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Molecular imprinted solid-phase extraction and analysis of Entecavir in presence of its induced degradation products and co-administered drug(s) in spiked human plasma, environmental three-color assessment and sustainability profiling

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A R T I C L E I N F O

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EVG
NQS

A B S T R A C T

Entecavir (ETV) is an antiviral drug that acts by blocking the active viral replication process due to its chemical similarity to guanine. Through this study, the extraction of ETV samples was described, for the first time, using a synthesized molecular imprinted polymer solid phase extraction (MISPE). The MISPE was characterized using TEM and FTIR analysis. A green RP- HPLC method was developed for the quantitation of ETV using C18 column and a mobile phase consisting of 0.1 % phosphoric acid in water and methanol in a gradient mode delivered at a rate of 1 ml/min at room temperature. The UV detection was carried out at 245 nm. The analytical method was validated according to ICH guidelines with a linear range of (5–250 µg/mL). The specific extraction of ETV was carried out successfully using MISPE in presence of its induced acidic, and basic degradation products. MISPE was also used for the extraction of ETV from spiked human samples containing the co-administered drug lamivudine. The MISPE showed excellent selectivity, reusability and high recovery percentages (>90 %) when compared to Oasis HLB cartridges. The analytical procedure was compared to the reported methods in terms of environmental three-color assessment (ETCA): greenness (using AGREE, AGREEMIP and ComplexMoGAPI), blueness (using BAGI), and whiteness (using RGB-12 algorithms). The proposed method transcended in saving energy, efficiency and applicability. The sustainability profile for the proposed method was established using the efficient-valid-green (EVG) framework displayed via its radar chart, that showed balance between the three pillars, and the NQS index that displays the excellent alignment of the method with the UN-17 SDGs.

1. Introduction

Entecavir (ETV) is an antiviral medication utilized for treating hepatitis B virus (HBV) infections where nearly 400 million people are chronically infected with HBV worldwide. ETV is a purine derivative, is chemically designated as 2-amino-9-[1S,3R,4S)-4-hydroxy-3-(hydroxymethyl)-2-methyledenecyclopentyl]-3H-purine-6-one. It is a potent guanosine nucleoside analog that is recommended as a first-line treatment for chronic HBV infection by inhibiting reverse transcription and DNA replication. Additionally, it is prescribed for managing chronic hepatitis B in adults co-infected with human immunodeficiency virus (HIV) [1]. A literature review described a few analytical methods reported for determining ETV in pharmaceutical forms and plasma. These include HPLC [2,3] UPLC MS/MS [4], electrochemical sensors [5], spectrophotometric methods [6], capillary zone electrophoretic method [7]. However prolonged monotherapy with ETV can lead to drug resistance. To enhance efficacy and reduce resistance, early combination therapy of ETV and lamivudine is preferred, supported by clinical evidence from HIV treatment and in vitro studies [8]. The simultaneous determination of ETV and lamivudine was reported using UPLC-MS/MS [9].

Molecularly imprinted polymers (MIPs) are 3D- highly cross-linked rigid tailor-made polymers that incorporate specially designed binding sites, thus known for their remarkable selectivity, like immunosorbents. They also exhibit significant physicochemical stability, predictable structure, reusability, and cost-effective to produce. MIPs are frequently used as sorbents in solid-phase extraction (SPE) or its miniaturized forms in food, environmental, biological, and pharmaceutical analysis

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[10–12]. Since the pioneering work of Sellergren and colleagues in 1994 [13], which focused on extracting pentamidine from human urine, molecularly imprinted solid-phase extraction (MISPE) has garnered significant interest in detecting various drugs in biological samples. However, due to the unique properties of MIPs, the enrichment capacity and selectivity of MISPE are generally superior [14]. MISPE provides several advantages such as simplicity, rapidity, specificity and high sensitivity due to the lock-and-key type of interaction.

Although MIPs attained mass production in academia and industry as they meet requirements of "sustainability" including reusability and selectivity, yet the impact of MIPs on the environment is still not concise, especially on two areas: human health and waste production (during and after synthesis) [15,16]. Thus, in order to bridge this gap between MIPs manufacture and the twelve principles of Green Chemistry, fourteen principles of sustainable molecular imprinting in the form of an acronym "GREENIFICATION" was introduced as shown in **Fig. 1SM.** The "GREENIFICATION" framework shall review the aspects of renewable starting materials, sustainable polymerization approaches, green strategies and signal accusation [17].

The extraction of ETV has been conducted using conventional techniques, such as protein precipitation [18], liquid-liquid extraction [19], cloud point extraction [20], and solid-phase extraction [21,22]. The reported extraction methods show several limitations when compared to MISPE including the addition of several interferents (as in salting out) [19,20], higher matrix effect, limited reusability [21,22], lower selectivity and extraction recovery, in addition to hindered ecological impacts [19–22].

No peer-reviewed studies have yet detailed the development and application of an MIP for detecting ETV in bulk or biological samples. Thus, this study aims to synthesize an ETV-MIP and develop a MISPE procedure for preparing plasma samples ahead of HPLC analysis. The study was evaluated against similar reported methods for ETV using environmental three colors assessment (ETCA): greenness, blueness and whiteness. In addition, the sustainability profile of the proposed study was generated using EVG (Efficient- Green -Valid) framework and NQS (Need-Quality-Sustainability) index.

2. Experimental

2.1. Materials and chemicals

EVA Pharma (Egypt) gifted the ETV standard with a purity of 99.89 ± 0.31. Analytical grade of dimethyl sulfoxide, methyl acrylate, 2,2′-Azobis (2-methyl propionitrile) 98 %, ethylene glycol dimethacrylate 98 %, sodium hydroxide, hydrochloric acid, phosphoric acid, and hydrogen peroxide were used. Methanol of HPLC grade was used. Tecavir® (containing 0.5 mg ETV) and Zeffix® (containing 100 mg of lamivudine) film-coated tablets were purchased from the local market. Plasma was purchased from VACSERA, Egypt.

2.2. Apparatus

Agilent 1260 Infinity-II liquid chromatographic system equipped with a gradient quaternary pump -model: VL G7111A, an autosampler – model: G7129A, a diode array detector – model: HS G7117C, and ChemStation software (USA). Thermo 6700 Fourier Transform Infrared FT-IR Spectrometer (USA). Jenway pH meter 3510 (US). JEOL transmission electron microscope (TEM) model JEM-2010 (Japan). KEMOLO FD-500 lyophilizer (China). BT Lab Systems Thermostatic Water Bath – model BT2303 (Japan). Carbolite AX60 Laboratory Oven 60 L (UK).

2.3. Synthesis of the molecular imprinted polymer (MIP)

Polymers were produced using a bulk polymerization method as detailed in previous research [23]. The molecularly imprinted polymer (MIP) was synthesized using ETV as the template molecule. In a sealed glass reactor, ETV (0.3 g) was dissolved in dimethyl sulfoxide (DMSO), followed by the addition of methacrylic acid (MAA, 0.34 g) as the functional monomer. The mixture was agitated for 15 min to facilitate self-assembly of the template-monomer complex. Ethylene glycol dimethacrylate (EGDMA, 0.164 g) and azobisisobutyronitrile (AIBN) were subsequently introduced as the cross-linker and radical initiator, respectively. The reaction system was deoxygenated via argon sparging (5 min), sealed under inert atmosphere, and polymerized at 60 °C for 24 h under continuous stirring. The corresponding nonimprinted polymers (NIP) were synthesized using the same procedure without the template molecule.

The resultant copolymer (MIP) was isolated and subjected to Soxhlet extraction with a methanol/acetic acid solution (9:1, *v*/v) until chromatographic analysis confirmed complete template removal. Residual impurities were eliminated by washing with deionized water, and the purified MIP was dried at 100 °C. Morphological and structural analyses included high-resolution transmission electron microscopy (HRTEM) was applied for surface topology evaluation, and Fourier-transform infrared (FTIR) spectroscopy (500–4000 cm^{-1}) was used to monitor functional group changes pre- and post-template extraction.

2.4. Loading MIP cartridges

Commercially available 6 mL polypropylene solid-phase extraction (SPE) cartridges were first emptied and subjected to sequential sonication in methanol and distilled water using an ultrasonic bath to ensure removal of contaminants. The cartridges were then thoroughly dried in a desiccator at an ambient temperature. Subsequently, 200 mg of the synthesized molecularly imprinted polymer (MIP) were suspended in methanol and carefully introduced into the cartridges utilizing a Phenomenex SPE vacuum manifold (Torrance, USA). To secure the sorbent within the cartridge, both lower and upper frits were employed. The assembled cartridges were stored in a desiccator at room temperature until further application.

2.5. Preparation of standard solutions and forced degradation products

A stock standard solution (1 mg/mL) of ETV was prepared in distilled water. Working solutions, with a final concentration of 250 µg/mL, were obtained by appropriate dilution of the stock solution using the mobile phase specified for the chromatographic analysis.

Forced degradation products were generated according to the reported methods [24,25] at a drug concentration of 1 mg/mL. Acidic and basic hydrolysis were carried out by refluxing with 1 M HCl and 1 M NaOH for 36 and 72 h, respectively, at 60 °C. The degradation products were confirmed using FT-IR spectrophotometry as shown in **Fig. 2SM.** Some differences were discovered by comparing the intact ETV IR spectra to the IR spectra of basic and acidic degradation products. The distinction lies in the existence of functional group N—O in acidic degradation at 1530 cm^{-1} and the absence of C═O functional group in basic degradation.

2.6. Chromatographic conditions

Elution was optimized using an Inertsil® ODS column (4.6 × 250 mm, 5 µm I·D). The mobile phase comprised 0.1 % phosphoric acid in water (solution A) and methanol (solution B), applied in a gradient mode. Prior to use, the mobile phase was filtered through a 0.45 µm Millipore membrane filter (Billerica, MA) to ensure purity. The flow rate was maintained at 1 ml/min under ambient conditions. A 10 µL sample was injected for analysis, with UV detection performed at 245 nm.

2.7. Applications using molecular imprinted polymer solid phase extraction (MISPE) and oasis HLB

For the assay of the dosage form, 10 tablets of Tecavir® 0.5 mg film-

coated tablets were ground into a fine powder and then the weight of one tablet was transferred to a 50-mL volumetric flask where the powder was dissolved in distilled water by vortex for 10 min, and the volume was then completed to the mark with distilled water to form working solution (A). For the spiked plasma samples, one mL of a plain plasma sample was mixed and vortexed for 60 s with aliquots of ETV and lamivudine to prepare a working solution (B) containing 10 μg/mL of each of ETV and lamivudine. For stress-testing, aliquots of ETV were mixed with aliquots of the prepared forced degradation products to form working solutions (C1-C5) containing different ratios of ETV, acidic, and basic hydrolysis products. The MISPE and Oasis HLB cartridges were initially preconditioned with 1 mL of methanol, followed by 1 mL of water. Subsequently, 1 mL of each working solution (A, B, and Cs) was loaded into three separate cartridges. A vacuum was applied to retain the samples. Each cartridge was then washed three times with 5 mL of methanol before elution.

For elution, each cartridge was subjected to a vacuum and rinsed three times with 3 mL of methanol. The eluates were collected in a 10-mL volumetric flask, which was filled to the mark with the same solvent. A rotary vacuum concentrator was employed to evaporate the eluent for 2 h at 1500 rpm and 60 °C. After re-constituting the samples with the mobile phase, they were injected into the HPLC system.

3. Results and discussion

3.1. MIP characterization and performance

In this investigation, the optimal ratio of the chosen monomer, methacrylic acid (MAA), to the template molecule, Entecavir (ETV), was determined to be 4:1 in accordance with established methodologies. Dimethyl sulfoxide (DMSO) was employed as the aprotic solvent due to its favorable properties, including non-toxicity and thermal stability.

The molecular imprinting process, along with morphological characterization and pore size evaluation of the synthesized molecularly imprinted polymer (MIP), was carried out using Fourier-transform infrared spectroscopy (FTIR) and transmission electron microscopy (TEM). Analysis of the TEM image of the leached MIP revealed a distinctive irregular morphology, which is indicative of the bulk polymerization technique, as depicted in Fig. 1.

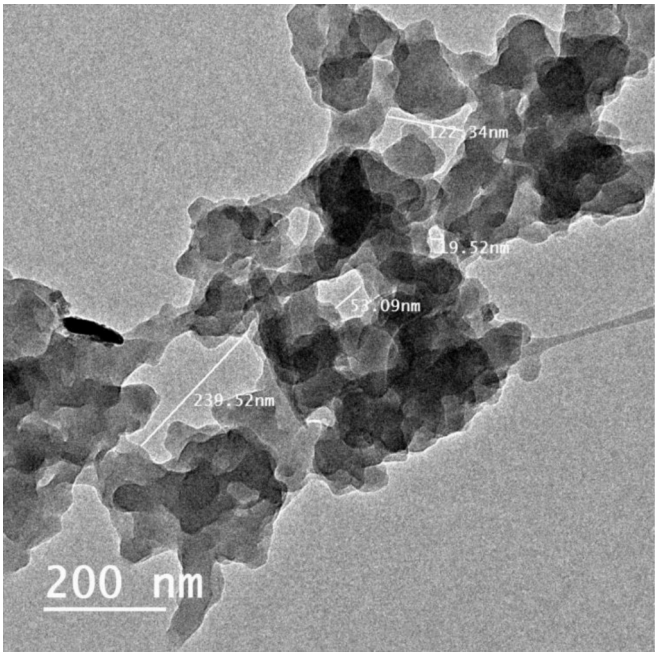


Fig. 1. High-resolution transmission electron microscope (TEM) image of the synthesized MIP.

FTIR analysis confirmed polymer formation by comparing the spectra of leached and unleashed polymers. The FTIR of the unleashed polymer showed O—H stretching at 3500 cm^{-1}, indicating interactions between the polymer and ETV. Peaks associated with the polymer matrix, such as the C═O stretching of carboxylic acid groups (~1700–1720 cm^{-1}) and C═C stretching (~1600–1650 cm^{-1}), remained unchanged after washing, confirming the integrity of the MIP. However, a decrease in intensity was observed at 1164 cm^{-1} and 1726 cm^{-1}. The IR spectra was shown in **Fig. 3SM.**

The adsorption performance of the polymers was evaluated through a dynamic adsorption curve and a static binding test. The proposed HPLC method was exploited to precisely assess the binding capacity. Specifically, 25 mg of MIP or NIP was added to 5 mL of a 250 μg/mL ETV solution. The binding capacities (Q) for both polymers were calculated using the equation:

$$Q = (C_i - C_f) \times V / M_{polymer}$$

Where **Q** is the binding capacity (μg g^{-1}), $\mathbf{C_i}$ and $\mathbf{C_f}$ are the initial and final free ETV concentrations (μg), respectively. **V** is the volume of solution **(mL)** and $\mathbf{M_{polymer}}$ is the mass of polymer (g). In the kinetic study, the adsorption capacity was measured from 15 to 90 min. The MIP's capacity increased initially but slowed as its specific surface holes were filled and mass transfer resistance increased, reaching saturation at approximately 30 min as shown in **Fig. 4SM.** The NIP, relying only on non-specific physical adsorption, showed a lower capacity and faster equilibrium. In addition, the imprinting factor (IF) was then deduced, using the following equation, based on the maximum binding capacity derived from Langmuir isotherm models:

$$IF = \frac{Q_{MIP}}{Q_{NIP}}$$

The IF of 3 obtained confirms the successful creation of specific binding cavities within the MIP that are complementary to the template molecule in both shape and chemical functionality, as opposed to the non-specific binding exhibited by the NIP.

3.2. HPLC method development

Several trials were performed to separate the drug of interest (ETV) from its co-administered drug (lamivudine) and its forced degradation products using the same elution conditions. The separation trials were conducted using the stationary phases C8 and C18, where the C18 column showed better resolution between ETV and its forced degradation products. Regarding the mobile phase composition, Acetonitrile failed to achieve proper resolution, where ETV showed a tailed peak. Thus, methanol was chosen as the organic part of the mobile phase together with 0.1 % phosphoric acid solution. The gradient mode, listed in Table 1, was employed to achieve the best separation between ETV and lamivudine and its forced degradation products on the other hand (Fig. 2). The mobile phase was delivered at a flow rate of 1 ml/min with an injection volume of 10 μL and detection at 245 nm. The retention times of ETV and lamivudine are 6.86 and 5.80 ± 0.0.1 min,

Table 1
The chromatographic conditions of the HPLC method.

Column	Inertsil® ODS column (4.6 × 250 mm, 5 μm I·D)
Mobile phase	Solution A: 0.1 % phosphoric acid in water Solution B: Methanol
Gradient program	0 to 8 min: Solution A (98 %): Solution B (2 %) 8 to 9 min: Solution A (40 %): Solution B (60 %) 9 to 12 min: Solution A (2 %): Solution B (98 %)
Temperature	25 °C
Flow rate	1 mL/min
Injection volume	10 μL
Detection	245 nm

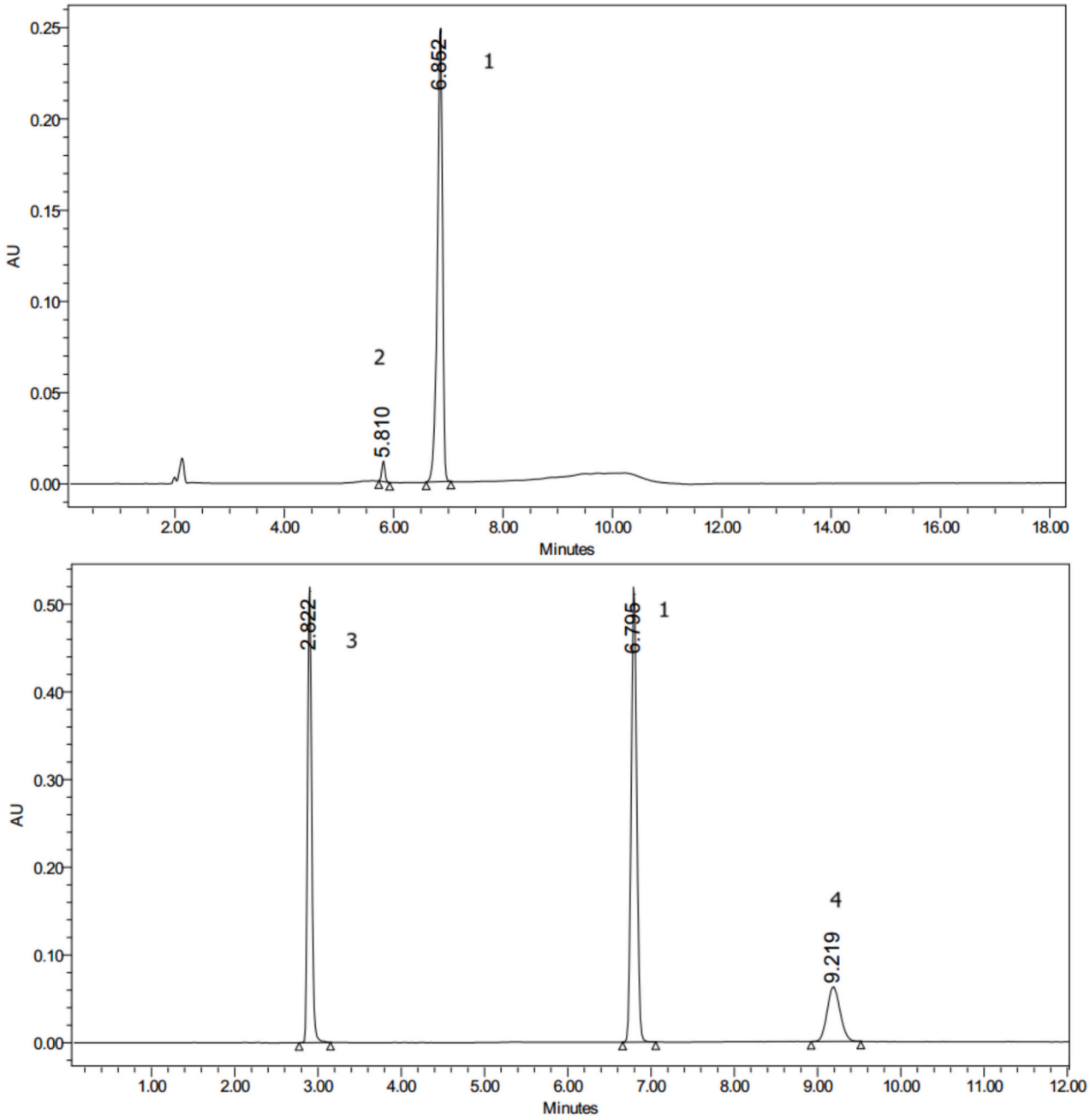


Fig. 2. Chromatogram using the adopted chromatographic conditions showing 10 µg/mL of (1) ETV R_t 6.86 min, (2) lamivudine R_t 5.81 min, (3) acidic degradation Rt 2.82 min, and (4) basic degradation Rt 9.42 min.

respectively. Likewise, the acidic and basic degradation products were eluted at 2.80 and 9.42 ± 0.02 min, respectively.

3.3. Method validation

Via ICH guidelines [26], method validation was carried out where all values were found within accepted reference range as listed in Table 2. The calibration curve was carried out for three days within concentration ranges of (5–250 µg/mL) using external standard. The ratio peak area [peak area/peak area of external standard (10 µg/mL)] was plotted versus the corresponding concentration of ETV where the correlation coefficient was used to assess linearity. Limits of detection and quantitation, LOD and LOQ, respectively, were estimated using the calculation method, using a ratio of 3.3and 10 of standard deviations of the blank to the slope of the calibration line, respectively. To assess accuracy, five blind concentrations of ETV were subjected to the procedure of linearity, and the results were expressed as recovery % ± standard deviation (SD). The precision study was carried out as a triplicate analysis of three concentrations of ETV (25, 100, 200 µg/mL) on the same day as intra-day precision, and on three consecutive days as inter-day precision. Both precision studies were estimated using relative standard deviation (RSD). For robustness testing, the separation was done with deliberate changes in elution conditions (flow rate and temperature) by 10 %. The robustness was expressed as RSD% for three concentrations of ETV. Tailing factor (T), capacity factor (k), theoretical plate (N), and resolution were calculated for 100 µg/mL standard solution as the system suitability parameters.

According to FDA bioanalytical validation [27], extraction recovery was calculated using the peak areas of ETV for post-extracted spiked human plasma compared to the peak areas of ETV for the pre-extracted

Table 2
Validation parameters and system suitability for the proposed HPLC method.

Validation Parameters for ETV		
Range (μg/mL)		5–250
Correlation coefficient (r)		0.9999
Slope		0.072
Intercept		0.3109
SD of residuals from line		0.0354
LOD (μg/mL)		1.625
LOQ (μg/mL)		4.923
Accuracy (Recovery % ± SD)		99.79 ± 0.82
Precision (RSD%)	Intraday	0.988
	Interday	1.113
Robustness		0.921
System suitability		
Tailing factor (T)		1.1
Capacity factor (K′)		2.48
Platelet number (N)		9813
Height equivalent to theoretical plate (cm plate $^{-1}$)		0.025
Resolution (Rs)		11.65[a], 9.88[b], 8.27[c], 2.88[d]

Rs between ETV and [a] lamivudine, [b] oxidative degradation product, [c] acidic degradation product, [d] basic degradation product.

samples of LQC (5 μg/mL) and HQC (100 μg/mL) in order to evaluate the extraction recovery. The average recovery after extraction was calculated as 88.32 %, which demonstrated the efficiency of the extraction process. Matrix effect was calculated using the peak areas of the post-extracted plasma compared to those of the neat solutions of ETV at the same concentration levels LQC and HQC levels. The average matrix effect was calculated as 91.71 %, which demonstrated that the co-eluted matrix's potential components had an insignificant effect on the ETV separation.

3.4. Specificity of MIPSE cartridges

3.4.1. Estimation of ETV in spiked plasma samples

Human plasma samples were spiked with both ETV and lamivudine to contain 10 μg/mL of each drug. The plasma samples were extracted using the synthesized MIP and commercial Oasis HLB solid phase cartridges. The extraction was performed as described under 2.7. in triplicate and compared for the 2 cartridges. The resulting chromatogram after plasma elution, using MIPSE, showed ETV peak only as shown in **Fig. 5SM**, confirming the elution of ETV using its retention time with no other eluted peaks. The average recovery % of ETV was calculated to be 89.14 ± 1.44, while lamivudine wasn't detected. On the other hand, the elution using commercial Oasis HLB showed elution of lamivudine, where the average recovery % of ETV and lamivudine was calculated to be 78.22 ± 2.54 and 63.44 ± 3.11, respectively. These results prove the high selectivity of the MIPSE towards ETV due to its distinct binding sites, while other interfering drugs were removed during the washing phase where ETV was not affected.

3.4.2. Estimation of ETV in presence of its degradation products

Competitive binding of ETV, basic and acidic degradation products to the polymer was done to assess the MIP's specificity towards extracting ETV in the presence of any other related compounds from any matrix other than plasma. Five synthetic mixtures (C1-C5) covering various percentages of ETV with its two degradation products were prepared by mixing the working solutions of ETV with its 2 degradation products solutions in the ratios listed in Table 3. The synthetic mixtures were extracted using the synthesized MIP and analyzed according to the previous chromatographic conditions. Adequate results were listed in Table 3 showing the high specificity of the MIP cartridges in extracting ETV in the presence of up to 90 % of its degradation products.

3.5. Reusability of MIP cartridges

One of the advantages of MIP cartridges over traditional silica-based cartridges is their reusable nature. Using the ETV standard, it was discovered that each cartridge may be utilized up to ten times with a reduction of 2 % in the recovery percentages. The cartridge can be refreshed three times using 3 mL of a 9:1 *v*/v mixture of methanol and glacial acetic acid.

Table 3
Determination of ETV in presence of its acidic and basic degradation products in laboratory prepared mixtures after MIPSE.

Laboratory prepared mixture %				Recovery %		
No.	ETV	Acidic DP	Basic DP	ETV	Acidic DP	Basic DP
C1	80	10	10	89.33	N·D	N·D
C2	60	20	20	87.44	N·D	N·D
C3	40	30	30	85.24	N·D	N·D
C4	20	40	40	84.10	3.33	N·D
C5	10	45	45	82.55	9.11	2.34
			Mean	85.72 ± 2.69		

DP: degradation product.
ND: not detected.

3.6. Environmental three-color assessment (ETCA)

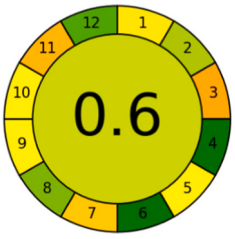
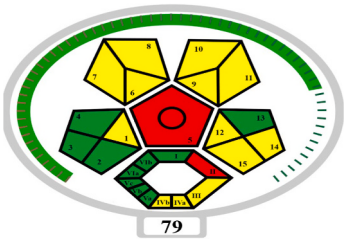
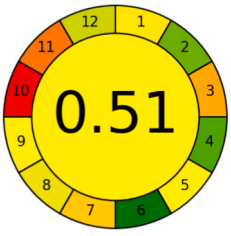
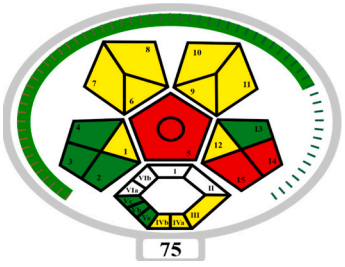
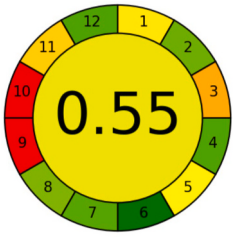
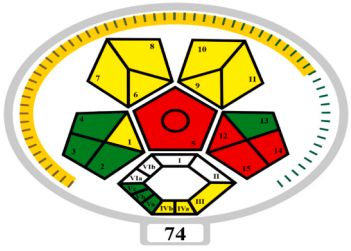
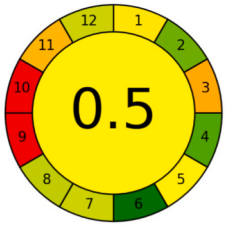
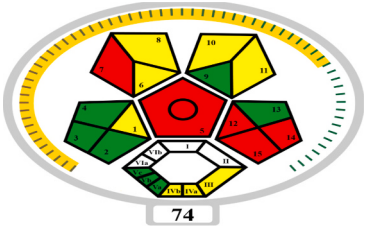
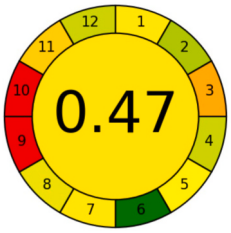
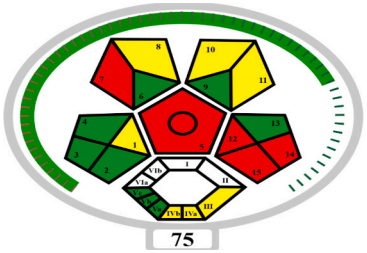
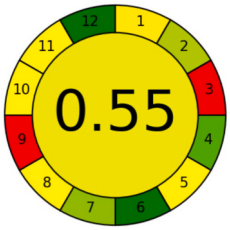
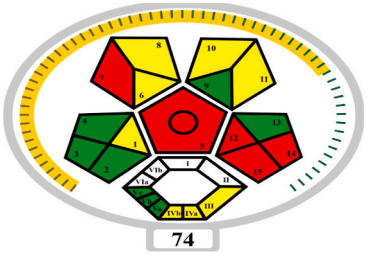
3.6.1. Greenness assessment

3.6.1.1. AGREE. The AGREE meter provides a pictogram that is divided into 12 sections, where each section is colored from deep green (highest) to deep red (lowest), representing each of the 12 Green Analytical Chemistry (GAC) principles. Finally, a total score is calculated, with one as the highest [28]. The proposed method showed a pictogram altered to a greener color with a score of 0.6. This can be correlated to the greener sections: 2, 4, 6, 8, and 12, showing a small sample size, integrated analytical process to save energy (HPLC-UV) and reagents, avoiding derivatization, multi-sample analysis (up to 5 components), and high operator safety. The drawbacks were the position of the analytical device and the use of methanol. The detailed AGREE report is shown **Table 1SM.** When comparing the reported methods to the proposed method, it was found that the reported method [2] showed a low score of (0.51) due to the use of acetonitrile as a major part of the mobile phase, while the rest of the reported methods [9,18,19,21,22] showed lower scores ranging from (0.47–0.55) due to the high-energy technique LC-MS/MS, in addition to the use of non-green solvents as acetonitrile in method [18] and formic acid in the method [19]. The AGREE pictograms for the proposed and reported methods were constructed as shown in Table 4.

3.6.1.2. AGREEMIP. This meter provides a pictogram that is divided into 12 sections to assess and the greenness of MIP synthesis where each section is colored resembling the shades of AGREE meter. The 12 sections correspond to the operator's safety, reagents, energy consumption and waste production. The input data of the 12 criteria are transformed into an overall score from 0 to 1 [29]. The synthesized MIP was assessed using AGREEMIP as shown in Fig. 3. The pictogram showed 8 green criteria out of 12 and an overall score of 0.65, which reveals good GREENIFICATION of the MIP synthesis process. The detailed assessment using this tool is listed in **Table 2SM**.

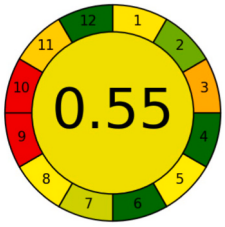
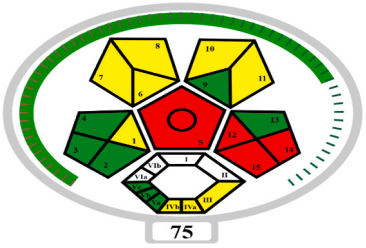
3.6.1.3. ComplexMoGAPI. ComplexMoGAPI was recently introduced for integrating the visual representation of ComplexGAPI tool with a numeric total score [30]. ComplexGAPI was formerly presented as a complementary tool for the recognized GAPI tool [31,32]. The tools represent green analytical chemistry (GAC) through 5 pentagrams divided into smaller sections. Moreover, both tools show the integration between GAC and green economy during the synthesis process. ComplexMoGAPI was a useful tool for the assessment of the synthesis step of the MIP. The impact of each GAC aspect is color-displayed from high

Table 4
Comparison of Greenness and Blueness assessment of the proposed method versus reported ones.

Method	AGREE	ComplexMoGAPI	BAGI
Proposed			
Ref [2]			
Ref [9]			
Ref [18]			
Ref [19]			
Ref [21]			

(continued on next page)

Table 4 (*continued*)

Method	AGREE	ComplexMoGAPI	BAGI
Ref [22]			

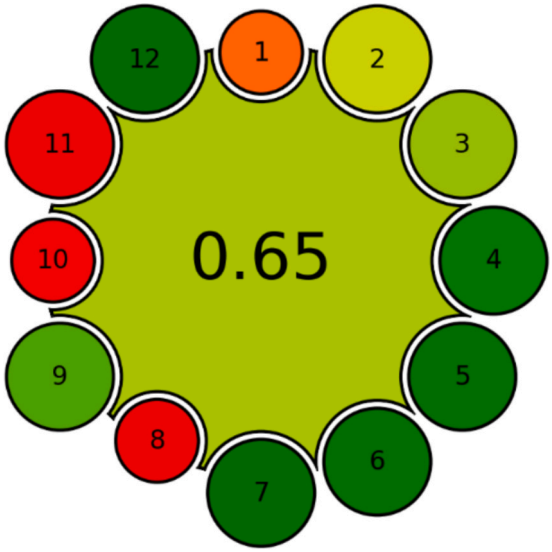


Fig. 3. AGREEMIP pictogram for the synthesized MIP.

(red) to medium (yellow) to low (green). The pentagrams in Table 4 reveal a greener profile for the proposed method (score 79) with a smaller number of red pentagrams when compared to the reported methods due to minimizing the use of non-green solvents during the synthesis of MIP or the analysis, and the use of energy-saving instrumentation. The reported methods [9,18,19,21,22] showed profiles ranging from yellow to green and scores (74 to 75) due to applying the high-energy technique LC-MS/MS, in addition to the use of greater amounts of non-green solvents.

3.6.2. Blueness assessment

The blue applicability grade index (BAGI) aims to assess 10 attributes regarding the feasibility, productivity and economic efficacy of the analytical method [33] as listed in **Table 3SM**. Each attribute is hued (for qualitative assessment) and record a score (for quantitative assessment) in the descending order: dark blue (10 points), blue (7.5 points), light blue (5 points), and white (2.5 points).

The proposed method recorded an overall score of 75, as shown in Table 4, which indicates the cost-effective and practicality of the procedure. It showed 3 dark blue hues (10 points) for the attributes (1, 7 and 8) which was reflected in the high selectivity and sensitivity of the method. Four blue hues (7.5 points) were recorded for the attributes (3, 6, 9 and 10) because of using simple semi-automated instrumentation with high output analysis and small size samples. Three light blue hues (5 points) were recorded for the attributes (2, 4 and 5) for the possible simultaneous sample analysis (up to 5 analytes) with a miniaturized extraction scale. Reported methods showed lower scores (65 and 67.5), due to the use of sophisticated apparatus (LC-MS/MS) in the methods [9,18,19,21,22] and single analyte analysis in the methods [2,18,21].

3.6.3. Whiteness assessment

White Analytical Chemistry (WAC) applies the RGB algorithm-12 which involves three aspects where each aspect ends up recording a score from (0−100), with (100) being the highest. The three aspects are: red (R1-R4), green (G1-G4), and blue (B1-B4) that corresponds to analytical performance, environmental impact, and economic efficiency, respectively. A method should agree with the three aspects to be as recognized "white" [34,35].

A comparison between the proposed versus reported methods showed differences in the three-color criterion. The proposed method recorded a red score of (92.5 %) due to their low sensitivity (lower LOD and LOQ values) but high validation parameters (accuracy and precision scores). In addition, the proposed method earned a higher green score (90 %) than LC-MS/MS methods [9,18,19,21,22] (76.7 % - 82.9 %) due to consuming less solvent and low- energy apparatus. The proposed method is considered as simple and economic when compared to the reported methods [9,18,19,21,22], making the blue criteria gain more score (85.4 %). The overall whiteness score of the proposed method is (89.3 %), which is almost equal to the whiteness score (89.7 %) of the highest reported method [2], but yet the proposed method beats it in the red score as shown in Fig. 4.

3.7. Sustainability profiling

3.7.1. EVG framework

The EVG framework displays its sustainability profile through its three pillars: (E) efficiency, (V) validation, and (G) greenness [36]. A score (from 0 to 3) is calculated for each pillar as an average from its five evaluation criteria (A-E) as shown in **Table 4SM**. Based on its average score, each pillar would be placed into one of four quartiles (Q1, Q2, Q3, or Q4), which are ordered descendingly. To ensure that the analytical process is sustainable through reliability, efficiency, and being eco-friendly, the three pillars should all fall into the same quartiles or even close ones in order to attain a high competency profile. A radar chart is used to represent the sustainability profile of the suggested method in order to determine whether a balance between the three pillars has been reached; if not, suggestions for changes should be made [37–39].

By applying the EVG framework, all three pillars of the proposed method lie in the second quartile which indicates a balance point of sustainability with the studied pillars as shown in Fig. 5. The efficiency pillar was assigned to (Q2) due to the method's capability to study ETV in the presence of its co-administered drug (lamivudine) in addition to its two degradation products. The validation pillar (Q2) was assigned to (Q2) due to high accuracy and precision values despite its relatively high LOQ. The greenness pillar was assigned to (Q2) due to applying several greenness tools which cover all aspects of GAC, in addition to low waste production, safe solvent, and low energy consumption.

3.7.2. NQS index

The sustainability of the proposed method was evaluated using the NQS Index against sustainable development guidelines. This innovative

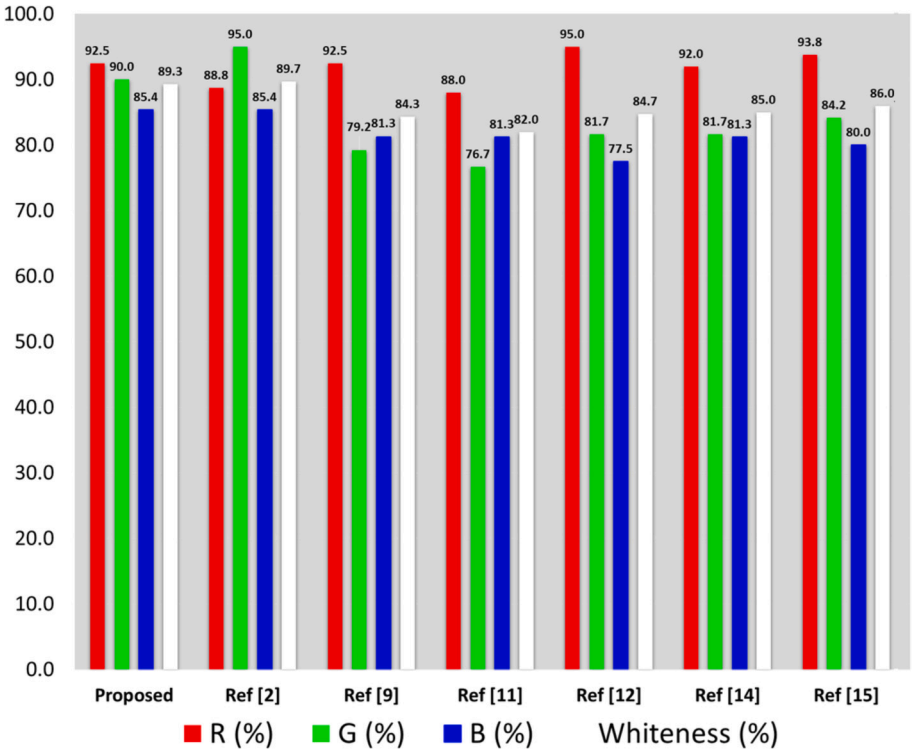


Fig. 4. RGB algorithm-12 and total whiteness score calculated for the proposed methods and compared to the reported methods [9,11,12,14,15].

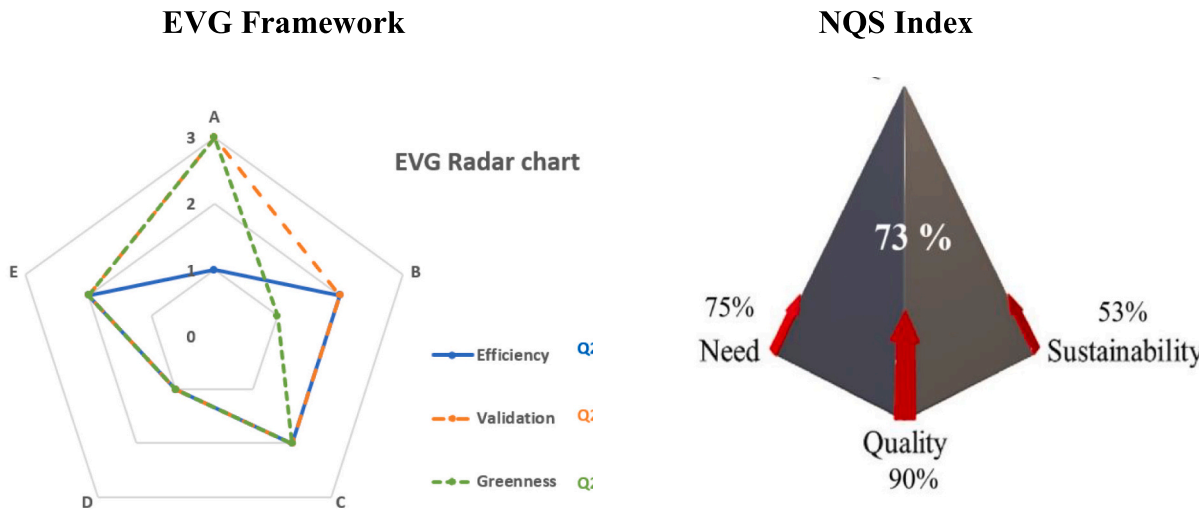


Fig. 5. Sustainability profile of the proposed method generated by EVG framework and NQS index.

index presents Koel's pyramid of the analytical method by combining three dimensions: "*Need*"," *Quality*", and "*Sustainability*" provide a thorough assessment of the method's overall performance. The *Need* % is estimated by giving a 100 % score to each of the four tiers of Koel's pyramid assigned at 25 %, 50 %, 75 %, and 100 %, respectively. *Quality* % is estimated based on average score of WAC using the RGB 12 algorithm. The *Sustainability* % considers the agreement of the analytical method with the 17 SDGs, then the number of agreements is added and divided by 17 which is the total number of SDGs [40,41].

The method showed an alignment with the UN-SDGs 3, 4, 5, 7, 9, 12,13,14, 15, and 17, focusing on goals such as health (SDG 3), quality education (SDG 4), affordable and clean energy (SDG 7), and responsible consumption and production (SDG 12), detailed in **Table 5SM**. The proposed method scored a high NQS Index rate of 73 % that is visually represented by the NQS triangular pyramid shown in Fig. 5, proving the balance between need, quality, and sustainability needed for a sustainable method. Applying the NQS Index in a novel way strengthens the method's impact on the scientific community's push for sustainable practices, aligning scientific novelty with global sustainability objectives.

4. Conclusion

A molecularly imprinted polymer (MIP) was synthesized and characterized for the selective extraction of Entecavir (ETV) from spiked human plasma, which also contained its co-administered drug, lamivudine. The synthesized MIP was further applied to isolate ETV in the presence of its acidic and basic degradation products. The specific

extraction process was followed by a robust and efficient RP-HPLC method developed for the quantification of ETV among potential interfering compounds. The MIP cartridges exhibited superior selectivity, reusability, and high recovery rates (>90 %) in comparison to commercially available alternatives. To evaluate the analytical approach, the method was assessed using an environmental three-color classification: greenness (AGREE, AGREEMIP and ComplexMoGAPI), blueness (BAGI), and whiteness (RGB-12 algorithms). Furthermore, the sustainability profile of the developed method was established based on the (EVG) framework and the NQS index. The results demonstrated that the proposed approach is environmentally sustainable, meeting the criteria of the UN-17 Sustainable Development Goals.

CRediT authorship contribution statement

Sarah S. Saleh: Writing – review & editing, Writing – original draft, Resources, Project administration, Investigation, Formal analysis. **Heba T. Elbalkiny:** Writing – review & editing, Writing – original draft, Validation, Methodology, Investigation, Conceptualization.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jchromb.2025.124843.

Data availability

Data will be made available on request.

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