
# Calcium Isotope Ratios in Milk by PlasmaQuant MS Elite

## Challenge

Precise, accurate, and interference-free detection of calcium isotopes for isotope ratio studies.

## Solution

Robust plasma performance using cold plasma conditions for reduced formation of interferences.

### Introduction

Calcium is one of the most-abundant elements in the earth's crust. The analysis of calcium isotope variations opens up new possibilities for the use of the calcium isotopic system to provide insight into biological, geological, and cosmic processes in which calcium plays an important role.[1]

In the geosciences, the use of high precision natural isotope analyses is already widely spread, and more recently, this analytical tool is increasing also in nutritional and disease-related research. One emerging approach is the use of highly precise isotopic techniques to understand isotope signatures in the human body to monitor nutritional status, unravel cellular mechanisms, and develop new probes for disease. For example, calcium isotopic analysis is being used to investigate bone metabolism: it has been shown that the calcium serum isotopic composition is related with myeloma (cancer of bone marrow). This correlation offers a potential new method to evaluate real-time myeloma-induced bone disease.[2]

Inductively coupled plasma mass spectrometry (ICP-MS) offers a high-precision and accurate isotope analysis technique, allowing for the determination of both the total elemental and isotopic content in a single analytical run. In this application note, the performance of a PlasmaQuant MS Elite ICP-MS is used to evaluate the possibility to use the variation in calcium isotope ratios to distinguish different types of milk. In the example of the analysis discussed here, highly precise and accurate isotope measurements of at least three calcium isotopes are

required. Therefore, the three calcium isotopes ($^{42}$Ca, $^{43}$Ca, and $^{44}$Ca) were selected for this study, which relates to the human metabolic isotope tracer.

The mass spectrum obtained from an ICP-MS shows that calcium isotopes are subject to a series of interferences based on the plasma and sample matrix (Table 1). These interferences pose one of the main challenges in precise and accurate isotope analysis. As can be seen in Table 1, the interferences are particularly problematic in the low mass range, where they are both numerous and significant. As a result, it is impossible to select three interference-free calcium isotopes. These polyatomic interferences can, however, be significantly and sufficiently reduced by using cold plasma conditions[3] and collision reaction cells[4], or can be resolved using HR-ICP-MS[5,6,7]. For HR-ICP-MS, the precision is typically not better than 0.25%.
In this application note it is shown that using the PlasmaQuant MS Elite ICP-MS, operating in cold plasma mode to eliminate polyatomic interferences, it is possible to achieve a precision of 0.09% to 0.5% for the calcium isotope ratio analysis to distinguish different types of milk.

Table 1: Potential isobaric, polyatomic, and doubly charged ion interferences and their abundance (%) in calcium isotopes.

| Isotope | Abundance (%) | Isobaric interferenes | Doubly charged interferences | Matrix-based polyatomic interferences | Gas-based polyatomic and isobaric interferences |
|---|---|---|---|---|---|
| $^{40}$Ca | 96.941 | $^{40}$K$^{+}$(0.01) | | $^{39}$K (93.3) $^{1}$H$^{+}$ (99.9)<br>$^{24}$Mg (78.99) $^{16}$O$^{+}$ (99.7) | $^{40}$Ar$^{+}$ (99.6) |
| $^{42}$Ca | 0.647 | | $^{84}$Sr$^{++}$(9.8) | $^{41}$K (6.7) $^{1}$H$^{+}$ (99.9)<br>$^{25}$Mg (10) $^{16}$O$^{+}$ (99.7)<br>$^{30}$Si (3.1) $^{12}$C$^{+}$ (98.9)<br>$^{28}$Si (92.2) $^{14}$N$^{+}$ (99.6) | $^{40}$Ar (99.6) $^{1}H_2^{+}$ (99.9)<br>$^{40}$Ar (99.6) $^{2}$H$^{+}$(0.01) |
| $^{43}$Ca | 0.135 | | $^{86}$Sr$^{++}$(7.0) | $^{27}$Al (100) $^{16}$O$^{+}$ (99.7)<br>$^{25}$Mg (10) $^{16}$O$^{+}$ (99.7) $^{1}$H$^{+}$ (99.9) | |
| $^{44}$Ca | 2.086 | | $^{88}$Sr$^{++}$(82.6) | $^{26}$Mg(11.0)$^{18}$O$^{+}$(0.2)<br>$^{28}$Si(92.2)$^{16}$O$^{+}$(99.7)<br>$^{30}$Si(3.1)$^{14}$N$^{+}$(99.6)<br>$^{12}$C(98.9)$^{16}O_2^{+}$(99.7)<br>$^{32}$S(95.0)$^{12}$C$^{+}$(98.9) | |
| $^{46}$Ca | 0.004 | $^{46}$Ti$^{+}$ (8.0) | $^{92}$Mo$^{++}$(14.8) | $^{30}$Si(3.1)$^{16}$O$^{+}$(99.7)<br>$^{14}$N(99.6)$^{16}O_2^{+}$(99.7) | |
| $^{48}$Ca | 0.187 | $^{48}$Ti$^{+}$(73.8) | $^{96}$Mo$^{++}$(16.7) | $^{24}$Mg(78.9)$^{24}$Mg$^{+}$(78.9)<br>$^{32}$S(95.0)$^{16}$O$^{+}$(99.7) | $^{36}$Ar(0.337)$^{12}$C$^{+}$(98.9) |

## Materials and Methods

### Instrumentation

A PlasmaQuant MS Elite ICP-MS, in combination with a Cetac ASX-560 autosampler, was used for calcium isotope ratio determination in milk samples after matrix removal. The ICP-MS was optimized for maximum sensitivity and lowest background for three calcium isotopes ($^{42}$Ca, $^{43}$Ca and $^{44}$Ca) using cold plasma conditions to improve the precision of isotope ratios measurements. All experiments were carried out in a routine analytical laboratory, and not under 'clean room' conditions. Instrument operating conditions are summarized in Table 2.

Table 2: PlasmaQuant MS Elite operating conditions.

| Parameter | Specification |
|---|---|
| Plasma gas flow | 9.0 L/min |
| Auxiliary gas flow | 1.25 L/min |
| Nebulizer gas flow | 0.80 L/min |
| iCRC condition | No gas |
| Plasma RF power | 0.60 kW |
| Dwell times | $^{42}$Ca – 80000 µs<br>$^{43}$Ca – 160000 µs<br>$^{44}$Ca – 40000 µs |
| Scans per replicate | 50 (peak hopping, 1pt/peak) |
| No. of replicates | 15 |
| Pump rate | 5 rpm – black/black PVC pump tubing (<1 mL/min) |
| Sample uptake time | 60 s |
| Stabilization delay | 45 s |
| Sampling depth | 6.0 mm |
| Nebulizer type | MicroMist™ (quartz concentric) |
| Ion optics | Auto-optimized |
| Spray chamber | Scott glas |
| Spray chamber temperature | 3 °C (Peltier cooled) |

## Samples and Reagents

The following high-purity reagents were used for the preparation of all solutions:

- Deionized water (>18.2 MΩ/cm, Millipore MiliQ)
- Calcium single element stock standard (1000 mg/L, CertiPUR®)
- Di-Sodium Oxalate (99,91%, Merck) at pH≈8
- 0.1 M Nitric acid Supra-quality 69% (ROTIPURAN® Supra)
- 6 M Hydrochloric acid ultra-quality 34% (ROTIPURAN® Ultra)

## Sample Preparation

A total of 13 different milk samples were treated according to the following procedure before calcium isotope determination with the PlasmaQuant MS Elite.

1 mL of milk samples was weighed into acid-cleaned quartz test tubes and dry-ashed overnight at 480 °C. The ash was dissolved in a minimum amount of 0.1 M nitric acid (≈ 2.5 mL) and the calcium separation was achieved by adding 1 mL of ammonium oxalate saturated solution at a pH of ≈8. After sitting at room temperature overnight, the samples were centrifuged (5 minutes at 3500 rpm) and the supernatant was discarded. The remaining calcium oxalate precipitate was washed twice with 2 mL of de-ionized water and then dissolved in 2 mL of 0.1 M nitric acid plus 5 drops of 6 M hydrochloric acid.

The samples, containing calcium oxalate, were diluted in 0.1 M nitric acid to a final concentration of about 20 mg/L calcium and the instrument was optimized to give a count rate of about 2 x $10^6$ counts/s for the $^{44}$Ca isotope at this concentration. Higher count rates were avoided since this could result in gain suppression, and could therefore affect the accuracy of the isotope ratio measurements.

Both isotope ratios $^{44}$Ca/$^{43}$Ca and $^{42}$Ca/$^{43}$Ca were measured. Every three subject samples were bracketed by unenriched calcium isotope standard of the same type and concentration. These standards were subjected to the same calcium separation procedure as the samples. This allowed for correction of instrumental mass bias and drift. Procedural blanks were subtracted from each reading, even though they typically contributed <0.2% of the sample signal.

## Results and Discussion

*Interferences*

Under normal plasma conditions, i.e. 1300 W, the signals for all calcium isotopes have overlap from interfering ions. According to Table 1, the most abundant isotope, $^{40}Ca$, has an isobaric interference from the most abundant isotope of argon, $^{40}Ar^{+}$. Titanium isotopes interfere with the minor calcium isotopes, $^{46}Ca$ and $^{48}Ca$, and strontium, with isotopes at m/z 84, 86, 87 and 88 interferes with $^{42}Ca$, $^{43}Ca$, and $^{44}Ca$, since strontium has a relatively low second ionization potential and forms doubly charged species. There are also polyatomic ions such as $^{40}Ar^{1}H_2^{+}$ and $^{12}C^{16}O_2^{+}$ that interfere with $^{42}Ca$ and $^{44}Ca$, respectively. With a nominal resolution of 1 amu, a quadrupole ICP-MS cannot resolve the Ca peaks from the interfering peaks. However, operated under cold plasma conditions (400–600W), the formation of polyatomic interferences can be dramatically reduced since the plasma energy is much lower than under normal conditions. For the same reason, doubly charged ions such as $Sr^{++}$ were not observed under cold plasma conditions. Figure 1 shows a typical background spectrum for 0.1 M nitric acid ($HNO_3$) under cold plasma conditions at the calcium masses 42, 43, 44, overlayed with the spectrum of a 20 mg/L Ca standard. The low background at 42, 43, and 44 under the cold plasma conditions makes the measurement of $^{44}Ca/^{42}Ca$, $^{43}Ca/^{44}Ca$ and $^{42}Ca/^{43}Ca$ isotope ratios possible.

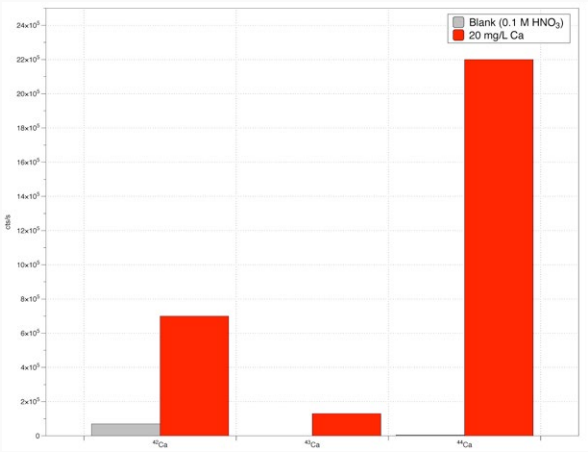


Figure 1: Typical background spectrum for 0.1 M nitric acid obtained for cold plasma conditions. Overlaid is the spectrum of a 20 mg/L Ca standard in 0.1 M nitric acid.

*Instrument mass bias*

Mass bias occurs in analysis by ICP-MS when ions of different mass are transmitted through the spectrometer with different efficiencies, resulting in non-uniform sensitivity across the mass range and inaccurate isotope ratio measurements. Among other effects, high concentrations of matrix elements result in an increased ion beam density, leading to greater space charge effects that exacerbate the loss of lighter ions from the ion beam, thereby enhancing the mass bias.

Most of the mass bias may originate in the field free regions between the sampler and the skimmer and/or immediately behind the skimmer. It may be small for heavier ions as they are better focused through the skimmer cone behind the sampler cone, but higher for lighter masses. In any case, mass bias should be considered during calculations to obtain good accuracy of measurement.

The fractionation coefficient deviation (β) can be defined as a function of the different masses studied. The true ratio of isotopes A and B (R) can be expressed from the ratio measured (r) by different relations called linear law (A.1), exponential (kinetic) law (A.2) or power law (A.3).

The bias per unit mass, α, can be determined by measuring a certified solution or a reference solution when using standard bracketing. In this study, a commercial calcium stock standard was used and the reference ratios between the measured isotopes were based on isotope natural abundances.

$$\frac{r}{R} = 1 + \alpha\Delta m \qquad (A.1)$$

$$\beta = ln\left(\frac{R}{r}\right)/ln(m_A/m_B) \qquad (A.2)$$

$$R = r\left(\frac{m_A}{m_B}\right)\beta \qquad (A.3)$$

To check the mass bias (Figure 2) and to correct for the calcium isotope ratios measured, a commercial calcium stock standard and the correspondent isotopes natural abundances were used. The instrument follows the exponential law for ion transmission.

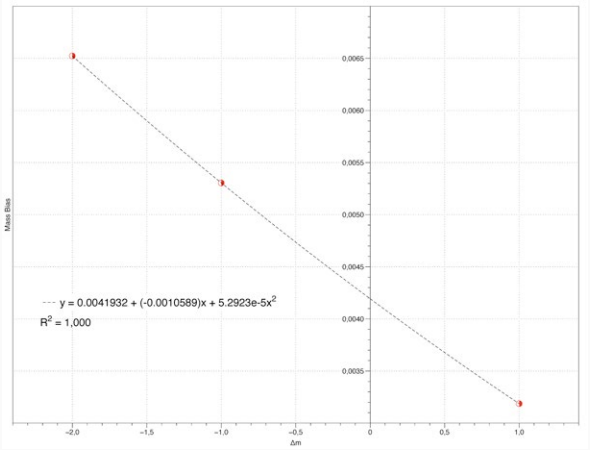


Figure 2: Mass bias for Ca isotopes measured in a 20 mg/L Ca commercial stock standard.

*Calcium isotope ratios*

After blank subtraction and mass bias correction, the final calcium isotope ratios in each of the samples were determined and are reported in Table 3.

Table 3: Results for Ca isotope ratios in milk samples.

| Ref. | $^{44}Ca/^{42}Ca$ | $^{43}Ca/^{44}Ca$ | $^{42}Ca/^{43}Ca$ | $\delta^{44}Ca$ | $\delta^{43}Ca$ | $\delta^{42}Ca$ |
|---|---|---|---|---|---|---|
| Milk 1 | 3,436 | 0,063 | 4,565 | 65,7733 | -31,3920 | -47,4477 |
| Milk 2 | 3,383 | 0,062 | 4,653 | 49,3603 | -34,9451 | -29,1549 |
| Milk 4 | 3,467 | 0,062 | 4,557 | 75,3462 | -36,56054 | -49,1782 |
| Milk 6 | 3,436 | 0,063 | 4,553 | 65,7703 | -29,1827 | -49,9457 |
| Milk 7 | 3,441 | 0,063 | 4,565 | 67,2255 | -30,2487 | -47,3912 |
| Milk 8 | 3,407 | 0,063 | 4,558 | 56,7534 | -20,4387 | -49,0111 |
| Soy Vanilla 1 | 3,483 | 0,062 | 4,541 | 80,3037 | -37,1531 | -52,5460 |
| Soy Vanilla 5 | 3,473 | 0,062 | 4,545 | 77,1002 | -36,0312 | -51,6402 |
| Milk w/o Lactose 3 | 3,474 | 0,062 | 4,556 | 77,6613 | -39,8823 | -49,4318 |
| Milk w/o Lactose 7 | 3,473 | 0,062 | 4,555 | 77,2396 | -38,8522 | -49,5479 |
| Soy Soft 6 | 3,477 | 0,062 | 4,537 | 78,3985 | -35,4222 | -53,4173 |
| Milk + Ca + Vitamin D 4 | 3,500 | 0,062 | 4,510 | 85,5865 | -35,0424 | -59,0145 |
| Milk + Ca + Vitamin D 8 | 3,497 | 0,062 | 4,511 | 84,7923 | -34,9457 | -58,7285 |

Variations between isotope ratios are usually small, therefore they are commonly measured relative to a reference material. Data are presented using the delta-notation (A.4) in parts per thousand (per mil, ‰).

$$\delta^{44/4x}Ca = \left[\frac{\left(\frac{^{44}Ca}{^{4x}Ca}\right)_{Sample}}{\left(\frac{^{44}Ca}{^{4x}Ca}\right)_{Std}} - 1\right] \times 1000 \qquad (A.4)$$

with $^{44}Ca/^{4x}Ca_{Sample}$ being different calcium isotope ratios measured in the samples and $^{44}Ca/^{4x}Ca_{Std}$ the isotope ratios in the calcium commercial stock standard used to check mass bias. A 1000 mg/L ICP calcium concentration standard from NIST (CertiPUR®) was used as the reference standard for the current work, because there is no certified calcium isotope standard. The NIST 915a used in other works is no longer commercially available.
The following isotopic diagrams (Figure 3 and 4) clearly show a distinct difference in the calcium isotope ratios for different types of milk.

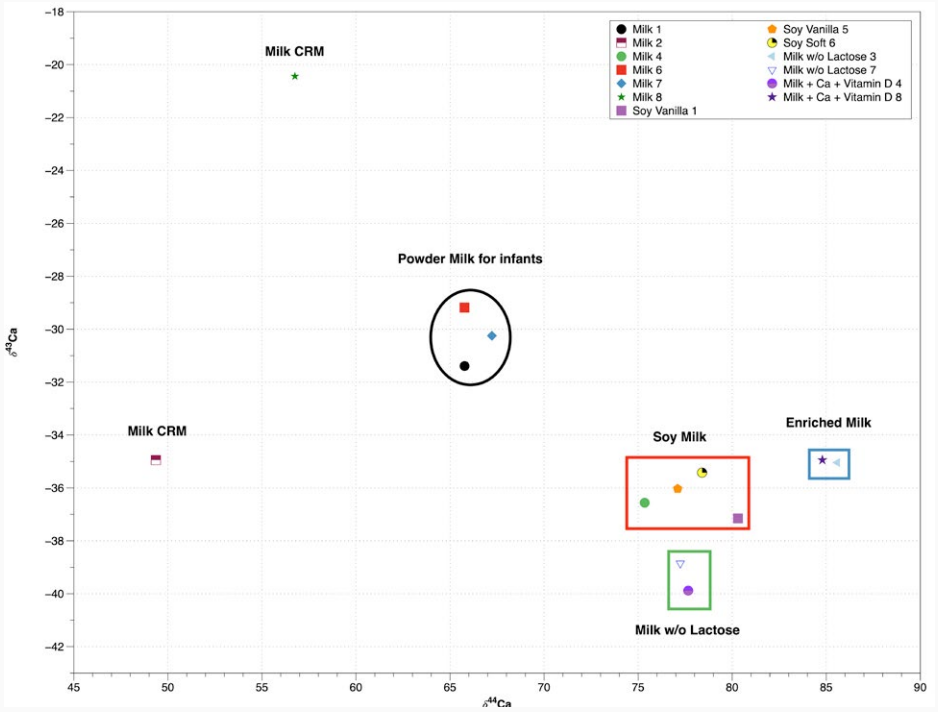


Figure 3: Isotopic composition of Ca in 13 milk samples measured. Ca isotope ratios are presented on a δ-scale ($\delta^{44}Ca$ vs. $\delta^{43}Ca$).

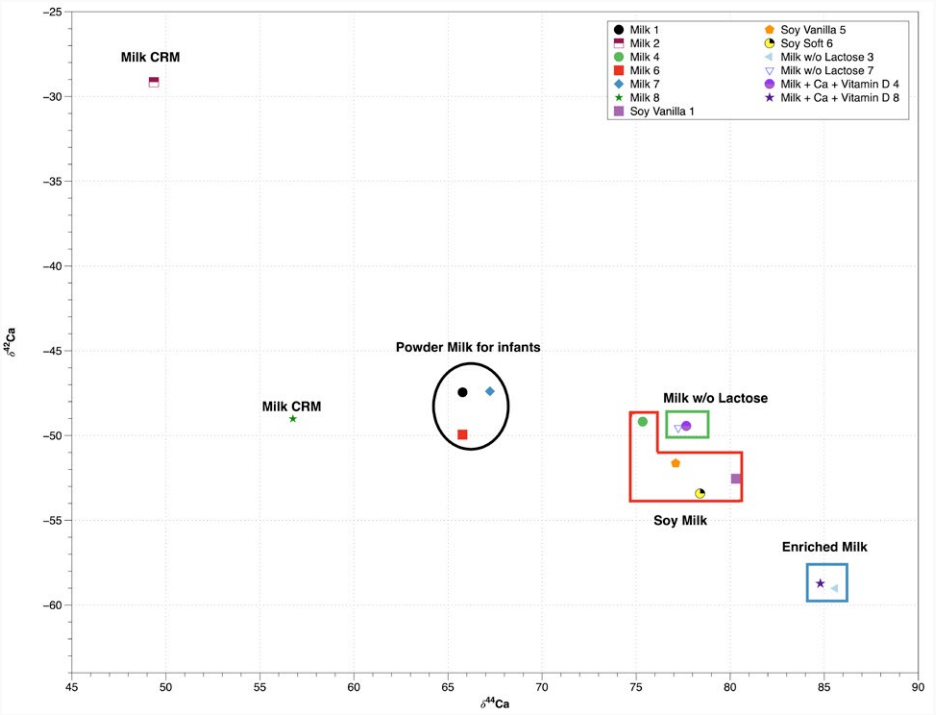


Figure 4: Isotopic composition of Ca in 13 milk samples measured. Ca isotope ratios are presented on a δ-scale ($\delta^{44}Ca$ vs. $\delta^{42}Ca$). More negative δ-values correspond to a lower $^{43}Ca/^{44}Ca$ isotope ratio, i.e., an enrichment of the heavier Ca isotopes, compared to the reference material of Ca stock standard.

Both isotopic diagrams clearly show five different groups of 13 milk samples measured. The differences among them are related to the nature of the milk (powder, soy, cow, etc.) and treatment (calcium and vitamin D enrichments and lactose removal). Furthermore, more negative δ-values correspond to a lower $^{43}Ca/^{44}Ca$ isotope ratio, i.e., an enrichment of the heavier calcium isotopes, compared to the reference material of calcium stock standard.
Measurements of ~20 mg/L calcium in the final sample/standard solutions were repeated 15 times, with achieved calcium isotope ratio precisions of between 0.09 and 0.50 (RSD%), which is in accordance with what is usually achieved using ICP-MS equipped with collision/reaction cells technology.

## Conclusion

A method was developed for the preparation and determination of calcium isotope ratios in 13 different types of milk by ICP-MS. The precision and accuracy of the measurement results allow for the clear distinction of the milk provenance and milk treatment. Polyatomic interferences with calcium isotopes were reduced to negligible levels using cold plasma conditions with only < 11 L/min of plasma argon gas.
Following matrix removal, samples were diluted to adjust calcium concentrations to approximately 20 mg/L. Matching the acid molarity and matrix between the samples and the calcium stock standard was essential to achieve accurate measurements. The matrix removal procedure did not affect isotope ratio accuracy of the measurements.
Using the PlasmaQuant MS Elite, accurate and precise calcium isotope ratios measurements, 0.09–0.50 (RSD%), in milk were achieved. The cold plasma conditions can also be used in other types of studies where calcium isotopes are fractionated, such as bones, urine, blood, soils, roots, leaves, etc.

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