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Preparation of a new composite based on multilayer fullerene with mesoporous carbon nitride and its application in the extraction of tacrolimus and everolimus from plasma prior to LC-MS/MS analysis†

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The development of new and efficient adsorbents for dispersive solid-phase extraction method, particularly prior to chromatography analysis, is increasing. In particular, this method is recommended for use before biological sample analysis. In this work, a new composite was prepared from mesoporous carbon nitrides and carbon nano-onions and was utilized for the extraction of tacrolimus and everolimus from plasma samples prior to high-performance liquid chromatography-tandem mass spectrometry analysis. To achieve this aim, first, mesoporous carbon nitrides and carbon nano-onions were synthesized separately and mixed at optimized proportions. Subsequently, a suitable amount of the prepared composite (5 mg) was added to 2 mL of sample solution containing the analytes under vortexing. Next, the extracted analytes were eluted using acetonitrile. The approach was linear within the ranges of 1.0–500 and 0.51–500 ng mL^{-1} for tacrolimus and everolimus, respectively. Sensitive limits of detection (0.31 and 0.15 ng mL^{-1} for tacrolimus and everolimus, respectively), acceptable relative standard deviations (intra- and inter-day precisions of ≤5.6% and high extraction recoveries of 71.0% and 83.0% for tacrolimus and everolimus, respectively) were obtained. The results showed that the method can be successfully applied in the simultaneous extraction of the studied analytes from plasma.

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1. Introduction

Tacrolimus and everolimus are immunosuppressive drugs and are frequently used by transplant recipients.[1,2] Tacrolimus prevents the advancement of the T-cell cycle by blocking interleukin-2 production, whereas everolimus plays a significant role in calcineurin inhibition and downregulates lymphocyte proliferation through the suppression of cytokine generation.[3,4] Although these drugs are prescribed alone, due to their synergistic effects, they are frequently given in combination after solid organ transplantation.[5] The merit of this synergism is that it leads to dosage reduction, thereby lowering adverse effects. Immunosuppressive drugs have narrow therapeutic ranges that rely on diverse parameters, including transplanted organs, co-medications, the age of patients, and the period after transplantation.[6] Alternatively, their dosage is strongly related to their adverse effects, and by observing any of the adverse symptoms of the drugs (renal, immunological, neurological, hepatic, or hepatic complications),[7] their dosage must be changed. Taking these points into consideration, therapeutic drug monitoring (TDM) of immunosuppressive drugs has been advised in the routine treatment of patients.[8] However, the accurate and precise monitoring of drug levels in biological samples represents a bottleneck in routine TDM.[9] It has been observed that in human blood flow, tacrolimus and everolimus are mainly situated inside erythrocytes or attached to plasma proteins.[10] Thus, the determination of these drugs in blood and plasma can be beneficial for TDM. In initial studies, immunological techniques were preferred for the analysis of these compounds, but overestimating their concentrations and cross-reaction of the drugs with some metabolites severely restrict their application. Recently, high-performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS) has been considered a powerful instrument for the analysis of these drugs, exclusively for their simultaneous monitoring at combined doses.[11] In spite

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of HPLC-MS/MS's ability for the accurate determination of drugs, their direct analysis in biological fluids is impossible as the presence of various compounds in the sample matrix disrupts the monitoring and determination procedures.[12,13] As a result, sample preparation is an essential step before analysis of the drugs using HPLC-MS/MS.

Dispersive micro solid-phase extraction (DμSPE), owing to its certain important benefits, like short analysis time, adaptability, plainness, and ease of use, is regarded as an appropriate procedure for sample preparation.[14] The basis of this technique is adding an adsorbent to the sample solution in the presence of mechanical agitation. In this procedure, improved contact of the adsorbent with a sample solution is provided by efficient extraction of the analytes.[15,16] It is obvious that the adsorbent type has a direct impact on the method's efficiency. Among the tested compounds, carbon-based materials have attracted significant attention to be used in DμSPE due to their high stability and efficiency and good dispersibility. Carbon nano-onions (CNOs) are a new type of carbon-based material with a multi-layered arrangement similar to the fullerene shell. They are extensively applied in diverse areas, including optical limiting materials, lubricants, additives for aerospace applications, catalysis, energy storage, electromagnetic shielding, fuel cells, and separation methods.[17] As well, mesoporous carbon nitrides (m-C_3N_4) are one type of carbon-based compound that have attracted considerable attention, due to their electronic, photo-electrochemical, and basic properties.[18,19]

The chief aim of the present study was to introduce and establish a DμSPE method for the simultaneous extraction and preconcentration of everolimus and tacrolimus from plasma samples prior to their quantification by HPLC-MS/MS. For the DμSPE, m-C_3N_4/CNO composite was prepared and was used as the adsorbent. The large surface area and relatively uniform pore diameters of m-C_3N_4 provide it with excellent physicochemical properties for extraction of the target analytes. In addition, its multilayer structure also enhances its adsorption ability. The presence of multiple nitrogens in the structure of composition facilitates the formation of hydrogen bonding interactions, which supports the extraction of the target analytes. Here, it is notable that the method was directly performed on diluted plasma samples and so there was a need for deproteinization.

2. Experimental

2.1. Materials and solutions

Standard materials of the studied drugs with a purity higher than 98% were bought from Sigma-Aldrich (St. Louis, MO, USA). A stock solution of tacrolimus, everolimus, and cyclosporine (as an internal standard) was prepared in acetonitrile (at a concentration of 100 mg L^{-1} of each drug) and then the requisite working solutions were prepared by diluting the stock solution with deionized water, properly. Analytical grade chemical compounds, including acetone, ethanol, acetonitrile, methanol, melamine, chitosan, sodium chloride, ammonium chloride, sodium hydroxide, phosphoric acid (85%), acetic acid, and hydrochloric acid solution (37%, w/w), were purchased from Merck (Darmstadt, Germany).

2.2. Apparatus

Determination of the analytes was conducted by applying a 2795 Waters HPLC separation system (Waters Milford, MA) equipped with a C_{18} column (15 × 4.0 mm; particle size of 5 μm, Agilent, USA). The analytes were detected by a triple quadrupole tandem mass spectrometer (Waters, USA) operating in a positive multiple reaction monitoring (MRM) mode. A mixture of acetonitrile and 0.1% formic acid in solution (90 : 10, v/v) was delivered at a flow rate of 0.5 mL min^{-1} into the column as the mobile phase in an isocratic elution mode. The MS detector was run under the following conditions: spray voltage, +3 kV; source temperature, 110 °C; desolvation temperature, 350 °C; capillary voltage, +2 kV; con voltage, +49 V; extractor, +3 V; electrospray mode, positive; desolvation flow rate, 600 L h^{-1}; and cone spray, 100 L h^{-1}. The tuning of the MS/MS transitions was performed by injection of the analytes (1 mg L^{-1} of each in the mobile phase with a flow rate of 50 μL min^{-1}). For quantitative analysis of the compounds, the MRM and MS conditions were optimized for each drug using a direct injection technique, and the corresponding parameters were optimized for each compound. The monitored transitions (corresponding collision voltage) were as follows: tacrolimus [826.3 → 763.8 (40 V)], everolimus [980.5 → 948 (40 V)], and cyclosporine as the internal standard (1226.0 → 1206 (40 V)). A 691 pH meter (Metrohm, Switzerland) was applied to adjust pH. Sorbent characterization was done by scanning electron microscopy (SEM) (Tescan Mira3, Brno, Czech Republic), Fourier transform infrared spectroscopy (FTIR) (PerkinElmer, Waltham, MA, USA), and X-ray diffraction (XRD) (Philips XRD diffractometer, Amsterdam, Netherlands) analyses. During the experiments, a vortex (Heidolph, Germany), an MIKRO 200 centrifuge (Hettich, Germany), and an analytical balance (±0.0001 g, Sartorius, Germany) were used.

2.3. Synthesis and preparation of the CNO/m-C_3N_4 composite

The preparation processes for CNO were done according to the reported method *via* a hydrothermal process.[20] Briefly, in the first stage, chitosan powder (1 g) and glacial acetic acid 1% (w/v) (20 mL) were vigorously stirred for 30 min at 60 °C, and chitosan gel was formed. Then, 1 g of the obtained chitosan gel, as a nitrogen and carbon source, was transferred into a Teflon-lined stainless-steel autoclave. The reaction solution was diluted to 10 mL by adding Millipore water. Subsequently, the autoclave was heated to 180 °C for 8 h and then allowed to cool to room temperature. The obtained powder was collected and washed with water and ethanol several times. Eventually, the washed product was vacuum-dried at 60 °C overnight. The bulk m-C_3N_4 was synthesized straightforwardly *via* heating melamine in a furnace. For this aim, 2 g of melamine was transferred into an alumina crucible with a cover and calcined at a heating rate of 2 °C min^{-1} up to 450 °C. After that, the process was followed by keeping it for 2 h at this temperature. In the end, the coarse m-C_3N_4 was grounded before characterization and use.

To obtain the composite, 25% CNO and 75% m-C_3N_4 were mixed and ground into a homogeneous powder.

2.4. Plasma samples

Blank plasma sample was obtained from the Iranian Blood Transfusion Organization (Tabriz, Iran), and kept in a freezer for use during the optimization and validation steps. Four plasma samples were obtained from healthy volunteers to check the method's accuracy and for the matrix effect studies. All participants signed a written consent form approved by the Ethics Committee of Tabriz University of Medical Sciences (approval code of IR.TBZMED.VCR.REC.1402.317). Plasma samples were diluted with deionized water at a ratio of 1 : 4 (v/v) in all procedures.

2.5. Extraction procedure

First, 5 mg of 75% m-C_3N_4/25% CNO as sorbent was added to 2 mL of diluted blank (or spiked) plasma that had been adjusted to pH 6. Then, the mixture was vortexed for 4 min. In the following step, the mixture was centrifuged at 5000 rpm for 3 min. Afterward, the supernatant was removed and 200 μL of acetonitrile was added onto the sorbent to elute the analytes by vortexing for 6 min. In the next step, the mixture was centrifuged to isolate the sorbent particles from the organic (acetonitrile) phase. Eventually, 50 μL of the solution was injected into the HPLC-MS/MS system for separation and determination of the analytes.

3. Results and discussions

3.1. Morphological and structural characterization of the sorbent

The morphology and structure of the particles of the composite and its components were investigated by SEM, XRD, and FTIR analyses. The results are shown in Fig. 1 and 2. The morphology of CNO and m-C_3N_4 can be seen in the SEM images (Fig. 1a–d). The quasi-spherical morphology of CNO (d = 53.02–61.59 nm) with aggregation of the nanoparticles is shown in Fig. 1a and b. Fig. 1c shows that the morphology of m-C_3N_4 appeared to consist of a porous structure. Also, Fig. 1d reveals that m-C_3N_4 was composed of a stacked layered morphology. The SEM images of m-C_3N_4/CNO in diverse magnifications are given in Fig. 1e–h. According to these images, the combination of m-C_3N_4 and CNO resulted in a morphological change of the m-C_3N_4/CNO porous walls, in which m-C_3N_4 and CNO particles were distributed in a homogenous pattern on the microporous walls of the m-C_3N_4/CNO composite. The functional groups in the sorbent were evaluated by FTIR. Fig. 2a illustrates the FTIR spectra of CNO, m-C_3N_4, and m-C_3N_4/CNO. The FTIR spectrum of CNO showed the presence of carboxylic and aromatic groups. The peak at 3400 cm^{-1} was related to the vibrational stretching of OH in the carboxyl group.[21,22] Considering the FTIR spectrum of m-C_3N_4, some peaks emerged at 1640, 1569, 1326, 1412, and 1240 cm^{-1}, which were associated with the vibrational stretching of C–C and C=C heterocycles. The broad band observed between 3000 and 3400 cm^{-1} corresponded to N–H vibrational stretching and the adsorbed H_2O molecules. The sharp band at 812 cm^{-1} could be indexed to the characteristic breathing mode of *s*-triazine units.[22] The FTIR spectrum of m-C_3N_4/CNO included the basic peaks for m-C_3N_4 and CNO. The XRD patterns of m-C_3N_4 and CNO are illustrated in Fig. 2b. The m-C_3N_4 pattern had a peak at 2θ = 27° (100 plane), which was related to the interlayer separation of the compound.[23,24] The existence of a broad peak in the CNO pattern revealed the lack of crystalline structure of the substance.

3.2. Optimization of the DμSPE parameters

DμSPE is a sample preparation technique used for the extraction and clean-up of analytes from complex matrices, such as food, environmental, and biological samples. It involves the

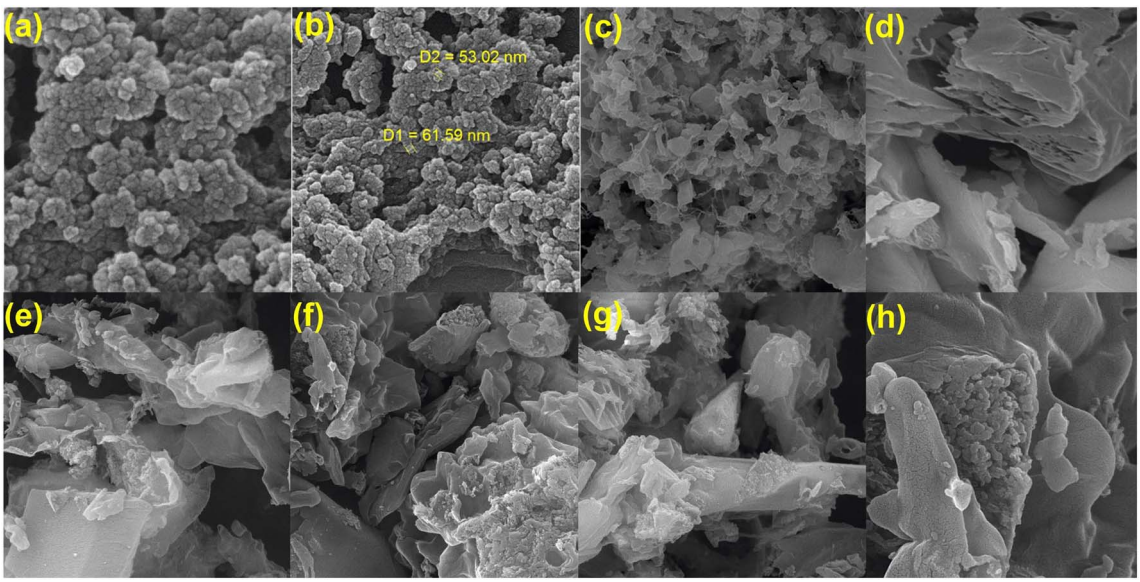

Fig. 1 SEM images of (a and b) the CNO; (c and d) m-C_3N_4, and (e–h) m-C_3N_4/CNO composite.

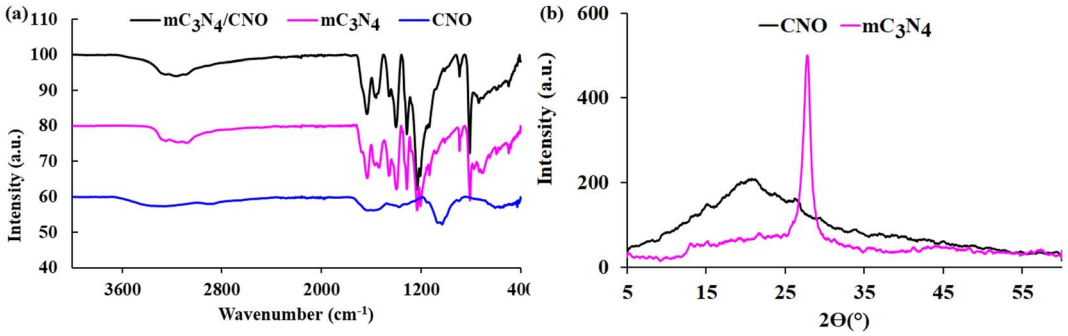

Fig. 2 (a) FTIR spectra of CNO, C_3N_4, and m-C_3N_4/CNO and (b) XRD of CNO and C_3N_4.

dispersion of sorbent particles into a sample matrix followed by their collection, which facilitates the separation of the analytes from interfering matrix components. This technique offers several advantages over traditional sample preparation methods, including a high extraction recovery (ER), low matrix effect, and reduced solvent consumption. DμSPE is an extraction method based on adsorption equilibrium that is established between the sorbent particles and analytes. Any factor that affects this equilibrium will impact the extraction efficiency. Thus, to assign the optimum extraction efficiency toward tacrolimus and everolimus, the DμSPE conditions, encompassing the sorbent composition, sorbent mass, ionic strength of the solution, eluent type, and volume, pH, adsorption and desorption times, and stirring type, were optimized.

3.2.1. Optimization of the sorbent composition. To investigate the influence of the sorbent composition on the extraction efficiencies of everolimus and tacrolimus from samples, CNO and m-C_3N_4 were mixed in various ratios with each other, including 100% m-C_3N_4, 75% m-C_3N_4/25% CNO, 50% m-C_3N_4/50% CNO, 25% m-C_3N_4/75% CNO, and 100% CNO. Fig. 3 shows the extraction performances for everolimus and tacrolimus from the sample solutions. It could be observed that the extraction efficiency of everolimus using 75% m-C_3N_4/25% CNO was significantly more than from the other compositions. Also, in the case of tacrolimus, the greatest signal was observed when 25% m-C_3N_4/75% CNO was used as the sorbent, which was nearly the same as the signal with 75% m-C_3N_4/25% CNO. Accordingly, 75% m-C_3N_4/25% CNO displayed the optimum extraction capacity toward the target analytes and it was thus selected as the sorbent for the following studies.

3.2.2. Optimization of the sorbent mass. The amount of sorbent affects the extraction efficiency of analytes *via* raising the contact surface area between the analytes and sorbent and increasing the number of available active sites on the surface of the sorbent. So, to verify the impact of sorbent mass on the extraction efficiency of the method, diverse doses of m-C_3N_4/CNO, including 2.5, 5.0, 7.5, 10, and 20 mg, were evaluated for the DμSPE of tacrolimus and everolimus, separately (Fig. 4a). As shown in Fig. 4a, for both analytes, as the sorbent mass increased from 2.5 mg to 5.0 mg, the adsorption amount of the analytes was enhanced too. Also, with the greater concentration of applied sorbent in the extraction processes, lower analytical

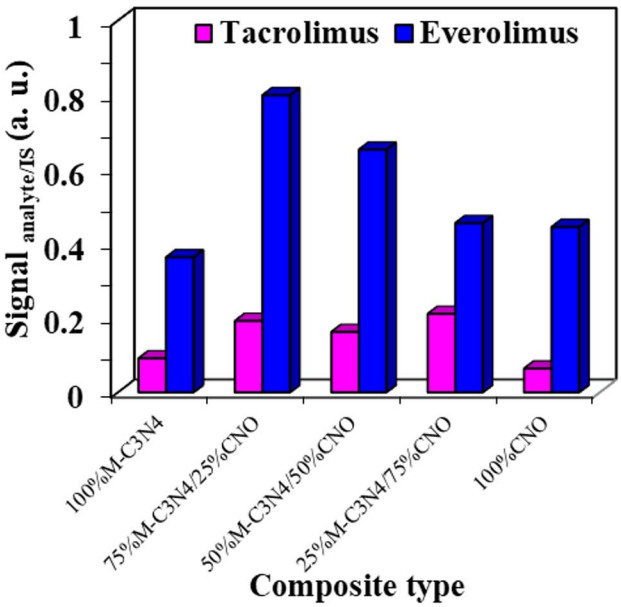

Fig. 3 Effect of the m-C_3N_4/CNO ratio on the extraction efficiency of the method (conditions: eluent type, acetonitrile; eluent volume, 200 μL; sorbent mass, 5 mg; pH, 4; adsorption time, 4 min; and desorption time, 4 min).

signals were obtained for the analytes. The highest extraction efficiency was observed with 5.0 mg of sorbent. Although, when raising the sorbent mass from 5.0 to 7.5 mg, the efficiency stayed nearly at the same level and further increasing the sorbent mass led to a decreasing extraction performance, which was more significant for everolimus compared to tacrolimus. CNO not only has a high surface area but also has functional groups and double bonds, which could aid its better absorption of the analytes, while the aggregation phenomenon might diminish its efficiency. Thus, 5.0 mg of the sorbent was chosen as the optimal amount of sorbent for the subsequent tests.

3.2.3. Study of the eluent type and volume. The eluent plays a key role in the desorption process of analytes from the sorbent surface, in which the interactions between the analytes and sorbent are hindered by using a small volume of eluent. Also, it is worth noting that the selected eluent should be compatible with the HPLC device. Thus, the performances of

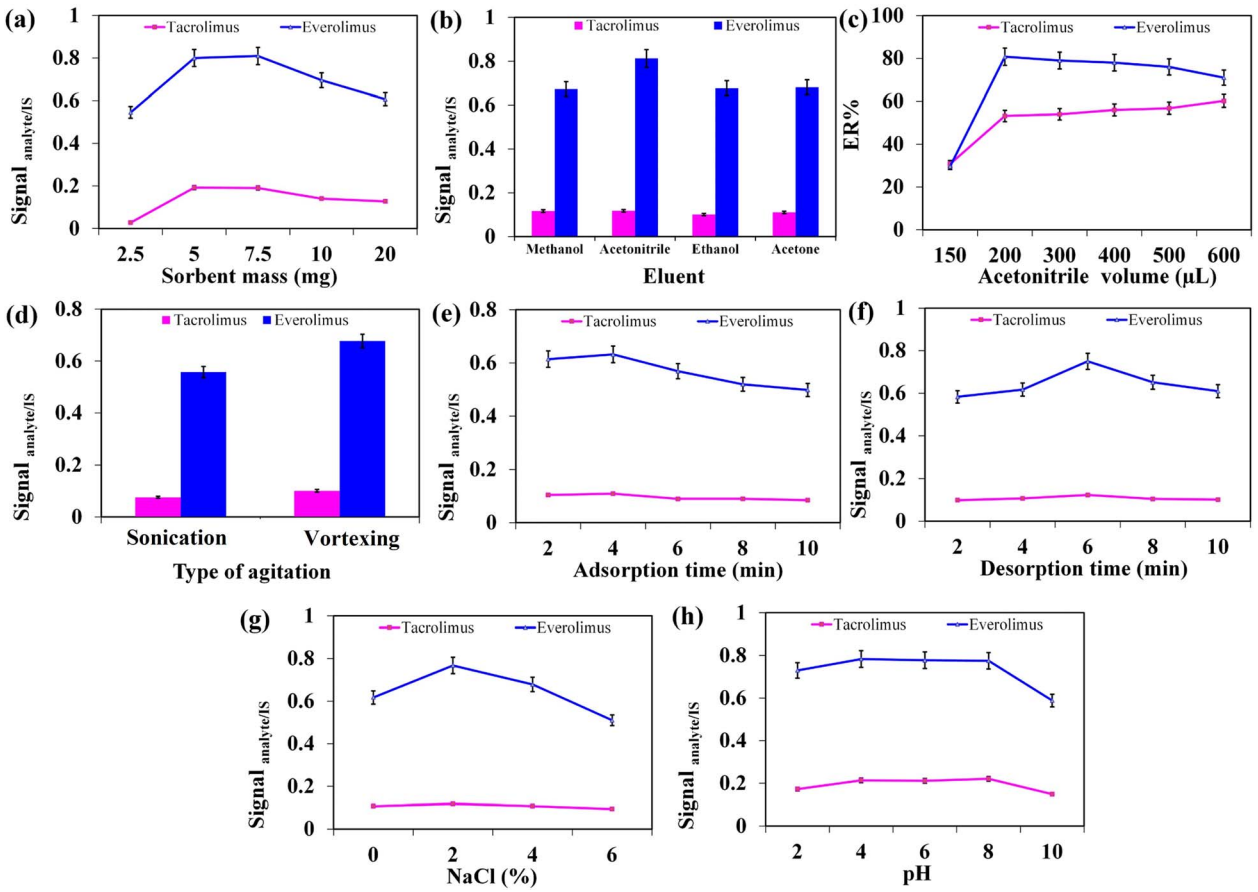

Fig. 4 Effect of (a) sorbent mass, (b) eluent type, (c) eluent volume, (d) type of agitation, (e) adsorption time, (f) desorption time, (g) salt addition, and (h) pH on analytical signals of the analytes.

four polar organic solvents, namely acetone, acetonitrile, ethanol, and methanol, were assessed in this work. Fig. 4b compares the efficiency of the mentioned solvents during the desorption mechanism. According to this figure, the studied analytes were better eluted from the m-C_3N_4/CNO surface by acetonitrile than the other solvents.

The volume of the desorption solvent makes a vital contribution to the extraction efficiency of the method; in this way, by increasing the volume of the desorbing solvent, numerous solvent molecules can take part in competition with the sorbent molecules over the sorption of the target analytes. Accordingly, the impact of acetonitrile volume on the desorption of everolimus and tacrolimus was explored in the range of 150–600 µL. As illustrated in Fig. 4c when increasing the desorption solvent volume up to 200 µL, the extraction efficiency of everolimus and tacrolimus improved from about 30% to 80% and 53%, respectively. However, although further addition of a higher solvent volume led to increasing the extraction efficiency of tacrolimus, the everolimus extraction decreased slightly. As a result, the next experiments were performed with 200 µL of acetonitrile.

3.2.4. Effect of the agitation type. Agitation can raise the sorbent surface contact area with the sample solution and increase the extraction efficiency of the DµSPE procedure. It can also reduce the extraction time and facilitate the procedure. In the following study, the impact of the stirring type was examined in the DµSPE technique. In this way, two various stirring modes, namely vortexing, and sonication, were employed separately in the DµSPE of everolimus and tacrolimus, while the other extraction conditions were kept constant. Fig. 4d compares the extraction efficiency of the target analytes by using the above-mentioned stirring modes during the extraction process. A higher extraction performance of the analytes was achieved by employing vortexing as the stirring mode. So, vortexing was chosen for the subsequent analyses.

3.2.5. Study of the adsorption time. In the DµSPE method, it is important to optimize the contact time between the sorbent and sample to achieve the maximum extraction efficiency. Thus, in this study, the influence of the adsorption time was investigated for everolimus and tacrolimus at 2, 4, 6, 8, and 10 min, and the results are shown in Fig. 4e. It can be seen that the adsorption of everolimus did not change in the studied times. However, as the adsorption time was increased from 2 to 4 min, the extraction performance for tacrolimus was improved. Nevertheless, the extraction efficiency for tacrolimus significantly decreased when increasing the time duration from 4 to 10 min. Here, if the contact time is too long, it may reach a point where the analyte starts to desorb from the sorbent surface and return to the sample solution, leading to a decreased extraction

efficiency. Therefore, 4 min was chosen as the optimal duration for the adsorptive process.

3.2.6. Study of the desorption time. It is worth mentioning that ER can be affected considerably by the desorption time. Thus, the effect of this parameter on everolimus and tacrolimus was investigated at different times, namely 2, 4, 6, 8, and 10 min (Fig. 4f). It could be noticed that, during the aforementioned times, the desorption of tacrolimus was nearly constant. However, in the case of everolimus, when increasing the desorption time from 2 to 6 min, the desorption increased at first and then declined. Hence, the desorption time was set at 6 min in the subsequent tests.

3.2.7. Salt addition in DμSPE. Salt addition can affect a DμSPE procedure in various ways. The addition of salt can change the solubility of the analytes in a sample. Also, the addition of salt can affect the adsorption sites of the sorbent. The presence of salt can also help to minimize the aggregation of the sorbent particles, which can improve the efficiency of the extraction process. However, it is important to note that the optimal conditions for DμSPE will vary depending on the specific analytes and the sample matrix being analyzed. Therefore, here we optimized the process by testing four solutions containing diverse amounts of NaCl (0%, 2%, 4%, and 6%, w/v). Considering the results in Fig. 4g, it is clear that the extraction efficiency of tacrolimus in the presence and absence of NaCl was almost equal. In contrast, the analytical signal of everolimus increased when adding 2% w/v, NaCl. This dramatic enhancement could be associated with the salting-out effect; whereas higher concentrations of NaCl caused lower efficiencies, which could be related to the enhanced viscosity of the solution. Therefore, the subsequent tests were carried out using 2% w/v NaCl.

3.2.8. Effect of the solution pH. The pH of the sample solution cannot only influence the chemical structure of the analytes and sorbent, but also might affect the intermolecular interactions between the sorbent and analytes. Accordingly, in this research, the effect of the sample solution pH was surveyed in the range of 2–10. Fig. 4h demonstrates that even though the extraction performance of both analytes was noticeably increased when changing the pH from 2 to 4, it remained constant in the pH range of 4–8. Also, when enhancing the pH from 8 to 10, the extraction efficiency significantly decreased. So, the pH was adjusted to 6 for the next analyses.

3.3. Method validation and analytical parameters

Method validation is a critical step in the development of an analytical method. Validation procedures typically involve evaluating various parameters, such as sensitivity, stability, selectivity, precision, accuracy, and linearity. In this part of the current research, verification of the established method efficiency for analyzing tacrolimus and everolimus from plasma samples was studied in terms of the coefficient of determination (r^2), linear range (LR), limit of detection (LOD), limit of quantification (LOQ), enrichment factor (EF), ER, and repeatability. As depicted in Table 1, a good linearity of the calibration curves was acquired in the ranges of 0.51–500 ng mL^{-1} for everolimus and 1.0–500 ng mL^{-1} for tacrolimus with r^2 of 0.992 and 0.994, respectively. The LODs, which were determined based on S/N = 3, were 0.15 and 0.31 ng mL^{-1}, for everolimus and tacrolimus, respectively. The precision of the method was determined *via* intra- and inter-day (n = 5) repetitive experiments at three different concentrations (1.0, 10, and 100 ng mL^{-1} of each drug). The results revealed that the relative

Table 2 Matrix effect study in real samples

		Tacrolimus		Everolimus	
Sample	C_{added} (ng mL^{-1})	RR[a] %	RSD% (n = 3)	RR%	RSD% (n = 3)
Plasma #1	10	88	5	90	6
	25	92	4	96	5
	250	97	4	104	3
Plasma #2	10	85	6	89	4
	25	90	4	93	4
	250	99	3	98	3
Plasma #3	10	90	5	93	5
	25	96	6	94	6
	250	102	4	104	4
Plasma #4	10	89	5	95	6
	25	93	4	100	4
	250	100	3	105	5

[a] Relative recovery.

Table 1 Quantitative features of the developed method for the analytes

					RSD[e] (%)							
					Intra-day precision at a concentration of			Inter-day precision at a concentration of				
Analyte	LOD[a]	LOQ[b]	LR[c]	r^{2d}	1 (ng mL^{-1})	10 (ng mL^{-1})	100 (ng mL^{-1})	1 (ng mL^{-1})	10 (ng mL^{-1})	100 (ng mL^{-1})	EF ± SD[f]	ER (%) ± SD[g]
Tacrolimus	0.31	1.0	1.0–500	0.994	5.6	5.0	4.0	5.6	5.3	5.1	71 ± 3	14 ± 0.6
Everolimus	0.15	0.51	0.51–500	0.992	5.2	4.8	3.9	5.4	5.0	4.5	83 ± 5	17 ± 0.9

[a] Limit of detection (S/N = 3) (ng mL^{-1}). [b] Limit of quantification (S/N = 10) (ng mL^{-1}). [c] Linear range (ng mL^{-1}). [d] Coefficient of determination obtained for the matrix-matched calibration curve. [e] Relative standard deviation for intra- (n = 6) and inter-day (n = 6) precisions. [f] Enrichment factor ± standard deviation (n = 3). [g] Extraction recovery ± standard deviation (n = 3).

standard deviations were lower than 5.6%, which indicates perfect repeatability. Also, the EFs ($EF = C_{org}/C_{aq}$; C_{org}: analyte concentration in the organic phase and C_{aq}: initial concentration of the analyte), and ERs ($ER\% = (V_{org}/V_s) \times EF \times 100$; V_{org} = volume of the organic phase and V_s = volume of sample solution) were calculated. To investigate the method stability, a blank plasma sample was spiked with target analytes at two concentrations (2 and 100 ng mL^{-1}, each analyte), independently. These samples were kept at various temperatures (freezer, room, and refrigerator temperatures) for 48 h. The obtained results regarding the target analytes concentration were compared with the data obtained from fresh samples. The deviation of the data was ≤±9%, which confirmed the good stability of the analytes. Furthermore, a standard solution of the analytes at a concentration of 250 ng mL^{-1} (each drug) was kept at room and refrigerator temperatures for 7 days. After direct injection of this solution into the HPLC-MS/MS system on each consecutive day, it was found that there was no significant variance in peak areas of the analytes. The absence of any interference peak at the retention times of the analytes confirmed the method selectivity. For evaluation of the method robustness, a series of experiments were done at different column temperature (38, 40, and 42 °C), mobile phase flow rate (0.48, 0.50, and 0.52 mL min^{-1}), and desolvation flow rate (580, 600, and 620 L h^{-1}). In all the experiments, the deviation in the results was less than 12%.

3.4. Real sample analysis

A difference in the slope of the calibration curves plotted using the standard solution and the matrix-matched one may occur due to a matrix effect. However, based on the results, there was no noticeable matrix effect, and it could thus be found that the method is applicable for quantifying the studied drugs in plasma samples. The accuracy of the approach was examined through the standard-addition method and the relative recovery (RR) rates of the experiments for the plasma samples were calculated. According to the obtained data (listed in Table 2), the RR% rates were in the range of 85–105% and the method accuracy is acceptable. The obtained chromatograms of the spiked plasma with the analytes (10 and 25 ng mL^{-1}) are shown in Fig. 5.

3.5. Comparison with other reported methods

The proposed method was compared with other procedures used for the determination of the selected analytes in biological

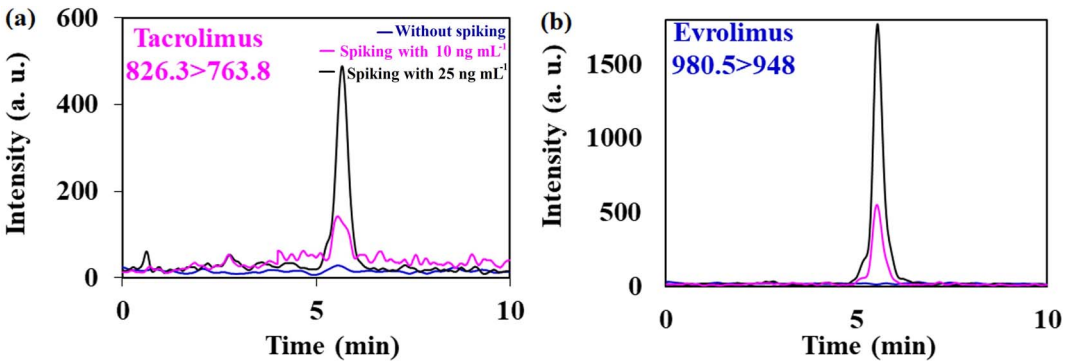

Fig. 5 Typical chromatograms of plasma after spiking 0, 10 and 25 ng mL^{-1} of each drug related to (a) tacrolimus and (b) evrolimus after performing the developed DμSPE under optimal conditions.

Table 3 Comparison of our method with other methods

Method	Analyte	Sample volume (μL)	Extraction time (min)	LOD[a]	LOQ[b]	LR[c]	RSD[d] (%)	Ref.
SPME-MOI-MS/MS[e]	Tacrolimus	200	≥60	0.3	0.8	1–50	2–6	25
SPE-PS-LC-MS/MS[f]	Tacrolimus	200	≥20	—	1.5	1.5–30	5–13	26
DμSPE-LC-MS/MS[g]	Tacrolimus	500	≥22	1.2	4.7	4.7–200	≤14	27
DLLME-LC-MS/MS[h]	Tacrolimus	—	≥12	—	1.0	—	—	28
SPME-LC-MS/MS[h]	Everolimus	—	≥300	—	2.5	—	—	
SALLE-LC-MS/MS[i]	Tacrolimus	50	≥12	0.2	0.4	0.4–85	≤4.1	8
	Everolimus			0.03	0.06	0.06–83	≤4.0	
DμSPE-HPLC-MS/MS	Tacrolimus	500	≥13	0.31	1.0	1.0–500	≤5.6	This
	Everolimus			0.15	0.51	0.51–500	≤5.6	study

[a] Limit of detection (ng mL^{-1}). [b] Limit of quantification (ng mL^{-1}). [c] Linear range (ng mL^{-1}). [d] Relative standard deviation. [e] Solid-phase microextraction–microfluidic open interface-tandem mass spectrometry. [f] Solid-phase extraction-paper spray-liquid chromatography-tandem mass spectrometry. [g] Dispersive micro solid-phase extraction-liquid chromatography-tandem mass spectrometry. [h] Dispersive liquid–liquid microextraction-liquid chromatography-tandem mass spectrometry. [i] Solid-phase microextraction-liquid chromatography-tandem mass spectrometry.

samples. For this comparison, several aspects of the methods and their figures of merits, including sample volume, extraction time, LOD, LOQ, LR, and RSD, were considered. The sample size required for analysis of the target analytes samples was higher than for the other methods, and only in the DμSPE-LC-MS/MS method was its volume similar to that needed for this method. The extraction time of the present study was shorter or comparable with other methods. The LODs and LOQs of this method and other procedures were comparable, except for the values obtained by the salting-out assisted (SA)-LLE-LC-MS/MS procedure.[8] In spite of the fact that the SA-LLE-LC-MS/MS method can analyze analytes at lower concentrations, a narrow LR and non-preconcentration of the analytes are its drawbacks. The listed results are presented in Table 3 in detail.

3.6. Study of the method greenness and practicality

The analytical eco-scale (AES) was used for the evaluation of the method greenness.[29,30] Considering AES, an ideal green method must have a score of 100. However, AES considers several penalty points for each step and these PPs are subtracted from 100. Methods having a score higher than 75 are considered as a green procedure. After subtracting the PPs of this method, the final score was 85 and the method is thus considered green (Table S1†). In the following, blue applicability grade index (BAGI) factors were considered for investigation of the method practicability.[31] BAGI considers the major factors of the extraction and analysis procedure and provides values in the range of 0 to 10 for each of them. An applicable method must have a BAGI of score of ≥65. Table S2† lists the values of the BAGI scores and confirms that the method is a good-applicable procedure.

4. Conclusions

A facile strategy to fabricate a porous adsorbent was developed by taking advantage of m-C_3N_4 and CNO, simultaneously. The resultant m-C_3N_4/CNO composite exhibited an exceptionally superior adsorption efficiency toward tacrolimus and everolimus. It was found that the composite could quantitatively preconcentrate low concentrations of tacrolimus and everolimus with LODs of 0.31 and 0.15 ng mL^{-1}, respectively, in plasma samples. Noteworthy, the method presented excellent linearity with $r^2 > 0.992$. This paper proposes a novel perspective to design and develop a composite of multilayer fluorene with m-C_3N_4 for its effective isolation and purification ability toward immunosuppressive drugs from various plasma samples.

Data availability

All of the data are confidential and are available on request.

Conflicts of interest

There are no conflicts to declare.

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