Development of a Cheap and Rapid Method to Determine Calcium in Milk Fractions in an Industrial Environment

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Abstract

Milk contains high concentrations of calcium. It occurs in two forms, a free ionic form, and calcium associated with milk proteins (caseins). The latter association is called colloidal calcium phosphate. New Zealand Dairy Foods of Takanini is marketing a range of commercial milks in supermarkets. The company uses ultrafiltration to concentrate milk proteins and calcium in different milk products. During ultrafiltration, the fraction that is retained by the membrane is rich in calcium bound to proteins and the portion that passes through the membrane is richer in the free ionic form. The company wanted to develop a quick and an economical method that can be applied in industrial settings to determine calcium in both these fractions and in other milk products.

This research aimed to develop a quick, wet chemistry method to measure calcium in milk fractions and to trial it in an industrial environment.

Two methods, the so-called EDTA method and the atomic absorption spectrophotometric method (AA) were trialled as potential reference methods against which to compare results obtained by the method to be developed. The AA method was chosen due to its ease, accuracy and precision. (This could not be selected as the industrial method for a number of reasons.)

A colorimetric method was favoured over other contenders. Two colorimetric dyes, Arsenazo I and o-cresolphthalein-complexone (CPC) were chosen to work with. Arsenazo I forms a purple complex with calcium in a suitable buffer at a range of pHs. o-Cresolphthalein-complexone also forms purple-coloured complexes at alkaline pHs.

During method development with Arsenazo I, different buffers were trialled and a NaOH/KCl buffer was selected for further development at pH 12. The method worked well during the development phase but with some inconsistent results at times. o-Cresolphthalein-complexone formed clear purple complexes with Clark and Lubs and 2-amino-2-methylpropanol (AMP) buffers. The key advantage of the CPC dye with AMP buffer was that when 8-hydroxyquinoline was included in the reaction mixture, it successfully masked coloured complex formation due to CPC with magnesium, which is present in milk at about 1/3 the calcium concentration. This effect did not work with Arsenazo I. However, the results obtained with the CPC method were lower than claimed values of most milks trialled during development. Both methods were compared for their precision and it was found that CPC method has better precision and was chosen for further development.

To improve the accuracy and precision, various denaturing reagents were used to (hypothetically) release calcium from the caseins. Trichloroacetic acid at 25 % was more effective than the several other denaturing treatments tested. The finalised CPC method, using trichloroacetic acid, AMP and 8-hydroxyquinoline, was then used to monitor calcium concentration over four months in three milk products, skim, *Xtra* (retentate) and permeate. For all milks, the CPC values were lower than the AA reference values, and the values reported by a commercial analytical laboratory. The reasons for this are discussed, as are other changes in calcium concentration in the three milks throughout the trial.

The correlation between the CPC and AA values was poor for *Xtra*, better for skim, and best for permeate. A chemical model to explain this is discussed.

The method developed is cheap and quick, and sample and reagent preparation is simple. The method could be applied in an industrial environment, but a proportionality factor would have to be applied to account for the difference in mean values between the CPC and AA methods.

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Statement of originality

I hereby declare that this submission is my own work and that, to the best of my knowledge and belief, it contains no material previously published or written by another person, nor material which to a substantial extent has been accepted for the qualification of any other degree or diploma of a university or other institution of higher learning, except where due acknowledgement is made.

Signed…………………………….

Date……………………………….

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The parties' rights to intellectual property owned or licensed to each at the date the student commenced research for the Company remain untouched. The student's thesis will be examined under confidentiality. Any publications from the thesis are subject to an embargo of 18 months after the date of thesis submission. This will allow the New Zealand Dairy Foods the time and opportunity to seek patent protection should it so choose. Any other disclosure of information related to this project in which the Company has a proprietary interest must be approved by the Company in writing.

1 Introduction

1.1 Statement of intent

This research aimed to develop a quick and economical method in industrial environment to measure unbound and bound calcium in milk for New Zealand Dairy Foods Limited (NZDF) Takanini, Auckland, New Zealand.

Calcium is an important mineral in human bones. In Western countries such as New Zealand, 60 % of dietary calcium is obtained from dairy sources, so it is important to dairy processors to be able to quantify the amount of calcium in dairy products, especially since consumers recognise the need for calcium to maintain bone health. The industrial environment also demands an economical, industrially viable and quick method, so that on-site decisions can be made.

Calcium exists in two main forms in milk. Roughly 66 % is present as colloidal calcium phosphate (CCP), where the calcium ion is ionically bound to phosphate that is in turn covalently linked to caseins at a number of serine residues. The remainder is present as free calcium ion, ionically balanced with phosphate, chloride, carbonate, bicarbonate, sulphate and citrate (Fox and McSweeney, 1998, p. 249-257)

However, the proportion of calcium as CCP and free ions varies from the 2:1 ratio in response to changes in overall concentration of calcium in milk, temperature, ionic strength, pH and the origins of the milk (diet, breed, time of year) (Fox and McSweeney, 1998, p. 243-246). Different milk processing methods such as ultrafiltration (UF) of milk, yoghurt and cheese manufacturing, can affect the amount of calcium in the final product. In particular, UF is used as a means to increase the calcium in specialty milk by concentrating the proteins, so in turn the bound calcium. As is discussed later there are two views on the importance of calcium in CCP versus free ions. Some research supports the view that calcium bound to proteins is more bioavailable than the ionic calcium. Other research contradicts this. Whatever the truth, NZDF wants a rapid method to determine calcium in its dairy products. There are two reasons for this. First, it suspects that cheese properties are affected by the free calcium and CCP concentrations. Second, it may wish to make promotional claims on the ratio of calcium in the two forms should the bioavailability differ.

The specific objectives of this investigation were:

- To research and review the current methods for determining bound and unbound calcium in milk.
- To develop a rapid and cheap method for bound and unbound calcium determination and to compare it against reference methods for total calcium.
- To use the developed method to monitor the concentration of calcium in ultrafiltration milk fractions in the Takanini facility, and to compare the results with those obtained by reference methods.

1.2 Milk synthesis and its biological role

Milk is a fluid secreted by the mammary glands of female mammals to meet the complete nutritional and several physiological requirements of the neonate. It contains a sugar, fat, anions and cations, and proteins and peptides, which include immunoglobulins, enzymes and enzyme inhibitors, binding or carrier proteins and peptides, growth factors and antibacterial agents (Fox and McSweeney, 1998, p. 1).

In a sense, milk can be viewed as modified secreted blood serum. Only single cell layer separates the capillaries of the mother from the alveolus lumen, the primary collection cavity of the mammary gland. Whereas this single cell layer is metabolically very active, some milk components are transmitted unchanged from serum to milk, and because of the close cellular connection between blood and milk, the two fluids have identical osmolarity (Swaisgood, 1996, p. 842-846). Significantly, however, milk contains no erythrocytes. The function of milk is nutrition, not oxygen transport.

Milk is a white or yellow-white, opaque liquid. The colour and opacity arise from scattering and absorption of light by milk fat globules as a discontinuous emulsion phase and protein micelles in colloidal suspension within the continuous aqueous phase. The physical properties of milk are similar to water but are modified due to the presence of various solutes in the continuous phase and by the degree of dispersion of the emulsified and colloidal components (Fox and McSweeney, 1998, p. 437).

Milk is principally comprised of about 3.2 % protein, 4.6 % carbohydrate (as lactose), 3.9 % fat, 0.2 % ash and rest water. It also contains organic acids such as citrate, and a range of vitamins that do not fit conveniently into the four major categories. Typical composition is summarised in Table 1 (Swaisgood, 1996, p. 846).

Table 1: Composition of bovine milk from Western cattle

Component	Average percentage[1]
Water	86.6
Fat	4.1
Protein	3.6
Lactose	5.0
Ash	0.7

[1]Values are mean values of range of percentages of different components of milk in different Western breeds of cows.

Because physiological and nutritional requirements of each species are different, the composition of milk shows marked interspecies differences. For example, the fat content of cold-adapted mammals (grey seal, 53 % and polar bear, 33 %) is much higher than that of others (cow 3.8 %, horse 1.9 %) presumably because of the greater energy-generating capacity of fat compared with lactose (milk sugar) and proteins (Fox and McSweeney, 1998, p. 2).

With the domestication of animals, it became possible to include milk of other species in the diets of adult humans as well as neonates. In much of the world, particularly in the Western world, bovine milk – commonly called cow milk – accounts for nearly all the milk processed for human consumption (Swaisgood, 1996, p. 842). In this thesis all investigation and discussion will refer to bovine milk.

1.3 Milk proteins

Milk contains 30 to 36 $g.L^{-1}$ of protein and it rates highly in nutritive quality (Swaisgood, 1996, p. 846). The milk proteins have very high proline content, and certain milk proteins are rich in lysine that is deficient in many plant proteins. The proline content of proteins makes them susceptible to proteolysis without heat denaturation, possibly an important characteristic for neonatal nutrition. However, lack of sulphur amino acids in most of the milk proteins (unlike egg albumin) limits their biological value (Fox and McSweeney, 1998, p. 166-167).

Milk proteins are usually classed as either caseins or whey proteins. When milk is acidified to pH 4.6 or exposed to the mammalian stomach enzyme chymosin (also known as rennin), about 80 % of total bovine milk protein precipitates out of suspension. This fraction is collectively termed casein, whereas the protein which remains in solution or suspension is called whey or serum protein, sometimes called non-casein nitrogen (Fox and McSweeney, 1998, p. 149-153). There is a direct analogy between casein precipitation by chymosin and

blood clotting. Proteinaceous matter (that includes red cells in the case of blood) forms clots under the influence of enzyme activity, yielding a soluble fraction termed serum. The name serum is sometimes used as the equivalent of whey.

Bovine caseins contain four main gene products, designated as α_{s1}, α_{s2}, β and, κ which represent approximately 37, 10, 35 and 12 % of the casein. All four kinds of caseins are phosphorylated to variable degrees but characteristic to each form. α_{s1} has eight or occasionally nine phosphorylated residues, α_{s2} may have 10,11, 12 or 13, β caseins five or occasionally four, and κ caseins can have one, two or perhaps three phosphorylated residues.

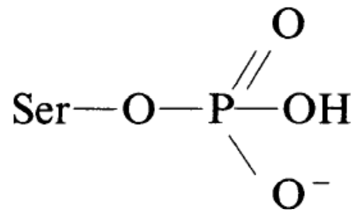


Figure 1: Serine residues esterified to phosphate in caseins (Fox and McSweeney, 1998, p. 173)

Phosphorus is mainly covalently bound to the caseins through ester bonds on serine residues. Those phosphates in turn bind calcium (Figure 1). The amino acid profiles of the caseins are unremarkable but the amino acid sequence of each is critically important to the functional roles of these proteins. The number and position of mainly serine residues in the primary structure is particularly important in respect of phosphorylation of caseins.

Phosphorylation occurs in Golgi membranes of the mammary cell, catalysed by two serine specific casein kinases. Only certain caseins are phosphorylated, requiring Ser-X-Y as recognition sites where Y is a glutamyl and occasionally an aspartyl residue. X may be any amino acid (Fox and McSweeney, 1998, p. 173).

In milk, proteins do not exist as individual molecules. Caseins, together with phosphate, calcium and traces of citrate form structures called casein micelles. These are roughly spherical particles with diameters up to 600 nm. A typical micelle contains 10^4-10^5 casein molecules (Coultate, 2002, p. 142). The biological importance of micelle structure relates to the comparative colligative properties of individual casein molecules on one hand and micelles on the other. If casein molecules were present as monomers, the viscosity of milk would prohibit secretion (Swaisgood, 1996, p. 856), and the osmolarity of milk would obviously exceed that of its progenitor, blood.

The structure of casein micelles has been subject of investigation for years. Various models of the casein micelles have been proposed and refined over the past 40 years. The widely accepted model is based on the concept; core coat, internal structure and subunit or sub micelles (Figure 2).

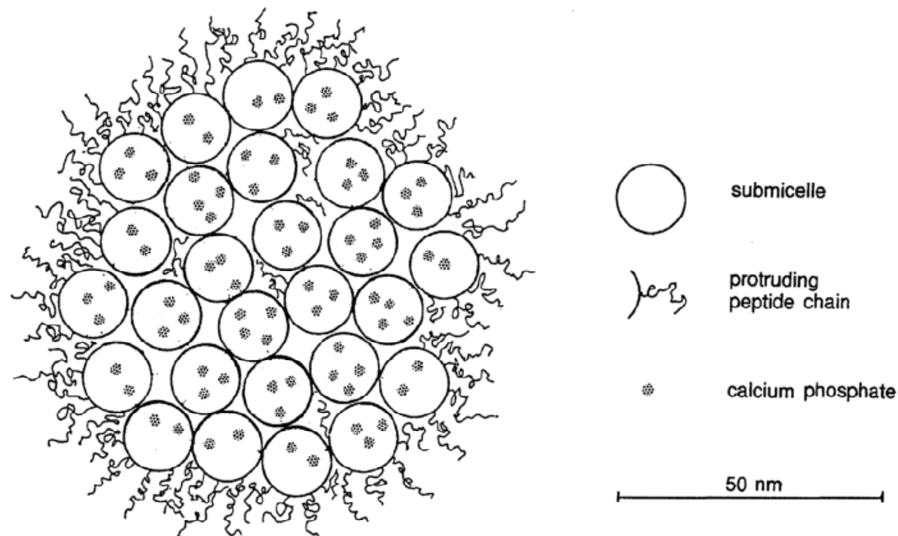


Figure 2: Submicelle model of casein micelle (Walstra and Jennes, 1999)

1.4 Salts in milk

Minerals in milk are represented by the ash fraction in Table 1**.** The ash content in Table 1 (0.7 %) does not truly represent all milk salts (Table 2), because organic salts of organic acids are also present in milk, principally citrate. Being carbohydrates these are destroyed on ashing. The quantities of milk salts are shown in Table 2 (Belitz and Grosch, 1999, p. 486).

Milk salts exist in many forms ranging from free ions and ion complexes to colloidal forms. The principle salts are chlorides, phosphates, citrates and bicarbonates of sodium, calcium and magnesium (Swaisgood, 1996, p. 853). Whereas the sodium and potassium are sufficiently soluble to be present almost entirely as free ions, other minerals, in particular calcium and phosphate are present in higher concentrations than that can be maintained in true solution at the normal pH of milk. Consequently, these exist partly in truly soluble form and partly in a colloidal form associated with caseins, the major milk proteins.

The division between soluble and colloidal form of milk salts is somewhat arbitrary but a fairly sharp separation between two phases is not difficult to achieve by physical processes

such as ultrafiltration, because insoluble salts are mainly associated with the colloidal casein micelles (Fox and McSweeney, 1998 , p. 249).

Table 2: Mineral composition of milk

Major minerals	$mg.L^{-1}$	Minor minerals	$mg.L^{-1}$
Potassium	1,500	Zinc	4,000
Calcium	1,200	Aluminium	500
Sodium	500	Iron	400
Magnesium	120	Copper	120
Phosphate	3,000	Molybdenum	60
Chloride	1,000	Manganese	30
Sulphate	100	Nickel	25
		Silicon	1,500
		Bromine	1,200
		Boron	200
		Fluorine	150
		Iodine	60

Among all the salts in milk, calcium has been the main focus of interest due to health issues (rickets, osteoporosis etc.). It is the second major salt present in milk after potassium. In the soluble phase, calcium exists mainly as a citrate complex bearing a single negative charge (Ca $Citr^{-1}$). Other soluble complex forms include $CaPO_4^{-1}$, and $CaHCO_3^{+1}$ (Fox, 2001).

Table 3: Colloidal and noncolloidal concentration of principal salts in milk (Swaisgood, 1996, p. 853)

Component	Mean[1] value ($mg.L^{-1}$)	Percentage ultrafiltrable or noncolloidal salt content	Percentage filterable or colloidal salt content
Total calcium	121	33	67
Calcium ion	8	100	0
Magnesium	13	64	36
Citrate	181	94	6
Inorganic phosphorus	65	55	45
Sodium	60	96	4
Potassium	144	94	6
Chloride	108	100	0
Lactose	4800	100	0

[1]Values are the means of range of values in different breeds of cows

About 67 % of calcium and 25 % of phosphate are in the colloidal phase (Table 3) and in this phase they are referred to as colloidal calcium phosphate (CCP). However, some potassium, sodium, magnesium and citrate are also present in the colloidal phase. CCP is closely associated with casein micelles and the nature of its association with the casein micelles has been intensely studied. To date there has been no agreement as the exact structure(s).

The most recent theory supports the concept of three different inorganic entities in bovine casein micelles (Kolar et al., 2000). These authors used isotopic exchange method (^{32}P) to demonstrate a ratio of 2.1:1.0:1.0. Because there are not three kinds of caseins, phosphoserine residues or residue clusters where this ratio occurs, the authors proposed these three inorganic phosphate entities are present within every single calcium phosphate-containing ion cluster (Figure 3).

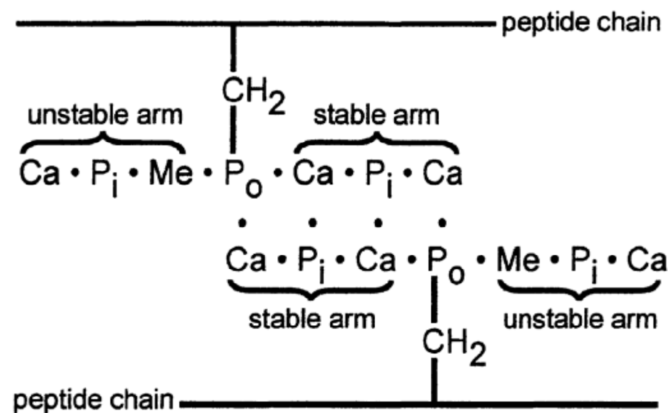


Figure 3: Three inorganic phosphate entities in single phosphate containing cluster (Kolar et al., 2002)

Whatever the exact structure, the close association of CCP with casein protects it from precipitation in the milk matrix. According to McGann et al. (1983), the storage of calcium and inorganic phosphate in mitochondria and casein micelles as a solid is a way of packing large amounts of these ions in a form which can be rapidly mobilized but which provide much higher ion concentrations than found in biological solutions. This is particularly relevant to the matter of bioavailability.

1.5 Bioavailability of bound and unbound calcium

Bioavailability can be broadly defined as the absorption and utilisation of nutrient, both of which may be affected by such host factors as gender, physiological state and coexisting pathologic conditions. It is a degree to which the amount of an ingested nutrient is absorbed and is available to the body. There are numerous factors that effect bioavailability of a nutrient, such as chemical form, the food or supplement matrix in which the nutrient is consumed and other foods in diet (Krebs, 2001).

Calcium absorption mainly occurs in the upper part of the small intestine (duodenum), because calcium needs a pH below 6 to stay in solution in an ionic state (Ca^{2+}). By the time acidic stomach contents reach the duodenum, they have been partially neutralised by the bicarbonates released by the pancreas, but are still acidic. This provides a suitable environment for calcium absorption. There is another factor, the presence of vitamin D hormone calciferol that also helps greatly in absorption of calcium. As food passes through small intestine it becomes progressively more alkaline so absorption of calcium also decreases as it moves to the lower part of small intestine (Wardlaw, 1999, p. 484).

As discussed earlier (Section 1.4) milk calcium exists as free divalent cation and in its colloidal form that is bound directly and indirectly to phosphate, citrate and caseins. The free form here will be referred to as unbound calcium (typically 1/3 of total) while the calcium in CCP – associated with caseins – will be referred to as bound calcium (2/3).

Some research supports the view that calcium bound to milk proteins is more bio available than the free calcium. As explained earlier (Section 1.3) the α_{s1}, α_{s2}, and β caseins have phosphorylated serine residues that bind calcium and phosphate. In the small intestine gastrointestinal proteinases hydrolyse caseins at certain residues (vulnerable lysine and arginine residues) to yield casein phosphopeptide fragments (Sato et al., 1983; Naito et al., 1972) that are soluble by virtue of their component amino acid environment. The argument is that this action maintains the calcium in a soluble, bioavailable form by inhibiting its precipitation as calcium phosphate (Mykkanen and Wasserman, 1980; Naito et al., 1972). In this respect, Delisle et al. (1995) found that the form of dietary milk and milk products (skim evaporated, yoghurt, cheese) affected calcium uptake, but in different ways in young and old rats.

In contrast, other researchers claim that the form of calcium is unimportant for bioavailability. (Ho et al., 2003) found that the uptake of calcium, supplied to rats as $^{45}CaCl_2$, had no effect on

calcium incorporation into bone. Likewise Weaver and Boushey (2003) reported that the availability of calcium from milk is not remarkably different than its availability from other sources like tofu and milks fortified with different calcium salts. They used a crossover design in Caucasian and Asian women to compare the absorption of calcium from milk and tofu. Within each ethnic group the absorption of calcium was the same for both foods. However, Caucasian women absorbed more than Asian women.

In summary, there is no established evidence that the calcium bound to caseins is more bioavailable than the ionic calcium.

1.6 Calcium in the human body and health issues

Calcium is the most abundant mineral in human body. It comprises about 40 % of the total mineral mass in body, translating to about 1.5 % of body mass. Ninety nine percent of it is located in bones and teeth where it has mainly a structural function. The remaining 1 % is present in intracellular and extra cellular fluids where it plays more functional roles like the release of neurotransmitters, contraction of muscles, regulation of heart beat, and the clotting of blood etc. (Wildman and Medeiros, 2000, p. 226). Calcium is lost due to normal metabolism, through urine, skin and faeces, and therefore the body needs to replenish calcium continuously (Nordin, 1997).

Mother's milk is unquestionably the best source of food for the neonate but its utility in older humans is less clear. Arguments that support life-long consumption of (ruminant) milk are generally accepted by nutritionists and population at large, but nonetheless must be seen in light of a huge global industry seeking profits.

Various health claims are made about, for example, calcium and bone health, and calcium intake and osteoporosis. Many are substantiated by research. These claims also extend to other milk nutrients, including bioactive peptides, which affect osteoblasts and osteoclasts directly. A study on bone health in children who were milk avoiders showed that they tend to be short and overweight and they fractured their bones more often than frequent and regular milk drinkers (Goulding, 2003). Thus, calcium-rich foods, particularly milk, have long been promoted in diets of children to enhance peak bone mass (bone density achieved at maturity) to its maximum within the genetic potential. If the diet is calcium deficient during skeletal growth it will decrease peak bone density. This has no immediate harmful consequences but is associated with increased chance of fractures in later life (Nordin and Heaney, 1990).

The USA's National Health and Nutrition Examination Survey (NHANES) between 1999 and 2002 was analysed by Wiley (2005) for correlation between milk consumption and height in American children. The analysis focused on the frequency of milk consumption in childhood and its relation to height in adulthood. The other analysis by the same team on children aged 5 to 18 yrs was related to the reported frequency of milk consumption and height. The results indicated that the adult height was positively correlated with milk consumption at ages 5 to 12 and 13 to 17 after adjusting data for gender, education and ethnicity.

Bone mineral status in children with cow milk allergies – and who avoided milk – was investigated by Jensen et al. (2004). They all had reduced bone mineral density and lower bone mineral content than equivalent children. They were all shorter than their siblings.

Osteoporosis is important health issue and is the single most common cause of fractures in the old and middle-aged costing health services millions of dollars every year. Loss of bone starts in women at the time of menopause and in men at about 55 years and increases the rate of fractures in both genders (Nordin, 1997). Murphy et al. (1995) studied the effect of historical milk consumption on current bone mineral density and found that the frequent milk consumption before age of 25 had a favourable influence on hip bone mass in middle-aged and elderly women. There is little evidence available about the relationship between osteoporosis and calcium intakes in elderly and middle-aged men. One such study by Owusu et al. (1997) showed that there is no consistent relationship between calcium intake and incidence of forearm or hip fractures in men.

1.7 Sources of calcium in the human diet

In the New Zealand population almost half the dietary calcium comes from milk (37 %) and cheese (11 %) (Russell et al., 1999). Other than dairy products, good sources of calcium include tofu, molasses, almonds, calcium fortified foods, and leafy vegetables, particularly brassica family plants like broccoli and turnip greens (Wildman and Medeiros, 2000, p. 226).

An analysis was recently reported by Cook and Friday (2003) on calcium intake by 18,000 persons in the U.S.A. Data were from food intake survey databases developed in the 1990s by the U.S. Department of Agriculture. The survey reported that milk, cheese and yoghurt – as separate food items in the dairy category – contributed 42 % of calcium in diet. An additional 21 % of dietary calcium came from dairy ingredients in mixed foods such as macaroni and cheese, pizza, sandwiches, and desserts. The remaining dietary calcium sources

were single grains (16 %), vegetables (7 %), meat, poultry and fish (5 %), fruit (3 %), and miscellaneous foods (7 %).

1.8 Production, processing and consumption of milk in New Zealand

Due to inherent low costs of production, an increasing world demand, the volume of milk produced in New Zealand has been steadily increasing from at least 1990 to the present day. Nearly all production is linked to the global cooperative Fonterra, headquartered in Hamilton. Most milk in New Zealand is produced for export, but New Zealand is minor producer when compared with E.U. and the U.S.A. (Johnson, 2005). Nonetheless, Fonterra is the world's largest trader of milk products.

Although New Zealand is a large producer on a population basis, it ranks only 5th in the world for consumption (Table 4). Countries like Ireland, Sweden and Austria are still the top consumers in the dairy products (Table 4).

Table 4: Per capita consumption of fluid milk in 1996

Country	Litre
Ireland	190
Sweden	155
Austria	142
Finland	135
New Zealand	128
U.K.	128
Australia	104
U.S.A.	100
France	75
Japan	41

Source: Scrimgeour (1998)

Milk consumption in New Zealand declined in last twenty years (1982 to 1997) (Wham and Worsley, 2002). The decreased consumption can be due to many factors like discontinuation of the subsidised school schemes in 1967, removal of government subsidy available on milk in 1985, and the impact of dairy export returns on milk pricing. The other influences may be the increased consumption of juices and soft drinks, which are cheaper to produce and are promoted and advertised on a large scale worldwide.

Faced with competition from the plethora of alternative drinks on the market, the New Zealand dairy industry has responded by its own promotional activity and a new range of fluid

milk products, such as *Mega*, *Xtra, Super Trim* etc. (Table 5). This has been accomplished with conventional cream separation to control fat content (appealing to the diet conscious), and the relatively new technology, ultrafiltration with membranes. Table 5 below displays different fat contents and calcium claimed in different milk products available in supermarkets in New Zealand marketed by NZDF, Takanini under the Anchor brand name.

Table 5: A range of fresh milk products marketed by NZDF

Milk	Claimed fat content $g.100\ mL^{-1}$	Claimed calcium $mg.100\ mL^{-1}$
Mega milk	2.5	160
Anchor Vital	1.0	165
Xtra	2.0	200
Super Trim	0.1	150
Trim	0.1	140
Lite	1.5	137
The milk	3.3	115

1.9 Ultrafiltration and calcium concentration in milks

Membrane technology is applied to fractionate and concentrate milk proteins. The technology involves ultrafiltration (UF) process which was developed for milk in the 1970s (Scrimgeour, 1998). The process involves forcing skim milk across and through porous membrane that allows only some of the water, lactose and minerals to pass through. During ultrafiltration, the fraction that is retained by the membranes is called retentate and the product that passes through the membrane is called permeate. Permeate is an aqueous solution of lactose, minerals, non-protein nitrogen, vitamins and other low molecular weight compounds (Rattray and Jelen, 1996).

Ultrafiltration results in a higher concentration of CCP (colloidal calcium phosphate) in the retentate because only free calcium ions can pass through the membrane. At New Zealand Dairy Foods Ltd (NZDF), Takanini, ultrafiltration is used to increase the CCP concentration so that milks can be marketed with a range of calcium concentrations. These are stated on the package label and those richer in calcium are promoted as such.

At this time, the question as to the relative bioavailability of calcium as a free ion and CCP is unimportant in the consumer mind. The claims made are simply to calcium concentration and this is a minimum legal requirement (Food Standards Australia New Zealand). No claims are

made as to the relative proportions of CCP and free calcium ion. As discussed earlier (Section 1.5) the relative bioavailability of calcium as a free ion and CCP is an unresolved question. But if it is eventually shown that CCP is a better source, then the high calcium milks could be promoted on that basis.

NZDF also produce a wide variety of cheeses, yoghurts and other milk products like sour creams, salad dips etc. When milk is processed into cheese, pH and temperature changes alter the balance between CCP and free calcium in curd and whey fractions (Singh, 2004). In the case of cheeses, NZDF suspects that cheese properties may be affected by free calcium and CCP concentrations.

In light of these calcium issues NZDF wants to develop a quick and easy method to measure calcium quantity in retentate and permeate the raw materials for the milks (Table 5) and other milk products. The industry reference method – described in the next chapter – is too laborious to be applied in a near-production line situation.

1.10 The problem

NZDF wants to develop a quick wet chemistry method to measure calcium either in its bound or unbound form in milk or calcium retained in the retentate and lost in the permeate during use of membranes in concentrating the milk proteins and also calcium concentrations. This method may be applied to fulfil regulatory demand, and fulfil consumer health demands, whether real or perceived. It may also be applied to industrial monitoring on the basis that calcium concentration may affect the attributes of various milk products.

2 Review of methods and choice of reference methods

2.1 Available methods

2.1.1 Inductively coupled plasma mass spectroscopy (ICP-MS)

This method can be used to measure metal ions in any milk product (Chen and Jiang, 2002). The advantage of the method is that sample preparation is simple. The sample is digested with HNO_3 and then passed through a concentric nebuliser with cyclonic spray chamber to the ICP-MS. However, ICP equipment is expensive and unavailable in milk processing factories, so this method was not examined.

2.1.2 Determination of calcium by precipitation as oxalate

Calcium is precipitated as the insoluble oxalate (Kirk and Sawyer, 1991, p. 32-33). The precipitate is solubilised with sulphuric acid and the oxalate is titrated with potassium permanganate. Although a cheap method, it involved ashing and various steps thus it was unsuitable as a rapid method.

2.1.3 Determinations of calcium by atomic absorption spectrophotometry (AA)

Methods are well documented for this technique but NZDF has no AA equipment.

2.1.4 Determination of calcium by calcium ion specific electrodes

The literature describes an ion selective field effect transistor (ISFET)-based calcium sensor (Bratov et al., 2000). This method is being used in medical and biological laboratories and also has a food industry application to control the calcium ion in cheese production. Research publications claim high accuracy and precision, but in industrial situation with relatively unskilled labour, data can often be unreliable (O.A Young, personnel communication) due to poor calibration, slow response time and electrode contamination.

2.1.5 Protein bound fraction of calcium by inductively coupled plasma optical emission spectrometry (ICP)

The method used 10 % trichloroacetic acid (TCA) and 5 % pepsin solution to precipitate the proteins out of bovine milk samples prior to determination by this advanced technique (Silva et al., 2001). The contents of calcium, zinc and magnesium were found to agree well with

values claimed in literature. However, the equipment involved is very expensive and not affordable industrially.

2.1.6 Calcium determination by sequential injection analysis

The method involves microwave-assisted sample preparation and can be used to introduce the sample for analysis as a suspension or as slurry (Oliveira et al., 2000). The method uses liquid chromatography equipment and involves about 20 steps. Because of the equipment requirement and the complexity of the procedure, the method is not industrially applicable.

2.1.7 Calcium determination by energy dispersive X-ray fluorescence (EDXRF)

Ekinci et al. (2004) have applied this method to determine calcium concentrations in human milk. The method is not suitable for industry due to the high capital cost involved.

2.1.8 Calcium determination by the EDTA method

EDTA is a dairy industry standard method for measuring calcium in liquid and powdered milks and was available from NZTM 3 Chemical Methods Manual. EDTA is widely used as a chelator and forms strong 1:1 complexes with most of the metal ions (Harris, 2003, p. 259). Method was trialled as a reference method.

2.1.9 Determination of calcium by a colorimetric method

Some spectrophotometric methods to determine calcium in different matrixes (Nyman and Ivaska, 1995; Tesfaldet et al., 2004; Stern and Lewis 1957) are available from literature. However in recent literature there is scant evidence for direct colorimetric methods applied to calcium determination in milk, other than one very old one by Stern and Lewis (1957). Though few methods were available in the literature involving direct colorimetric determinations, it was still a cheap and rapid option. The capital outlay can be less than $10,000 for rugged colorimeters or spectrophotometers, and the use of cheap disposable cuvettes was a further attraction.

The Sigma catalogue (2002-2003) offers two diagnostic kits both yielding purple complexes that can be measured in a colorimeter (cheap option) or spectrophotometer. The instrument operates by passing a beam of light of a selected wavelength through a sample and measuring the amount of light absorbed. At first sight these rapid methods appear to be entirely suitable, but their applicability to potentially opaque samples remains unknown. Interestingly one test

requires an alkaline environment and the other an acid environment suggesting scope for clarifying opaque samples. Both mediums were tested for milk samples.

2.2 Choice of reference method

A suitable reference method is necessary to compare the results of the rapid method(s) to be developed. Two methods were tested for suitability, the EDTA method and atomic absorption spectrophotometry (AA), both noted above. In the EDTA method, which is the current industry standard method available from NZTM 3: Chemical Methods Manual (2000), calcium is determined by complexometric titration with the chelating chemical ethylenediaminetetraaceticacid (EDTA). This is applied to liquid milks, milk powders and protein products. However, it is a laborious method and is therefore unsuited to routine industrial applications.

The AA method used here is derived from Methods for Chemical Analysis of Water and Wastes from manual of U.S. Environmental Protection Agency (1983) and from general chemical methods in Pearson's Composition and Analysis of Foods (Kirk and Sawyer, 1991, p. 33-34).

2.2.1 The EDTA method

2.2.1.1 Principle

The method is based on the titrimetric method discussed in Vogel's Textbook of Quantitative Analysis (Bassett et al., 1978, p. 223-324). The principle of the method is as follows: Hydrochloric acid is used to liberate metal ions from milk. Subsequently an excess of EDTA solution is added which will serve to complex calcium along with other divalent cations like magnesium. Magnesium sulphate solution is then added. The pH is then adjusted to about 10 by addition of concentrated sodium hydroxide. Under these conditions all the magnesium is present as $Mg(OH)_2$, with none as an EDTA complex because calcium forms a more stable complex with EDTA than does magnesium. All the excess EDTA is free to complex with the back titrant, $CaCl_2$ solution. The excess EDTA does not react with the magnesium (present as $Mg(OH)_2$) until all the free calcium and the calcium-indicator complex have been complexed with EDTA. At this point the indicator changes colour from blue to pink. This is the end point. The calculation involves four values: the volume of the standard EDTA solution added; the titre to achieve the end point colour change; the weight of original sample; and a conversion factor relating calcium equivalents to EDTA. Details of the method are given in

Appendix 1. All chemicals used were analytical grade from recognised suppliers like BDH, Merck, and Aldrich Sigma, and are not detailed in this thesis except where the chemical is not in common use.

2.2.1.2 Analysis of milks by the EDTA method

The EDTA method was used to determine calcium in seven different milk products made by NZDF, most with a claimed calcium concentration.

The milks were tested in quadruplicate. The quantity of EDTA added to the different milks required a molar excess as noted in the previous section. Thus between 3 and 7.5 ml of EDTA solution were used on the basis of the claims. The official method requires that the milks be sampled by weight as was done in these tests.

2.2.2 The atomic absorption (AA) method

2.2.2.1 Principle

Because of the time required to perform the EDTA method, another reference method was developed. AA was found to be rapid, easy and reliable. In AA a small quantity of sample is introduced to very hot flame via a nebuliser. The solvent evaporates in the flame and metal atoms are produced. The vast majority of these atoms are in the 'ground state' that is, the electrons are in orbitals that give the lowest possible energy to the atom (Fifield and Kealey, 1995, p.320 -333).

It is possible to excite an electron to a higher energy orbital by supplying energy to it in the form of light. Because the electronic orbitals are quantised, i.e. have discrete energy levels, atoms will only absorb light that has energy exactly equal to the energy difference between the orbitals. The simplest way to ensure that the light is of the correct energy is to use a lamp made with the metal being analysed. In the case of calcium the wavelength is exactly 422.7 nm (Fifield and Kealey, 1995 p. 320-333).

The more metal atoms there are in the flame, the more light from the lamp is absorbed. A light detector measures the decrease in light caused by the absorbance of the metal atoms. The absorbance is proportional to the concentration of the metal in solution (Mcguire, 2004).

2.2.2.2 Preparation of standards and solutions

As with the EDTA method above, all chemicals were analytical grade from recognised suppliers.

$CaCO_3$ was dried to constant weight at 180°C. A stock solution of 1000 $mg.L^{-1}$ Ca^{2+} was prepared by dissolving 2.497 g of $CaCO_3$ in a minimum amount of 1 M HCl then made to 1 L with deionised water. Trichloroacetic acid solution (TCA) (25 % w/v) was prepared as was lanthanum chloride solution. The latter at 5 % (w/v) was prepared from $LaCl_3.7H_2O$. The concentrations of trichloroacetic acid solution and lanthanum solution were not critical. TCA in excess serves to precipitate the milk proteins.

In AA, chemical interference can originate during dissociation of the analyte in the flame, where it is volatilised at a different rate than the standard. One method of alleviating this interference is through the addition of a releasing agent, a metal or salt, which forms a more stable compound with the interferent than the analyte (Van Loon, 1980, p. 41-42). In calcium analysis the addition of lanthanum, which acts as a releasing agent, reduces interference with the calcium peak from Al, Si, PO_4^{3-}, and SO_4^{2-} (Trudeau and Freier, 1967). When in excess it serves to bind phosphates that would otherwise complex with calcium (Van Loon, 1980, p. 41).

A reagent blank for standard solutions was made by adding 20 mL of 25 % TCA solution and 20 ml of 5 % lanthanum chloride solution to 1L with deionised water. A series of standard calcium solutions containing 2, 4, 6, 8, 10, 12, 14 and 16 ppm Ca^{2+} in volumetric flasks were prepared from the stock solution of calcium using the reagent blank as diluent.

2.2.2.3 Instrumentation

The AA instruments used were a Perkin Elmer 3110 (Wellesley, Massachusetts, U.S.A.) for initial work, and later a GBC Avanta (GBC Scientific, Dandenong, Victoria, Australia). The inlet on the Perkin Elmer 3110 proved to be narrow to the point that samples could block it, so leading to erroneous results. This was not a problem with the GBC Avanta.

2.2.2.4 AA conditions

The lamp for calcium was used (422.7 nm) to pass light through an air-acetylene flame. The lamp current was set to 7 mA (Perkin Elmer) or to 5 mA (GBC Avanta). Readings were taken in continuous absorption mode. The dynamic range was linear between 0 and 16 ppm calcium, linearly translating to absorptions between 0 and 0.8.

2.2.2.5 Procedure for atomic absorption as reference method

Initially the procedure followed involved sampling a small volume of milk, 0.3 mL, to which TCA solution was added followed by lanthanum solution. This was then made up to 50 mL.

This approach was deleted for two reasons. The small volume of the relatively thick milk suspension was difficult to pipette accurately and the fixed volume led to off-scale results for calcium-rich milks as the calcium varied from 1150 to 2000 mg.L^{-1} in different milks to be tested.

The following protocol was adopted and varied slightly to account for different calcium concentrations. By way of example, 5 mL of *The Milk*, which contained a claimed 1150 mg.L^{-1} of calcium, was diluted to 50 mL in volumetric flask. A 3 mL aliquot of this suspension was treated with 1 mL of TCA solution, allowed to stand for 10 min, shaken, mixed with 1 mL of lanthanum solution, and then finally diluted to 50 mL. The resulted treated mixture contained approximately 6 to 7 mg.L^{-1} calcium, which was in the middle of the dynamic range. About 10 mL of this mixture was centrifuged at 1500 gravities (3000 rpm) for a period of 10 min in a Heraeus 400e centrifuge (Hanau, Germany) to let the precipitates settle at the bottom of centrifuge tube and obtain the clear supernatant for tests. The supernatant solution was immediately used for calcium determination.

A calibration curve was prepared from the standard calcium solutions described in Section 2.2.2.2 (Figure 4). Quadruplicate samples of milks to be tested were prepared as described above and analysed straight after. The results were adjusted to account for dilutions.

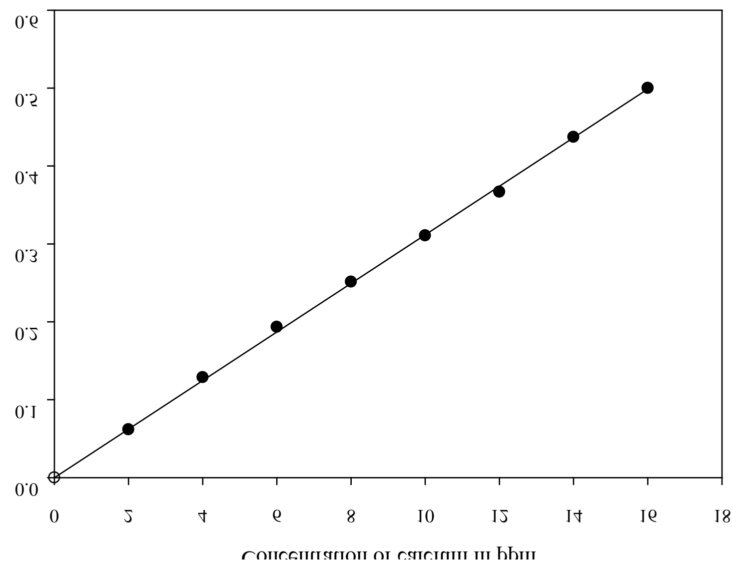


Figure 4: The calibration curve of calcium standard with AA Elmer Perker

2.2.3 Results of the EDTA and AA analyses of seven milk products

Ideally a comparison of the EDTA and AA methods should be done with the same bottle of milk for a number of milk products. This was not possible due to time constraints and the perishability of milk. Thus the data for EDTA and AA were obtained on separate batches on different days. Two features of the results were of primary interest. Are the results plausible (a rough measure of accuracy) and is precision acceptable?

In Table 6 the units for both results are not same because in EDTA method the milks analysed were weighed in during the procedure whereas in AA method the milks were measured by volume.

Table 6: Calcium concentration of milks marketed by NZDF by two reference methods

Milk name	Claimed calcium concentration ($mg.L^{-1}$)	Calcium concn. By EDTA method ($mg.kg^{-1}$)		Calcium concn. By AA method ($mg.L^{-1}$)	
		Mean[1]	SD[2]	Mean	SD
The Milk	1150	1060	40	1150	10
Lite	1370	1090	10	1190	20
Supertrim	1500	1280	20	1440	9
Xtra	2000	1970	30	2310	60
Raw milk	NC[3]	1230	30	1270	60
Permeate	NC	320	30	390	20
Retentate	NC	2020	72	1970	40

[1] Values are means of quadruplicates. [2] SD, standard deviation. [3] NC, no claims

Although results in Table 6 were calculated in different units, they were still a good measure of precision and accuracy against the claimed calcium by NZDF in the milk products tested. The last three products are not being marketed by NZDF as such; therefore no claims made were available for them. However retentate ultimately is marketed as *Xtra.*

In the EDTA method most of the milk solutions turned clear during the procedure other than *Lite* milk. The clarity of the milk sample solutions was important in obtaining the clear endpoints. Acid digestions and prolonged heatings were recommended to overcome this problem. *Lite* milk was allowed to stand for longer periods than the others but clarity of the sample mixture could not be achieved. As method was time consuming and laborious acid digestions were not tried.

2.3 Conclusion

Although the AA method is more expensive as a reference method it was still selected as the method of choice looking at the ease of use, precision and accuracy of results as compared to EDTA.

3 Colorimetric method development

3.1 Introduction

For this research a colorimetric method was chosen as the best approach for routine calcium determination in milk processing factories. The basis for this decision was cost and simplicity.

In colorimetric methods light usually of a defined wavelength or wavelength range, is passed through the sample solution. The difference between the incident and transmitted light is due to the analyte.

Two different dyes were selected for this work. This chapter will discuss the principle of the colorimetric determinations, and describe in detail the two dyes and method development with them.

3.2 Principle of colorimetric determinations

When light of a suitable wavelength passes through a solution containing an analyte, some of the photons may be absorbed by the analyte. An analyte can be a molecule or element whether in its native form or complexed in some way. The difference between the light intensity before and after passage through the sample is a measure of the absorbance due to the analyte. For light of a single or very narrow wavelength, the light absorbed by the analyte varies with the concentration of the analyte. This technique is called spectrophotometry and its behaviour can be described mathematically according to the Lambert-Beer's Law (Skoog et al., 1994, p.403-406):

$$A=\log_{10} (I_0/I_t) =\varepsilon cl$$

Where

I_0 is the intensity of the incident light
I_t is the intensity of the transmitted light
A is absorbance, a dimensionless quantity
ε is the molar absorptivity constant ($L.mole^{-1}.cm^{-1}$) that is constant for a given analyte
c is the concentration of the analyte in the solution ($mole. L^{-1}$) and
l is the length of the light path through the solution (typically cm).

Since spectrophotometer determinations are carried out in cuvettes for which the path length is constant (usually 1 cm), and the molar absorptivity is also a constant for a given analyte under set conditions, then according to the above equation the absorbance due to analyte is linearly proportional to its concentration. Linearity usually, but not always, occurs when absorbance values are between 0 and 2 (Higson, 2003, p. 116). Determinations of unknown concentrations of an analyte are made after a standard curve is prepared from a concentration series of the analyte. It is important to note that spectrophotometry is valid only for clear solutions, and as will be seen this has important consequences in this research.

3.3 Colorimetric dyes

3.3.1 Arsenazo I

According to the Sigma catalogue (2002-2003) Arsenazo dyes form purple complexes with calcium ions in an acidic environment. Two forms are available, Arsenazo I and Arsenazo III. Arsenazo III forms three kinds of complexes with calcium (Palade and Vergara, 1983; Dorogi and Neumann, 1981; Clemen et al., 1988; Gratzer and Beaven, 1977) whereas Arsenazo I forms a simple 1:1 complex (Dorogi et al., 1982). An extensive literature reports that Arsenazo III forms coloured complex with calcium in a pH range from neutral to highly alkaline (Blasco et al., 1999; Gratzer and Beaven, 1977; Malcik et al., 2005; Dorogi and Neumann, 1981). Contrary to the claim in the Sigma catalogue that coloured complexes form in acidic solutions, most research reports that a pH around 7 is the most suitable for calcium complexation with Arsenazo III (Clemen et al., 1988; Dorogi and Neumann, 1981; Ohnishi, 1979). Similarly Dorogi et al. (1982) reported the complexation of Arsenazo I with calcium at neutral pH.

Arsenazo I was chosen over Arsenazo III because of the reported simplicity of its complexation with calcium (Dorogi et al., 1982). No clear representation of the 1:1 complex was presented in that paper, or in others listed above, but it seems very likely that the single arsenate group is involved on the basis that Arsenazo III has two arsenate groups and forms more than one type of complex (three reported).

Arsenazo I (Figure 5) has the chemical formula $C_{16}H_{11}As\ N_2\ Na_2\ O_{11}S_2$ with a molecular weight of 592 $g.mole^{-1}$. It was bought from Sigma Aldrich, Steinheim under the name Neothorin. The claimed purity was 99.9 %.

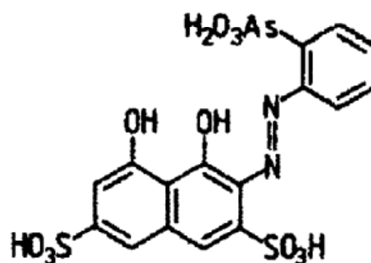


Figure 5: Structure of Arsenazo I (Dorogi et al., 1982)

The literature was searched to get a starting point for suitable method development. Among the many methods reported for calcium and Arsenazo I and III that by Dorogi et al. (1982) was the clearest and so was chosen.

3.3.2 o-Cresolphthalein complexone

o-Cresolphthtlein complexone (CPC) (Figure 6) also forms a purple colour complex with calcium. It is generally recognised that the complex forms only in alkaline solution.

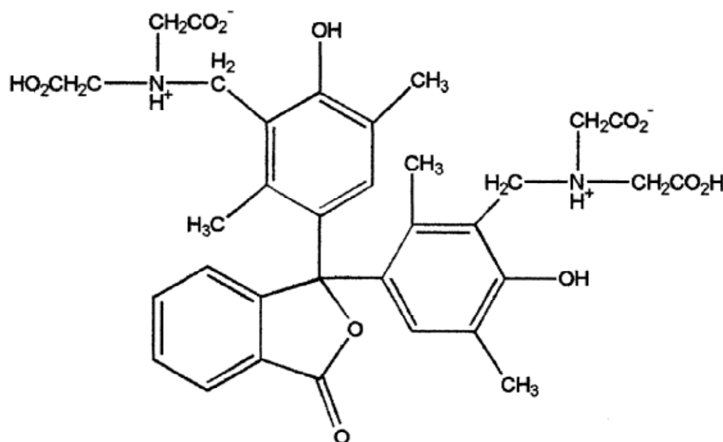
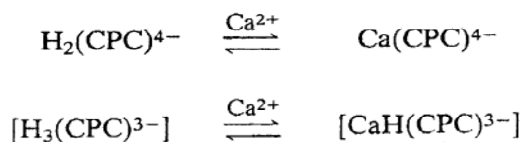


Figure 6: Structure of o-cresolphthalein-complexone (Dorey and Draves, 1998)

It is one of the most sensitive metallochromic ligands available for the determinations of alkaline earth metal ions. Toei (1988) used CPC as a component of an ion-exchange eluant and applied the system to the separation and detection of alkaline earth metals. For this work stable metal complexes were determined by absorption at 572 nm at pH 10.2.

CPC forms the complexes: $Ca(CPC)^{4-}$, $CaH(CPC)^{3-}$, and $Ca_2(CPC)^{2-}$ with Ca^{2+}. A weakly absorbing complex $H_2(CPC)^{4-}$ is also formed. According to Staden and Rensburg (1990) the colour formed by the CPC complex is due to the lactone ring in the phthalein molecule, but in

exactly what way is not clear from their paper or other research publications. The reaction between CPC and calcium between pH 10 and 11 can be written as:



CPC has chemical formula $C_{32}H_{32}N_2O_{12}$ and molecular weight of 637 g.mole^{-1}. It was bought from Sigma Aldrich, Steinheim. The dye was kept at room temperature in the dark.

3.4 Method development with Arsenazo I

3.4.1 Materials and methods

3.4.1.1 Development philosophy

Given that an industrial method was being developed, simplicity and ease of use were important. Several decisions were made at the outset. Because the absorbance is in the visible range for aqueous solutions, disposable plastic cuvettes for a conventional 1 cm-path length spectrophotometer were deemed to be adequate. The spectrophotometer was defined to be a simple single beam instrument with only visible range capability. In every case the final volume was to be 3 mL, of which 2 mL would be buffer and the other 1 mL of reagents. Generally the final diluted and treated with reagents, sample volume to be tested was 0.5 mL, a convenient volume for the sorts of pipette guns common in quality control laboratories. It was decided that the concentration of the dye in the cuvette would never be less than double than the concentration of calcium whether in a test or calibration.

3.4.1.2 Choice and preparation of reagents

A stock solution of 15 mM Arsenazo I was prepared by dissolving the dye in deionised water at the rate of 0.886 g.100 mL^{-1}. It was held in the dark at room temperature. Analytical-grade calcium carbonate from BDH, Poole, dried to a constant weight was used to make 7.5 mM calcium standard (half the concentration of Arsenazo I). To prepare this, 0.1875 g of $CaCO_3$ was dissolved in minimum amount of 1 M HCl then made to 250 mL in a volumetric flask with deionised water.

Two sources of magnesium ion were used, analytical grades of $MgSO_4.7H_2O$ and $4MgCO_3.Mg(OH)_2\,5H_2O$ both from BDH, Poole. The standard solution for the former was prepared by dissolving 0.1845 g in deionised water by making the final volume to 100 mL.

For the latter 0.0724 g was dissolved in deionised water adding several drops of 1 M HCl until fully dissolved and made upto final volume of 100 mL.

The lithium standard was prepared by dissolving 0.032 g of analytical grade LiCl (BDH, Poole) in deionised water and then making the final volume to 100 mL. The potassium standard was similarly prepared from analytical grade KCl (BDH, Poole) by dissolving 0.060 g in deionised water to final volume of 100 mL. All these standards were made as stock solutions of 7.5 mM then dilutions were made as required. The standards were used to test the interference of other monovalent and divalent cations during the complexation of calcium with Arsenazo I dye.

Trichloroacetic acid 25 % was prepared from analytical grade $CCl_3.COOH$ (Ajax Chemicals N.S.W.). The acid was dissolved in deionised water and made to 100 mL.

Pipes buffer, piperazine-N N'-bis-2-ethane sulphonic acid (BDH, Poole) was prepared at 0.03 M by titration with sodium hydroxide to pHs 5.9, 6.9 and 8.0. Tris-maleate buffer (0.05 M) was prepared by dissolving 24.2 g of tris-(hydroxymethyl)-aminomethane (Serva Fine, Heidelberg) and 23.2 g maleic acid (May and Baker, Dagenham) in 1 litre. To 25 mL of this were added varying volumes of 0.2 M NaOH to yield pHs 6.0, 7.0 and 8.0. Other buffers specified in Table 7 were also prepared according to the methods described by Dawson et al. (1986, p. 417-449). Five buffers were chosen to cover the pH range of 3.2 to 12.0 (Table 7).

Table 7: Buffers used to test colour of Arsenazo I at different pHs

Buffer system	Concentration in cuvettes, mM	pH values
Pipes	20 (Pipes)	3.2, 4.2, 5.1, 5.9, 6.9, 7.9
Tris-maleate	33 (Tris), 33 (maleate)	6.0, 7.0, 8.0
Clark and Lubs	33 (KCl), 33 (borate)	9.0, 10.0
Phosphate	16 (Phosphate)	11.0
NaOH/KCl	33 (KCl)	12.0

From Dawson et al. (1986)

Cuvettes were prepared as follows. Two millilitres of a prepared buffer solution was added to cuvettes, followed by 0.5 mL of Arsenazo I dye and 0.5 mL of the salt standards. The final concentrations of buffers in cuvettes are given in Table 7. The final cation concentrations and Arsenazo I were changed at times according to claims made in literature and to get absorbances between zero and unity.

3.4.1.3 Choice of spectrophotometer

An Ultraspec 2100 Pro UV/visible spectrophotometer from Biochrom (Cambridge) was used for spectrophotometric titrations. This is a single beam spectrophotometer.

3.4.1.4 Sequence of reagent addition and basic data handling

In all cases the spectrophotometer was zeroed with water. Other cuvettes were filled in the sequence: buffer, dye then standard or test solutions, to 3 mL. Absorbances were read directly after mixing. The mean absorbances of the duplicate cuvettes containing no calcium were subtracted from all values. Thus the curves pass through zero by definition. Absorbances were measured at single wavelengths in the visible range and scans were made as required.

3.4.1.5 Calcium determinations in commercial milks

Different commercial milks like *The Milk, Bluetop, Lite, Super Trim* and *Trim* were tested with the Arsenazo I method. All the milks claim different amounts of calcium in them (Table 5). The milks were routinely diluted with water according to the calcium content claimed on the bottles to bring their calcium concentrations within the dynamic range of the calibration curve.

Four replicates of each milk were used and from each replicate three cuvettes were prepared. Thus 12 measurements contributed to the mean. Results were manipulated on a spreadsheet to calculate the estimated calcium concentration in the original milk.

3.4.1.6 Denaturisation of milk proteins

Because about 2/3 of calcium in milk is bound to protein, it was thought that liberation of calcium from this matrix by protein denaturation might increase the measured values.

Two ways were tried to denature proteins in milk with TCA. In first method, to 0.5 mL of *The milk* and *Trim*, 0.5 mL of 25 % TCA was added. The mixture was shaken and allowed to stand for 10 minutes and final volume was made to100 mL. In second treatment, *Trim* milk was diluted in different ways (Table 11) to avoid the clumping of precipitates after treating with TCA. In each trial, for every 0.5 mL of *Trim* milk, 0.5 mL of 25 % TCA was used for protein denaturation and final volumes were made to 100 mL. From the diluted TCA - treated sample mixtures 0.5 mL was taken for absorbance readings.

3.4.2 Results for Arsenazo I

3.4.2.1 Behaviour of Arsenazo I around neutrality

Contrary to claims made in the literature (Clemen et al., 1988; Dorogi and Neumann, 1981; Ohnishi, 1979) the absorbance due to complexation of calcium with Arsenazo I was poor around neutrality.

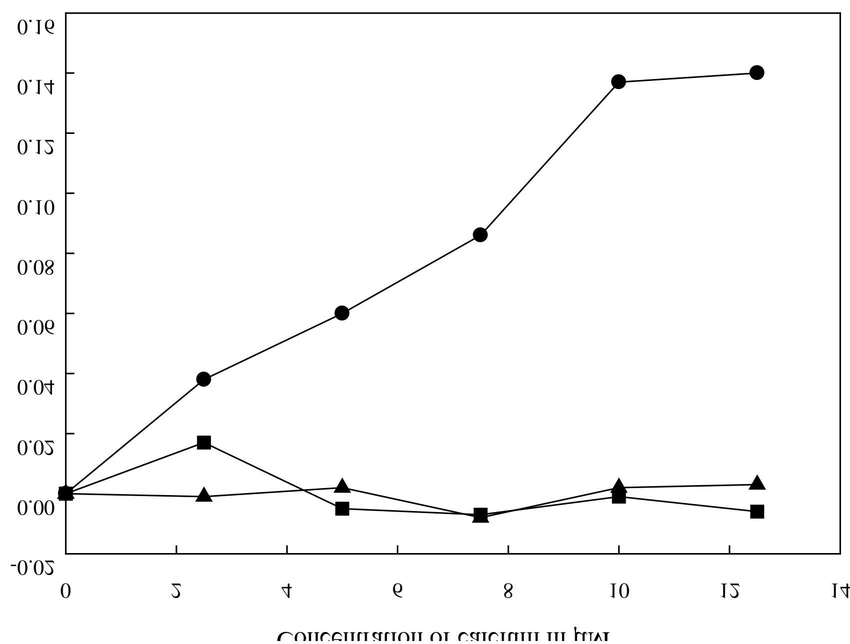


Figure 7: Absorbances of calcium with Arsenazo I dye at 562 nm in 0.03 M Pipes buffer at pH 5.9 ■, 6.9 ▲, and pH 7.9 ●

At up to a final cuvette concentration of 12.5 µM calcium (25 µM Arsenazo I) the absorbance was negligible at pHs 5.9 and 6.9 (Figure 7). However, at pH 7.9 there was clear absorbance due to increasing calcium concentration.

Another buffer (Tris maleate) was tested in the same pH range (Grater and Beaven, 1977), with similar results (Figure 8), except that the absorbance at pH 8 was comparatively lower than the approximately equal value in Pipes buffer (Figure 7).

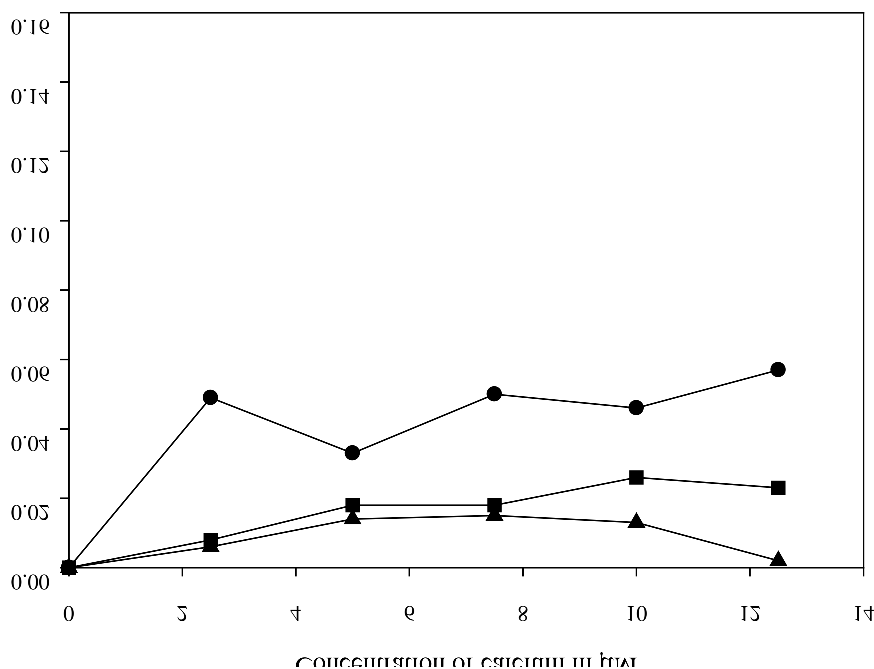


Figure 8: Absorbances of calcium complexed to Arsenazo I dye at 562 nm with Tris Maleate buffer at pH 6.0 (■), pH 7.0 (▲), and pH 8.0 (●)

At this point it was thought possible that the dye might be heavily contaminated with calcium or other alkaline earth metal, so causing it not to respond to added calcium although (the claimed purity was 99.9 %). The concentration of calcium in the Arsenazo I was tested by atomic absorption spectrophotometry. It was found that the mole ratio of calcium to Arsenzo I was a negligible 1 to 1920. This was clearly not the cause of the problem. Attention was turned to pH.

3.4.2.2 Behaviour of Arsenazo I at alkaline pHs

In the previous section, increasing concentrations of calcium in both the buffers used showed increasing absorbance at 562 nm to around 0.05 to 0.14 (Figure 7 and 8) at slightly alkaline pHs with final calcium concentration in the cuvette 12.5 µM.. Preliminary trials showed that this absorbance could be greatly increased at pH 9.0 with Pipes buffer (results not shown). The absorbances were about 10 fold higher.

However, Pipes does not buffer well at pH 9, so it was decided to explore the colour changes of Arsenazo I dye over a wide range of pH values for cations significant in milk and for lithium.

3.4.2.3 Exploration at a range of pH values

Figure 9 is a panoramic view of the three of the five treatments (color change for lithium and potassium ion not shown) in cuvettes taken with hand-held digital camera. A potentially useful change in colour occurred at higher pHs for both calcium and magnesium.

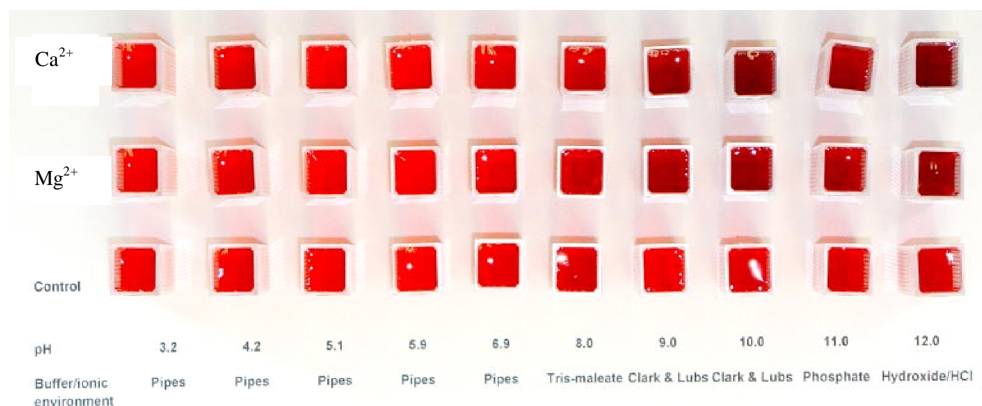


Figure 9: Calcium and magnesium complexed with Arsenazo I dye at a range of pHs from highly acidic to strongly alkaline conditions showing significant colour change at highly alkaline pHs where cation concentration were 37.5 µM each and Arsenazo I 75 µM

In the case of calcium, Figure 9, pHs 9, 10 and 12 gave useful differences. pH 11 appeared to break a trend from pH 9 to 12, and this may be due to a specific buffer effect (Table 7). Lithium and potassium behaved rather like the control except that there was some colour change in the acidic range (not shown) but not at higher pH values, as will be examined later.

Difference spectra (figures shown on next two pages) showed that potentially useful colour changes could be obtained at the higher alkaline pH values for calcium (Figure 10). Similar results were obtained for magnesium (Figure 11).

However for magnesium, absorbances were higher than calcium at the wavelength of interest, 562 nm. Moreover at pHs 5.9, 6.9, and 8.0, the colour changed little but the absorbance was high (Figure 11). As with calcium, the more acidic pHs showed no colour change (Figure 10).

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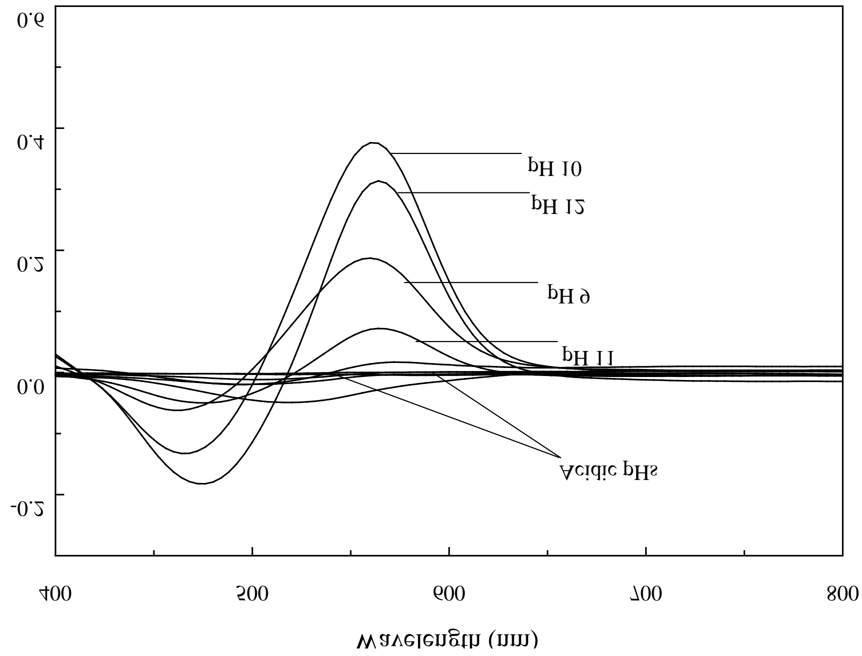

Figure 10: Absorbances of calcium due to complexation with Arsenazo I at various pHs ranging from highly acidic to highly alkaline from 400 to 800 nm. The calcium concentration was 37.5 µM with Arsenazo I at 75 µM

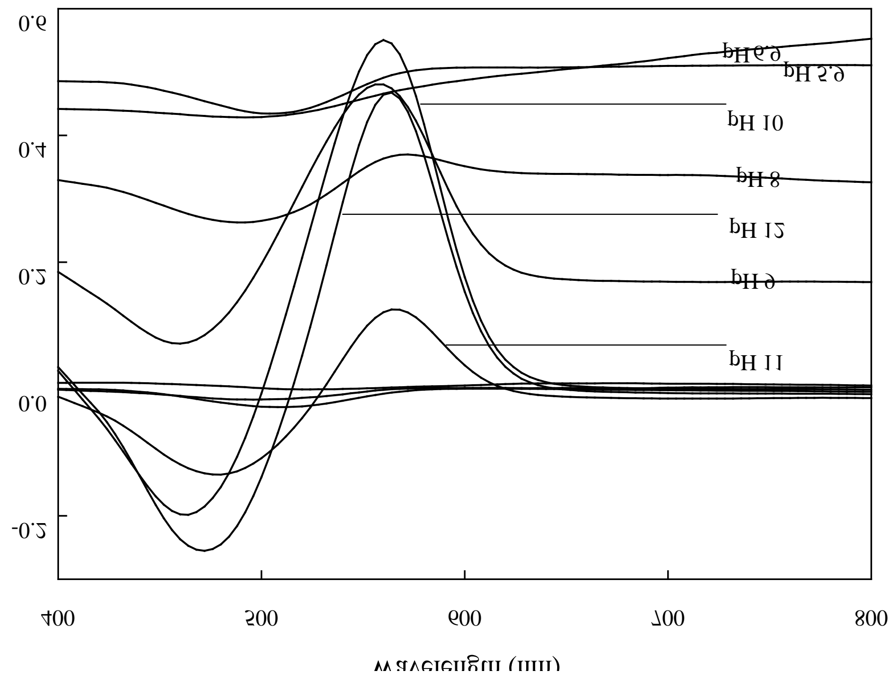

Figure 11: Absorbances of magnesium due to complexation with Arsenazo I at various pHs ranging from highly acidic to highly alkaline from 400 to 800 nm. The magnesium concentration was 37.5 µM with Arsenazo I at 75 µM

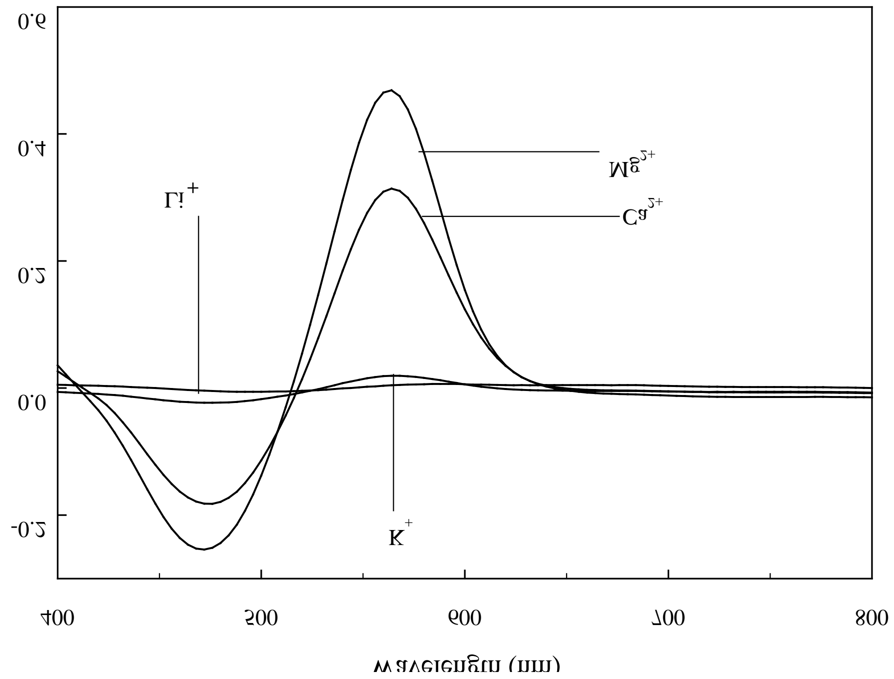

Figure 12: Absorbance spectra of lithium, potassium, magnesium and calcium cations at pH 12 with NaOH/KCl buffer. The concentration of Arsenazo I was 75 µM and the cation concentrations were each 37.5 µM.

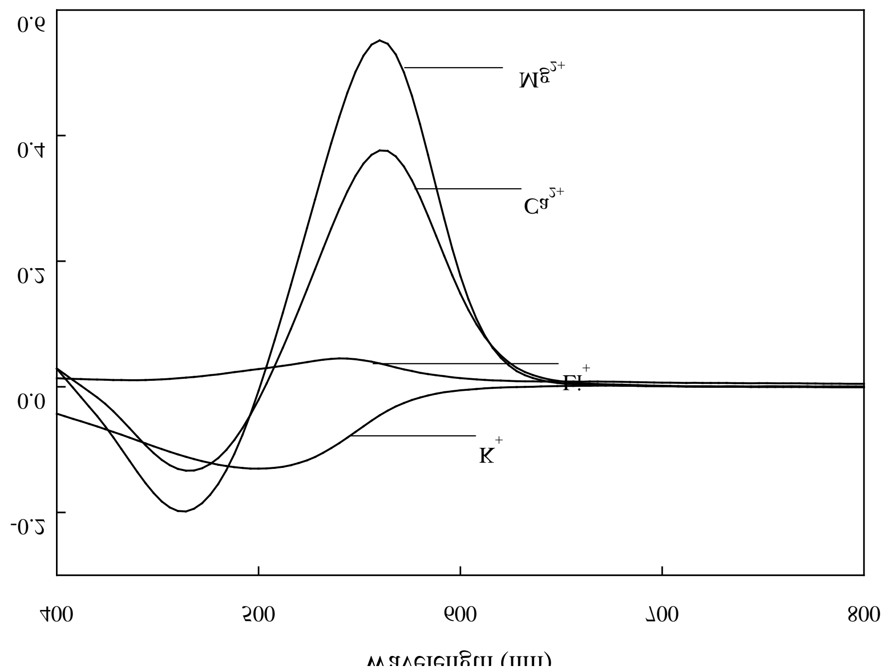

Figure 13: Absorbance spectra of lithium, potassium, magnesium and calcium cations at pH 10 with Clark and Lubs buffer. The concentration of Arsenazo I was 75 µM and the cation concentrations were each 37.5 µM

The most useful absorbance differences were obtained at pH 12 and 10 (Figure 12 and 13) so further work was focused on these pHs. Figure 12 (pH 12) shows that the magnesium complex has the highest absorbance. Lithium was unresponsive, whereas potassium showed a loss of colour over a range of wavelengths at pH 12. At pH 10 (Figure 13) potassium showed negative absorbance and lithium showed some absorbance when compared to pH 12 (Figure 12), where both were non-responsive.

Both pH 10 and 12 looked equally good for calcium absorption but both these pHs showed similar colour response for magnesium. At pH 12, both lithium and potassium were almost non-responsive so pH 12 was selected for further trials. At wavelength 562 nm, which has been used by Dorogi et al. (1982), both showed maximum absorption for calcium and magnesium.

3.4.2.4 Standard curves at pH 12

As noted in Section 3.4.1.2, two magnesium salts were used in method development. As work progressed it was realized that the magnesium salt initially used was not completely homogeneous suggesting variable waters of crystallization.

Therefore in subsequent work, the dry salt ($4Mg\ CO_3\ Mg\ (OH)_2\ 5H_2O$) was used. Absorbances obtained in standard curves do not exactly equal absorbances obtained in various figures presented earlier.

Figure 14 shows the calibration curves obtained for calcium and magnesium at 562 nm at pH 12 with new magnesium salt used. On occasions, the calibration curves showed a slight tendency to saturate, but generally a linear fit was best.

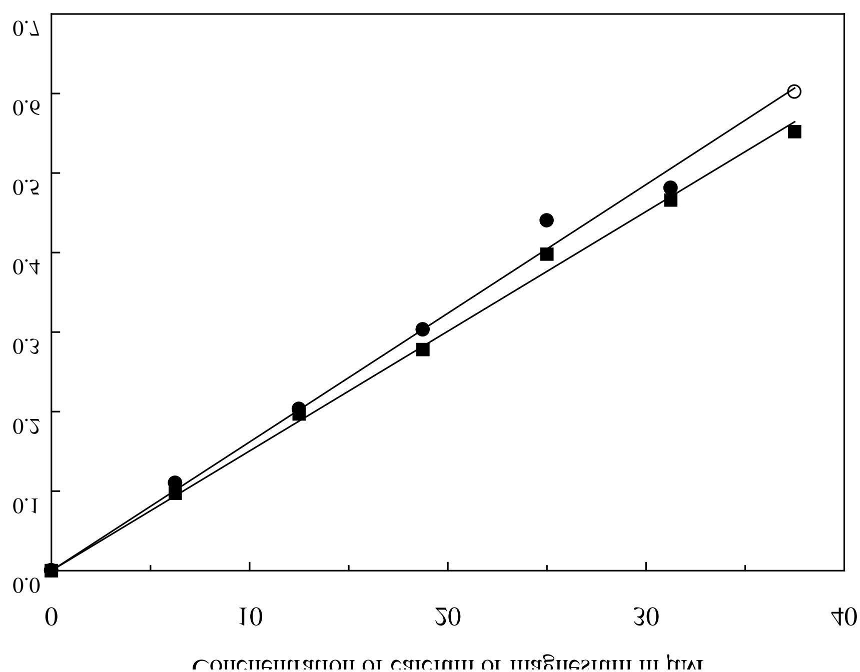

Figure 14: Calibration curve for magnesium ■ and calcium ● (maximum molarities for both in cuvettes 37.5 µM) when complexed with 75 µM Arsenazo I dye in NaOH/KCl buffer at pH 12 at wavelength 562 nm.

3.4.2.5 Effect of magnesium on calcium absorbance

The standard curves above show that the magnesium-Arsenazo I complex has a very similar absorbance to that due to calcium. According to Malcik et al. (2005), interference from competing divalent cations during calcium Arsenazo III complexation at pH 9 is not significant until the competing cation concentration is very high compared with calcium.

The molar concentration of magnesium in milk is about 1/3 that of calcium, and has the potential to compete with calcium during complexation with Arsenazo I. This raised the question of how magnesium would interfere with calcium complexation with Arsenazo I. To test this, a series of cuvettes were assembled where the calcium concentration was maintained at constant 9.37 µM, and the concentration of magnesium was varied from zero to 3.13 µM (about 1/3 the calcium concentration) (Table 8). In both experiments the Arsenazo I concentration was in excess at 37.5 µM. Table 8 shows that the effect of magnesium was not as great as would be expected if each ion acted independently, and in this respect Arsenazo I appears to behave similarly to Arsenazo III.

Table 8: Effect of magnesium ions on the absorbance of calcium Arsenazo I complex at pH 12

Concentration of calcium ion (µM)	Concentration of magnesium ion (µM)	Absorbance obtained	Predicted absorbance if each ion acted independently
9.37	0.00	0.182	0.182
9.37	0.63	0.186	0.192
9.37	1.25	0.193	0.201
9.37	1.88	0.185	0.211
9.37	2.50	0.190	0.220
9.37	3.13	0.209	0.230

[1]The Arsenazo I concentration was in excess, 37.5 µM.

3.4.2.6 Trial determinations of calcium in commercial milks with Arsenazo I

The method was then tested with commercial milk samples diluted in water with no denaturation treatment. The samples were also measured by AA according to the reference method discussed earlier in Section 2.2.2.2 to 2.2.2.5. The results paralleled the claimed calcium concentrations but were higher, especially as measured by the Arsenazo I method (Table 9).

Table 9: Calcium concentration of commercial milks determined with the Arsenazo I and AA methods

Milk name	Claimed calcium concentration ($mg.L^{-1}$)	Calcium concentration by AA method ($mg.L^{-1}$)		Calcium concentration by Arsenazo I method ($mg\ L^{-1}$)	
		Mean[1]	SD[3]	Mean[2]	SD
The Milk	1150	1205	14	1424	73
Lite	1370	1265	22	1297	106
Supertrim	1500	1345	10	1408	104
Trim	1400	1482	11	1595	85

[1] Values are means of quadruplets. [2] Values are means of 12. [3] SD, standard deviation.

3.4.2.7 Denaturation with trichloroacetic acid

As discussed earlier, about 2/3 of calcium in milk is bound to proteins. The AA method adopted here includes a denaturing step with concentrated trichloroacetic acid (TCA), the idea

being to liberate calcium from the protein (Rodriguez et al., 2001). It was thought that TCA might similarly be useful in the Arsenazo I procedure. This idea was explored with *The Milk* and *Trim.*

When 25 % TCA was mixed with an equal volume of undiluted milk, severe clumping was observed. The results obtained after treating these milk samples with TCA were low (Table 10).

Table 10: Effect of TCA on the measured concentration of calcium in milks using the Arsenazo I method

Milk name	Claimed calcium concentration. ($mg.L^{-1}$)	Calcium concentration after TCA treatment($mg.L^{-1}$)		Calcium concentration. with no TCA treatment ($mg.L^{-1}$)	
		Mean[1]	SD	Mean	SD
The milk	1150	797	55	1354	43
Trim	1400	924	36	1426	29

[1]Values are means of 12.

This issue was further explored with the same sample of *Trim* milk but used TCA applied in different ways (Table 11). This time the results for TCA treatment were good and again higher than the claimed calcium with all the methods tried. There appears to be an inherent inconsistency.

Table 11: Calcium concentration found in *Trim* milk after treating with TCA in different ways using Arsenazo I Method

	Calcium claimed by NZDF ($mg.L^{-1}$)	No TCA treatment of *Trim* milk ($mg.L^{-1}$)		0.5 mL of TCA and .5 mL of *Trim* milk then diluted ($mg.L^{-1}$)		2 mL water and 0.5 mL of *Trim* milk then 0.5 mL of TCA and diluted ($mg.L^{-1}$)		0.5 mL of *Trim* milk diluted to 99 mL then 0.5 mL of TCA added ($mg.L^{-1}$)	
		Mean[1]	SD	Mean	SD	Mean	SD	Mean	SD
Trim milk	1400	1486	41	1416	38	1492	37	1506	25

[1]Values are means of 12

3.4.3 Review of Arsenazo I method results

Arsenazo I dye has been found pH sensitive in above trials. It complexed with both cations of interest in milk, but was slightly sensitive to magnesium at the wavelength used. In milk where magnesium is present about 1/3 of calcium this dye may give incorrect measures of calcium due to its competing tendency for magnesium. No other monovalent cations were found to complex with the dye at the pH and buffer used to influence the results which was good because there are relatively high concentration of potassium in milk. The trials on commercial milks gave inconsistent results between tests (some results shown). Some of the results for commercial milks were reasonably good and in close agreement with the reference method (Table 9), but when TCA was used for protein denaturation results varied considerably in the same milk for same techniques used (Table 10 and 11). At this stage attention was switched to test the practicability of another dye.

3.5 Method development with o-cresolphthalein complexone (CPC)

3.5.1 Methods and materials

3.5.1.1 Development philosophy

The literature was searched for a colorimetric method available for the dye, but only one was found first described by Stern and Lewis (1957). In contrast to the experiments with Arsenazo I where the choice of buffer was made empirically, the buffers used here were as defined for CPC and calcium by earlier researchers. These were Clark and Lubs buffer (Stern and Lewis, 1957; Paull et al., 1997) and 2-amino-2-methyl-1-propanol (AMP) buffer (Moorehead and Biggs, 1974; Mesquita and Ranngel, 2004; Stedan and Rensburg, 1990). Moorehead and Biggs (1974) reported that interferences from magnesium could be removed by using 8-hydroxyquinoline in presence of AMP buffer.

3.5.1.2 Reagents

The stock solution of 15 mM CPC was prepared by dissolving 0.9549 g in minimum amount of 5 M HCl and then making the final volume to 100 mL with deionised water. Dilutions were made as required.

8-Hydroxyquinoline (analytical grade) was obtained from Beijing Chemical Works, Beijing. To prepare CPC solution with 8-hydroxyquinoline, the latter was mixed with CPC prior to the dissolution procedure with HCl and water.

A stock solution of AMP buffer from 2-amino-2-methyl-1-propanol (J.T. Baker chemicals, Phillisburg) (1.2 M) was prepared by titration with HCl to pH 10.5. (The researchers mentioned in Section 3.5.1.1 used high concentrations of this buffer routinely and there was no compelling reason to change it from this practice). The final concentration of Clark and Lubs buffer in cuvettes was as in Table 7, whereas the final concentration of AMP was very much higher at 800 mM. Calcium standard solutions were as described for work with Arsenazo I. The magnesium standard was prepared from $4MgCO_3.Mg(OH)_2.5H_2O$ (BDH, Poole).

3.5.1.3 Sequence of reagent addition and basic data handling

As for Arsenazo I work, the reference was water. Cuvettes were prepared as follows. Two millilitres of a prepared buffer solution was added to cuvettes, followed by 0.5 mL of CPC dye or CPC plus 8-hydroxyquinoline of required molarity and 0.5 mL of the salt standards or test solutions. Absorbances were read at 575 nm (Paul et al., 1997) directly after mixing.

3.5.1.4 Experiments with AMP buffer and 8-hydroxiquinoline

CPC and 8-hydroxyquinoline solutions made according to Herraro et al. (1992) were used to see the effect on magnesium masking with AMP buffer.

3.5.1.5 Preliminary trial of the CPC method on commercial milks

Several milks were diluted in water to ensure a calcium concentration in the dynamic range of the assay. The details were as for the Arsenazo 1 method Section 3.4.1.5.

3.5.2 Results for CPC

3.5.2.1 Experiments with Clark and Lubs buffer

When Clark and Lubs buffer and CPC were mixed with calcium or magnesium ion, the clear colourless solutions became clear purple resulting in linear calibration curves. The typical calibration curves were generated with 25 µM maximum calcium standard and 50 µM of CPC dye (Figure 15).

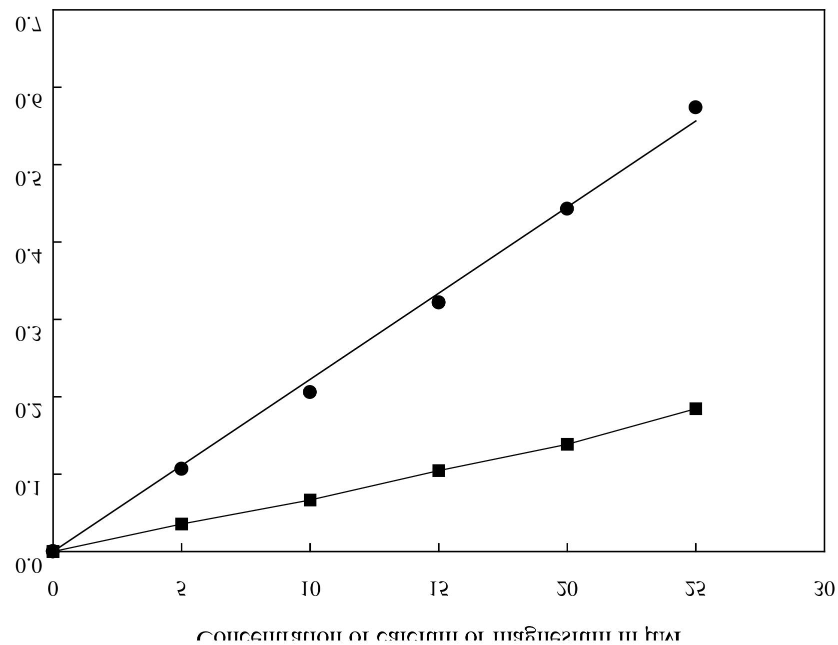

Figure 15: Calibration curves for calcium (●) and magnesium (■), when maximum concentrations for each were 25 µM and CPC 50 µM at pH 10.2 with Clark and Lubs buffer

The literature claims that magnesium can be masked with 8-hydroxyquinoline in the presence of AMP buffer (Section 3.5.1.1). This was tested with Clark and Lubs buffer (concentration of CPC and 8-hydroxyquinoline used according to Herraro et al. 1992). Over the cation concentration range 0 to 6.25 µM, 1.15 mM 8-hydroxyquinoline quenched the usual colour change for both calcium and magnesium with Clark and Lubs buffer (Figure 16).

The absorbance for calcium at 6.25 µM without 8-hydroxyquinoline was about 0.14 (Figure 15), but fell to a low 0.04 with 8-hydroxyquinoline (Figure 16) so rendering the assay useless. Attention was therefore switched to AMP buffer.

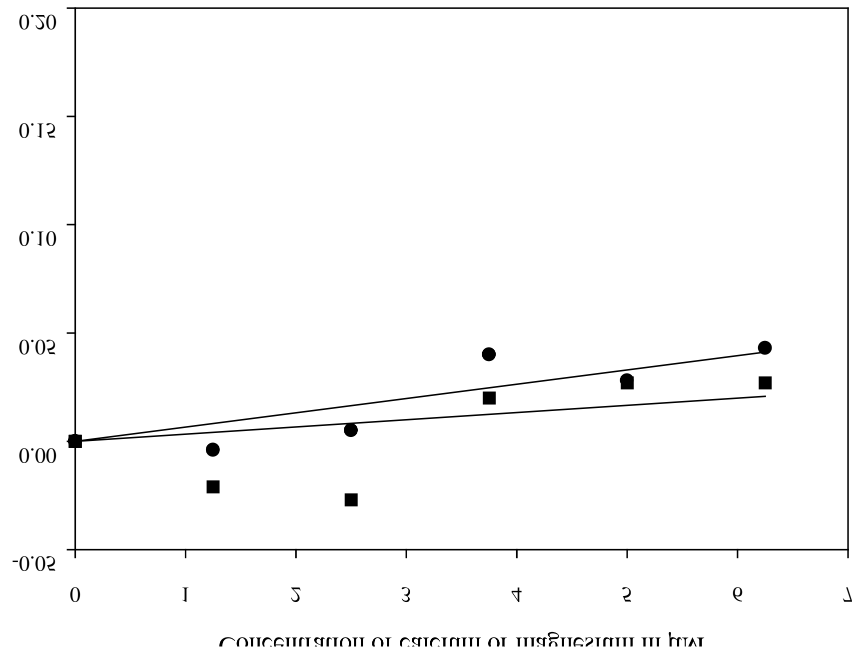

Figure 16: Absorbances for calcium and magnesium (6.25 µM maximum concentration for both) in the presence of 8-hydroxyquinoline with CPC (12.5 µM concentration in the cuvettes) at pH 10.2 with Clark and Lubs buffer

3.5.2.2 Experiments with AMP buffer and 8-hydroxyquinoline

Figure 17 shows the relationships between calcium and magnesium concentration and absorbances at 575 nm in the absence of 8-hydroxyquinoline with AMP buffer.

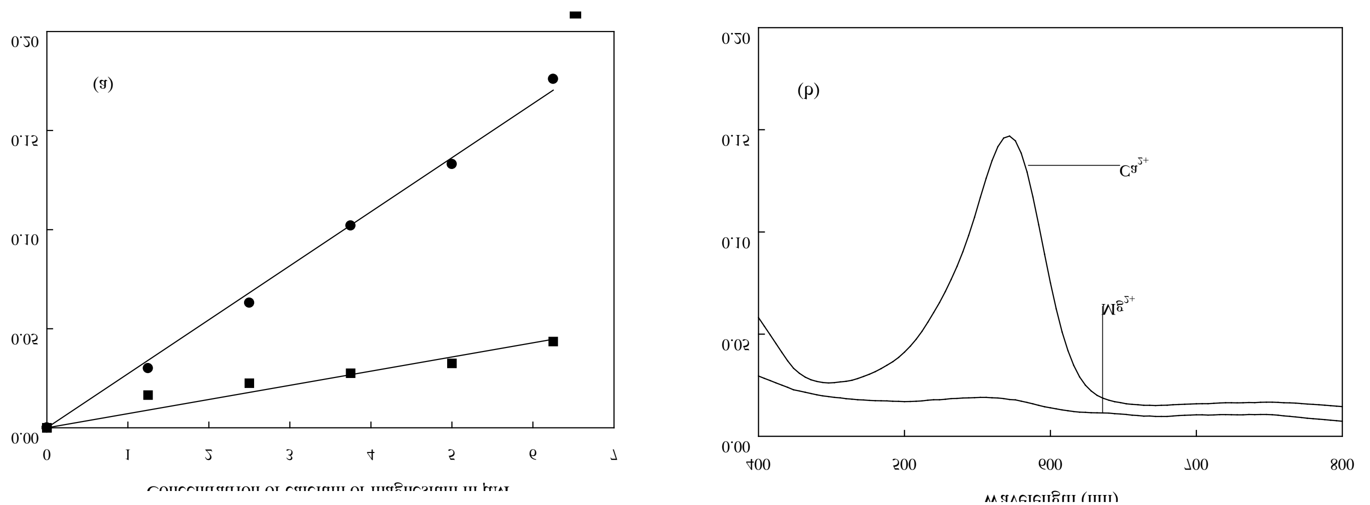

Figure 17: **a**. Absorbances due to calcium (●) and magnesium (■) with CPC in AMP buffer at pH 10.5. The CPC concentration was 12.5 µM. **b**. Spectral scans at maximum cation concentration, 6.25 µM.

Addition of 1.15 mM 8-hydroxyquinoline almost completely masked absorption due to magnesium with AMP buffer (Figure 18). The negative absorbance in Figure 18 b is unexplained.

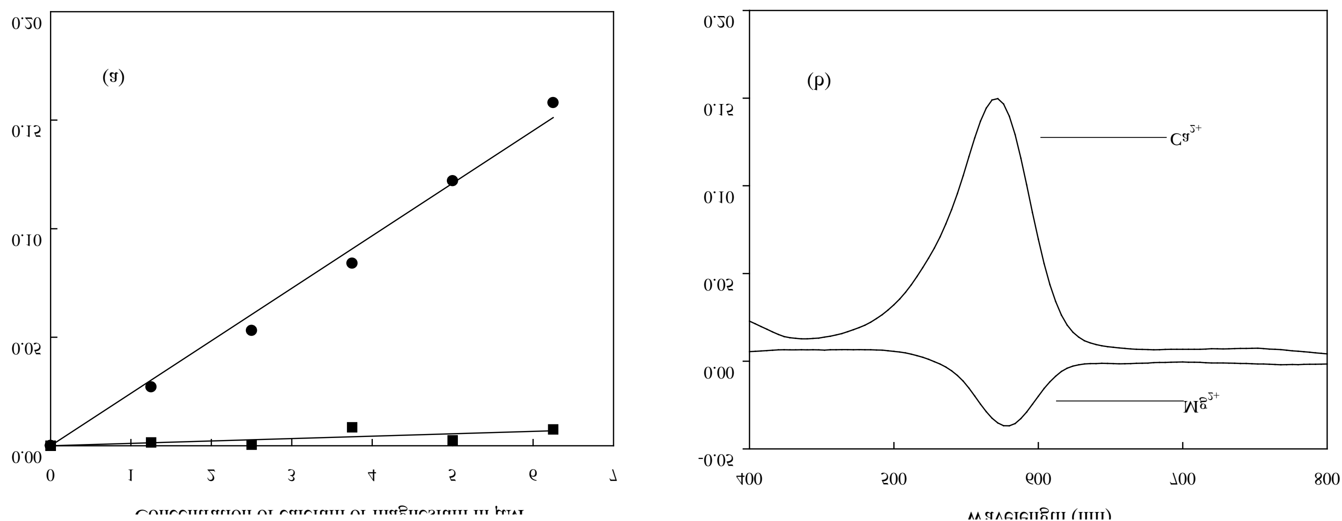

Figure 18: **a**. Absorbances due to calcium (●) and magnesium (■) with CPC in AMP buffer at pH 10.5 in the presence of 1.15 mM 8-hydroxyquinoline. The CPC concentration was 12.5 µM. **b**. Spectral scans at maximum cation concentration, 6.25 µM.

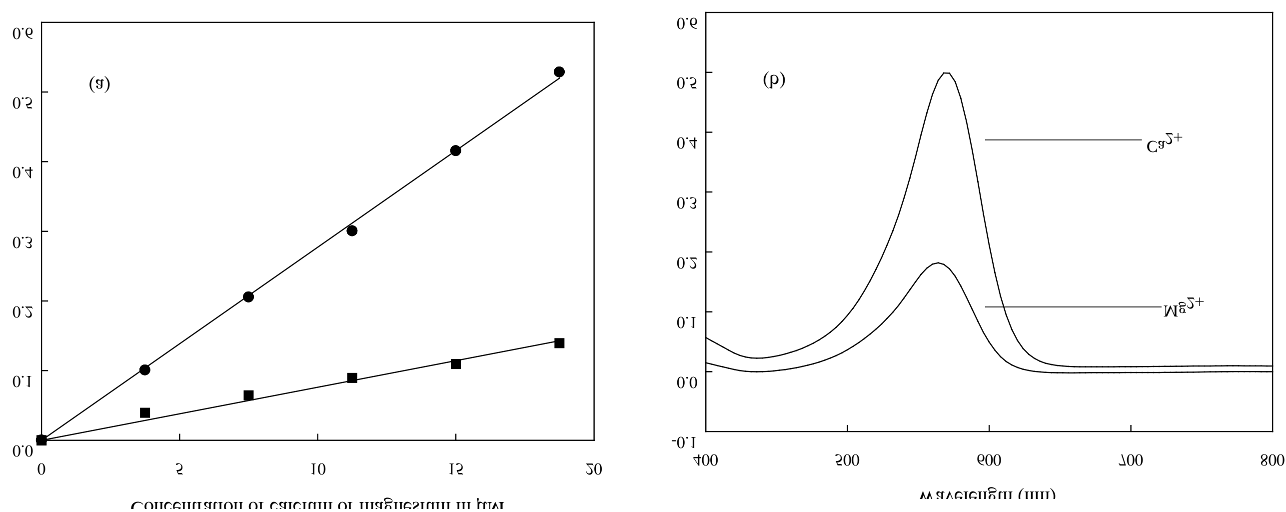

Figure 19: **a**. Absorbance for calcium (●) and magnesium (■) with CPC in AMP buffer at pH 10.5. The CPC concentration was 37.5 µM. **b**. Spectral scans at maximum cation concentration, 18.7 µM.

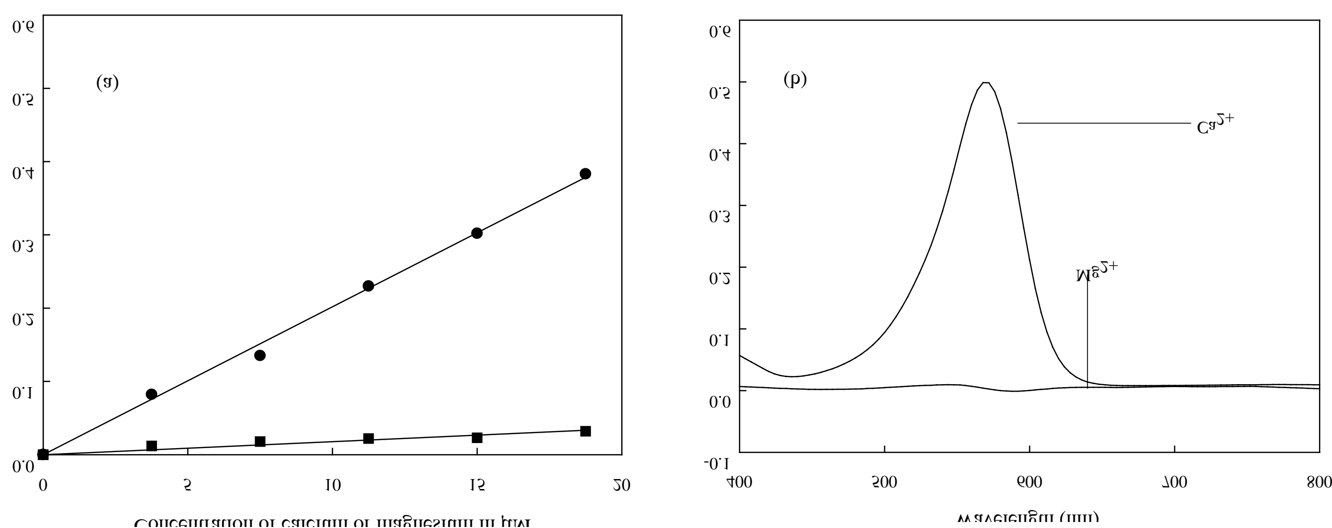

Figure 20: **a**. Absorbances for calcium (●) and magnesium (■) with CPC in AMP buffer at pH 10.5 in the presence of 3.45 mM 8-hydroxyquinoline. The CPC concentration was 37.5 µM. **b**. Spectral scans at maximum cations concentration, 18.7 µM.

Figure 19 and 20 compares the absorption for both cations at higher concentrations of CPC and 8-hydroxyquinoline as the cation concentrations were increased. The figures showed the similar trend as before in previous two figures (Figure 17 and 18). No negative absorption observed in the latter scans.

3.5.2.3 Preliminary trial of the CPC method on commercial milks

Table 12 compares the claimed concentration in milks by NZDF with the results of the CPC method. For all the milks the value determined by the CPC method was lower than the claimed value, in three cases markedly so. Standard deviations were also high considering the large number of replicates.

Table 12: Calcium concentration in different commercial milks determined by the CPC method

Milk	Claimed calcium concentration ($mg.L^{-1}$)	Calcium concentration by CPC method ($mg.L^{-1}$)	
		Mean[1]	SD[2]
Trim milk	1400	1061	41
The Milk	1150	1064	24
Super Trim	1500	925	57
Lite	1370	814	46

[1] Values are means of 12. [2] SD standard deviation

3.5.3 Review of CPC results

With CPC dye both buffers gave good linear calibration curves, but behaved very differently with 8-hydroxyquinoline. The advantage of the CPC method over the Arsenazo I dye was the successful masking of magnesium with AMP buffer, and consistency in calibration curves. However the CPC method gave lower results than those claimed for the milks trialled.

3.6 Conclusion

The origin of the claimed values in the milk brands tested in both methods is unknown, but is likely to stem from determinations made at the time the product and its process was defined. At this point of the research the fact that the CPC value was markedly lower than the claimed was disturbing but interesting. The Arsenazo I method suffered from inconsistent calibration. Neither assay was suitable for long term monitoring of calcium in milk. It was decided to compare both methods for precision, even if accuracy was still poor.

4 Method selection and further improvement

4.1 Introduction

The previous chapter described the development of two colorimetric assays for calcium in milk. Both suffered interference from magnesium, but with one assay, o-cresolphthalein complexone (CPC), this could be minimised by the addition of 8-hydroxyquinoline to the assay mixture. At the outset it appeared that CPC assay would be more useful than the Arsenzo I assay, but that had to be tested.

Thus, the first part of this chapter compares the accuracy and precision of the three methods – atomic absorption (AA), Arsenazo I, and CPC – on several milks replicated over five days. This experiment served to identify the colorimetric assay that would be used to monitor the calcium content of ultrafiltered milks over a period of months.

Once identified (and it was the CPC method) it was developed further. Those results are reported here, in the second part of this chapter.

4.2 Determination of calcium in several milks by three methods

4.2.1 Materials and methods

4.2.1.1 Milks

The Milk, *Trim*, *Mega*, *Xtra*, raw milk and permeate were obtained from NZDF, Takanini for five consecutive days, each from a different production run. *Budget* whole milk powder was bought from Foodtown supermarket, Mt Wellington. A sample was sent to Gribbles Analytical Laboratories, Auckland for calcium determinations. They used NZTM3: 9.21 the standard dairy industry method to determine calcium in whole milk powder. The milk was intended to use as a reference milk at times as the liquid milks were perishable and were unable to be preserved for longer periods.

4.2.1.2 Assays

The AA assay protocol was as described in the Section 2.2.2.2 to 2.2.2.5. The Arsenazo I and CPC assay protocols followed the order of addition described in Section 3.4.1.4, with final cuvette concentrations as follows: the final concentration of Arsenazo I and CPC were both

37.5 µM and the maximum final calcium concentration was 18.75 µM. The final 8-hydroxyquinoline concentration in the CPC assay was 3.45 mM. The final buffer concentrations were 0.03 M NaOH/KCl and 0.80 M AMP for Arsenazo I and CPC, respectively.

The milk powder was reconstituted according to the recipe on the pack, and then diluted to get the calcium concentration in the dynamic range of the calibration curve.

Liquid milk dilutions were made according to the claimed or known calcium concentrations, again to match milks to the dynamic range of the calibration curve. For each liquid milk, four replicate dilutions were made for Arsenazo I and CPC assays, and three replicate determinations were made on each, making 12 values for every milk. For AA milk four replicates were used for each milk tested.

4.2.2 Results and discussion

4.2.2.1 Whole milk powder tested by four methods

The analysis of whole milk powder (Table 13) showed reasonable agreement between the several methods. AA showed the lowest mean value, but the lowest percent coefficient of variation (CV %) of three methods. The highest CV % was 7 % for the CPC method, but the mean was close to the Gribbles mean, which is the industry standard.

Table 13: Determination of calcium in reconstituted whole milk powder by three methods

Method	Calcium concentration claimed by different methods (mg.Kg^{-1})	
	Mean	CV %[1]
AA	8367	3
Arsenazo I	9260	4
CPC	8929	7
Gribbles	8800	Not given

[1] Coefficient of variation percent

In principle, these results from reconstituted milk powder showed that any of the methods might be expected to work well with whole fresh milks. This was tested in the next section.

4.2.2.2 Several liquid milks tested by three methods

Analysis of six liquid milks over five days of sampling explored means and within-day and between-day variation for three analytical methods (Table 14). The within-day percent

Table 14: Calcium determined by AA, the Arsenazo I and the CPC method in different commercial milks to compare precision of both methods

	The milk		*Trim*		*Xtra*		*Mega*		Raw milk		Permeate	
	Mean $mg.L^{-1}$	CV %[1]	Mean $mg.L^{-1}$	CV %	Mean $mg.L^{-1}$	CV %	Mean $mg.L^{-1}$	CV %	Mean $mg.L^{-1}$	CV %	Mean $mg.L^{-1}$	CV %
AA[2]	1134	2	1362	2	1923	2	1487	2	1045	2	291	1
AA	1262	0	1297	1	2344	2	1564	2	1200	2	336	0
AA	1027	2	1232	2	1828	3	1290	3	1158	0	298	2
AA	1092	1	1267	3	1878	0	1388	2	1114	3	285	2
AA	1114	2	1286	1	1945	2	1378	1	1134	1	309	3
Day-to-day CV %		8		5		4		7		10		7
Arsenazo I[3]	2160	5	1648	6	2556	6	2672	2	2193	3	611	8
Arsenazo I	267	7	190	8	309	25	270	12	265	14	68	15
Arsenazo I	1794	7	1592	8	2382	6	2017	9	1711	4	484	10
Arsenazo I	1773	4	1544	8	2301	6	2027	4	1859	6	483	15
Arsenazo I	713	6	605	7	931	6	779	9	301	7	221	5
Day-to-day CV %		60		72		60		59		60		64
CPC	590	6	857	4	1379	3	1042	7	868	5	227	5
CPC	889	6	922	7	1764	9	1317	4	1043	4	255	7
CPC	868	5	771	15	1254	4	1047	7	948	5	192	19
CPC	900	5	860	8	1335	5	1043	4	964	9	182	8
CPC	886	4	855	6	1307	4	1006	7	987	9	196	10
Day-to-day CV %		16		7		6		14		15		12

[1]CV % is coefficient of variation percentage; [2]AA Atomic absorption means are averages of four values; [3]Arsenazo I mean values for Arsenazo I and CPC method are average of 12 values. Claimed calcium for *The Milk*, *Trim, Xtra* and *Mega* were 1150, 1400, 2000 and 1600 $mg.L^{-1}$ respectively. No calcium claims were made for raw milk and permeate.

coefficients of variation for the AA method were low and consistent across all milks. By contrast the equivalent values for the Arsenazo I and CPC methods were high and variable.

The mean values for CPC determinations were always lower than the same-day AA determinations for all milks. Summed over five days, the mean percent difference was 27 %, with a range of 15 to 34 %. The between-day coefficients of variation for CPC were up to twice as high as those for AA. Thus by any measure the AA method was better than the CPC method. However, the CPC method performed far better than the Arsenazo I method.

Although the Arsenazo I method returned high and variable within-day coefficients of variation, these were less than the extremely high between-day variability. Percent coefficients of variation ranged between 59 and 72. This variability was totally unacceptable. What is surprising was that although the method worked reasonably well during development, it failed in this comprehensive trial. The CPC method was chosen for subsequent work, principally a long term monitoring experiment in a commercial environment.

Prior to starting the monitoring trial, some further refinements were made to the CPC method in an effort to reduce variability.

4.3 Further improvement of the CPC method

It was thought that the release of calcium from caseins might be the cause of variation, so the following experiments were done to identify suitable denaturing reagents, if any, that might aid the release of calcium from caseins.

4.3.1 Choice of denaturating agents

Glucono-δ-lactone has been used in many milk research papers as a progressive acidification agent for the release of calcium from caseins (Roesch et al., 2004; Dalgleish et al., 2004 and Dalgleish et al., 2005). Herraro et al. (2001) used acetate buffer to precipitate caseins from milk samples. Sodium tungstate and sulphuric acid was used by Sarkar and Chauhan (1967) for protein removal in the milk samples.

Highly alkaline 8 M NaOH used in EDTA method almost dissolved the milk proteins during EDTA procedure in this investigation, so it was tested as well. Trichloroacetic acid was already used in Arsenazo I method where trichloroacetate interacts with positive protein groups $R\text{-}NH_3^+$ producing a white precipitate to denture the proteins (Rodriguez et al., 2001;Silva et al., 2001). Milk was heated to see any effect on calcium measurement. Concentrated nitric acid and perchloric acids were used for digestion of milk proteins.

4.3.2 Materials and methods

4.3.2.1 Preparation of reagents and denaturation

CPC (0.1432 g) and 8-hydroxyquinoline (3 g) were dissolved in a minimum amount of 5 M HCl and then made to 1 litre. A calcium standard was prepared again as described in Section 2.2.2.2. The final concentrations of CPC and calcium in the cuvettes were 37.5 µM and 18.7 respectively with 3.45 mM 8-hydroxyquinoline.

Aqueous sodium tungstate solution (10 %, w/v) was prepared from $Na_2WO_4.2H_2O$ (Scharlau, Chemie, Barcelona). H_2SO_4 (0.3 M) was prepared from concentrated acid. Perchloric and nitric acid were used as supplied, 11.7 M and 15.9 M, respectively. Aqueous glucono-δ-lactone (1.5 % w/v) was prepared from solid glucono-δ-lactone (Sigma Aldrich, Steinheim). 0.2 M sodium acetate trihydrate (Sharlau Chemie, Barcelona) solution was made by dissolving 2.72 g of it in deionised water and final volume made to 100 mL. To 7 mL of 0.2 M NaOAc solution 3 mL of glacial acetic acid (BDH, Poole) was added to make pH 5 sodium acetate-acetic acid buffer.

A single sample of three milks was selected for this work, skim, *Xtra* and permeate. (Pasteurised skim milk is ultrafiltered into a casein- and calcium-enriched fraction, *Xtra*, and a depleted permeate.)

Except for two treatments (heat, wet digestion) the three milk products were first diluted before any denaturing treatment. Three millilitres of *Xtra* and 5 mL of skim and permeate were each diluted to 50 mL in volumetric flasks. All denaturing treatments were done in quadruplicate followed by three determinations from each denaturation, making 12 determinations for each milk product.

The treatments were as follows. For the control, the milks were diluted by a further 1 in 50 by volume. For the heat treatment, undiluted milks were heated on hot plates and magnetically stirred continuously to boiling. The milks were then accurately diluted to match the dynamic range before determination.

For one TCA treatment, 1 mL of diluted milks was mixed with 1 mL of 25 % TCA in volumetric flasks. The mixture was shaken thoroughly, allowed to stand for 10 minutes and was made to 50 mL. An aliquot was centrifuged at 1500 gravities and the determinations were made on the supernatant. In a variation of this treatment, the TCA-treated samples were held overnight before centrifugation.

For wet digestion, 3 mL of *Xtra* and 5 mL of skim and permeate were mixed with 25 mL of the concentrated nitric and 5 mL of the concentrated perchloric acid. The mixtures were heated to boiling on a hot plate until fumes appeared. Final volumes were made to 50 mL with water after adjusting the pH to neutrality with 2 M NaOH. Further dilutions were made before calcium determinations. There was no need to centrifuge because the solutions cleared on digestion.

The alkali treatment employed 1:1 dilutions of initially diluted milks with 8 M NaOH. After 10 minutes at room temperature, the mixture was clear and further dilutions were made. The mixtures were neither neutralised nor centrifuged.

Glucono-δ-lactone (GDL) treatment employed 1:1 dilutions of initially diluted milks with 1.5 % GDL. The mixture was shaken and held for 10 minutes before further dilution and centrifugation prior to determinations. For the sodium tungstate treatment, 1 mL of initially diluted milks were mixed with 2 mL of 10 % sodium tungstate and 2 mL of 0.3 M sulphuric acid, shaken and held for one minute. After dilution to 50 mL, the mixture was centrifuged before determinations on the supernatant.

For the combined acetic acid and sodium acetate treatments, 1 mL of each of the initially diluted milks was mixed with 1 mL of sodium acetate and acetic acid buffer, shaken and held for 10 minutes before the dilution. After centrifugation determinations were performed.

4.3.3 Results and discussion

The means for the CPC method across the several denaturation treatments were usually different from the equivalent means determined by the AA method, the reference in this experiment (Table 15). At this time however, accuracy was not as important as precision, on the basis that the means for two methods may prove to be well correlated.

In Table 15, the AA results had low percent coefficients of variation, about 1 %, and markedly better than those for the control (no denaturation treatment) with CPC, ranging between 4 and 19 %. Those for the wet digestion treatment were also poor (6 to 16 %) and completely unacceptable for an analytical method.

Looking at the results (Table 15) it was seen there were two to three possible options that could be used to collect final data in the monitoring trial. A short treatment with TCA appeared to be the best across the three milks, with a coefficient of variation range between 3 and 5 %. Highly alkaline conditions also worked well.

Table 15: Effects of various treatments on calcium concentration in different milks by the CPC method and compared to AA method

	Mean calcium concentration ($mg.L^{-1}$) and percent coefficient of variation (CV %)					
Treatment[1]	*Xtra*		Skim		Permeate	
Control	1766	19	1278	4	335	11
Heat treatment	1789	5	998	18	363	17
TCA short time	2003	4	1270	3	401	5
TCA overnight	1889	3	1393	23	401	8
Wet digestion	2227	16	1216	11	395	6
NaOH treatment	1900	3	1265	7	373	5
Glucono- δ -lactone	1903	6	1299	3	469	10
Sodium tungstate	1808	7	1222	3	275	7
HOAc with NaOAc	1829	7	1277	4	389	4
AA (reference method)	1968	1	1196	1	369	1

[1]All treatments were done on same batch of milk to compare difference for calcium determined by each.

The decision was made to choose the quick TCA treatment over NaOH denaturation because the coefficients of variation were lower and TCA has previously been used to denature protein in calcium determimation (Rodriguez et al., 2001; Silva et al., 2001).

4.4 Conclusion

The CPC method proved to be more precise than the Arsenazo I method, where very high day-to-day variation was seen. The Arsenazo I method worked well with the reference whole milk powder, but with liquid milks its variability was totally unacceptable for routine testing. Both the methods were quick and easy, but possibly acceptable precision was only achievable by the CPC method. The denaturation of milk proteins improved the results in *Xtra* milk over those obtained in a previous trial (Table 14). The CPC method was chosen over the Arsenazo I method for long term monitoring of calcium in milk.

5 Long term monitoring of calcium in three milks

5.1 Introduction

The previous chapter defined a rapid colorimetric method to measure calcium in milk. The method is here applied to mid-season milk supply in NZDF's Takanini plant to test the method's utility in an industrial setting. At this plant, pasteurised milk is first separated into cream and skim milk. The skim milk is then passed over ultrafiltration membranes to concentrate the milk proteins at the expense of the permeate. The concentrate fraction of the skim milk is NZDF's *Xtra* brand. The permeate is low in caseins and other proteins but high in free calcium and other salts. The three fractions tested were thus skim milk, *Xtra*, and permeate.

The aim was to compare the results of the AA and CPC methods from mid of September to mid January, 2005/06, with periodic monitoring by the Gribbles reference method up to mid November, 2005.

5.2 Materials and methods

5.2.1 Reagents preparation for CPC and AA

To cope with the expected wider range of calcium concentrations in this monitoring trial, the final cuvette concentration of CPC and 8-hydroxyquinoline were doubled for this work. There was no loss of linearity in calibration curves.

Reagents and standards were prepared for AA as detailed in Section 2.2.2.2 and the AA procedure were followed in as discussed in subsequent Sections 2.2.2.3 to 2.2.2.5 in Chapter 2.

5.2.2 Milks collection and analysis

Milk samples of skim, *Xtra* and permeate were collected daily from NZDF Takanini, on typically Monday through Thursday when the dairy prepared *Xtra*. The milks were refrigerated in 1 L bottles, the content of which were analysed twice weekly.

5.2.3 Analysis of calcium by a commercial laboratory

Milk as sampled off the production line was sent to Gribbles Analytical Laboratories, Auckland, weekly to mid-November 2005. The laboratory determined calcium by AOAC 984.27, the official food industry method for determining calcium in milk.

5.2.4 Milk dilution and protein denaturation

Two sequential dilutions were made for each milk. In the primary dilution, 3 mL of *Xtra*, 5 mL of skim and of permeate, were made to 50 mL. This applied to both the CPC and AA methods.

For CPC, quadruplicate 2 mL aliquots of each primary dilution were treated with 1 mL of 25 % TCA, shaken and held for 10 minutes before final volumes were made to 50 mL.

For the AA method, quadruplicate 5 mL aliquots of each primary dilution were mixed with 1 ml of 25 % TCA, shaken and held for 5 minutes before the addition of 1 mL of 5 % lanthanum chloride solution and further standing for 5 minutes. Final volumes were made to 50 mL.

For CPC and AA, 10 mL from each final dilution (i.e. 3 milks x 4 fully-diluted replicates) were centrifuged at 1500 gravities for 10 minutes and the supernatants recovered.

5.2.5 Calcium determinations

On each analysis day, calcium was determined without delay after centrifugation. For each milk, the four replicate dilutions were sampled in triplicate for the CPC method while single determinations were made by the AA method. Thus, each data point for CPC represents the mean and standard deviation of 12 values and each for AA represents four values.

To 1 cm-path length cuvettes, reagents and the sample were added in this order: 2 mL of AMP buffer (1.2 M) 0.5 mL of CPC reagent (450 µM) (includes 8-hydroxyquinoline, 40 mM) and 0.5 mL of the milk sample from the second dilution. For calibration curves, 0, 100, 200, 300, 400 and 500 µL of calcium standard (225 µM) was added in lieu of milk, and the final volume made to 3 mL with deionised water. Absorbances were measured at 575 nm.

For AA, light from a calcium lamp (422.7 nm) was directed through an air-acetylene flame. The lamp current was set to 5 mA. Readings were taken in continuous absorption mode.

5.3 Results

5.3.1 Longitudinal monitoring of three products

Figure 21 shows the results of the monitoring trial for calcium in three milk products, skim *Xtra*, and permeate between mid-September 2005 and mid-January 2006. The ultrafiltation process is clearly effective because in the monitoring period, there was consistent and very large differences between the calcium content of *Xtra* and of permeate (Figure 21, Table 16).

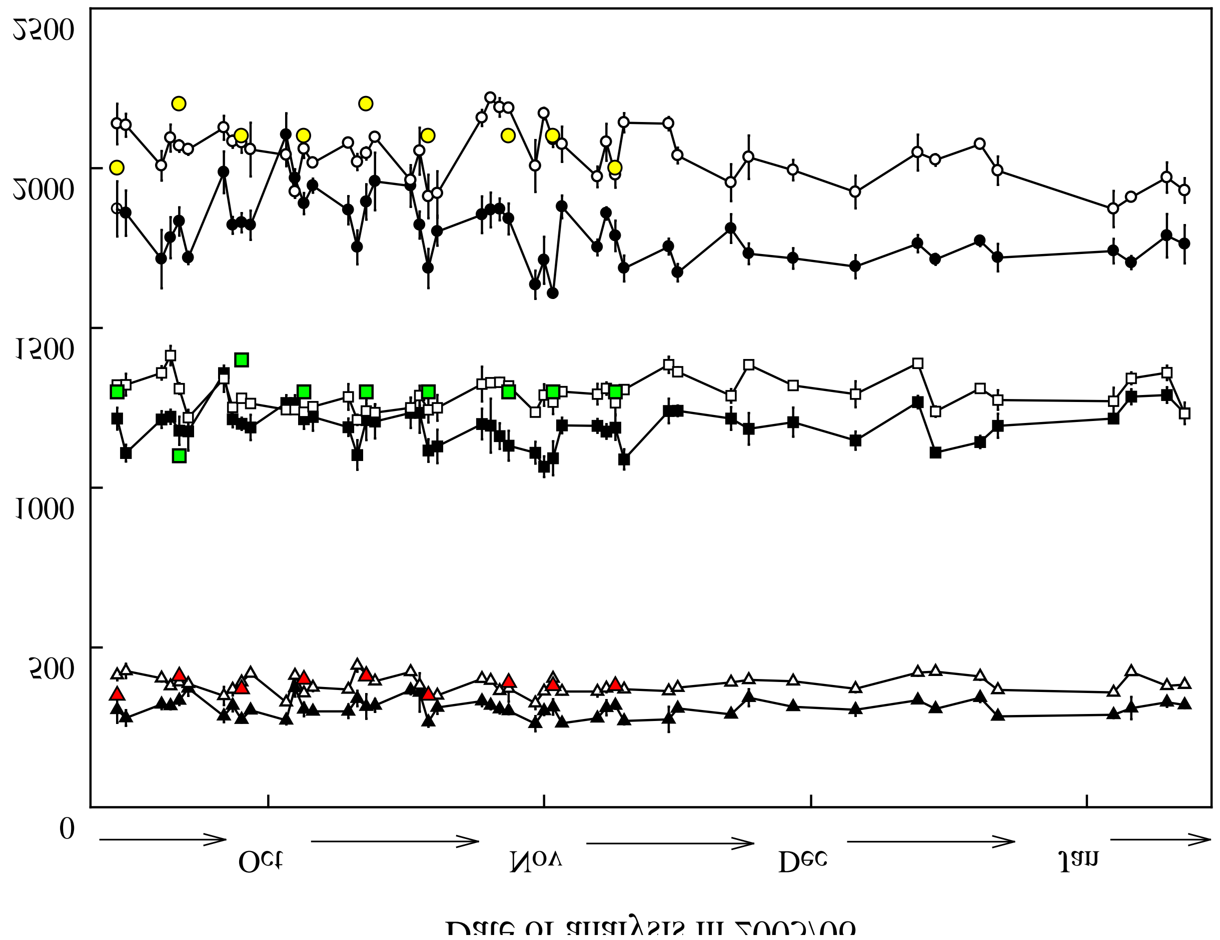

Figure 21: Monitoring of calcium in three milk products during the 2005/06 season by the CPC and AA methods, with periodic comparison to a commercial laboratory method. For AA, ○ is *Xtra*, □ is skim and Δ is permeate. For CPC, ● is *Xtra*, ■ skim and ▲ permeate. Gribbles results are in colour. Standard deviations are vertical lines.

Inspection of the Figure 21 shows that AA values agree well with the results from the Gribbles Analytical Laboratory for all three products. Values for the CPC method were generally lower than for AA and Gribbles.

Close inspection of Figure 21 suggests that the differences between AA and CPC values in *Xtra* and skim were less in the period late September to mid-October, but there was scant evidence for this in permeate. In January, there was possibly a similar narrowing of the gap between AA and CPC values for *Xtra* and skim.

There was some day-to-day variation in calcium concentrations, the cause of which is not known. Because volume data for the three products were not obtained, it has not been possible to compare mass balances of calcium as determined by the three methods on a daily basis.

Inspection of Figure 21 also suggests that there is a gradual long-term decline in calcium concentration in *Xtra* as the season progressed. This decline was not evident in skim or permeate.

5.3.2 Mean values for calcium measured in three products by both methods

The mean values for calcium measured by both methods in all three products were significantly different (Table 16). The mean value obtained by the AA method was close to the claimed value, but the mean CPC value was significantly lower. At first sight it appears that the CPC values in permeate are much closer to the AA values than for skim and *Xtra*. On an absolute value basis this is true, but is not true when data are expressed on a ratio basis. Thus, the CPC:AA ratio as extracted from Table 16 were 0.88 for *Xtra*, 0.92 for skim and 0.79 for permeate.

Table 16: Mean values of calcium concentration measured by AA and CPC method in three milk products summed over the monitoring period

Method	*Xtra*			Skim			Permeate		
	Mean[1] mg.L^{-1}	SD[2] mg.L^{-1}	CV %[3]	Mean mg.L^{-1}	SD mg.L^{-1}	CV %	Mean mg.L^{-1}	SD mg.L^{-1}	CV %
CPC	1796	44	2	1190	35	3	307	17	5
AA	2040	37	2	1291	22	2	384	10	3
Claimed Calcium	2000	NG[4]	NG	NC[5]	NG	NG	NC	NG	NG

[1]Means are average values of 51 sets of data. [2]SD is standard deviation of 51 sets of data. [3]Percent coefficient of variation. Within each milk, the means of CPC and AA were significantly different at $P < 0.001$. [4]Standard deviation is not given (NG) for any milk sample by NZDF. [5]No claims (NC) have been made for calcium in skim and permeate.

5.3.3 Correlation plots

The within-day, between-method correlation graphs plotted for *Xtra*, skim and permeate, Figures 22, 23 and 24, show that there was no significant correlation for *Xtra* ($r^2 = 0.05$ and $P < 0.11$), an improving correlation for skim ($r^2 = 0.08$ and $P < 0.048$), and significant correlation for permeate ($r^2 = 0.33$ and $P < 0.0001$).

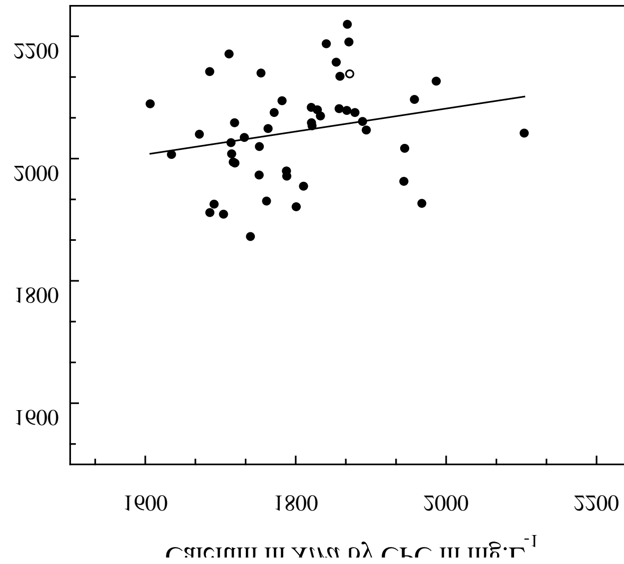

Figure 22: Correlation between AA and the CPC methods for calcium determination in *Xtra* milk

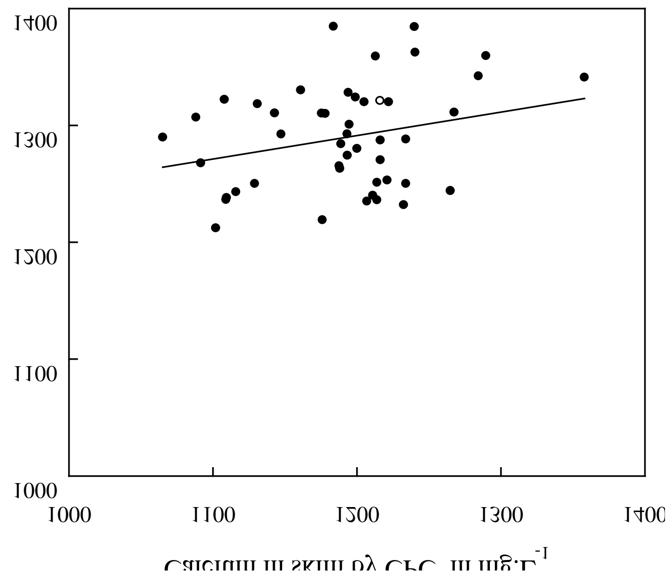

Figure 23: Correlation between AA and the CPC methods for calcium determination in skim milk

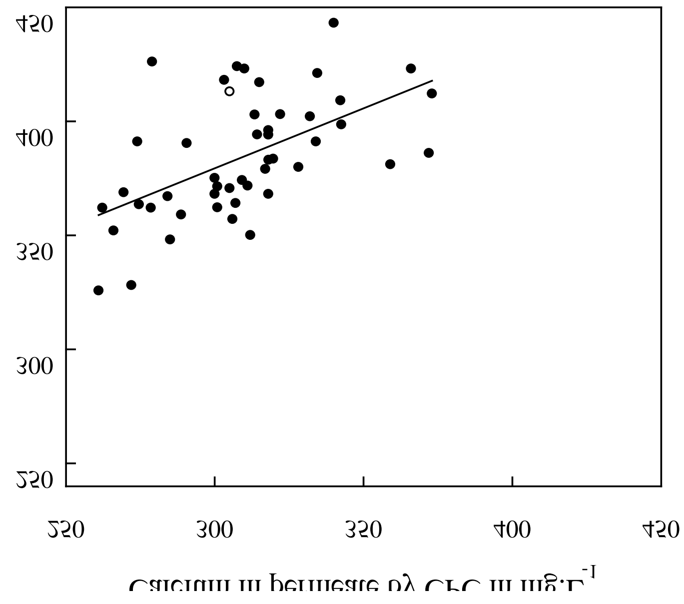

Figure 24: Correlation between AA and the CPC methods for calcium determination in permeate

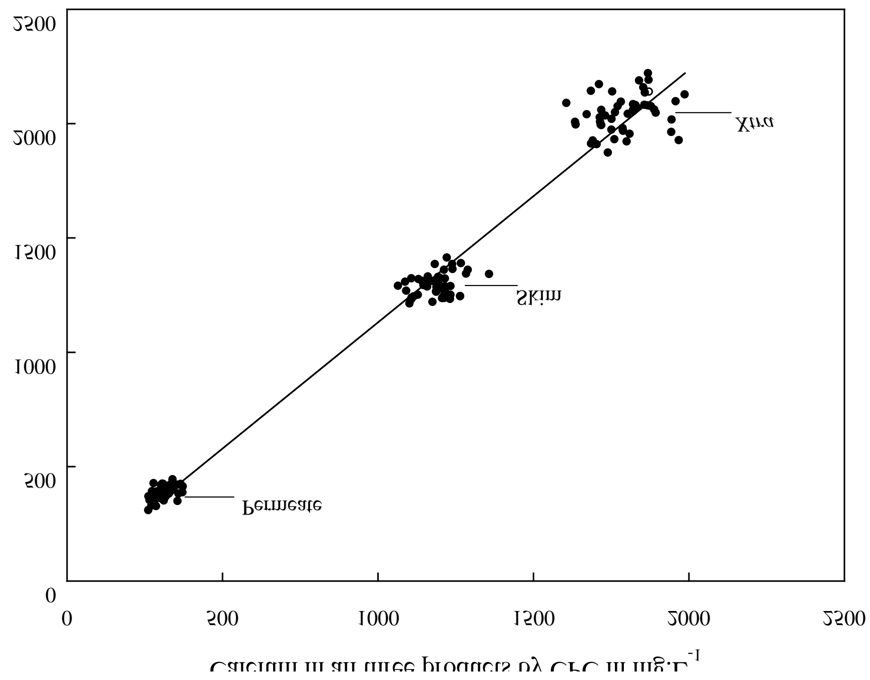

Figure 25: Correlation between the AA and CPC methods for calcium determination in all three products

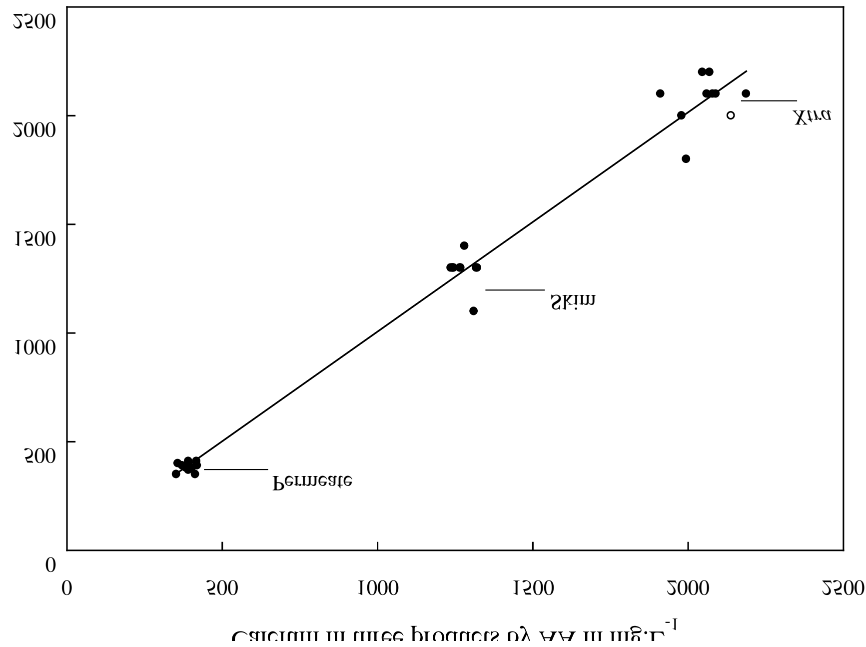

Figure 26: Correlation between the Gribbles and AA method for calcium determination in all three products

A rather different picture emerged when all AA data were plotted against all CPC data (Figure 25), where there was a strong correlation between the two methods ($r^2 = 0.98$, $P < 0.0001$). This plot also reveals the better correlation in permeate than in skim than in *Xtra.*

An equivalent correlation plot between the AA method and the Gribbles results showed a similar pattern in that the correlation with *Xtra* was the poorest and with permeate the best (Figure 26).

5.4 Discussion

There are two main issues that emerge from the data in Figure 21. The first is an explanation as to why the values obtained by the CPC method are lower than those obtained by AA. The second is why the within-day, between-method variability as revealed by the correlation plots is greatest for *Xtra* and least for permeate. The two issues may also be related.

Consider the results for *Xtra*. According to Fox and McSweeney (1998, p. 257) about 33 % of the calcium in milk is present as free ions and the remaining 67 % is bound to caseins in the form of colloidal calcium phosphate. The fraction of calcium partitioning in the permeate fraction is about 18 % (calculated from Table 16), indicating that about half the free calcium in the original skim is retained in *Xtra* on a concentration basis. But after ultrafiltration,

nominally all the milk proteins – with caseins dominating – will be present in the *Xtra* fraction. All the colloidal calcium phosphate will therefore be in *Xtra*. In the AA method, there is a denaturation step with TCA followed by exposure to transient very high temperatures in the acetylene flame. These events may be sufficient to fully liberate the colloidal calcium. In the CPC method there was a similar denaturation step with TCA, but no exposure to extreme temperature. Arguably, not all the colloidal calcium phosphate may be liberated by the CPC method, so accounting for the low CPC:AA ratio of 0.88. Progressing now to skim, where there is relatively more free calcium, the ratio improved to 0.92, as would be expected from this model. However, the model is not tenable for permeate, where nominally all the calcium will be free. The equivalent ratio for permeate was worse at 0.79.

The correlation plots show that the within-day, between-method variability is greatest for *Xtra* and least for permeate. This explanation for this may be the proportion of colloidal calcium phosphate. When it is relatively low, as in permeate, the results will more reflect the concentration of free calcium, for which there are no complexities due to protein.

Thus the proportion of colloidal calcium appears useful to explain within-day, between-method variability, but not the ratio of results from the two methods for the three milks. The reason remains unknown.

The AA method is not the standard reference method, but was used in this research as an alternative to the laborious EDTA method. The AA data closely agreed with Gribbles data, obtained with their official food industry method AOAC 984.27. The fact that the same pattern in variability occurred between AA and Gribbles (compare Figure 25 and 26) is not inconsistent with colloidal calcium model discussed above. However, it is worth noting that Gribbles results appeared to be rounded to nearest 100 for *Xtra* and skim and for permeate to the nearest 10 $mg.L^{-1}$. Correlations would be closer for unrounded data.

The concentration of calcium in skim milk was essentially constant throughout the monitoring period. According to Fox and McSweeney (1998 p .244) the composition of milk salts is influenced by a number of factors including breed, individuality of the cow, stage of lactation, feed, mastitic infection and season of the year. However, calcium concentrations are usually constant if early and late lactation periods are excluded as is the case in the present monitoring trial. Again according to Fox and McSweeney (1998, p. 244), diet has little effect on minerals in milk because the skeleton acts as a reservoir. However, Yves (1995) reported a marked increase in the concentration of colloidal and free calcium during winter months in bovine milk in Quebec, Canada, when temperatures drop markedly. Rodriguez et al. (2001) reported

almost constant levels of calcium during the 12 months of sampling milks where the temperature ranged from 18 to 26°C. Therefore the data obtained from milk from grazing cows, mid-season in a temperate zone, are consistent with the literature.

With no trend in calcium concentration in skim as received in the period under study, the apparent decline in *Xtra*'s calcium concentration has to be explained in another way. Factors linked to ultrafiltration performance may be involved, or the association of calcium between free and various bound forms may vary subtlely at different times of the year. In this respect the narrowing of differences between AA and CPC values in *Xtra* and skim between the period late September to mid-October is interesting. However, an ultrafiltration effect cannot be excluded.

6 Conclusion

The requirement from New Zealand Dairy Foods was to develop a cheap, simple and rapid method to measure calcium in a variety of product forms, principally skim milk – the basis of all processed products – and the ultra filtration products *Xtra* and permeate. *Xtra* is enriched in caseins and thus bound calcium, while permeate is correspondingly low in bound but higher in free calcium. In this research four methods were used to determine calcium.

Initially the atomic absorption (AA) and EDTA methods were trialled for precision and accuracy, so that one of them could be adopted as a reference method for this research. For both methods the results agreed well with the claimed calcium concentration in commercial milks. The EDTA method was, however, laborious and time consuming whereas AA was quick and easy. The AA method was chosen over EDTA method.

Colorimetric methods were chosen for development over more costly and complex methods involving expensive equipment. Two colorimetric dyes Arsenazo I and o-cresolphthalein complexone (CPC) were selected. These formed coloured complexes with calcium that could be used to determined calcium concentrations in a spectrophotometer.

For the Arsenazo I method, different buffers were first tested to find a suitable buffer and pH. Of the various buffers trialled, NaOH/ KCl buffer at pH 12 was found to be suitable. In colorimetric methods it is important to work with clear samples. Although the milk samples used in the tests were very dilute, they were not fully transparent due to presence of milk proteins in colloidal suspension. Trichloroacetic acid was thus used to denature the proteins (and was routinely used in the AA method as well). The Arsenazo I method worked well during the development phase but when it was applied in routine to test its precision, inconsistent results were obtained. Day-to-day coefficients of variation were unacceptably high.

The CPC dye was complexed with calcium at a pH of 10.5 with AMP buffer. The method developed here gave consistent calibration curves. Magnesium is present in milk at about 1/3 the concentration of calcium, and also produces a similarly coloured complex with CPC, although the molar extinction about 1/2 that of calcium. The reaction with magnesium could be also completely masked with 8-hydroxyquinoline included in the reaction mixture. The results during the early trials with CPC method were consistently lower than claimed values on several liquid milk products.

Both methods were then trialled on reconstituted milk powder, and the results compared with those of the AA method and the method used by Gribbles. Agreement was good among all methods. However, when the colorimetric methods were compared with AA in six liquid milks over five days, the Arsenazo method proved highly variable. CPC results had acceptable precision, but the means were variably lower for each liquid milk. Because these milks contained varying concentrations of calcium-binding caseins and fat, it was reasoned that agreement with the AA method might be improved by denaturation of caseins, and removal by centrifugation before colour development. Of the seven methods tried in three milks, the best results were obtained with brief treatment with 25% TCA. The means were close to the AA means, and the percent coefficients was low.

The long term monitoring trial on three milk products, skim, *Xtra* and permeate, showed clear differences in the amount of calcium in all three products as expected, but there were no clear long term changes in calcium concentration in any of the fractions. CPC results were consistently lower than AA results, but this was probably not due to the concentration of casein and thus bound calcium. Nonetheless, the best correlations between AA and CPC results were obtained for permeate, suggesting that the state of the calcium was important in some way to the success of the CPC method.

The correlations obtained were statistically significant only for permeate, but this does not negate the value of the method because there was no long term change in calcium concentration. Thus within-fraction plots cover a narrow range of calcium concentration. A clearer example of the method's utility is seen when data are pooled. The resulting plot with three clusters of data (skim, *Xtra*, permeate), shows very strong correlation with AA. In short the method works, but with a consistent accuracy bias.

The CPC method here can be applied in an industrial situation. The method is cheap and involves simple steps and minimal sample preparation. It uses a calcium standard, a TCA solution, CPC plus 8-hydroxyquinoline, and AMP buffer, all being shelf-stable. The equipment required is a single beam ,visible-range spectrophotometer or a colorimeter with a suitable filter, disposable plastic cuvettes, and possibly a bench top clinical centrifuge.

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Appendix 1

8 M sodium hydroxide was prepared by dissolving 64 grams of sodium hydroxide pellets in deionised water making the solution up to 200 mL. 0.01 M ethylenediaminetetraacetic acid (EDTA) solution was dried to constant weight at 80° C for two hours weighing every hour until constant weight was obtained. 3.7224 g of the dried EDTA was dissolved in 1 L of water. 1.0007g calcium carbonate 99.9 % also dried to its constant weight was dissolved in minimum amount of dilute HCL about 1 M and then made to 1 litre. 1.2005 g of $MgSO_4$ $.7H_2O$ was dissolved in 100 mL of water to make 0.05 M magnesium sulphate solution. Patten and Reeder's indicator, 2- hydroxy -1-(2-hydroxy-4-sulpho-1-naphthylazo)-3-naphthoic acid was prepared by grinding finely a mixture of 0.5 g of indicator and 50 g of anhydrous sodium sulphate in a mortar and pestle. The indicator was then stored in the airtight container. 0.1 M HCl was made by diluting the 12 M concentrated HCL. A weighing balance in all the weight measurements was used weighing up to .0001 g.

EDTA Titration procedure:

Liquid milk (any milk sample from different brands being marketed by NZDF) was weighed 4 grams in 150 mL erlenmeyer flask to 2 decimal places. 25 mL of reagent grade water (25 mL) for liquid milks for every 4 grams was added and the mixture swirled well to disperse the sample.

For each milk 10 mL 0.1 M hydrochloric acid (HCL) was added, heated to 60° C and the flask was swirled until the entire sample was dissolved. The mixture was then cooled to room temperature.

10 ml of EDTA was required for every 1-% m/m of calcium in the sample. The volume of EDTA (V) was calculated for different milks according to the Ca content claimed in the brand of the milk

A volume (V) of 0.010 M EDTA, which was 5-10 mL in excess of that, required to complex all the calcium present in the sample was added by means of a pipette.

1 mL of 0.05 M magnesium sulphate solutions was added. The mixture was swirled to dissolve the product as completely as possible, then 2 mL of 8.0 M sodium hydroxide added. The mixture swirled thoroughly and allowed to stand for about half an hour shaking occasionally until the mixture cleared.

Sufficient Patten and Reeder's indicator was added to produce a distinct purple blue colour. The mixture was titrated immediately with 0.010 M calcium chloride solution (T1) until a pink colouration persisted on standing for at least 15 seconds.

Calculations:

Volume of calcium chloride solution used to titrate the mixture = T1

Volume of EDTA used = V ml

Weight of sample used = W

Conversion factor based on 1 ml of 0.01 M EDTA being

Equivalent to 0.4008 mg of calcium = 0.4008

% of Calcium (m/m) = $[(V-T_1)*0.4008*100]/ (W * 1000)$

Calcium reported to the nearest 0.01 %

The EDTA method was repeated using same amount of 0.1 M trichloro acetic acid in place of 0.1 M HCl to get the clear solution but the results were not found to be reliable.