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Towards circular analytical chemistry: Optimization of colorimetric assay for sustainable and selective dopamine detection in pharmaceutical formulations using green solvents

Doretta Cuffaro [a,*], Fabio Spiaggia [a,b], Enrico Crispino [a], Andrea Mezzetta [a], Elisa Nuti [a], Vincenzo Calderone [a], Lorenzo Guazzelli [a], Pasquale Palladino [c,1], Maria Minunni [a,*]

[a] Department of Pharmacy, University of Pisa, Via Bonanno 6, 56126 Pisa, Italy
[b] Département de Pharmacochimie Moléculaire, Université Grenoble Alpes, UMR 5063, Grenoble, France
[c] Department of Chemistry 'Ugo Schiff', University of Florence, Via della Lastruccia, 3, 50019 Sesto Fiorentino FI, Italy

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ABSTRACT

The drive towards sustainability in analytical chemistry has prompted the replacement of hazardous solvents with greener alternatives, in line with circular chemistry principles. We here report a rapid, low-cost, and sustainable colorimetric assay for dopamine (DA) detection in pharmaceutical formulations. Building on our previously developed dimethyl sulfoxide (DMSO)-based melanochrome (MN) assay, three bio-based solvents—Cyrene™, γ-valerolactone (GVL), and N-butylpyrrolidone (NBP)—were evaluated as DMSO substitutes. Screening indicated that DMSO and NBP supported effective MN formation, whereas GVL and Cyrene were unsuitable. NBP was selected for optimization using a Design of Experiments (DoE) strategy to assess pH, solvent fraction, and temperature effects. Temporal contributions and interactions of parameters were analyzed through dynamic DoE modeling, with curvature effects evaluated via triplicate center-point measurements. Model predictivity was verified at the center and at the predicted maximum absorbance region, achieving the highest signal at 30 min. The optimized assay (NBP:buffer 1:4 v/v, pH 10.0, 45 °C) exhibited excellent linearity ($R^2 = 0.9998$) across 11–80 μg/mL, a low detection limit (LOD 1.35 μg/mL), and quantification limit (LOQ 4.59 μg/mL). Compared to the reference DMSO system, the NBP assay showed improved sensitivity and reduced solvent usage, minimizing environmental impact. Selectivity was confirmed against adrenergic drugs (adrenaline, noradrenaline, ephedrine, etilefrine, dobutamine), none of which produced MN chromophore. Thes results demostrated method robustness in clinically relevant contexts. Overall, the proposed NBP-based assay integrates eco-friendly solvent selection with DoE-guided optimization, delivering sensitivity, selectivity, and practicality for pharmaceutical quality control. Beyond simple solvent substitution, this study presents a systematic green re-evaluation of a validated analytical protocol, effectively balancing sustainability, functionality, and analytical performance.

List of Abbreviations

AGREE – Analytical GREEnness metric
A – Adrenaline
BAGI – Blue Applicability Grade Index
DA – Dopamine
DOBU – Dobutamine
DoE – Design of Experiments
DMSO – Dimethyl sulfoxide
EE – Etilefrine
Ef – Ephedrine
GAC – Green Analytical Chemistry
GVL – γ-Valerolactone
KAT – Kamlet–Abboud–Taft parameters
LOD – Limit of Detection
LOQ – Limit of Quantification
MN – Melanochrome
NA – Noradrenaline

* Corresponding authors.
E-mail addresses: doretta.cuffaro@unipi.it (D. Cuffaro), pasquale.palladino@unifi.it (P. Palladino), maria.minunni@unipi.it (M. Minunni).
[1] Co-last.

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NBP – N-Butylpyrrolidone (1-butylpyrrolidin-2-one)
OVAT – One-Variable-At-a-Time
RSD – Relative Standard Deviation
RSM – Response Surface Methodology
UV–Vis – Ultraviolet–Visible spectroscopy

1. Introduction

The growing emphasis on sustainability in analytical chemistry has prompted a global reassessment of traditional methodologies, particularly in relation to environmental impact and safety concerns. Colorimetric assays are experiencing a renaissance [1,2], reimagined into greener, more sustainable formats without compromising sensitivity or versatility. These eco-friendly adaptations substitute toxic reagents with natural or less hazardous alternatives while minimizing waste, energy usage, and the safety risks posed by organic solvents [3,4].

In recent years, transition to sustainability has boosted the search/quest for alternatives to traditional organic solvents, which raise several concerns related to both their environmental and health impacts [5]. Indeed, they are obtained or prepared from non-renewable fossil sources and display safety issues due to their decomposition, disposal or incineration [6]. Moreover, especially for polar aprotic solvents (i.e. DMF, NMP), significant health risks such as damage to fertility or even to the unborn child are well-known [7–9]. Dimethyl sulfoxide (DMSO) belongs to polar aprotic solvents family but shows an unusually safe toxicity profile which makes it suitable even in medical applications such as interstitial cystitis treatment, drug delivery systems or cryopreservation [10]. However, DMSO still presents safety issues due to its autocatalytic decomposition, which generates high heat and gas and possible explosion, which is why Janssen discourages the use in its plants, and produces SO_2 after its incineration, thus ultimately contributing to the increase of rain acidity [11,12]. Furthermore, DMSO is industrially produced from dimethyl sulfide, in turn obtained from methanol and hydrogen sulfide, a toxic, corrosive, and flammable gas [13]. Also, the appraised enhancing skin penetration properties of DMSO may also cause the absorption of toxins or unwanted contaminants if not properly handled [14].

Considering the application fields of DMSO and its yet unsolved problematic aspects, there is the urge to investigate the suitability of proposed alternatives. In our recent work, we developed colorimetric assays based on the formation of Melanochrome (MN) for detecting dopamine (DA), norepinephrine (NE), and levodopa [15]. These assays were performed using a 1:1 (v/v) DMSO-to-buffer mixture, with the buffer consisting of 150 mM $Mg(OAc)_2$ and 150 mM NH_4Cl in aqueous solution at pH 9.4, enabling detection in urine samples [16], and pharmaceutical formulations [15].

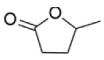
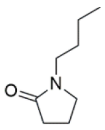
Following the main principles of green analytical chemistry [17], and to further improve the sustainability of our well-established colorimetric assay for DA detection, we explore here the replacement of DMSO with three bio-based solvents with comparable solvatochromic parameters: Cyrene™ (dihydrolevoglucosenone) as a sustainable alternative to traditional dipolar aprotic solvents, γ-valerolactone (GVL), and 1-butylpyrrolidin-2-one (NBP) [18–20]. However, it is important to analyze *pro* and *cons* of their use in real cases to better understand the practical suitability of the proposed new solvents (see Table 1). All the selected solvents can be produced from bio-renewable materials. For example, GVL can be easily prepared from levulinic acid, itself obtained from the acid breakdown of cellulose and hemicellulose. It is a polar aprotic organic solvent with a five carbons cyclic ester ring that shows high boiling point (207–208 °C), low flammability (f.p 96 °C) and low toxicity (LD50 Oral-rat = 8800 mg/kg) [18]. Similarly, Cyrene can also be obtained from cellulose via a solvent-free two-step process with > 90 % product yield, involving the formation of levoglucosenone (LGO) and its subsequent reduction to Cyrene. The Kamlet-Abboud-Taft (KAT) parameters and Hansen solubility parameters (HSP) of Cyrene are similar to those of DMSO, making it an excellent sustainable alternative to the latter. Finally, NBP is another aprotic polar solvent that can be prepared from renewable sources [19]. It is a viable alternative to *N*-methyl-2-pyrrolidinone [16] being non-toxic to reproduction (according to the OECD414 test method), non-mutagenic (OECD471) and inherently biodegradable (OECD 302B) [21].

In this work, we present the first fully green and systematically optimized MN-based colorimetric assay for DA detection in pharmaceutical formulations. Building on our previous DMSO-based colorimetric assay, rather than performing a simple solvent substitution, we integrate bio-based solvent selection (Cyrene™, GVL and NBP), Design of Experiments and time-resolved kinetic modelling to identify an operating window that maximizes analytical performance while minimizing environmental impact.

The analytical performances obtained with DMSO and NBP were then compared. Optimization of assay conditions was carried out using a Design of Experiments (DoE) approach, unlike conventional one-variable-at-a-time (OVAT) methods, DoE enables the simultaneous evaluation of multiple parameters and their interactions, facilitating the identification of optimal conditions with a reduced number of trials. Specifically, the Sequential Experimentation approach [27] was adopted in this study. This methodology begins with two-level full-factorial designs. The inclusion of center points allows detecting possible curvature, which, only if present, indicates the need to transition to Response Surface Methodology (RSM).

Furthermore, to evaluate the kinetics of MN formation, a full-factorial model was independently built at each time point. This strategy, here termed Dynamic DoE Modeling, enabled the temporal resolution of factor effects (Temperature, pH, and green solvent percentage) and their interaction terms on the absorbance response. The optimized assay relies on MN formation under alkaline conditions in the presence of a green solvent system, providing a rapid, inexpensive, and instrument-free colorimetric platform for DA detection. Importantly, the method demonstrated high selectivity against structurally related adrenergic drugs commonly used in emergency and intensive care,

Table 1
Physicochemical and safety properties of NBP, GVL, Cyrene, and DMSO relevant to sustainable analytical applications, including structure, boiling point, polarity, origin, and toxicity.

Property	Cyrene™ [22]	γ-Valerolactone (GVL) [23,24]	*N*-Butylpyrrolidone (NBP) [25]	Dimethyl sulfoxide (DMSO) [10,26,27]
Structure				
Boiling Point (°C)	227	207	245	189
Polarity (δ_P)[21]	10.6	4.7	9.9	16.4
Sources	Bio-based – from cellulose	Bio-based – from levulinic acid	Bio-based – pyrrolidone derivatives	Fossil-derived – from dimethyl sulfide
Toxicity	Low – non-mutagenic, biodegradable	Low	Low/Moderate – biodegradable	Low/Moderate – skin penetration risks

underscoring its robustness and translational potential.

To the best of our knowledge, this study is the first to establish a fully green, MN-based colorimetric assay for DA detection, specifically tailored for pharmaceutical applications. Its scope extends well beyond conventional solvent substitution by integrating innovative solvent selection based on environmental criteria, systematic optimization through Design of Experiment (DoE), and validation in real-world pharmaceutical formulations. The resulting NBP-based platform offers a novel and sustainable alternative to traditional DMSO-based methods, achieving comparable or superior analytical performance while significantly reducing the environmental footprint. While many analytical methodologies focus primarily on sensitivity enhancement, fewer studies systematically integrate solvent sustainability, solvent volume reduction, and statistical optimization within a single analytical workflow.

Overall, this study illustrates the principles of circular and green analytical chemistry by integrating solvent innovation, DoE-based optimization, and selective colorimetric detection into a practical and sustainable strategy for neurotransmitter analysis.

2. Materials and methods

2.1. Chemicals and reagents

Water, Dimethyl sulfoxide (DMSO), γ-Valerolactone (GVL), *N*-Butyl-2-pyrrolidone (NBP), Dihydrolevoglucosenone (Cyrene) were HPLC grade. All of them were from Merck (Merck, Darmstadt, Germany), as well as the commercial analytical standards of DA, $Mg(OAc)_2$ and NH_4Cl. All solutions were prepared using water obtained from Milli-Q Water Purification System (resistivity ≥ 18 MΩcm) (Merck, Darmstadt, Germany). Green solvents: Cyren (≥98,5 %) and γ -valerolactone (>98 %) were purchased from Sigma Aldrich while 1-butylpyrrolidin-2-one (≥99.5 %) was obtained from Supelco (Merck, Darmstadt, Germany). Pharmaceutical-grade drugs were purchased as commercial vial formulations: dopamine (DA, Galenica Senese, Siena, Italy), adrenaline (A, Galenica Senese, Siena, Italy), noradrenaline (NA, Galenica Senese, Siena, Italy), ephedrine (E, Salf, S. p. A. Bergamo, Italy), etilefrine (EE, Serb S.A., Paris, France), and dobutamine (DOB, Bioindustria Laboratorio Italiano Medicinali S.p.A, Novi Ligure, Italy).

2.2. Optimization using Design of Experiments (DoE)

A 2^3 full-factorial design was initially performed to evaluate the main effects of the experimental factors (pH, temperature, and green solvent percentage (% v/v in water) and their two-factor interactions. Three replicated center-point experiments were included to assess the presence of curvature. The results confirmed the absence of second-order effects, therefore supporting the adequacy of a first-order model and excluding the need for response surface methodology (RSM). This design-efficient strategy enabled reliable screening and optimization while minimizing experimental burden.

To characterize the kinetic evolution of the system, the full-factorial model was independently fitted at each regularly spaced time point of the absorbance-time profile. In this Dynamic DoE Modeling approach, absorbance at each time point served as the response variable, enabling time-resolved estimation of factor effects and their interactions. This allowed us to identify how the contributions of temperature, pH, and green solvent percentage changed throughout the reaction, ultimately revealing the most informative measurement time for analytical quantification.

The experimental ranges used in the design are listed in Table 2 and were: pH 9.00–10.00 (selected based on MN stability under alkaline conditions), temperature 25–45 °C (corresponding to the operating limits of the spectrophotometric system), and green solvent content 25–75 % v/v (chosen to avoid both excessive use of green solvent and insufficient solubility of dopamine in the assay medium). The full experimental matrix (**Table S1**) was executed in randomized order to prevent systematic bias, using the "random.shuffle" function from the NumPy Python module. Each experimental condition was performed in triplicate to ensure reproducibility and allow statistical estimation of model coefficients and overall significance.

Table 2
Investigated factors and the corresponding levels at which the experiments were conducted.

Variable	Units	+1	−1	0
pH	$-\log(a_{H+})$	10.00	9.00	9.50
Temperature	°C	45	25	35
% green solvent	%(v/v)	75	25	50

The absorbances at each investigated time served as the output variables for our models, which is of the following type:

$$Y = \beta_0 + x_1\beta_1 + x_2\beta_2 + x_3\beta_3 + x_1x_2\beta_{12} + x_1x_3\beta_{13} + x_2x_3\beta_{23} \quad (1)$$

where the x values in eq. (1) are the investigated factors (represented in Table 2) and the β values are the coefficients of the model, and Y is the output variable. The estimator of the coefficients of each model was determined by solving the following matrix equation:

$$Xb = \widehat{Y} \quad (2)$$

where X is the experimental matrix comprising all the values normalized at the levels expressed in Table 2, b is the matrix of the estimator of the coefficients, and $\widehat{Y}$ is the matrix comprising all the responses for each experiment. Since X is not a squared matrix, the matrix of the coefficients b was found as:

$$b = (X'X)^{-1}X'\ \widehat{Y} \quad (3)$$

where X' is the transpose of the X matrix.

2.3. Instrumental analysis

2.3.1. Spectrophotometric analysis of DA in bio-based solvents using optical analysis

DA stock solutions (1.0 mg/mL) were prepared in 0.5 mL of DMSO, Cyrene™, GVL, or NBP. The aqueous buffer (300 mM $Mg(OAc)_2$, 300 mM NH_4Cl, pH 9.4) was adjusted with 1 M NaOH, and $Mg(OH)_2$ precipitate was removed by centrifugation (10,000 × *g*, 5 min). For assays, 100 μL of DA stock solution in the proper solvent was mixed with 100 μL of buffer in a 96-well plate. Reactions were carried out in triplicate. Control wells contained solvent diluted 1:1 with buffer (final concentration: 150 mM $Mg(OAc)_2$, 150 mM NH_4Cl, pH 9.4) for background correction. Optical measurements were performed on a SPECTROstar® Nano microplate reader (BMG Labtech, Ortenberg, Germany). Full UV–Vis spectra (250–650 nm) were recorded, and the MN absorption peak at 595 nm was monitored at 25 °C after 25 min.

2.3.2. DA calibration curve in DMSO-based assay

DA stock solutions (160 μg/mL) were prepared in 0.5 mL of DMSO. The aqueous buffer (300 mM $Mg(OAc)_2$, 300 mM NH_4Cl, pH 9.4) was adjusted with 1 M NaOH, and $Mg(OH)_2$ precipitate was removed by centrifugation (10,000 × *g*, 5 min). For assays, 100 μL of DA stock solution in the proper solvent was mixed with 100 μL of buffer in a 96-well plate (final concentration: buffer: 150 mM $Mg(OAc)_2$, 150 mM NH_4Cl, pH 9.4; DA 7–80 μg/mL). Reactions were carried out in triplicate. Control wells contained solvent diluted 1:1 with buffer for background correction. Optical measurements were performed on a SPECTROstar® Nano microplate reader (BMG Labtech, Ortenberg, Germany). Full UV–Vis spectra (250–650 nm) were recorded, and the MN absorption peak at 595 nm was monitored at 25 °C after 25 min. The limit of detection (LOD) and the limit of quantification (LOQ) were calculated

according to the standard deviation (σ) of replicate blank measurements, using the conventional criteria of 3σ/slope and 10σ/slope, respectively.

2.3.3. Optimization of spectrophotometric analysis of DA in NBP based on DoE approach

The evaluation of MN formation from DA in NBP was evaluated at different temperatures (25, 35, 45 °C), buffer pH values (7.78–12.20), and NBP fractions (8–75 %) according to DoE design. DA solutions in NBP (final concentration 0.5 mg/mL) were assayed considering the different conditions. Control wells contained only NBP and buffer (150 mM $Mg(OAc)_2$, 150 mM NH_4Cl) without DA. Absorbance at 595 nm was monitored kinetically for up to 60 min using the SPECTROstar® Nano. Optimal conditions were identified as 25 % NBP, pH 10.0, and 45 °C, and were used for subsequent assays.

2.3.4. DA calibration curve in NBP-based optimized assay

DA stock solutions (49.5–360 μg/mL) were prepared in 0.5 mL of NBP. For analysis, 50 μL of each stock solution was mixed with 150 μL of buffer (200 mM $Mg(OAc)_2$, 200 mM NH_4Cl, pH 10; NBP/buffer 1:3, v/v), yielding final concentrations of DA in the range 11–80 μg/mL and 150 mM $Mg(OAc)_2$ 150 mM of NH_4Cl in the buffer solution. Reactions were carried out in triplicate. Control wells contained solvent diluted 1:4 with buffer for background correction. Optical measurements were performed on a SPECTROstar® Nano microplate reader (BMG Labtech, Ortenberg, Germany). Full UV–Vis spectra (250–650 nm) were recorded, and the MN absorption peak at 595 nm was monitored kinetically at 45 °C up to 60 min. The LOD and the LOQ were calculated according to the standard deviation (σ) of replicate blank measurements, using the conventional criteria of 3σ/slope and 10σ/slope, respectively.

2.3.5. Spectrophotometric analysis of adrenergic agonists (EE, E, DOB, A and NA) using optical testing

A panel of adrenergic agonists, commonly employed in emergency and intensive care medicine, was selected for analysis. Stock solutions of DA, EE, E, DOB, A and NA (2 mg/mL) were prepared in NBP from their commercial formulations. For analysis, 50 μL of each stock solution was mixed with 150 μL of buffer (200 mM $Mg(OAc)_2$, 200 mM NH_4Cl, pH 10; NBP/buffer 1:3, v/v), yielding final concentrations of 0.5 mg/mL (DA, EE, E, DOB, A and NA) and 150 mM $Mg(OAc)_2/NH_4Cl$. All assays were performed in triplicate with solvent/buffer controls for background correction. Absorbance at 595 nm was measured after 30 min incubation at 45 °C using the SPECTROstar® Nano.

2.3.6. Greenness assessment

The environmental performance and practical applicability of the analytical methods were evaluated using the Analytical GREEnness (AGREE) index and the Blue Applicability Grade Index (BAGI) within the White Analytical Chemistry framework.

AGREE (version 0.5, University of Warsaw, Poland) [28] was used to assess compliance with the twelve principles of Green Analytical Chemistry. The software generated individual scores for each principle and an overall greenness score ranging from 0.00 (non-green) to 1.00 (ideal green method). The default weighting scheme was applied. The evaluation was performed for both the DMSO-based reference assay and the newly developed NBP-based procedure, and the resulting circular diagrams were exported as graphical outputs for comparison (see Fig. 6).

BAGI (https://bagi-index.anvil.app) was used to evaluate the practical applicability of the analytical procedures. The index is based on ten criteria describing key operational aspects, including sample throughput, analysis time, reagent consumption, cost, instrumentation requirements, degree of automation, and procedural complexity. Each method was scored according to the BAGI guidelines, and the overall BAGI values were calculated using the built-in algorithm of the software.

The application of both tools ensures a comprehensive assessment consistent with the framework of White Analytical Chemistry. This combined approach confirms that the method is not only environmentally responsible but also efficient and feasible for routine analytical use.

3. Results and discussion

3.1. DA detection assay development with bio-based solvents: screening of bio-based solvents

In this study, we report a sustainable, rapid, and cost-effective method for selective DA detection in pharmaceutical formulations. Building on our previous DMSO-based colorimetric protocol [15], this assay is based on the oxidative conversion of DA to MN, a stable purple/blue chromophore that affords a simple visual readout and can be quantitatively detected at 595 nm. The original method, developed for DA determination in Parkinson pharmaceutical drugs, provided robust sensitivity and straightforward execution relying on DMSO as the organic co-solvent mixed 1:1 with specific buffer (150 mM $Mg(OAc)_2$, 150 mM NH_4Cl, pH 9.4).

To advance greener chemistry within a circular chemistry framework, we expanded that approach by exploring three bio-based, renewable-sourced solvents, Cyrene, GVL, and NBP. These solvents originate from cellulose/hemicellulose-derived feedstocks via low-waste, energy-efficient routes; for example, GVL is produced from levulinic acid and Cyrene from levoglucosenone via a solvent-free process. Their physicochemical properties (e.g., boiling point and polarity see Table 1) broadly mirror those of DMSO, positioning them as sustainable candidates for substitution. The colorimetric assay was therefore redeveloped using these green solvents, with conditions tuned to match or exceed the analytical performance of the reference DMSO-based method.

Testing the three selected green solvents under the previously reported alkaline conditions (1:1 v/v organic solvent: buffer (150 mM $Mg(OAc)_2$, 150 mM NH_4Cl, pH=9.4, at 25 °C) revealed marked differences in assay performance (Fig. 1). The DMSO control consistently afforded the most intense and stable color development, confirming its role as a robust benchmark for MN-based DA detection. When DMSO was replaced by NBP, the characteristic chromophore also developed efficiently, yielding a clearly visible and reproducible signal suitable for quantitative analysis. This outcome suggests that NBP sustains the oxidative pathway leading to MN formation, plausibly by providing polarity and solvation characteristics comparable to DMSO. Importantly, NBP acts exclusively as a solvent in this system, which is solely governed by the intrinsic dimerization reactivity of DA under this specific alkaline conditions.

In contrast, assays performed in GVL remained essentially colorless, with no evidence of MN generation. This indicates that GVL may suppress the initial oxidation or destabilize key intermediates, hindering chromophore build-up. Cyrene, despite its promising profile, caused precipitation under the alkaline conditions, which interfered with chromophore formation and impeded optical readout, consistent with either limited miscibility at high pH or side interactions involving its ketone functionality.

Overall, these results underscore how solvent–analyte compatibility governs the performance of green analytical assays. While DMSO ensures the highest sensitivity, NBP emerges as a viable bio-based alternative that preserves reliable visual detection without compromising assay integrity. Conversely, the lack of signal in GVL and the instability in Cyrene point to the need for careful solvent selection and further mechanistic probing if broader DMSO replacement is sought.

Building on these findings, we next focused on optimizing the NBP-based assay by evaluating the fundamental parameters that govern analytical performance, namely pH, the percentage of biobased solvent (NBP), and temperature. These variables are universally recognized as critical in assay development because they modulate both the kinetics of MN formation and chromophore stability. To efficiently capture main effects and interactions while minimizing experimental burden and

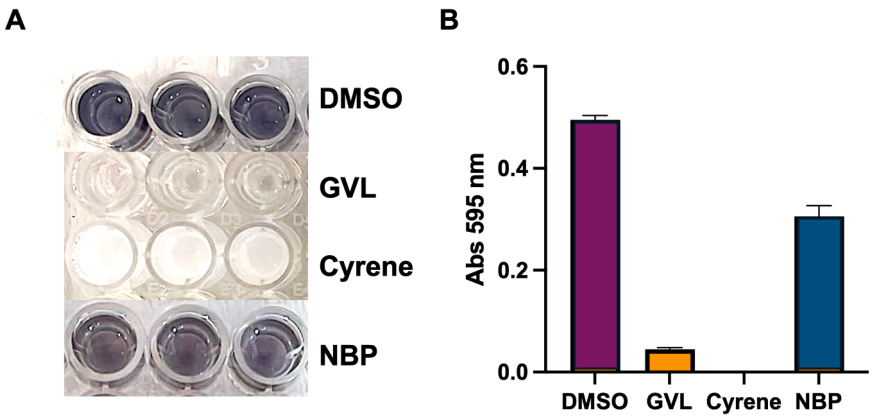


Fig. 1. Colorimetric dopamine assay in conventional (DMSO) and bio-based solvents: visual MN formation and absorbance response at 595 nm. (A) Blue/purple color development due to MN formation in 96well with DA (0.5 mg/mL) in previously reported assay conditions (1:1(v/v) solvent: buffer (150 mM $Mg(OAc)_2$, 150 mM NH_4Cl, pH=9.4), at 25 °C after 25 min of incubation performed in DMSO (control) and in the three bio-based solvents (NBP, GVL, Cyrene) ($n = 3$). (B) Absorbance values recorded at 595 nm for the DA colorimetric assay performed in DMSO (control) and in the three bio-based solvents (NBP, GVL, Cyrene) ($n = 3$).

resource use, we adopted a Design of Experiments (DoE) strategy to identify the optimal operating window for routine application and extended the analysis along the kinetic curve to assess the temporal evolution of factor effects.

3.2. Design of experiment approach (DoE) and optimization

Design of Experiments (DoE) enables a systematic exploration of the experimental domain, allowing researchers to identify key factors and uncover potential interactions [29]. In this study, a full factorial design was chosen for the initial screening due to its simplicity and interpretability [30]. Furthermore, the suitability of full factorial designs in describing the response variable with respect to the factors can be confirmed by evaluating responses at the center of the experimental domain. Indeed, if no curvature effects are present, the response at the center of the experimental domain should be equal to the average of the experimental runs [31–33].

A Design of Experiments (DoE) approach was here employed to optimize the green solvent-based assay. To achieve this, a 2^3 full factorial design, representing a first-order empirical model, was implemented to evaluate the linear effects of each factor as well as their interactions. Specifically, three key factors were selected for optimization: pH, reaction temperature, and the percentage (v/v) of green solvents in water.

3.2.1. Model of Time-resolved modeling of MN formation via dynamic DoE

Absorbance at 595 nm was continuously monitored throughout the reaction, and the kinetic profile was analyzed using a Dynamic DoE approach, whereby a full-factorial model was fitted at each experimental time point. This strategy provided a time-resolved description of factor influence, enabling identification of the earliest time at which stable and reliable responses were achieved across all experimental conditions. The coefficients for each model at the distinct time points were determined using eq. (3) and a *t*-test was conducted to evaluate the significance of each coefficient. Both values and *t*-tests are reported in Table 3.

To assess the overall significance of the models, an analysis of variance (ANOVA) was conducted at 5 % significance level. Summary of the results from the ANOVA tests and R^2, R^2_{adj}, and R^2_{pred} are reported in **Table S2**.

By plotting the coefficients at each time point, it is possible to more accurately observe their temporal contribution to each DoE model (Fig. 2). Confidence interval on the coefficients were computed from the standard errors of the coefficients and the t value for a significance level of $\alpha = 0.05$ and N – P degrees of freedom, where N is the number of experiments used for the calibration model (24 for the experiments carried out from 0 to 45 min and 23 for the experiment carried out at 60 min) and $P = 7$. Determination of the standard error for each coefficient ($S(\beta)$) was calculated as $S(\beta) = \sqrt{\hat{\sigma}^2 C_{jj}}$ where $\hat{\sigma}^2$ is the mean residual sum of squares and C_{jj} is the *j*th diagonal element of the $(X'X)^{-1}$ matrix. Such approach may allow to gain insight into the way each experimental factor contributes to the kinetics absorbance. Several interesting information can be obtained from Fig. 2.

The coefficient describing the linear contribution to temperature ("T") shows an increase in the value up to approximately 30 min and then decays after 60 min. Essentially, the positive effect due to temperature reaches an optimum response at approximately 30 min and then it gradually decays. After 30 min, therefore, an increase of temperature does not lead to a positive increase in the absorbance, likely due to a degradation effect on MN formation. The effect due to green solvent percentage (%), on the other hand, shows a continuous negative effect with time, meaning that an increase in green solvent percentage leads to worse performance as the reaction kinetics progress. What was even more interesting to notice is that both the linear term linked to pH and the interaction term between % and pH are constant over the entire

Table 3
Values and *t*-tests for the model coefficients for each model determined at the given time points. Critical t value for a significance level $\alpha = 0.05$ and $N - P = 17$ degrees of freedom (with N being the number of total runs (24) and $P = 7$ being the number of parameters) is $t_{critical} = 2.11$. For the model obtained at 60 min, a critical value of $t = 2.12$ should be considered as one experiment was discarded being an outlier ($N - P = 16$ degrees of freedom).

		0 min		15 min		30 min		45 min		60 min	
Coefficient estimator	Physical quantity	Value	*t*-test	Value	*t*-test	Value	*t*-test	Value	*t*-test	Value	*t*-test
b0	Intercept	0.377	-	0.553	-	0.710	-	0.747	-	0.819	-
b1	T	0.056	6.703	0.068	6.523	0.069	7.771	0.063	7.623	0.041	5.265
b2	%	−0.110	−13.167	−0.110	−10.494	−0.161	−18.182	−0.176	−21.383	−0.192	−24.733
b3	pH	0.034	4.064	0.035	3.333	0.042	4.698	0.042	5.119	0.046	5.907
b12	T x%	−0.008	−0.986	−0.011	−1.069	−0.026	−2.903	−0.025	−3.049	−0.004	−0.572
b13	T x pH	0.000	−0.050	−0.002	−0.207	−0.004	−0.451	−0.005	−0.555	−0.003	−0.433
b23	% x pH	0.021	2.470	0.034	3.222	0.028	3.176	0.028	3.412	0.034	4.355

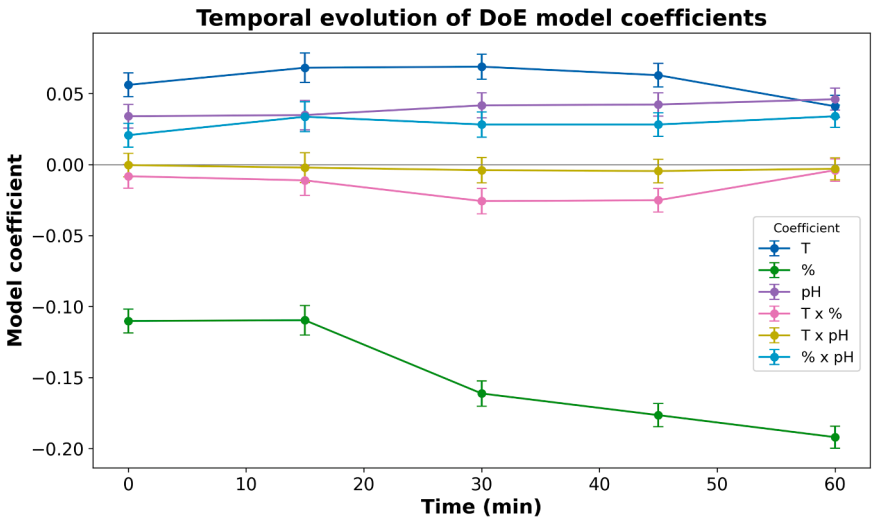


Fig. 2. Temporal evolution of the coefficients for each DoE model. Error bars refer to the confidence interval.

reaction kinetics and mildly contribute to the absorbance. The interaction terms between T and pH, quite curiously, are not significant (see also Table 4) throughout the entire reaction kinetics. These quite surprising results may be explained by the fact that the green solvent /water mixture may show a different behavior between pH and temperature when compared to classical water-based systems. Finally, it is interesting to note that the interaction term "$T \times$ %" showed no statistical significance and contributed little to the models at 0 and 15 min but showed its maximum contribution at 30 min. At 60 min, however, this term no longer contributed significantly to describing the response. We may point out that the factors temperature and green solvent percentage exerted their maximum transient synergistic effect at around 30 min of incubation.

To evaluate the descriptive and predictive capabilities of the models, the values of R^2, R^2_{adj}, and R^2_{pred} were determined for each model and plotted in function of time (Fig. 3).

As it can be observed from **Fig. S1**, the models reach stable descriptiveness and predictivity after only 30 min of incubation, as a stabilization in the values of R^2, R^2_{adj} and R^2_{pred} could be reached only after this time. In any case, all the models showed no curvature effects when a triplicate experiment was performed at the center of the experimental domain, monitored over time and compared with the response predicted at the center of each model (Fig. 3). This lack of curvature effect demonstrates that for each time point the simple full factorial models suffice in describing the responses in line with sequential experimentation [27]. The best experimental condition, therefore, is given by a suitable combination of parameters that yield the highest absorption. Since no change in sign is present for the models at each time point (see Fig. 2), the combination of experimental parameters that give the best absorbance are Temperature = 45 °C, % of green solvent = 25 % and pH = 10.00. By computing the highest absorbance

Table 4
Comparative analytical performance of the newly developed NBP-based method versus the conventional DMSO-based protocol for dopamine (DA) quantification.

Parameters	1:4 (v/v) NBP:buffer*	1:1 (v/v) DMSO:buffer *
Linear range (μg/mL)	11–80	11–80
Slope (m)	0.0061 ± 0.0005	0.0050 ± 0.0001
Intercept (a)	−0.0095 ± 0.0038	0.0171 ± 0.0058
Correlation coefficient (R^2)	0.9998	0.9983
LOD (μg/mL)	1.35 ± 0.38	1.98 ± 0.30
LOQ (μg/mL)	4.59 ± 0.44	6.00 ± 0.82

* Buffer: 150 mM $Mg(OAc)_2$, 150 mM NH_4Cl at pH 10.

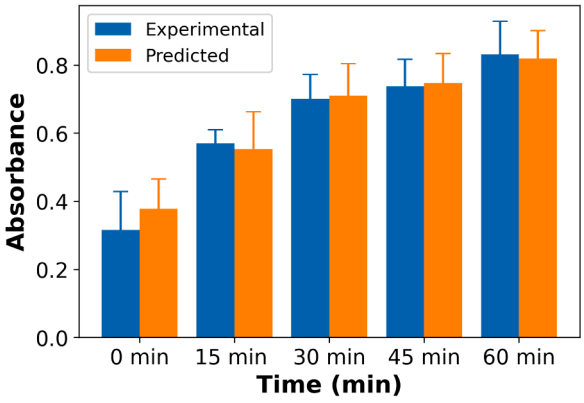


Fig. 3. Comparison between experimental results at the center of the experimental domain (blue) and responses predicted by the model (orange). Bars refer to confidence interval determined at a significance level α = 0.05. Experiments were carried out as triplicates ($N = 3$).

from the models at each time point with the set of statistically significant parameters, it is possible to obtain a prediction of the absorbance together with a confidence interval that can be computed according to the methodology described by Myers [34]. Comparison with experimental data (**Fig. S2**) shows acceptable agreement between the predicted and experimental values after 30 min. It is also very worthwhile to point out that the predicted absorbances for the models at 30 min and 60 min are 1.0 ± 0.1 and 1.06 ± 0.09, respectively. On the other hand, the experimental values at the same time point are 0.94 ± 0.05 and 1.08 ± 0.09, respectively. On a practical note, and given the fact that the absorbances are quite close to each other, it may be considered reasonable to use an absorbance reading at 30 min and therefore halving the assay time even though this may result in a slightly reduced absorbance value.

3.3. Development and validation of a green colorimetric assay for DA using NBP as a bio-based solvent under optimized conditions defined by DoE

Following the optimization of reaction conditions via a Design of Experiments (DoE) approach, the focus shifted to developing a colorimetric assay for DA detection using N-butylpyrrolidone (NBP) as the selected bio-based solvent. Under optimized conditions being NBP: buffer ratio of 1:4 (v/v), buffer composition of 150 mM $Mg(OAc)_2$ and

150 mM NH_4Cl, pH 10, and incubation at 45 °C for 30 min, MN formation was readily detectable through the emergence of an intense purple/blue signal. To ensure optimal assay performance, the kinetics of MN formation were evaluated at a DA concentration of 0.50 mg/mL (Fig. 4A). The kinetic profile revealed a rapid increase in absorbance at 595 nm, with over 85 % of the final signal reached within 30 min. The subsequent plateau confirmed the absence of side reactions or degradation, demonstrating the assay's specificity and reliability. Accordingly, a 30-minute incubation was selected, supported by DoE, as a good compromise, balancing rapid signal generation with reproducibility. The fast chromophore development and stable signal further underscore the method's practicality for routine pharmaceutical analysis, including use in laboratories with limited instrumentation. Fig. 4B shows the full spectra of DA at increasing concentrations, revealing a distinct absorption maximum at 595 nm attributable to MN formation. The chromophore intensity scaled proportionally with DA concentration, confirming the suitability of this wavelength for quantitative detection. The absence of significant spectral interferences or secondary peaks further supports

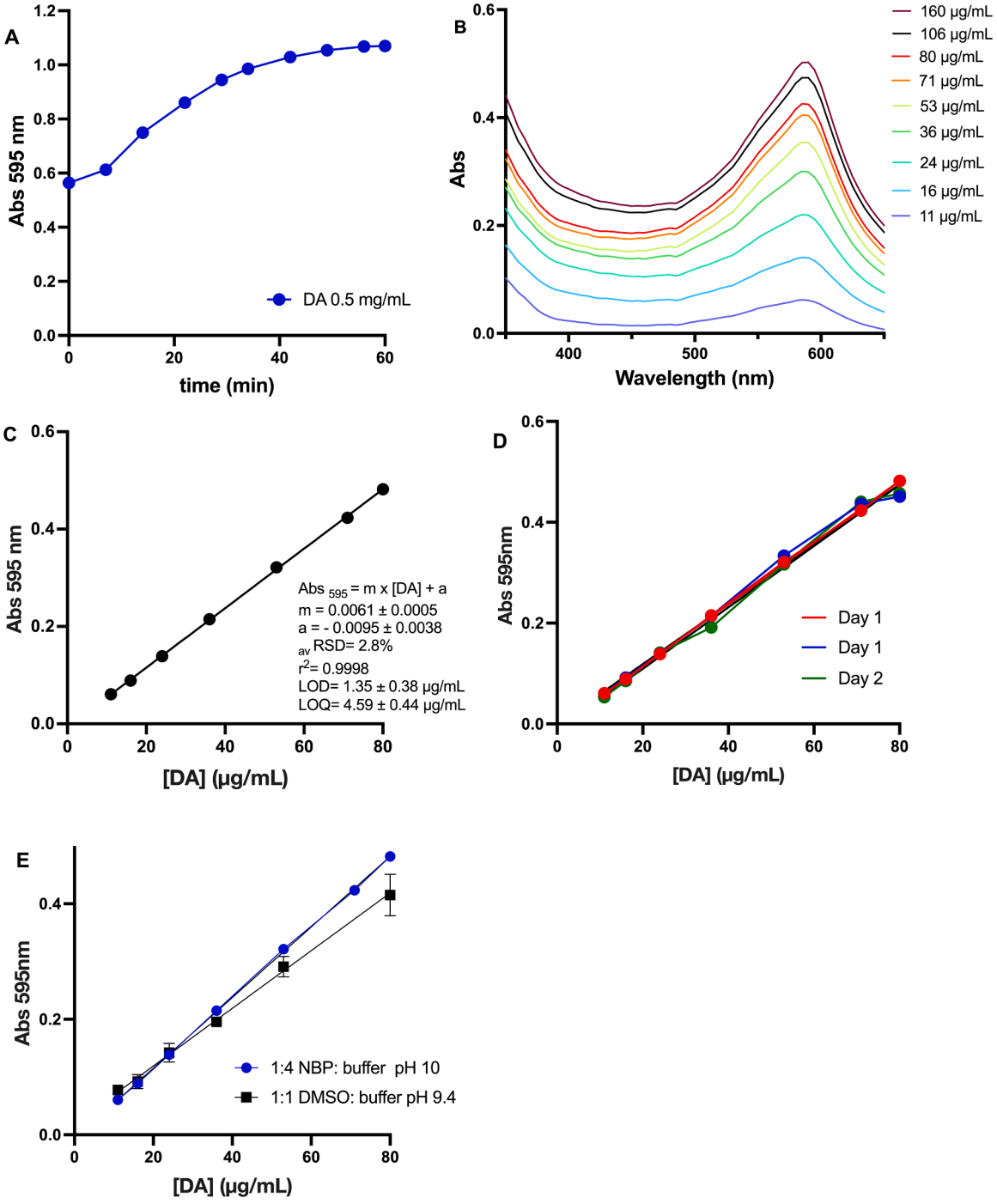


Fig. 4. Kinetic Profile, Spectral Characterization, and Calibration Performance of the NBP-Based Colorimetric Assay for Dopamine (A) Time-dependent oxidation of dopamine (DA, 0.5 mg/mL) in a 1:4 (v/v) NBP:buffer system (150 mM $Mg(OAc)_2$, 150 mM NH_4Cl) at pH 10 and 45 °C over a 0–60 min interval. (B) UV–Visible spectra and (C) calibration curve for DA (11–80 µg/mL) under the same conditions after 30 min of incubation. (D) Intraday analysis of DA calibration curves. (E) Comparison of calibration curves for DA quantification using the newly developed NBP-based assay (blue) versus the conventional DMSO-based/our reference method (black).

the assay's selectivity. A calibration curve for DA in the range of 11–80 μg/mL was constructed by measuring absorbance at 595 nm under optimized conditions (Fig. 4B–C). The resulting regression equation was:

$$Abs_{595} = 0.006 \times [DA] + 0.009$$

with an excellent correlation coefficient (R^2 = 0.9994), confirming excellent linearity across the tested range. Analytical performance was further evaluated in terms of precision and sensitivity. The average relative standard deviation (%avRSD) across replicates was 2.8 %, indicating good intra-assay reproducibility. The calculated limits of detection (LOD) and quantification (LOQ) were 1.35 ± 0.38μg/mL and 4.59 ± 0.44 μg/mL, respectively. Intra- and inter-day precision studies confirmed robustness, with %RSD values consistently below 5 % and 7 %, respectively. The analytical performance of the NBP-based colorimetric method was compared with the conventional DMSO-based protocol (Fig. 4E and Table 4). Both solvent systems provided an identical linear dynamic range (11–80 μg/mL), ensuring comparable sensitivity and applicability for routine measurements. Correlation coefficients were very good for both solvents (R^2 = 0.9998 for NBP vs R^2 = 0.9983for DMSO), showing respectively excellent and strong linearity. The average relative standard deviation ($\%_{av}$RSD) across replicates was 2.8 %, confirming very good intra-assay reproducibility. However, the LOD and LOQ values demonstrated a marked improvement with the NBP-based system: LOD decreased more than twofold (1.35 ± 0.38 μg/mL vs 1.98 ± 0.30 μg/mL), and LOQ was similarly reduced (4.59 ± 0.44 μg/mL vs 6.00 ± 0.82 μg/mL). These results indicate that NBP enhances analytical sensitivity and lowers the threshold for reliable DA quantification. Intra- and inter-day precision studies further confirmed robustness, with %RSD values consistently below 5 % and 7 %, respectively. A key practical advantage of the NBP-based protocol is its reduced solvent consumption. The optimized assay employs an NBP: buffer ratio of 1:4 (v/v), compared to the 1:1 (v/v) ratio required by the conventional DMSO-based method. This results in a substantial reduction in organic solvent usage, making the NBP-based approach not only analytically superior but also more sustainable and cost-effective. Taken together, these findings demonstrate that the optimized NBP-based assay is rapid, sensitive, and reproducible, offering a robust green alternative to conventional solvent-based spectrophotometric methods for dopamine (DA) detection. The synergistic effects of Mg^{2+} and NH_4^+ ions under alkaline conditions likely accelerate oxidation kinetics, contributing to the fast and stable formation of MN (MN) observed under the optimized assay parameters.

3.4. *Application and validation of the NBP-Based colorimetric assay in pharmaceutical formulations: selectivity toward DA detection*

To further demonstrate the practical applicability and robustness of the developed assay, we extended its application on a commercial DA pharmaceutical formulation (see Materials and methods). This step was undertaken to evaluate potential matrix effects arising from excipients and other components present in the formulation. Fig. 5A shows the superposition of calibration curves obtained from standard DA solutions (blue circles) and the pharmaceutical vial (green triangles). Linear regression fitting revealed excellent overlap, confirming minimal, if any, interference from the formulation matrix and validating the assay's suitability for real-sample applications.

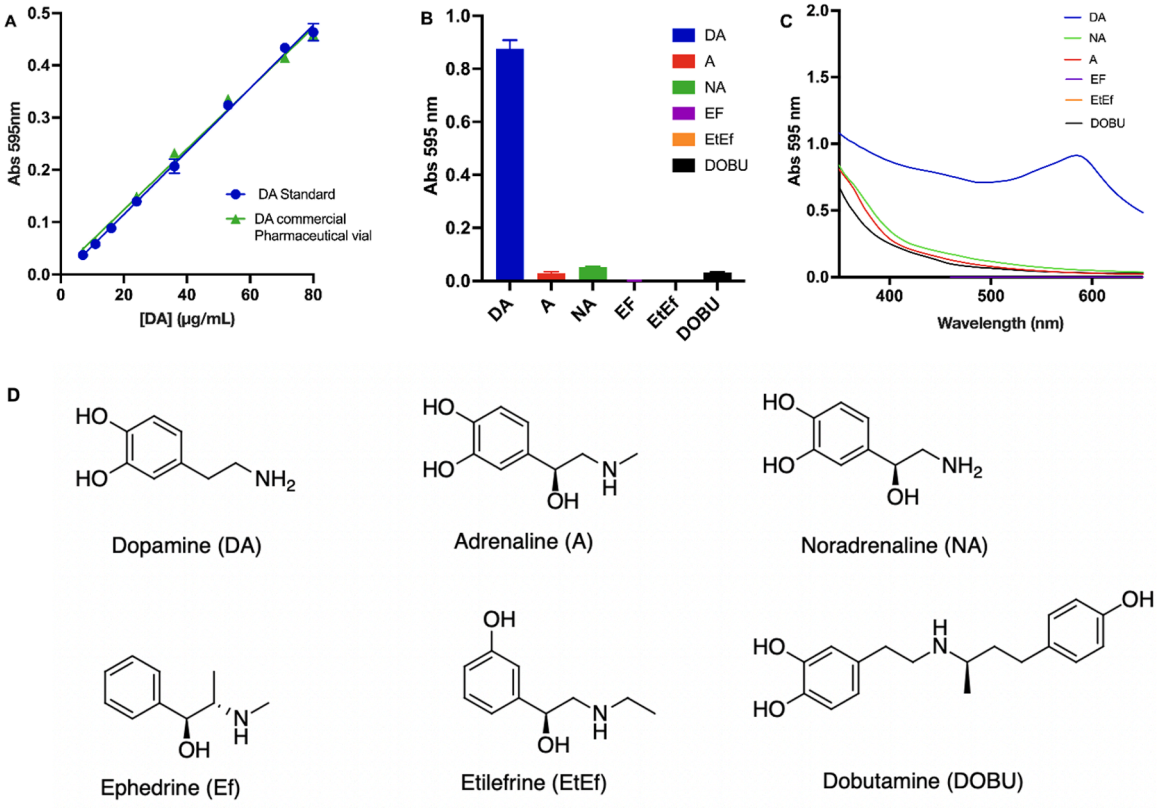


Fig. 5. Calibration, absorbance response, and spectral selectivity of the NBP-based dopamine assay. (A) Comparison of calibration curves for dopamine (DA) standard solutions (blue) and a commercial pharmaceutical vial (green), obtained by NBP-based assay (1:4 (v/v) NPB: buffer (150 mM $Mg(OAc)_2$, 150 mM NH_4Cl) at pH 10 and 45 °C after 30 min incubation); (B) Absorbance at 595 nm indicating MN formation under the same assay conditions. (C) UV–Visible spectra recorded by NBP-based assay after 30 min of incubation for dopamine (DA), adrenaline (A), noradrenaline (NA), ephedrine (Ef), etilefrine (EtEf), and dobutamine (DOBU), each at 0.5 mg/mL, highlighting selective chromophore development for DA. (D) Chemical structures of all adrenergic compounds evaluated in this study: dopamine (DA), adrenaline (A), noradrenaline (NA), ephedrine (Ef), etilefrine (EtEF), and dobutamine (DOBU).

The selectivity of the optimized NBP-based colorimetric assay was further evaluated by comparing the response of DA to that of other adrenergic drugs commonly used in emergency or intensive care settings in vial formulation, namely adrenaline (A), noradrenaline (NA), ephedrine (E), etilefrine (EE), and dobutamine (DOBU) (Fig. 6). Among all tested compounds, only DA generated a clear purple/blue chromophore with a distinct absorption maximum at 595 nm, whereas no significant absorbance signals were detected for the other analytes under identical assay conditions (Figs. 5B and C). These findings demonstrate excellent assay selectivity, with DA being uniquely susceptible to MN oxidative chromophore formation in the tested solvent/buffer system.

The molecular basis of this selectivity can be rationalized by considering the structural features of DA compared to the other adrenergic compounds (Fig. 5D). DA contains a catechol moiety directly connected to a primary amine via a two-carbon side chain, a structural arrangement that readily undergoes oxidation due to DA dimerization under alkaline conditions to form reactive ortho-quinones. These intermediates can further polymerize, leading to the stable *Mn species* responsible for the absorbance at 595 nm. In contrast, A and NA also possess a catechol ring, but the presence of an additional hydroxyl group at the β-carbon of the side chain increases polarity and intramolecular stabilization, reducing their propensity to undergo auto-oxidation and subsequent chromophore formation under the assay conditions. EF and EtEF, although adrenergic agonists, lack the catechol motif and therefore cannot generate the reactive quinonoid intermediates necessary for MN formation. DOBU, while containing a catechol substituent, features a more sterically hindered and substituted side chain, which limits its oxidation potential due to both electronic and steric effects.

The observed selectivity is consistent with the established mechanism of MN formation, as previously described in our earlier works [15, 16,35]. Dopamine undergoes oxidation to ortho-quinone intermediates, which then dimerize and polymerize to yield the characteristic MN chromophore. Structural variations such as the β-hydroxyl group in adrenaline and noradrenaline, despite sharing the catechol moiety, contain a βhydroxyl substituent that alters their oxidation potential and intramolecular stabilization. Under the alkaline conditions employed here, this structural difference prevents the same chromophore formation, explaining the absence of interference.

The use of pharmaceutical formulations in this study was a deliberate and strategic choice, enabling a rigorous evaluation of the method's analytical performance within a well-defined and industry-relevant matrix. By focusing on pharmaceutical samples, the study ensures high reproducibility, minimal matrix interference, and direct applicability to quality control environments. This choice not only aligns with the practical objectives of green analytical chemistry but also reinforces the robustness and reliability of the proposed assay as a sustainable alternative for routine pharmaceutical analysis.

This selective reactivity not only confirms DA as the sole target of the assay but also excludes interference from clinically relevant sympathomimetics, reinforcing the method's robustness for pharmaceutical and biomedical applications. This selective response is advantageous for analytical applications, as it ensures that other adrenergic agents, even those with structural similarities, do not interfere with DA detection.

This outcome underscores the true innovation of the present work: the dopamine assay developed with NBP exemplifies the systematic redesign of a well-established colorimetric method into a green and circular analytical process. The introduction of NBP as an eco-friendly solvent is not a mere substitution, but part of a broader strategy that integrates: a) **Solvent greenness assessment**, replacing DMSO with a safer, bio-based alternative; b) **Solvent volume reduction**, minimizing environmental burden (1:4 vs 1:1); c) **Preservation of analytical precision**, ensuring robust quantification, and d)**Validation on real pharmaceutical formulations**, confirming practical applicability.

Together, these elements demonstrate that environmental sustainability and scientific rigor are not mutually exclusive. Supported by DoE-based optimization and full analytical validation, this framework bridges high-performance assay development with ecological responsibility, moving toward truly circular analytical chemistry. Ultimately, this assay not only delivers analytical excellence but also reflects a broader commitment to sustainable innovation, demonstrating how thoughtful method redesign can align environmental responsibility with real-world performance. In this framework, we moved on to assess the sustainability.

3.5. *Sustainability assessment of the NBP-Based colorimetric assay*

Quantifying the sustainability of analytical procedures remains challenging due to their inherently multifactorial nature. Unlike synthetic chemistry, where the environmental impact of a reaction product can be directly assessed, analytical chemistry lacks a single, well-defined

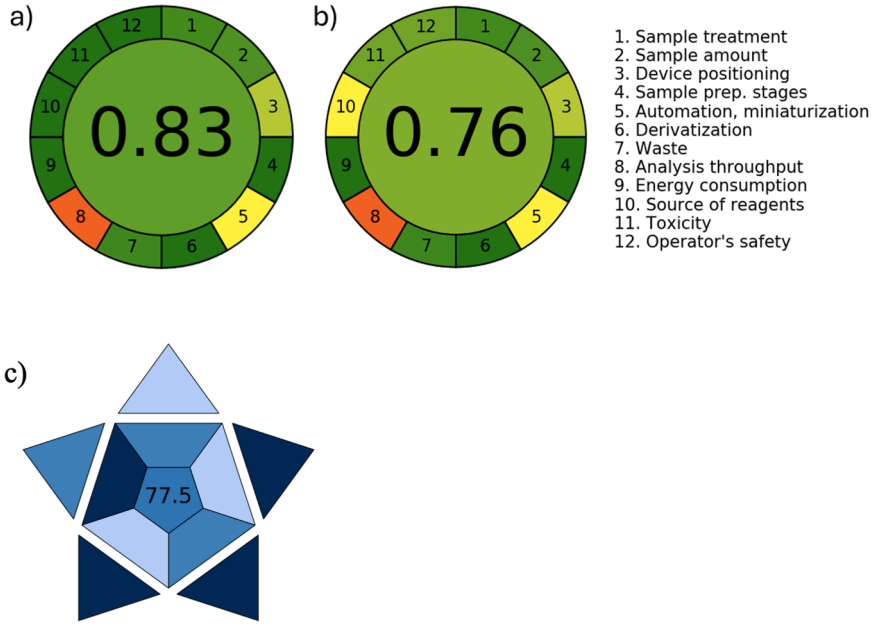


Fig. 6. a) and b) Results of AGREE analysis of the a) NBP-based assay and b) DMSO. c) Results of BAGI analysis of the NBP-Based Colorimetric Assay.

output with an associated ecological burden. To address this gap, the 12 Principles of Green Analytical Chemistry (GAC) were established, providing a comprehensive framework for designing and evaluating environmentally responsible analytical methods.

Because no single metric can fully capture the complexity of analytical sustainability, complementary tools have been developed to support practical implementation of the GAC principles across diverse laboratory contexts [17,36,37]. Among these, the Analytical GREEnness (AGREE) metric [28] metric has emerged as a rapid, intuitive, and effective tool. AGREE evaluates each of the 12 principles individually and integrates the results into a circular pictogram, with a final score derived from the weighted average of all parameters (see Fig. 6a and b).

For the NBP-based assay developed in this study, the AGREE score was **0.83**, indicating strong alignment with green analytical principles. In comparison, the previous DMSO-based method yielded a lower score (0.76). Notably, replacing DMSO with a bio-based solvent and reducing the overall solvent volume (1:4 vs 1:1, v/v) improved the AGREE score by **0.07** (Fig. 6a and 6b). A detailed breakdown of the scoring is provided in the Supporting Information (**Figs. S3** and **S4**).

It is worth noting that parameters 3 (*device positioning*) and 6 (*avoidance of derivatization*) were slightly underweighted, as the present study did not involve in situ analysis or derivatization steps. Nonetheless, the overall sustainability profile of the NBP-based method confirms its environmental advantage and supports its adoption in laboratories committed to green and circular analytical practices.

However, environmental sustainability alone does not guarantee suitability for routine application. To complement AGREE, the Blue Applicability Grade Index (BAGI) assesses operational performance, including throughput, cost, simplicity, and overall applicability (Fig. 6c). BAGI is a metric tool that can be used to evaluate the practicality of a method in analytical chemistry. It ranges from 25 to 100, with a higher score indicating greater practicality. The applicability of a selected method can be quickly assessed this way allowing to identify strong and weak features. Fig. 6c shows the BAGI analysis for the method developed in this work. The supporting information includes the 10 parameters set for the analysis (**Fig. S5**). Deep blue highlights the strengths of the method [38], while the lighter the blue shade the higher its weakness. The overall evaluation of the method gives a score of 77.5, which is higher than the minimum value of 60 considered necessary for a method to be considered "practical". The score suggests that the method performs well across most applicability criteria, such as operational simplicity, robustness, and resource requirements, while highlighting a few moderate limitations that could be optimized to further enhance its practicality. The combined use of AGREE and BAGI aligns with the principles of White Analytical Chemistry, ensuring that the evaluated method is not only environmentally responsible but also efficient, practical, and feasible for routine analytical use.

3.6. Comparison of the proposed method with colorimetric assays reported for DA detection

Beyond the numerical performance, the key novelty of the proposed method lies in the fact that it is the only assay in which environmental sustainability, analytical sensitivity and practical applicability are optimized simultaneously within a unified design strategy. In contrast to existing colorimetric methods, which rely on hazardous or persistent reagents and were developed without sustainability-by-design criteria, the present work demonstrates that a classical dopamine assay can be systematically re-engineered into a green and circular analytical platform without sacrificing analytical quality. As shown in Table 5, the NBP-based MN assay clearly stands out due to its use of a green solvent, distinguishing it from all other methods [39] that rely on conventional or potentially hazardous organic solvents, such as DMSO or 2,6-dichloroquinone derivatives. The method exhibits excellent analytical performance, with a linear range of 11–80 μg/mL and a low LOD of 1.35 μg/mL, suitable for pharmaceutical formulations. Notably, all methods reported in Table 5 are applied to pharmaceutical matrices, yet only the NBP-based approach combines high sensitivity with environmental sustainability. Similarly, 2,6-dichloroquinone 4-chloroimide involves halogenated quinones [40], which are highly reactive, potentially hazardous, and non-biodegradable. 2,4-dinitrophenylhydrazine contains a hydrazine moiety, a class of compounds recognized for toxicity, mutagenicity, and strong environmental persistence. The dopamine-based inhibition of thionine oxidation relies on thionine, a synthetic dye known for limited biodegradability and aquatic toxicity. Finally, the ferricyanide/potassium ferrocyanide system, while effective analytically, involves cyanide complexes that require careful disposal due to environmental and safety concerns. Collectively, these conventional methods, although applicable to pharmaceutical matrices, do not address green chemistry principles. The NBP-based method, by contrast, achieves comparable or superior analytical performance (linear range 11–80 μg/mL, LOD 1.35 μg/mL) while minimizing ecological impact, demonstrating that environmentally benign solvents can replace hazardous reagents without compromising analytical quality. This approach exemplifies the integration of high-performance analysis with true sustainability in pharmaceutical applications. By avoiding toxic solvents, it aligns with the principles of Green Analytical Chemistry, reducing ecological impact and improving laboratory safety. Its robustness in complex formulations, broad linear range, and low detection limits demonstrate that green solvents can achieve analytical quality comparable to traditional methods. Overall, this work highlights the feasibility of incorporating environmentally benign solvents in routine pharmaceutical analysis, providing a model for future sustainable method development. To the best of our knowledge, none of the previously reported colorimetric assays for dopamine combine bio-based solvent use, solvent volume reduction, full validation, and sustainability assessment within a single analytical strategy.

Table 5
Comparison among colorimetric assays for DA detection in pharmaceutical formulations, biological and food samples.

Method reaction	λ_abs (nm)	Linear Range (μg/mL)	LOD (μg/mL)	Matrix	Reference
MN (NBP-based)	595	11.0 – 80.0	1.35	Pharmaceutical formulations (injectable)	This work
MN (DMSO-based)	595	11.0 – 80.0	1.98	Pharmaceutical formulation (tablets)	[15]
2,6-Dichloroquinone 4-chloroimide	470	5.0 – 45	2.5	Pharmaceutical formulation (bulk and injectables)	[40]
2,4-Dinitrophenylhydrazine	560	5 – 50	0.32	Pharmaceutical formulations (injectable)	[41]
Inhibition of thionine oxidation	601	0.20 –103	0.06	Pharmaceutical (injectables) and biological samples (urine and serum)	[42]
$KFe^{III}[Fe^{II}(CN)_6]$	735	0.1 – 8	0.04	Pharmaceutical (injectables), biological (serum) and food samples (banana)	[43]
$KMnO_4$ reduction	610	0.190 - 4.362	0.1	Pharmaceutical formulation (bulk and injectables)	[44]

4. Conclusions

In this study, we developed and validated a colorimetric assay for dopamine (DA) detection in pharmaceutical formulations, integrating analytical performance with environmental responsibility. The assay conditions were efficiently optimized using a statistically robust and Time-Resolved DoE modeling approach, which proved superior to traditional one-variable-at-a-time methods. Notably, evaluation of the first-order model suitability allowed us to avoid the use of star-designs and central composite designs, streamlining the optimization process and minimizing time, energy and reagent consumption in line with green analytical chemistry principles. The resulting NBP-based assay demonstrated rapid kinetics of MN formation, high sensitivity in the low micromolar range, excellent reproducibility, and strong selectivity toward DA, while benefiting from the use of a bio-based and safer solvent. A linear response was observed across the 11–80 μg mL^{-1} concentration range, with very good precision (%RSD ≈ 2.8 %) and low detection and quantification limits (LOD: 1.35 ± 0.38 μg mL^{-1}; LOQ: 4.59 ± 0.44 μg mL^{-1}), confirming its suitability for quantitative analysis of commercial pharmaceutical products and representing a clear analytical advantage over the conventional DMSO-based protocol.

Selectivity experiments further revealed that structurally related adrenergic agents, including adrenaline, noradrenaline, ephedrine, etilefrine, and dobutamine, did not produce detectable chromophore formation at 595 nm under the optimized conditions. This highlights DA's unique reactivity and ensures reliable detection without interference from clinically relevant analogues, which is a key advantage for pharmaceutical quality control where multiple sympathomimetics may be present.

By combining methodological simplicity, bio-based solvents, and analytical robustness, the assay offers a sustainable and practical alternative to chromatographic techniques for DA quantification. Its rapid response, minimal instrumentation requirements, reduced solvent consumption (1:4 instead of 1:1, v/v), and high specificity make it a promising candidate for routine monitoring in pharmaceutical and decentralized analytical settings, where cost, safety and environmental impact are critical.

The successful validation on pharmaceutical vials provides a solid foundation for future extensions to more complex biological matrices, which may require additional stabilization and pre-treatment steps to control matrix effects and oxidation pathways; nevertheless, the green solvent platform and DoE-driven optimization strategy described here offer a flexible framework to address these challenges. These developments are envisioned as part of the ongoing greening of this analytical platform.

The novelty of this work lies in its integrated approach to sustainability: rather than designing a new assay from scratch, we re-engineered a proven analytical method within the framework of Green Analytical Chemistry, achieving improved sensitivity, reduced solvent consumption, and enhanced environmental compatibility. To the best of our knowledge, this is the first colorimetric dopamine assay fully optimized using a bio-based solvent system (NBP) and validated for pharmaceutical formulations, setting a precedent for future green redesigns of classical analytical methods. Overall, this work demonstrates how an established analytical protocol can be systematically transformed into a green, circular, and validated analytical solution, supporting the translation of Green Analytical Chemistry from principles to practice.

CRediT authorship contribution statement

Doretta Cuffaro: Writing – review & editing, Writing – original draft, Investigation, Conceptualization. **Fabio Spiaggia:** Writing – review & editing, Investigation. **Enrico Crispino:** Investigation. **Andrea Mezzetta:** Writing – review & editing, Conceptualization. **Elisa Nuti:** Writing – review & editing, Resources. **Vincenzo Calderone:** Writing – review & editing, Resources. **Lorenzo Guazzelli:** Writing – review & editing, Resources, Conceptualization. **Pasquale Palladino:** Writing – review & editing, Writing – original draft, Supervision, Conceptualization. **Maria Minunni:** Writing – review & editing, Writing – original draft, Supervision, Funding acquisition, Conceptualization.

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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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.greeac.2026.100329.

Data availability

Data will be made available on request.

References

[1] P. Palladino, M. Lettieri, S. Scarano, M. Minunni, The golden age of colorimetry in diagnostics and drug analysis: focus on the rising star of metabolite-based assays, TrAC Trends Anal. Chem. 180 (2024) 117947, https://doi.org/10.1016/j.trac.2024.117947.

[2] D. Cuffaro, P. Palladino, M. Digiacomo, S. Bertini, M. Minunni, M. Macchia, Fast, sensitive, and sustainable colorimetric detection of chlorogenic acid in artichoke waste material, Food Chem. 463 (2025) 141505, https://doi.org/10.1016/j.foodchem.2024.141505.

[3] A.T. Bişgin, Effective and selective type V deep eutectic solvent based microextraction of E127 in foodstuffs and drugs, J. Food Compos. Anal. 132 (2024) 106343, https://doi.org/10.1016/j.jfca.2024.106343.

[4] D. Kaçanbüre, A.T. Bişgin, Selective microextraction of erythrosine (E127) in foodstuffs using a new generation high-density type-V deep eutectic solvent, Food Chem. 463 (2025) 141273, https://doi.org/10.1016/j.foodchem.2024.141273.

[5] V. Hessel, N.N. Tran, M.R. Asrami, Q.D. Tran, N.V.D. Long, M. Escribà-Gelonch, J. O. Tejada, S. Linke, K. Sundmacher, Sustainability of green solvents – review and perspective, Green. Chem. 24 (2022) 410–437, https://doi.org/10.1039/D1GC03662A.

[6] J.D. Chea, A. Christon, V. Pierce, J.H. Reilly, M. Russ, M. Savelski, C.S. Slater, K. M. Yenkie, Framework for solvent recovery, reuse, and recycling In industries, in: S.G. Muñoz, C.D. Laird, M.J. Realff (Eds.), Computer Aided Chemical Engineering, Elsevier, 2019, pp. 199–204, https://doi.org/10.1016/B978-0-12-818597-1.50032-1.

[7] S.-J. Hong, X.-N. Zhang, Z. Sun, T. Zeng, The potential health risks of N,N-dimethylformamide: an updated review, J. Appl. Toxicol. 44 (2024) 1637–1646, https://doi.org/10.1002/jat.4590.

[8] C.R. Kirman, B.R. Sonawane, J.G. Seed, N.O. Azu, W.T. Barranco, W.R. Hamilton, T.J. Stedeford, S.M. Hays, An evaluation of reproductive toxicity studies and data interpretation of N-methylpyrrolidone for risk assessment: an expert panel review, Regul. Toxicol. Pharmacol. 138 (2023) 105337, https://doi.org/10.1016/j.yrtph.2023.105337.

[9] O. US EPA, Risk management for n-methylpyrrolidone (NMP), (2016). https://www.epa.gov/assessing-and-managing-chemicals-under-tsca/risk-management-n-methylpyrrolidone-nmp (accessed September 5, 2025).

[10] J.-C. Xiang, Q.-H. Gao, A.-X. Wu, The applications of DMSO. Solvents As Reagents in Organic Synthesis, John Wiley & Sons, Ltd, 2017, pp. 315–353, https://doi.org/10.1002/9783527805624.ch7.

[11] Y. Deguchi, M. Kono, Y. Koizumi, Y. Izato, A. Miyake, Study on autocatalytic decomposition of dimethyl sulfoxide (DMSO), Org. Process Res. Dev 24 (2020) 1614–1620, https://doi.org/10.1021/acs.oprd.0c00113.

[12] J. Galvao, B. Davis, M. Tilley, E. Normando, M.R. Duchen, M.F. Cordeiro, Unexpected low-dose toxicity of the universal solvent DMSO, FASEB J 28 (3) (2014) 1317–1330, https://doi.org/10.1096/fj.13-235440.

[13] K.-M. Roy, Sulfones and sulfoxides. Ullmann's Encyclopedia of Industrial Chemistry, John Wiley & Sons, Ltd, 2000, https://doi.org/10.1002/14356007.a25_487.
[14] P. Karande, S. Mitragotri, Enhancement of transdermal drug delivery via synergistic action of chemicals, Biochim. Biophys. Acta (BBA) - Biomembr. 1788 (2009) 2362–2373, https://doi.org/10.1016/j.bbamem.2009.08.015.
[15] M. Lettieri, R. Emanuele, S. Scarano, P. Palladino, M. Minunni, MN-based colorimetric assay for quantitative detection of levodopa in co-presence of carbidopa and its application to relevant anti-parkinson drugs, Anal. Bioanal. Chem. 414 (2022) 1713–1722, https://doi.org/10.1007/s00216-021-03804-8.
[16] M. Lettieri, M. Spinelli, L. Caponi, S. Scarano, P. Palladino, A. Amoresano, M. Minunni, Sensing of catecholamine in Human urine using a simple colorimetric assay based on direct MN and indolequinone formation, Sensors 23 (2023) 3971, https://doi.org/10.3390/s23083971.
[17] A. Gałuszka, Z. Migaszewski, J. Namieśnik, The 12 principles of green analytical chemistry and the SIGNIFICANCE mnemonic of green analytical practices, TrAC Trends Anal. Chem. 50 (2013) 78–84, https://doi.org/10.1016/j.trac.2013.04.010.
[18] M. Khalid, M.G. Mesa, D. Scapens, A. Osatiashtiani, Advances in sustainable γ-valerolactone (GVL) production via catalytic transfer hydrogenation of levulinic acid and its esters, ACS Sustain. Chem. Eng 12 (2024) 16494–16517, https://doi.org/10.1021/acssuschemeng.4c05812.
[19] C.J. Clarke, W.-C. Tu, O. Levers, A. Bröhl, J.P. Hallett, Green and sustainable solvents in chemical processes, Chem. Rev 118 (2018) 747–800, https://doi.org/10.1021/acs.chemrev.7b00571.
[20] T.M. Lammens, M.C.R. Franssen, E.L. Scott, J.P.M. Sanders, Synthesis of biobased N-methylpyrrolidone by one-pot cyclization and methylation of γ-aminobutyric acid, Green. Chem. 12 (2010) 1430–1436, https://doi.org/10.1039/C0GC00061B.
[21] A. Jordan, C.G.J. Hall, L.R. Thorp, H.F. Sneddon, Replacement of less-preferred dipolar aprotic and ethereal solvents in synthetic organic chemistry with more sustainable alternatives, Chem. Rev 122 (2022) 6749–6794, https://doi.org/10.1021/acs.chemrev.1c00672.
[22] D. Kong, A.V. Dolzhenko, Cyrene: a bio-based sustainable solvent for organic synthesis, Sustain. Chem. Pharm. 25 (2022) 100591, https://doi.org/10.1016/j.scp.2021.100591.
[23] F. Kerkel, M. Markiewicz, S. Stolte, E. Müller, W. Kunz, The green platform molecule gamma-valerolactone – ecotoxicity, biodegradability, solvent properties, and potential applications, Green. Chem. 23 (2021) 2962–2976, https://doi.org/10.1039/D0GC04353B.
[24] F. Valentini, G. Brufani, B. Di Erasmo, L. Vaccaro, γ-valerolactone (GVL) as a green and efficient dipolar aprotic reaction medium, Curr. Opin. Green Sustain. Chem. 36 (2022) 100634, https://doi.org/10.1016/j.cogsc.2022.100634.
[25] J. Sherwood, H.L. Parker, K. Moonen, T.J. Farmer, A.J. Hunt, N-butylpyrrolidinone as a dipolar aprotic solvent for organic synthesis, Green. Chem. 18 (2016) 3990–3996, https://doi.org/10.1039/C6GC00932H.
[26] Y. Lv, J. Wu, J. Zhang, Y. Niu, C.-Y. Liu, J. He, J. Zhang, Rheological properties of cellulose/ionic liquid/dimethylsulfoxide (DMSO) solutions, Polym. (Guildf) 53 (2012) 2524–2531, https://doi.org/10.1016/j.polymer.2012.03.037.
[27] S.N. Politis, P. Colombo, G. Colombo, D.M. Rekkas, Design of experiments (DoE) in pharmaceutical development, Drug Dev. Ind. Pharm. 43 (2017) 889–901, https://doi.org/10.1080/03639045.2017.1291672.
[28] F. Pena-Pereira, W. Wojnowski, M. Tobiszewski, AGREE—Analytical GREEnness metric approach and software, Anal. Chem 92 (2020) 10076–10082, https://doi.org/10.1021/acs.analchem.0c01887.
[29] M. Otto, Chemometrics: statistics and computer application in analytical chemistry., 2023.
[30] D.E. Booth, Chemometrics: data analysis for the laboratory and Chemical Plant, Technometrics. (2004), https://doi.org/10.1198/tech.2004.s738.
[31] R. Gheshlaghi, J.M. Scharer, M. Moo-Young, P.L. Douglas, Application of statistical design for the optimization of amino acid separation by reverse-phase HPLC, Anal. Biochem 383 (2008) 93–102, https://doi.org/10.1016/j.ab.2008.07.032.
[32] G. Vicente, A. Coteron, M. Martinez, J. Aracil, Application of the factorial design of experiments and response surface methodology to optimize biodiesel production, Ind. Crops. Prod. 8 (1998) 29–35, https://doi.org/10.1016/S0926-6690(97)10003-6.
[33] D. Harvey, Modern Analytical Chemistry, McGraw-Hill, Boston, 2000.
[34] R.H. Myers, D. Montgomery, G.G. Vining, T.J. Robinson, Generalized Linear Models: with Applications in Engineering and the Sciences: Second Edition, John Wiley and Sons Inc., 2012, https://doi.org/10.1002/9780470556986.
[35] M. Sugumaran, Reactivities of quinone methides versus o-quinones in catecholamine metabolism and eumelanin biosynthesis, Int. J. Mol. Sci. 17 (2016) 1576, https://doi.org/10.3390/ijms17091576.
[36] S.I. Kaya, G. Ozcelikay-Akyildiz, S.A. Ozkan, Green metrics and green analytical applications: a comprehensive outlook from developing countries to advanced applications, Green Anal. Chem. 11 (2024) 100159, https://doi.org/10.1016/j.greeac.2024.100159.
[37] N.P. Koppuravuri, S. Yenduri, V.H. N, Judging the greenness of analytical method using ecological foot prints: a green metric approach, Res. J. Pharm. Technol. 17 (2024) 6132–6136, https://doi.org/10.52711/0974-360X.2024.00930.
[38] Blue applicability grade index (BAGI) and software: a new tool for the evaluation of method practicality - Green Chemistry (RSC Publishing), (n.d.). https://pubs.rsc.org/en/content/articlelanding/2023/gc/d3gc02347h (accessed January 12, 2026).
[39] V. Sliesarenko, U. Bren, A. Lobnik, Fluorescence based dopamine detection, Sens. Actuators Rep. 7 (2024) 100199, https://doi.org/10.1016/j.snr.2024.100199.
[40] * R.M.O. Ali Idris, E.A. Gadkariem, K.E.E. Ibrahim, A. Mohamed Magdi, Kinetic method for determination of dopamine hcl in bulk & injectable forms, Int. J. Chem. Sci. Technol. (2011) 218–223.
[41] M.A.A. Ramadan, I. Almasri, G. Khayal, Spectrophotometric determination of dopamine in bulk and dosage forms using 2,4-dinitrophenylhydrazine, Turk. J. Pharm. Sci. 17 (2020) 679–685, https://doi.org/10.4274/tjps.galenos.2019.25902.
[42] M.R. Shishehbore, A. Asgharpoor, N. Nasirizadeh, A novel kinetic spectrophotometric method for the determination of dopamine in biological and pharmaceutical samples, J. Chem. (2013) 819460, https://doi.org/10.1155/2013/819460, 2013.
[43] L. Guo, Y. Zhang, Q. Li, Spectrophotometric determination of dopamine hydrochloride in pharmaceutical, banana, urine and serum samples by potassium ferricyanide-Fe(III), ANAL. Sci. 25 (2009) 1451–1455, https://doi.org/10.2116/analsci.25.1451.
[44] N.O.A. Al-Salahi, E.Y. Hashem, D.A. Abdel-Kader, Spectrophotometric methods for determination of dopamine hydrochloride in bulk and in injectable forms, Spectrochim Acta. Mol. Biomol. Spectrosc 278 (2022) 121278, https://doi.org/10.1016/j.saa.2022.121278.