Vol.10, No.1 pages 59-72 *Food Agricultural Sciences and Technology (FAST)* *Research Article*

Optimization and validation of experimental designs for selenium analysis via the iodometric method in food evaluation

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Received: 13th October 2023**, Revised:** 8th December 2023**, Accepted:** 12th December 2023

Abstract - This study delved into the realm of experimental design methodologies for Selenium (Se) analysis using the iodometric method, with a particular focus on comparing the applicability of completely randomized design (CRD) and factorial experimental design (FED). The research revealed distinctive optimal conditions for Se analysis: the CRD prescribed conditions of 0.1 M HCl, 0.2 M KI, and 0.02% w/v starch, while the FED formulated alternative conditions, characterized by 0.15 M HCl, 0.15 M KI, and 0.03% w/v starch. Nevertheless, both experimental designs provided statistically robust Se quantification results through UV-visible spectrophotometry. This underscored the pivotal role of experimental design in result determination. The CRD provides efficient processes but may overlook factor interactions, whereas the FED, although more resource-intensive, furnishes a comprehensive dataset. The choice between CRD and FED should be guided by the specific research objectives, finely balancing simplicity, and the capture of intricate factor interactions in the relentless pursuit of optimal conditions. Furthermore, the analysis of Se content in Se-enriched food samples under the conditions of iodometry using

Citation: Thosaikham, W., Wongkam, N., Hemathurin, R., Chantiratikul, P., Chantiratikul, A., Sakong, P. (2024). Optimization and validation of experimental designs for selenium analysis via the iodometric method in food evaluation. *Food Agricultural Sciences and Technology, 10*(1). 59-72. https://doi.org/10.14456/fast.2024.5

CRD and FED conditions showed no significant differences with the results of HG-AAS ($p > 0.05$). This robust concordance emphasizes the reliability and cost-effectiveness of both CRD and FED iodometric methods, offering versatile utility, including in the food quality assessment. This approach has the potential to lower quality control expenses in contrast to alternative methods, all the while ensuring consistent and reliable outcomes.

Keywords: Selenium, iodometry, completely randomized design, factorial experimental design, Se-enriched foods

1. Introduction

Selenium (Se) crucial trace element, is essential for human well-being, contributing to functions such as antioxidant defense and immune system support (Chantiratikul *et al*., 2021). The production of Se-enriched foods is a specialized process designed to increase the Se content in a variety of food items, providing health advantages to consumers. This production procedure encompasses the selection of a Se source, fortifying the food with Se via meticulous supervision, stringent quality assurance, and appropriate labeling for marketing purposes (Maneetong *et al*., 2013). The availability of Se-enriched food products in diverse forms, ranging from grains to dairy items, offers a convenient method for individuals to augment their Se intake, especially in regions characterized by naturally low soil Se levels (Maneetong *et al*., 2013; Zhang *et al*., 2021). Nevertheless, it is imperative to maintain a balance, as excessive Se consumption can lead to health concerns (Leamsamrong *et al*., 2019). Therefore, rigorous oversight and regulation are essential throughout the production of Se-enriched foods to ensure consumer safety and well-being (Trombetti *et al*., 2022).

Se-enriched food production rests on meticulous quality control, especially concerning Se content analysis. Accurate Se determination plays a vital role in ensuring product safety, efficacy, and regulatory compliance (Qiu *et al*., 2021; Zhang *et al*., 2021). Regular monitoring and verification of Se content empowers producers to guarantee their products meet both labeled nutritional claims and established safety standards. This rigorous quality control process safeguards consumer health and bolsters the market standing of Se-enriched food (Hamdan *et al*., 2022; Trombetti *et al*., 2022). The quality control measures typically rely on advanced analytical techniques, such as hydride generation-atomic absorption spectroscopy (HG - AAS) and inductively coupled plasma mass spectrometry (ICP - MS), due to their superior precision and sensitivity (Hegedus *et al*., 2008; Khan *et al*., 2013; Mollo *et al*., 2013).

Although these methods can effectively determine Se in Se-enriched products, they are often expensive. An alternative, cost-effective method is iodometry, where selenious acid (H_2SeO_3) reacts with iodide ions to produce iodine (I_2), which then forms a blue I_3^-/starch complex when reacting with starch solution (Kulkarni *et al*., 2018; Mörschbächer *et al*., 2018; Sekine *et al*., 1968). The absorbance of I_3^-/starch complex can be measured with a UV-visible spectrometer at 600 nm, making iodometry a straightforward and economical choice for Se analysis (Pesek *et al*., 2022).

Analytical chemistry is a vital field in scientific research and industry, providing accurate data for diverse purposes such as quality control, environmental monitoring, and scientific discovery. However, the quality of analytical chemistry results heavily depends on experimental design (Araujo & Brereton, 1996). Proper experimental design is important for improving accuracy and achieving analytical objectives. It involves considering factors like environmental conditions, instrument precision, and potential risks (Ferreira *et al*., 2018). Setting up experiments and selecting appropriate methodologies are essential for obtaining reliable results. There are various experimental design approaches available to determine the suitable condition, such as completely randomized design, simplex experimental design, Box-Behnken design, and factorial experimental design. It is imperative to note that distinct experimental methodologies can yield distinct sets of optimal conditions for analysis, emphasizing the need for a thoughtful and tailored approach (Bezerra *et al*. 2019).

Completely randomized design (CRD) and factorial experimental design (FED) are contrasting methods in experimental research. CRD is simple, involving random subject allocation to treatment groups, typically with one independent variable (Araujo & Brereton, 1996; Ferreira *et al*., 2018). Conversely, FED is more intricate, examining the combined influence of multiple independent variables and their interactions. While CRD suits basic and single-variable studies, FED provides a holistic view of the interplay among diverse factors, making it suitable for complex research. The efficiency of FED in exploring interactions distinguishes it from CRD, which may require numerous separate experiments for equivalent insights. The choice depends on research goals and variable complexity (Leardi, 2009; Prajapati *et al*., 2022; Tarley *et al*., 2009). Therefore, this research aimed to investigate the optimal condition for iodometry-based Se analysis, comparing CRD and FED for application to determine Se content in food samples with cost-effective and acceptable validation. This study is expected to demonstrate its ability to reduce quality control costs in Se-enriched food production when compared to other analytical methods and provide consistent and reliable results.

2. Material and methods

2.1 Instrument

A UV-Vis spectrophotometer used was model UV-1601, Shimadzu (Japan). Sample digestion was achieved using a graphite heating block from Seal Analytical (USA) as the heating source. The atomic absorption spectrophotometer used was model 240FS AA from Agilent Technologies (USA). It was equipped with an electrothermal atomizer, controlled using an electrothermal temperature controller (ETC-60). Se hydride generation was accomplished using the vapor generation apparatus (VGA 77). The Se hollow cathode lamp (HCL) was acquired from Hamamatsu (Japan).

2.2 Reagents and materials

Hydrochloric acid (HCl), nitric acid (HNO_3), potassium iodide (KI), sodium hydroxide, and soluble starch were analytical grade reagents from Carlo Erba (Italy). Sodium selenite and Se standard solution (AAS grade) were purchased from Fisher

Chemical (UK), while sodium borohydride was obtained from HiMedia (India). The Se-enriched sprout samples used in this research, including Se-enriched Chinese daikon sprouts, Se-enriched Chinese kale sprouts, and Se-enriched Chinese mustard sprouts, were produced through hydroponic cultivation, as demonstrated by Maneetong *et al*. (2013). The supplement samples, referred to as Supplement A and Supplement B, were obtained from a pharmacy in Sakon Nakhon Province.

2.3 Completely randomized design

The iodometry method for Se analysis is influenced by various factors, including the concentrations of HCl, KI, and starch in the mixture. Optimization of these factors is essential for effective I_3^-/starch complex production. Initially, the concentration of HCl was optimized in the range of 0.00 - 0.40 M, while the concentrations of Se, KI, and starch were held constant at 0.50 mg L^{-1}, 0.20 M, and 0.02% w/v, respectively. The mixture was prepared by combining 2 M HCl, 10 mg Se L^{-1}, and 2% w/v KI stock solutions in a volumetric flask. After incubation at 25 ºC for 2 min, selenite reacted with I^- ions to form I_2. A stock starch solution was then added, leading to the formation of the blue I_3^-/starch complex. The absorbance of the solution was measured using UV-visible spectrophotometry at 600 nm. The optimal HCl concentration was selected for subsequent experiments. Afterward, the concentration of KI was optimized within the range of 0.00 -0.40 M under the established optimal HCl conditions. Lastly, the effect of starch concentration was explored in the range of 0.00 - 0.04% w/v, with HCl and KI concentrations held at their optimum levels. The sequential optimization of these factors resulted in the identification of optimal conditions for I_3^-/starch complex production in the iodometry method for Se analysis. This optimized method can be used to determine the Se content of various samples accurately and reliably.

2.4 Factorial experimental design

The optimal conditions for Se determination using the iodometry method were investigated through a comprehensive full factorial experimental design. The concentrations of HCl, KI, and starch in the mixture, represented by factors A, B, and C, respectively, were combined in a full factorial design (A x B x C), resulting in 5 x 5 x 5 = 125 combinations (n = 3). Factor A represented HCl concentration (A_1 = 0.00 M, A2 = 0.05 M, A_3 = 0.10 M, A_4 = 0.15 M, and A_5 = 0.20 M). Factor B denoted KI concentration (B_1 = 0.00 M, B_2 = 0.05 M, B_3 = 0.10 M, B_4 = 0.15 M, and B_5 = 0.20 M). Factor C indicated starch concentration (C_1 = 0.00% w/v, C_2 = 0.01% w/v, C_3 = 0.02% w/v, C_4 = 0.03% w/v, and C_5 = 0.04% w/v). Each treatment was prepared by mixing with Se concentration at 0.50 mg L^{-1} for the final concentration of the mixture. The absorbance of the I_3^-/starch complex at 600 nm was measured using the UV-visible spectrophotometer. The full factorial design allowed for the simultaneous evaluation of the main effects and interactions of the three factors on the absorbance of the I_3^-/starch complex. This information can be used to identify the optimal conditions for Se determination using the iodometry method.

2.5 Sample digestion

The samples were prepared using the wet acid digestion method which was demonstrated by Agilent Technologies (2023). A 0.10 g sample was digested in tubes (n = 3) with 5 ml of HNO_3 (65% w/v) at 135 °C until reduced to 1 - 2 ml. After cooling, 5 ml of HCl (37% w/v) was added and heated again until it remained 1 - 2 ml. The digest was then adjusted to the final volume with deionized water and stored at 4 °C. The Se concentration of the solution was determined using the optimal conditions for CRD and FED iodometry.

2.6 Se determination with HG-AAS

Hydride Generation-Atomic Absorption Spectroscopy (HG-AAS) was employed to determine Se content in Se-enriched samples. The results obtained through HG-AAS were utilized to assess the accuracy and efficiency of both iodometric methods. The processed sample solutions underwent Se content analysis using HG-AAS, following the specific conditions demonstrated by Agilent Technologies (2023). The generation of hydrogen selenide (H_2Se) involved using 6.0 M HCl solution, and 6.0% w/v $NaBH_4$ mixed with 0.6% w/v NaOH solution. The H_2Se vapor was isolated and transported into a quartz cell using N_2 as the carrier gas. It was then atomized in an electric thermal atomizer at 980 ºC. Subsequently, the absorption of the spectral wavelength line at 196.03 nm, originating from HCL, was quantified. These findings will be comprehensively compared with the iodometry results obtained from both experimental designs to facilitate an in-depth analysis.

2.7 Method validation

The investigation of the linear range of the calibration curve involved the preparation of Se standard solutions in the range from 0.0 to 1.0 mg L^{-1}, employing experimental designs like CRD and FED. Each of these Se standard solutions was subsequently subjected to UV-visible spectrophotometry analysis at a wavelength of 660 nm, allowing for the construction of a scattering curve depicting the relationship between Se concentration and absorbance. This scatter curve was pivotal in defining the linear range, which was then utilized to establish the calibration curve, along with calculating the linear equation and the linearity (r^2).

The methods' sensitivity was expressed in terms of the limit of detection (LOD) and limit of quantitation (LOQ). These values were determined using the linear equations: LOD = 3.3σ / S and LOQ = 10σ / S, where σ represented the standard error of the calibration curve or the standard deviation of the y-intercept, and S denoted the slope of the calibration curve.

Precision was rigorously evaluated under ideal conditions for both experimental designs, focusing on two aspects: repeatability and reproducibility. Repeatability, specifically concerning intra-day performance, involved the analysis of the mixture with 15 replicates (n=15) using UV-visible spectrophotometry. In contrast, reproducibility was assessed within the context of inter-day performance, involving the analysis of Se with five replicates (n = 5) over a span of three days. Precision was quantified by calculating the relative standard deviation (RSD) based on the absorbance values.

The recovery test was used to evaluate the accuracy of both iodometric methods. It was investigated for two parts of the determination procedure such as sample digestion and iodometric analysis. The recovery of the sample digestion procedure was achieved by spiking 2.0 mg Se standard into kale sprouts sample (non-Se-enriched sprouts) while it was prepared with the wet acid digestion. The digest was determined by both iodometric methods. For the iodometric methods, the Se standard was added into the mixed solution of HCl, KI, and starch by using the digested kale sprouts as the matrices prior to determining with UV-visible spectrophotometry.

2.8 Statistical analysis

The data analysis was performed using SPSS version 19.0 (SPSS Inc.). One-way ANOVA was used to compare groups within the completely randomized design, followed by Duncan's multiple comparison test. Three-way ANOVA was used to assess differences among groups in the factorial design. Differences were considered statistically significant at $p < 0.05$. Regression analysis was used to calculate coefficients.

3. Results and discussions

3.1 Completely randomized design

In the pursuit of optimizing the analysis of Se using the iodometric method, a meticulous CRD experiment was employed. This procedure involved a systematic exploration of the influence of various factors, with a primary focus on the concentrations of HCl, KI, and starch (Mörschbächer *et al.*, 2018).

In the study of the impact of HCl concentration on Se analysis using the iodometric method, optimization was first conducted within the range of 0.00 to 0.40 M. This was studied by fixing the Se standard, KI, and starch concentration in the mixture at 0.5 mg L^{-1}, 0.1 M, and 0.1% w/v, respectively. The results showed that HCl concentrations from 0.00 to 0.10 M significantly affected the increase in absorbance of the I_3^-/starch complex solution at wavelength 600 nm ($p < 0.05$) (Figure 1). However, there was no significant difference in absorbance ($p > 0.05$) when the HCl concentration exceeded 0.10 M. Therefore, an HCl concentration of 0.10 M was deemed suitable and selected for the subsequent optimization.

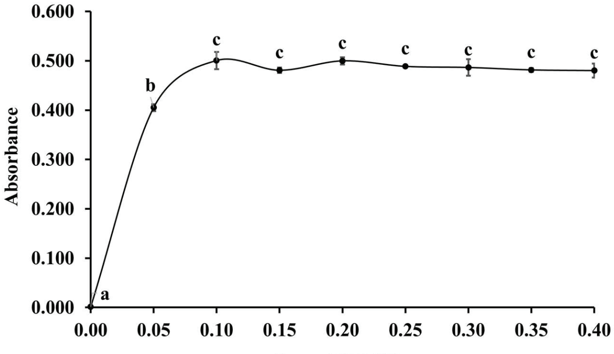

Figure 1. Effect of HCI concentration on Se analysis by iodometry. a, b, and c denote statistically significant differences in absorbance ($p < 0.05$).

The effect of KI concentration on the formation of the I_3^-/starch complex was investigated by varying its concentration from 0.00 to 0.40 M. The result found that an increase in KI concentration from 0.00 to 0.20% w/v significantly affected the formation of the I_3^-/starch complex in the mixture ($p < 0.05$), resulting in an increase in the absorbance of the mixture (Figure 2). There was no significant difference when the KI concentration ranged from 0.20 to 0.40 M. Thus, the optimal KI concentration was determined to be 0.20 M.

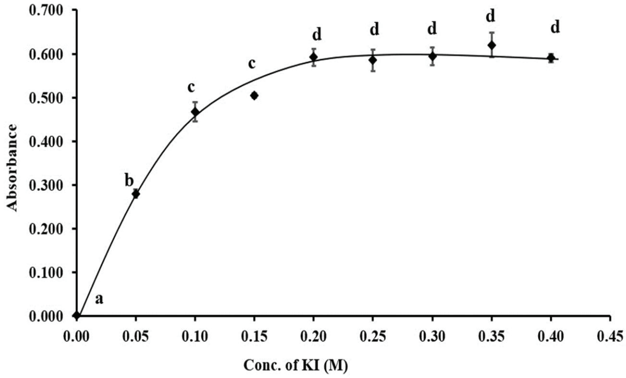

Figure 2. Effect of KI concentration on Se analysis by iodometry. a, b, c, and d denote statistically significant differences in absorbance ($p < 0.05$).

In terms of optimizing starch concentration within the range of 0.00 to 0.040% w/v, it was observed that an increase in starch concentration from 0.00 to 0.02% w/v significantly impacted the absorbance of the I_3^-/starch complex in the mixture (Figure 3). Nevertheless, absorbance remained consistent ($p > 0.05$) when the starch concentration exceeded 0.02% w/v. The optimal starch concentration, found to be 0.02% w/v, established the ideal conditions for Se analysis using the iodometric method through CRD optimization: HCl at 0.10 M and KI at 0.20 M. However, validation is essential to confirm their analytical suitability before application.

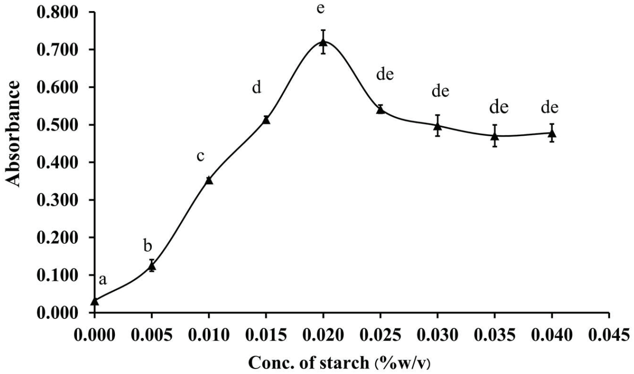

Figure 3. Effect of starch concentration on Se analysis by iodometry. a, b, c, d, and e denote statistically significant differences in absorbance ($p < 0.05$).

The application of CRD played a pivotal role in systematically investigating and optimizing the factors influencing Se analysis using the iodometric method. The assignment of experimental units through CRD facilitated a data-rich exploration of HCl, KI, and starch concentrations, resulting in the identification of optimal conditions: HCl at 0.10 M, KI at 0.20 M, and starch at 0.02% w/v. These findings, supported by statistical significance, promise to enhance the accuracy and efficiency of Se determination. However, it is essential to validate these conditions before practical implementation, underscoring the fundamental role of CRD in systematic optimization in analytical chemistry (Araujo & Brereton, 1996; Mörschbächer *et al*., 2018; Sekine *et al*., 1968).

3.3 Factorial experiment design

The results obtained from the factorial experimental design (FED) analysis offer valuable insights into the impact of HCl, KI, and starch concentrations on Se determination using the iodometric method, employing response surface methodology (RSM). The regression model equation for estimating the response surface graph was $Y= 0.423+0.063(A)+ 0.068(B) + 0.069(C) + 0.021(AB) + 0.021(AC) + 0.025(BC) + 0.008(ABC) - 0.037(A^2) - 0.033(B^2) - 0.030(C^2)$. It was created from coefficient values of each variable. Figure 4 displays 3D response surface plots depicting the relationships between HCl and KI, HCl and starch, and KI and starch. Based on these findings, it was evident that the concentrations of HCl, KI, and starch in the mixture had risen. This increase was attributed to the improved reactivity of Se^{4+} with I^-, leading to a higher formation of the I_3^-/ starch complex.

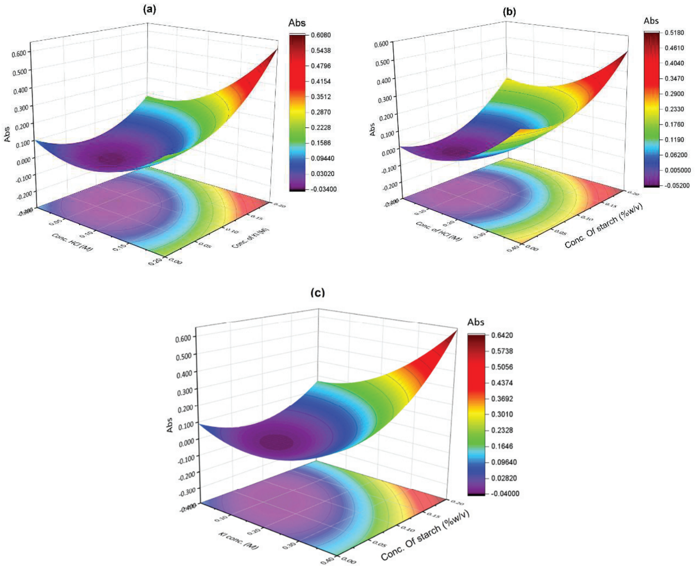

Figure 4. The response surface plots for the determination of Se using iodometry under FED optimization; HCl vs. KI concentration (a), HCl vs. starch concentration (b), and KI vs. starch concentration (c).

Moreover, the three-way ANOVA analysis emphasized the statistical significance ($p < 0.05$) of these concentrations in relation to the formation of I_3^-/starch, thereby illustrating their pivotal role in the experiment. Furthermore, this analysis underscores that all three variables jointly exert statistically significant effects on Se analysis using the iodometric method (Table 1).

Table 1. p-values for the main effects and interactions on RSM amplitude of HCl, KI, and starch concentrations in Se determination with the iodometric method (n = 3).

Effects	***p* - values**		**Partial Eta Squared**
	RSM	**Three-way ANOVA**	
HCl	0.000	0.00	0.9959
KI	0.000	0.00	0.9960
Starch	0.000	0.00	0.9958
HCl × KI	0.000	0.00	0.9867
HCl × Starch	0.000	0.00	0.9838
KI × Starch	0.013	0.00	0.9864
HCl × KI × Starch	0.000	0.00	0.9623

Significant effects and interactions at $p < 0.05$

The process of determining the suitable conditions was conducted by evaluating both the RSM and three-way ANOVA results. It was found that the most effective concentrations for HCl, KI, and starch were 0.15 M, 0.15 M, and 0.30% w/w, respectively. These concentrations yielded high absorbance values and exhibited no significant difference compared to higher concentrations of these factors ($p > 0.05$). This finding indicates that these specific concentrations are optimal for facilitating the complete I_3^-/starch complex reaction (Mörschbächer *et al*., 2018; Pesek *et al*., 2022; Sekine *et al*., 1968).

Moreover, the integration of FED alongside three-way ANOVA and RSM is pivotal in deciphering the intricate interconnections among the concentrations of HCl, KI, and starch in the analysis of Se. This methodology evaluated the independent and collective influence of these factors, simplifying the identification of the most advantageous conditions for precise Se determination. These results carry the promise of substantially enhancing the accuracy and effectiveness of the iodometric approach in Se analysis (Bezerra *et al*., 2019; Ferreira *et al*., 2018; Tarley *et al*., 2009).

3.4 Method validation

The validation of the Se analysis method using iodometric techniques with the optimal conditions obtained from both CRD and FED experiments is presented in Table 2. The linear range for the Se concentration graph remained consistent under both CRD and FED conditions, spanning from 0.05 to 0.7 mg L^{-1}. This concentration range was employed to construct the calibration curve.

It was found that the linear equation for the CRD condition was y = 0.9854x + 0.039, with the r^2 value of 0.9983. Moreover, the FED condition yielded the linear equation y = 0.9146x + 0.0657, with the r^2 value of 0.9977. These results indicated that both conditions produced linear equations well within the acceptable range of linearity for iodometric method (Kulkarni *et al*., 2013; Rebary *et al*., 2010).

Table 2. Analytical characteristics of Se determination using iodometry under optimum conditions of CRD and FED.

Parameters	Values	
	CRD	FED
Linear range (mg L^{-1})	0.1 - 0.7	0.1 - 0.7
Linear equation	y = 0.9854x + 0.039	y = 0.9146x + 0.0657
Linearity (r^2)	0.9983	0.9977
Precision (%RSD)		
- Repeatability	6.37	3.20
- Reproducibility	7.50	3.57
Sensitivity (mg L^{-1})		
- Limit of detection (LOD)	0.064	0.047
- Limit of quantitation (LOQ)	0.12	0.11
Percentage recoveries (% ± SD)		
- Sample preparation	94.01 ± 10.10	96.56 ± 5.43
- Iodometry procedure	95.01 ± 5.16	96.87 ± 7.21

The precision of Se analysis for both the CRD and FED experimental conditions was assessed in terms of both repeatability and reproducibility. The results were expressed as the relative standard deviation (%RSD). Under the CRD condition, the repeatability and reproducibility were 6.37% and 7.50%, respectively. Meanwhile, the FED condition exhibited repeatability and reproducibility values of 3.20% and 3.57%, respectively. These findings indicate that the FED condition demonstrated superior precision compared to the CRD condition.

The sensitivity of Se analysis was assessed under both experimental conditions. In the CRD condition, the LOD and LOQ were determined to be 0.064 mg L^{-1} and 0.12 mg L^{-1}, respectively. Meanwhile, in the FED condition, the LOD and LOQ were found to be 0.047 mg L^{-1} and 0.11 mg L^{-1}, respectively. Consequently, the FED condition exhibited superior sensitivity for Se analysis using the iodometric method compared to the CRD condition. Hence, these results indicated that FED was a preferable experimental design when optimizing the iodometric method for Se analysis due to its superior precision and sensitivity. Researchers and analysts in the field of analytical chemistry may benefit from using FED to achieve more reliable and accurate results in Se analysis.

3.5 Determination of total Se in Se-enriched foods

The results of the experiments showed that the Se content determined by the optimum condition of CRD and FED iodometry methods was close to that determined by HG-AAS in all samples, and there was no significant statistical difference ($p > 0.05$) (Table 3). This result indicates that both iodometry methods are accurate and reliable for the analysis of Se in Se-enriched food supplements and plants. This suggested that both CRD and FED iodometry methods are reliable for Se content analysis in these samples, offering a cost-effective alternative to the more expensive the HG-AAS method for various applications, including food quality assessment (Mörschbächer *et al*., 2018). The high standard deviation in the analysis of selenium in some samples may be attributed to inadequate sample homogenization, improper sample preparation, or insufficient purity of the employed chemicals and reagents. Future studies should prioritize controlling these factors to enhance analytical precision.

Table 3. Se contents in samples analyzed by both iodometric methods and HG-AAS.

Samples	**Se content (mg Se kg^{-1})**			***p* - value**
	CRD	**FED**	**HG-AAS**	
Se-enriched Chinese daikon sprouts	1072.09 ± 23.48	1023.63 ± 56.83	1024.34 ± 34.21	0.316
Se-enriched Chinese kale sprouts	1093.21 ± 54.87	1089.67 ± 63.93	1044.63 ± 43.54	0.518
Se-enriched Chinese mustard sprouts	496.48 ± 32.51	468.40 ± 10.99	460.60 ± 25.23	0.249
Supplement A	756.68 ± 32.51	782.06 ± 6.54	769.71 ± 26.32	0.259
Supplement B	687.21 ± 18.74	722.48 ± 19.17	714.32 ± 25.57	0.188

In the context, the authors showed p>0.05, then please indicate “ns” in Table 3.

4. Conclusion

This research directly compared the effectiveness of CRD and FED in optimizing the conditions of the iodometry method to analyze Se content in Se-enriched foods. The CRD provided conditions of 0.1 M HCl, 0.2 M KI, and 0.02% w/v starch, while the FED led to different conditions: 0.15 M HCl, 0.15% w/v KI, and 0.03% w/v starch. Despite these differences, both approaches provided statistically valid results for Se quantification using UV-visible spectrophotometry. This highlights the significance of experimental design in determining outcomes, with the CRD simplifying the process but potentially overlooking factor interactions, while the FED, though more resource-intensive, offers a comprehensive dataset. The development of analytical methodology should choose between CRD and FED based on their specific objectives, considering the balance between simplicity and capturing intricate factor interactions in the quest for optimal conditions. Moreover, the results of Se content analysis in Se-enriched foods using iodometry under CRD or FED conditions were not significantly different from those obtained with HG-AAS ($p > 0.05$). Hence, the iodometry method is a technique that can be applied in the quality control processes of Se-enriched food production. This method can result in a reduction in quality control costs compared to other techniques. Meanwhile, it still delivers dependable results.

Acknowledgments

We are very grateful for the laboratory, equipment, and instrument from Sakon Nakhon Rajabhat University

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