THE COMPOSITION OF MILK OF THE NEW ZEALAND SEA LION (*PHOCARCTOS HOOKERI*)

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Abstract

The composition of milk collected from 321 New Zealand sea lions (*Phocarctos hookeri*) found on the Auckland Islands during January and February in 1999, 2000, 2001 and 2002 were examined. The mean composition of milk was 21.7 % fat, 9.97% protein and 31.97% total solids. The milk composition varied significantly from 1999 to 2002. The average fat percentage decreased each year from 24.08% in 1999 to 11.02% in 2002. Protein percentages fluctuated each year from 8.36% in 1999, 11.07% in 2000, 10.57% in 2001 and 9.14% in 2002.

Once the milk composition was determined additional biochemical tests were performed on the same milk samples. A total of 17 sea lion samples were selected for fatty acid analysis. These samples indicated that N.Z. sea lion milk consists of primarily mono-unsaturated (47%) and saturated (34%) fatty acids. The most prevalent fatty acids found in the milk included C18:1n-9 (26.28%) and C16:0 (22.5%).

Lastly, thirty-one samples were selected for protein analysis. Preparative methods for bovine milk using gel electrophoresis, with dilution alterations accommodating total protein percentages proved adequate for the separation of whey and caseins found in N.Z. sea lion milk. These samples were compared to bovine indicated that Alpha, beta and kappa caseins had molecular weights around 2300 and beta and alpha lacto-globulin whey proteins had molecular weights around 1800. Proteins comprise approximately 10% of the total milk composition, 36.04% of which are caseins and 29.26% were whey.

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Table of Contents

Chapter 1

Literature Review

1.1 Introduction .

The New Zealand (Hooker's) sea lion (*Phocarctos hookeri*), one of the world's rarest pinnipeds, lives on the Auckland Islands, located 322 kilometers south of New Zealand. Once found from the sub-antarctic to the western shores of the North Island of New Zealand, today the sea lion is restricted to the sub-antarctic zone (Childerhouse and Gales, 1998). With a relatively stable world population estimated between 11,600 and 15,200, the New Zealand sea lion is classified as vulnerable by the IUCN Seal Specialist Group and it is protected under New Zealand's Marine Mammals Protection Act 1978 (Gales, 1998; Reijnders, 1993).

This study analyses NZ sea lion milk and is the first step in an effort to better understand the foraging ecology of lactating N.Z. sea lions through the composition of their milk. The growth and survival of a young sea lion is dependent on the supply and nutritive quality of its mother's milk. A previous study that examined the composition of milk from the Steller sea lion (*Eumetopias jubatus*) found that milk composition related to pup survival (Calkins *et al.*, 1998). The pup population suffered because of nutritional stress. Pups that received adequate quantities of milk with appropriate nutrients were more likely to survive than pups that received either too little milk or poor quality milk (Calkins *et al.*, 1998).

The ratio of fat, protein, ash and carbohydrate in an animal's milk are related to environmental conditions, life history and feeding behavior. This study will review our current knowledge of the life history and the ecology of lactation of the New Zealand sea lion. It will present new data on the composition of NZ sea lion milk and will discuss the importance and relevance of these recent results in terms of the species reproductive success and foraging behaviour over a four-year period.

1.2 Life History

The New Zealand sea lion, like all Otariids, has marked sexual dimorphism. Males, which are dark brown with a rough mane, and long hair on their neck and chest, weigh between 318 and 410kg and grow to be 2 to 2.5m long. Females are silvery gray dorsally and cream ventrally and tend to be smaller than males ranging from 136 to 230kg and 1.6 to 2 m in length (Gaskin, 1972; Crawley and Cameron, 1972; King, 1983). Pups have dark brown hair at birth and range from 70 to 100cm in length. When they are about 2 months old, pups shed their natal pelage and become dark gray with cream markings on the top of the head, nose, tail and base of the flippers (Walker and Ling, 1981). As the pups grow their coats change depending on their sex, males attaining the adult blackish-brown color and females retain the cream and gray colors of their mother (Marlow, 1975).

The NZ sea lion is coastal all year round but spends a significant amount of time foraging at sea (Gales, 1998; Childerhouse and Gales, 1998). Rookery and haul sites vary and include sandy beaches, reef flats, dense bush and solid rock (Cawthorn, 1981). In general these sites are protected from, yet have easy access to the sea. Mothers tend to move their pups inland where vegetation is more dense and provides better protection (Gales, 1995).

Breeding colonies are found in the sub-antarctic, between the latitudes 48°S and 52°S, on the Auckland Islands and Campbell Island. Ninety five percent of the pups are born at one of the four colonies on the Auckland Islands. Dundas Island hosts the largest colony (~2,000 pups), which accounts for fifty-seven percent of the total pup production. Enderby Island hosts two colonies of sea lions, one found on Sandy Bay (~400 to 500 pups) and another colony found at South East Point (~40 pups) while Figure of Eight Island has the smallest colony with only 100 pups on average (Cawthorn, 1986b; Falla and Taylor, 1979; Falla, 1986).

Haul out sites are widespread and include Macquarie Island, Stewart Island, the islands of the Foveaux Strait and Otago Peninsula. Sightings of single males have also been made as

far north as Plimmerton and on the south-west corner of the North Island of New Zealand (Cawthorn, 1986; Childerhouse and Gales, 1998; Crawley and Cameron, 1972; Hawke 1986; Hawke, 1993; Mitchell and Ensor, 1980).

The sea lion breeding season starts at the end of November when males space themselves along beaches and establish territories. Pregnant cows start to concentrate on beaches and form harems at the beginning of December. Pupping begins in the first two week of December and ceases by the third week of January (Cawthorn, 1993). From the third week of January or after suckling for two weeks, females begin to take pups from the rookery areas and leave them in the bush where they can be easily relocated when females return from feeding excursions (Cawthorn, 1993). Between February and October females will continue to alternate between foraging trips to sea and periods on land suckling their pups (Falla, 1986; Gales, 1995). Mothers continue to suckle their pups for approximately one year, however pups are introduced to solid food well before the end of this period (King, 1983). When a female reaches three years of age she is capable of reproducing and she is then presumed to pup annually for the rest of her life (Wilkinson pers. comm., 2001). Males take longer to mature and are sexually mature at 4 or 5 years but do not achieve behavioural maturity (i.e. hold a place on the breeding beach) until 8 or 9 years old (Wilkinson pers com, 2001).

The life expectancy of the NZ sea lion is unknown, however Cawthorn *et al.* (1985) estimated that males live to be 23 and females 18 years of age. There are no published data with life tables or estimates of age or sex specific moralities (Gales, 1995). It is likely that pups are subject to similar causes of mortality as other Otariids, such as, drowning, starvation, predation, disease and parasitism. Furthermore, netting and plastic or other marine debris can entangle and kill young pups (Gales, 1995).

The New Zealand sea lion swims to great depths to obtain food in the Southern Ocean and will dive up to 210 meters to obtain food (Gales and Mattlin, 1997). Due to their high blood volume they are physiologically better equipped for prolonged continuous diving than any

other Otariid (Gales and Mattlin, 1997). Between January and February 1995, Gales and Mattlin (1997) recorded the diving behaviour of 14 lactating females on the Auckland Islands. Each female made 73 trips to sea with a total of 19,720 dives for all 14 females. They dove almost continuously at a rate of 7.5 dives/hour and spent on average 45% of their time submerged (Costa *et al.*, 1998; Gales and Mattlin, 1997). The diving behavior of these females may reflect successful physiological adaptation to exploiting benthic prey and/or a marginal foraging environment in which diving behaviour is close to physiological limits (Gales and Mattlin, 1997; Gentry and Kooyman, 1986).

Despite their unique diving abilities NZ sea lions are opportunistic feeders, and tend to be fairly catholic in their tastes. The most common foods include krill and other crustaceans, mollusks and hollothurians (Cawthorn *et al.*, 1985). Squid is the main diet for breeding sea lions, which forage far out to sea and dive to great depths to locate their prey (Lalas, 1992). Scats of sea lions were collected in 1999 on Macquarie Island and analysis showed that prey items included 14 taxa of teleost fish, cephalapods, gastropods, and crustaceans, algae, octopus, squid and bivalves (McMahon *et. al.*, 1999). Sea lions are also known to occasionally eat fur seals, elephant seals, penguins and various other birds but this has little impact on the populations of these species (Gales, 1995, Bradshaw *et. al.*, 1998). Furthermore, Wilkinson (2000) recorded cannibalism of pups by adult males.

1.3 Milk Composition

Mammalian females must acquire, process and transfer sufficient nutrients to offspring to support postnatal growth up to weaning. This process, called lactation, is energetically demanding and costly. Females require an appropriate quality and quantity of food in order to synthesise milk without compromising their own survival. As a result different lactation strategies evolved to cope with the ecological stresses, the nutritive requirements of the young and biochemical and physiological potential of the lactating female (Gentry and Kooyman, 1986).

Milk is the most complete single food and is essential for reproductive success. Neonates depend on milk, a nutritionally balanced natural food, to provide sufficient energy for rapid growth over a short period, and is their only source of nutrition during the postpartum period. This can range from a few days, as the case of guinea pigs, to months as with monotremes. Though some milk may be deficient in certain essential constituents, such as iron and vitamin D, it contains most of the essential nutrients for neonate growth (Arnould *et al.*, 1996).

At the time of secretion milk comprises two liquid phases, an aqueous phase and a lipid phase, in which at least forty chemical compounds are partitioned. In the aqueous phase, lactose, minerals and water-soluble vitamins are present in simple solution while proteins and other ions are present in colloidal suspension. The lipid phase contains fat soluble vitamins and compounds such as phospholipids, sterols, and cartenoids (Mepham, 1983; Oftedal, 1984)

Though milk composition varies between and among mammalian species the major milk components of most species include fat, casein, milk serum protein, lactose and calcium (see Table 1.1). The percentage of these milk components relate to characteristics such as stage of maturity at birth, the period of lactation, and environmental factors (Mephan, 1983). There are many similarities in the composition of milk from distantly related species and from animals eating very different diets but the proportions of carbohydrates, fat and protein varies greatly. The concentrations of the principal carbohydrate, lactose, varies amongst species. Specifically, lipid concentrations varies 30 fold, being high in most marine species and some rodents and low in most primates. The protein and mineral content of milks are relatively constant and independent of the mother's dietary intake of these minerals (Blaxter, 1961; Jenness, 1974).

The main proteins found in milk include casein, milk serum proteins and immunoglobulins (Jenness, 1974). The protein contents of milks of various species range from 1 to 20% (Jenness and Sloan, 1970). Caseins are one of the few naturally occurring proteins that

contain phosphorus. They consist of a mixture of different proteins (i.e. alpha and beta casein) and proteins specifically used by the organs of the body. The caseins synthesized by different mammals are closely related though differences are found in the proportions of the fractions (Swaisgood, 1992). Milks of ruminants, buffalo, cow and goat contain at least 2.5% casein whereas human milk, mare and donkey milk is low in casein (Ling, 1961). Milk serum proteins include several kinds of protein, some of which are organ specific (such as alpha-lactalbumin), while others (such as immunoglobulins and serum albumin) are identical with blood proteins. Alpha-lactalbumin is present in virtually all milks due to the fact it helps in the catalysis of a crucial step in the pathway of lactose synthesis (Mephan, 1983; Davies *et al.*, 1983). Immunoglubulins are a heterologous group of glycoproteins synthesized by cells of lymphocytic lineage. The relative concentration of the different immunoglobulins is highly species-variable and the concentration also varies throughout lactation being higher in colostrum than in mature milk (Butler, 1974; Davies *et al.*, 1983; Ling, 1961; Mehpan *et al.*, 1992). In some animals such as artiodactyls, the young are born without circulating antibodies and other animals such as primates and harbor and gray seals receive low concentrations of immunoglobulins by placental transfer (Jenness, 1974). Thus in most species, immunoglobulins found in milk are vital for the protection of neonates from infectious agents.

Sarwar et al. (1998) reported that milk of pinnipeds contain the highest levels of total free amino acids. Taurine was the most abundant amino acid and histidine was the second most abundant. Studies that looked at the immunoglobulin concentrations in the milk of seals indicate that serum IgG, IgM and IgA are also present in variable concentrations (Davies *et al.*, 1983; King, 1983).

The most variable component of milk is fat. It differs in its concentration and chemical composition among and within species. The milk of some species, such as horses (*Equus spp.)* and black rhinoceros (*Diceros bicornus*) has little or no fat while other species such as the Northern fur seal (*Callorhinus ursinus*) have high fat concentrations that are over 53.3% of the total composition (Ashworth *et. al.*, 1975). Although the fat concentration vary

among species the main class of fats found in all mammalian species are triaglycerols (TG), which may account for over 98% of the total lipids. The variation in composition that is present among species is mainly due to the wide range of fatty acids. Four hundred and thirty seven fatty acids have been isolated from cow's milk. If all of these were randomly esterified among the three positions of the glycerol backbone, a total of 8.3×10^7 individual TG species would be possible. Overall, there are approximately 14 fatty acids in the TG fraction that are most important in quantitative terms (Mephan, 1983).

In general, most milks contain a much higher proportion of short-chain and medium-chain length fatty acids than are present in the other tissues of the same species. Furthermore, milks of ruminants have a relatively higher concentration of short chain fatty acid than milks of non-ruminant species. The particular fatty acid composition of milk TG is dependent on the nature of the diet of the lactating animal (Ling, 1961; Davies *et al.*, 1983; Mephan, 1983).

Numerous factors influence lipid concentrations in milk. The lipid content of milks varies with diet, stage of lactation, number of lactation days and season (Davies *et al.*, 1983). In some mammals, such as the tammar wallaby (*Macropus eugenii*), lipid concentrations vary with the stage and growth of the young (Green *et. al.*, 1998). These researchers found that in the tammar wallaby increased growth efficiency was associated with massive increase in lipid energy content of the milk from 26 weeks onwards. Lipid concentrations in phocids also vary during lactation and are positively correlated with lactation length. An example of this in the harp seal (*Phoca groenlandica*)) in which there is an increase in fat and energy content during lactation (Nordoy *et. al*, 1993; Kovacs, 1991).

Lactose is the principal and most distinctive carbohydrate of most milks. In marine mammals and monotremes lactose and carbohydrate concentrations are low or non-existent. Pilson and Kelly (1965) reported that milks of the entire superfamily Otariodea (fur seals, sea lions and walruses) lacked lactose but traces of lactose were found in milk of the northern fur seal (Schmidt *et al.*, 1971). Milk also contains a number of other carbohydrates

but at much lower concentrations than lactose. These include monosaccharides (glucose and galactose), oligosacharides, inositol, glycopeptides and glycoproteins. Lactose and other carbohydrate concentrations vary among milks of different species but the variation within species is usually very small (Jenness, 1974).

In addition to the major nutrients considered, milk contains a wide range of constituents present in low concentrations, such as cellular metabolites (eg. amino acids, urea UDP glucose, and vitamins), trace elements (eg Zn Cu, Mn), hormones (prolactin) and ions (K, Na, Ca, Mg and Cl). Some of these may have no particular significance for the growth and development of the young but others have important nutritional consequences. Calcium, for example, plays a vital role in bone growth and the regulation of energy production by activating glycogenolysis (Baumrucker, 1978). Milk also contains cells including polymorphonuclear leucocytes, macrophages, lymphocytes and degenerating mammary epithelial cells. Some of these constituents may be important for immunity against pathogens (Head and Beer, 1978).

In summary, there is a basic qualitative similarity in the composition of milks of different species in that they all contain distinctive types of fat, proteins and carbohydrates but there is considerable interspecies variation in the precise nature of these substances and their relative concentrations. Water, fat, protein and ash comprise the major components of mammalian milks. Cetaceans have high dry matter and fat content, while primates are low in solids and gross energy. Proboscidea have moderate dry matter and gross energy. Fat and sugar comprise about 30-40% of the dry matter. Perissodactyls are low in dry matter and gross energy. In general approximately 22% of the dry matter in Perissodactyl milk is protein and the remaining percentage is made up of sugar (Oftedal, 1984) (see Table 1.2 for additional examples). Ursid milk tends to be high in dry matter with a large proportion of energy as fat and red deer milk is high in energy content as well (Loudon and Kay, 1984). Overall, relative to other mammals carnivores, especially carnivorous marine mammals, secrete milk of moderate to high energy density (Gittleman, 1987).

1.4 Factors That Influence Milk Composition

In general there are three interactive factors that govern the chemical composition of milk. These are genetic, environmental and behavioral factors. Genetic factors relate to the biochemical and physiological potential of the lactating females and/or nutritive requirements of the young. Behavioural factors include the length of the sucking period of the young, the age of pups which begin eating solid food, and abruptness of weaning (see Table 1.3). Ecological factors include latitude where the animal lives, and weather conditions, both of which can influence food availability.

Though each of the above factors can play an independent integral role in determining milk composition it is more likely that these factors do not work in isolation. In fact it is more accurate to state that each of these factors coincide and influence one another. For example the genetic factor determines the mother's metabolic rate and her resulting capacity for lactation. In addition to physiological abilities determined through genetic factors, environmental factors such as resource availability can predict the energy budget of a lactating female hence determine biochemical and physiological potential. Furthermore the nutritive requirements of the young can influence the mother's energy output (Merchant, *et al.*, 1996; Young, 1976; Bonner, 1984; Oftedal *et. al*, 1987b)

There are a number of physiological, behavioral and environmental factors that determine a neonate's nutritive requirements. For example the Antarctic fur (*Arctocephalus gazella*) seal, must develop quickly over a relatively short period of time due to environmental stresses. Mothers must rear their young over a four-month period during which there is a highly predictable food supply. During this time the mothers milk will contain a significantly high percentage of fat which is needed to satisfy the energy requirements of the young during the short suckling period. The duration of sucking, therefore, can be viewed as a behavioral factor related to neonate nutritive requirements. Newborns that suck over a shorter period of time require more nutrients in one dose of milk than those that spend significantly more time sucking. (Merchant *et al.*, 1996). For example mice suckle at

approximately 20 minute intervals, pigs hourly, rabbits once daily, tree shrews once every two days and northern fur seals once a week (Martin, 1984). Despite the influence of other factors there is clearly a direct relationship between the concentration of nutrients in milk and suckling frequency (Oftedal *et al.*, 1987b). Furthermore, behavioural factors such as time spent foraging by the mother can also influence milk composition. If a mother forages over a long period and does not return to feed her young frequently during foraging then it is necessary that the young receive very rich milk.

Mammals, such as marine mammals and desert species, that live in environments where food availability is limited or seasonal (i.e. regions with below freezing temperatures) generally have high milk fat concentrations. Thus, high fat content is important for aquatic (e.g. whales and seals) and arctic (e.g. arctic fox and reindeer) species, in facilitating subcutaneous fat deposition in the suckling young thus reducing heat loss (Young, 1976; Bonner, 1984, White and Luick, 1984). Moreover, the high energy value of fat serves to maintain the high metabolic rates demanded by low environmental temperatures. In species which live in arid environments like the camel and ibex, the high fat content represents a means of conserving maternal water (Maltz and Schkonik, 1984). The low water intake of desert mammals may be surpassed by that of phocid seals, which abstain from feeding and drinking during several weeks of lactation, so that all water for milk secretion must come from metabolism of body stores, chiefly in the form of fats. Furthermore animals that tend to fast during lactation such as desert mice and phocid seals depend on high fat stores to supply energy for survival of mother and young (Fletcher *et al.*, 1982; Oftedal, 1984; Kovacs, 1991).

1.5 Pinniped Lactation and Milk

Pinnipeds, though marine mammals, have retained the basic mammalian pattern of parturition and suckling. Unlike ceteceans and sireans, pinnipeds mate, give birth and suckle their young on land or ice but forage in the ocean, thus they developed lactation strategies to accommodate an amphibious life strategy. Each species of pinniped developed a slightly

different lactation strategy (Table 1.3). For example, one lactation strategy occurs amoung phocid females. They minimize the lactation period, between 3 and 30 days and Otariid species tend to have longer lactation periods lasting from two months to a year (Rieter *et al.*, 1978; Gentry and Kooyman, 1986)

The location and latitude of reproduction is significant in determining the period of lactation in phocids. Ice breeding phocids breed on large blocks of ice with little shelter and forage in rich feeding grounds. As a result the lactation period is the shortest of all pinnipeds, ranging from 4 days, Hooker Sea Lion (*cystapharacristata*) to 50 days, Weddel Sea lion (*leptomychotes weddelli*) (Bowen *et al.*, 1985; Condy, 1980; King 1983). Land breeding phocids such as the southern elephant seal (*Mirounga leonina*) breed on sandy beaches or rocky shores and their lactation periods range from 23 days to 489 days (see table 1.3) (Anon, 1979).

There is considerable debate as to whether the duration of lactation in Otariids is related to latitude. Oftedal *et al.* (1987b) suggests that there is no reliable evidence that lactation length is determined by latitude whereas Gentry and Kooyman (1986) argue that high latitudes with harsh weather and strong but predictable seasonality seem to favor short lactation, and low latitudes with less seasonality but unpredictable environmental oscillation such as those caused by El Nino seem to favor longer lactation. Regardless of the correlation with latitude, otariids have longer periods of lactation than phocids, ranging from 117 days for the Antarctic fur seal (*Arctocephalus gazella*) to 365 days for the Galapagos fur seal (*Arctocephalus glapagoensis*) and most species (except the Antarctic, Galapagos and northern fur seals) suckle their young for 9-12 months (Doidge, 1986; Trillmich and Lechner, 1986; Renouf, 1991). Furthermore, unlike phocids there are no ice-breeding otariid species (Renouf, 1991).

During lactation mothers divide their time between suckling young and foraging in the ocean. otariid mothers leave for their first foraging trip about one week after birth and continue to alternate between extended periods of feeding at sea and visits to the rookery to

feed their young over periods ranging from four months to several years (Renouf, 1991). Phocid mothers, on the other hand, tend to fast or reduce food intake during an abbreviated period of lactation in which they rarely leave their pup for more than several hours (Horn and Baker, 1971; Renouf, 1991; Boness *et al.*, 1994; Merrick and Laughlin, 1997).

1.6 Pup Behaviour

The behaviour of both otariid and phocid seal pups is similar during the first week following birth. Newborns rarely leave their mothers during the first few days and spend their time suckling and getting to know their mother by vocalizing or identifying one another by smell. Phocid pups spend little time suckling and spend most of their time resting. Unweaned phocid pups do not usually interact with other pups (Renouf, 1991). Otariids, on the other hand spend most of their time suckling before the mother leaves the rookery on her first of many feeding trips. In the absence of their mothers pups tend to form pods in protected areas and are much more social and playful than phocid pups (Cawthorn, 1981; Kovacs, 1987; Hood and Onon, 1997; Gentry and Kooyman1986; Melin *et al.*, 2000).

In Pinnipeds, weaning occurs when the offspring graduate from milk to solid food. This process may be an abrupt cessation of suckling (which perhaps may involve abandoning the pup and allowing it to find solid food on its own), a gradual reduction of suckling before solid food is taken, or a gradual shift from milk to solid food (Cameron, 1998). Weaning is typically abrupt in phocids and gradual in otariids. Most phocid pups undergo a post-weaning fast which can last from four days in hooded seals or several weeks in the southern elephant seal (Rieter et al., 1978; Bowen et al., 1985, Wilkinson, 2000). Several factors such as food availability, latitude and the amount of energy stored during lactation could influence the duration of post-weaning fasting (Condy 1980; Lavigne *et al.*, 1981; Renouf 1991). Otariids begin to consume solid food prior to being weaned (Trillmich and Lechner, 1986). Therefore, unlike phocid pups, they are able to develop foraging skills much more gradually and during a time when their mother's milk is still available.

1.7 Costs of Lactation

Lactation is an energetically demanding and costly component of mammalian reproduction. The actual amount of energy transferred from mother to pup during lactation varies among species. In pinnipeds, for example, lactation accounts for 60-80% of total maternal energy budget. This includes energy spent foraging, giving birth and attending young (Reilly *et al.*,1996; Costa *et al.*, 1986).

Behavioural factors such as length of time for weaning can also effect the energy demands placed on the mother. The shorter the weaning period the greater the energy demand over a shorter period of time. For example, gray seal (*Halichoerus grypus*) females transfer large amounts of energy to their pups during a brief lactation period. Fedak and Anderson (1982) studied the transfer of energy from mother to pup in gray seals. They found that the mother uses up to 30,000 kcal/day to maintain herself and produce milk. Pups required up to 3 litres of milk/day and account for 80% of the mother's energy expenditure.

The sex of a pup can influence the amount of energy the mother puts towards pup survival. The reproductive success of males is much more variable than females, due to the fact it costs more to raise a male than a female. Anderson and Fedak (1987a) studied the energetics of female gray seals and found that 10% more energy was required to feed male pups to weaning as opposed to female pups.

In order to meet the nutrition requirements for milk production and produce enough energy to support mother and young, mothers consume considerably more food than when she is not lactating (Vernon and Flint, 1984). If females do not consume adequate quantities of food milk production will decrease and this will lead to poor nutrition and will hinder reproductive success (Boyd *et al.*, 1991; Arnould, 1997).

1.8 Changes of Milk Composition during Lactation

Because pinniped milk tends to have high concentrations of fat, small amounts of lactose, low percentages of carbohydrate and ash and varying protein concentrations, most pinniped milk studies concentrate on the compositional change, particularly the change in fat, of the milk during lactation (see Table 1.4). Fat constitutes between 40-50% of most pinniped milk. In the Weddell seal (*Leptonychotes weddelli*) and Antarctic fur seal fat constitutes 40% and 51% of the milk respectively (Peaker and Jane, 1978, Kooyman and Drabek, 1968). In some phocid species, such as the Northern elephant seal, fat content fluctuates throughout lactation. Seal pups of all phocid species are born without a layer of blubber therefore they require milk high in fat so that they can re-deposit it as blubber which provides thermal insulation and energy storage. The fat content of harp seal milk increases from 23% at the start of lactation to 40% towards the end (Lavigne *et al.*, 1981). In addition during the first 21 days of lactation for the northern elephant seals the fat content of the milk rises from 15% to 55% and remains constant until weaning at 28 days (Riedman and Ortiz, 1979). Similar changes have been shown in the Weddell seal, although in these animals the fat content of the milk decreased just before weaning (Kooyman and Drabek, 1968).

In general milk composition changes throughout lactation in otariids, however there are some species that do not support this observation. In the Australian sea lion (*neophora cinerea*) the stage of lactation seems to have little influence on milk composition (Gales *et al.*, 1996) and little compositional change has been found in samples from early lactation in Galapagos fur seal (Trillmich and Lechner, 1986), and in California sea lions (Oftedal *et al.*, 1987a).

In contrast, lipid concentrations in the Australian fur seal (*Arctiocephalus pusillus doriferus*) increase from 30% during parturition to 50% at 230 day postpartum and then decrease to 45% near weaning (Arnould and Hindell, 1999). Similar increases in lipid, protein and gross energy have been found in Antarctic fur seals and South American fur seals (*Arctocephalus*

australis) (Ponce de Leon, 1984; Arnould and Boyd, 1995). In northern elephant seals fat content decreased between parturition and the first foraging trip and increased thereafter with each trip to sea until the original fat level is achieved (Costa *et al.*, 1986). Arnould and Boyd *et al.* (1996) found that the stage of lactation, maternal mass and year of sampling accounted for 27% of the observed variability in gross energy content in Antarctic fur seals. Changes in milk composition during lactation are not as well documented in otariids as in phocids. Therefore additional studies should be conducted on the inter- and intra- annual variation in milk composition. These studies could provide valuable information on the energy requirements of mothers and their pups during lactation and factors that could influence their survival thus leading to better conservation strategies particularly for stable or decreasing otariid populations.

1.9 Aims of Study

The NZ sea lion population is stable or perhaps slowly declining. It is possible that the status of the sea lion population is related to poor nutritional status of pups. This study will examine the lipid, protein, mineral, ash, carbohydrate and water content of lactating females over a four-year period (1999- 2002). It will look at changes over the four-year period that could be related to genetic, behavioral and/or environmental factors. Furthermore this study could lead to additional studies that examine the biochemistry of sea lion milk in finer detail.

Table 1.1 Major Milk Components.

Species	Total Solids	Fat %	Casein %	Whey Protein	Lactose	Ash	Reference
Echidna	-	9.6	7.3	5.2	0.9	-	Griffiths (1965)
Virginia opossum	24.4	7.0	2.8	2.0	4.1	-	Bergman and Housely (1968)
Longnose bat	12.1	1.7	4.4	5.4	0.6	-	Huibregtse (1966)
Squirrel monkey	-	5.1	3.5	6.3	0.3	13	Buss and Cooper (1967)
African Elephant	20.9	9.3	-	5.1	3.7	0.7	Cook and Baker (1969)
Harp Seal	61.6	52.5	3.8	2.1	0.9	0.5	McCullagh and Widdowson (1970)

Table 1.2 Composition of Milk of different species

Species	*Fat (%)*	*Protein (%)*	*Carbohydrate (%)*	*Ash (%)*	*Data Source*
MARSUPALIA					
Tammar Wallaby	-	23	53	-	Green *et al.*, 1998
Red Kangaroo	21	28	43	6	Lemon & Barker, 1966
PRIMATES					
Baboons	33	9	55	2	Buss, 1968
Humans	33	7	55	2	Jenness, 1979
LAGOMORPHA					
Rabbit	49	32	6	6	Cowie, 1969
RODENTIA					
Guinea-pig	33	36	28	5	Oftedal, 1981
House mouse	45	31	10	5	Jenness & Sloan, 1970
CARNIVORA					
Arctic fox	47	38	10	4	Dubrovskaya, 1967
Dog	41	33	17	5	Oftedal, 1981
Northern fur seal	53.3	9.6	0.1	0.5	Ashworth *et. al.*, 1975
PERISSODACTYLA					
Black rhinoceros	2	12	75	3	Gregory, 1965
ARTIODACTYLA					
Reindeer	41	34	20	7	Luick et al., 1974
Red deer	40	34	21	7	Arman, 1974
Ibex	52	24	19	5	Maltz and Shkonik, 1984
Cow	30	26	37	6	Mephan, 1983

Table 1.3 Lactation strategies in Pinnipeds.

Species	*Whelping Habitat*	*Duration of lactation (days)*	*% mother with pup during lactation*	*Age (d) pup first enters the water*	*Weaning*	*Source*
PHOCIDAE						
Hooded seal	floe-ice	4	100	5	abrupt	Bowen et al., 1985
Harp seal	floe-ice	12	28	10-12	abrupt	Kovacs, 1986
Crabeater seal	floe-ice	28	-	-	abrupt	Shaughnessy & Kerry, 1989
Weddell seal	fast-ice	50	-	8-20	gradual	Jenness & Sloan, 1970
Southern elephant seal	land	23	100	>35	abrupt	Condy, 1980
Northern elephant seal	land	28	100	40	abrupt	Le Boeuf, 1972
OTARIIDAE						
New Zealand sea lion	land	365	-	21-28	gradual	Cawthorn, 1993
Galapagos sea lion	land	180-365	73	7	gradual	Trillmich & Lechner, 1986
Australian sea lion	land	365	-	-	gradual	Marlow, 1975
Antarctic fur seal	land	117	-	-	gradual	Doidge, 1986
ODOBENIDAE						
Walrus	land	730	-	-	gradual	Mansfield, 1958

Table 1.4 Milk composition in Pinnipeds.

Species	Stage of lactation	Solids (%)	Lipid (%)	Protein (%)	Sugar (%)	Ash (%)	Source
PHOCIDAE							
Harp seal	Early	38.39	28.14	6.8		0.56	Lavigne et al., 1981
	Middle	45.87	35.38	6.8		0.56	Lavigne et al., 1981
	Late	53.35	42.62	6.8		0.56	Lavigne et al., 1981
N. elephant seal	Early	37	24	7.6			Riedman & Ortiz. 1979
	Middle	62	47	7.6			Riedman & Ortiz. 1979
	Late	67	54	7.6			Riedman & Ortiz. 1979
Hooded seal	Early	66	56.3	6.2	0.86		Oftedal et al., 1993
	Middle	69.7	61	4.7	1.05		Oftedal et al., 1993
	Late	79.8	61.1	5.1	0.99		Oftedal et al., 1993
OTARIID							
Southern sea lion	Early		25.8	8.6		1.1	Werner *et al.*, 1996
Antarctic fur seal	unknown	57.36	44.1	11.44	0.13	0.65	Arnould et al., 1994
	parturition		30	10			Arnould, 1996
	day 230		50				Arnould, 1996
	postpartum		45	12			Arnould 1996
Galapagos fur seal	day 1-8		32.4	12.1			Trillmich & Lechner, 1986
	day 12 +		25.1				Trillmich & Lechner, 1986
California sea lion	Middle day 30-45	41	30.7	8.6	0.3		Oftedal , 1981

Chapter 2

2. Abstract

The composition of milk, collected in 1999, 2000, 2001 and 2002 during January and February, of 321 NZ sea lions was determined. During the time of collection age and a body condition score was given (1 = the poorest condition and 5 = the best condition). Animals ranged from six to fourteen years old and averaged 8.42 years. The majority of the animals received a score of 3 or 4 for body condition. The mean composition of milk for 321 samples was 21.27% fat, 9.97 % protein and 31.97% total solids. The milk composition had a remarkable range from 4.2% fat to 52.07% fat, and 4.11% protein to 18.9% protein. The yearly milk composition was calculated. In 1999 the average fat 24.08 % and protein was 8.36%; 23.09% fat and 11.07% in 2000; 19.03% fat and 10.67% in 2001 and 11.02% fat and 9.14% in 2002. Fat percentages and protein percentages were inversely related as fat increased protein percentages decreased. There was a significant relationship between year and milk composition. Fat percentages gradually decreased from 24.08 % in 1999 to 11.02 % in 2002. Protein percentages increased from 8.36% in 1999 to 9.11.07% in 2000 and decreased to 9.14% in 2002. Body condition nor age effected the percentage fat found in the samples, however body condition influenced the protein percentages in 3% of the data. As Body condition increased protein percentages increased.

2.1 Introduction

There is a basic qualitative similarity in the composition of milks of different species in that they all contain distinctive types of fat, proteins and carbohydrates but there is considerable interspecies variation in the precise nature of these substances and their relative concentrations. The percentage of fat in milk varies from a little over 1% to greater than 50% in mammals. Aquatic mammals tend to have higher percentages of milk fat than terrestrial mammals (Jenness, 1974).

There are pronounced differences in the proportions of individual carbohydrates from milk of different species. Lactose ranges from only trace amounts, as found in seals, to just less than 7% as seen in donkeys. Numerous qualitative data show that some carbohydrates are present in some animals but completely absent in others (Jenness and Sloan, 1970).

The constituent protein molecules found in milk vary in number and kind depending on the species. Proteins that may be present in all milks include caseins, blood serum albumin, immunoglobulins and alpha lactalbumin. Total protein varies considerably ranging from 1% to 14%. Generally protein is positively correlated with milk fat percentages (Jenness, 1974).

In addition to inter species differences there are often drastic intra species differences. For example the southern elephant seal has an average of 25.8% milk fat in comparison to the Hooded seal with 61% milk fat (Werner et al., 1996; Oftedal, 2000). Such variations can be a result of life history and stage of lactation of the animal according to the changing nutritional requirements of the offspring.

Although fur is an excellent insulator for animals that live on land, fur loses most of insulative value when wet (Gittleman, 1989), thus most marine mammals rely on subcutaneous fat or blubber instead. Effective insulation in cold water is especially necessary as water transports heat away from submerged animals body about twenty five-times as fast as air (Fricke et al., 1984; Young, 1976). Furthermore when the animal hauls out on the land or on ice, the thick blubber can be a great barrier to heat

dissipation. Sea lions can lose up to 25% of the metabolic heat formed (Horn and Baker, 1971; Green and Merchant, 1980; Oftedal, 1984)

N.Z. sea lion pups are born on land with a hairy coat and no subcutaneous fat (Cawthorn, 1981). Once they start feeding on their mother's milk they quickly develop a thick layer of blubber to protect them from the harsh environmental conditions and to provide an efficient energy source when their mother is off foraging. As the pup matures, the blubber develops, the pups increase their tolerance to cold sea water or external temperatures and the blood circulation transfers less heat to the surface tissues where it would be lost (Green et al., 1993).

In order for the mother sea lion to provide efficient energy to her pup her milk must contain the appropriate percentage of fat in her milk. Lipid rich milk with low water content provides more energy per gram than any other food source. Furthermore because the mother must make 4-8 foraging trips each day she must acquire energy and nutrients as efficiently as possible, which is in the form of fat. The lightness and high energy yield of fat relative to carbohydrate or protein makes it the favored storage form for potential energy. The energy value of triacylglycerols to the body is about 38 kJ/g (9 kcal/g) compared to 17 kJ/g for proteins and 16 kJ/g for carbohydrates. As storage fuels, triacylglycerols have the advantage that they can be stored in anhydrous form, representing a more concentrated form of energy than complex polysaccharides such as body glycogen, which are highly hydrated (Patton and Keenan, 1975).

The appropriate percentage of milk fat in N.Z. sea lion milk is unknown however previous studies of milk fat of other otariids indicate that the average milk fat percentage ranges from 25.8% in the Southern sea lion to 50% in the Antarctic fur seal (Werner et al., 1996; Arnould and Hindell, 1999). This study examines the milk composition of the NZ sea lions, located on the Auckland Islands.

2.2 Methods

Sample Collection in the field

Milk samples were taken from 321 individual lactating sea lions located on the Auckland Islands were sampled in 1999, 2000, 2001 and 2002. Of the 321 females sampled, 309 females were sampled in only one year and 9 females were sampled over two years and three females were sampled over three years.

Only, females seen with a pup were chosen for capture. The female was caught using a hand held net and once inside the net, restrained on the ground by two people. Anesthesia was induced using isofluorane/O_2 administered by a facemask from a portable anesthetic machine. Oxytocin (1ml) was injected by intramuscular injection and a milk sample was collected within 10 minutes. Milk (up to 20ml was taken from the caudal teats by either manual massage or by using a milk pump. The milk was aliquotted into cryovials (1.8 ml or 4 ml) and stored in liquid nitrogen (-196°C) until taken to the laboratory where it was transferred to a –80°C freezer.

Laboratory Analysis

Samples were removed from the – 80°C freezer and placed in a rack. All samples were heated to 32°C in a hot water bath, prior to being tested for their percentage fat, protein and total solid. Once the samples reached the appropriate temperature they were removed from the hot water bath. One ml of each sample was placed in a separate plastic test tube and was diluted with 23 ml of water. The sample was then placed in the FTIR MilkoScan FT 120 a machine that employs FTIR interferometer (Fourier Transform Infrared Spectroscope), which relies on the principle of light interference to determine milk composition. In order to receive accurate results, the MilkoScan FT 120 was calibrated for sea lion milk. Fifteen samples were randomly selected from the pool of milk samples and tested using traditional methods. Fat was determined using Rose-Gotlieb and ASE (Accelerated Solvent Extraction); Protein was determined using Kjeltec method and Carbohydrates using Gas Chromatography. (See appendix I, for a more detailed account of the methods and the principle of the MilkoScan along with the steps taken to calibrate the MilkoScan for sea lion milk). Once the MilkoScan was calibrated 321 samples were analyzed. Approximately 50 samples were analyzed per day. Statistical analysis, were then performed on the data using the statistical programs SPSS and Minitab.

2.3 Results

Total Milk Composition:

The range of the concentration of fat in the New Zealand sea lion was 4.2% to 52.07% with a mean of 21.27% (and deviation of ± 10.17%). Protein varied from 4.11% to 18.9% with an average of 10.09% (±2.76%). Total Solids varied from 13.8% to 59.23% with an average of 31.96% (± 9.56%) (see Table 2.1 and Fig. 2.1).

Due to the lack of carbohydrates found in sea lion milk (< 0.005%) total solids reflected the amount of protein and fat found in the milk. There is an inverse relationship between the protein and fat (i.e. as fat percentages increase protein percentages decrease). Thus for each sample, fat percentages plus protein percentages equaled total solid percentages. Trace amount of minerals (i.e. K, Mg, Ca, Na, and P), and Nitrogen were also found in the milk. Trace minerals contributed between 1 to 2 % of the total solids of each sample.

In 1999, 99 samples were taken, within these 99 samples the maximum fat was 52.07% with an average of 24.08%. In 2000, 91 samples were taken. There was a slight decline in the percentage of fat in 2000 when compared to 1999. The average fat percentage was 23.09%. The maximum was 45.03% and minimum 4.2%. Fat percentages continued to decline in 2001. Out of 121 samples there was an average of 19.03% fat. The maximum fat percentage was 40.52% and minimum 7.52%. Finally in 2002 there is yet another drop in fat percentages with an average of 11.02% (N=34). The maximum fat percentage was 22.31% and the minimum was 6.44%. (Table 2.2 and Fig. 2.2 (a))

In 1999 the average protein was 8.36% and with a maximum of 16.79% and a minimum of 6.4%. Protein percentages increased slightly from 1999 to 2000 averaging 11.07% with a maximum of 18.89 % and a minimum of 5.16%. Protein percentages decreased slightly from 2000 but where higher than 1999's average. The average protein was 10.67% with a maximum of 17.63% and a minimum of 6.25%. Protein levels continued to remain level

when compared to previous years. The average protein percentages for 2002 was 9.14% with a maximum of 14.03% and a minimum of 4.6%. (Table 2.2 and Fig. 2.2(b))

Life history, which included age, body condition and the year the sample was taken, was available for sea lions that were sampled. Age data was available for 288 samples. The minimum age recorded was 4 and the maximum age was 17. The average age was 8.9. (Fig. 2.3 (b).

The Body Condition score is a numeric representation of a scale based on visual assessment. Body condition data was analyzed using the statistical assumption categorical data can be used the same way as numerical data. The Body condition ranged from 1 to 5. Out of 275 individuals the majority of the body conditions for the animals scored a 3 or 4. Body condition data was analysed under the assumption categorical data such as body condition can be analysed as if it were a continuous variable. The average Body condition was 3. (Fig. 2.3(a))

The body condition for sea lions sampled in the years 1999, 2000, 2001 and 2002 was determined. The majority of the animals remained between 3 and 4 years of age. In 1999 and 2000 there is a range of individuals with different body conditions however in 2001 and 2002 there were only 2 (in 2001) or 3 (in 2002) animals that were not scored a 3 for body condition The average body condition remained relatively stable in 1999 and 2000 however it decreased slightly in 2001 and 2002. (2.4 (a))

The average age over the three years (1999 to 2002) was determined. The average age increases slightly from 7.5 in 1999 to 8.5 in 2000 and remains at 8.5 in 2001 yet increases to 10.4 in 2002. It is obvious that the animals age each year. However, only 20 animals were sampled over consecutive years, thus these results do not necessarily reflect the same animals aging. Also, note the number of animals with age data available each year. In 2002 there were only 24 animals for which age was recorded. (Fig. 2.4(b)

Fat and protein percentages were divided into groups based on year (1999 to 2002) and body condition (B.C.=2, 3,4 and 5). The majority of the animals fell under body condition 3 or 4. Within these categories fat decreased slightly each year for body condition three and four. Protein increased for body condition three and four from 1999 to 2000 but decreased there after (Fig. 2.5).

Fat and protein percentages for each age group in 1999, 2000, 2001 and 2002 were analyzed. The majority of the animals were 7 or 8 years old, thus it is most accurate to look at the trend of animals for this age group only. The protein percentage for animals that were 8 years old increased from 1999 to 2000 but decreased slightly in 2001. The fat percentages for this same age group increased from 1999 to 2000 and decreased from 32% in 2000 to 19% in 2001.

There is a significant relationship between fat, protein and total solids with year (Table 2.3). As fat percentages increase Protein percentages decrease (P=-300). This was valid for 9% of the data (r ^2 =.09) As Fat percentages decreased as year increased (p= -.292). This was present for approximately 8.5% of the data (r^2=.085). Protein percentages increased with year (p=.223) and body condition (p=.186). There was no significant relationship between age and fat, protein or total solids, thus the fat or protein percentages did not relate to the age of the animal. The body condition of the animal did significantly correlate with the age of the animal. In addition there was no significant relationship between the fat percentages and the body condition however there was a significant relation ship between body condition and protein percentages (Table 2.4).

As mentioned previously the protein and fat percentages have an inverse relationship, as fat percentages increase protein percentages decrease. Further tests such as the Pearson correlation support this by illustrating a significant relationship between fat and protein percentages (Table 2.4).

Finally by performing a multivariant test, using year, body condition and percentage fat, and protein, the test suggests that year affects the compositional profile of fat and protein (Table 2.5 (b)). The results of the multivariant test is more clearly outlined from the trend shown by the least squares method. The least squares mean for 1999 is 25.9%, 2000 (23.1%), 2001 (19.0%) and 2002 (11.02%). Therefore there is not only a correlation between year and fat percentage but there is also a significant yearly decrease in milk fat as demonstrated by the least square means.

Table 2.1

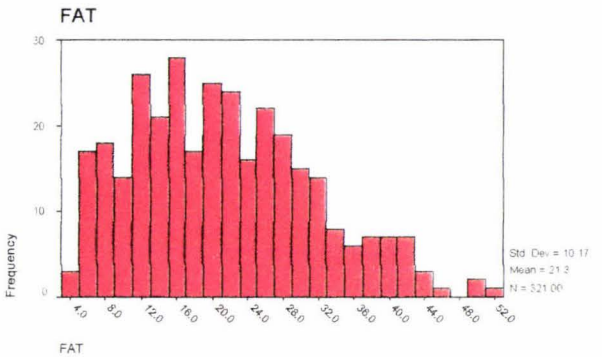
Total milk composition of the New Zealand sea lion from 1999 to 2002

%	N	Minimum	Maximum	Mean	Std. Dev.
Fat	321	4.20	52.07	21.27	10.17
Protein	321	4.11	18.90	9.97	2.76
TS	321	13.80	59.23	31.97	9.7

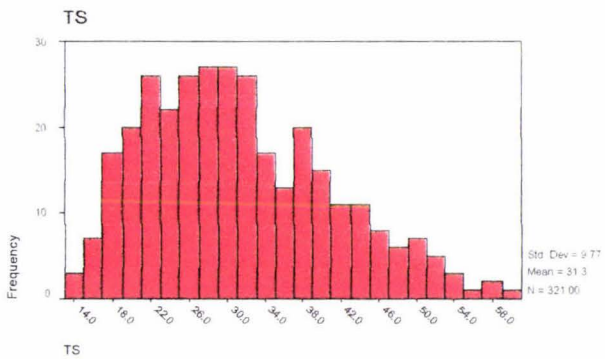
Fig. 2.1

Distribution of fat, protein and total solids in sea lion milk from 1999 to 2002.

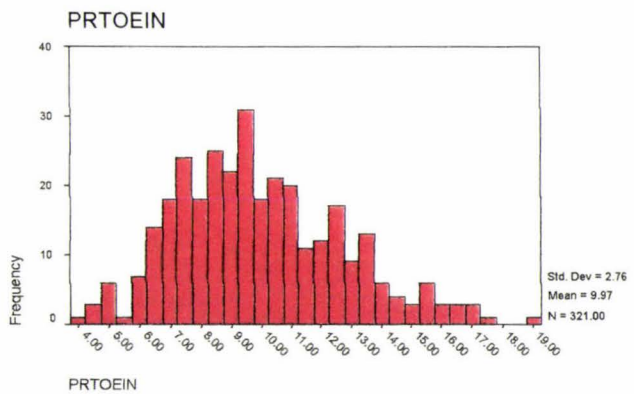
(x-axis = the percentage of fat (graph (a)), total solids (graph (b)), and protein (graph (c)) ; y-axis = the frequency of animals.)



(a)



(b)

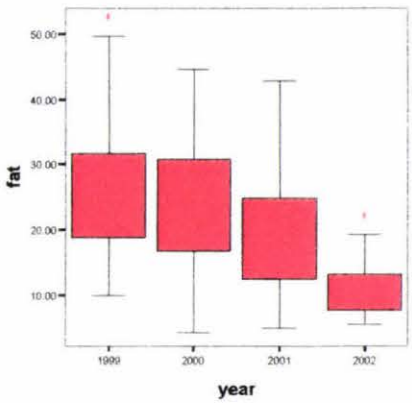


(c)

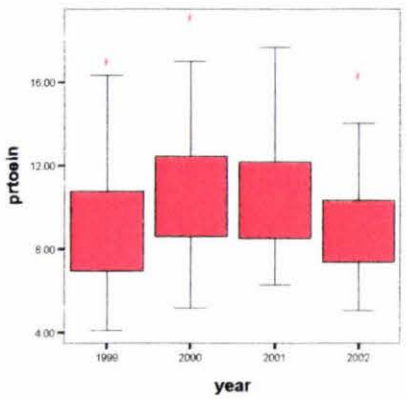
Fig. 2.2

Boxplot of the percentage fat (a) and protein (b) in the milk of the New Zealand sea lion for the years 1999, 2000, 2001, and 2002.

(N= 99 in 1999, N= 91 in 2000, N = 121 in 2001, and N= 34 in 2002; ° = outliers).



(a)



(b)

Table 2.2

Mean, minimum and maximum milk composition in New Zealand sea lion milk for each year (1999, 2000, 2001 and 2002).

		Mean %	Maximum %	Minimum %
1999	Fat	24.08	52.06	4.1
	Protein	8.36	16.79	6.4
2000	Fat	23.09	45.03	4.2
	Protein	11.07	18.89	5.16
2001	Fat	19.03	40.52	7.52
	Protein	10.67	17.63	6.25
2002	Fat	11.02	22.31	6.44
	Protein	9.14	14.03	4.6

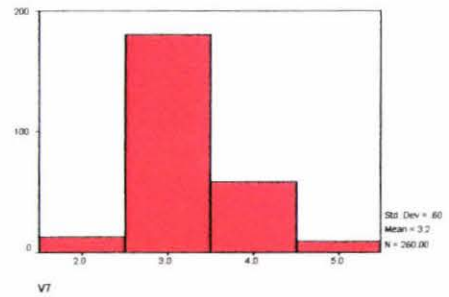
Fig 2.3

Histograms of (a) body condition and (b) age of sea lions.

(a) Body condition of sea lions: (N=265); x-axis represents the body condition score 1 being the lowest score and 5 being the highest score, and the y-axis represents the number of animals.

(b) Age of sea lions: (N=288); x-axis represents the age of the sea lion (4-18 years old), and the y-axis represents the number of animals.

(a)



(b)

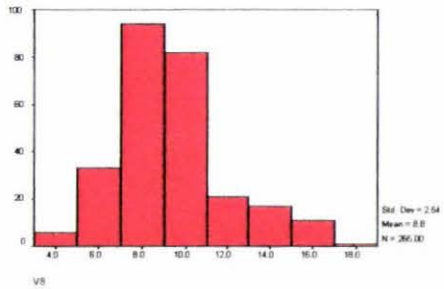


Fig. 2.4 (a).
Box plot of body condition between 1999 and 2002. (N= 82 in 1999, N=80 in 2000, and N=99 in 2001, and N= 33 ; * = outliers)

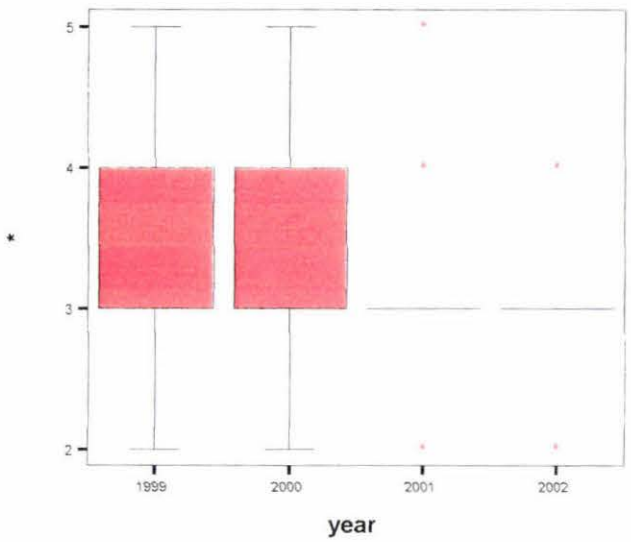


Fig 2.4 (b)
Box plot of the age range of animals sampled between 1999 and 2001. (N= 86 in 1999, N=85 in 2000 and N=93 in 2001 and N=24 in 2002; = outliers)

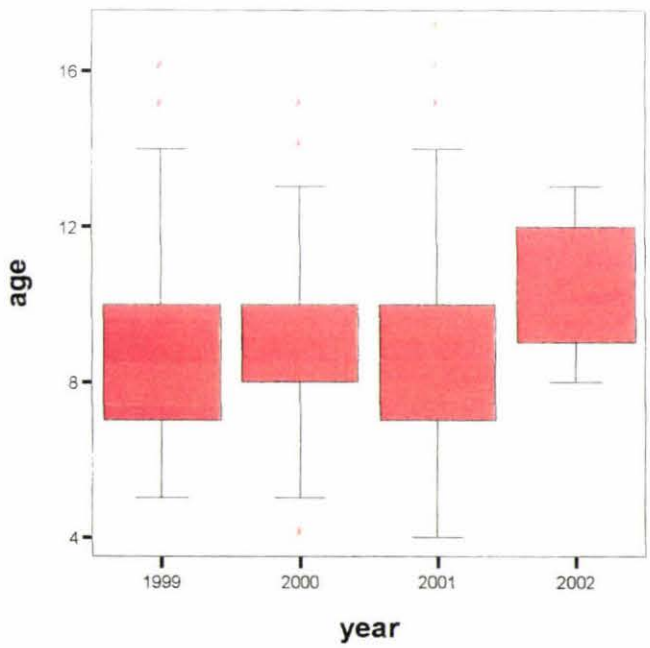


Fig. 2.5 (a)

Fat percentages for 1999, 2000, 2001 and 2002 for body conditions 3, 4, and 5; x-axis represents year and number of samples present y-axis represents percentage fat.

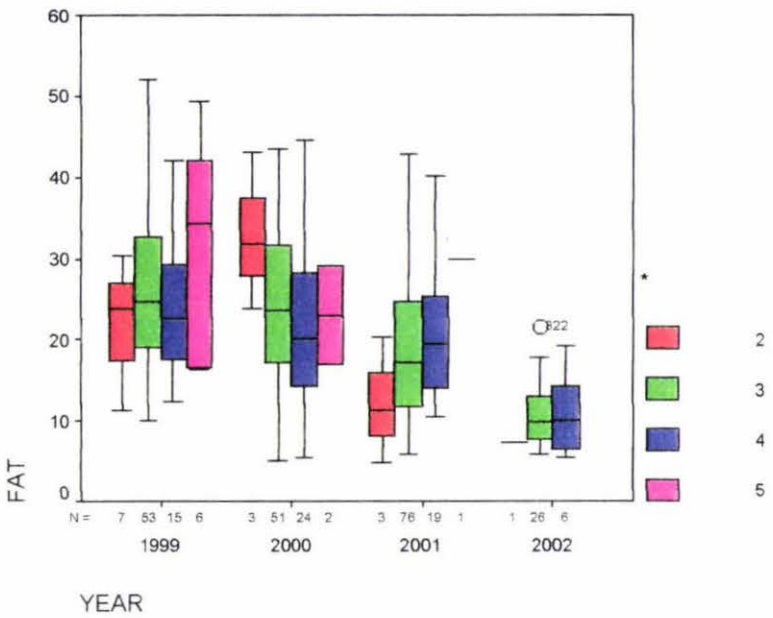


Fig. 2.5 (b)

Percentage protein for 1999, 2000, 2001 and 2002 for body conditions 2, 3, 4, and 5.

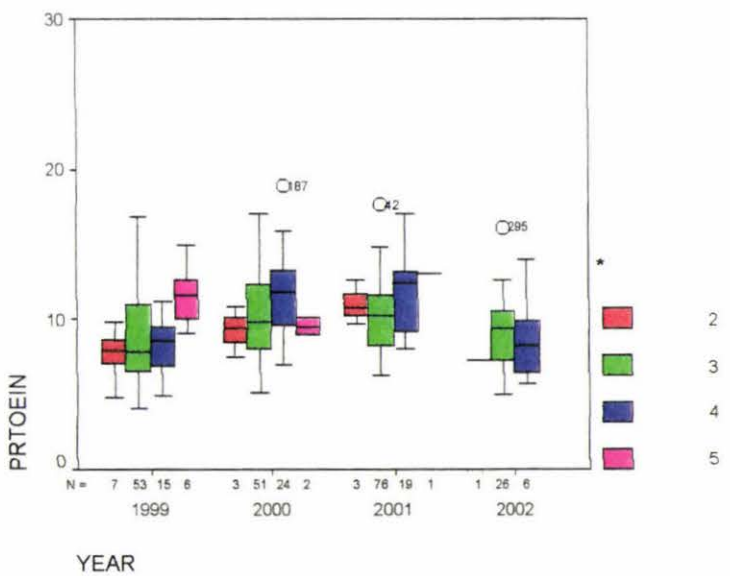


Table 2.3

One-way analysis of variance with year as the independent factor and fat, protein and total solids as the dependent factors.

		Df	F	Sig.
Fat %	Between Groups	2	13.43	**.000**
	Within Groups	286		
	Total	288		
Protein %	Between Groups	2	10.89	**.000**
	Within Groups	286		
	Total	288		
TS %	Between Groups	2	9.40	**.000**
	Within Groups	286		
	Total	288		

Table 2.4

Below is a table of correlations between fat %, protein %, total solid %, body condition (B.C), year and age. Significant values are in bold.

		Protein %	Year	B.C.	Age
Fat %	Pearson Correlation	-.300	-.292	.066	.057
	Sig. (2-tailed)	**.000**	**.000**	.289	.359
	N	289	289	260	265
Protein %	Pearson Correlation		.223	.186	.039
	Sig. (2-tailed)		**.000**	**.003**	.525
	N		289	260	265
Year	Pearson Correlation			-.050	-.048
	Sig. (2-tailed)			.425	.437
	N			260	265
B.C.	Pearson Correlation				.223
	Sig. (2-tailed)				**.001**
	N				241

** Correlation is significant at the 0.01 level (2-tailed).

**Correlation is significant at the 0.05 level (2-tailed).

Table 2.5 (a)

Description of data used for multi-variant analysis.

		N
YEAR	1999	99
	2000	91
	2001	119
	2002	30
BODY CONDITION		
	2	13
	3	191
	4	59
	5	11

Table 2.5 (b)

Multivariant test between year, body condition and percentage fat, protein and total solids. Significant values are in bold.

Multivariate Tests

Effect		Value	F	Error df	Sig.
YEAR	Pillai's Trace	.058	2.54	338	**.040**
	Wilks' Lambda	.942	2.55	336	**.039**
	Hotelling's Trace	.061	2.55	334	**.039**
	Roy's Largest Root	.052	4.37	169	**.014**
BC	Pillai's Trace	.067	1.96	338	.071
	Wilks' Lambda	.934	1.95	336	.072
	Hotelling's Trace	.070	1.95	334	.072
	Roy's Largest Root	.052	2.93	169	**.035**
AGE	Pillai's Trace	.147	1.03	338	.425
	Wilks' Lambda	.856	1.04	336	.408
	Hotelling's Trace	.165	1.06	334	.392
	Roy's Largest Root	.139	1.81	169	**.046**

a Exact statistic

b The statistic is an upper bound on F that yields a lower bound on the significance level.

c Design: Intercept+YEAR+BC+AGE+YEAR * BC+YEAR * AGE+BC * AGE+YEAR * BC * AGE

2.4 Discussion

There are three issues of concern presented by the results of this study. Firstly, the mean fat percentages of the New Zealand sea lion is lower than that reported of other Otariid species (Gales et al., 1996; Arnould and Hindell, 1999; Oftedal, 1984). The mean fat in New Zealand sea lion milk is 21.27%, which is lower than other Otariid species such as the Australian sea lion milk which, reported approximately 30.82% lipids (Gales et. al., 1996). Secondly, mean fat percentages in NZ sea lion milk gradually decreased from 24.08% in 1999 to 11.02% in 2002. Finally there is a wide range of fat percentages among the 321 individuals (minimum = 4.20%; maximum = 52.07%), which deserves investigation.

Life history information was recorded for each of the animals of which milk samples were taken. Using this information it was determined that there was no relationship between the age or the condition of the animal and its fat percentages. Therefore additional hypothesis have been made as to why fat percentages have declined over the years and why fat percentages vary greatly between individuals.

There are numerous potential explanations for the results of this study. However it cannot be certain why there is a decline in fat percentages from 1999 to 2002, and a wide range of fat percentages among individuals. It is possible that the decline in fat percentages in between 1999 and 2000 is a result of a mass mortality of pups that took place in 1998. However the decline in 2001 and 2002 requires further investigation.

The first year of sampling took place in 1999, which was a year following a mass mortality event at the Auckland islands which may have been caused by a viral or bacterial infection, biotoxin poisoning, oil or chemical pollution or some natural imbalance in environmental conditions (Baker, 1999). In 1998 at least 53% of the pups died. Given that lactation is energetically demanding in pinnipeds it is likely that females in 1999 were in good body condition as a result of being relieved of the burden of a pup (most only lactated for 2 months). In the following two years there was no pup mortality to the extent of 1998, and consequently females spent almost all year weaning pups thus the associated energetic costs were placed upon the female. As a result fat

percentages could have declined during these years as mothers had to feed pups where as in 1998 they did not have pups to feed.

It is possible that low fat percentages found in 2002 were a result of poor foraging. This is supported by the fact that male pups during this year weighed at 4 weeks were lighter than that recorded in the past seven years. This, however, was not the case with female pups, which were similar weights to previous years. However as mentioned previously male pups require more energy for growth than female pups, thus mothers could have been capable of providing enough energy for females pups but not so for males pups.

Environmental factors such as climate change and weather conditions resulting from climate change could influence food availability and thus sea lion milk fat percentages. On Australia's sub-Antarctic Macquarie and Heard Islands, elephant seal and penguin populations have fallen by 50% in recent years (NOAA, 2002). Scientists from the Australian Antarctic Division and the University of New England have suggested that increasing temperatures and changes in food supply may be responsible. Furthermore Vergani (2001) studied the possible effects of "El Nino" Southern Oscillation (ENSO) components and "La Nina" on populations of Southern elephant seals over a ten year period (1985 to 94). Vergani found that the weaning mass of elephant seals was higher during La Nina years and lower in El Nino years. The study suggests that seal pups weaning mass is an indicator of changes in food availability (Vergani, 2001).

The samples in this study were taken in 1999, 2000, 2001, and 2002, which were La Nina years. In order to make a comment on the effects of weather patterns, resulting from El Nino or La Nina years, on food availability and its effect NZ sea lion milk additional milk samples should be taken during an El Nino period. El Ninos take place ever three to seven years. Nineteen-ninety eight ended the most recent El Nino period (NOAA, 2002)

Recent concerns of rising temperatures resulting in global warming could however influence the availability of food for NZ sea lions. Temperature directly impacts marine productivity, which would therefore affect the abundance and breeding success of the sea lion. Few studies on the effects of global warming and sea lions have been made however there is potential for future studies on this topic. NZ sea lions are highly

localized thus the consequences of major environmental change is very serious (Vergani, 2001).

Humans may have a significant impact on NZ sea lion prey. Competition for food may have arisen from competing fishing industries, particularly the squid industry, in the area. The sea lion population feeds off the Auckland islands, an area renowned for its high squid population. In 1978 the MAF Fisheries Statistics Unit in New Zealand reported fishing trawls caught between 9,500 and 34,355 tons of squid in this area. In 1992/93 only 1500 tons of squid were caught. This could be an indication of falling squid populations due to exploitation of the squid by the squid fishery industry. A more in depth study investing the effects of the fishing industry on sea lion prey is needed.

Milk composition can vary tremendously depending on the stage of lactation. Such changes were found in southern elephant seal and the harp seal (Fayolle et al., 2000; Fletcher et al., 1982). Though the NZ sea lion milk composition study took place over a four-year period all samples were taken during the same stage of lactation in January and February, thus lactation stage probably did not have an effect on the variation of the milk composition. However an alternative explanation involves the individual's situation in relation to foraging and feeding. An animal that has just recently returned from a foraging trip will have higher milk fat percent than that which has just finished feeding its young (Costa, 1988). Because these samples were taken at random the feeding status of the mother is unknown. Individuals could have recently finished feeding their young and thus be drained of her milk supply and therefore on the verge of going on a foraging trip when milked. In contrast the individual with a high fat percentage could have just returned from a foraging trip and was yet to feed its young. However based on the randomness of the sampling and the number of samples present the time of foraging should not effect the outcome of the results.

In the Northern fur seal fat content of milk declines during the 7 day parental period and during the subsequent 2-day suckling intervals (Renouf, 1991). The milk fat content slowly recovers to the postpartum levels (requiring at least four foraging trips) indicating that suckling is very demanding. Studies by Anderson and Fedak (1987a) have also shown that energy requirements of the gray seal pup vary based on age, mass and sex. This implies that energy available in the mothers may be influenced by the

demands of her pup. For example an older male pup would require more energy than a young female pup thus a mother with an older male pup must supply more energy to her pup hence could have a higher fat percentage in her milk.

Finally a potential, yet less easily assessed, source of error could originate in the sampling of the sea lions. Oftedal (1984) presents the potential for unequal distribution of milk fat in mammary contents in many species. A pronounced rise in the fat content of successive portions of milk removed during milking has been indicated for cows, sheep, water buffalo, eland, moose, horses and humans (Oftedal, 1984). The fat content of the first portion drawn may be but one-third or less of the level found in the last or residual portion. Thus incomplete mammary evacuation during milk collection may yield samples atypically low in fat. By contrast, residual milk remaining after suckling will contain a disproportionate amount of fat so that samples collected shortly after the young have suckled may overestimate fat levels. Furthermore, oxytocin may influence milk composition. Oxytocin apparently allows an exchange of ions and small molecules (including lactose) to take place between the aqueous phase of milk and the extra-cellular fluids. This may exaggerate or underestimate lactose content and may influence the amount of minerals found in the milk (i.e. cause trace minerals to be reported when in fact minerals are present) (Katoaka *et al*, 1972).

Fortunately there are numerous studies of pinniped milk composition that support the results, there are trace amounts of lactose and minerals present in sea lion milk. The NZ sea lion recorded trace amounts of carbohydrate. This is similar to other studies such as the Northern fur sea which also reported trace amounts of carbohydrates (Arnould *et al*., 1975)

In regards to potential errors in sampling methods, efforts to replicate suckling were made when taking samples. Ideally milk sampling should replicate normal suckling behaviour both in the interval allowed for milk to accumulate before sampling and in the amount of milk removed at milking. Though this did not take place, the teats of the mammals were carefully massaged and efforts to evacuate the mammary gland as completely as possible were made.

Though there is potential for numerous external influences such as food availability, environmental factors and foraging status of the mother, a very likely explanation to the range of fat percentages can simply be linked to the genetic characteristics of the individual. Such findings are present in different breeds of cattle. Guernsey and Jerseys produce milk averaging higher in fat and exhibiting greater variability among individuals than do Holsteins, Fiesians and Ayrshires (Jenness, 1974). Perhaps some individual sea lions are more inclined to convert more of its energy into milk fat than others.

Fat is a valuable source of energy to both the mother and her young. Without appropriate energy sources the fate of mother and young is threatened. It is therefore important to further investigate the nutritional status of the mother and young, evaluate the health of the N.Z. sea lion population and eliminate or reduce threatening factors to their survival.

Chapter 3

3. Abstract

Lipids provide a major source of energy and essential structural components of the cell membranes of newborn mammals. The energy values of medium- and short-chain fatty acids are considerably lower than those of longer-chain fatty acids. The New Zealand sea lion has 47% mono-unsaturated and 34% saturated (long-chain) fatty acids. The most prevalent fatty acid found in the milk of the N.Z. sea lion are C16:0 (22.5%), and C18:1(n-9) (26.81%). The remaining fat consisted of mainly C14:0 (5.8%), C16:1 (6.6%), C18:0 (3.2%), C18:1(n-7) (4.9%) C20:1(n-9) (7.6%) and C22:6(n-3) fatty acids. The most common fatty acids found in Krill (*Euphausia superba*) are also found in high percentages in N.Z. sea lion (i.e. C16, C18:1(n-9) and C22:6(n-3) (Fricke et al., 1984). There was a relationship between the percentage fat and the fatty acid composition.

3.1 Introduction

Lipids provide a major source of energy and essential structural components of the cell membranes of newborn mammals. They are small molecules that have a strong tendency to associate through noncovalent forces and are characterised by a hydrophilic (polar) head connected to a hydrophobic (nonpolar) tail (Masaro, 1968).

The simplest lipids are the fatty acids, which are constituents of many more complex lipids. There are two types of fatty acids, saturated and unsaturated. In saturated fatty acids the carbons of the tail are all saturated with hydrogen atoms. Unsaturated fatty acids contain one or more double bonds. Most naturally occurring fatty acids have an even number of carbon atoms (Mathews and Van Holde, 1996).

Fatty acids are very extraordinarily efficient for energy storage because they contain carbon in a fully reduced form and therefor yield a maximum amount of energy on oxidation. Storage of fatty acid in organisms is largely in the form of triacylglycerols or fats (Mathews and Van Holde, 1996). Triacylglycerols are the major lipid class found in all species studied to day accounting for 97-98% of the total lipids (Chrisite, 1994). They are derived from three primary sources, 1. The diet; 2. *De novo* biosynthesis; and; 3. Storage depots in adipocytes (Davies et al., 1983; Mathews and Van Holde, 1996).

There are numerous other components in milk such as phospholipids, glycerolipids, sterols, hydrocarbons, lipid soluble vitamins and prostaglandins. Phospholipids, which are found mainly in the milk fat globule membrane, are a small but important fraction of the milk (Morrison, 1968). Glycerolipids have distinctive fatty acids or other alkyl constituents that affect the chromatographic properties and permit separation. Lipid and fat-soluble compounds are present in small amounts but include several components of potential physiological importance to the newborn including hormones and vitamins and organoleptic properties of milk.

Lipid soluble vitamins A, D, and E which have great nutritional importance for the newborns is found in milk, along with prostaglandins E and F and carnetine, an essential cofactor for the oxidation of fatty acids in animal tissues (Bannon et al., 1986; Jensen *et al.*, 1991; Christie, 1994; Iverson et al., 1997)

The major sterol component (at least 95%) of most milk is cholesterol. Small amounts of other sterols and a number of steroidal hormones, such as progesterone, oestrogens and corticosteroids, have also been found in milk of various species (Davies et al., 1983).

The composition and content of lipids from milks of different species vary with such factors as diet, stage of lactation and environment. The fatty acid composition depends on species type, genetic factors, nutritional and lactional factors (see Table 3.1.1). Ruminants have short-chain fatty acids such as butyric and hexonoic acids, which are rarely found in the milk of non-ruminants (Davies et al., 1983; Glass and Jenness, 1967). Medium-chain fatty acids are present in the milk of many disparate groups of animals. Unlike ruminants most non-ruminants obtain and absorb considerable amounts of polyunsaturated fatty acids such as linoleic and linolenic acid (18:3(n-3)) (Christie, 1994; Glass and Jenness, 1967). Marine animals that subsist on a diet of fish and invertebrates such as krill tend to contain a high proportion of C20 and C22 fatty acids and relatively low proportions of C18 polyunsaturated fatty acids; hence the presence of these fatty acids is related to the diet of the sea lion (Ackman et al., 1971; Adams et al., 1997).

Few studies have examined the fatty acid composition of pinnipeds and no studies have examined the fatty acid composition of the milk of the New Zealand Seal lion. The following study examines the fatty acids found in milk from seventeen different sea lions over a three-year period (1999-2001).

Table 3.1

The fatty Acid Composition of milk fats from milk taken from various species.

T= trace; - = not detected, measure in mol% of fatty acids.

Species	4:0	6:0	8:0	10:0	12:0	14:0	16:0	16:1	18:0	18:1	18:2	18:3	18:3 +	Reference*
Seal	-	-	-	-	-	5	23	21	2	35	2	1	13	Anderson et al. (1971)
Monkey	-	7	9	8	2	2	20	1	5	27	18	-	-	Glass et al. (1967)
Man	-	-	-	2	4	6	21	6	5	42	12	1	-	Glass et al. (1967)
Mouse	-	-	T	6	9	13	33	5	2	29	9	-	-	Smith et al. (1970)
Elephant	-	-	13	67	15	1	2	T	-	2	T	T	-	McCrae (unpub.)
Cow	12	4	2	4	4	10	24	2	12	24	2	1	-	Glass et al. (1967)

* Based on Table from Christie (1994).

3.2. Methods

Seventeen NZ sea lion milk samples were selected from a large pool of sea lion milks. Samples were picked based on their percentage fat previously determined. Five to six samples were selected from a pool of samples with high fat percentages, average fat percentages and low fat percentages. Once samples were selected they were thawed and stirred. Using Gas Chromatography fatty acid profiles were determined (see Appendix II). Once profiles were made the data was put into an excel spread-sheet. The data were analyzed using Microsoft excel and Minitab statistical program.

3.3 Results

The fatty acid composition of seventeen carefully selected animals indicate that C16:0 (22.5%) and C18:1(n-9) (26.8%) are the most present fatty acids in sea lion milk (see Table 3.1). The remaining fat primarily consists of C14:0 (5.8%), C16:1 (6.6%), C18:0 (3.2%), C18:1(n-7) (4.9%) C20:1(n-9) (7.6%) and C22:6(n-3) fatty acids. Fig. 3.1 illustrates the distribution of each of the above fatty acids for the seventeen individuals sampled. The majority of the fatty acids fit a normal shape curved.

Mono-unsaturated fats comprise 47% and saturated 34% of the total fatty acid composition (see Fig. 3.3 (a) and (b)). There is a significant relation ship between the total fat composition and the proportion and frequency of fatty acids present. The higher the total fat percentage, the higher the frequency of fatty acids and the proportion of fatty acids (see Table 3.3).

The fatty acids found in high percentages in N.Z. Krill are also found in high percentages in sea lion milk. For example C16 is 23% of the total fatty acids in NZ sea lions and 27% for krill; and C18:1(n-9) is 27% of total fatty acids in NZ sea lion and 16% for Krill (Fig. 3.2). Furthermore many of the same fatty acid are found in the milk of other species such as the Antarctic fur seal and Elephant seal (Table 3.2). For example C18 is found in NZ sea lions (3.15%), Antarctic fur seal (2.12%), Hooded seal (2.8%), Crab eater seal (1.86%) and Elephant seal (4.4%). (Adams, 1997; Jangaard and Ke., 1968; Green, 1993; Fayolle, 2000; Fricke et al., 1984).

Table 3.1:

Fatty acid composition of milk fat from N.Z. sea lions. A total of 17 samples were analysed using GC methods. Results are displayed in percentage of total fats found. Unknown represents unidentifiable fatty acids. Fatty acids with notable fat percentages are present are in bold.

Fatty Acid	Percent fatty acid
C4:0	0.03
C6:0	0.06
C8:0	0.00
C10:0	0.02
C10:1	0.00
C12:0	0.15
C12:1	0.003
C13:0 Br	0.01
C13:0	0.05
C14:0 Br	0.00
C14:0	**5.79**
C14:1	0.19
C15:0 Is	0.23
C15:0 an	0.013
C15:0	0.58
C16:0 Br	0.09
C16:0	**22.51**
C16:1	0.56
C17:0 Is	0.34
C17:0 an	0.18
C17:0	0.48
C17:1	0.60
C18:0	**3.15**
C18:1n-9	**26.81**
C18:1n-7	4.9
Unknown	0.12
C18:2n-6	1.25
Unknown	0.22
C18:3n-3	0.43
C18:2 cn	0.01
C18:4n-3	0.56
C20:0	0.07
C20:1n-1	0.61
C20:1n-9	**7.56**
C20:2n-6	0.29
C20:3n-6	0.053
C20:4n-6	0.73
C20:3n-3	0.21
C20:4n-3	**1.09**
C20:5n-3	**3.96**
C22:0	0.000
C22:1n-1	**1.24**
C22:1n-9	0.66
C22:4n-6	0.00
C22:5n-6	0.00
C22:5n-3	1.56
C24:0	0.02
C22:6n-3	**6.29**
C24:1	0.36

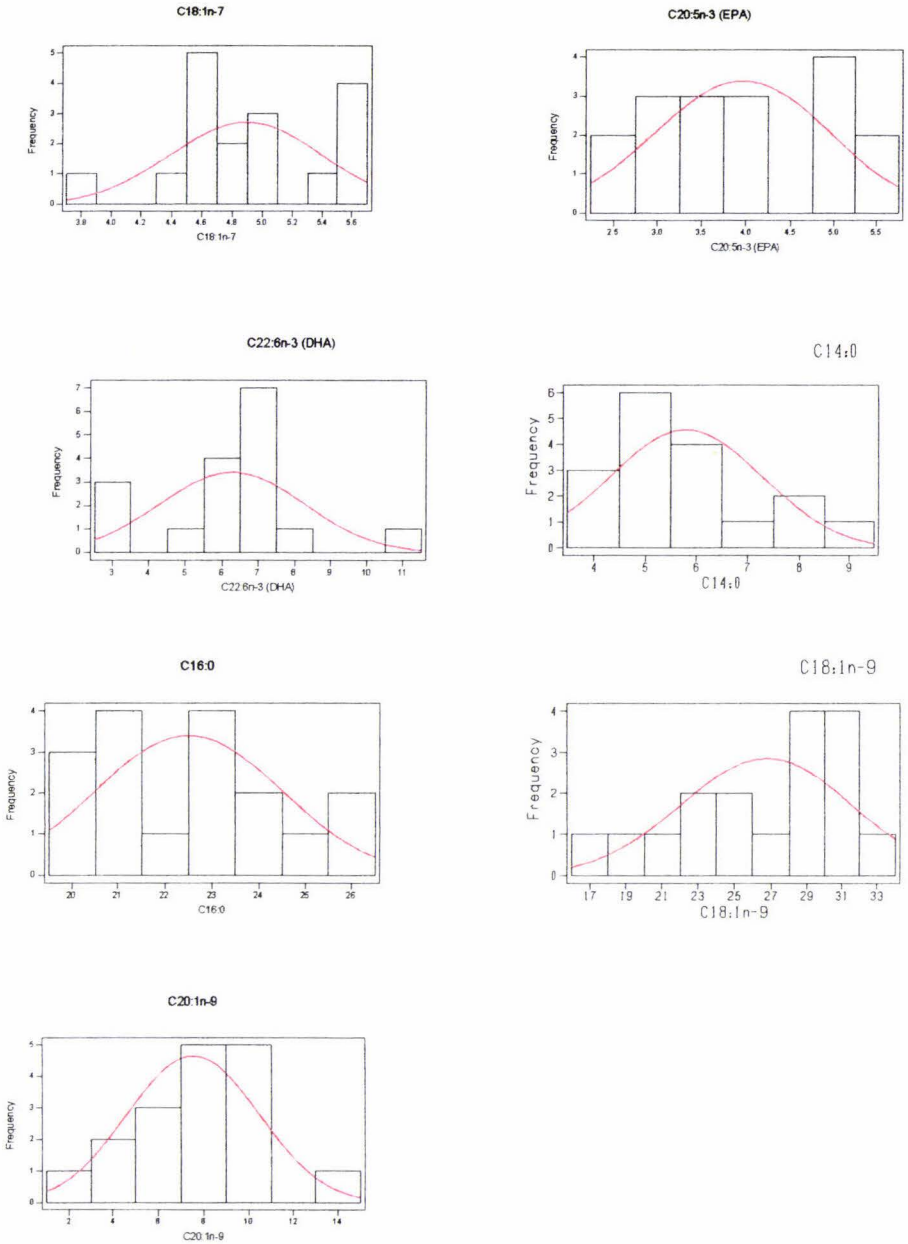


Fig. 3.1

Histograms of the 7 most common fatty acid (C20:1n-9, C16:0, C14:0, C18:1n-9, C18:1n-7, C20:5n-3, C22:6n-3) from 17 randomly selected samples of N.Z. sea lion milk (x-axis is the percentage of the fatty acid present ; y-axis is the frequency of animals that had that percentage of fatty acid present).

Fig.3.3(a)

Box-plot, where the box represents the 25th and 75th quartile and the whiskers are the range, of saturated, mono-, and poly-unsaturated fatty acids in the N.Z the milk of 17 randomly selected N.Z. sea lions.

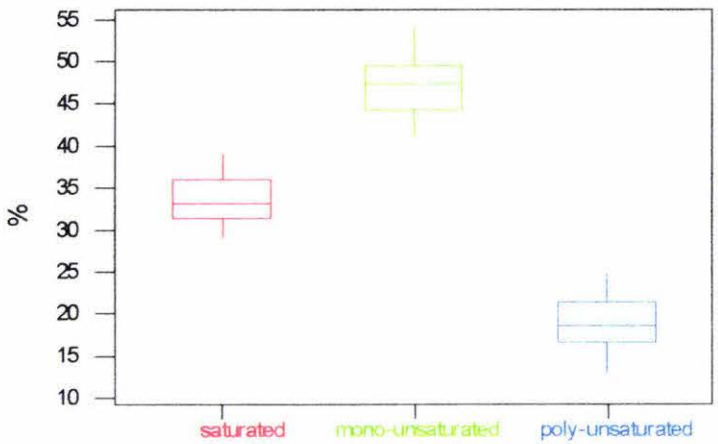


Fig. 3.3(b)

Percentage of saturated, mono- and poly-unsaturated fatty acids found in the milk of the N.Z. sea lion.

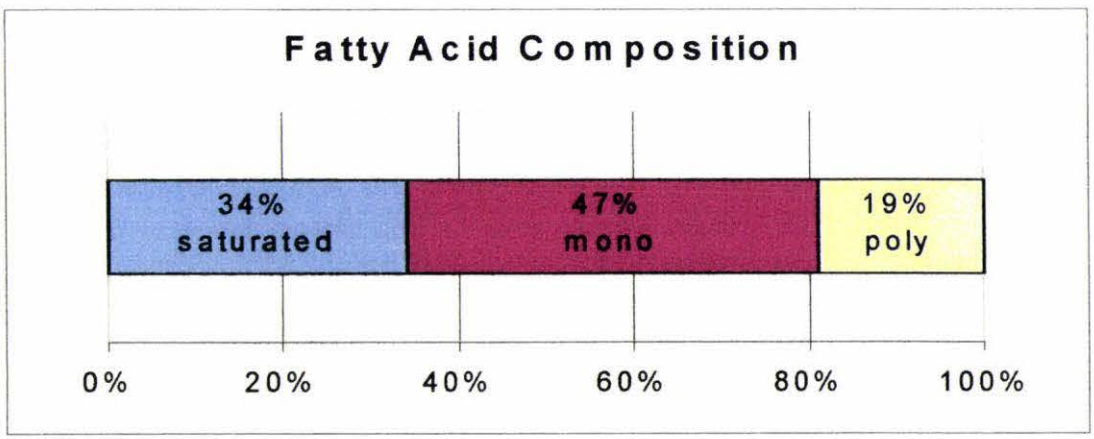


Table 3.2:

Fatty Acid Composition N.Z. Sea lion milk, krill (*Euphausia superba*)and some Phocid and Otariid seals (data in percentages).

	N.Z Sea Lion	Antarctic Fur Seal (Adams, 1997)	Hooded Seal (Jangaard and Ke 1968)	Crabeater Seal (Green, 1993)	Elephant Seal (Fayolle, 2000)	Krill (Fr icke et al. 1984)
C16	22.5	-	9.5	15.11	16.75	25.9
C18	3.15	2.12	2.8	1.86	4.4	1.2
C20	0.07	-	-	-	-	-
C24	6.29	-	-	-	-	-
C18:1n-9	26.81	29.86	-	18.08	-	10.1
C20:5n-3	0	4.77	6.8	10.37	-	12.7
C22:5	-	-	-	-	1.1	-
C22:6	-	10	-	-	4.15	-
C22:6n-3	0.35	7.58	3.9	8.7	-	5.4

Fig. 3.2:

Comparison of major fatty acids in the N.Z. Sea Lion and Krill. Data for krill represents an average of 7 values obtained from the literature for whole krill (Body, 1983; Lee et al., 1991; Fricke et al., 1984; Virtue et al., 1993).

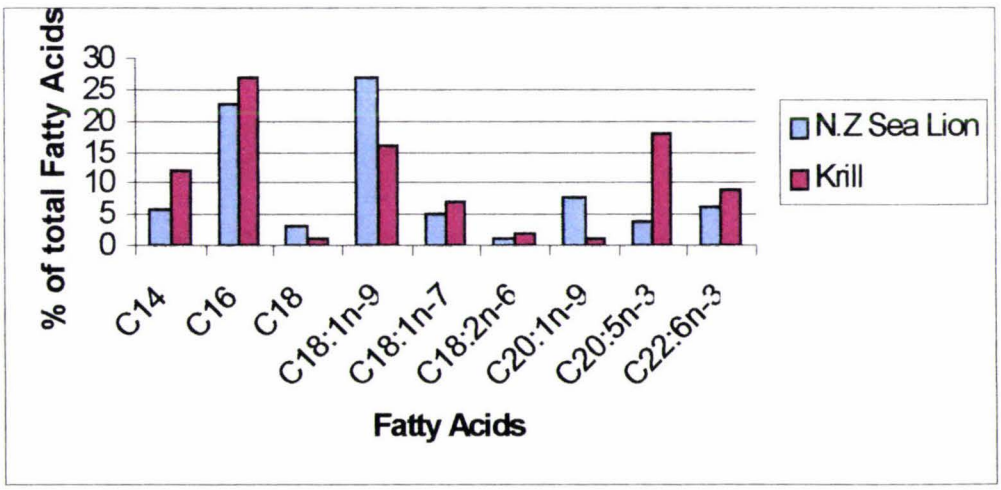


Table 3.4:

Relationship between the percentage of fat in the milk of the N.Z. sea lion and the fatty acid composition (p is significant if p> .456)

Fatty Acid	P-value	Fatty Acid	P-value
C12:1	0.572	C18:4n-3	0.551
C14:1	0.465	C20:0	0.747
C15:0	0.772	C20:1n-1	0.639
C16:0	0.849	C20:1n-9	0.851
C17:0 iso	0.56	C20:2n-6	0.724
C17:0	0.887	C20:3n-6	0.805
C17:1	0.772	C22:1n-9	0.669
C18:1n-9	0.965	C24:0	0.572
Unknown	0.982	C22:6n-3	0.512
		C24:1	0.475

3.4 Discussion

Mono-unsaturated fats make up 47% of the total fatty acid composition (Fig. 3.2 a & b). Mono-unsaturated fatty acids are found in the highest proportions when compared to saturated fatty acids and poly-unsaturated fatty acids. For example, C19:1n-9 makes up 26.81% of the fatty acid profile. Additional mono-unsaturated fatty acids are found in the following porportions: C20:1n-9 (7.56%), C22:6n-3 (6.3%) and C18:1n-7 (4.9%).

Saturated fatty acids make up 34% of the total fatty acid composition (Fig 3.2b). Twenty-two and half percent of the total fatty acid profile consists of C16:0 and 5.79% of the total profile consist of C14:0 . However most other saturated fatty acids comprise less than .07% of the total fatty acid profile. Only 19% of the total fatty acid composition is made up of poly-unsaturated fats.

Food preference in carnivores can be examined using fatty acid profiles (FA). This technique can be particularly useful for examining food preferences in animals such as the N.Z. sea lion for which stomach contents or observations of feeding are difficult to obtain. In monogastric animals, dietary fatty acids remain intact during digestion and are deposited into blubber stores or mobilized for milk production in a relatively unchanged form (Adams et al., 1997).

Lipids in the marine food chain are characterized by a complex and unusual array of fatty acids, which have been found to occur in specific relationships depending upon the prey species and their geographical location (Ackman et al., 1971; Iverson et al., 1993), and have direct effects on the fatty acid patterns of their predators (Iverson et al., 1997). In rapidly fattening seals, ingested fatty acids appear to be deposited in adipose tissue in proportion to their intake, so blubber tissue may be a mirror for the diet (Iverson et al., 1997). In lactating females, milk fatty acids are derived largely from blubber mobilization during fasting, but are likely derived primarily from the diet when the animal is feeding (Iverson et al., 1992; Iverson et al., 1993; Iverson et. al., 1997). As a result, in lactating otariids, milk secreted in between foraging trips should reflect the dietary intake during the lactation period (Iverson et al., 1997). The milk samples taken for this study were taken in January and early February thus represent a period in lactation when females are feeding young in between foraging trips.

The N.Z. sea lion are opportunistic feeders and subsist on a diet of mainly fish, krill and other mollusks (Cawthorn et al., 1985). Deep sea pelagic fish species such as the alfonsino (*Beryx splendens*), cardinal fish (*Epigomus* sp.), and javelin fish (*Lepidorphynchus denticulatus*) and Antarctic krill (*Euphausia superba* Dana) have fatty acid profiles similar to that found in N.Z. sea lion milk (Adams et al., 1997; Body, 1983). The lipid concentrations in pelagic fish examined by Body (1983) indicate that the most prominent fatty acids are 16:0, 18:1, and 22:6. Similar fatty acids are prevalent in krill, with the major fats being 14:0, 16:0, 16:1(n-7), 18:1(n-9), 18:1(n-7), 20:5(n-3) and 22:6(n-3). (Vileg *et al.*, 1993; Body, 1983). In comparison, the major fatty acids found in the milk of N.Z. sea lions are 16:0, 18:1(n-9), 20:1(n-9), 22:6(n-3). (see Table 3.2)

A general feature of maternal strategies in otariids is the deposition of moderate sized-energy stores as blubber prior to parturition in order to support milk secretion during the first week post partum. Subsequently, females make intermittent foraging trips to sea during which body stores are replenished to support continued milk secretion (Gales and Mattlin, 1997).

Numerous studies such as Iverson et. al (1997) demonstrate significant changes in the fatty acid composition of seal's milk across lactation stages. When a female sea lions comes ashore to give birth, her blubber stores will represent an integration of dietary history, but will probably be most influenced by the previous months of fattening in advance of parturition (Iverson et al., 1993; Iverson et al., 1997). During the initial prenatal fasting period, these blubber lipids will be mobilized to largely support milk fat secretion, however when the female begins foraging trips, dietary fatty acids will probably be directed first to mammary gland by lipoprotein lipase (Iverson et. al., 1997), second to blubber stores that are being replenished, and ultimately again to the milk during several-day suckling bouts, when blubber is again mobilized. The samples for this study were taken during the same stage of lactation in January and February each year. Hence an appropriate continuation of this study would be to examine the milk content during different stages of lactation, thus study changes in foraging diet during lactation.

This study is an introduction to the fatty acid profile of the N.Z. sea lion. Based on 17 individual sea lion samples, forty-seven percent of the sea lion milk consists of mono-unsaturated fatty acids and 34% saturated fatty acids. Many of the most prevalent fatty acids (such as 16:0, 18:0 and 18:1(n-9)) found in the N.Z. sea lion were common in other studies of otariids as well as phocids such as the Antarctic fur seal, Harp seal, Hooded sea and Crabeater seal. Finally, the N.Z. sea lion fatty acid profile highlights the same fatty acids found from previous studies on the fatty acid signature of Antarctic Krill. Further studies that compare the sea lion fatty acid signature to other potential marine prey of the N.Z. sea lion will enhance our understanding of the feeding ecology of the N.Z. sea lion.

Chapter 4

4. Abstract

Proteins are found in all mammalian milk and contribute significantly to the nutritional requirements of neonates. Most of the non-nutritional functions of milk are served by proteins and peptides, which include immunoglobulins, enzymes, enzyme inhibitors, binding or carrier proteins, growth factors and antibacterial agents (Fox and Flynn, 1992)

Preparative methods for bovine milk using gel electrophoresis, with dilution alterations accommodating total protein percentages, proved adequate for the separation of whey and casein from the milk of 31 N.Z. sea lions. There were nine identifiable proteins present on all 23 gels and two additional unidentified bands found on two gels. The unidentified proteins were not a result of phosphorolation or poor testing techniques.

There are two major groups of proteins, caseins and whey proteins, and some minor components such as enzymes and the proteins which form an integral part of the milk fat globule.

Proteins comprise approximately 10% of N.Z. sea lion milk, 37% of which are Caseins 29.1% are Whey. *Alpha, beta* and *kappa* caseins had molecular weights of approximately 2300 and beta and alpha lacto-globulin whey proteins had molecular weights around 1800. These proteins were present in all 31 samples.

4.1 Introduction

Proteins are found in all mammalian milk and contribute significantly to the nutrition requirements of neonates. Most of the non-nutrition functions of milk are served by proteins and peptides, which include immunoglobulins, enzymes, enzyme inhibitors, binding or carrier proteins, growth factors and antibacterial agents (Fox and Flynn, 1992). There are two major groups of proteins, caseins and whey proteins, and some minor components such as enzymes and the proteins that form an integral part of the milk fat globule (Hambraeus, 1992)

Caseins are a group of phosphoproteins which are specific to milk. There are four different designated caseins found primarily in cow's milk but also in other species which include alpha, beta, gamma and kappa caseins. Most of the caseins found in milk of species studied in detail exist in colloidal micelles. Both beta and alpha proteins are insoluble at 36°C (Fox and Flynn, 1992). These caseins are most probably present in sea lions milk as well.

Whey proteins remain in solution when milk is acidified to around pH 4.6 at 20°C. This distinguishes it from caseins. Types of whey (or serum) proteins include beta-lacto globulins, immunoglobulins, alpha-lacto globulins, proteose-peptones, serum albumin, lactoferrin, and transferrin (Mephan et al., 1992)

Both caseins and whey proteins have specific biological roles. Caseins are capable of binding metal ions strongly which results in charge neutralization. Calcium is the principal metal involved; phosphopetides derived from caseins stimulate the absorption of calcium. In addition a substantial portion of the zinc in milk is also chelated by the phosphate residues of caseins and iron is also bound by phosphoserine residues although the influence of this binding on the bioavailability of iron is ambiguous (Fox and Flynn,1992; Hambraeus, 1992).

Whey proteins also have important metal binding properties. Lactoferrin is the most significant of the specific metal-binding proteins. Related proteins are found in the blood of

cetaceans and prochordates. Lactotransferrin binds iron very strongly which indicates two roles for this protein: iron absorption and protection against enteric infection in the neonate. Beta-lacto globulin is capable of binding several types of hydrophobic molecules. Furthermore Beta globulin may be involved in phosphorus metabolism. Finally, immunoglobulins are vital for the protection of neonates from the influence of infection and toxic agents (Fox and Flynn, 1992; Hambraeus, 1992; Mephan et al., 1992)

Proteins make up four to eighteen percent of the total solids of N.Z. sea lion milk (the remaining composition consist of water and fat) and they play a significant role in the nutrition and functional requirements of neonates (see chapter two). Numerous studies examined the total composition of milk of seals and sea lions however very few studies have examined the specific proteins found in Otariids. The following study is an introduction to the specific proteins found in N.Z. sea lion milk

4.2 Methods

Thirty-one sea lion milk samples were examined using the sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) method. The SDS-PAGE method is a good general method for quantifying whey proteins and caseins. It is used extensively in biochemistry to gain information about the approximate weights of proteins in solution. The samples were run using the standard procedure for Gel Electrophoresis (see Appendix III). The only alteration made to the standard procedure for sea lion milk, was in the sample preparation. Between 14 and 48 micro-litres of sample were taken. The amount of sample taken was determined by the overall protein percentage found in the sample. Samples were then diluted with the appropriate amount of Buffer (see Appendix III for complete detail of sample preparation). Once gels were complete they were scanned into the computer and protein bands were analyzed using the appropriate computer program.

4.3 Results

A total of ten bands appeared on the SDS-PAGE gels. Eight bands were present on all lines of all gels, however ten bands appeared on two lines of two different gels. The extra bands are bands 6 and 10 in Fig. 4.1. An estimated molecular weight for some of the bands was determined by comparing the sea lion milk protein bands to those of bovine milk. It is estimated that bands 3, 4, and 5 have a molecular weight around 2300 and bands 7 and 8 have a molecular weight around 1800. It is hypothesised that bands 3, 4, and 5 are caseins: band 3 is an alpha-protein, band 4 a beta-protein and band 6 is a gamma protein. Band 7 and 8 are hypothesised to be whey proteins (band 7 is beta-lactoglobulin and band 8 is an alpha-lactoglobulin), (see Fig. 4.1 (a) and (b)).

In order to determine the average intensity of each protein band a ratio (protein band : total protein composition) was determined for each band of each individual and then the mean for each, for all 31 samples was calculated. Band three, six and seven were the most intense bands resulting in 35.35%, 15% and 12.89% respectively of the total percentage of proteins present (see table 4.1).

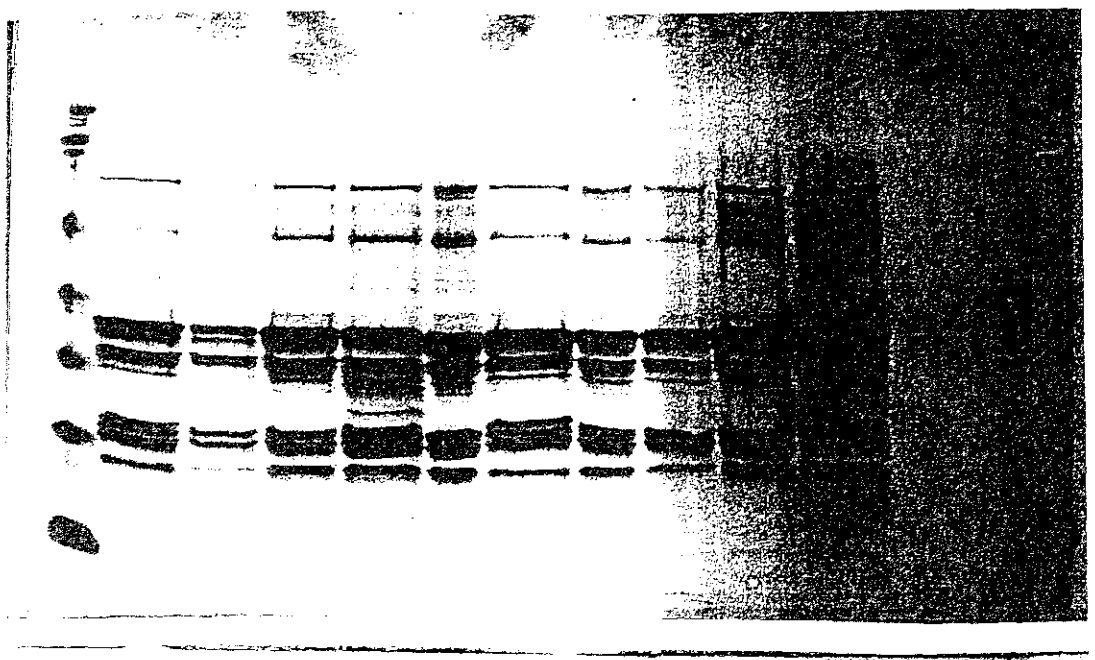
The relationship in band intensity and the total percentage of proteins found in NZ sea lion milk indicates that bands with the highest intensity also have the highest composition of protein in the milk (Fig. 4.2). There seems to be a direct relationship between band intensity and total protein percentage. Band 5 and 7 had a band intensity of 15% and 12.89% respectively and have the highest total proteins present (16% and 15% respectively). Furthermore, band 10 has the lowest band intensity of 3.93% thus has the lowest percentage of total proteins which is approximately 4%.

Correlations were calculated between proteins found in NZ sea lion milk and the following variables: year, body condition, total fat % and total protein %. Correlations were significant between different bands and different variables (see Table 4.2 (b)). Bands 1, 4, 5, 8 and 9 had a significant correlation with total protein percentage. Bands 3, 5 and 8 significantly correlated with total fat percentages. Bands 3, 4, 6, 7, and 9 had a significant correlation with year. Finally bands 3 and 4 significantly correlated with body condition.

Fig. 4.1.

Gel electrophoresis pictures of sea lion milk samples. Each Gel had a molecular weight indicator in cell 1. A standard of sea lion milk from animal 1034 ran in cell 2 or 3 of all the gels. Fig. 4.1 (a) is an example of the nine typical bands found on all the samples. Cell 3 and 7 in Fig 4.1 (b) are the two sea lion milk samples that resulted in two unidentified bands.

(a)



(b)

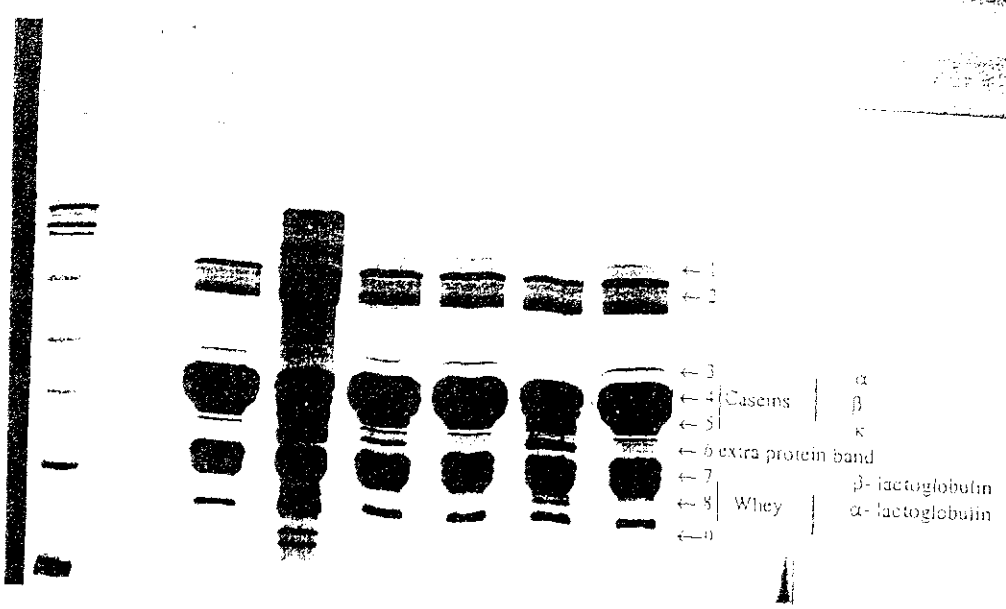


Table 4.1

Descriptive statistics for individual protein bands found in the N.Z. sea lion. Each band number represents a different molecular weight (1 represents the highest weight and 10 represents the lowest molecular weight). The mean represents the percentage each protein band was present out of the total number of proteins found in the milk.

Band	N	Mean	Median	St.Dev
1	31	6.59	6.98	1.70
2	31	5.08	5.05	1.25
3	31	35.25	36.71	6.46
4	31	9.61	9.79	3.89
5	31	4.40	4.29	1.31
6	31	15	14.85	4.04
7	31	12.89	13.43	5.77
8	31	9.15	8.01	3.41
9	31	6.87	6.16	3.87
10	31	3.93	3.93	2.87

Fig. 4.2

This chart illustrates the intensity of each protein band in relation to % of total protein found in the milk.

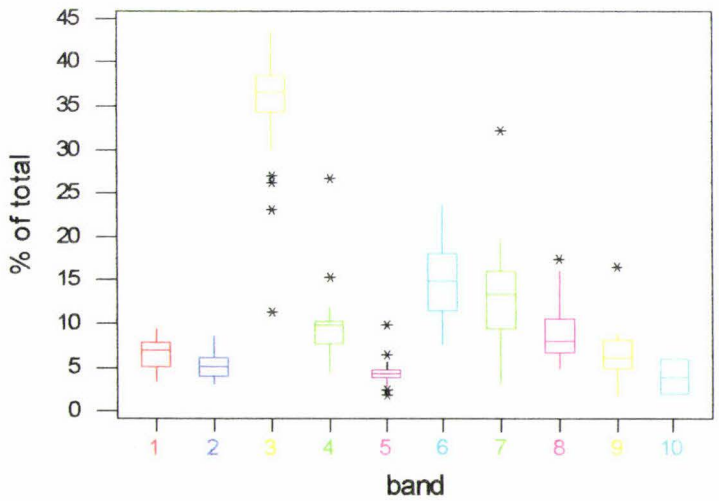


Fig. 4.3

Means (histograms) of total fat and protein composition of the 31 animals used for SDS-PAGE gel protein analysis.

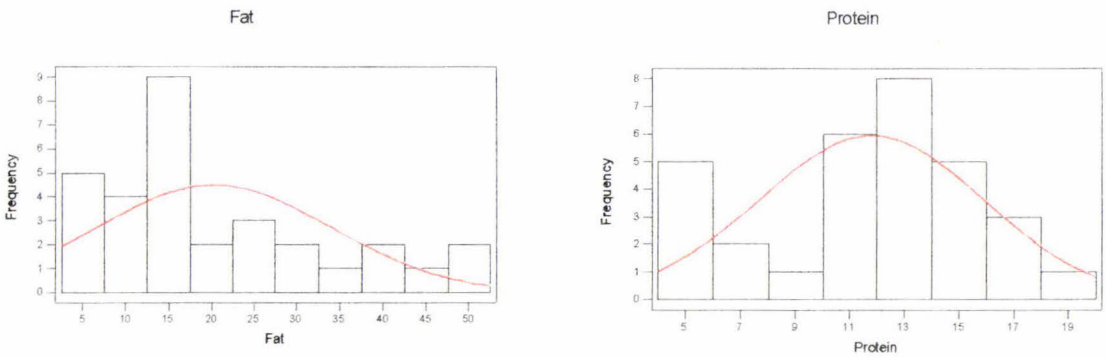


Table 4.2 (a):

Description of the fat %, protein %, and body condition of the31 animals used for protein analysis.

	N	Mean	Median	StDev
Fat	31	20.3	14.7	13.7
Protein	31	11.8	13.2	4.2
Body Condition	19	3.2	3	0.5

Table 4.2 (b):

Correlations between protein bands and: year, body conditions, total fat% and total protein %. (N=31, Significant if p>.394; * = Not significant)

Band	Fat	Protein	Year	Body Condition
1	*	**.489**	*	*
2	*	*	*	*
3	**.712**	*	**.875**	**.525**
4	*	**.902**	**.464**	**.718**
5	**.970**	**.874**	*	*
6	*	*	**.751**	*
7	*	*	**.514**	*
8	**.588**	**.630**	*	*
9	*	**.688**	**.829**	*
10	*	*	*	*

4.4 Discussion

Preparative methods for bovine milk using gel electrophoresis, with dilution alterations accommodating total protein percentage, proved adequate for the separation of N.Z. sea lion whey and caseins. The electrophoresis gels indicate that there were 8 identifiable proteins that were found in all 31 samples, however there were two samples that resulted in two different additional unidentified protein. Due to repeatability and careful sample preparation it was concluded that these samples were not a result of phosphorolation.

Due to the lack of studies in sea lion protein composition there were no studies found on protein composition in otariids that could be used to compare to the N.Z. sea lion protein composition. However in comparison to bovine milk and a Biorad molecular weight indicator that ran on each of the sea lion milk gels, estimates of proteins present in the milk can be made. Alpha, beta and kappa caseins with molecular weights around 2300, and beta and alpha lacto globulin whey proteins with molecular weights around 1800, were present in all samples. A specific molecular weight for each protein band could not be determined in this experiment. However a guesstimate of molecular weights could be determined. Furthermore caseins comprised 37.04% of the total proteins and whey proteins comprised 49.26%.

The diet of an animal effects the composition of their milk (Jenness et al., 1964). The diet of the NZ sea lion consists of fish, crustaceans, krill and other food found in the southern ocean (Cawthorn, 1986). This is a completely different diet to that of bovines therefore it is not suprising that sea lion milk would contain proteins that are not typical found in bovine milk. The sea lion milk was compared to bovine milk strictly because of availability and convenience. It would be more enlightening to compare the proteins found in sea lion milk to other marine mammals. This milk however was not available.

The signifcance of band intensity relates to the overall presences of a particular protein. If a band is more present then there is more of that protein present in the sample. This could relate to diet, in the sense that if an individual ate a certain fish or crustacean then this could possibly increase the intensity of a certain band. In contrast if an individual

did not eat this same fish or crustacean then the band could be non existent or simply at a low intensity. It is possible that the most intense bands found in this experiment (bands 3, 7, and 8) are the most important proteins needed by sea lions thus it is the most intense band present. When the band intensity is compared to the percentage of total protein found in the sea lion milk, it is concluded that the greater the percentage total the greater the band intensity. For example band 6 has a average intensity of 15% and it has an average of 16% total protein present. Perhaps the intensity of a band is simply related to the overall presence of total proteins in the individual.

Some bands showed a significant correlation with one of the following variables (total fat %, total protein %, year, or body condition). The reasons behind these correlations could not be determined in this experiment. However, these results deserve further investigation and could explain the variation in band intensity and the presence of particular proteins in different individuals.

Protein comprises approximately 10% of the total milk composition of sea lions and is essential for specific nutritional and physiological functions (Hambraeus, 1992). From a strictly nutritional view point, proteins are used for the transport of certain nutrients as well as the metabolism of other metabolites of nutritional interest. Their role in the defense mechanisms against infections is, however, not essentially a nutritional problem although there is a close relationship between malnutrition and infection (Hambreaus, 1992). The relatively high protein composition in N.Z. sea lion milk indicates it has significant nutritional value. Further investigation on the latter and additional studies investigating the relationship between the protein composition and its relation to the mother and pups health status are needed.

The overall objective of the above experiment was to test the potential of using SDS PAGE gel elctrophoresis with sea lion milk. In addition it was set up to investigate the possible proteins present in NZ sea lion milk. The results of this experiment are not concrete therefore it is important to recognize that they are simply an introduction to protein analysis of sea lion milk. This does not represent a thorough study on proteins in NZ sea lions.

Conclusion

Methodology

The FTIR Milko scan proved to be an efficient, quick and reliable method for determining milk composition in N.Z. sea lion milk. The traditional methods, such as Rose-Gotolieb and ASE for fat composition and Kjeltec for protein, used to analyse milk composition require a significant amount of time to prepare and process the samples. Thus to analyse 100 samples would take many days. The Milko scan overcomes this problem by allowing scientist to analysis 100 samples in just 200 minutes. Furthermore, by using the Milko Scan a scientist can minimize the volume of sample used i.e. this experiment used only 1g of sample. Prior to this study the Milko Scan had not been used to study milk composition of any other mammal other than bovine milk. This technological advancement introduces a new method for analysing large numbers and low volumes of mammalian milk.

Milk Composition

The results of the total composition of the sea lion milk were interesting and prompted many questions. When compared to other pinnipeds the average fat, 21.27%, and protein 9.97% for 321 animals remained at a healthy level. However the range or difference among individuals sampled during same years was quite overwhelming. Fat ranged from 4.2% to 52.07% and protein ranged from 4.11% to 18.9%. The reasons behind this discrepancy could not be determined in this study and thus would be an important study for the future. There are numerous questions that arise from this result, such what was the overall health of the animal and what was its feeding behaviour prior to being sampled?

The average fat percentage declined form 24.08% in 1999 to 11.02% in 2002. Once again the reasons for this decline are unknown, however there are numerous potential explanations (such as environmental or behavioural reasons) for this decline. Continuing

studies should focus on the behaviour, feeding ecology and environmental threats of the N.Z. sea lion. Declining fat percentages could be an effect of decreasing prey for the sea lions, which is a result of competition between sea lions and man or it, could be a result of an environmental disaster such as global warming. In addition to studies on the feeding behaviour and ecology of the sea lion it is important to collect more detailed life history information on each individual animal. Measurements such as weight, length, girth and radio tagging and daily monitoring of individuals would be useful when trying to predict the relationship between fat percentages and individuals. Finally a study that examines the composition of milk of the N.Z. sea lions over its entire lactation period would also help to determine the overall health of the sea lions.

Fatty Acid Analysis

As expected the fatty acid composition of the N.Z. sea lions that were selected for sampling consisted of primarily unsaturated fatty acids (47%). The most prevalent fatty acids included C16:0 (22.5%) and C18:1n-9 (26.81%) Furthermore these fatty acids are also found in Krill and most fish. This fatty acids study can be taken to a much higher level. The exact diet of the sea lion can be determined using the fatty acid composition of the milk and comparing it to the fatty acid composition of fish and krill found in the feeding area of the N.Z. sea lion. This is a much more advanced and accurate method of analysing diet of mammals than the traditional scat analysis.

Protein Analysis

Preparative methods for bovine milk using gel electrophoresis, with dilution alterations accommodating total protein percentages, proved adequate for the separation of whey and casein found in N.Z. sea lion milk. Thirty-one sea lion milk samples were used using the SDS PAGE method. The gel electrophoresis results, presented nine identifiable proteins and two additional unidentifiable proteins. In order to determine the exact molecular weights of these proteins additional experiments using Mass Spectrometry is necessary. Additional research that analysis the proteins of the N.Z. sea lion milk in more detailed would provide a more complete picture of the biochemistry of mammalian milk.

Proteins contribute significantly to the nutritional requirements of neonates, thus the more known about the protein composition in sea lions the more accurate the conclusion will be on their overall health status.

This study was an introduction to the milk composition of the N.Z. sea lion. It opens the door to many additional studies and experiments on the biology, ecology and biochemistry of the N.Z. sea lion. Furthermore it introduces new methods that can be used for other mammalian milk studies.

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Appendix I

2.2.1 Materials and Methods for Determination of Fat Content

Determination of Fat Content by The Rose-Gottlieb Extraction Method

Test Principle

The fat content is gravimetrically determined by extraction of the fat from an ammonical alcoholic solution with diethyl ether and light petroleum ether, evaporation of the solvents and weighing of the residue, according to the principle of Rose-Gottlieb.

Aparatus

1. Analytical balance
2. Mojonnier tubes with silicon rubber stoppers (a set of 8)
3. Erlenmyer flaks, (set of 10) 250 mL. These flasks are only to be used for fat analysis.
4. Centrifuge - which mojonnier tubes can be spun.
5. Evaporation apparatus - system enabling solvents and ethanol to be evaporated from Erlenmeyer flasks after extraction. The water bath is to be kept at a temperature not exceeding 70 °C. This takes place in a fume cupboard in the solvent room.
6. Electric drying oven; set to operate at 102°C ± 2°C and adapted with ventilation parts for use with solvents. The oven must be fitted with a calibrated thermometer of which the temperature should be read and recorded before use daily.
7. Vent bench for extraction procedures.
8. boiling chips (to facilitate boiling).
9. Water bath - for warming of powder solutions 65°C.

Reagents

1. Ammonia solution - 25% m/v (density 0.91 kg/L)

2. Ethanol - >94%, redistilled in all glass system.
3. Diethyl ether - peroxide free.
4. Light petroleum ether - boiling range between 30-60°C.
5. Congo red indicator 1% m/v in water.
6. NaCl 0.5% m/v - used as a diluent.

Procedure

I. Erlenmeyer Flasks

1. After washing in detergent, rinsing with water and ethanol, 5 boiling chips are added to the 250 mL Erlenmeyer flasks and dried in an oven at 102 ± 2°C for 1 h. (these flasks are numbered 1-8) for samples, and also include a solvent blank and a humidity blank).
2. Remove from oven and cool on shelves for 1h.
3. Accurately weight, cooled flasks on 4 decimal place balance (including blanks and record the weights. The solvent blank and humidity blank weights must be indicated as the first weight.

II. Mojonnier flasks or tubes

1. Sets of 8 clean tubes are removed from the oven to cool to room temperature. These are then placed into corresponding numbered segments in the Mojonnier bucket before the weighing in of samples.

III. Sea Lion Milk Samples

1. Bring sample to 20°C. Mix thoroughly by repeated inversion to ensure a homogeneous mixture of the fat throughout the sample. Do not agitate vigorously as this causes the sample to be aerated and may result in the churning of fat.
2. Invert bottle of prepared sample and immediately weigh into Mojonnier tube. This is done on a 2 decimal place balance and the weight of sample is recorded.
3. Add 2 mL of ammonia (by auto pipette) and mix well.

IV. Extraction Procedures

1. Add 10 mL of ethanol by dispenser (2 dispensing of 5 mL). Mix gently.
2. Add 25 mL diethyl ether 9by dispenser) mix gently and then close Mojonnier tubes (using stopper wettened by H_2O). Mix with inverting for 1 min.
3. Add 25 mL diethyl ether to solvent blank flask Erlenmeyer flask) (see blank determination procedures).
4. Remove stoppers carefully and rinse stoppers into Mojonnier tubes using mixed ethers. Add 25 mL of petroleum ether (by dispenser) add 1 drop of 1% Congo red indicator. Replace rubber stoppers and mix by inverting for 30 seconds.
5. Add 25mL Petroleum Ether to solvent blank flask (Erlenmeyer flask).
6. Centrifuge for 2 min in centrifuge with the setting of 600 rpm.
7. Remove stoppers, rinsing them and the inside the neck of the flasks with a few mL of petroleum ether.
8. Carefully transfer as much as possible of the supernatant layer to the corresponding numbered erlenmyer flasks. Rinse the neck of the Mojonnier flask with a few mL of petroleum ether at completion of transfer.
9. Add a further 5 mL of ethanol to Mojonnier tubes and mix.
10. Add 25 mL diethyl ether and 25 mL petroleum ether. Stopper tubes and shake/mix with inverting for 30 seconds. Repeat procedures 6, 7, and 8.
11. Add 25 mL of each ether to the solvent blank flask (Erlenmeyer flask).
12. Add a further 25 mL diethyl ether and 25 mL petroleum ether, stopper tubes, shake/mix for 30 seconds and repeat procedures 6,7, and 8. Rinse the neck of the Erlenmeyer flask with petroleum ether to ensure no fat is left.
13. Add 25 mL of each ether to solvent blank flask (Erlenmeyer flask).
14. Erlenmeyer flasks are then placed in heated waterbed in the solvent room where the solvent is carefully evaporated off
15. When the solvents are completely distilled off,, they are removed from the water bath with tissues. The external surfaces of the flasks are wiped with a tea towel to ensure that no residue from the water is present. Each flask is then blown with clean filtered air to remove solvent fumes. They are then placed in the oven for 1.5 hours at 102 ± 2°C.
16. Flasks are removed from the oven and cooled for a minimum of 1 hour before reweighing on the same balance used before. Weights are recorded.

IV. Blank determination procedures

A solvent and humidity blank are used in the procedure for calculation of purity of solvent and humidity present.

Solvent Blank

1. At each addition of solvent (diethyl, petroleum ether) to sample solutions, an equal amount of the same is dispensed into the Erlenmeyer flask marked solvent blank. This blank should have representative treatment as the sample solutions and therefore follow the same procedures as the sample flasks (should not exceed 0.5 m in comparison to humidity blank).

Humidity Blank

1. The humidity blank indicates changes in atmospheric conditions and therefore must have nothing added.

V. Calculations of Fat %

Equation (IDF 9C: 1987)

$$\frac{(m1-m2) - (m4 - m3) \times 100}{m0}$$

m0 = sample weight taken
m1 = fat and flask weight
m2 = flask weight
m3 = reagent flask 1st weighing
m4 = reagent flask 2nd weighing

2.2.2 Materials and Methods for determining total solids

Ash Determination - 550°C

Test Principle

The ash content refers to the non-carbonaceous mineral matter, which remains after the sample has been charred by heating , then oxidised at high temperature in a muffle furnace. The ash material consists generally of the calcium, sodium, potassium and magnesium phosphates and oxides. The ash does not contain any of the anions normally found in milk as these are volatilised during the ashing process. Although the ash content does not represent the natural salt system (it includes elements such as phosphorus form the proteins, but excludes volatile minerals) it does reflect the total content of inorganic matters.

Apparatus

1. Balance; reading to four decimal places
2. Bunsen burners
3. Drying dessicator (medium size), silica gel dessicant
4. Drying oven - set at 102 ± °C
5. Fume hood
6. Heat resistant gloves
7. Metal tray
8. Muffle - set at 550 °C
9. Pateur pipettes
10. Silica crucibles - 50 mm diameter
11. Spatula
12. Tongs

Sample Preparation

Mix to get a homogeneous sample.

Liquid sea lion milk sample - invert the sample gently several times before use. For milk and cream; bring the sample to 20°C, mix thoroughly to ensure a homogeneous mixture of fat throughout the sample. Do not agitate vigorously, cool the sample quickly to room temperature.

Procedure

I. Weighing

1. Weigh 1 gram of sample.

II. Drying

1. Once all the samples are weighed out, they are put into the drying oven. The oven is set at 102 ± 2°C, leave overnight.

III. Charring

1. Remove the tray from the oven
2. turn on the gas, and light the Bunsen burners.
3. Turn on the extraction fan and lights.
4. Using metal tongs remove the crucibles from the tray. Place them over the Bunsen burners on the stand directly above the Bunsen.
5. Crucibles are left on the stand to 'charr' off, until no more smoke is emitted from the samples. Take care not to lose any of the ash.
6. Using tongs, remove the crucibles form the heat and place on a fibrolite board.
7. As the fibrolite board fills up, transfer the crucibles to the muffle set at 550°C.

IV. Ashing

1. Start the muffle.
2. Samples are left in the muffle for 5 hours and after this stage should be white (free of carbon).
3. After 5 hours, turn the muffle off.
4. Using long handled metal tongs, remove the crucibles from the muffle. Place them on the fibrolite mat.
5. Leave the crucibles out to cool for 5 minutes.
6. Collect a dessicator.
7. Using tongs put the crucibles into a dessicator, and make sure a good seal forms. Ensure the desicator has enough sealant on the lid.
8. Leave to cool for a minimum of 1 hour.
9. When cooled, turn the tap on the dessicator slowly to release the vacuum seal. Note: opening too fast will disturb the ash.
10. Reweigh the crucibles on the balance initially used using the balance program.

Calculations

$$\%w/w\ Ash = \frac{M_3 - M_1}{M_4 - M_1} \times 100$$

Where:

M_1 = mass of crucible only

M_2 = mass of crucible and initial sample

M_3 = mass of crucible and ashed sample

2.2.3 Materials and Methods for determining mineral content

Elemental Analysis Using Inductively coupled Plasma Optical Emission Spectrometry

Purpose

This method was developed for the analysis of Ca, Mg, Na, K and P in various products using coupled Plasma Optical Emission Spectometry (ICP-OES) (also known as Atomic Emission Spectrometry or ICP-AES).

Test Principle

An acidic solution of a product is prepared by dry ashing followed by dissolution in nitric acid. This solution is aspirated into the ICP-OES using a glass concentric nebuliser.

Apparatus

Varian vista CCD Simultaneous ICP-OES with auto sampler
Auto pipette 1-5mL
Balances weighing 2 and 4 decimal places
Beakers (various sizes)
Fume Cupboard
Muffle furnace controlled to approximately 500°C
Microwave digester, Mars 5
Hot plate, approximately 80°C
Volumetric flasks (various sizes)
Volumetric pipettes (various sizes)

Chemicals

Hydrochloric acid, HCl (37% sp.gr. 1.18) Analar Grade
Nitric acid concentrated, HNO_3 (69% sp. Gr. 1.42) AnalaR Grade
Triton X-100 (laboratory Grade)
Caesium chloride, CsCl AnalaR Grade
Scandium standard (1000 ppm) Aristar Grade
Milli-Q water
Calcium standard (1000ppm) Aristar Grade
Potassium standard (1000ppm) Arsitar Grade
Sodium standard (1000ppm) Arsitar Grade
Phosphorus standard (1000ppm) Arsitar Grade
Magnesium standard (1000ppm) Arsitar Grade
Manganese standard (1000ppm) Arsitar Grade

Reagents Preparation

1 Triton X-100, 0.1% w/v

Triton X-100	1 g
Milli-Q water	approx. 1L

Weigh approximately 1 g of Triton X-100 into the 0.1% Triton reagent bottle. Add approximately 400 mL of Milli-Q water and warm the solution under hot running water until the Triton is in solution. Cool, make up to the mark with Milli-Q water and mix well.

2 Caesium chloride, 50% w/v

Caesium Chloride	50g
Milli-Q water	100mL

Weigh approximately 50g ± 0.1g caesium chloride into a 100mL volumetric flask. Dissolve and make up to the mark with Milli-Q water. Mix well.

3 Scandium (10 ppm)/Caesium (1%) mix

Using a volumetric pipette add 10 mL of scandium standard (stock solution, 1000 ppm) and 20 mL of caesium chloride (stock solution, 50%) to a 1 L volumetric flask. Make up to the mark with Milli-Q water. Mix well.

4 Rinse solution

0.1% Triton X-100	20 mL
Nitric acid, concentrated	20 mL

Add approximately 20 mL of 0.1% Triton X-100 into a litre bottle. Then add 20 mL of nitric acid using the dispenser. Make up to the mark with Milli-Q water. Mix well.

5 Torch Alignment solution, 5 ppm

Add 0.5 mL of manganese standard (stock solution, 1000 ppm) add 2 mL of nitric acid (concentrated) into a 100 mL volumetric flask. Make up to the mark with Milli-Q water. Mix well.

6 10% Nitric acid

Add 10 mL concentrated nitric acid to a 100 mL volumetric flask containing approximately 50 mL Milli-Q water. Mix then make to volume with Milli-Q water. Mix well.

7 Reagent blank solution

Add 2 mL concentrated acid and 2 mL of 0.1%. Triton X-100 to a 100 mL volumetric flask containing approximately 50 mL Milli-Q water. Mix then make to volume with Milli-Q water. Mix well.

Standards Preparation

1 Template 1 standards

i. Calcium, Potassium, Sodium and Phosphorus intermediate solution, 200 ppm

BDH Aristar grade calcium, potassium, sodium and phosphorus 1000-ppm standard Stock solutions. Pipette 50.0 mL of each stock solution into a 250 mL volumetric flask and dilute with Milli-Q water. Mix well.

ii. Magnesium intermediate solution, 50 ppm

BDH Aristar grade magnesium 1000 ppm standard Stock solution. Pipette 10.0 mL of stock solution into 200 mL volumetric flask and dilute with Milli-Q water. Mix well.

iii. Working Standards Solutions

Use 200 mL volumetric flasks. After the dilutions, as shown in Table 1, add 4 mL of nitric acid (concentrated and 4 mL of 0.1% Triton X-100 then make to volume with Milli-Q water. Mix well.

Table 1. Template 1 - Working Standard solutions

Std. No.	Ca, K, Na, & P 200 ppm soln. ML	Final Conc. Ppm	Mg 50 ppm soln. ML	Final Conc. Ppm
1	**2.5**	**2.5**	**1**	**0.25**
2	**5**	**5**	**2**	**0.5**
3	**10**	**10**	**4**	**1**
4	**20**	**20**	**8**	**2**
5	**40**	**40**	**20**	**5**

2 Template 2 Standards

i. Calcium intermediate solution, 100 ppm

BDH Aristar grade calcium 1000 ppm standard Stock solution. Pipette 20.0 mL of stock solution into a 200 mL volumetric flask and dilute with Milli-Q water. Mix well.

ii. Potassium intermediate solution, 100 ppm

BDH Aristar grade potassium 1000 ppm standard Stock solution. Pipette 20.0 mL of stock solution into a 200 mL volumetric flask and dilute with Milli-Q water. Mix well.

iii. Magnesium intermediate solution, 25 ppm

BDH Aristar grade magnesium 100 ppm standard Stock solution. Pipette 5.0 mL of stock solution into a 200 mL volumetric flask and dilute with Milli-Q water. Mix well.

iv. Sodium intermediate solution, 250 ppm

BDH Aristar grade sodium 100 ppm standard Stock solution. Pipette 50.0 mL of stock solution into a 200 mL volumetric flask and dilute with Milli-Q water. Mix well.

vi. Working Standard solutions

Use 100 mL volumetric flasks. After the dilutions as shown in Table 2 add 2 mL of nitric acid (concentrated) and 2 mL of 0.1% Triton X-100 then make to volume with Milli-Q water. Mix well.

Table 2. Template 2 - Working Standard solutions

Std. No.	*Ca 100 ppm mL*	*Final Ca ppm*	*K 100 ppm mL*	*Final K ppm*	*Mg 25 ppm mL*	*Final Mg ppm*	*Na 250 ppm mL*	*Final Na ppm*	*P 5 ppm mL*	*Final P ppm*
1	1	1	0.5	0.5	1	0.25	0.5	1.25	1	0.05
2	2.5	2.5	1	1	2	0.50	2	5	2	0.10
3	5	5	2.5	2.5	4	1	4	10	5	0.25
4	10	10	5	5	10	2.5	10	20	10	0.50
5	20	20	10	10	20	5	20	50	20	1
6	40	40	20	20	-	-	30	75	-	-

Sample Preparation

Liquid samples

Weigh 1 g of sample into a 100 mL flask.
Dilute to approximately half the volume of the volumetric flask with Milli-Q water, mix thoroughly then add 2 mL amount of concentrated nitric acid and the same amount of 0.1% Triton X-100. Make up to the mark with Milli-Q water. Mix well.

Procedure

Refer to 'Operating Instructions for the Varia Vista ICP-OES'.

Calculations

The ICP-OES software calculates the results based on the standard curve, sample weight and dilutions as mg/kg.

2.2.4 Materials and Methods for determination of Carbohydrates

Analysis of Lactose and Hydrolyzed Lactose Products by Gas Chromatography

Purpose

The following method separates lactose form other disaccharides and has been evaluated for lactose but good separations of fructose, glucose, galactose, sucrose, galaxies biose, allo-lactose and trisaccharides have been obtained as well.

Principle

The sample is dissolved in water and then heated in a water bath at 60°C for 30 minutes. The proteins are precipitated using barium hydroxide and zinc sulphate. After centrifugations 1 mL of supernatant is pipetted into a 4 mL sample vial and then dried under a stream of nitrogen at 50-70°C.

The dried residue is treated with hydroxylamine hydrochloride in pyridine to form the oxime derivatives of reducing sugars and trimethylsilyl (TMS) ethers of non reducing sugars. The sugars are determined by gas chromatography using phyenyl-ß-D-glucopyranoside as an internal standard.

Apparatus

Dessicator
4 mL vials with septum caps and teflon-lined septa
1 mL glass bulb pipette, gross error checked
Nitrogen supply
Block heater
Disposable pasteur pipettes
Autopipettor, adjustable, set to 500 µL
2 mL auto sampler vials
50 and 100 mL volumetric flasks, gross error checked
Balance, 4 decimal places

Balance, 2 decimal places

Thermometer, calibrated at 70 or 80°C

Centrifuge tubes: Blue Max™ 50mL graduated conical polypropylene screw capped centrifuged tubes. Becton Dickson, obtained from Health Care Auckland

Centrifuge

Water bath at 60°C

GC instrument GC17A Shimadzu

GC column: DB5 20 m x 0.25 mm x 0.25 μm from J & W Scientific, INC. (available from Alltech or Shimadzu)

Chemicals/Media

All chemicals are analytical Reagent (AR) grade unless otherwise indicated.

ß-D-Fructose

D-Glucose

Sucrose

Lactose monohydrate

Galactose

Maltotriose

Hydroxylamine hydrochloride ($NH_2OH.HCl$) - (BDH 10129)

Phenyl-ß-D-glucopyranoside, (Sigma P-6876)

Pyridine. Stored over KOH pellets to keep dry.

Hexamethyldisilazane (HMDS) - (Alltech 18006 or Sigma H-4875)

Trifluoroacetic acid (TFA) - (Sigma T- 6508)

Zinc sulphate heptahydrate

Barium hydroxide octahydrate

n-Octanol (laboratory reagent grade)

De-ionised water

Reagents/Media preparation

1 Barium Hydroxide (1.44%)

Weigh 3.6 g barium hydroxide octahydrate into a 250 mL volumetric flask and make up with water. The solution will be slightly turbid due to the formation of insoluble barium carbonate.

2 Zinc Sulphate (3%)

Weigh 7.5 g zinc sulphate heptaydrate into a 250 mL volumetric flask, dissolve and then make up to volume with water.

3 STOX reagent - (Internal Standard/Hydroxylamine Hydrochloride)
Into a clean, dry 100 mL volumetric flask, weigh 0.6 g of phenyl ß-D-glucopyranoside (internal standard). Record the weight to 4 decimal places. Add 2.5g ± 0.05 g of hydroxylamine hydrochloride. Dissolve in pyridine and make to volume. To a 100 mL amber bottle with teflon-lined stopper, add 0.5 g anhydrous sodium sulphate, then pour the STOX solution into the bottle. Keep firmly stoppered at all times to exclude moisture. Discard after one month. Record the concentration of the internal standard to 2 decimal places as mg/mL.

Standards Preparation

Lactose Standard Solutions

1 Lactose monohydrate stock solution (2.5 mg/mL)
Accurately weigh approximately 0.5g lactose monhydrate (4 decimal places) into a 200 mL volumetric flask. Dissolve the lactose in water and make up to the mark with water at 20°C.

2 Working lactose standard solutions (0.025, 0.10, 0.25, 0.50, and 0.75 mg/mL)
Pipette 0.5, 2.0, 5.0, 10.0 and 15.0 mL of lactose stock solutions into 50 mL volumetric flasks. Add 19.5, 18.0, 15.0, 10.0 and 5.0 mL of water respectively.

Then make the solutions up to approximately 1 mL below the mark using methanol or acetonitrile. Place in water bath at 20°C for 20 minutes and make up to the mark with methanole or acetonitrile. Store in freezer.

3 Before use, thaw the frozen standard solutions in a 20°C water bath. Thoroughly mix the separated solvent layers. Check whether all lactose is in solution If not, warm in hot water (50-70°C) until all lactose is redissolved and then cool to 20°C.

Accurately pipette 1 mL of each solution in 4 mL sample vials and dry in a heater block at 70°C under a gentle stream of nitrogen.

Standard solution for sugar profile

1 Weigh 0.2 g each of fructose, galactose, glucose, sucrose and lactose and 0.05 g of maltotiose into 50 mL volumetric flask and make the volume up with water.

2 Pipette 0.1, 0.4, 0.8, and 1.2 mL of this sugar solution into 4 mL sample vials using automatic pipette.

Dry in a heater block at 70°C under a gentle stream of nitrogen.

Sample Preparation

1 Weigh a screw capped graduated polypropylene centrifuge tube on a top loader balance.

2 Weigh 1.0 g sample into tarred centrifuge tube.

3 Fill the tube to the 40 mL mark with warm water (50-60°C) and firmly cap the tube. Shake the tube vigorously to break up any lumps.

4 Add one drop of n-octoanol to suppress any foam formation.

5 Place the firmly capped tube in a water bath at 60°C for 30 min and then cool to room temperature in a water bath.

6 Add 5 mL of 1.44% barium hydroxide solution using an automatic pipette mix by

gentle inversion.

7 Add 5 mL of 3% zinc sulphate solution using an automatic pipette and mix by gentle inversion.

8 Centrifuge the tube at 2000 rpm for 10 min.

9 Pipette 1.00 mL supernatant into a 4 mL vial and dry in a heater block at 70°C under a gentle stream of nitrogen.

10 Ensure there is no moisture remaining in the vials. Cap firmly with a new septum if not proceeding immediately with the derivatisation.

Derivatisation

1 Pipette 1.00 mL of STOX reagent into a vial of the dried supernatant, cap firmly and heat at 50 to 70°C for 30 minutes. Do not heat at or above 75°C.

2 Allow to cool

3 Add 0.5 mL HMDS (autopipette) and 5 drops of TFA (pasteur pipette), cap the vial and swirl gently.

4 Shake vigorously occasionally. Stand overnight.

5 Allow the precipitate to settle and transfer 1-2 mL of the clear supernatant into a 2mL auto sampler vial, using a pasteur pipette.

6 Analyse on the GC. Trimethylsilyl derivatives are stable in solution up to 5 days if kept free of moisture.

Calculations

1 Calculate the sample dilution factor D = (Wf - Wt)/0.99821

2 For the standard at each level plot on the y axis (peak area sugar)/(peak area ISTD) versus (amount of sugar in extract)/(amount of ISTD in extract) on the x-axis.

3 Calculate the slope of the graph (n)

4 For each sample calculate the ration - Y = (peak area sugar)/(peak area ISTD)

5 $$\text{Sugar (g/100g)} = \frac{(1/n)\, xY \times Ai \times D}{10\ W}$$

Where

Ai is amount of internal standard in mg

D is the sample dilution factor

W is the sample weigh in g

10 is a calculation factor to convert mg/g to g/100g

2.2.5 Materials and methods for determining Protein

Measurement of Nitrogen using Kjeltic

Apparatus

1 Kejeltec Auto Sampler System 1035 Analyser.
(Tecator AB, Box 70, S-263, Höganäs, Sweden.)

2 Kjeltec Auto Sampler System 1038 Sampler (Tectator).

3 Tecator Digestion System 2020.

4 Tecator Scrubber Unit 2001.

5 Tectator Lift System 2015.

6 Analytical Balance, reading to 4 decimal places.

7 Tecator Weighing Terminal 1036.

8 Balance, 10 kg for weighing caustic.

9 Normal laboratory glassware as specified.

10 Volumetric pipettes 5.0 mL, 10.0 mL.

11 Automatic pipettes for NCN/NPN preparation.

12 Automatic Dispensers for dispensing sulphuric acid and hydrogen peroxide.

13 Digestion tubes, 250 mL.

14 Digestion tube racks.

15 Stand for rapid cooling of tubes.

16 Retainer plate for washing tubes.

17 Printer, star LC-100 colour and interface converter.

18 Ultraturrex T25 high speed blender.

19 Refrigerated centrifuge capable of 10,000 rpm (12,100 g).

Chemicals

1 Caustic soda, laboratory grade.

2 Sodium thiosulphate, laboratory grade $Na_2S_2O_35H_2O$.

3 0.1 mol hydrochloric acid solution (AnalaR volumetric).

4 30% Hydrogen peroxide, AnalaR grade (1 liter).

5 Conc. Sulphuric acid, laboratory grade.

6 Orthoboric Acid, H_3BO_3 AnalaR grade, minimum assay 99.8%.

7 Acetic acid glacial, CH_3COOH, AnalaR grade.

8 Ammonium sulphate, $(NH_4)2SO_4$, AnalaR grade, minimum assay 99.5%.

9 L-Lysine monohydrochloride, $NH_2(CH_2)4CH(NH_2)COOH.HCl$, Biochemical grade, minimum assay 99%.

10 Sucrose, $C_{12}H_{22}O_{11}$ AnalaR grade 0.002% nitrogen maximum.

11 Methyl red.

12 Bromocresol green, BDH product No. 20012.

13 Methanol, CH_3OH, AnalaR grade, minimum assay 99.8%.

14 Sodium acetate anhydrous, CH_3COONa, AnalaR grade, minimum assay 99.0%.

15 Trichloroacetic acid (TCA), CCl_3COOH, AnalaR grade, minimum assay 99.5%.

16 High purity water prepared by Milli-Q system.

17 RO water, water purified by Reverse Osmosis system.

18 Sodium hydroxide, NaOH, AnalaR grade, minimum assay 99%.

19 Mercury kjeltabs (3.5 g K_2SO_4 + 0.17 g HgO).

20 Copper Kjeltabs (3.5 g K_2SO_4 + 0.5 g $CuSo_4$).

21 Sodium carbonate anydrous, Na_2CO_3, GPR grade, minimum assay 99%.

Reagents/Media Preparation

Caustic Sodium Thiosulphate Solution

- Use a large measuring cylinder to obtain 5 litres of RO water
- Place a bucket on 10 kg balance, weigh 2 kg NaOH, and 300 g of $Na_2S_2O_3$
- Put bucket into fume cupboard. Turn on the fan.
- Carefully add the 5 litres of RO water from the cylinder into the bucket and stir well making sure there are NO lumps on the bottom. Stir well making sure all NaOH and Na_2S2O_3 is dissolved.

- Leave in fume cupboard to cool.

1% Boric Acid

- Dissolve 50 g of boric acid in 3 litres of high purity water in a 5 litre glass flask.
- Add 50 mL bromocresol green solution (500 mg in 500 mL methanol in a volumetric flask, heat to dissolve), using a measuring cylinder.
- Add 35 mL methyl red solution (500 mg in 500 mL methanol in a volumetric flask, heat to dissolve), using a measuring cylinder.
- Add 10-15 mL 0.1 M Na OH (The alkali is necessary to achieve a positive blank value, a blank value of 0.05-0.15 is required). If a blank value of less than 0.05 is obtained more 0.1 M NaOH needs to be added.
- Make up to 5 L with high purity water.
- Date and initial the flask.
- Store in a cupboard away from direct light.

15% Trichloroacetic Acid

- To 10 L volumetric flask add 2 L high purity water.
- Pour high purity water directly into 500 g bottle of trichloroacetic acid, wait for air bubbles to disappear.
- Gently (using large funnel pour into 10 L volumetric flask. Rinse bottle until all acid is in volumetric flask.
- Repeat this, using in total three 500 g bottles of trichloroacetic acid.
- Ensure that all trichloroacetic acid is dissolved. Mix gently but well.
- make up to the mark with high purity water.

Acetic Acid, 10% (v/v)

- To a 100 mL volumetric flask add 10 mL acetic acid (graduated pipette) and make up to the mark with high purity water.

Sodium Acetate, 2 M

- To a 100 mL volumetric flask add 16.4 g sodium acetate and add approximately 50 mL high purity water. Mix to dissolve. Make up to the mark with high purity water.

Sodium Hydroxide, 1.5 M

- To a 100 mL volumetric flask add 6 g sodium hydroxide and add approximately 50 mL high purity water. Mix to dissolve. Make up to the mark with high purity water.

Sample Preparation

Total Nitrogen

Weigh 1 g of sample into a digestion tube. Digestion time is 60 min/Hg (2 kjeltabs).

Procedure

Sample Weight Entry

- Fill in Kjeltec Sample Record sheet.
- Turn distillation unit on.
- Press Weigh
- Enter batch no (1-40). Press Enter.
- Enter tube No. (1-20). Press Enter.
- Enter Factor 1. Press Enter.
- Select Result type '%N'.
- Select %,record and enter a theoretical N%.
- Enter weigh via the balance interface.
- Place a tube on the balance and tare.
- Weigh 1 g of sample into the tube.
- Press the white confirm button on the interface to send the weight to the Kjeltec.

Digestion Preparation

- Once a batch was completed weight entry place the rack on rapid cooling stand in fume cupboard and place heat shield in place.
- Add appropriate Kjeltabs and 12 mL concentrated Sulphuric Acid (automatic dispenser). (Note: Acid must be added to all tubes (even empty ones) to prevent undue thermal stress on tubes).
- Set lift arms at a suitable height.
- Position tube rack on lift arms and raise the lift up so that tubes and exhaust manifold dock.

Digestion

- Check scrubber unit. The flask to the right should contain a solution of 212 g sodium carbonate (GPR grade) and water filled up to the 1.2 L mark maximum.
- Start controller.
- Choose step # 1 on the controller.
- Press run/stop to begin the digestion cycle.
  If the digestion s stared at step # 1 the digestion block begins to heat up to 420°C. When it reaches the set temperature the lift will lower the complete assembly into the block. At the same time the scrubber unit will start.

Digestion will proceed for the programmed time (60 min), after which, the controller switch to step # 2, commanding the digestion to stop. The assembly is raised out of the block and the block then cools. The scrubber unit will continue to operate for 10 min drawing off any residual fumes.

The same cycle is followed when digestion is stared at step # 3. However, instead of cooling the block when digestion is complete step # 4 holds the block temperature at 420 °C when the assembly is raised.

This is an advantage when the operator wants to begin digestion another batch immediately.

Titration

- Remove air bubbles from top of burette.
- Load Sampler.
- Enter Warm-Up Tube data.
- commence Analysis.

Calculations

The Kjeltec will calculate results using the following calculations:

$$\% \text{ Nitrogen} = \frac{(\text{mL} - \text{Blank}) \times M \times 1.401 \times F}{\text{wt(g) sample}}$$

Where:

M = Exact molarity of the 0.1 M HCl as entered into Kjeltec.

F = 1 (Dilution factor)

$$1.401 = \frac{14.01 \text{ (m.2.nitrogen)} \times 100\%}{1000 \text{ mg/g}}$$

Which converts moles of nitrogen to % nitrogen.

The weight taken is entered into the Kjeltec at the time of weighing the original sample along with the factor for that sample.

2.2.6 Materials and Methods for determination of Fat composition using ASE

Fat Content using Accelerated Solvent Extraction

Purpose

Gravimetric fat determination using direct extraction with the ASE-200 (dionex Corp, Sunnyvale, CA) as a semiautomatic automated alternative to manual gravimetric fat methods.

Test Principle

Fat is extracted directly from the sample with a mixture of organic solvents at elevated pressure and temperature. The absence of an alkaline or acid digestion step (for protein) results in intact lipids, suitable for further lipid analyses.

Apparatus

1 ASE-200 (Dionex Crop. CA, USA), with 11 mL extraction cells and 40 mL collection vials.

2 Analytical balance, readable to 0.0001 g, 150 g capacity.

3 A heated dry block with temperature control from 60-110°C, with a vapour purge accessory.

4 Spoon spatula (to fit inside extraction cells).

5 Vented bench.

Chemicals

Petroleum ether, 40-60°C B.P.
Acetone
Isopropanol

Moisture absorbent "Hydromatrix" (Varian, CA, USA).

Reagents

The extraction solvent is made up in volume ratio of 3:2:1, for a total volume of one litre. Volume tolerance is ± 10 mL for petroleum ether; ± 5 mL for other solvents.

500 mL petroleum ether
335 mL acetone
165 mL isopropanol

Sample Preparation

1 Ensure extraction cells have a filter circle in place at the bottom of the cell.

2 Record all sample weights to 0.0001 g.

3 Warm samples to 40°C and mix well prior to sub sampling 0.5 g.

4 Add 0.7 g of hydromatrix to each extraction cell.

ASE

1 For all products:

Pressure - 1500 psi,
Purge time - 40 sec.
Temperature - 120°C.
Tatic time - 2 min.
Cycles - 3
Flush - 100% of volume.

Procedure

1 Handle vials with tissues or by the screw thread area only.

2 With a vivid marker, write and identify number on a vial, in the halfway position.

3 Allow empty vials to equilibrate (10 min) next to the balance before weighing.

4 To a clean extraction cell with bottom cap screwed on , insert a filter circle.

5 Perform 2 solvent rinses on the ASE, or 3 rinses if changing solvent.

6 Place ASE collection vials in a heat block (60°C or more) for approx. 10 min then allow to cool and equilibrate at room temperature (10-15 min).

7 Weigh the empty vials, including a blank, recording weight to 0.0001 g.

8 Cap the vials and place them in order on the lower ASE carousel.

9 Prepare samples in extraction cells as above, screw up the top caps finger tight (avoid over tightening) and place the cells in order on the upper carousel. Blanks must contain hydromatrix where this is used for sample extractions. Place the blank first into the carousel.

10 Check that the correct method or schedule is loaded before starting the extraction sequence.

11 When extractions are complete, transfer the collection vials to the solvent evaporator, a heating block.

12 Star heating block at 65°C to avoid boiling and bumping of the solvent.

13 Raise the temperature to 110°C at 1°C/min ramp from 65 to 110°C followed by 20 min at 110°C. Total time to remove solvent is 65 minutes, to ensure that the less volatile isopropanol is completely removed and to completely dry the fat sample.

14 Remove the vials and allow them to cool on vented bench.

15 Allow vials to equilibrate (approx. 10 min) before weighing.

Calculations

Fat content is given by: fat, wt% = {(w2 - w1) - (b2 - b2)} x 100/m

where:
w1 = weight of vial
w2 = weight of vial + fat
b1 = weight of blank vial before extraction
b2 = weight of blank vial after extraction
m = sample weight

Specifications

Repeatability is similar to that of gravimetric fat methods Rose-Gottlieb and SBR.

2.2.7 Materials and methods: Milk Composition determined using FTIR method

Purpose

This method was developed for the analysis of Total Fat, protein and solids in milk using FTIR (Fourier Transform Infrared Spectroscopy), an instrument that measures raw material and milk products with minimum sample treatment prior to analysis. This method replaces traditional Kjeltec, Rose-Gotolieb, ASE, and Ash methods used to determine total protein, fat and total solids (respectively).

Test Principle

The MilkoScan FT 120 employs a purpose built FTIR interferometer (Fourier Transform Infrared Spectroscopy). It relies on the principle of light interference, which modulate the amplitude of a signal as a function of the path differences between two interfering sources, an interferometer records the light intensity caught by the detector as a function of path differences generated by sliding a moving mirror. The minute displacement of this mirror is measured by means of a laser beam, which follows the same path as an IR beam. The infrared beam from the IR source hits the beam-splinter, which send half the beam to a fixed mirror and the other half to a moveable mirror. From the mirrors, the IR beams reflect and recombine before they reach the detector. All the IR frequencies travel through the interferometer at the same time and rapid small distance movements of the mirror enables simultaneous generation of the entire IR spectrum. Using the Fourier transform principle calculations are made using the latest PC technology. The interferogram is collected by the spectrometer, process through the Fourier calculations and converted into a full spectrum of the sample.

Apparatus

FTIR MilkoScan FT 120 (consists of two parts: the measuring unit and the PC for control of the overall operations)

Balance weighing 4 decimal places

Volumetric pipettes

Beakers

Volumetric flasks

Heat bath

Sample preparation

1 Heat samples to 35°C prior to sampling to ensure homogeneity.

2 Tare weight of volumetric flask.

3 Pipette 1 g of sample into volumetric flask. Record sample weight.

3 Dilute sample by adding 25-27 g of water. Record total weight.

Machine Preparation

1 Callibrate milcoscan, using the calibration master which adjusts the slope/intercept, for sea lion milk by entering fat, protein, and total solid percentages from preliminary traditional tests (ASE, Kjeltec, and Total Ash). Select "Analysis Set Up" from the menu of the Milkoscan FT 120 program and the program will guide through the calibration procedure. When calibrating the Milkoscan FT activate the dilution option by pressing the dilution button next to the Sample Identification field button.

Sample Analysis

1 Zero the machine by pressing Zero button.

2 Select sea lion milk calibration.

3 Shake sample thoroughly.

4 Place sample into machine and press enter.

5 Milkoscan FT automatically determines milk composition.

Appendix II

3.1 Materials and Methods

Fatty Acid composition by Gas Chromatography of Fatty Acid Methyl Esters

Purpose

The fatty acid profile of a fat gives an indication of its origins (species of animals or plant) its purity (whether adulterated), and its properties (hard or soft).

Scope

Milk fat and other common fats and oils of animal and vegetable origin containing triglyceride fatty acids in the range C4-C20,

Principle

Methyl esters are prepared by direct trans-esterfication of an anhydrous fat sample with sodium methoxid/methanol. Individual fatty acids are determined by gas chromatography of the methyl esters.

A certified Reference material (AMF or soya/maize oil) is the primary standard for calibration

A secondary reference anhydrous milk fat (AMF or soya/maize oil) is used as a quality control sample for dairy fats.

A secondary reference soya bean oil is used as a quality control sample for vegetable oils high in polyunsaturated fatty acids.

Apparatus

1. Tweezers
2. Screw cap tubes, 16 ml
3. Sample corer (8 mm i.d.)
4. Auto sampler vials, 2 ml
5. Balance (readable to 0.001 g)
6. Block heater with evaporator attachment
7. Auto pipette (1 ml)
8. Centrifuge
9. Filter Paper
10. Glass pasteur pipettes
11. Scalpel knife

Chemicals

All chemicals are Analytica Reagent (AR) grade unless otherwise indicated.

1. Sodium metal, GPR grade (e.g. BDH 30101 3E)
2. Methanol, CH3OH
3. Sodium sulphate, anhydrous, granular, Na2SO4
4. 2,2,4-trimethylpentane
5. Diethyl ether.
6. Hexane or hexane distillation fraction.
7. Nitrogen (oxygen-fee grade)
8. Petroleum ether (Boiling range 40-60°C)
9. Potassium dihydrogen phosphate (KH2PO4)
10. Sodium chloride, NaCl

Reagents Preparation

1. Neutralizing solution: dissolve 100 g potassium dihydrogen phosphate (KH2PO4) and 150 g sodium chloride (NaCl) in 1 L 'Milli-!' water.
2. Esterfiying reagent (0.5 M sodium methoxide in methanol as a 20% solution in ether/isooctane).

(a) Dry the diethyl ether by shaking well with anhydrous sodium sulphate (5g/100 ml) for 2 min. Rinse a 100 ml brown glass bottle with dry diethyl ether and add 40 ml of dry ethyl ether and 40 ml of isooctane.
(b) Using tweezers wash a small piece of sodium metal in hexane or petroleum ether to remove grease, place on a filter paper circle and scrape off any encrusted oxide layer with a scalpel. Weight 0.25 –0.30 g onto a filter paper. Place the weighed sodium in a beaker containing 20 ml methanol and allow to dissolve.
(c) Add the sodium/methanol solution to the bottle of ether/isooctane and mix. Reagnets will form one phase. Keep firmly stoppered with an airtight stopper. Store at room temperature. Discard if the solution becomes cloudy.
(d) Dispose of sodium scraping.

Sample Preparation

1. Using a corer sub sample 1 g into a screw cap tube and add 10 ml hexane. Cap, shake vigorously (approximately 10 s_ and allow to settle. Separation may be assisted by centrifuging. Withdraw 2-3 ml of the (cloud) hexane layer into a clean tube containing 1 g anhydrous sodium sulphate and shake. The solution should become clear. Transfer 0.5 ml to a clean tube. Evaporate the solvent in the heater block (50°C) under a gentle stream of nitrogen.

Procedure

1. Warm the dry fat sample (35-40°C) and add 1 ml esterfying reagent. Mix until all the fat has dissolved and leave 2-4 min.
2. Add 10 ml of hexane, mix and leave 2 min.
3. Add 1 ml of KH2PO4: NaCl buffer solution, mix thoroughly by shaking the tube r vortex mixing for 10 s, and centrifuge briefly or allow to stand until clear (1 h).
4. Withdraw 1 ml of the upper layer, transfer to a 2 ml autosampler vile and cap immediately.
5. Transfer several 1 ml aliquots of the quality control AMF solution of methyl esters to auto sampler vials as above and place at eh start, finish and after nine samples throughout the run.

Calculations

$$\text{Wt\% of fatty acid} = \frac{\text{peak area x correction factor}}{\Sigma_{n/1=1}\ (\text{peak area x correction factor})}$$

Calculations were performed by the data-handling software.

Reference

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Appendix III

4.1 Material and Methods

Gel Electrophoresis- SDS PAGE Method

Scope

This is a good general method for quantifying whey proteins and caseins. SDS PAGE is used extensively in biochemistry to gain information about the approximate molecular weights of proteins in solution.

Principle

SDS (sodium dodoecyl sulphate) is a negatively charged surface-active substance. It is used to disrupt the non-covalent bonds, through its ability to absorb to hydrophobic and positively charged sites on proteins. Different proteins bind almost the same amount of SDS on a mass basis. Thus, once coated with SDS, the proteins can be separated by electrophoresis on the basis of the molecular size of the protein- SDS complexes.

Samples are usually reduced by treatment with the 2-mercaptoethanol, which breaks the disulphide to further dissociate the protein interaction (but not lysinoalanine types).

Non-reduced samples are also run on SDS gels. This method disrupts only the non-covalently bonded aggregates.

Both reduced and non-reduced samples can be run on the same SDS gels to reveal information about he type of bonding in the sample.

Reagents

Acrylamide/Bis 30% T
Dissolve 30 g of acrylamide/Bis mixture 37.5:1 (2.6% C) in approximately 60 mL of Milli-Q water. Bring to volume in a 100 mL volumetric flask. Store solution in an amber bottle at 4°C. (This solution should be polymerised and discarded if older than 1 month.)

10% APS (make up fresh each day)
Dissolve 100 mg of APS in 1.0 mL of Milli-Q water.

Bromophenol Blue 0.4% (w/v)
Dissolve 1.6g of Bromophenol Blue in approximately 7 mL of 0.1 M NaOH. Make up to 400 mL in a measuring cylinder.

SDS Buffer Preparation

10% SDS
Dissolve 10 g of SDS in Milli-Q water with gentle stirring and bring to volume in a 100 mL volumetric flask. Store at room temperature.

SDS Sample Buffer

Measure
500 mL Milli-Q water

125 mL 0.5 M Tris-HCl buffer (pH 6.8)
100 ml Glycerol
200 ml 10% (w/v) SDS
25 mL 0.4% (w/v) Bromophenol Blue solution
Mix well. The total volume is 950 mL.

SDS Resolving Buffer: 1.5 M Tris-HCl Buffer
In a 100 mL beaker, weigh 18.15 g of Tris base. Add about 60 mL of Milli-Q water, mix and adjust the pH to 8.8 with 6 M HCl. Bring voume in a 100 mL volumetric flask. Store at 4°C.SDS Stacking Buffer: 0.5 M Tris-HCl

In a 100 mL beaker, weigh, 6.0 g of Tris base. Add about 60 mL of Milli-Q water, mix and adjust eh pH to 6.8 using 6 M HCl. Bring to volume in a 100 mL volumetric flask. Store at 4°C.

SDS Elecrode Stock Buffer 95 x Concentration
In a 1 L beaker, weigh

15 g Tris (hydroxymethyl) mehtylamine
72 g Glycine
5 g SDS

Bring to 1 L volume with Millie-Q water. Check the pH is 8.6 (± 0.2). Store at 4°C. Do Not Adjust the pH. Dilute 80 mL (5 x) stock to 400 mL with Milli-Q water for one electrophoresis run.

SDS Gel Preparation

(a) SDS Resolving Gel

1. Measure the quantities described in Table 1a(I) into a 100 mL Buchner flask and degas with stirring for 15 min.

2. While the resolving gel is degassing, prepare the APS solution.
3. To the Buchner flask, add the 10% SDS solution, the TEMED and then the APS (see Table 1a(ii), swirling the flask gently to mix after each addition.
4. Using an autopipette, quickly transfer 2.20 mL of gel buffer solution in between the two glass plates down the side of the grey divider strip. It is important to keep the flow of gel buffer constant while pipetting so as not to introduce air bubbles into the gel.
5. Run 200 μL of Milli-Q water down both sides of the plate with an autopipette to form a layer over the gel solution. This wil remove the meniscus from the sides and edges of the gel
6. After the gel has set (about 15-30 min), decant off the layer of water. Place casting stand with gel sandwich on its side then remove the last traces of water using a piece of filter paper placed carefully between the two glass plates just above the gel line. Do not allow the filter paper to touch the gel.

Table 1a Preparation of SDS resolving Gel

	SDS-RESOLVING GEL	
REAGENTS	2 GELS	4 GELS
(i)		
Milli-Q water	1.00 mL	3.00 mL
1.5M Tris-HCl buffer	2.50 mL	3.75 mL
Acrylamide/Bis (30% T)	5.30 mL	7.95 mL
	DEGAS FOR 15 MIN	
(ii)		
10 % SDS stock	100 μL	150 μL
TEMED	5 μL	7.5 μL
APS (10%)	50 μL	75.0 μL

(b) SDS Stacking Gel

7. Measure the quantities required (refer Table 1b(I)) into a 100 mL Buchner flask and degas the solution for 15 min.
8. Add the 10% SDS, the TEMED and APS, (refer Table 1b(ii)) swirling the flask gently to mix after each addition.
9. Using an auto-pipette, quickly pipette the gel buffer solution down the center in between the two glass plates unit it reaches the top and slightly overflows.
10. Immediately fit the slotted comb between the two plates so that the teeth are resting in the gel buffer (The teeth form the sample wells in the stacking gel.) Make sure that there are no bubbles trapped around the teeth. Let the gel stand for at least 1 h at room temperature.
11. Store gels in cold-room or freezer. Gels may be stored in cold-room up to 4 days.
12. Remove the comb before use.

Table 1b Preparation of SDS stacking gel

	SDS –STACKING GEL	
REAGENTS	2 GELS	4 GELS
(i)		
Milli-Q water	3.05 mL	6.10 mL
0.5 M Tris-HCl buffer	1.25 mL	2.50 mL
Acrylamide/Bis (30% T)	0.65 mL	1.30 mL
	DEGAS FOR 15 MIN	
(ii)		

10% SDS stock	50μL	100 μL
TEMED	5 μL	10 μL
APS (10%)	25μL	50 μL

SDS Sample Prparation

Sample preparation for SDS reduced is carried out in 2 pars: (a) sample are made up to appropriate dilutions in SDS sample buffer (see Table 2), and (b) they are reduced with 2-mercaptoethanol just prior to being loaded to SDS Reduced PAGE. Note the samples for SDS Non-reduced are not treated with 2-mercaptoethanol.

Sample and Dilution:

Sample ID	% Protein	Sample (μL)	Buffer (μL)
986	17.64	13	987
1029	10.64	60	940
1446	16.99	28	972
1127	4.87	48	952
1022	12.42	20	980
1085	4.55	48	952
1082	5.45	48	952
1118	4.8	48	952
1422	10.54	22	978
1405	13.75	20	980
1467	15.71	15	985
1435	18.9	13	987
1005	12.9	18	982
1442	13.33	18	982
941	4.02	17	983
949	13.92	17	983
944	16.99	14	986
954	13.97	17	983
1172	10.44	24	976
1374	11.66	20	980

1475	6.24	40	960
1084	16.99	34	966

Sample & Standard Dilution

A standard was run on every gel.

Standards

Animal ID 1082 ran on all four gels.

Molecular weight standard 'Biorad' ran on all four gels.

SDS Reduced Sample Treatment

1. Pipette 1 mL of sample into a microcentrifuge tube and treat with 20 μL of 2-mercaptoethanol. Vortex to mix and place the tubes in a suitable rack.
2. Heat the tubes for 4 min in a boiling water bath.
3. Allow the samples to cool, vortex and then load 10μL of sample per well.

SDS Electrophoresis Conditions

Set the power pack to deliver.

Program	=T/V-H
Volts	= 210 V
Current	= 70 mA
Power	= 6.5 W
Time	= 1 gel 0.9 h
	= 2 gel 1.1 h

Reference:

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