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Determination of Zinc by Solid Surface Fluorescence in Natural Adaptogen Samples

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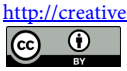

Abstract

Zinc is an essential element for living organisms which plays an important role in the metabolism of proteins and nucleic acids, participating in the activity of approximately 100 enzymes and collaborating in the proper functioning of the immune system. Deficiency of Zn(II) is associated with growth retardation, impaired immune response, premature birth, weight loss and anorexia. On the other hand, adaptogens are a unique group of herbal ingredients used to improve the health of the adrenal system, which is responsible for managing the body's hormonal response to stress. Dietary supplements are often combined in clinical practice to achieve synergistic effects and consequently greater health benefits. The objective of this study was to develop a new method for monitoring Zn(II) in natural adaptogens and to demonstrate a potential magnifying effect on health benefits. It is proposed the Zn(II) determination based in the exaltation of the fluorescent signal of o-Phenanthroline (o-phen) and the dye eosin (eo), using filter paper as a solid supported (without pretreatment) by solid surface fluorescence at λ_{em} = 440 nm (emission), using λ_{ex}t = 370 nm (excitation). A multivariate optimization strategy based on Design of Experiments (DoE) was employed. A full factorial design 2^3 was first applied to screen the significant variables, followed by a Central Composite Design (CCD) to find the optimal conditions. Under optimal experimental conditions, selective and quantitative retention of the metal was achieved, with a detection limit of 0.12 ng L^{-1} and a linearity range from 0.43 to 7.55 × 10^5 ng L^{-1}. The methodology showed high sensitivity, good selectivity and adequate tolerance to possible interferents. It was applied to the determination of Zn(II) in a natural adaptogens samples with satisfactory results, representing a novel alternative to conventional methods for analysis of trace metals.

Subject Areas

Analytical Chemistry

Keywords

Zinc, Adaptogens, o-Phenanthroline, Eosin, Solid Surface Fluorescence, Design Experimental

1. Introduction

Adaptogens are a unique group of herbal ingredients used to improve the health of the adrenal system, which manages the body's hormonal response to stress. It is important to highlight that some adaptogens, along with other botanical supplements, may have concerning interactions with certain prescription medications, including antidepressants, antipsychotics, and immunosuppressants [1] [2]. In some cases, they may decrease or enhance the effects of these medications in the body, causing side effects [3] [4].

Particularly, the co-existence of Zn(II) with an adaptogen may enhance its mechanism of action in the body and amplify its health benefits. Therefore, monitoring the presence of the metal in these samples is of vital importance [5] [6]. This necessarily entails a challenge in the development of new analytical methodologies with high sensitivity and adequate selectivity, which facilitate the determination of metals at concentrations on the order of ultratrace levels in complex matrices [7].

While it is indisputable that the methodologies of choice for the determination of low concentrations of metals continue being atomic spectroscopies [8] [9], sometimes economic limitations restrict access to these instruments, which in addition to being expensive, require costly maintenance. It is at this point that luminescent methods position themselves as a real alternative that allow for the determination of analytes at trace levels, using medium-cost instruments, accessible in control laboratories, without losing analytical quality [10] [11].

Molecular fluorescence is characterized by limited selectivity due to its band-like emission mode, making it susceptible to interference from spectral overlap. However, this disadvantage is relative, as not all substances are fluorescent. In the specific case of metal ions, only a small minority exhibit native fluorescence. Zn(II) in particular belongs to the group of non-fluorescent metals, which is why it must be properly derived in order to be determined by this instrumental methodology [12] [13].

In recent years, researchers have successfully combined fluorescence with simple strategies such as solid-phase extraction, which allows for the proper isolation of the analyte from the matrix components and its preconcentration [14]. This significantly improves the selectivity of the methodology, along with an additional improvement in sensitivity.

This work proposes a simple luminescent methodology for determining trace amounts of Zn(II), based on its complexation with the reagents o-phen and eo. The fluorescent product formed is filtered through an adequate solid support, presented to the instrument in a solid-phase sample holder, and its solid surface fluorescence (SSF) is determined. A multivariate optimization strategy based on Design of Experiments (DoE) was employed. First, preliminary univariate studies were conducted to narrow the experimental domain. Then, a full factorial design 2^3 was applied to screen the significant variables affecting the fluorescence signal. Finally, a Central Composite Design (CCD) was used to optimize the critical factors and build a response surface model. Given the importance of this topic, the optimized methodology was applied to the quantification of trace amounts of Zn(II) present in widely consumed natural adaptogens in our country.

2. Experimental

2.1. Chemicals and Apparatus

Reagents

Stock solutions of Zn(II) were prepared by dilution of 100 μg·mL^{-1} standard solution plasma-pure (Leeman Labs, Inc., Hudson, NH, USA). The standard stock solution was stored in a glass bottle at 4˚C in the dark. Lower concentration standards were obtained weekly by dilution of the stock solutions.

Solution of eosin (H.E Daniel Ltd., UK 1×10^{-3} mol·L^{-1}) and o-Phenanthroline (Merck & Co., Inc., 1×10^{-3} mol·L^{-1}) were prepared weekly by dissolving the appropriate amount of each reagent in ultrapure water. The stability of the solutions was checked using a spectrophotometer. All glass materials were previously rinsed with a 10% (v/v) HNO_3, and then with ultrapure water. All reagents were analytical grade.

Blue ribbon filter papers (FPs) (Whatman, England) 2 - 5 μm pore size and 4.5 cm diameter were used in sorption studies.

Tris-(hydroxymethyl)-aminomethane (Mallinckrodt Chemical Works, St. Louis, USA HTAB, 1×10^{-2}), sodium tetraborate (Merck & Co., Inc., HTAB, 1×10^{-2}), solution was prepared. This solution was adjusted to the desired pH, with aqueous HCl (Merck, Darmstadt, Germany) or NaOH (Mallinckrodt Chemical Works) using a pH meter (Orion Expandable Ion Analyzer, Orion Research, Cambridge, MA, USA) Model EA 940.

The stability of solutions was checked by spectrophotometric measurements. All used reagent were analytical grade.

2.2. Apparatus

All spectrofluorimetric measurements were made using a Shimadzu RF-5301 PC spectrofluorophotometer equipped with a 150 W Xenon lamp and devices for solid supports. Instrument excitation and emission slits both were adjusted to 5/5 nm. (λ_{em} = 440, λ_{exc} = 370).

2.3. Sample Collection and Treatments

The proposed methodology was applied to the analysis of nine samples: three of aswanda, three of reishi, and three of lion's mane.

The adaptogen samples were acquired from health food stores and selected based on the products most commonly consumed by the Argentine population in these specific regions of the country. The samples were selected considering the main products consumed by population segments with different dietary requirements according to their age and lifestyle. To ensure sample representativeness, a random sampling strategy was used; three samples of the same brand/origin were acquired for each product. The whole products were homogenized and reserved for sample preparation.

2.4. General Procedure

A 500 µL Eosin (Eo) solution (1×10^{-8} mol·L^{-1}), 200 µL o-phen solution (1×10^{-7} mol L^{-1}), Zn (II) sample/standard (0.62, 1.25 and 1.80 ng·L^{-1}), 100 µL Tris buffer (0.1 mol L^{-3}, pH 10.5 were placed in a volumetric flask. The mixture was diluted to 5 mL with ultrapure water and was filtrated across blue ribbon filter using a vacuum pump and dried at room temperature. Zn (II) was determined on the solid support by SSF at λ_{em} =440 nm and λ_{exc} = 370 nm, using a solid sample holder (Figure 1).

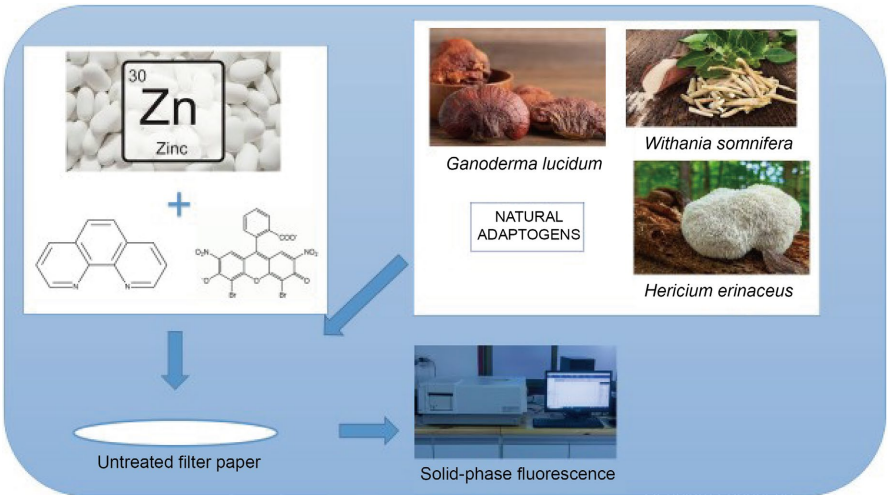

Figure 1. Representative outline of the general procedure.

2.5. Preliminary Univariate Studies

In order to narrow the experimental domain and identify the most relevant factors and their ranges, preliminary univariate studies were conducted. Factors such as pH, type and concentration of buffer, nature of solid support, and reagent concentrations were evaluated one at a time while keeping the others constant. These experiments allowed us to establish approximate optimal regions and select the factors to be included in the multivariate optimization stage. The results of these preliminary studies are presented in Section 3.1.

2.6. Multivariate Optimization: Experimental Design

Once the critical variables and their working ranges were identified through univariate assays, a multivariate optimization strategy based on Design of Experiments (DoE) was implemented using Minitab® software (version 18). The optimization was carried out in two sequential stages: screening and response surface methodology (RSM).

2.6.1. Screening Stage: Full Factorial Design 2^3

A full factorial design 2^3 with two replicates and three central points (total 19 runs) was employed to identify the factors that significantly affect the fluorescent signal.

Table 1 shows the experimental matrix with the factors and levels evaluated, where (−1) and (+1) represent the low and high levels, respectively, and (0) represents the central point."

The selected factors and their levels were:

Table 1. Ranges of the factors studied in the 2^3 factorial design.

Factor	Low level (−1)	High level (+1)
A: Phosphate buffer concentration (mol L^{-1})	0.005	0.015
B: o-Phenanthroline volume (µL)	100	300
C: Eosin volume (µL)	400	600

pH was fixed at 7.0 based on the preliminary studies. The response variable was the relative fluorescence intensity (%). Experiments were performed in random order to minimize systematic errors. The significance of main effects and interactions was evaluated by analysis of variance (ANOVA) and Pareto chart.

2.6.2. Optimization Stage: Central Composite Design (CCD)

Based on the screening results, factors A (buffer concentration) and B (o-Phenanthroline volume) were found to be statistically significant ($p < 0.05$), while factor C (eosin volume) and its interactions were not significant. Therefore, a Central Composite Design (CCD) 2^2 + axial points + central points was performed to optimize A and B. The experimental ranges were:

Factor	$-\alpha$	**−1**	**0**	**+1**	$+\alpha$
A: Buffer concentration (mol L^{-1})	0.0016	0.005	0.010	0.015	0.0184
B: o-Phenanthroline volume (µL)	32	100	200	300	368

The CCD consisted of 13 runs: 4 factorial points, 4 axial points ($\alpha = 1.414$), and 5 central points to estimate pure error and evaluate curvature. The experimental data were fitted to a second-order polynomial model by multiple regression. The quality of the model was evaluated by ANOVA, lack-of-fit test, and coefficient of determination (R^2).

2.7. Interferences Study

Different amounts of foreign ions, which may be present in samples, (1/10, 1/100, 1/500 and 1/1000 Zn(II)/interferent ratio) were added to the test solution containing 1.25 ng·L^{-1} Zn(II) and the 2.4 General Procedure was applied.

2.8. Dilution Test

In order to establish the proper volume of each sample for realizing Zn(II) determination, several sample volumes were assayed. The adequate dilution for each sample was that signal which intensities fall into the linearity range of the developed methodology. Dilution test was assayed of 100 µL to 25 µL depending of the sample characteristics. These dilution factors were adopted for the following studies. Zn(II) contents were determined by the proposed methodology, employing the obtained volume samples through test dilution.

2.9. Accuracy Study

Volumes of 0.100 mL of samples were spiked with increasing amounts of Zn(II) (0.62, 1.25 and 1.80 ng·L^{-1}). Zinc contents were determined by proposed methodology.

2.10. Precision Study

The repeatability (within-day precision) of the method was tested for adaptogen samples replicate samples (n = 6) spiked with 0.62, 1.25 and 1.80 ng·L^{-1} of Zn(II) and metal contents were determined by proposed methodology.

3. Results and Discussion

Previous research has demonstrated the feasibility of forming ternary o-phen/eo/metal ion complexes [15]-[18] and their subsequent determination by molecular fluorescence.

The fluorescence of the eo/o-phen/Zn(II) system was initially explored in an aqueous medium without obtaining satisfactory results in terms of signal enhancement and stability.

Subsequently, the fluorescent emission of the eo/o-phen/Zn(II) system was explored by SSF using different solid supports. As a preliminary study, the fluorescent signal of the eo/o-phen/Zn(II) complex was evaluated on different membrane materials. The results obtained showed a significant signal enhancement when Blue Ribbon filter paper was used in the retention process. This fact reinforces the formation of an eo/o-phen/Zn(II) complex, with the added advantage of greater sensitivity to the luminescent response and a linear signal enhancement as a function of analyte concentration (**Figure 2**).

3.1. Preliminary Studies

To establish approximate optimal regions for the quantification of trace amounts of Zn(II) using SSF, sequential univariate investigations were carried out on

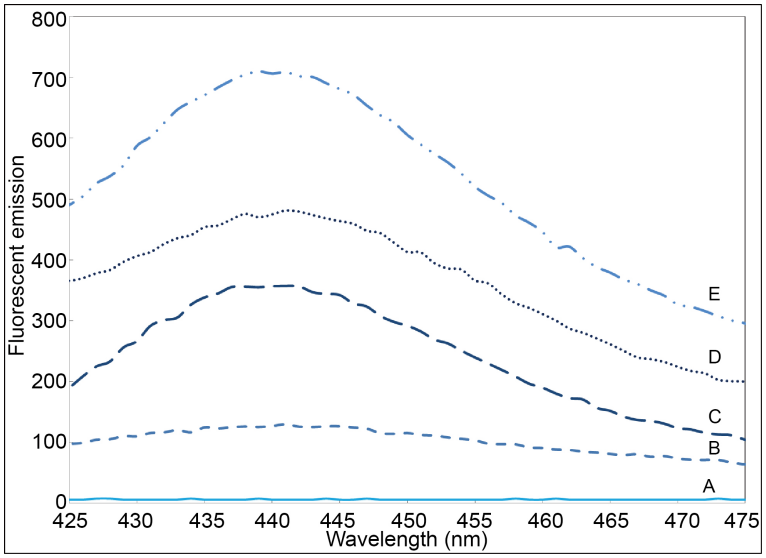

Figure 2. Emission fluorescent spectra of eo/o-phen/Zn(II) complex. (A) Filter paper; (B) Reagent blank: Filter paper with o-fen and eo; (C) Same B with Zn(II) 0.62 ng·L^{-1}; (D) Same B with Zn(II) 1.25 ng·L^{-1}; (E) Same B with Zn(II) 1.80 ng·L^{-1}.

experimental parameters such as pH, nature buffer solution, and nature of the solid support. These preliminary experiments allowed us to narrow the experimental domain for the subsequent multivariate optimization.

3.2. Optimization of Variables

To establish the optimal experimental conditions for the quantification of trace amounts of Zn(II) using SSF, sequential investigations were carried out on experimental parameters such as the nature and concentration of samples and the nature of the solid support, pH, and the nature and concentration of the buffer solution, through the analysis of their spectral behavior.

To ensure the retention of the ternary complex eo/o-phen/Zn(II) on the solid support, tests were performed using membrane filters of different types. The retention levels for each material tested were evaluated by the intensity of the fluorescent emission (λ_{exc} = 370 nm; λ_{em} = 440 nm).

The solid support for the SPE stage was chosen considering quantitative analytical retention and the lowest background fluorescent emission. The retention of the complex was verified by measuring the fluorescent intensity of the filtered solution. The best results were obtained using blue ribbon filter paper.

The pH value plays an important role in the formation of associations with metals. The results are illustrated in **Figure 3**; near pH 7.0, a maximum enhancement of the fluorescent signal was obtained. Due to this behavior, the pH value of 7.0 was selected as the working value for the following experiments.

The effect of different pH buffering agents was studied, several tests were conducted in which all experimental variables were kept constant except for the type of the buffer solution. The system's behavior was studied for Tris, Phosphate, Sodium Tetraborate, Potassium Biphthalate, and Acetic Acid/Acetate buffers in a

buffer concentration of 1×10^{-4}. The best results in terms of stability and sensitivity were obtained with phosphate buffer.

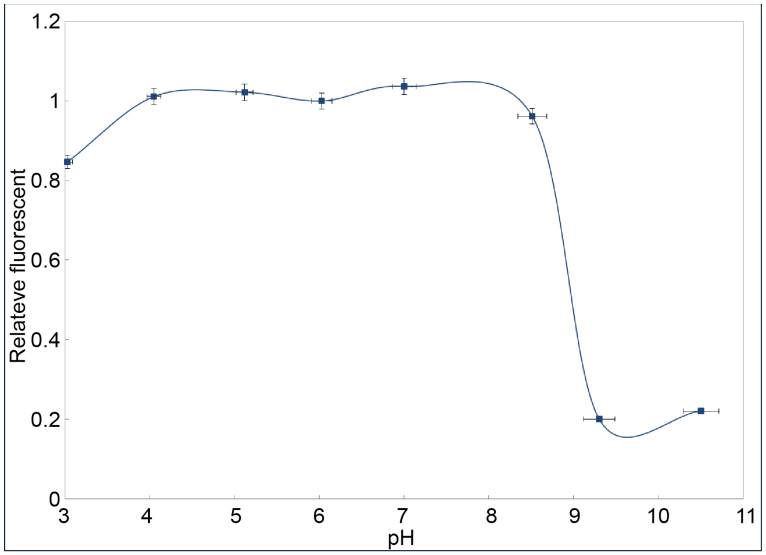

Figure 3. Influence of pH on the signal emission fluorescent of the ternary complex eo/o-phen/Zn(II).

To study the effect of the buffer concentration on the system, concentrations in the range of 1×10^{-5} to 5×10^{-4} mol·L^{-1} were analyzed by multivariate optimization. The best results regarding system stability and sensitivity were obtained at a concentration of 2.5×10^{-4} mol·L^{-1}.

3.2.1. Screening: Identification of Significant Factors

Once the preliminary ranges were established, a full factorial design 2^3 was applied to identify which factors significantly affect the fluorescence signal. **Table 2** shows the experimental matrix and the relative fluorescence responses obtained for each run. Statistical analysis was performed using both ANOVA and Pareto chart. The Pareto chart (**Figure 4**) shows that factors A (buffer concentration) and B (o-Phenanthroline volume) exceed the t-value limit (dashed line), indicating a statistically significant effect, while factor C (eosin volume) and all interactions (AB, AC, BC) do not. ANOVA corroborated these findings, yielding p-values for A and B factors (both < 0.05), and p-values > 0.05 for factor C and all interactions. Based on these results, factor C was fixed at its central level (500 µL) for subsequent experiments.

3.2.2. Optimization: Response Surface Methodology (CCD)

The significant factors A (buffer concentration) and B (o-Phenanthroline volume) were optimized using a Central Composite Design. The experimental results were fitted to a second-order polynomial model. The ANOVA (**Table 2**) revealed that the quadratic model was highly significant ($p < 0.0001$), with a non-significant lack-of-fit ($p > 0.05$), indicating good predictive capacity.

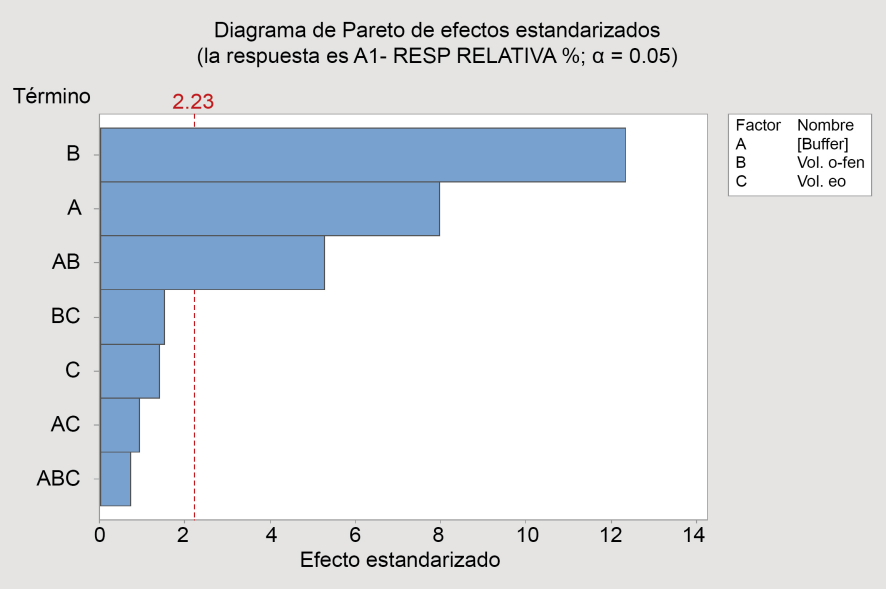

Figure 4. Pareto chart of the standardized effects from the 2^3 factorial design.

Table 2. ANOVA for the quadratic model from the CCD.

Source	Sum of squares	df	Mean square	F-value	p-value	
Model	1243.07	5	248.61	8.44	0.0071	significant
A-buffer concentration	36.29	1	36.29	1.23	0.3037	
B-vol. o-fen	400.58	1	400.58	13.60	0.0078	
AB	2.18	1	2.18	0.0738	0.7937	
A^2	479.21	1	479.21	16.27	0.0050	
B^2	429.48	1	429.48	14.58	0.0066	
Residual	206.24	7	29.46			
Lack of fit	114.84	3	38.28	1.68	0.3083	not significant
Pure error	91.40	4	22.85			
Cor total	1449.31	12				

The response surface and contour plots (Figure 5) were constructed from the fitted model. The optimal conditions predicted by the model were:

- Phosphate buffer concentration: 0.010 mol·L^{-1}
- o-Phenanthroline volume: 250 μL

The response surface methodology (RSM) was applied to model the relationship between the significant factors (buffer concentration, A; o-Phenanthroline volume, B) and the relative fluorescence response (Y). The experimental data were fitted to a second-order polynomial equation. The final model in terms of coded factors (after removing non-significant terms) was:

***R1 = 16.24 + 6770.83 × [Buffer] + 0.3703 × o-phen + 1.475 × [Buffer] × o-**

$$\text{phen} - 3.32 \times 10^{5} \times [\text{Buffer}]^{2} - 7.86 \times 10^{-4} \times \text{o-phen}^{2*}$$

where:

- $R1$ = Relative fluorescence intensity (%)
- [*Buffer*] = Phosphate buffer concentration (mol·L^{-1})
- *o-phen* = o-Phenanthroline volume (µL)

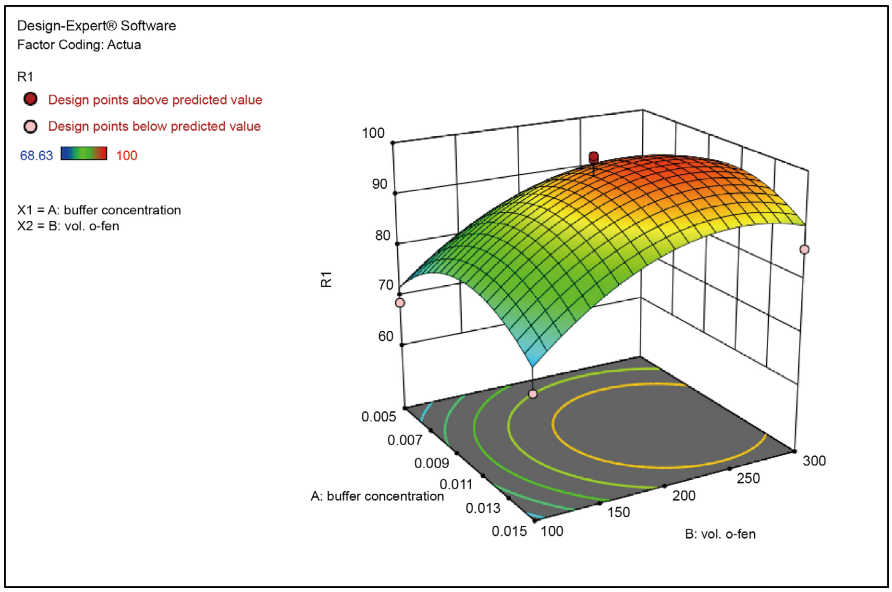

Figure 5. Response surface (3D) for the optimization of buffer concentration and o-Phenanthroline volume.

3.3. Analytical Parameters

Under optimal working conditions (buffer phosphate 0.010 mol·L^{-1}, o-Phenanthroline 250 µL, eosin 500 µL, pH 7.0, Blue Ribbon filter paper), a limit of detection (LOD) of 0.12 ng·L^{-1} and a limit of quantification (LOQ) of 0.43 ng·L^{-1} were achieved, with a linearity range of 0.43 - 7.55 × 10^{5} ng·L^{-1} (**Table 3**). Precision (repeatability intra-day variation) was 0.228 CV and reproducibility (inter-day variation) was 0.243 CV. The accuracy of the method was verified by applying the standard addition method, obtaining satisfactory results with recoveries close to 100%.

Table 3. Experimental conditions and figures of merit.

	Parameters	Studied range	Optimal condition
Analytical parameters	pH	4 - 10	7.0
	Phosphate buffer concentration	5 × 10^{-5} - 5 × 10^{-4} mol·L^{-1}	2.5 × 10^{-4} mol·L^{-1}
	Nature of solid support	Nylon, acetate, mixed esters, immobilon, filter paper: black, white and blue ribbon	Blue ribbon filter paper
Figures of merit	LOD	-	0.12 ng·L^{-1}
	LOQ	-	0.43 ng·L^{-1}
	Linearity range	-	0.43 - 7.55 × 10^{5} ng·L^{-1}.
	R^2	-	0.9983

The limit of detection (LOD) was calculated as 3.3 s/m [19], where s is the standard deviation of 10 successive means of the blank and m is the slope of the calibration curve (calibration sensitivity). The limit of quantification (LOQ) was calculated as 10 s/m. The range of linearity was evaluated by checking the linear regression coefficient (R^2) of the calibration curve. The linearity of the calibration curve was considered acceptable when $R^2 > 0.9983$.

3.4. Interference Study

The effect of the presence of potentially interfering ions on the quantification of Zn(II) was studied. A given ion was considered interfering when it generated a variation in the analyte's fluorescent signal greater than ± 5%. Under optimal conditions, Na^+, K^+, Cl^-, Fe^{3+}, Co^{2+}, Mn^{2+}, CO_3^{2-}, SO_4^{2-}, NO_3^-, Cd^{2+}, Ca^{2+}, Mg^{2+}, Sb^{3+}, As^{3+}, Ni^{2+}, and Cu^{2+} can be present up to a 1000:1 excess with respect to Pb^{2+} without interfering. Table 4 shows the tolerance results obtained for a group of ions commonly present in the analyzed samples.

The results obtained demonstrate the good tolerance of the proposed methodology.

Table 4. Tolerance limits of selected interfering species in Zn(II) determination.

Interferent/Zn(II) mole ratio	Interferent specie
1000:1	Na^+, K^+, Cl^-, Fe^{3+}, Mn^{2+}, Cd^{2+}, Ca^{2+}, Mg^{2+}, Sb^{3+}, Al^{3+}, As^{3+}, Co^{2+}, CO_3^{2-}, SO_4^{2-}, NO_3^-, Ni^{2+}, Cu^{2+}
100:1	Pb^{2+}

3.5. Applications

The proposed methodology was applied to the analysis of nine adaptogen samples: lion's mane, reishi, and ashwagandha. Six portions of each sample were analyzed using the developed method, with the average Zn(II) concentration obtained in the determinations serving as the baseline. Increasing amounts of the metal were then added to the samples, and the concentration was determined using molecular fluorescence.

Table 5 shows the analyte concentrations found and their corresponding coefficients of variance. Reproducibility was evaluated by replicating the proposed procedure six times for each level of super-addition. The samples were successfully analyzed, achieving average recoveries close to 100%.

Table 5. Recovery studies Zn(II) determination in adaptogen samples.

Sample	Zn(II) added[a] ($\mu g \cdot L^{-1}$)	Proposed methodology		
		Zn(II) found ± CV[c] ($\mu g \cdot L^{-1}$)	% recovery[b] (n = 6)	Zn(II) found ± CV ($\mu g \cdot g^{-1}$)
1	-	2.87 ± 0.04	-	542.33± 0.04
	0.62	3.51 ± 0.02	103.22	
	1.25	4.13 ± 0.08	100.80	

Continued

	-	3.09 ± 0.01	-	
2	0.62	3.74 ± 0.01	104.83	602.55± 0.01
	1.25	4.32 ± 0.04	98.40	
	-	2.96 ± 0.03	-	
3	0.62	3.56 ± 0.06	96.77	571.62± 0.03
	1.25	4.23 ± 0.04	101.60	
	-	1.58 ± 0.05	-	
4	0.62	2.22 ± 0.04	103.22	301.57 ± 0.05
	1.25	2.81 ± 0.05	98.40	
	-	1.33 ± 0.08	-	
5	0.62	1.96 ± 0.05	101.61	209.77± 0.08
	1.25	2.57 ± 0.02	99.20	
	-	1.47 ± 0.07	-	
6	0.62	2.08 ± 0.03	98.38	270.96± 0.07
	1.25	2.70 ± 0.02	98.40	
	-	0.68 ± 0.04	-	
7	0.62	1.32 ± 0.05	103.22	37.57± 0.04
	1.25	1.90 ± 0.01	97.60	
	-	0.51 ± 0.06	-	
8	0.62	1.12 ± 0.07	98.39	27.15± 0.03
	1.25	1.77 ± 0.04	100.80	
	-	0.54 ± 0.06	-	
9	0.62	1.15 ± 0.03	98.39	34.38± 0.06
	1.25	1.79 ± 0.02	100.00	

1-3: Lion's mane; **4-6:** Reishi; **7-9:** Ashwagandha root. [a]Mean ± standard deviation for six determinations; [b]%Recovery = 100 * (analyte concentration in fortified sample – analyte concentration in the unfortified sample)/analyte concentration added in the unfortified sample; [c]Coefficient variation = (SD/mean).

4. Conclusions

This study presents an alternative, simple, precise, and economically viable methodology for the detection of trace amounts of Zn(II) using solid-phase fluorescence. The application of molecular fluorescence in this research has demonstrated multiple analytical advantages, such as high sensitivity, adequate selectivity, and a wide dynamic range. The retention and preconcentration of Zn(II) on filter paper have proven to be effective tools for the accurate determination of this analyte in the analyzed samples. The implemented solid-phase extraction strategy has made it possible to eliminate matrix effects in complex samples, enabling the quantification of the analyte with recoveries close to 100%. The excellent tolerance to high concentrations of potential interferents highlights the selectivity and ver-

satility of the proposed methodology. The sensitivity achieved with this methodology is comparable to that of more expensive atomic spectroscopic techniques, highlighting the effectiveness of this sustainable and efficient approach. It aligns with the principles of green chemistry by minimizing waste generation, using mostly non-toxic reagents, and employing a relatively inexpensive instrument such as a spectrofluorometer. This underscores the importance of developing environmentally friendly and economically viable analytical techniques. The successful application of this methodology to samples of natural adaptogens has revealed that zinc is a mineral present in very low concentrations in ashwagandha root. Therefore, to enhance its effects, it would be beneficial to combine it with supplements containing zinc and vitamin C. Furthermore, lion's mane and reishi mushrooms are rich in essential minerals, including zinc. This innovative approach opens the door to future applications in the detection of other metals in different matrices, thus expanding its potential in the field of analytical chemistry.

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Conflicts of Interest

The authors declare no conflicts of interest.

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