Article

Verification of The Method for Determining Calcium Content in Animal Feed

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Abstract

Calcium is one of the essential macromineral elements in animal feed. To ensure that animal feed meets nutritional and safety standards, accurate and validated analytical methods are needed to determine calcium levels, specifically using the ICP-OES (Inductively Coupled Plasma Optical Emission Spectroscopy) technique, which is known for its high sensitivity, enabling the rapid and precise detection of calcium elements at low concentrations. This study aims to verify the method for analyzing calcium content in animal feed, referring to method verification parameters, including linearity, accuracy, precision (repeatability test), limit of detection (LoD), method detection limit (MDL), and limit of Quantitation (LOQ). The verification process was conducted using calcium standard solutions at various concentrations and analysis of animal feed samples according to SNI 3148.2:2009 using Inductively Coupled Plasma (ICP-OES) with a linearity test parameter coefficient of correlation (r) value of 0.9991, precision with a % RSD value of 2.18%, accuracy (recovery) reaches 90-99%, with a detection limit (LoD) of 0.0745 mg/L and a Limit of Quantitation (LoQ) of 0.9060 mg/L, all of which meet the acceptance criteria set by AOAC. These results prove that the ICP-OES technique is suitable for use as a test method for determining the calcium content in animal feed, can be adopted as a standardized routine procedure.

Keywords: *Animal Feed, calcium, ICP-OES, method verification*

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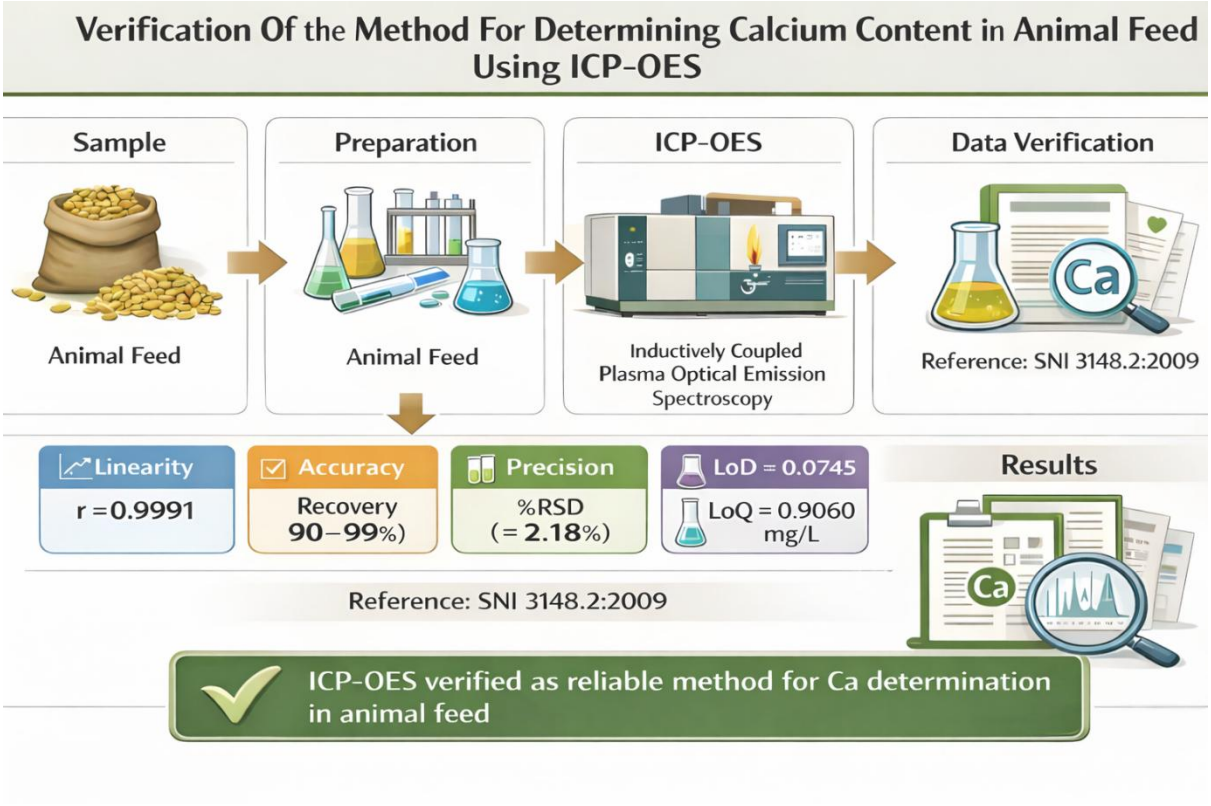
Graphical Abstract



Introduction

Animal feed is one of the most important components in modern livestock production systems [1]. Global animal protein consumption increased by approximately 14% over the past two decades. This underscores the need for feed quality assurance, including essential nutrient content such as calcium (Ca) [2]. Calcium is one of the essential macromineral elements in animal feed, playing a primary role in bone formation, muscle contraction, nerve transmission, and blood coagulation [3]. Calcium requirements vary greatly depending on the livestock species, age, and physiological stage. Therefore, accurate determination of calcium levels is crucial in feed formulation [4].

In practice, the analysis of mineral content such as calcium in feed materials requires methods that are not only sensitive and accurate, but also precise, selective, and reproducible [5]. One of the analytical methods currently relied upon is Inductively Coupled Plasma Optical Emission Spectroscopy (ICP-OES). This technology allows for the simultaneous detection of minerals with high sensitivity and low detection limits, making it highly suitable for complex matrices such as animal feed [6]. However, the use of the ICP-OES method for determining mineral elements must first undergo a verification stage to meet analytical validity standards. Method verification is an important process in ensuring that the method used meets performance specifications such as accuracy, precision, linearity, limit of detection, and limit of quantitation [7]. International regulatory bodies such as AOAC and SNI have emphasized the importance of analytical method verification processes in ensuring the quality of laboratory test result.

Various previous studies have developed methods to measure calcium in various food materials using ICP-OES, such as in milk [8], nuts [9], persian tahini (ardeh) ([10] and sorghum [11] but there are no publications that comprehensively examine the verification of this method's performance parameters in the context of animal feed. Some other studies are still limited to determining the general levels of mineral elements without emphasizing the verification aspects of the methods used. This research gap underscores the importance of conducting a study to verify the ICP-OES method for determining Ca levels in animal feed. Therefore, verifying this method is of significant value both scientifically and practically, especially in supporting the livestock feed industry, which relies on accurate and reliable analytical data [12].

This research not only determines the Ca content using the ICP-OES technique but also conducts a thorough verification of the method's performance parameters according to SNI 3148.2:2009 guidelines, which have not been systematically studied in previous research. This standard also includes verification parameters such as linearity, accuracy, precision, method detection limit (LOD), and limit of Quantitation (LOQ), which serve as a reference for validating laboratory test results. Linearity parameter verification is performed to ensure that the instrument's emission signal is directly proportional to the calcium concentration within a specific range, while the limit of the linearity curve is needed to determine the maximum limit of the calibration curve [13]. Precision indicates the reproducibility of results under the same testing conditions [14], while accuracy describes the closeness between the test result values and the true value [15]. Meanwhile, LOD and LOQ are important for knowing the minimum concentration limit that can be detected and measured with a certain degree of confidence and the method detection limit (MDL) to determine the actual detection limit of the entire analytical method [16].

This research also contributes scientifically by presenting empirical data that fills a gap in the literature regarding the application and validation of ICP-OES in livestock feed matrices. Additionally, the results of this study will serve as a reference for testing laboratories, certification bodies, and feed manufacturers in establishing accountable mineral testing protocols. With this background, the main objective of this research is to verify the method for determining the calcium (Ca) content in animal feed using ICP-OES based on the parameters of linearity, limit of linearity, precision, accuracy, limit of detection, method detection limit, and limit of Quantitation, as regulated in SNI 3148.2:2009 and to confirm that the ICP-OES technique meets AOAC criteria for calcium determination in animal feed. The results of this research are expected to provide theoretical benefits in the development of analytical science, as well as practical benefits for testing laboratories and the feed industry in ensuring the quality of their products.

Materials and Methods

Materials

The equipment used in this experiment consists of two types: main equipment and supplementary equipment. The main equipment used is the ICP-OES instrument (PerkinElmer Optima 8300) with a plasma power of 1400 W. Plasma gas flow 12 L/min, Auxiliary gas flow 1 L/min, Nebulizer gas flow 0.8 L/min and axial view observation mode. Calibration was internal using a single-element calibration curve. Meanwhile, the

supplementary equipment includes the Mettler Toledo analytical balance, furnace, 100 mL and 200 mL volumetric flasks, micropipette, 10 mL measuring pipette, porcelain crucible, funnel, stirring rod, spatula, compressor, 0.45 µm filter paper, 50 mL beaker, rubber bulb, and spray bottle. The materials used in the experiment consist of test materials and chemicals. The test materials include livestock feed samples and a standard calcium solution with a concentration of 1000 mg/L. The chemicals used include concentrated nitric acid (HNO_3), HCl solution with concentrations of 0.5 M and 3 M, demineralized water (distilled water), and argon gas.

Preparation of 100 mg/L Calcium Standard Solution

A total of 10 mL of standard calcium solution with a concentration of 1000 mg/L was taken using a pipette, then placed into a 100 mL volumetric flask. Next, the volume of the solution was adjusted with distilled water until it reached the mark, then shaken until homogeneous.

Preparation of Calcium Standard Series Solutions (0; 2; 4; 8; 16; 20) mg/L

5 mL of concentrated nitric acid (HNO_3) was pipetted into each 100 mL volumetric flask that contained a small amount of distilled water. After that, a standard calcium solution with a concentration of 100 mg/L was added to each volumetric flask in amounts of 0, 2, 4, 8, 16, and 20 mL, respectively.

Test Sample Preparation

A total of 2 g of feed sample was weighed and placed into a porcelain crucible, then burned in a furnace at 550 °C for 4 h until all the material turned to ash. The ash from the combustion was allowed to cool, then 10 mL of 3 M HCl solution was added, covered with a watch glass, and heated for 10 minutes to dissolve the remaining minerals. The solution was then cooled and filtered using 0.45 µm filter paper. The obtained filtrate is placed into a 100 mL volumetric flask and diluted with distilled water to the mark.

Preparation of Precision and Accuracy Test Solution

The prepared test sample of 95 mL was placed into a 100 mL volumetric flask and 5 mL of a 10 mg/L standard solution was added. For precision and accuracy testing, the test solution was prepared in ten replicates.

Preparation of Limit of Detection Test Solution

The instrument's detection limit test is conducted using a solvent blank. The preparation of the blank involves using 5 mL of concentrated HNO_3, which is added to a 100 mL flask that has previously been supplemented with a small amount of distilled water, then brought to the mark with distilled water and homogenized. The Limit of Detection test solution was made in ten replicates.

Preparation of Method Detection Limit and Limit of Quantitation Solutions

A 20 mg/L standard solution of 5 mL was placed into a 100 mL volumetric flask, and adjusted to the mark with a sample of known concentration. The test solution for the method detection limit and Limit of Quantitationwas made in ten replicates.

Preparation of Confirmation Limit of Quantitation Solution

A total of 9 mL of standard solution with a concentration of 10 mg/L was added to a 100 mL volumetric flask, then the volume of the solution was adjusted to the mark using a sample with a known concentration. The test

solution for confirming the Limit of Quantitation was prepared in ten replicates.

Linearity Test

The standard working solution of Ca (0; 2; 4; 8; 16; 20) mg/L that has been prepared was measured using the ICP-OES 422.673 nm instrument. The measurement results were statistically processed to obtain the correlation value, slope, and intercept. The analysis results are then compared with the acceptance criteria set by AOAC. Data processing is calculated using Microsoft Excel.

Limit Test of Linearity Curve (LoL)

The working solution of the standard Ca with the lowest concentration of 2 mg/L and the highest concentration of 20 mg/L was measured ten times using the ICP-OES instrument at a wavelength of 422.673 nm. The measurement results were statistically processed to obtain the Fcalculated value. The Fcalculated value obtained was compared with the acceptance criteria set by AOAC. Data processing is calculated using Microsoft Excel.

Precision Test (Repeatability)

The precision test solution, which has been prepared ten times, was measured using the ICP-OES instrument at a wavelength of 422.673 nm. The test results were statistically processed to determine the precision value or relative standard deviation (%RSD), and then compared with the acceptance criteria set by AOAC. Data processing is calculated using Microsoft Excel.

Accuracy Test

The accuracy test solution, which has been prepared ten times, was then measured using the ICP-OES instrument at a wavelength of 422.673 nm. The test results were statistically processed and then compared with the acceptance criteria set by AOAC. Data processing is calculated using Microsoft Excel.

Limit of Detection Test

The prepared blank solution was analyzed using ICP-OES at a wavelength of 422.673 nm. The obtained data were statistically processed and subsequently compared with the acceptance criteria established by AOAC. All data processing and statistical calculation were performed using Microsoft Excel.

Method Detection Limit Test and Limit of Quantitation

The prepared solutions for the Method Detection Limit (MDL) and Limit of Quantitation (LOQ) determination were analyzed using an Inductively Coupled Plasma-Optical Emission Spectrometer at a wavelength of 422.673 nm. The reading data were then statistically processed to determine the relative standard deviation (%RSD), percent recovery, signal-to-noise ratio (S/N), as well as the LDM and LOQ values. All evaluation results were compared with the acceptance criteria established by AOAC. All statistical calculation and data processing were calculated using Microsoft Excel.

Confirmation Limit of Quantitation Test

The confirmation solution for the Limit of Quantitationthat has been prepared was analyzed using the ICP-OES instrument at a wavelength of 422.673 nm. The measurement data were then statistically analyzed to calculate the relative standard deviation (%RSD) and recovery percentage. These values are then evaluated by comparing them against the acceptance limits set by AOAC. Data processing is calculated using Microsoft Excel.

Result and Discussion

Method verification for calcium (Ca) content analysis in animal feed was conducted using the ICP-OES instrument. The basic principle of this technique is the measurement of the intensity of radiation emitted by atoms during energy transitions due to excitation [17]. ICP-OES operates based on the characteristic of atoms that absorb light energy and trigger electron excitation from the ground state to a higher energy level [18]. Based on the method verification tests that have been conducted, the results include linearity parameters, Linearity Curve Limit (LoL), Limit of Detection (LoD), Method Detection Limit (MDL), Limit of Quantitation (QL), Precision, and Accuracy. The results of the method verification test for determining calcium in animal feed can be seen in Table 1.

Table 1. Data Results of Verification of the Calcium Content Determination Method in Animal Feed Using ICP-OES

No	Parameters	Results	Acceptance Conditions	Source	Conclusion
1	Linearity (r) (0-20) mg/L	r = 0.9991	r ≥ 0.9950	AOAC (2020)	Meet The Requirements
2	Limit Curve Linearity (2 and 20) mg/L	0.03 < 3.18	$F_{count} < F_{table}$	AOAC (2020)	Meet The Requirements
3	Precision (Repeatability)	%RSD = 2.18 ½ CV Horwitz = 7.92 (2.18≤ 7.92)	%RSD ≤ ½ CV Horwitz	AOAC (2020)	Meet The Requirements
4	Accuracy (Recovery)	%Recovery = (90-99)%	%Recovery = (80-115)%	AOAC (2020)	Meet The Requirements
5	Limit of Detection (LoD)	0.0745 mg/L	Positive Response	AOAC (2020)	Meet The Requirements
6	Method Detection Limit (MDL) and Limit of Quantitation (LoQ)	Theoretical MDL= 0.8079 mg/L S/N = 9.08 %Recovery = (86-113)% LoQ = 0.9060 mg/L	Reads positive 2.5-10 %Recovery= (80-115)% Positive Response	AOAC (2020)	Meet The Requirements
7	Confirmation Limit of Quantitation	%RSD = 10, 2/3 CV Horwitz = 10.2 10≤10.2 Recovery = (86-113)%	%RSD≤2/3 CV Horwitz Recovery = (80-115)%	AOAC (2020)	Meet The Requirements

Linearity

Linearity describes the extent to which an analytical method can produce a response that is proportional to the concentration of the analyte within a certain range. This linear relationship is usually indicated by the correlation coefficient (r) [19]. The linearity calculation results are shown in Table 2, and the calibration curve for the Ca standard series obtained is shown in Figure 1.

Based on the graph, the linearity test results show that the intensity of the calcium signal is directly proportional to the calcium concentration. The regression equation obtained is y = 293,320x – 163,402 with a correlation coefficient (r) value of 0.9991, which is close to 1. This indicates a strong and proportional linear relationship between the concentration of the element Ca and the instrument response. The value of this correlation coefficient also meets the AOAC acceptance criteria, which is r ≥ 0.9950. Linearity evaluation was performed using six calibration points (0.00–20.00 mg/L), and the regression results showed excellent linearity for Ca determination using ICP-OES. The regression model yielded a coefficient of determination (R^2) value of 0.99842, indicating that over 99% of the variation in the optical signal can be explained by changes in Ca concentration.

Table 2. The linearity calculation results

No	Concentration of Ca (mg/L) (Xi)	Intensity (Yi)	(Xi)*(Yi)	Xi^2	Yi^2
1	0.00	0.00	0.00	0.00	0.00
2	2.00	343,348.52	686,697.04	4	117,888,206,186.19
3	4.00	931,870.22	372,7480.88	16	868,382,106,922.85
4	8.00	2,124,684.91	16,997,479.28	64	451,428,596,6781.71
5	16.0	4,559,671.64	72,954,746.24	256	20,790,605,464,620.30
6	20.00	5,726,023.29	114,520,465.8	400	32,787,342,717,622.40
Amount	50.00	13,685,598.58	208,886,869.2	740	59,078,504,462,133.50
Slope (b)			293,320.25		
Intercept (a)			–163,402.32		
r			0.9991		
R^2			0.9984		
(Sy/x)			104,897.86		

In linear regression analysis, the relationship between variables is expressed through the equation $y = bx + a$, where b is the slope of the line, a is the intercept, x represents the analyte concentration, and y denotes the instrument response [20]. The correlation coefficient (r) is used to assess the strength of the linear relationship between two datasets [21]. An ideal relationship occurs when the intercept value approaches zero and r approaches +1 or −1, depending on the direction of the correlation.

From the obtained curve, the intercept value (a) is -163,402.32. An intercept close to zero

indicates that the instrument does not provide a signal when a blank solution is tested [22]. However, the presence of interference, noise, contamination, or other instrument effects often causes a signal to still be read even for a blank. A slope value (b) of 293,320.25 indicates the sensitivity level of the method. The larger the slope value, the higher the method's sensitivity to the analyte being tested [23].

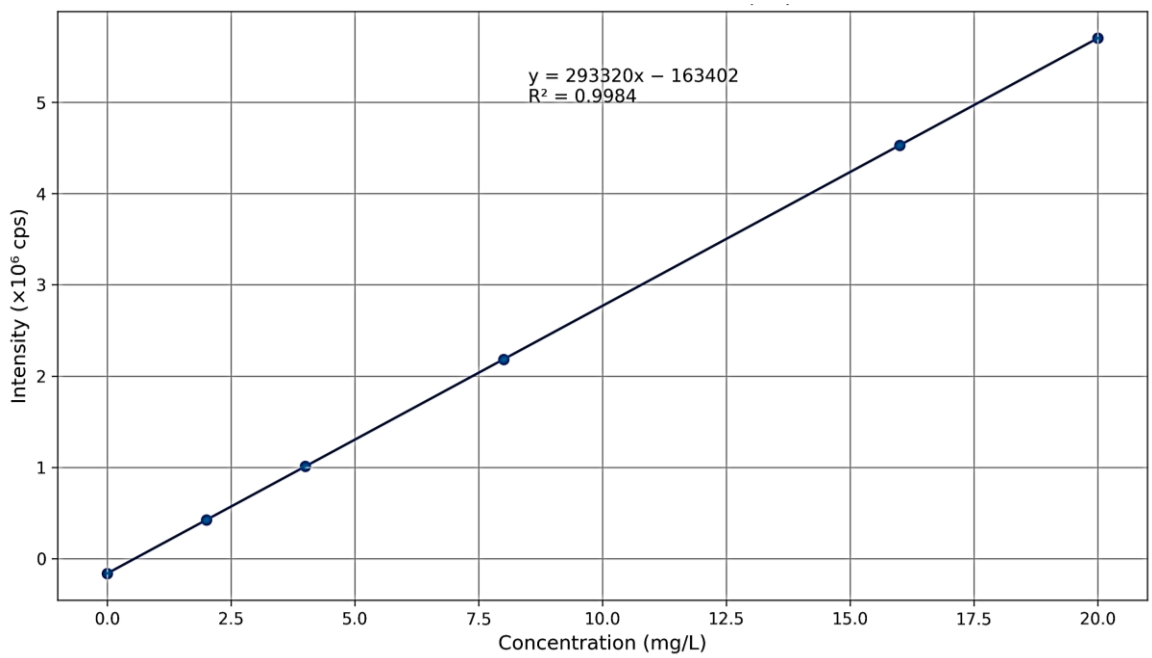


Figure 1. Curve of Calcium Concentration vs. Intensity

Residual analysis showed a random distribution around the zero line with no observable systematic pattern, indicating absence of lack-of-fit and confirming that the linear model is adequate over the tested concentration range. The standard error of regression (Sy/x) value of 104,897.86 demonstrates good precision in the relationship between analytical intensity and analyte concentration. The 95% confidence interval for the slope (277,123.40–309,517.10) indicates stable instrument sensitivity, while the confidence interval for the intercept (–343,277.29 to 16,472.63) suggests that the intercept does not significantly differ from zero, indicating the absence of systematic bias at the zero concentration level. Although measurements were performed without replication at each concentration level, the calibration design remains appropriate for initial method verification. Overall, these statistical parameters confirm that the calibration curve satisfies the linearity requirements recommended by AOAC and ISO/IEC 17025, demonstrating that the method is suitable for the determination of Ca in animal feed.

Limit Curve Linearity

The linearity limit of the curve was measured by comparing with the standard solutions of Ca using concentrations 2 mg/L and 20 mg/L, each 10 times. The results of the two standard deviations obtained are compared. The data limits for the linearity curve and the F-table are shown in Table 3. The comparison of the standard deviation of the data from the repeated tests is in the form of F-count. The calculated F count is 0.018. Based on degrees of freedom df1=df2=n-1 and a confidence level of 95% (α = 0.05), the value of F-tabel is obtained as 3.18. From the calculation data, it is known that F-count < F-

table, so H_0 is accepted, meaning the precision level of the lowest working solution intensity is not significantly different from the precision level of the highest working solution intensity [24]. Based on the results obtained, with a confidence level of 95% (α = 0.05), 2 mg/L – 20 mg/L is a linear regression and 20 mg/L is the highest concentration detectable by the instrument.

Table 3. Data limits for linearity curve and F-Table

Sample	Intensity	
	Lowest Standard (2 mg/L)	**Highest Standards (20 mg/L)**
1	350,226.9894	5,773,543.8410
2	332,253.1689	5,741,227.0290
3	349,590.3771	5,697,047.2310
4	350,098.3865	5,579,630.9190
5	347,700.5273	5,658,376.0490
6	332775,5831	5,652,233.3640
7	333,089.9671	5,689,108.3010
8	343,347.0374	5,658,277.0960
9	347,605.1231	5,625,120.0700
10	338,383.0410	5,624,015.7550
SD	8,935.336	51,372.977
SD^2	79,840,222.0	2,639,182,775.4
F_{count}	0.018	
F_{table} (df = n-1, α = 5%)	3.18	

Precision (Repeatability)

Precision testing is an essential parameter in analytical method validation, intended to evaluate the degree of agreement among repeated measurements obtained under identical analytical conditions. Precision reflects the magnitude of random errors in an analytical procedure and provides insight into the short-term reproducibility of the method. In this study, precision was assessed through a repeatability test, in which replicate analyses were performed using the same analyst, instrumentation, reagents, and experimental conditions within a limited time frame [25].

The repeatability of the method was expressed as the relative standard deviation (%RSD), a statistical parameter that describes data dispersion relative to the mean value. The use of %RSD allows for objective comparison of method precision across different concentration levels, with lower values indicating higher consistency and analytical reliability. The experimental results yielded a %RSD of 2.18%, which was evaluated against the Horwitz coefficient of variation (CV) of 7.92%. The Horwitz CV, derived from an empirical model, serves as a widely accepted benchmark for assessing acceptable precision in chemical analysis. According to the acceptance criteria established by AOAC, a method meets repeatability requirements when the observed %RSD does not exceed 0.5 × CV Horwitz.

In this case, the obtained %RSD was significantly lower than the allowable limit (3.96%), confirming that the analytical method demonstrates satisfactory repeatability. These results indicate that

random variability is well controlled, supporting the method's reliability and suitability for routine quantitative analysis [26]. The relative standard deviation value obtained from the precision test indicates that the precision test results fall into the medium accuracy category.

RSD ≤ 1% : very accurate (1)

1% < RSD ≤ 2% : high accuracy (2)

2% < RSD ≤ 5% : moderate accuracy (3)

RSD ≥ 5 % : low accuracy (4)

Table 4. Precision test data and calculations

Test	C Sampel (mg/L)	C Spike (mg/L)	V Spike (mL)	V Sampel (mL)	C Target (mg/L)	Intensity	Actual Concentration of Ca (mg/L)
1	0.6600	10	5	95	1,1270	132,409.6	1.022381
2						136,044.6	1.03481
3						140,934.6	1.051531
4						145,760.1	1.068031
5						145,603.9	1.067497
6						149,725.7	1.08159
7						143,037.5	1.058721
8						151,478	1.087582
9						153,487.3	1.094452
10						149,311.4	1.080174
Amount							10.64677
Average							1.064677
SD							0.023
% RSD							2.18
0,5 CV Horwitz							7.92

Accuracy (Recovery)

Accuracy is a key parameter in analytical method validation that describes the closeness of agreement between the measured value and the true or accepted reference value of an analyte [27]. It reflects the trueness of an analytical method and indicates the presence of systematic error. In quantitative analysis, accuracy is commonly evaluated through recovery studies, in which a known amount of analyte standard is spiked into the sample matrix and analyzed using the proposed analytical method. This approach allows evaluation of matrix effects, analyte loss during sample preparation, and potential analytical bias. Accuracy is generally expressed as the percentage recovery (%Recovery) of the added analyte using a standard solution with a known concentration. The results of the accuracy test and the corresponding calculations are presented in Table 5.

The obtained %Recovery values fall within the acceptable limits recommended by the AOAC guidelines for analytical method validation, indicating that the method produces reliable and unbiased results. Based on ten replicate analyses, the %Recovery values ranged from 90% to 99%, with an average recovery of 94%. These results comply with the acceptance criteria established by AOAC (2002), which specify an acceptable recovery range of 80–115% for quantitative analytical methods. Therefore, the method.

Table 5. Accuracy Data and Calculation

Test	C Sampel (mg/L)	C Spike (mg/L)	V Spike (mL)	V Sampel (mL)	C Target (mg/L)	Intensity	Actual Concentration Calcium (mg/L)	% Recovery
1	0.6600	10	5	95	1.1270	132,409.6	1.0224	90
2						136,044.6	1.0348	91
3						156,432.9	1.1045	98
4						140,934.6	1.0515	93
5						159,879.9	1.1163	99
6						145,603.9	1.0675	94
7						153,487.3	1.0945	97
8						143,037.5	1.0587	93
9						126,101.8	1.0008	88
10						162,660.6	1.1258	99
Average							1.0677	94
Accuracy Range Value							(90-99) %	
Acceptance Conditions							(80-115) %	

Limit of Detection (LoD)

The data and LoD calculations are presented in Table 6. The limit of detection is defined as the lowest concentration of an analyte in a sample that produces a signal significantly different from that of the blank [28]. This parameter reflects the minimum sensitivity of an analytical method. The LoD can be determined by measuring blank samples, which are matrices free of the analyte, to confirm that the instrument response originates from the presence of the analyte rather than background noise [29].

Table 6. Data and LoD Calculations

Test	Intensity	Concentration (xi) (mg/L)	(xi-$\bar{X}$)	(xi-$\bar{X}$)^2
1	17,400.6287	0.3443	0.04207	0.0018
2	16,400.4184	0.3408	0.03853	0.0015
3	3,920.1495	0.2966	-0.00562	0.0001
4	821.1895	0.2856	-0.01658	0.0003
5	1,259.3401	0.2872	-0.01503	0.0002
6	2,038.0774	0.2899	-0.01228	0.0002
7	338.3124	0.2839	-0.01829	0.0003
8	285.8692	0.2837	-0.01848	0.0003
9	235.6276	0.2836	-0.01866	0.0003
10	12,393.7679	0.3022	0.02435	0.0006
Amount	3.0223			0.0056
Average (mg/L)	0.3022			
SD	0.0248			
LoD (mg/L)	0.0745			

In this study, the theoretical LoD value obtained was 0.0745 mg/L, representing the lowest concentration of Ca that could be reliably detected by the instrument. The LoD evaluation met the acceptance criteria established by AOAC (2002), as it produced a positive and distinguishable analytical response

Method Detection Limit

Determining the method's detection limit reflects both the laboratory's capabilities and limitations in applying an analytical method to detect analytes at low concentrations. The method's limit of detection is defined as the concentration of an analyte that can be detected thru all stages of the analysis procedure with 99% confidence, where the signal produced can be clearly distinguished from the blank signal [30]. Limit of Quantitation (LOQ), often referred to as the reporting limit, is the lowest concentration of an analyte in a sample that can still be determined quantitatively with acceptable levels of precision and accuracy, under agreed-upon test conditions [31]. The LOQ value and method detection limit were determined by measuring the prepared solutions using the analyte addition (spiking) technique. Data and Calculations for the Detection Limit of the Method and Limit of Quantitationare shown in Table 7.

In this test, the theoretical concentration values for MDL and LoQ were obtained. The method's limit of detection is acceptable if the data from the test repetitions meet several acceptances following limit formula 5-9.

%RSD < 0,67 Horwitz (5)
% Recovery = 80-115 % (AOAC, 2002) (6)
The signal to noise ratio (S/N) = 2,5-10 (7)
MDL < Spike < 10MDL (8)
LoQ ≤ BML (9)

Table 7. Data and Calculation of Method Detection Limit and Limit of Quantitation

Test	C Sampel (mg/L)	C Spike (mg/L)	V Sampel (mL)	V Spike (mL)	C Target (mg/L)	Intensity	C Calcium (mg/L)	Recovery (%)
1						9,762,972 ,568	2.9500	113 %
2						1,006,484,546	2.8900	111%
3						1,047,067,863	2.3000	88%
4						1,092,602,128	2.2800	87%
5	0.6600	20	90	10	2.5940	1,129,398,123	2.6000	100%
6						1,125,396,591	2.6500	102%
7						1,150,724,794	2.2500	86%
8						1,179,239,004	2.8900	111%
9						1,554,535,717	2.8600	110%
10						2,011,566,989	2.3500	90%
Amount							26.0200	
Average							2.6020	
SD							0.2864	
SD' SD /$\sqrt{n}$							0.0906	
S/N							9.08	
MDL theoretical (mg/L)							0.8079	
10 MDL (mg/L)							8.079	
LoQ theoretical (mg/L)							0.906	

The method detection limit (MDL) must satisfy acceptable precision and accuracy criteria. Precision is commonly expressed as the coefficient of variation, also referred to as the relative standard deviation (%RSD). The %RSD obtained from repeated

measurements during the verification process should not exceed 0.67 times the Horwitz coefficient of variation (CV) value [32]. In addition, the recovery test (%Recovery) results must fall within the acceptable range of 80–115%.

The signal-to-noise ratio (S/N) is used to assess the level of random error that may occur during the testing process and to estimate the precision of repeated test results which is calculated using Microsoft Excel. If the S/N ratio is less than 2.5, this indicates that the random variation in the replicates is quite high and may cause the method detection limit (MDL) to be large. In such conditions, the concentration of the added analyte (spike) needs to be increased to produce a stronger signal. Conversely, if the S/N exceeds 10, it means the amount of analyte added is too high, so its concentration needs to be reduced to remain within the ideal working range [33].

When determining the method detection limit (MDL), the choice of spike concentration must be made carefully to ensure the results obtained are within an acceptable range. The precision level in determining the MDL is highly influenced by the concentration of the spike used. If the obtained MDL value exceeds the spike level, it will be statistically difficult to distinguish between the spike value and the blank value, resulting in low precision. Therefore, the determination of spike levels should consider the lower limit of the concentration range being verified, as well as follow the recommended ratio, which is MDL : LoQ = 4 : 10.

When the method detection limit (MDL) meets the required statistical acceptance criteria, the obtained value may be compared with relevant environmental laboratory quality standards. In routine practice, laboratories that have verified their analytical methods may also compare the baseline method limit (BML) with the corresponding limit of quantitation (LoQ). This approach is based on the established working range of the method, in which the lower limit is defined by the LoQ and the upper limit by the limit of linearity (LoL). Consequently, the LoQ serves as a critical parameter for determining the applicability and reliability of an analytical method for quantitative measurements [34].

Based on the limit of quantitation test results, the theoretical LoQ was determined to be 0.9060 mg/L, representing the lowest analyte concentration that can be quantified with acceptable precision and accuracy. This theoretical value required confirmation to ensure compliance with method performance requirements. Therefore, a LoQ confirmation test was conducted. The results demonstrated that the method met the required precision and accuracy criteria and complied with the acceptance standards established by the AOAC (2002), confirming the suitability of the method for quantitative analysis

Confirmation Limit of Quantitation (LoQ)

The data and calculations for the confirmation of the limit of quantitation (LoQ) are presented in Table 8. Based on the quantitation test results, the theoretical LoQ was determined to be 0.9000 mg/L, which represents the lowest analyte concentration that can be quantified reliably and therefore requires confirmation to ensure that the method meets the required precision and accuracy criteria [35]. LoQ confirmation was carried out by analyzing a calcium standard solution with a concentration of 10 mg/L using ICP-OES. The precision of the measurements was evaluated in terms of the relative standard deviation (%RSD), and the confirmation test yielded an %RSD value of 10%. This value satisfies the applicable acceptance criterion, namely %RSD ≤ 2/3 CV

Horwitz, indicating that the analytical method demonstrates adequate precision at the quantitation limit and that the confirmed LoQ is suitable for reliable quantitative determination of calcium at low concentration levels.

Table 8. Data and Calculation for Confirmation Limit of Quantitation

Test	C^a Sample (mg/L)	C (mg/L)	V^c (mL)	V^d (mL)	C Target (mg/L)	Intensity	C Ca actual (mg/L)	*Recovery* (%)	xi-$\bar{x}$	(xi-$\bar{x}$)2
1	0.6600	10	91	9	1.5006	371,290	1.3014	86	-0,0864	0.0075
2						367,644	1.2876	85	-0,1002	0.0101
3						377,557	1.3252	88	-0,0627	0.0039
4						385,331	1.3546	90	-0,0333	0.0011
5						385,191	1.3541	90	-0,0338	0.0011
6						391,908	1.3795	91	-0,0083	0.0001
7						376,094	1.3196	87	-0,0682	0.0047
8						366,178	1.2821	85	0,1058	0.0112
9						479,541	1.7114	114	0,3234	0.1046
10						440,481	1.5635	104	0,1755	0.308
Amount							13.8790			
Average							1.3879			
SD							0.1395			
% RSD							10.0			
% *Recovery*							(85-114) %			
2/3 CV Horwitz							10.2			

Conclusions

The verification results demonstrate that the ICP-OES method based on SNI 3148.2:2009 provides adequate analytical performance for the determination of calcium in livestock feed matrices. The method exhibited excellent linearity, stable precision, and satisfactory accuracy within the acceptance limits recommended by the AOAC, indicating a consistent and proportional instrument response to variations in calcium concentration. Furthermore, the low values of the limit of detection (LOD), limit of quantitation (LoQ), and method detection limit (MDL) confirm that the method is sufficiently sensitive to detect and quantify calcium at low concentration levels, supporting its applicability for feed quality testing across diverse matrix compositions. Overall, the verification confirms that ICP-OES is a reliable and suitable analytical technique for routine calcium analysis in animal feed, both for quality control and regulatory compliance purposes. However, this study has certain limitations, particularly the absence of a comprehensive measurement uncertainty evaluation. Therefore, further research is recommended to assess the influence of different feed matrices and to perform structured measurement uncertainty calculations in accordance with ISO/IEC 17025. Addressing these aspects would further strengthen method validity and broaden its application in industrial-scale laboratory testing.

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Author Contributions

The contribution of each author to this article is : "Conceptualization, Syafrinal and Nurmaliza.; Methodology, Syafrinal and Nurmaliza; Software, Hafnimardiyanti.; Validation, Syafrinal., Nurmaliza and Selfa Dewati Samah.; Formal Analysis, Pevi Riani.; Investigation, Renny Futeri.; Resources, Syafrinal.; Data Curation, Nurmaliza.; Writing – Original Draft Preparation, Syafrinal and Nurmaliza; Writing – Review & Editing, Syafrinal.; Visualization, Hafnimardiyanti.; Supervision, Pevi Riani.; Project Administration, Selfa Dewati Samah.; Funding Acquisition, Renny Futeri.

Conflict of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

Ethical Standards

This article does not contain any studies involving human or animal subjects.

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