Simultaneous Determination of [45]Calcium and [65]Zinc Uptake by Caco-2 Cells

Paz Etcheverry,[†] John C. Wallingford,[‡] Dennis D. Miller,*,[†] and
Raymond P. Glahn[§]

Institute of Food Science, Cornell University, Ithaca, New York 14853; Wyeth-Ayerst
Pharmaceuticals, Philadelphia, Pennsylvania 19101; and U.S. Plant, Soil and Nutrition Laboratory,
Agricultural Research Service, U.S. Department of Agriculture, Ithaca, New York 14853

A simple method for simultaneously determining cell-associated Ca and Zn in Caco-2 cells is described. Calcium and zinc uptake was measured via radioisotopes [45]Ca and [65]Zn. Preliminary studies revealed that [65]Zn, a positron (β^+) and gamma emitter, contributed to [45]Ca counts in a liquid scintillation counter (LSC). However, [45]Ca, being a true beta emitter, did not contribute to the counts in a gamma counter (γC). To differentiate the counts of [45]Ca from those of [65]Zn, first a [65]Zn-labeled cell suspension was read in a γC and an LSC, thus obtaining the relationship between the radioactive counts obtained from the γC and LSC. This information defined the linear relationship between γC [65]Zn counts per minute (CPM) and LSC [65]Zn CPM. Because the [45]Ca and [65]Zn counts obtained in the LSC are additive, giving total LSC CPM, the value of LSC [45]Ca CPM was obtained by subtracting LSC [65]Zn CPM from total LSC CPM for the dual-labeled cell sample, obtaining then LSC [45]Ca CPM. To determine the absolute activity or disintegrations per minute (DPM) of each isotope in the dual-labeled sample, the linear relationship between DPM and CPM was determined for each isotope. The method is simple and straightforward for the determination of [45]Ca counts from a sample also containing [65]Zn, using gamma and liquid scintillation counters.

KEYWORDS: Calcium; zinc; liquid scintillation counter; in vitro digestion; Caco-2 cells; quenching

INTRODUCTION

The use of radioisotopes in mineral nutrition has proven to be extremely powerful. Through the use of radioisotopes, we can get a better understanding of how minerals are absorbed, distributed, stored, and excreted by the body. Stable isotopes can also be used as isotopic tracers. They offer several advantages over radioisotopes including the lack of health risks to subjects and workers and avoidance of difficult and costly disposal issues. Furthermore, they can be given to pregnant women, infants, and young children, groups especially susceptible to the hazards of radioisotopes. However, they are more expensive than radioisotopes, and their detection is costly and laborious and requires highly trained personnel (*1*, *2*).

Radioisotopes, depending on their form of decay, are detected by gamma counters (γC) or liquid scintillation counters (LSC). In the latter, we have to account for quenching, which is the process that results in a decrease in the intensity of the flashes of light produced by the beta particles or electrons in the liquid scintillator. Put another way, quenching is the phenomena that

results in a reduction of the count rate. Regardless of the quenching mechanism, which could be chemical, color, or optical, almost any radiolabeled sample can act as a quenching agent. It is important therefore that we have an understanding of the counting efficiency (E_{co}) of the LSC in the presence of the sample and that we report the counts obtained in absolute levels of activity.

Whereas detecting a single isotope in a sample is quite simple, detecting multiple radioisotopes can be complicated. It is considerably more difficult when the energy spectra of the isotopes involved overlap with one another.

In this study two isotopes were used, [45]Ca and [65]Zn. Whereas [45]Ca decays by negatron emission, [65]Zn decays via electron capture and positron decay. Negatron decay involves the transformation of a neutron into a proton, a negative electron ($_{-1}\beta$), and a massless neutral particle called a neutrino (ν) (*3*). In the case of [45]Ca, this gives rise to scandium, an ejected beta particle, and ν:

$$^{45}_{20}\text{Ca} \rightarrow {}^{45}_{21}\text{Sc} + {}^{0}_{-1}\beta + \nu$$

[Superscripts represents *mass number* = number of protons + number of neutrons. Subscripts are the *atomic number* = number of protons (=number of electrons). The 0 mass number for β indicates that electrons and positrons have an insignificant mass.]

* Author to whom correspondence should be addressed [telephone (607) 255-7900; e-mail ddm2@cornell.edu].
[†] Cornell University.
[‡] Wyeth-Ayerst Pharmaceuticals.
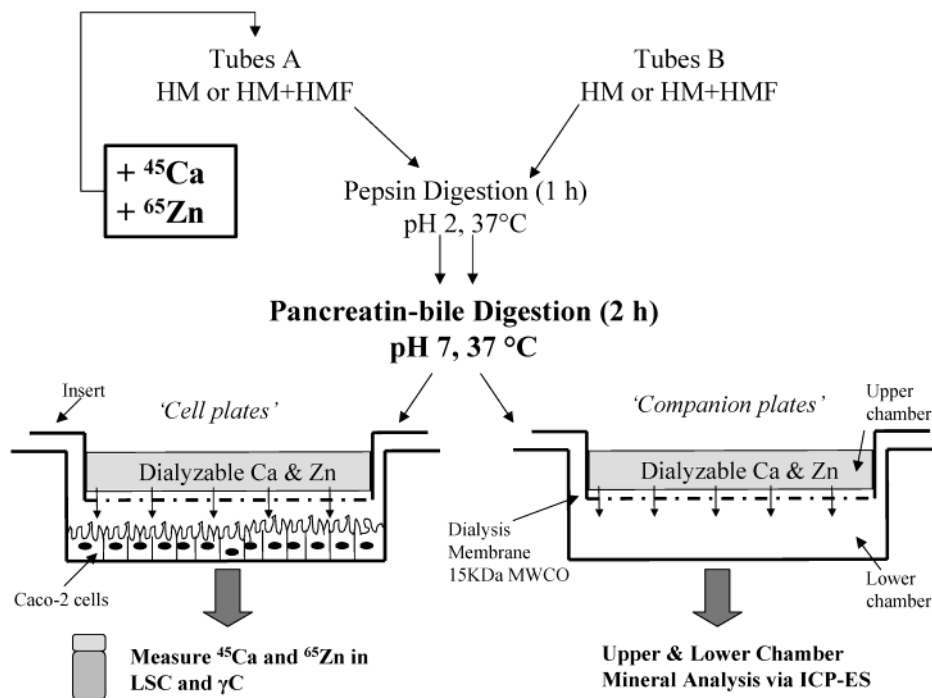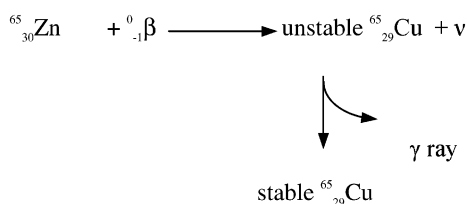[§] U.S. Department of Agriculture.

Figure 1. Diagram of in vitro digestion/Caco-2 cell culture model.

Electron capture, as the words imply, involves the capture of an orbital electron by the nucleus and the subsequent release of a γ-ray (*3*).

$$^{65}_{30}\text{Zn} \quad + \, ^{0}_{-1}\beta \longrightarrow \text{unstable } ^{65}_{29}\text{Cu} + \nu$$

$$\gamma \text{ ray}$$

$$\text{stable } ^{65}_{29}\text{Cu}$$

During positron decay, a proton in the nucleus is converted into a neutron, a positive electron (positron, $_{+1}\beta$), and ν (*3*).

$$^{65}_{30}\text{Zn} \rightarrow \, ^{65}_{29}\text{Cu} + \, ^{0}_{+1}\beta + \nu$$

Although the simultaneous counting of calcium and zinc radioisotopes has been reported (*4*−*6*), a detailed systematic description of sample preparation and quench correction has not been presented. In this study we describe a simple way of detecting ^{45}Ca from ^{65}Zn by using liquid scintillation and gamma counting while taking into account quenching effects. The method was applied to the study of the uptake of the two minerals from human milk fortifiers by Caco-2 cells, an intestinal epithelial cell line, using an in vitro digestion model.

MATERIALS AND METHODS

Cell Cultures. Caco-2 cells, obtained from the American Type Culture Collection (Rockville, MD), were used at passages 30−35. Stock cultures were maintained in Dulbecco's Modified Eagle Medium (DMEM) (GIBCO, Grand Island, NY), pH 7.4, with 10% (v/v) FBS (GIBCO), 25 mmol/L HEPES (Sigma, St. Louis, MO), and 1% antibiotic−antimycotic solution (GIBCO). The cells were cultured at 37 °C in an incubator with a 5% CO_2 and 95% air atmosphere at constant humidity. The medium was changed every 2 days. For uptake experiments, cells were grown in collagen-treated six-well plates at an initial seeding of 50000 cells/cm^2, where confluency was observed 4 days postseeding. Experiments were conducted at 16 days postseeding.

Human Milk Samples. The samples consisted of human milk (HM) with and without various human milk fortifiers (HMF): S-26/SMA from Wyeth International Nutritionals Inc. (St. Davids PA); colostrum from GalaGen Inc. (Minnetonka, MN); casein phosphopeptides (CPP) from DMV International Nutritionals (Delhi, NY); α-lactalbumin from Avonmore Foods (Kilkenny, Ireland); and a combined colostrum/CPP/α-lactalbumin. They were prepared on the same protein basis [One packet of Wyeth S-26/SMA (2 g), which provides 0.5 g of protein and 45 mg of Ca, was dissolved in 50 mL of HM. The amount of the other HMFs that contributed 0.5 g of protein were dissolved in 50 mL of HM.] and with added calcium in the form of $CaCl_2$ [The HMFs had calcium added, in the form of $CaCl_2$, so that they would contain the same level as S-26/SMA.] except for HM. Levels of zinc and iron were different among the samples.

In Vitro Digestion/Caco-2 Cell Uptake. Details of the in vitro digestion method have been described elsewhere (*7*). This study introduces a slight modification to the method as it incorporates radioisotopes. A diagram of this method is shown in **Figure 1**. Briefly, on the first day, cells were treated with 2 mL of Minimum Essential Medium (MEM, GIBCO). To 6.54 mL of each human milk sample were added 3.46 mL of 140 mM NaCl and 5 mM KCl were added (tubes A). Half of this volume was transferred to other sterile tubes (tubes B). Radioisotopes were added to tubes A: 20 μL of ^{45}Ca (420 μL of ^{45}Ca stock + 280 μL of H_2O; stock 370 MBq/mL $^{45}\text{CaCl}_2$ in 1 mL of H_2O; NEN Life Science Products Inc., Boston, MA) and 20 μL of ^{65}Zn (70 μL of ^{65}Zn stock + 630 μL of 0.1 M HCl; stock 77.46 MBq/mL $^{65}\text{ZnCl}_2$ in 2 mL of HCl; NEN Life Science Products Inc.). Tubes were allowed to equilibrate with the isotope in the refrigerator overnight. On the second day of the experiment, 0.25 mL of pepsin solution (0.2 g in 5 mL of 0.1 N HCl) was added to tubes A and B after the pH of the samples had been brought to 2 and after treatment with Chelex-100 (Bio-Rad, Hercules, CA), an ion-exchange resin. The samples were left in an incubator at 37 °C for 1 h.

Inserts, carrying a dialysis membrane with an MWCO of 15 kDa (Spectra/Por Regenerated Cellulose, Fisher, Pittsburgh, PA) were aseptically placed in the wells of cell plates and companion plates (six-well plates devoid of cells used to study the mineral dialysis across the membrane.) Following the 1-h incubation, samples were adjusted to pH 6.0 with 1 M $NaHCO_3$ and 1.25 mL of pancreatin/bile solution
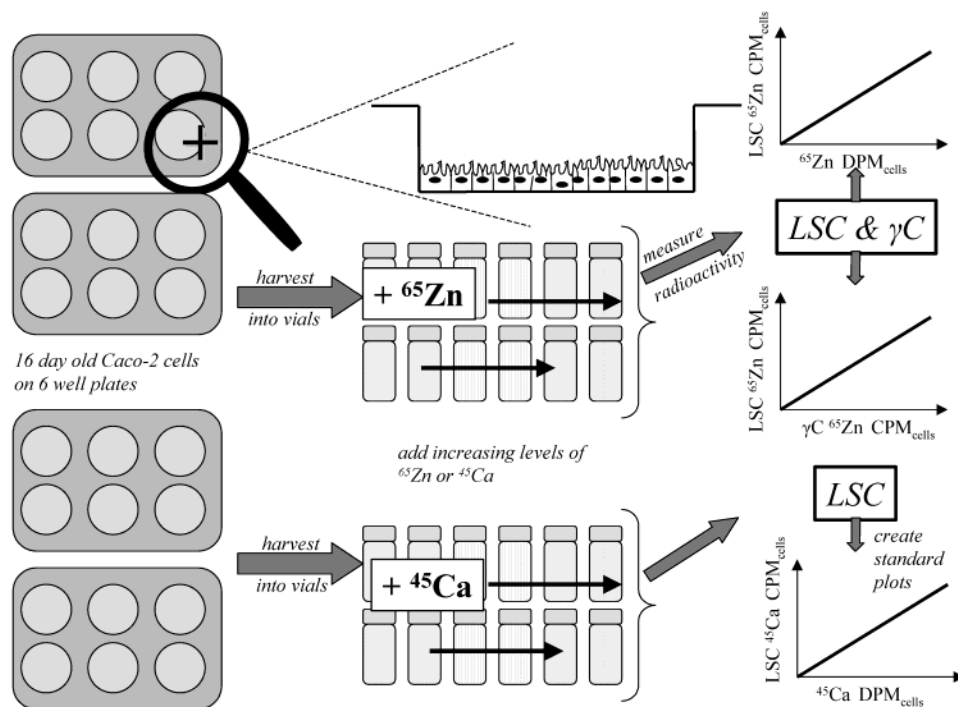
45Ca and 65Zn Uptake by Caco-2 Cells

J. Agric. Food Chem., Vol. 50, No. 22, 2002   **6289**

Figure 2. Diagram of 45Ca and 65Zn measurement in the presence of Caco-2 cells. Caco-2 cells growing on six-well plates were harvested 16 days postseeding into vials. Various amounts of 65Zn or 45Ca were added, and activity was measured in the LSC and γC (65Zn) or exclusively in the LSC (45Ca). Plots showing the relationship between (a) LSC 65Zn CPM and γC 65Zn CPM, (b) LSC 65Zn CPM and 65Zn DPM, and (c) LSC 45Ca CPM and 45Ca DPM in the presence of cells were constructed. Six levels of activity for each isotope were added to the cells in duplicate (12 vials). This experiment was replicated four times.

(0.05 g of pancreatin and 0.3 g of bile extract in 25 mL of 0.1 M NaHCO$_3$). The tubes were adjusted to pH 7.0 with 1 M NaHCO$_3$ and brought to a volume of 10 mL with 140 mM NaCl and 5 mM KCl. To the upper chamber of each insert was added 2.5 mL of digest: tubes A were added to the cell plates and tubes B to the companion plates. The plates were transferred to the incubator at 37 °C for 2 h.

Following the incubation, the inserts were removed and 1 mL of MEM was added to each lower chamber. The lower chamber contents of the companion plates were transferred to sterile tubes and stored in the −20 °C freezer for future mineral analysis. Tubes A were saved in the refrigerator for upper chamber radioactive count; tubes B (which represent the upper chamber contents) were saved for future mineral analysis in the −20 °C freezer. The cell plates were placed back in the incubator for a further 22 h. The cells were washed with 2 mM EDTA to ensure removal of nonspecifically bound 45Ca and 65Zn and sonicated for 15 min at 4 °C in 2 mL of 18 MΩ water. Each well was scraped and mixed to obtain a homogeneous cell suspension. Of this cell suspension, 1.8 mL were transferred to vials containing 8 mL of scintillation fluor. [Ecoscint liquid scintillation fluor consists of 70−85% chiral phenylalkanes; the rest is composed of nonionic surfactants and solvents (Ecoscint, National Diagnostics, Atlanta, GA).] Tube A contents were also transferred to vials (2.5 mL + 8 mL of scintillation fluor). Radioactivity was read in a gamma counter (γC) (Packard Auto-Gamma model 5530, Packard Instruments, Downers Grove, IL) and in a liquid scintillation counter (LSC) (Wallac 1410, Pharmacia, Turku, Finland), both under the 65Zn window. The mineral content of the upper chamber and lower chamber contents were measured via inductively coupled argon plasma emission spectrometry (ICP-ES) (ICAP model 61E trace analyzer, Thermo Jarrell Ash Corp., Franklin, MA).

45Calcium and 65Zinc Measurements. *In the Presence of Caco-2 Cells.* Sixteen days postseeding, Caco-2 cells growing on six-well plates were prepared for counting. DMEM was aspirated from the top of the cells, and the cell monolayers were washed twice with 2 mL of 130 mM NaCl, 5 mM KCl, and 5 mM PIPES, pH 6.7. Then they were sonicated in a bath sonicator at 4 °C for 15 min in the presence of 2 mL of 18 MΩ water and then transferred to scintillation vials. Various

amounts of either 65Zn or 45Ca isotope were added to each vial and measured in the γC and/or LSC. Plots showing the relationship between LSC 65Zn CPM and γC 65Zn CPM, between LSC 65Zn CPM and 65Zn DPM, and between LSC 45Ca CPM and 45Ca DPM in the presence of cells were constructed. A diagram summarizing these steps is shown in **Figure 2**.

In the Presence of HM and HM + HMF Digests. To study the quenching, that is, reduction in LSC 65Zn CPM or LSC 45Ca CPM caused by the presence of digested samples, the HMFs were prepared on the same protein basis and with added calcium as described for the in vitro digestion section, except that no plates with cells or companion plates were involved. Following the 2-h incubation period in the presence of pancreatin/bile, the tubes were removed from the incubator and stored overnight in a refrigerator. A 1:2-diluted 65Zn solution was prepared by adding 240 μL of 65Zn to 240 μL of 0.5 M HCl, and a 1:2 45Ca solution was prepared by adding 280 μL of 45Ca to 280 μL of water. Increasing activities of 65Zn and 45Ca were added to vials containing 2.5 mL of the digest and 8 mL of scintillation fluor. 65Zn was measured in the γC and LSC, and 45Ca was measured only in the LSC. Plots showing the relationship between LSC 65Zn CPM and γC 65Zn CPM, between LSC 65Zn CPM and 65Zn DPM, and between LSC 45Ca CPM and 45Ca DPM in the presence of HM and HM + HMF digests were constructed. A diagram of this procedure is shown in **Figure 3**.

Statistical Analysis. Linear regression analyses were performed with the Prism software (GraphPad Software, Inc., San Diego, CA).

In vitro digestion/Caco-2 cell uptake experiments were replicated five times. Each replication was carried out on separate days and was done in duplicate, meaning that there were two sets of plates with cells and two sets of companion plates. The position of each experimental treatment in the multiwell plate was random for each replication.

For the 45Ca and 65Zn measurement in the presence of cells, two six-well plates were used for each isotope. For this study, the cells were seeded on different days and addition of isotope took place 16 days postseeding. Six levels of activity for each isotope were added to duplicate wells. This was replicated four times. For the 45Ca and 65Zn
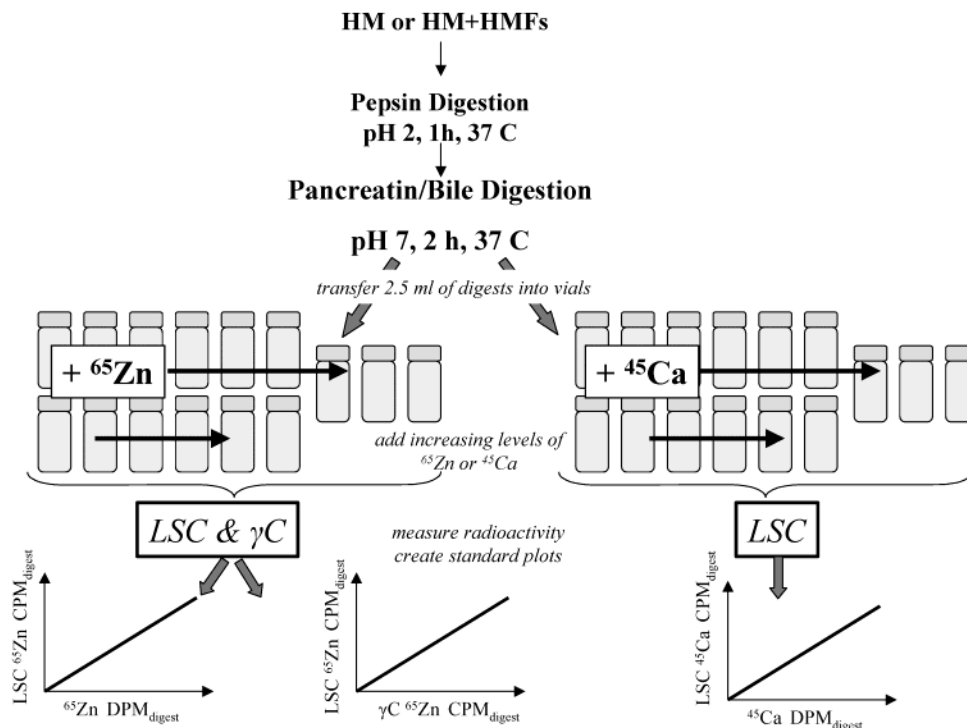
6290   *J. Agric. Food Chem.,* Vol. 50, No. 22, 2002

Etcheverry et al.

Figure 3. Diagram of ^{45}Ca and ^{65}Zn measurement in the presence of HM and HM + HMF digests. HM or HM + HMFs samples were first digested with pepsin followed by pancreatin/bile. Exactly 2.5 mL of digest was placed into scintillation vials. Various amounts of either ^{65}Zn or ^{45}Ca were added, and the activity was measured in the LSC and γC (^{65}Zn) or exclusively in the LSC (^{45}Ca). Plots showing the relationship between (a) LSC ^{65}Zn CPM and γC ^{65}Zn CPM, (b) LSC ^{65}Zn CPM and ^{65}Zn DPM, and (c) LSC ^{45}Ca CPM and ^{45}Ca DPM in the presence of each digest were constructed. Five levels of activity for each isotope were added to the digests in triplicate. This experiment was replicated three times for ^{65}Zn and twice for ^{45}Ca.

measurement in the presence of digests, digestions of the samples were carried out on three different days. To construct the regression curves, five levels of activity for each isotope were added to the digests in triplicate.

RESULTS

As described before, ^{45}Ca is a pure negatron emitter ($_{-1}\beta$), whereas ^{65}Zn is a positron ($_{+1}\beta$) and gamma (γ) emitter. Preliminary studies revealed that when ^{45}Ca and ^{65}Zn were present together, ^{65}Zn contributed to the ^{45}Ca counts in the LSC, making it very difficult to distinguish between the two. However, the counts contributed by both isotopes were found to be additive; that is, the total counts obtained in the LSC were equal to the counts contributed by ^{65}Zn and those contributed by ^{45}Ca (equation 1).

$$\text{total LSC CPM}_{\text{cells}} = \text{LSC }^{65}\text{Zn CPM}_{\text{cells}} + \text{LSC }^{45}\text{Ca CPM}_{\text{cells}} \quad (1)$$

Data obtained from the in vitro digestion/Caco-2 cell uptake experiments are total LSC CPM$_{\text{cells}}$ from the LSC and γC ^{65}Zn CPM$_{\text{cells}}$ from the gamma counter. As ^{45}Ca is a pure β emitter there is no contribution to the counts in the gamma counter. Thus, by counting samples containing both isotopes in this counter, only the counts of ^{65}Zn are obtained. Therefore, LSC ^{45}Ca CPM$_{\text{cells}}$ cannot be obtained directly from the LSC.

Because the ^{45}Ca and ^{65}Zn counts obtained in the LSC are additive (eq 1), the value of LSC ^{45}Ca CPM can be obtained by knowing the relationship between the ^{65}Zn counts obtained in the γC (γC ^{65}Zn CPM) and those counts obtained in the LSC (LSC ^{65}Zn CPM).

As an example, if LSC ^{65}Zn CPM is explained by the regression equation LSC ^{65}Zn CPM $= a + b(\gamma\text{C }^{65}\text{Zn CPM})$ (where *a* and *b* are the intercept and slope of the regression equation, respectively)
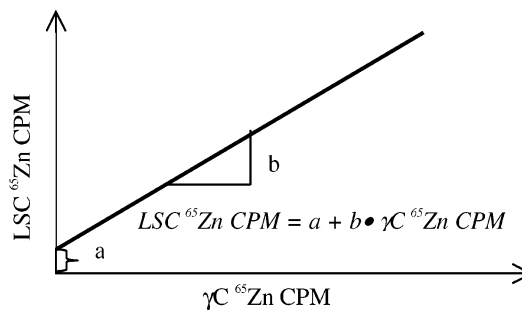
then eq 1 can be rewritten into

$$\text{total LSC CPM}_{\text{cells}} = [a + b(\gamma\text{C }^{65}\text{Zn CPM}_{\text{cells}})] + \text{LSC }^{45}\text{Ca CPM}_{\text{cells}} \quad (2)$$

LSC ^{45}Ca CPM$_{\text{cells}}$ can be calculated by simply rearranging eq 2.

$$\text{LSC }^{45}\text{Ca CPM}_{\text{cells}} = \text{total LSC CPM}_{\text{cells}} - [a + b(\gamma\text{C }^{65}\text{Zn CPM}_{\text{cells}})] \quad (3)$$

Preliminary work revealed that when the counts of LSC ^{45}Ca were calculated in this way and compared to the CPM readings of known ^{45}Ca activity, nonsignificant differences were observed (**Table 1**).

**Table 1.** Percent Difference between Observed [45]Ca CPM and Calculated [45]Ca CPM (from Equation 3)

γC [65]Zn CPM	LSC [65]Zn CPM[a]	total LSC CPM[b]	calcd LSC [45]Ca CPM[c]	obsd LSC [45]Ca CPM[d]	% diff[e]
2853	4313.41	12849.8	8536.39	8456.2	−0.95[f]
3079	4667.17	12787.4	8120.23	8407.3	+3.41
3004	4549.77	21330.2	16780.43	17053	+1.60
2983	4516.90	21274.6	16757.70	16951.1	+1.14
2947	4460.55	21090.2	16629.65	17197.3	+3.30
3021	4576.38	55136.7	50560.32	51061.9	+0.98
2926	4427.68	55080.6	50652.92	51762.8	+2.14
2955	4473.07	72886.6	68413.53	68386.2	−0.04
2710	4089.57	72154.1	68064.53	69054.9	+1.43

[a] Preliminary work showed that LSC [65]Zn = −152.39 + 1.5653(γC [65]Zn CPM). [b] As obtained from the liquid scintillation counter, total LSC CPM = LSC [65]Zn CPM + LSC [45]Ca CPM. [c] *Calculated* LSC [45]Ca CPM = total LSC CPM − [−152.39 + 1.5653(γC [65]Zn CPM)]. [d] Readings obtained from the LSC, in the absence of [65]Zn, were *observed* LSC [45]Ca CPM. [e] Percent difference = (obsd LSC [45]Ca CPM − calcd LSC [45]Ca CPM) × 100%. [f] Differences between the two columns were not significant, $p > 0.05$. Vials containing [65]Zn and [45]Ca were read in the γC (obtaining γC [65]Zn CPM) and LSC (obtaining Total LSC CPM). The calculated [45]Ca CPM was then obtained. Vials with the same activity of [45]Ca but devoid of [65]Zn were read in the LSC (obtaining observed [45]Ca CPM).

Another equation that is important is

$$DPM_{cells} = LSC\ CPM_{cells}/E_{co} \quad (4)$$

which shows the relationship between DPM_{cells} and LSC CPM_{cells} for either isotope, also known as the quench curve.
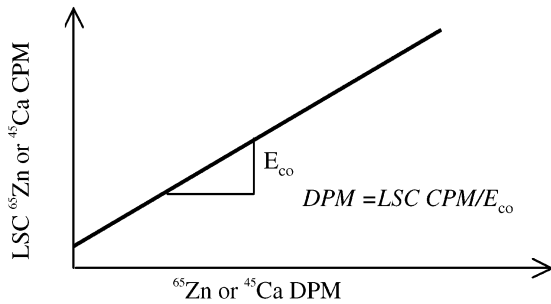
Just like for cells, the same equations in the presence of digests are needed:

$$total\ LSC\ CPM_{digest} = LSC\ ^{65}Zn\ CPM_{digest} + LSC\ ^{45}Ca\ CPM_{digest} \quad (5)$$

$$total\ LSC\ CPM_{digest} = [a + b(\gamma C\ ^{65}Zn\ CPM_{digest})] + LSC\ ^{45}Ca\ CPM_{digest} \quad (6)$$

$$LSC\ ^{45}Ca\ CPM_{digest} = total\ LSC\ CPM_{digest} - [a + b(\gamma C\ ^{65}Zn\ CPM_{digest})] \quad (7)$$

$$DPM_{digest} = LSC\ CPM_{digest}/E_{co} \quad (8)$$

Finally, assuming that the isotopes added had equilibrated with the endogenous content of either mineral and that their contribution to the endogenous content was negligible, then the conversion of DPM_{cells} to the actual amount of Ca and Zn from the digests taken up by the cells can be calculated.

$$total\ Ca\ or\ Zn\ uptake = [(total\ Ca\ or\ Zn\ in\ upper\ chamber)/DPM_{digest}] \times DPM_{cells} \quad (9)$$

$$LSC\ ^{65}Zn\ CPM = -217.8 + 1.147\ (\gamma C\ ^{65}Zn\ CPM)$$
$$R^2 = 0.9828$$
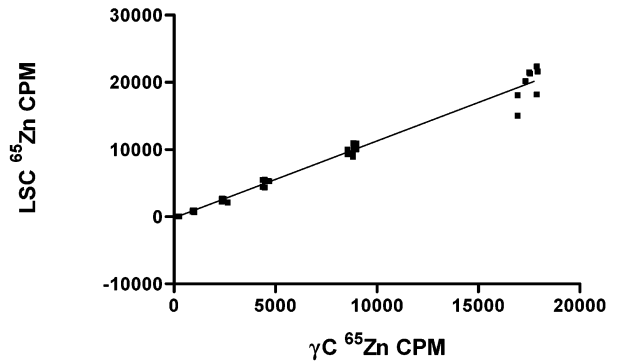
Figure 4. Relationship between [65]Zn CPM obtained in the LSC (LSC [65]Zn CPM) and the [65]Zn CPM obtained in the γC (γC [65]Zn CPM) in the presence of a 2 mL Caco-2 cell suspension. $n = 48$.

$$water: LSC\ ^{65}Zn\ CPM = -61.70 + 0.2805(^{65}Zn\ DPM)$$
$$R^2 = 0.9714$$
$$cells:\ LSC\ ^{65}Zn\ CPM = 174.5 + 0.2182(^{65}Zn\ DPM)$$
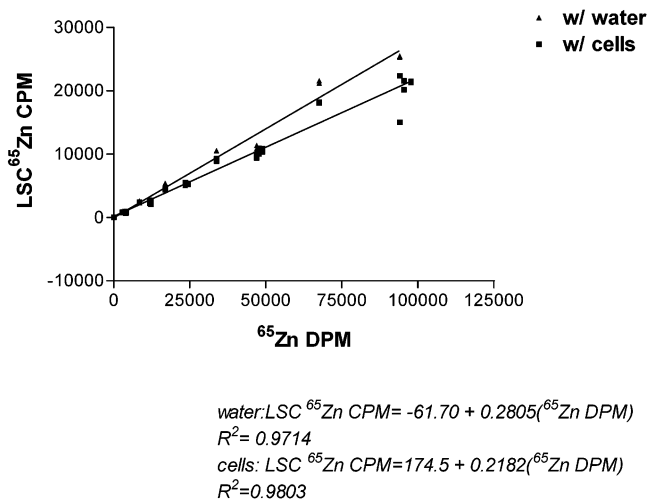$$R^2 = 0.9803$$

Figure 5. Degree of quenching by a 2 mL sonicated Caco-2 cell suspension on [65]Zn CPM measured in the LSC. Water was included for reference. $n = 48$.

Figure 4 shows a regression curve that relates γC [65]Zn counts to LSC [65]Zn counts in the presence of a 2 mL Caco-2 cell suspension. The isotopes were studied in the presence of 2 mL of Caco-2 cell suspension because this represents the volume of radioactively labeled cells that are going to be measured in the LSC and γC following the in vitro digestion/Caco-2 cell uptake experiments. **Figures 5** and **6** show the degree of quenching in the presence of Caco-2 cells for [65]Zn and [45]Ca, respectively. The graphs are useful for determining the absolute activity or disintegrations per minute (DPM) of either isotope in the presence of cells. Water did not have as much of a quenching effect on [65]Zn counts as the presence of cells did. However, for [45]Ca the water and cell curves seem to almost overlap, which suggests that there is no difference in the degree of quenching between the two samples for this particular isotope. In this study, the efficiency in counting [65]Zn in the LSC was 28% in the presence of water and 22% in the presence of cells, whereas for [45]Ca it was 27% in the presence of either water or cells.

Figure 7 shows the relationship between LSC [65]Zn CPM and γC [65]Zn CPM in the presence of the HM and HM + HMF digests. The degree of quenching of the human milk fortifier digest on either isotope is shown in **Figures 8** and **9**. These graphs are useful for determining the efficiency and absolute activity of either isotope in the presence of the digests. Again,
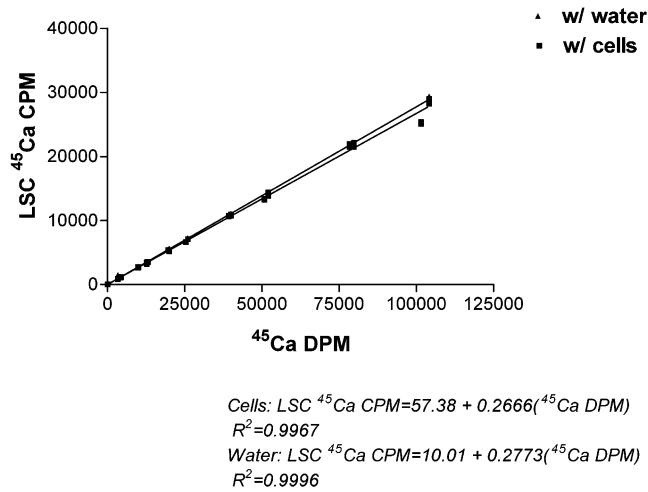
Cells: LSC ^{45}Ca CPM=57.38 + 0.2666(^{45}Ca DPM)
R^2=0.9967
Water: LSC ^{45}Ca CPM=10.01 + 0.2773(^{45}Ca DPM)
R^2=0.9996

Figure 6. Degree of quenching by a 2 mL sonicated Caco-2 cell suspension on ^{45}Ca CPM measured in the LSC. Water was included for reference. $n = 48$.

water gave the highest counts. On the other hand, HM + SMA gave the lowest counts in the presence of either isotope.

DISCUSSION

Although radioisotope tracer techniques are an important tool for the study of mineral absorption, the simultaneous determination of multiple radioisotopes in biological samples is not a simple procedure. Complications arise mainly due to spectrum overlap and quenching. This study introduced a simple method for the determination of ^{45}Ca counts from a sample also containing ^{65}Zn, using gamma and liquid scintillation counters.

The fact that ^{45}Ca is a pure β emitter was crucial in the separation of the count contribution by both isotopes. The counts obtained from the γC data are exclusively from ^{65}Zn decay, because β particles cannot penetrate the sodium iodide crystal present in this counter. On the other hand, the LSC is considered the optimum counting system for low-energy β particle emitters including ^{3}H, ^{14}C, and ^{35}S, all with E_{max} lower than that of ^{45}Ca. The LSC is also applicable to the measurements of α particle emitters, of isotopes that decay by electron capture resulting in the emission of the low-energy X-rays or γ-rays characteristic of the daughter element and of β particle emitters of high energies (8). Consequently, the LSC detects both β and γ emitters. Because γ-rays can be detected in the LSC by a process that will be described later on, ^{65}Zn counts obtained in this
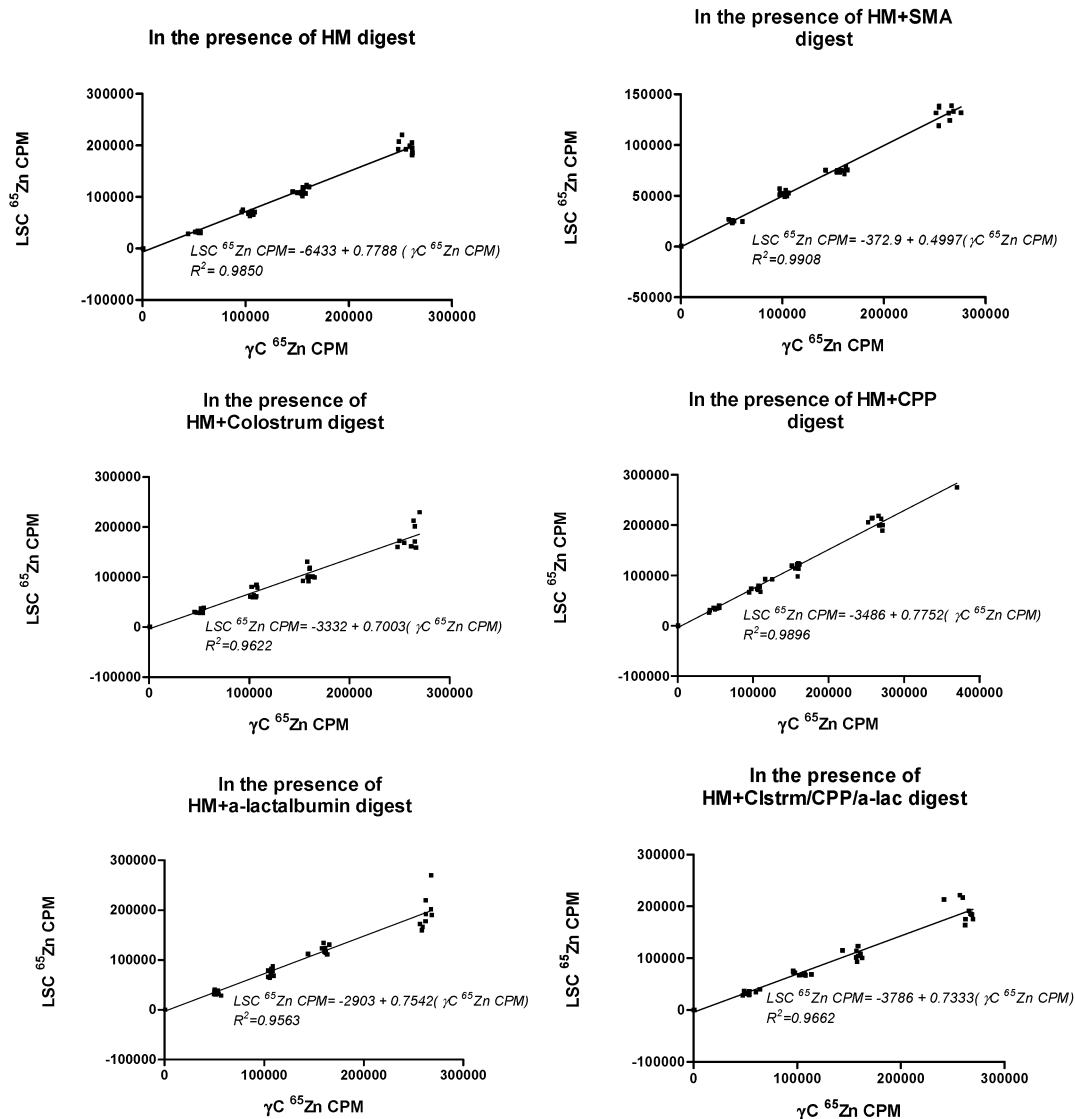

In the presence of HM digest

LSC ^{65}Zn CPM= -6433 + 0.7788 (γC ^{65}Zn CPM)
R^2= 0.9850

In the presence of HM+SMA digest

LSC ^{65}Zn CPM= -372.9 + 0.4997(γC ^{65}Zn CPM)
R^2=0.9908

In the presence of HM+Colostrum digest

LSC ^{65}Zn CPM= -3332 + 0.7003(γC ^{65}Zn CPM)
R^2=0.9622

In the presence of HM+CPP digest

LSC ^{65}Zn CPM= -3486 + 0.7752(γC ^{65}Zn CPM)
R^2=0.9896

In the presence of HM+a-lactalbumin digest

LSC ^{65}Zn CPM= -2903 + 0.7542(γC ^{65}Zn CPM)
R^2=0.9563

In the presence of HM+Clstrm/CPP/a-lac digest

LSC ^{65}Zn CPM= -3786 + 0.7333(γC ^{65}Zn CPM)
R^2=0.9662

Figure 7. Relationship between ^{65}Zn CPM obtained in the LSC (LSC ^{65}Zn CPM) and the ^{65}Zn CPMs obtained in the γC (γC ^{65}Zn CPM) in the presence of HM and the different HM + HMF digests. $n = 45$.

HM:LSC ^{65}Zn CPM= -3442 + 0.0853(^{65}Zn DPM), R^2= 0.9569

$HM+SMA$:LSC ^{65}Zn CPM=517.9 + 0.0562(^{65}Zn DPM), R^2=0.9695

$HM+Colostrum$:LSC ^{65}Zn CPM=681.1 + 0.0766(^{65}Zn DPM), R^2=0.8931

$HM+CPP$:LSC ^{65}Zn CPM=-3185 + 0.0914(^{65}Zn DPM), R^2=0.9352

$HM+a-lac$:LSC ^{65}Zn CPM=-756.9 + 0.0851(^{65}Zn DPM), R^2=0.9375

$HM+Clstrm/CPP/a-lac$:LSC ^{65}Zn CPM=-2919 + 0.0818(^{65}Zn DPM), R^2=0.9301

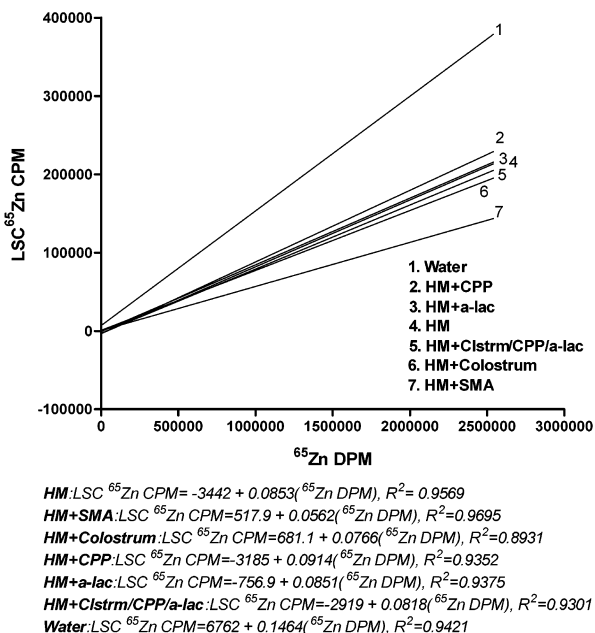$Water$:LSC ^{65}Zn CPM=6762 + 0.1464(^{65}Zn DPM), R^2=0.9421

Figure 8. Degree of quenching by HM and the different HM + HMF digests on LSC ^{65}Zn CPM. Water was included for reference. $n = 45$. Data points were not included to simplify the figure. Standard deviations of the data points for the same *X* value were within 10% of the mean.
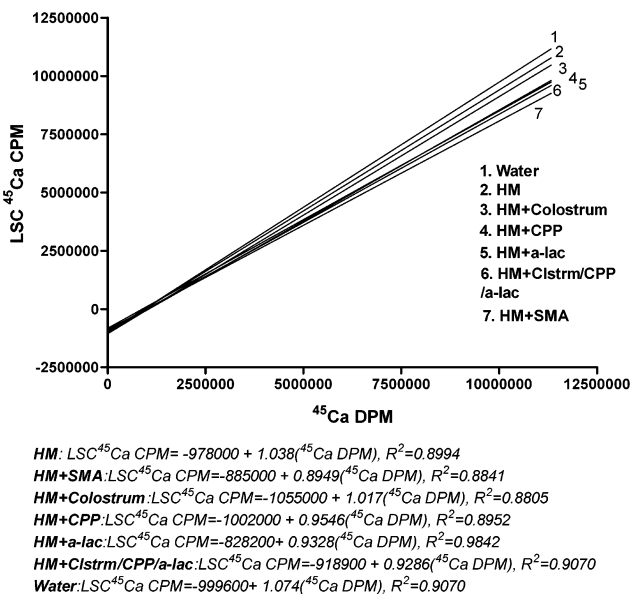

HM: LSC^{45}Ca CPM= -978000 + 1.038(^{45}Ca DPM), R^2=0.8994

$HM+SMA$:LSC^{45}Ca CPM=-885000 + 0.8949(^{45}Ca DPM), R^2=0.8841

$HM+Colostrum$:LSC^{45}Ca CPM=-1055000 + 1.017(^{45}Ca DPM), R^2=0.8805

$HM+CPP$:LSC^{45}Ca CPM=-1002000 + 0.9546(^{45}Ca DPM), R^2=0.8952

$HM+a-lac$:LSC^{45}Ca CPM=-828200+ 0.9328(^{45}Ca DPM), R^2=0.9842

$HM+Clstrm/CPP/a-lac$:LSC^{45}Ca CPM=-918900 + 0.9286(^{45}Ca DPM), R^2=0.9070

$Water$:LSC^{45}Ca CPM=-999600+ 1.074(^{45}Ca DPM), R^2=0.9070

Figure 9. Degree of quenching by HM and the different HM + HMF digests on LSC ^{45}Ca CPM. Water was included for reference. $n = 30$. Data points were not included to simplify the figure. Standard deviations of the data points for the same *X* value were within 10% of the mean.

counter were then named LSC ^{65}Zn instead of β ^{65}Zn, to indicate the fact that not only β emissions are detected by this type of counter.

To understand why ^{65}Zn emissions, other than β, may be detected in the LSC, a brief review of how this type of counter works is necessary. In an LSC, emitted β particles interact with the liquid scintillation fluor to produce photons. Fluors usually consist of a solvent, such as toluene, xylene, or methoxybenzene, and primary and secondary scintillators, such as naphthalene and anthracene (*9*). As the electron or β particle collides with a solvent molecule, it transfers its energy to this molecule, raising it to an excited state. The energy embodied in the excited solvent molecule may be transferred to another solvent molecule

or emitted as light. That is where the primary and secondary scintillators play a major role. The primary fluor absorbs the light emitted by the excited solvent and reemits it at a second, longer wavelength. Some old instruments, however, require a wavelength that is even longer than that emitted by the primary scintillators, so secondary scintillators absorb light at the wavelength emitted by the primary scintillator and emit it at longer wavelengths. Primary and secondary scintillators, due to their ability to emit light at a longer wavelength than that at which it was absorbed, are known as fluorescent compounds (*9*). The light emitted is detected by a photomultiplier tube and registered in a scaler.

How X- or γ-ray emitting compounds, such as ^{65}Zn, may be detected in the LSC may be attributed to Compton scattering and to the photoelectric effect. During Compton scattering, part of the energy from an incident X- or γ-ray is transferred to an electron in the absorbing medium. The electron is then ejected, behaving as a β particle, which can then interact with the scintillation fluor. During a photoelectric interaction, the entire energy of the ray is transferred to the electron, thus also resulting in its ejection (*10*).

In the LSC, quenching corrections are imperative, because counts obtained in this counter are greatly affected by the type of material the isotope is in. The fact that HM + SMA had the most quenching in the presence of either isotope could be attributed to color quenching as this product contains β-carotene and riboflavin and thus is slightly yellow in color, compared to the other samples, which are white. Another possible reason could be attributed merely to the fact that in the case of HM + SMA there was more material dissolved in the HM compared to the other HMFs. All of the samples were prepared on the same protein basis, which means that in order to achieve the same level of protein of SMA, which provides 0.5 g of protein per 2 g of product, less sample had to be dissolved in human milk for the other fortifiers, which were exclusively protein-based. Thus, it is likely that concentration and dilution effects played a major role in the counts obtained.

There are several methods for making quench corrections, all of which aim at estimating E_{co} of the instrument for the sample. The most common methods involve the preparation of internal or external standards and the channels ratio method, which can be tedious when one is dealing with a large number of samples. This study introduced another method for correcting for quench. Because the amount of quenching material, which in this experiment were the cell suspension and the digest, was constant, by adding increasing activity of either isotope, the relationship between the absolute activity and the counts from the LSC could be determined and the counting efficiency could be obtained.

Sample preparation for quenching curves was labor intensive and time-consuming because in order to have better accuracy in the results, the samples to be used in a quench curve had to be subjected to the same conditions as the samples in the experiment. However, time can be saved by preparing extra samples for the in vitro digestions, which can then be used for future quenching curves. It is possible that this method will give increased accuracy in data collection, as the samples used in the quenching studies are the same as those used in the experiments. Alternatively, the activity present in the digests can be obtained by knowing the amount of isotope added and the activity this corresponds to. This obviously requires the basic assumption that the correct amount of activity was indeed pipetted onto the samples, which can be difficult to do when one is working with small volumes.

Regardless of the way the true activity in the presence of digests is determined, it is crucial to determine the activity in the presence of cells. It is likely that calibration, maintenance routines, and the mere use of the counter can alter its counting efficiency with time. Consequently, it is essential not only to determine the efficiency in the presence of cells for each study but also to determine it on the same day that the samples are measured and possibly with same stock of cells that were used in the in vitro study.

The measurement of dual-labeled samples with ^{45}Ca and ^{65}Zn has been documented (*4, 5*). In these studies, ^{65}Zn activity was measured in a gamma counter and ^{45}Ca activity was measured in a LSC. These studies, however, failed to take into account the contribution of ^{65}Zn counts to ^{45}Ca counts when the samples were measured in the LSC. This means that the ^{45}Ca counts were probably higher in the presence of ^{65}Zn than in the absence of this isotope. Hansen et al. (*6*) studied the effect of different milk proteins on the calcium and zinc absorption from phytate-containing meals in rat pups. ^{65}Zn- and ^{47}Ca-labeled diets were given to rat pups, and radioactivity was measured in various organs in a gamma counter. Radioactivity was measured immediately after the animal had been sacrificed and 4 weeks later to allow for ^{47}Ca decay (^{47}Ca half-life = 4.5 days), and thus ^{65}Zn and ^{47}Ca activity could be determined. Although this method is straightforward, it suffers from some disadvantages: it can be too slow in some clinical/biological situations; it is not applicable to the measurement of ^{45}Ca, which has a half-life of 165 days; unlike ^{47}Ca, ^{45}Ca does not emit gamma rays; and this method can be costly as a constant supply of ^{47}Ca is necessary as this decays very quickly. Furthermore, due to the short half-life of this isotope, there is a limitation in the flexibility that is desired and needed when consecutive experiments are conducted.

While this study introduced a method for simultaneously determining ^{45}Ca and ^{65}Zn in a sample, iron can also be measured by determining Caco-2 cell ferritin formation and, thus, represents a third mineral that can be concurrently measured. Caco-2 cell ferritin formation has been shown to be a highly sensitive marker of iron bioavailability (*7*). To allow for ferritin formation, cells should remain in the presence of digested compounds that dialyzed through for 24 h past the start of the intestinal digestion period. At this point, cells are harvested for ferritin analysis and ^{45}Ca and ^{65}Zn radioactive counting. Ferritin formation can be assessed via a one-stage, two-site radioimmunoassay, which involves ^{125}I, a gamma emitter. We have seen that the presence of ^{45}Ca and ^{65}Zn does not interfere with the measurement of ^{125}I and hence determination of cell ferritin formation.

ABBREVIATIONS USED

γC, gamma counter; LSC, liquid scintillation counter; CPM, counts per minute; DPM, disintegrations per minutes; E_{co}, LSC counting efficiency; γC ^{65}Zn CPM, ^{65}Zn counts obtained from the gamma counter; LSC ^{45}Ca CPM, ^{45}Ca counts obtained from the liquid scintillation counter; LSC ^{65}Zn CPM, ^{65}Zn counts obtained from the liquid scintillation counter.

LITERATURE CITED

(1) Weaver, C. Isotopic tracer methodology: potential in mineral nutrition. In *Trace Minerals in Foods*; Smith, K. T., Ed.; Dekker: New York, 1988; pp 429−454.

(2) Van Campen, D. R.; Glahn, R. P. Micronutrient bioavailability techniques: accuracy, problems and limitations. *Field Crops Res.* **1999**, *60*, 93−113.

(3) Hendee, W. R. Radioactive decay. In *Radioactive Isotopes in Biological Research*; Wiley: New York, 1973; pp 13−33.

(4) Bagheri, S. M.; Guéguen, L. Effects of wheat bran on the metabolism of calcium-45 and zinc-65 in rats. *J. Nutr.* **1982**, *112*, 2047−2051.

(5) Schwartz, R.; Nivens, P. Effects of phytate reduction, fat extraction and level of Ca on Ca and Zn bioavailability. *Biol. Trace Elem. Res.* **1989**, *19*, 93−106.

(6) Hansen, M.; Sandström, B.; Lönnerdal, B. The effect of casein phosphopeptides on zinc and calcium absorption from high phytate infant diets assessed in rat pups and Caco-2 cells. *Pediatr. Res.* **1996**, *40* (4), 547−552.

(7) Glahn, R. P.; Lee, O. A.; Yeung, A.; Goldman, M. I.; Miller, D. D. Caco-2 cell ferritin formation predicts nonradiolabeled food iron availability in an in vitro digestion/Caco-2 cell culture model. *J. Nutr.* **1998**, *128*, 1555−1561.

(8) Stubbs, R. D. Liquid scintillation counting. In *Radioisotope Laboratory Techniques*; Wiley: New York, 1973; pp 209−231.

(9) Cooper, T. G. Radiochemistry. In *Tools of Biochemistry*; Wiley: New York, 1977; pp 65−135.

(10) Hendee, W. R. Interactions of X radiation and γ radiation. In *Radioactive Isotopes in Biological Research*; Wiley: New York, 1973; pp 44−58.