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Analytical Methods Section

Validation of chemical measures of calcium with bioassay of calcium-fortified cottage cheese

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Chemically measured calcium was correlated with bioavailability of calcium by rat bioassay. Calcium-fortified cottage cheese was evaluated by both chemical and bioassay tests. Cheese was fortified at four levels of calcium with or without added guar to increase acceptability. Chemical tests on laboratory digests and non-digested samples were by two different methods: (1) dialyzed and ionic dialyzed calcium, and (2) soluble and ionic soluble calcium. Calcium added to cheese significantly increased % calcium availability. Calcium availability from rat diets containing the same cheese with and without guar correlated well with bioassay results. Ionic dialyzed, ionic soluble and soluble calcium correlated with bioassay measures. Ionic dialyzed calcium in non-digested diets was the best chemical test for bioavailability. Digestion did not improve accuracy. These chemical tests accurately measured bioavailability from a calcium-fortified cheese.

INTRODUCTION

Chemical tests for mineral bioavailability are needed. In-vitro methods are rapid and inexpensive compared to bioassays. It is possible to control experimental conditions tightly and to avoid variability experienced with bioassays. In-vitro procedures may also indicate changes in bioavailability during processing and storage. These in-vitro tests of calcium bioavailability must be validated by correlation with in-vivo data.

Studies of the last 15 years support the concept that chemical tests may accurately determine bioavailability of certain minerals. The present authors introduced in-vitro tests for chemical forms of iron and their changes during food processing, storage or fortification (Lee & Clydesdale, 1979). Miller *et al.* (1981) used an innovative simulated gastrointestinal digestion to measure iron and other minerals by dialysis. Dialyzed iron as a predictor of iron bioavailability was validated by correlation with in-vivo data (Schricker *et al.*, 1981). Dialyzed zinc also correlated with in-vivo data (Sandstrom & Almgren, 1989) but Hunt *et al.* (1989) found that simulated digestion did not estimate zinc bioavailability.

Chemical tests for calcium are difficult since calcium solubility depends on pH. It is also difficult because of soap formation with fatty acids. Sheikh *et al.* (1987) reported solubility of calcium salts at the pH of the duodenum did not predict absorption in humans. They hypothesized that acid dissolution affected calcium absorption. Schwartz and Nevins (1989) found that calcium solubility after simulated digestion did not correlate with in-vivo data. Extraction of fat and lower phytate levels increased calcium solubility, yet these treatments did not affect calcium absorption in rats. The method of Miller *et al.* (1981) was also used by Keane *et al.* (1988) to study chemical forms of calcium in foods. Kim and Zemel (1986) found that soluble calcium in milk and spinach differed according to known bioavailability. Walsh *et al.* (1989) studied the passage of calcium in casein digests through ultrafiltration membranes. The observations in these last three studies were not supported by bioassay. The purpose here was to evaluate soluble, dialyzed and ionic calcium in calcium-fortified cottage cheese and rat diets prepared from these foods. Results were further correlated with in-vivo data to find possible predictors of calcium bioavailability.

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MATERIALS AND METHODS

Design

Two separate methods were used in parallel to evaluate available calcium, consisting of dialyzed (D), ionic dialyzed (ID), soluble (S) and ionic soluble (IS) calcium. D- and S-Ca represent both ionic and bound calcium. Both methods were used to measure calcium availability in cottage cheese and rat diets prepared with cottage cheese. Cottage cheese with 0·28% guar gum and fortified at four levels with calcium lactate (0, 50, 100 and 130 mg calcium added per 100 g cheese) was prepared at the UW-Madison Dairy Plant (Puspitasari & Lee, 1991). Rat diets were prepared at the UW-Madison Department of Nutritional Sciences with freeze-dried cottage cheese (Kaup *et al.*, 1991). Control diets contained calcium lactate at four levels and cottage cheese without guar. Freeze-dried samples were kept in vacuum-packed containers at −40°C until needed. Total solids were consistently 97·15 ± 0·20% (n = 8).

Both chemical methods were used to test (1) dialysis for D- and ID-Ca, and (2) solubility for S- and IS-Ca. Available calcium was measured in digested and non-digested samples. Digestion was a simulated gastrointestinal digestion. Calcium availabilities of diets were compared to rat bioassay data from the same diets.

Dialyzed calcium (D and ID)

The in-vitro method of Miller *et al.* (1981) as modified by Keane *et al.* (1988) was adapted to measure dialyzed (D) and ionic dialyzed (ID) calcium. Dry samples were hydrated with deionized, distilled water and kept at 4°C overnight for all experiments. For digested samples, slurries (62 ml) containing 5% solids were acidified to pH 2·00 ± 0·02 and incubated with 3 ml 0·1 M HCl containing 63 mg pepsin per g cheese protein (Sigma Chemical Co., St Louis, MO) at 37°C for 2 h in a shaking water bath. Pepsin digests were kept at −20°C overnight. Dialysis bags (Spectropor 1, 6000–8000 dalton cut-off) contained 20 ml freshly prepared sodium $NaHCO_3$ solution of concentration (0·14–0·32 M) sufficient to raise the pH to 7. Bags were immersed into thawed pepsin digest and incubated at 37°C with agitation. A 0·1 M $NaHCO_3$ solution (15 ml) containing 33 mg pancreatin per g cheese protein (Sigma) and 52 mg bile per g cheese protein (Sigma) was added when the digest pH was 5 (about 30 min). This avoided enzyme inactivation. After 2 h of digestion, dialysates were weighed, pH measured and calcium analyzed. The amount of $NaHCO_3$ needed to raise digest pH to 7 was determined separately for cheese and diets by varying $NaHCO_3$ concentration. This allowed for digestion products and $NaHCO_3$ lost as CO_2.

Non-digested samples were dialyzed without acidification and digestion. Hydrated samples (80 ml) containing 5% solids were incubated at 37°C for 2.5 h with shaking. Dialysis bags contained 20 ml deionized distilled water. All experiments were repeated three times.

Soluble calcium (S and IS)

The in-vitro procedures of Kim and Zemel (1986) and Schwartz and Nevins (1989) were changed to measure soluble (S) and ionic soluble (IS) calcium. Samples were hydrated in sealed screw cap test tubes to give a final 5% solid content. Samples were acidified with 0·1 M HCl containing 63 mg pepsin per g cheese protein (Sigma). The pH was adjusted to 2·00 ± 0·02 with HCl and the samples incubated at 37°C for 1 h in a shaking water bath. $NaHCO_3$ was added to raise the pH to 6·5 and about 1 h allowed for the pH to rise due to loss of CO_2. A 1 ml solution of 0·1 M $NaHCO_3$ containing 33 mg pancreatin per g cheese protein (Sigma) and 52 mg bile per g cheese protein (Sigma) was added, pH made to 6·5 and volume adjusted to 30 ml. The tubes were sealed to prevent loss of CO_2. Samples were digested for 1 h at 37°C in a shaking water bath. The pH was measured and the digests immediately centrifuged at 18 000 g for 20 min at 5°C. Supernatants were weighed, filtered (Whatman no. 4) and analyzed for calcium.

S- and IS-Ca were also measured in hydrated samples without pH or enzyme treatments (non-digested samples). Samples were incubated for 2 h at 37°C with shaking. All experiments were repeated three times.

Mineral analysis

A calcium-selective electrode (Model 93–20, Orion Research, Inc., Boston, MA) measured both ID and IS calcium. Ionic strength of standards and samples was adjusted to 0·08 M with KCl before measurement. Ionic calcium was measured in dialysates and supernatants the same day as samples were digested or extracted. Samples for D calcium content were dry-ashed at 520°C for about 18 h in a muffle furnace. The ash was re-wetted, heated until dry, re-ashed at 520°C for 1–2 h, dissolved in HNO_3, heated until dry and redissolved in HCl. Supernatant fluids for S calcium were diluted with 0·1 M HCl. Calcium and iron were measured with a Perkin Elmer model 2380 double-beam atomic absorption spectrophotometer with air–acetylene flame, 4·25 inch 3-slot burner head and Pt–Rd nebulizer. Sodium was measured by atomic emission on the same instrument. Calcium- and iron-specific hollow cathode lamps and calcium and iron standard, 1000 ppm (VWR), were used. Calcium samples contained 0·05% lanthanum chloride (J. T. Baker, Inc., Phillipsburg, NJ). Results were confirmed by a standard reference skim milk powder no. 1549 (National Institute of Standards and Technology, Gaithersburg, MD). Solutions were prepared with deionized, distilled water in acid-washed glassware. All reagents were reagent grade. These equations were used in calculation in D, ID, S and IS forms of calcium, respectively:

$$\% \text{ D–Ca} = \frac{\dfrac{\text{mg Ca}}{\text{g dialysate}} \times (\text{g dialysate}) \times 100\%}{\text{total mg Ca}}$$

$$\% \text{ ID–Ca} = \frac{\frac{\text{mg ionic Ca}}{\text{g dialysate}} \times (\text{g dialysate}) \times 100\%}{\text{total mg Ca}}$$

$$\% \text{ S–Ca} = \frac{\frac{\text{mg Ca}}{\text{g supernatant}} \times (\text{g supernatant}) \times 100\%}{\text{total mg Ca}}$$

$$\% \text{ IS–Ca} = \frac{\frac{\text{mg ionic Ca}}{\text{g supernatant}} \times (\text{g supernatant}) \times 100\%}{\text{total mg Ca}}$$

Statistical evaluation

Significant differences between cheese or diets at different calcium levels were determined by Fisher's protected least significant difference (LSD) test. A polynomial trend test showed changes in calcium availability as calcium content of samples increased. Two-way analysis of variance was used to measure effects of digestion, diet preparation and guar. Pearson's first-order correlation coefficients were calculated correlating calcium availability data and rat bioassay measures from the same diets. Analysis of covariance using the general linear model determined if guar influenced correlations. Difference between correlation coefficients were tested for significance using the z-transformation (Snedecor & Cochran, 1980). An SAS program (SAS Institute, Cary, NC) was used for the LSD test and the test of polynomial trend. A Minitab program, version 7·2 for the PC (State College, PA), was used for all other statistical calculations.

RESULTS

Calcium availability

Percent dialyzed (D), ionic dialyzed (ID), soluble (S) and ionic soluble (IS) calcium in digested and non-digested cottage cheese are shown in Table 1. About half of the total calcium in non-digested cottage cheese containing guar was IS-Ca. S-Ca was 18–20% higher than IS-Ca, the difference representing calcium bound to other compounds. D-Ca was higher than ID-Ca; thus some D-Ca was bound by low-molecular-weight compounds. The proportion of bound D-Ca increased after digestion. The percent calcium availability in cottage cheese increased ($p < 0·05$) as calcium lactate was added, showing that proportionally more calcium was available at higher levels of fortification. The overall calcium availability was less ($p < 0·05$) in digested than in non-digested cottage cheese. D- and S-Ca were about half, ID-Ca about 1/5 and IS-Ca about 1/10 after digestion compared to non-digested. Although absolute values were less, the rank order remained consistent.

Rat diets containing cottage cheese were made with guar (test) or without guar (control). Table 2 compares calcium availabilities in non-digested diets. Guar lowered ($p < 0·05$) % D-, % S- and % IS-Ca in non digested diets compared to controls without guar. The decrease in calcium was no more than 14% of calcium available in controls. As was seen for cottage cheese, the calcium availability in non-digested diets increased ($p < 0·05$) as calcium lactate was added. This was a significant linear increase.

Calcium availability in rat diets after digestion is shown in Table 2. In the presence of digestion products, guar lowered ($p < 0·05$) % D- and % ID-Ca relative to controls without guar. Only % ID- and % IS-Ca increased significantly in digested diets as calcium lactate increased. Percent D-, % ID- and % S-Ca increased ($p < 0·01$) during digestion of diets (Table 2).

Comparison of the calcium in cottage cheese alone and rat diets containing cottage cheese shows the effect of diet preparation, which is sometimes not controlled in bioassays. The calcium availability in non-digested diets was lower ($p < 0·01$) compared to non-digested cheese (Tables 1 and 2). However, diet preparation increased ($p < 0·01$) the calcium availability in digested diets compared to digested cheese (Table 1). IS-Ca was 3–10 times higher in digested diets than in digested cheese.

Calcium availability was markedly affected by pH. Large changes in dialyzed calcium occurred in the pH

Table 1. Calcium availability in calcium-fortified cottage cheese containing guar. Hydrated cottage cheese contained 5% solids. Values represent means ± SD for three observations.

Total Calcium content (mg per 100g)	Available Ca, Method 1, Keane *et al.* (1988)		Available Ca, Method 2, Kim & Zemel (1986)	
	Ionic dialyzed Ca (ID) (%)	Ionic dialyzed Ca (D) (%)	Soluble Ca (IS) (%)	Soluble Ca (S) (%)
Non-digested:				
14·9	$8·76 \pm 0·04^{b}$	$11·2 \pm 0·72^{c}$	$45·8 \pm 0·7^{b}$	$64·6 \pm 1·6^{b}$
25·3	$9·73 \pm 0·32^{a}$	$12·1 \pm 0·52^{bc}$	$51·4 \pm 1·4^{a}$	$69·9 \pm 1·5^{a}$
36·0	$9·54 \pm 0·32^{ab}$	$12·6 \pm 0·65^{ab}$	$50·3 \pm 2·1^{a}$	$69·1 \pm 3·0^{a}$
43·5	$9·85 \pm 0·85^{a}$	$13·4 \pm 0·63^{a}$	$52·6 \pm 2·7^{a}$	$72·7 \pm 2·1^{a}$
Digested:				
14·9	$2·05 \pm 0·38^{e}$	$5·41 \pm 0·65^{f}$	$1·10 \pm 0·28^{f}$	$26·6 \pm 4·5^{f}$
25·3	$2·86 \pm 0·45^{d}$	$6·43 \pm 0·11^{e}$	$2·61 \pm 0·27^{e}$	$36·3 \pm 1·6^{e}$
36·0	$3·12 \pm 0·21^{cd}$	$6·36 \pm 0·74^{e}$	$4·06 \pm 0·87^{d}$	$43·0 \pm 3·8^{d}$
43·5	$3·66 \pm 0·34^{c}$	$7·56 \pm 0·15^{d}$	$5·50 \pm 0·97^{c}$	$50·5 \pm 3·6^{c}$

$^{a-f}$ Means in the same column with the same superscript are not significantly different ($p < 0·05$).

Table 2. Calcium availability in cottage cheese diets fed in a rat bioassay of calcium bioavailability. Hydrated diets contained 5% solids. Data are means ± SD, $n = 3$ for available calcium and $n = 6$ for bioassay

	Available calcium, Method 1		Available calcium, Method 2		Bioassay		
Calcium content (mg per 100 g)	Ionic dialyzed Ca (ID) (%)	Ionic dialyzed Ca (D) (%)	Soluble Ca (IS) (%)	Soluble Ca (S) (%)	Tibia Ca (mg g^{-1})	Tibia Ca (mg per bone)	Tibia weight (g)
Non-digested guar diets:							
11·3	2·42 ± 0·03^{e}	4·10 ± 0·32^{d}	15·8 ± 1·7^{d}	35·7 ± 1·9^{e}	107 ± 4^{c}	29·0 ± 2·9^{c}	0·271 ± 0·030^{c}
14·6	3·83 ± 0·21^{d}	4·97 ± 0·20^{c}	21·0 ± 1·1^{c}	44·3 ± 1·8^{d}	119 ± 4^{b}	37·6 ± 3·2^{b}	0·316 ± 0·026^{b}
18·0	4·45 ± 0·43^{c}	5·26 ± 0·28^{c}	25·4 ± 1·4^{b}	47·6 ± 0·7^{c}	126 ± 7^{a}	44·0 ± 4·5^{a}	0·350 ± 0·035^{ab}
20·7	5·27 ± 0·40^{a}	6·41 ± 0·48^{b}	31·5 ± 1·0^{a}	52·9 ± 1·5^{ab}	123 ± 3^{ab}	46·9 ± 4·0^{a}	0·380 ± 0·029^{a}
Non-digested control diets:							
11·1	2·54 ± 0·05^{e}	4·03 ± 0·22^{d}	16·5 ± 2·2^{d}	37·5 ± 1·4^{e}	101 ± 6^{c}	28·9 ± 1·9^{c}	0·286 ± 0·014^{bc}
15·0	3·69 ± 0·12^{d}	5·29 ± 0·66^{c}	21·9 ± 0·9^{c}	46·0 ± 1·5^{cd}	119 ± 5^{b}	37·1 ± 3·3^{b}	0·312 ± 0·033^{b}
18·3	4·87 ± 0·14^{b}	6·10 ± 0·67^{b}	27·3 ± 1·8^{b}	51·5 ± 1·2^{b}	123 ± 7^{ab}	45·1 ± 4·6^{a}	0·367 ± 0·038^{a}
21·3	5·33 ± 0·07^{a}	7·39 ± 0·49^{a}	33·3 ± 1·2^{a}	55·0 ± 1·9^{a}	126 ± 5^{a}	46·3 ± 4·2^{a}	0·368 ± 0·041^{a}
Digested guar diets:							
11·3	5·78 ± 0·88^{e}	10·4 ± 1·29^{ab}	10·8 ± 0·5de	63·7 ± 3·9^{abc}	107 ± 4^{c}	29·0 ± 2·9^{c}	0·271 ± 0·030^{c}
14·6	6·35 ± 0·91de	10·3 ± 0·83^{ab}	12·5 ± 2·0^{cd}	68·2 ± 2·3^{abc}	119 ± 4^{b}	37·6 ± 3·2^{b}	0·316 ± 0·026^{b}
18·0	7·02 ± 0·68^{cd}	10·7 ± 0·82^{ab}	13·0 ± 0·9^{cd}	65·9 ± 4·3^{abc}	126 ± 7^{a}	44·0 ± 4·5^{a}	0·350 ± 0·035^{a}
20·7	7·68 ± 0·98^{bc}	10·2 ± 0·50^{b}	16·5 ± 1·5^{ab}	70·0 ± 4·2^{ab}	123 ± 3^{ab}	46·9 ± 4·0^{a}	0·380 ± 0·029^{a}
Digested control diets:							
11·1	6·30 ± 0·54de	11·0 ± 0·80^{ab}	8·9 ± 1·5^{e}	60·6 ± 6·7^{c}	101 ± 6^{c}	28·9 ± 1·9^{c}	0·286 ± 0·014^{bc}
15·0	6·92 ± 0·63^{cde}	11·6 ± 1·26^{a}	11·6 ± 2·3de	61·9 ± 6·3^{bc}	119 ± 5^{b}	37·1 ± 3·3^{b}	0·312 ± 0·033^{b}
18·3	8·38 ± 0·46^{ab}	11·3 ± 0·16^{ab}	14·6 ± 0·3^{bc}	65·5 ± 6·5^{abc}	123 ± 7^{ab}	45·1 ± 4·6^{a}	0·367 ± 0·038^{a}
21·3	9·09 ± 0·24^{a}	11·2 ± 0·17^{ab}	17·4 ± 2·3^{a}	72·4 ± 6·4^{a}	126 ± 5^{a}	46·3 ± 4·2^{a}	0·368 ± 0·041^{a}

^{a} ^{e}Means in the same column with the same superscript are not significantly different ($p < 0·05$).

range 5·5–6·5. At the final pH of digested cottage cheese, about pH 7, the rate of change of dialysis was less. Calcium solubility increased linearly with decreasing pH in digests. Final pH of digests did not differ between treatments. Final pH of dialysates was 7·19 ± 0·20 ($n = 24$) for digested diets and 7·25 ± 0·05 ($n = 12$) for digested cottage cheese. The final pH values of supernatant fluids were 7·20 ± 0·12 for digested diets ($n = 24$) and 6·99 ± 0·09 ($n = 12$) for digested cheese.

The pH of non-digested samples was not adjusted since the aim was to evaluate the calcium in unmodified food. A slight decrease in pH was expected as calcium lactate was added. The pH differed by no more than 0·18 units between added calcium levels in cheese or diets. The pH difference between levels was significant for both diets ($p < 0·01$) and cheese ($p < 0·05$). The pH of hydrated cottage cheese and diets was 5·01 ± 0·05 ($n = 12$) and 5·64 ± 0·09 ($n = 24$), respectively.

Correlation with bioassay

Calcium availability data were well correlated with rat bioassay data. This provides a chemical indicator of calcium bioavailability. This study has the advantage of measurements on the same diets as were used in a rat bioassay. Data compared to bioassay measurements include D-, ID-, S- and IS-Ca (both as an absolute concentration and as a percentage of the total) and calcium content of samples. Analysis of covariance showed that guar had no influence on correlations except in one case, so data were pooled. Highly significant correlations between calcium availability in diets (except % D-Ca) and calcium concentration in rat tibia (mg per g bone), calcium in tibia (mg per bone) and tibia weight were found (Table 3).

As expected, total calcium content of rat diets correlated well with bioassay measurements. Calcium

Table 3. Pearson's correlation coefficients (r for means, $n = 8$) between chemical measures in diets and rat bioassay measurements. Correlation coefficients in boldface represent a better correlation than control (Ca content) with bioassay

Chemical measures in diet	Rat bioassay measurements		
	mg Ca per g tibia	mg Ca per tibia	mg tibia
Control (mg Ca per g)	0·899**	0·979**	0·972**
Calcium in non-digested diets:			
% Ionic dialyzed (ID)	**0·913****	**0·990****	**0·984****
% Dialysed (D)	0·820*	0·897**	0·894**
% Ionic soluble (IS)	0·853**	0·954**	0·959**
% Soluble (S)	0·899**	0·971**	0·964**
Calcium in digested diets:			
% Ionic dialyzed (ID)	0·715*	0·836**	0·861**^{a}
% Dialysed (D)	0·096 NS^{b}	0·065 NS^{b}	0·058 NS^{b}
% Ionic soluble (IS)	0·830*	0·912**	0·907**
% Soluble (S)	0·714*	0·756*	0·734*

*$p < 0·05$.
**$p < 0·01$.
^{a} Correlation underestimated due to a significant ($p < 0·05$) guar effect.
^{b} NS = Not significant.

availability data correlating **better** than total calcium content (control) are in **boldface** in Table 3. These measures, with coefficients larger than the control, are desirable predictors of calcium bioavailability. In general, ID- and IS-Ca are better correlated to bioassay measurements than D- or S-Ca. ID-Ca in non-digested diets is consistently better correlated than the control, although no significant differences were detected between correlation coefficients. ID-Ca in non-digested diets was the best predictor of calcium bioavailability. These trends were found when the calcium availability of cottage cheese, rather than rat diets containing cottage cheese, were correlated. Correlations were improved when logarithmic or negative reciprocal data were used for the calcium availability and control. Correlations did not improve by digestion of samples. Dialysis simulated gastrointestinal digestion more closely than solubility measures. However, D-Ca in digests did not correlate better than S-Ca with in-vivo data. These results show that simulation of gastrointestinal conditions may not yield correct results.

DISCUSSION

Guar reduced bitter flavor in cottage cheese fortified with calcium lactate (Puspitasari & Lee, 1991). Guar is a neutral polymer and was not expected to bind calcium ionically. The low pH of cottage cheese (pH 5·0) supports high calcium solubility, making cottage cheese a good candidate for calcium fortification. There is complete loss of colloidal calcium as colloidal calcium phosphate which occurs from casein micelles at pH 5. Some ionic calcium may still be bound (Walstra & van Vliet, 1986). Comparison of rat diets containing cottage cheese with or without guar shows slight binding of calcium. Guar did not affect calcium when the same diets were fed to rats (Kaup *et al.*, 1991). Camire and Clydesdale (1981) found that 10% calcium from a standard solution was bound to guar compared to 94·5% bound to pectin. The minor effect of guar on calcium availability in cottage cheese was not measurable by the bioassay.

Fatty acids form insoluble soaps with calcium. It is assumed that calcium-soap formation lowered free calcium in digested samples. Fat was intact in non-digested samples and soaps were not expected. The final pH of digests (about pH 7) was much higher than the pH of hydrated samples (pH 5·0–5·6). This explained lower calcium solubility and lower calcium dialysis in digested cheese. This did not explain different calcium availabilities in digested and non-digested diets.

Nutrient bioavailability tests sometimes assume that the mixing of animal diets has no effect. This assumption was tested here. The addition of diet components to cottage cheese raised pH from 5·0 to 5·6. This accounts for lower calcium availability in non-digested diets compared to cheese. Hydrated cheese and diets contained 1·2 and 0·6% fat, respectively. The fat composition differed as corn oil was added. Gacs and Barltrop (1977) reported that solubility of calcium soaps depended on fatty acid composition. Thus addition of corn oil may alter calcium availability. We found diet preparation significantly altered the calcium availability of cottage cheese.

Geerts *et al.* (1983) showed minerals in milk did not interfere with calcium measurements by electrode. Our samples contained higher concentrations of minerals than milk. Based on selectivity coefficients, sodium and iron are the most probable interfering ions. The highest concentrations found in digested or non-digested samples, 11·1 mg sodium per 100 ml and 0·2 mg iron per 100 ml, did not affect our data. Thus mineral interferences were negligible.

Calcium equilibrium was not attained after dialysis of samples for 2·5 h since concentration of ionic calcium on both sides of a dialysis membrane differed. This was due to insufficient time for equilibrium and possibly the Donnan effect (large charged molecules, like proteins, on one side of a semipermeable membrane cause unequal distribution of calcium ions across the membrane). D- and ID-Ca were calculated for dialysates only. These measures are calculated by multiplying the reported values in Tables 1 and 2 by the dilution factor 5 (100 ml total per 20 ml dialysate). This shows that not all soluble calcium was dialyzed. However, more ionic calcium was dialyzed than was soluble in digests. It is likely that calcium was released from soaps as calcium diffused into a dialysis bag. This was tested for digested whole milk and skim milk. Percent IS-Ca was lowered 8-fold in digested whole milk compared to skim milk, but percent ID-Ca was about the same in both samples. We found dialysis of calcium is less affected by soaps than calcium solubility.

Chemically available calcium estimated calcium bioavailability from cottage cheese. Correlation analysis showed ID- and IS-Ca are the best predictors of calcium bioavailability. Correlations were not improved by simulated digestion. Two in-vitro tests for calcium bioavailability are recommended: estimation of calcium availability as ionic calcium in (1) non-digested samples or (2) pepsin digests after adjustment of pH to simulate duodenal conditions. With this approach, formation of calcium-soaps is avoided and rapid, accurate results are possible.

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