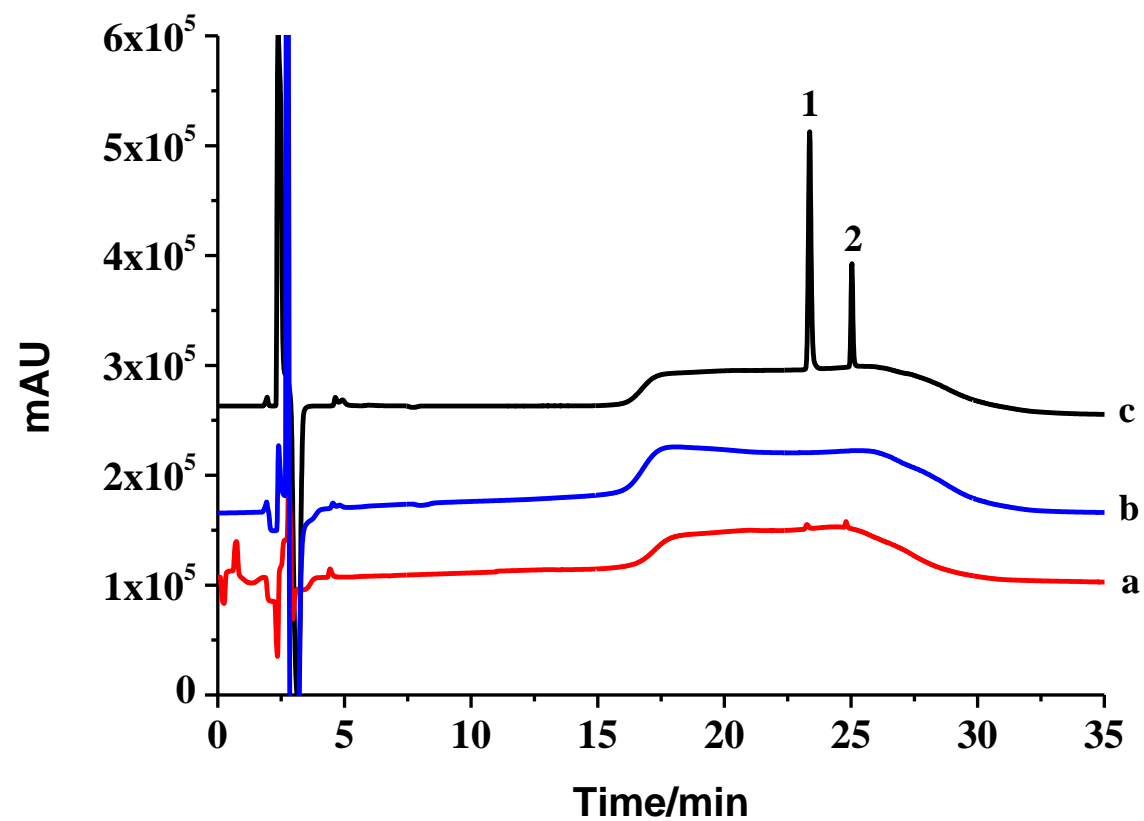


Fig. S2. Chromatograms obtained by (a) direct HPLC analysis of a standard solution containing 1.0 μM of NT and NmN; (b) MIM-CME/HPLC analysis of blank sample; (c) MIM-CME/HPLC analysis of a standard solution containing 1.0 μM of NT and NmN. Peaks: (1) NT (2) NmN. Other conditions as in Fig. 5.