

# Determination of Calcium in Serum Samples by AAS Using a Fuel Lean Flame

## Application Note

Clinical Research

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### Introduction

Many methods have been proposed for determination of calcium in serum, but only atomic absorption has the necessary specificity and sensitivity to accomplish this. Atomic absorption relies on the principle that electrons in the outermost shell of a particular atom absorb radiant energy and become excited. The energy absorbed by the atom is generally in the form of a very narrow absorption line either within the ultraviolet or visible spectrum<sup>5</sup>. The flame is primarily used to disperse and release the atoms from their molecular states.

Calcium in serum is found 46% free, 32% bound to albumin, 8% bound to globulins, and 14% associated in freely diffusible complexes. Calcium present in protein or inorganic complexes is detectable by flame atomic absorption only when steps are taken to release calcium from these complexes. Acid is used to dissociate calcium from protein while lanthanum reduces interferences from phosphates, or anticoagulants such as oxalates, citrates, and heparin.

Many methods were published during the 1960s outlining the proper conditions needed to measure serum calcium accurately by atomic absorption. The majority of these methods recommend that the acetylene should be set to produce a slightly fuel rich flame, with a yellow haze. This provides greater sensitivity, accuracy and precision. The accepted reference method utilizes the flame conditions described by Pybus, Feldman and Bowers [3], which also recommends a slightly fuel rich flame. Our studies have shown that these conditions result in consistent under-recovery of serum based standards. We, therefore, began a search for flame conditions that could be used for accurate determinations of calcium in serum samples. The criterion set forth during this study was to develop a method that would consistently recover values for SRM 909 a1, a2 and other serum based control materials within published ranges. Further, the maximum allowable error was set at < 2%.



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## Method

### Materials

An Agilent SpectrAA-20 Plus Atomic Absorption Spectrometer, fitted with a Mark VI burner, was used to perform all calcium determinations. The Hi-Vac nebulizer was set to the high vacuum position and the adjustable glass impact bead was set closer to the nebulizer for lower noise levels and better precision. Additionally, twin mixing paddles were installed inside the spray chamber to ensure thorough mixing of sample and gases thus providing better precision. The burner, spray chamber, and nebulizer were removed each day and sonicated in a water bath containing de-ionized water and 0.01% Triton X-100. These parts were thoroughly rinsed with de-ionized water prior to assembly. The flame was ignited and allowed to burn for 15 minutes before analysis. All glassware used to prepare standards, modifiers or to dilute serum samples was acid washed twice in 2N HCl for 30 minutes and rinsed a minimum of 5 times with de-ionized water prior to use.

Stock solutions of sodium chloride and potassium chloride were prepared at concentrations of 1400 and 50 mEq respectively. Sodium chloride and potassium chloride from this stock were added to the blank and standard solutions to more closely match ionic conditions present in serum. A stock lanthanum oxide solution was formulated by dissolving 58.64 g of lanthanum oxide (99.99% purity) with approximately 100 mL of de-ionized water and 250 mL of concentrated HCl. This mixture is diluted to 1 litre with additional de-ionized water. Working lanthanum diluent was prepared by diluting the stock 25 fold with de-ionized water. Lanthanum, in the presence of HCl, eliminates the effects of phosphate in both aqueous and serum samples. In addition, the lanthanum acts as a releasing agent enhancing the disassociation of calcium from serum proteins [5].

A stock calcium solution was prepared by dissolving 0.2497 g of calcium carbonate (SRM 915), previously dried at 200 °C for 4 hours, in a few mL of de-ionized water and 1 mL of concentrated HCl. This mixture is brought to a total volume of 100 mL with additional de-ionized water. A series of working standards were prepared from the calcium stock as shown in Table 1.

Table 1. Preparation of Working Calcium Standards

Stock calcium (mL)	Stock Na, K diluent (mL)	Final calcium concentration (mg/dL)
0	10	0
6	10	6
8	10	8
10	10	10
12	10	12
14	10	14

Both the standards and serum samples were diluted 50 fold with working lanthanum diluent prior to analysis. Concentrations of standards which are lower and higher than the samples were used for bracketing. The final result was calculated using the following equation:

$$C = \frac{S_1 + (A_x - A_{S1}) (S_2 - S_1)}{(AS_2 - AS_1)}$$

Where:

- C = Sample concentration, mg/dL
- S<sub>1</sub> = Concentration of the lower standard, mg/dL
- S<sub>2</sub> = Concentration of the higher standard, mg/dL
- A<sub>x</sub> = Mean absorbance of the sample
- A<sub>S1</sub> = Absorbance of the lower standard
- A<sub>S2</sub> = Absorbance of the higher standard

## Results

Table 2 shows results for measurements of calcium in serum using a fuel rich flame (yellow tinge) as described by Pybus, Feldman and Bowers [3]. SRM 909 a1 and a2 are serum based standards obtained from the National Institute of Standards and Technology (NIST). Values assigned to these standards were determined by either isotopic dilution mass spectroscopy or inductively coupled plasma. SRM 3109 is an aqueous calcium standard.

As can be seen, the conditions recommended by Pybus et. al., in our hands, resulted in under-recovery of serum based standards. This effect was not seen with aqueous based materials (SRM 3109) and measurement of calcium yielded values that were only 0.6% from target. The under-recovery of SRM 909 a1 and a2 was attributed to some interference in the serum matrix.

Table 2. Measurement of Calcium Concentrations in Various Standards. The Number of Replicates was 3–6

Day	SRM 909 a1 (mg/dL) SD	SRM 909 a2 (mg/dL) SD	SRM 3109 (mg/mL) SD
1	8.96 0.09	12.80 0.13	
2	9.14 0.07	12.96 0.08	10.06 0.05
Grand Mean	9.05 0.13	12.91 0.078	10.06
Target	9.31	13.4	10.00
Range	9.15–9.47	13.2–13.6	

Based on these results we began a search for flame conditions that could yield both accurate and precise calcium measurements. The conditions that were found to be optimum are shown in Table 3.

Table 3. Flame Conditions Used to Measure Calcium in Serum Samples

Flame	Lean-blue
Air	~14 L/min
Acetylene	~2.0 L/min
Burner height	5-6 mm
Wavelength	422.7 nm
Slit width	0.5 nm
Ca lamp	Photron HC
Type	P809
Lamp current	3 mA
Aspiration rate	5 mL/min
Delay time between readings	5 seconds
Measurement time	3.0 seconds
Replicates	4
Background correction	Off

Flame profiles using lean and slightly fuel rich conditions were determined during the course of the study (Figure 1). The relationship between height of the flame and atomic absorption signal is called the flame profile. The degree of absorption is a direct measure of the number of atoms in the light path, and therefore the flame profile is a measure of the atomic population in various parts of the flame. The absorbance signal for calcium atoms in a lean flame is greatest when the burner is closest to the beam produced by the hollow cathode lamp. This suggests that the formation of calcium atoms in the region near the burner must be high. This may be attributed to lower expansion of flame gases and higher temperatures in that region of the flame. Hotter temperatures are expected in a lean fuel flame because of the

higher ratio of oxygen (air) to fuel (acetylene). The higher temperatures should enhance evaporation of droplets and increase the formation of free calcium atoms by providing more energy to break molecular bonds.

As Figure 1 shows, the absorbance signal decreases rapidly as the distance between the optical beam and the burner increases, presumably due to rapid formation of refractory oxides resulting from an abundance of oxygen in a fuel lean flame [1]. Conversely, ground state atoms are formed higher up in a fuel rich flame. The decrease in signal occurring between 15–18 mm may be explained by calcium oxide formation. As gases rise from the burner, they expand and become diluted with air and therefore, formation of calcium oxides could be expected to occur. Figure 1 also demonstrates that sensitivity of the method using a fuel lean flame was better than that achieved using a fuel-rich flame.

The precision and accuracy of the method, was determined using SRM 909 a1 and a2 together with various MILES TESTpoint and SETpoint serum based controls. The results of this study are shown in Table 4. The grand mean of 45 replicate values obtained for SRM 909 a1 and a2 coincided exactly with values assigned by NIST. Total coefficient of variation for SRM, TESTpoint and SETpoint controls ranged from a low of 0.75% to a high of 1.6%. Also shown are the within run, run to run and total standard deviations. These results demonstrate that this method, together with the conditions recommended, produced highly accurate and precise determinations of calcium in serum based materials.

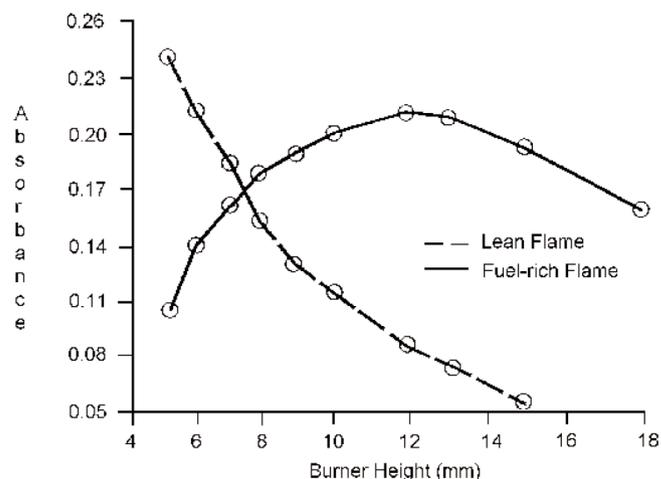


Figure 1. Flame profiles of calcium absorbance in various flame conditions (serum samples).

Table 4. Summary and Statistical Analysis of Data from Several Days for the Calcium Reference Method. The Flame was Air-Acetylene Set to Produce an Oxidizing, Fuel Lean Condition

Grand mean	SRM X 909 a1 9.31 mg/dL	SRM 909 a2 13.39 mg/dL	TP1 V05623 7.14 mg/dL	TP2 V05602 11.40 mg/dL	TP1 V13393 7.36 mg/dL	TP2 V13395 10.84 mg/dL	TP1 V29433 7.27 mg/dL	TP2 V29442 11.42 mg/dL	STPT T09713 9.88 mg/dL
SD <sub>WR</sub>	0.035	0.055	0.013	0.138	0.031	0.056	0.044	0.05	0.013
SD <sub>RR</sub>	0.073	0.094	0.075	0.09	0.101	0.164	0.032	0.082	0.132
SD <sub>TO</sub>	0.081	0.109	0.076	0.164	0.106	0.173	0.055	0.096	0.133
CV <sub>TO</sub>	0.874	0.815	1.068	1.441	1.44	1.595	0.754	0.838	1.348
n	45	45	8	8	6	6	6	6	6
Range	9.31 ± 0.1	13.4 ± 0.2	7.2 ± 0.15	11.2 ± 0.2	7.4 ± 0.20	10.9 ± 0.2	7.2 ± 0.11	11.3 ± 0.2	9.8 ± 0.22

WR: Within Run, RR: Run to Run, TO: Total

Linearity of the method using a fuel lean flame was tested between 0 and 14 mg/dL which is actually 0 to 2.8 µg/mL, (if one remembers that both standards and samples were diluted 50 fold prior to analysis). The method proved to be linear, yielding a correlation coefficient of 0.9999, a slope of 0.0188 and an intercept of  $8.65 \times 10^{-4}$  (see Figure 2).

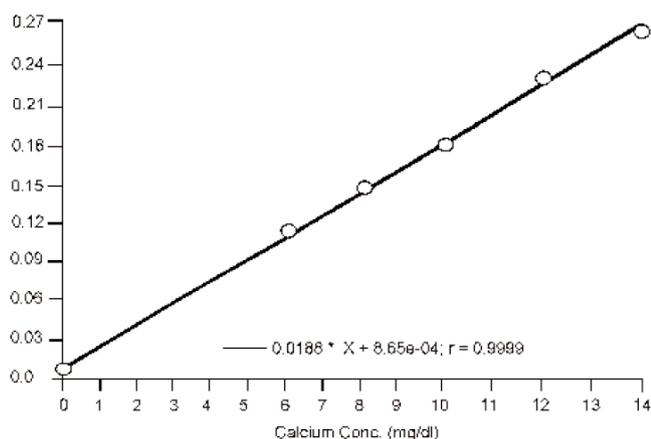


Figure 2. Linearity of the calcium reference method using a fuel lean air-acetylene flame.

If a fuel lean mixture produces a hotter flame which reduces interferences related to measurement of calcium in serum, then the use of a nitrous oxide-acetylene flame should also reduce interferences and improve sensitivity. Ramakrishna, West and Robinson<sup>4</sup> state that chemical interferences decrease in a nitrous oxide-acetylene flame. Messman, O'Haver and Epstein [2] state that the hotter temperatures and reducing environment produced in a nitrous oxide-acetylene flame diminish the effect of chemical interferences and thus make calcium absorbances less sensitive to subtle changes in flame conditions. We examined the possibility of using a nitrous oxide-acetylene flame as an alternative to air-acetylene.

As can be seen in Tables 5 and 6, when comparing nitrous oxide acetylene flame to air-acetylene flame, sensitivity is 2 times better but precision is nearly 5 times worse. Sensitivity is a measure of the absorption produced by a given sample concentration. Detection is defined as the minimum sample concentration needed to produce a signal that is distinguishable from zero [5]. The most common misconception is the belief that an increase in sensitivity automatically improves detection limits. This is not necessarily true since detection limit is a function of precision as well as signal. Clearly, nitrous oxide-acetylene flame has better sensitivity but, due to poorer precision, the air acetylene flame provides a better detection limit. But what is responsible for the higher noise observed using nitrous oxide-acetylene flame? Messman, O'Haver and Epstein [2] state that in a nitrous oxide-acetylene flame the emission from calcium exceeds the intensity of the calcium hollow cathode lamp and becomes the dominant noise source. Analyte emission in an air-acetylene flame is reduced and therefore, precision is improved.

Table 5. Comparison of Sensitivities Achieved Using Various Flame Conditions.

Calcium conc (mg/dL)	Air-acetylene (lean)	Air-acetylene (rich)	Nitrous oxide acetylene
0	0.000	0.001	0.000
6	0.115	0.097	0.230
8	0.152	0.128	0.285
10	0.188	0.162	0.353
12	0.228	0.192	0.417
14	0.263	0.224	0.482

Table 6. Comparison of Precision and Accuracy Using Air-Acetylene and Nitrous Oxide Acetylene Flames.

Conditions	Statistics	SRM909a1 (mg/dL)	SRM909a2 (mg/dL)	SET point V63230
Air-acetylene (lean)	Mean	9.37	13.35	10.41
	SD	0.027	0.027	0.038
	%CV	0.28	0.20	0.37
	n	6	6	6
Nitrous-oxide acetylene	Mean	9.46	13.29	10.32
	SD	0.13	0.20	0.11
	%CV	1.36	1.50	1.08
	n	6	7	6
Target		9.31	13.4	NA
Range		9.15–9.47	13.2–13.6	NA

## Conclusion

Conditions have been optimized for accurate determinations of calcium in serum samples using flame atomic absorption spectroscopy. The method utilizes a lean acetylene-air flame and linearity has been demonstrated in the range of 0 to 2.8 µg/mL in diluted samples. A correlation coefficient of 0.9999 was achieved indicating that linearity for this method is excellent. The lean fuel condition produces higher flame temperatures which, in turn, reduce or eliminate interferences present in the serum matrix. This data is supported by Hwang and Sandonato, who state that interferences from various cations are reduced by the hotter conditions that prevail in a lean air-acetylene flame. Conversely, a slightly fuel rich air-acetylene flame results in under-recovery of calcium concentrations when measured in a serum matrix.

Sensitivity of a lean air-acetylene flame was better than the sensitivity achieved using a fuel rich flame. Accuracy and precision of this method has proven to be excellent. Average values of 45 replicate measurements of NIST controls fell well within certified ranges and a total coefficient of variation of less than 2% was achieved for all samples tested.

Examination of a nitrous oxide-acetylene flame as an alternative to air-acetylene proved unproductive since precision was poor. This was attributed to high analyte emission which apparently exceeds the output of the hollow cathode lamp and therefore, becomes the dominant source of noise during measurement of calcium.

This study represents a significant update on specific conditions essential for proper measurement of calcium in serum samples by flame atomic absorption, especially when accurate determinations with good precisions are required.

## References

- J. Y. Hwang, and L. M. Sandonato. Some chemical interferences of calcium in atomic absorption spectrophotometry. *Anal. Chim.* 48, 188 (1969).
- J. D. Messman, T. C. O'Haver, M.S. Epstein. Precision of wavelength-modulated atomic absorption measurements of high calcium concentrations in a nitrous oxide-acetylene flame. *Anal. Chem.* 60, 2707 (1988).
- J. Pybus, F. J. Feldman, G. N. Bowers, Measurement of total calcium in serum by atomic absorption spectroscopy, with use of a strontium internal reference. *Clin. Chem.* 16(12), 998 (1970).
- T. V. Ramakrishna, P. W. West, J. W. Robinson. The determination of calcium and magnesium in acetylene flame. *Anal. Chim. Acta* 40, 347 (1968).
- J. Robinson, *Atomic Absorption Spectroscopy*. 2nd Ed. Marcel Dekker, Inc. New York, N.Y. (1975).

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