NOTE TO USERS

SPECIATION OF IODINE IN BOVINE MILK BY NEUTRON ACTIVATION ANALYSIS

by

Keila Isaac-Olivé

Submitted in partial fulfillment of the requirements

for the degree of Doctor of Philosophy

at

Dalhousie University

Halifax, Nova Scotia

February, 2005

Your file Votre référence
ISBN: 0-494-00971-3
Our file Notre référence
ISBN: 0-494-00971-3

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To Eunice Olivé-Alvarez and Miguel Angel Isaac-Tejera

Because from "mami" I learned about the fascinating world of radioactive isotopes and from "papi" I learned that the only chemistry that I actually want to do is analytical chemistry.

This thesis is for them and because of them.

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ABSTRACT

Iodine is considered one of the essential trace elements for humans. Iodine deficiency disorder is common in Africa and Asia. In countries such as Canada and the United States there are concerns over the high levels of iodine intake. There is an increasing interest in the speciation analysis of iodine in bovine milk which is one of the main sources of this element for humans.

One of the objectives of this thesis was to develop neutron activation analysis (NAA) methods of high precision, accuracy and sensitivity for measuring the iodine content of milk. Instrumental NAA (INAA), epithermal INAA (EINAA), and pseudo-cyclic INAA (PCINAA) methods using conventional and Compton suppression (CS) gamma-ray spectrometry were developed. The lowest detection limit of 0.02 $\mu g.mL^{-1}$ was obtained using PCINAA-CS followed by 0.06 $\mu g.mL^{-1}$ by both INAA-CS and EINAA-CS methods. Both relative and k_0 methods for calculating concentrations gave high precision and accuracy. The expanded uncertainty of the relative method was estimated as 4.5%.

One of the main objectives of this thesis was to do speciation analysis of iodine in bovine milk. A solvent extraction method was first employed for the separation of extractable organic compounds such as lipids. The organic extract was further separated using solid phase extraction into apolar, less polar and polar groups. The apolar group was then fractionated into hydrocarbons, tri-acylglycerides, di- and mono-acylglycerides, and free fatty acids. Ion exchange chromatographic methods were developed for the separation of inorganic iodine species.

In summary, the total iodine content of milk was found to be 0.457±0.008 $\mu g.g^{-1}$. The major iodine species was iodide ions with a concentration of 0.333±0.009 while iodate was 0.064±0.003 $\mu g.g^{-1}$. Amounts of 0.034±0.003 and 0.013±0.003 $\mu g.g^{-1}$ were protein- and lipid-bound iodine, respectively. Of the total protein-bound iodine, casein had 0.026±0.001 $\mu g.g^{-1}$ and the rest (0.011±0.006 $\mu g.g^{-1}$) was whey proteins. Fractionations of the lipid-bound iodine revealed that the amounts of apolar, less polar and polar classes were 11±3, 1.3±0.4, and 0.074±0.005 $ng.g^{-1}$, respectively. Further fractionation of the apolar extract gave 3.4±0.3, 2.9±0.3, 2.0±0.2, and 3.3±0.4 $ng.g^{-1}$ of hydrocarbons, triacylglycerols, di- and mono-acylglycerols, and free fatty acids, respectively.

LIST OF ABBREVIATIONS AND SYMBOLS USED

AAS	Atomic absorption spectrometry
ANOVA	Analysis of variance
AOAC	Association of analytical chemists
b	barn
b.w	bodyweight
BCA	Bicinchoninic acid
BSA	Bovine serum albumin
CNAA	Cyclic neutron activation analysis
COMA	Committee on Medical Aspects
CS	Compton suppression
CumNAA	Cumulative neutron activation analysis
D.L	Detection Limit
DDW	De-ionized distilled water
DEAE	Diethyl amoniethyl
DUSR	Dalhousie University SLOWPOKE-2 Reactor
ECD	Electron capture detector
EDTA	Ethylene diamine tetraacetic acid
EINAA	Epithermal instrumental neutron activation analysis
ENAA	Epithermal neutron activation analysis
eV	Electron volt
FAO	Food and Agriculture Organization
FWHM	Full width at half maximum
GC	Gas chromatography
HPGe	High pure germanium
HPLC	High performance liquid chromatography
ICP	Inductively coupled plasma
IDD	Iodine deficiency disorders
IDMS	Isotope dilution mass spectrometry
INAA	Instrumental neutron activation analysis
ISE	Ion-selective electrode
JECFA	Joint Expert Committee on Food Additives
keV	kilo electron volt
MeV	Mega electron volt
MF	Milk fat
MS	mass spectrometry
NAA	Neutron Activation Analysis

NIST	National institute of standards and technology
PAA	Photon activation analysis
PCNAA	Pseudocyclic neutron activation analysis
PENAA	Preconcentration epithermal neutron activation analysis
PMTDI	Provisional Maximum Tolerable Daily Intake
PMTs	Photomultiplier tubes
PNAA	Preconcentration neutron activation analysis
QCM	Quartz crystal microbalance
RDA	Recommended dietary allowance
RNAA	Radiochemical neutron activation analysis
RNI	Recommended nutrient intake
RP	Reversed phase
RSD	Relative standard deviation
SEC	Size Exclusion Chromatography
SFC	Super fluid chromatography
SPE	Solid phase extraction
SPME	Solid phase microextraction
SRMs	Standard reference materials
$\sigma_{\eta\tau}$	Thermal neutron cross section
T_3	3,5,3'-triiodothyronine
T_4	Tyroxine
tc	Counting time
td	Decay time
Tetrabase	4,4'-tetramethyldiaminodiphenylmethane
ti	Irradiation time
TMAH	Tetramethylammonium hydroxide
TSH	Thyroid-stimulating hormone
UK	United Kingdom
UNICEF	United Nations Children's Fund
USSR	Union of Soviet Socialist Republics
UV	Ultra violet
WHO	World Health Organization
XRF	X-ray fluorescence spectrometry

ACKNOWLEDGMENTS

I would like to thank my supervisor Professor A. Chatt, for giving me the opportunity of learning neutron activation analysis by doing experiments with my own hands and not from a book. His guidance and encouragement throughout these years were crucial in the completion of this work.

Thanks to Dr. Ragunath Acharya, who I named my second supervisor, for sharing with me all his knowledge in neutron activation. His help and encouragement were decisive for me, especially at the beginning of this project.

Many thanks to Dr. Jiri Holzbecher, for his valuable help in my experimental work, for his useful suggestions and for "taking care of my babies" (my samples) in each experiment.

Thanks to present and former members of SLOWPOKE group: to Dr. Weerasinghe Indrasena (Indra) for giving me advice on lipid separation; to Dr. Michiko Fukushima for sharing with me her experience in dealing with proteins; to Dr. Joe Kiceniuk for his always valuable help in many areas; to Blaine Zwicker who arrived at SLOWPOKE at the right and precise moment bringing friendship and helping me every time that I needed it.

Special thanks to Erick Gonzalez, Sonia Hevia and Wilber Menendez for being more than my co-workers, my friends. Thanks to all my Cuban and non-Cuban friends in Canada especially to Lorenzo Vega and Perla Delagado for being like my family during these years in Canada. Thanks also to Tijana Stojkovic, Federica Belluccini, Gianna Aleman, David Iza and Irene Forsey for their friendship and strong support in my last period in Halifax. Thanks to all my friends and family back home. Their support might seem little in terms of "neutron activation", but to me it has meant a lot.

The financial assistance from the Natural Sciences and Engineering Research Council (NSERC) of Canada, Faculty of Graduate Studies of Dalhousie University, Department of Chemistry of Dalhousie University, and Instituto Superior de Ciencias y Tecnología Nucleares (High Institute of Nuclear Science and Tecnology), Havana, Cuba, is gratefully acknowledged.

To all of them: Thank you very much!!!!
Muchas Gracias!!!

CHAPTER 1

INTRODUCTION

1.1 Discovery of iodine

Once upon a time in 1811, during the French war, a French chemist named Bernard Curtois (1777-1838) worked for Napoleon producing gunpowder. The production of gunpowder used large quantities of sodium carbonate, which was extracted from wood ash in metal containers. Curtois leached the ash with water and after evaporation sodium carbonate precipitated, as predicted. After several extractions in the same metal container, a thick layer of insoluble material started to build up and Curtois cleaned out the vessel by means of acid and heat. Curtois substituted wood ash with ash of burned seaweed (kelp), and one day during the acid cleaning process of the metal container Curtois accidentally added more sulfuric acid than the usual amount. He noted then that an intense violet-colored vapor formed. The vapor condensed on the top part of the vessel (cooler area) and he saw the formation of dark-lustrous metal-like crystals [1]. Curtois had just discovered, by accident, the element fifty-three of the periodic table through the following reaction:

$$2I^- + H_2SO_4 = I_2 + SO_3^{2-} + H_2O$$

Curtois was investigating the properties of this new chemical element, but in 1812 he abandoned this task for financial reasons. He passed the investigation to two of his colleagues, Charles Bernard Desormes (1777-1862) and Nicolas Clément (1779-1841). Joseph Gay-Lussac (1778-1850) and André M. Ampére (1775-1836) joined Curtois's two colleagues, and as a group continued characterization of this new chemical element. A small sample of the new element was given mysteriously to Sir Humphry Davy (1778-1829), a very well known English chemist. He also started to characterize this new element. Then started a quarrel between the French and English chemists for the copyright of the investigation although everybody recognized that Curtois discovered the

element. Davy named the element iodine; the first part of the name "iod" comes from the Greek language *ioeides*, which means violet colored. The last part of the name "ine" is for the resemblance of this element to chlorine and bromine [1].

1.2 Iodine in nature

Oceans are the earth's major reservoir of iodine [2, 3]. They contain nearly 70% of the earth's total iodine [3]. Different hypotheses have been proposed for the pathways of iodine from ocean to atmosphere [2-5]. These include the photochemical oxidation of iodide in seawater resulting in iodine release into the atmosphere [2], the production of iodine by the reaction of iodide with atmospheric ozone [2], and the conversion of iodide and iodate present in seawater into organic iodine through a metabolic action of marine and terrestrial bacteria [2-5]. The last hypothesis has been most widely accepted [4, 5]. Thus iodine is released from seawater in the form of organic iodide (mainly methyl iodide) and then it undergoes either photolysis or oxidation by ozone to produce inorganic iodine. The amount of iodine transferred from the ocean to the atmosphere is about 2.4×10^5 ton per annum [3].

Once iodine is in the atmosphere, 70% of it is expected to return to the ocean (1.68×10^5 tons a^{-1}) and the remaining 0.7×10^5 tons a^{-1} is deposited on land via rain or dry deposition of aerosols [3]. The amount of terrestrial iodine decreases with increasing distance from the ocean [6], hence there is a higher probability of iodine deficiency in continental regions. Recently, it has been proven that plant material releases organic iodine back to the atmosphere [7]. Iodine is also transported back to the ocean *via* rivers (about 1.1×10^5 tons a^{-1}) [2]. However, the net input of iodine into the ocean ($1.68 \times 10^5 + 1.1 \times 10^5 - 2.4 \times 10^5 = 0.38 \times 10^5$ ton a^{-1}) is not sufficient for maintaining the relatively high concentration of iodine in oceans. The net input of iodine into the ocean should be 2.1×10^5 ton a^{-1} in order to balance the geochemical cycle [3].

The lack of sensitive techniques for iodine determination might be one of the reasons for this gap in the geochemical iodine cycle [6]. Recently, studies have been

carried out with more sensitive analytical techniques, such as inductively-coupled plasma in conjunction with mass spectrometry (ICP-MS) and neutron activation analysis (NAA) for checking the previously compiled data and to investigate this apparent break in the iodine cycle [2-9]. One of the aims for obtaining information on the iodine cycle is to facilitate the tracking and accumulation of the man-made long-lived radionuclide ^{129}I in the environment and to estimate its hazard to humans [2-9].

It has been previously mentioned that about 30% of the iodine transferred from the oceans reaches the land mostly in the iodide form [4]. It has been reported that the iodine concentrations in soils are much higher than those in crustal rock [8, 9]. The iodine concentration in crustal rock, including marine sediment, is about 0.1 ppm whereas in Japanese soil this value can reach between 5 and 60 ppm [8, 9]. Iodine concentrations in several foodstuffs such as milk, cereals, and vegetables are related to iodine level in soils [9], hence it is also related to iodine intake by humans and animals.

1.3 Biological importance

Iodine is a very important trace element for all animals, including man, for thyroid tissues [1, 10]. Twenty percent of all the iodine in humans is found in the thyroid gland. Free iodine and iodate are reduced to iodide in the intestinal wall of the thyroid gland which converts iodide to organic iodine by coupling to the amino acid L-thyrosine, which is a precursor of the thyroid hormones, namely thyroxin (T_4) and 3,5,3'-triiodothyronine (T_3) [1]. One of the functions of these hormones is to accelerate cellular reactions and increase metabolic rate. They are essential for energy metabolism, neuromuscular function, and protein synthesis [1, 10]. Iodine is mainly excreted through renal filtration, and its partial reabsorption is proportional to its concentration in the plasma [1]. Since only a very small amount of iodine is lost in the feces, its urinary excretion indirectly reflects intake or body iodine status [1, 10].

Iodine deficiency results in a lower production of T_3 and T_4 hormones leading to the release by the pituitary gland of the hormone that induces the synthesis of

T_3 and T_4 hormones. This inducing hormone is known as thyroid-stimulating hormone (TSH). As a result of the TSH release, the activity in the thyroid gland increases causing its enlargement. This enlargement of the thyroid gland, observed as a swelling at the front of the neck is called goiter (Latin: *guttur*, throat), and it is the main human disease associated with iodine deficiency [1, 10, 11]. Since the early days of civilization, Greeks, Chinese, and Egyptians used seaweed and burnt sponge for treatment of goiter [1].

Iodine deficiency not only produces goiter, but also mental, psychomotor and growth abnormalities. These consequences of iodine deficiency are known under the general name of iodine deficiency disorders (IDD) [10, 11]. Deficiency in pregnant women can lead to stillbirth, miscarriages and congenital abnormalities. Cretinism is a condition resulting from a maternal iodine deficiency, and is common to children born in iodine-deficient areas [1, 10, 11]. It is characterized by mental deficiencies, deaf-mutism, poor bone and muscle development, hypothyroidism, and dwarfism. The disease severely affects mental and physical development. Iodine deficiencies either in children or adults can also lead to reduced mental function. Observed effects include reduced academic performance, and impairment of decision making and initiative taking skills [10, 11].

According to the World Health Organization (WHO), the recommended dietary allowance of iodine for humans is 50 $\mu g\ d^{-1}$ from 0-6 months, 90 $\mu g\ d^{-1}$ from 6 months to 6 years, 120 $\mu g\ d^{-1}$ from 7-10 years, 150 $\mu g\ d^{-1}$ during adolescence and adulthood, and 200-300 $\mu g\ d^{-1}$ during pregnancy and lactation [10]. Health Canada has set the recommended nutrient intake (RNI) of iodine as 160 $\mu g\ d^{-1}$. The RNI is the minimum intake required to prevent diseases related to deficiency [12]. The United States has set the recommended dietary allowance (RDA) for iodine as 150 $\mu g\ d^{-1}$ [12]. In the United Kingdom (UK) the recommended value by U.K Department of Health's Committee on Medical Aspects of Food Policy (COMA) is 140 $\mu g\ d^{-1}$ [11, 12].

The WHO has estimated that nearly 30% of the Earth's population, that is around 1.6 billion people, is afflicted by IDD including 655 million with goiter, 26

million with severe brain damage and 56 million with endemic cretinism. From this data it appears that in Europe approximately 140 million people are at risk of IDD and 97 million have goiter [10]. This situation is certainly worst in developing countries such as Africa, Latin America, and Asia. The WHO and the United Nations Children's Fund (UNICEF) have made the treatment and prevention of IDD a worldwide priority [10, 11]. Iodine deficiency can be prevented or treated by increasing the intake of iodine through diet [1, 10, 11]. Treatment of existing IDD is generally more effective if iodine supplementation is accompanied with hormone therapy. Iodine supplementation achieved through the use of iodized salt, as a food additive for farm animals, and as iodized oil. Apart from table salt and seafood, milk and dairy products are the most important sources of dietary iodine [1, 10, 11]. Iodine deficiency constitutes a major public health problem and at the same time it is one of the most common preventable causes of mental retardation in the world today [10].

On the other hand, in some countries such as the United States, Canada, and United Kingdom, there are concerns over the high levels of iodine intake [10, 11]. Excessive exposure to iodine can inhibit the function of the thyroid gland and produces iodine disorders as well [1, 10]. To protect against the toxic effects of excessive iodine exposure, a Provisional Maximum Tolerable Daily Intake (PMTDI) has been recommended by the Joint Expert Committee on Food Additives (JECFA) of the Food and Agriculture Organization (FAO) and the WHO. The PMTDI is an estimate of the amount of a substance that can be ingested over a lifetime without appreciable risk. The dose for iodine is 17 $\mu g\ kg^{-1}$ of bodyweight (b.w) per day, which is equivalent to 1020 $\mu g\ d^{-1}$ for a 60 kg person. For children (11-17 kg), the PMTDI would be in the range 180-290 $\mu g\ d^{-1}$ [10, 11].

The reasons for the increased iodine intake by select population groups in several countries are not very clear. Some people suggest there is a relationship between increased intake and the iodine concentration of milk. Milk is estimated to provide 16-30% of dietary iodine intake. In some countries such as the U.K, iodine in cow milk is

the primary source of dietary iodine [1, 11, 13]. The increased consumption of milk with high iodine concentration may account for some of the observed trends.

The major cause of high iodine levels in milk might be the use of organic iodine compounds for the prevention and treatment of foot rot disease in dairy cattle [11, 14, 15, 16]. Significant amounts of iodine may also be introduced in milk through the disinfection of the teats by iodine containing agents [17]. Iodine solutions are often used to sterilize the mechanical systems employed to extract milk from cows; any residual iodine may be passed on to the milk. Therefore, there is an increasing interest in the determination of total iodine in milk. In addition, any identification of iodine species may help explain their role in the iodine cycle. Therefore, rather than the determination of the total iodine in milk, the identification as well as quantification of various iodine species in milk will be a big step forward in solving iodine-related problems described above.

1.4 Iodine determination in milk

Milk is a source of high nutritional value and of several dietary trace elements. It is also one of the most important food sources for children. For these and other reasons, the iodine content of milk continues to be an important topic in the scientific community. About 320 references under the key words "iodine determination in milk" were found in the SciFinder database on 2004 September 20. After rejection of the papers related to the determination of radioiodine and of iodine-number for fat identification, 170 papers remained. The distribution of these papers over time is shown in Fig. 1.1. The figure shows that the determination of iodine in milk has and continues to be an area of active research. In general it can be said that it is a steady topic. During 1990-1994 most of the work on iodine in milk was related to the determination of radioiodine after the Chernobyl accident in the former USSR. This perhaps accounts for the apparent decline in publications related to the non-radioactive iodine content of milk during that period.

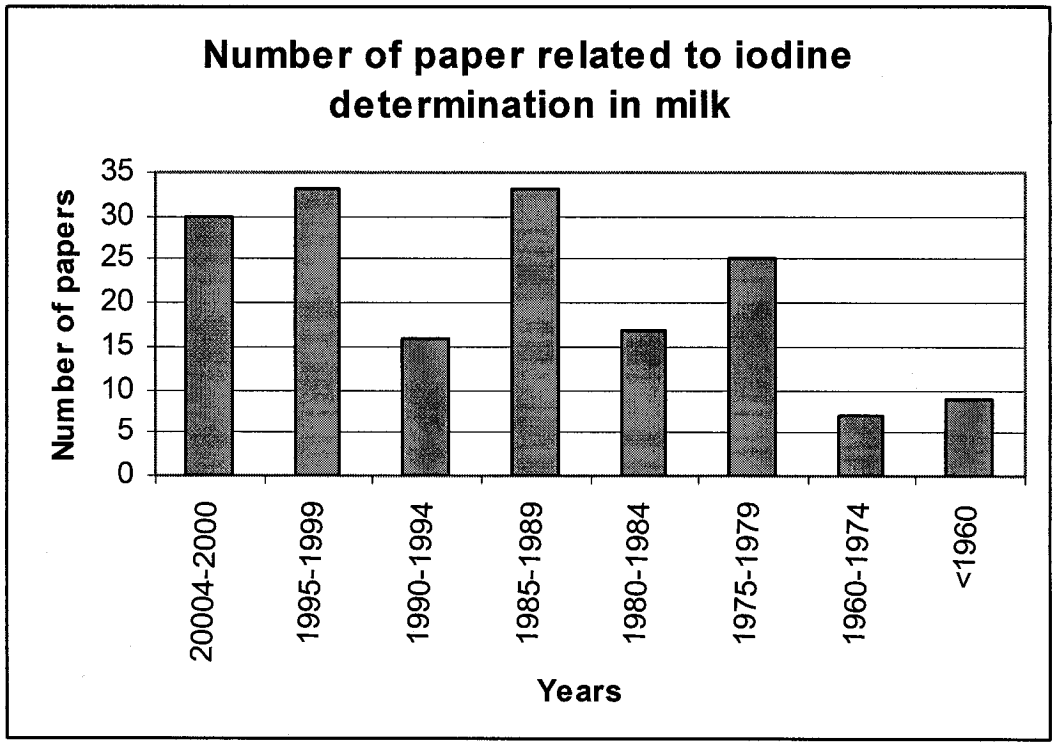

Fig 1.1. Distribution by years of papers related to iodine determination in milk.

The first paper on iodine determination in milk reported in this database is from 1929 [18]. Areas of interest have varied over the years investigating topics such as the estimation of iodine intake via milk [19, 20], the study of the iodophor teat disinfection process and its implications on iodine concentration in milk [21], the establishment of geographical conditions (*i.e.* iodine intake relationships in some areas) [22], the assessment of IDD in some regions [23, 24], the study of iodine bioavailability [25], and in a few cases the study of iodine speciation in milk [13, 16, 26].

Different techniques used in the 170 publications cited above on the iodine determination in milk are summarized in Fig. 1.2. Among the variety of techniques used the most common ones are colorimetry, ICP-X (includes all combinations of ICP and other techniques such as AES, MS) and NAA. These three techniques account for 71% of all published papers. Colorimetry has been the most popular technique and it is still being used. It is surprising that electrochemical techniques have not been as extensively used as colorimetric ones, especially since polarography is very sensitive.

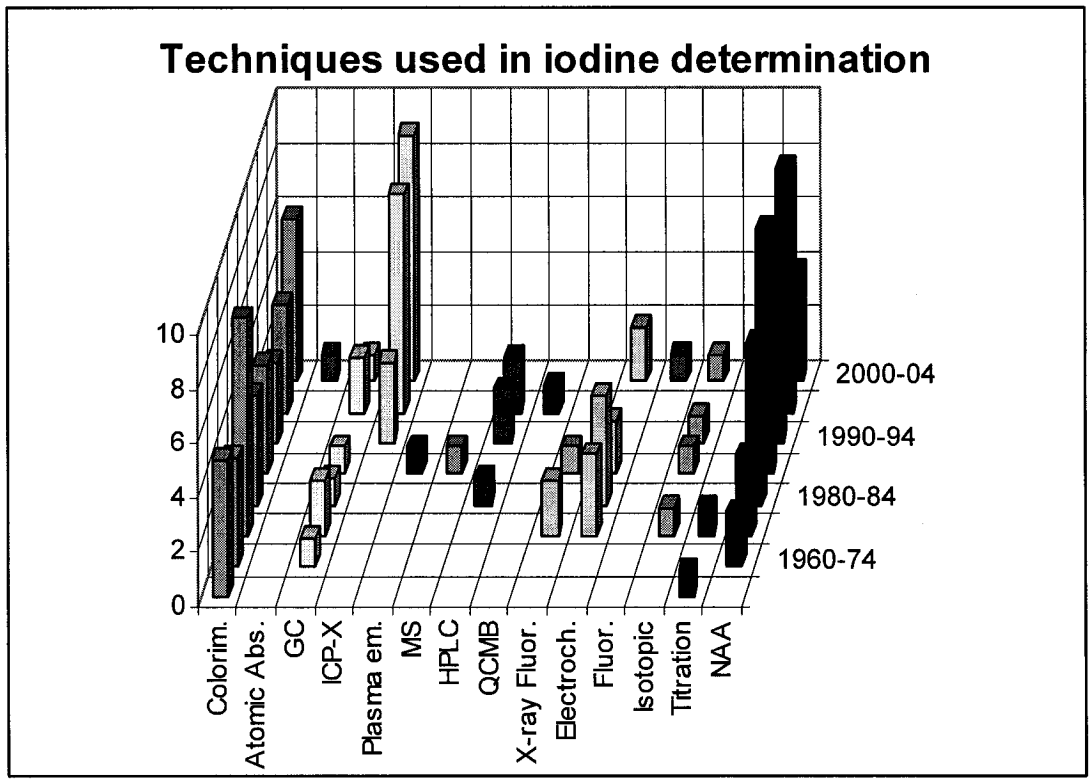

Fig. 1.2. Techniques used for iodine determination in milk during 1960-2004.

Legend:
Colorim. = Colorimetric techniques;
Atomic abs. = Atomic absorption spectrometry (AAS);
GC = Gas chromatography;
ICP-X = Inductively coupled plasma – X (X = other technique);
Plasma em. = Plasma emission;
MS = Mass spectrometry;
HPLC = High performance liquid chromatography;
QCMB = Quartz crystal micro balance;
X-ray fluor. = X-ray fluorescence;
Electroch. = Electrochemical techniques;
Fluor. = Fluorescence;
Isotopic = Isotope dilution, radiotracers, radio-immuno assay (RIA); and
NAA = Neutron activation analysis.

Neutron activation analysis has been extensively used, the main reasons being the high sensitivity of iodine and speed of analysis. NAA does not require, unlike most of the other techniques, digestion of the sample prior to analysis. However, NAA is declining in popularity and over the past 10 years publications have decreased because of

the closure of several reactor facilities. NAA is quickly being replaced by ICP-X (any of the possible combinations of ICP) which is also a very sensitive technique. Although it generally requires sample digestion and is very matrix-dependent, it appears to be the method of choice currently and for the foreseeable future.

The techniques discussed so far dealt with the determination of total iodine in milk. However, the situation of iodine speciation is different. Despite the importance of speciation analysis, there are only a few papers that deal with this topic. The papers where iodine speciation was examined are listed in Table 1.1.

Table 1.1. List of publications on iodine speciation.

Iodine species determined / identified	**Analytical technique**	**Reference**
Free iodine (I_2), iodide and tightly bound organic iodine	Gas chromatography after derivatization	27
Total inorganic iodine	Gas chromatography after derivatization	28
Total inorganic iodine	Gas chromatography after derivatization	29
Protein bound iodine	RNAA followed of protein separation on sephadex G-25	30
Protein bound iodine	Protein precipitation, ashing and colorimetry	31
Iodide	Milk passed through a membrane filter and iodide determined by HPLC	32
Iodide, iodine bound to organic species in the whey fraction	Size exclusion chromatography and ICP-MS	16
Iodide and iodate	Anion exchange chromatography and NAA	26
Iodide and iodate	Anion exchange chromatography and ICP-MS	13
Iodine in whey fraction	Size exclusion chromatography and ICP-MS	33
Free iodine, iodide, iodate	Solid phase microextraction and GC-MS	34

From Table 1.1 it can be seen that 11 of the 170 (6%) papers published dealt with iodine species in some way. In these papers, some iodine species were determined, but there is not one paper where the iodine distribution in the milk was investigated thoroughly. Although there are papers where the milk composition was determined but the iodine present in the different milk components is still unknown. These observations show the necessity to develop methods capable of filling the gaps in knowledge. Given the fact that milk is a very important source of food as well as the essential micronutrient iodine, the speciation of this element is indeed a necessity.

1.5 Objectives of this thesis

The need for the determination of total as well as species of iodine in milk with high precision and accuracy is obvious from the above introduction. Although several techniques have been used in the past for total iodine (details given in "Literature Review" in Chapter 2), NAA is a suitable technique for determining iodine levels in milk due to its intrinsically high sensitivity, among others properties. It is also highly selective, matrix effects are negligible, and there is no need for extensive sample preparation. There is only one naturally occurring isotope of iodine, ^{127}I with 100% abundance, and it has a high cross-section for neutrons, making it ideal for NAA. Because of its relatively high cross-section for epithermal neutrons compared to that of interfering elements such as potassium, sodium, chlorine and manganese, iodine is suitable for irradiation in a site shielded from thermal neutrons. The technique, called epithermal neutron activation analysis (ENAA), suppresses the activity due to the interfering nuclides and improves the detection limit for iodine. The 442.96-keV gamma-ray of ^{128}I lies in the low-energy region of the spectrum where the main interference is due to Compton scattering from the interfering nuclides. Compton suppression gamma-ray spectrometry (CS) is a powerful technique that reduces the Compton background of the radionuclides with high gamma-ray energies. Therefore, CS gamma-ray spectrometry can also improve the detection limit for iodine. The combination of EINAA and CS can give suitable sensitivity and detection limits capable of determining iodine instrumentally at very low levels. One of the objectives of this thesis was to develop both INAA and

EINAA methods using conventional and CS gamma-ray spectrometry, and to optimize conditions for measuring the iodine content of milk. It was also intended to explore the possibility of using pseudo-cyclic INAA for improving the detection limits for iodine. These are described in detail in Chapter 3 under "Iodine Determination in Milk by Neutron Activation Analysis".

Cow's milk is the most widely used type of milk in the world. The research work presented in this thesis involves only cow's milk, which is commonly referred to as milk. In order to carry out a credible speciation analysis it is necessary first to determine various components of milk, such as vitamins, carbohydrates, water, minerals, proteins, and lipids. The methods for the first are straight forward and well established. However, it is not so for proteins and lipids. Consequently, one of the objectives of this thesis was to evaluate various available methods for proteins and lipids, and modify them if necessary. Extensive work was carried for the separation and determination of proteins and lipids in milk (described in Chapter 4 under "Composition of Milk"). The capability of analyzing solid, liquid or oil by INAA without affecting sensitivity made this technique very attractive for the determination of iodine in milk lipids, proteins and in aqueous phases.

One of the main objectives of this thesis was to do speciation analysis of iodine in bovine milk. Since iodine can exist in both inorganic and organic forms, it would be necessary to separate them prior to their determinations. The separation of inorganic iodine species using ion exchange chromatography in both batch and column modes is given in Chapter 5 entitled "Separation of Inorganic Iodine Species". Concentrations of total inorganic iodine species, iodide and iodate ions were measured in this work.

In order to identify and quantify organic iodine species in milk it was necessary to develop various separation schemes and evaluate the merits and disadvantages of each scheme. A total of 7 schemes was developed for the sequential separation of organic and inorganic iodine species. It is often argued in speciation

analysis that there exists a possibility of transformation of species during the separation process. Therefore a simultaneous speciation analysis scheme was developed. The details of the separation and determination of total organic, lipid-bound, protein-bound, and inorganic anionic iodine species as well as iodide and iodate ions, casein and whey proteins containing iodine, and fractionation of apolar, less polar and polar lipid-bound iodine are given in Chapter 6 entitled "Separation Schemes for Iodine Species".

In today's world of analytical chemistry no method development is acceptable without validation; neither is a measurand without the calculation of its expanded uncertainty. The general procedure for validating a method is to analyze control samples. A number of reference materials (RM) and standard reference materials (SRM) were analyzed in this work as control samples as well as for measuring the accuracy of the methods developed. Additionally, an uncommon approach of comparison of two methods, namely the conventional relative NAA and the so-called k_0-NAA, was taken in this thesis for the purpose of validation. Both experimental and results are discussed in Chapter 3.

Considering the importance of uncertainty measurement and the amount of work needed to be done the entire Chapter 7 called "Calculation of Expanded Uncertainty" is devoted to the topic. Many experiments were carried out to estimate the error involved in each measurement step. Calculations were done to estimate uncertainty in each step. Finally, the expanded uncertainties of both the relative and k_0-NAA methods for the determination of total iodine in milk were calculated. Conclusions of all the work done in this thesis and recommendations for future work are given in Chapter 8.

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CHAPTER 2

LITERATURE REVIEW

2.1 Analytical methods for iodine determination

Analytical techniques are being continually developed with a view of detecting very small amounts of the analyte of interest with high precision and accuracy. More classical techniques such as gravimetry, volumetry, spectrophotometry, fluorometry, electrochemistry, *etc.* have been to some extent replaced by X-ray fluorescence (XRF) and atomic absorption spectrometry (AAS), inductively-coupled plasma spectrometry (ICP), mass spectrometry (MS), neutron activation analysis (NAA), high performance liquid chromatography (HPLC), and gas chromatography (GC) among others. Finally, the combination of some of these techniques has given rise to what is known as hyphenated techniques such as HPLC-MS, ICP-MS, and GC-MS. At present, this last family of techniques is being developed and further improved in many laboratories.

The determination of an analyte can be performed using either direct or indirect methods depending on the role of the analyte in the reaction and mechanism or process that is being studied. If the analyte participates in the detection process, then the method is direct. On the other hand, if the analyte is not involved in the detection process but it reacts or interacts with one of the chemical species in the system, then the method is an indirect one.

Iodine can be determined using almost all of the above techniques, directly or indirectly depending on the technique. The reason for selecting one technique over the other depends mainly on the purpose of the study, accessibility to the technique, complexity of the matrix, the expected iodine levels in the matrix, and the kind of analytical determination required. For instance, total iodine in milk has been determined using some of the above techniques. However, iodine speciation analysis at low levels is a

different problem because it requires the use of very sensitive techniques in conjunction with good separation methods. In this chapter, a review of the analytical methods used for the determination iodine in milk is presented.

2.1.1 Indirect methods

2.1.1.1 Catalytic colorimetric reactions

It has been noted in Chapter 1 that colorimetric methods have been most consistently used for iodine determination in milk since the 1930s. Out of a total of 38 papers on colorimetric methods cited in the SciFinder data base, 30 involve catalytic reactions. Out of these 30 papers, 7 use the catalytic effect of iodide ions in the reaction of $FeSCN^{2+}$ ions with nitrite ions. One uses the catalytic effect of iodide ions in the oxidation of tetrabase by chloramine T. The other 22 papers described iodine determination by the catalytic effect of iodide ions on the Ce(IV)-As(III) reaction, making it the most widely used method for total iodine determination.

Ce(IV)-As(III) reaction: This method, discovered in 1934, is known as the Sandell-Kolthoff reaction, in honor of the two chemists who developed it [1]. This method relies on the catalytic action of iodide ions in the Ce(IV)-As(III) reaction. Without the presence of iodide ions, the above reaction occurs very slowly, requiring 36 hours or more, but traces of iodide ion increase the reaction rate. Thus, the iodide determination is achieved by noting the time required for the disappearance of the yellow color due to Ce(IV) ions. The absorbance at 420 nm allows one to follow the reaction. The users have reported results of high precision and accuracy using this method [1-13]. Ions such as Cu(II), Cr(III), Ni(II), Co(II), Fe(III), and Zn(II) have been reported to interfere to some extent but Ag(I), Hg(II), Os(IV), Ru(VI) could cause serious interferences [1]. The method is still in use and was recently applied to the determination of iodine in cattle milk with concentrations as low as 24 $\mu g\ L^{-1}$ [2]. However, it has been reported by another researcher that concentrations lower than 2.5 $ng\ L^{-1}$ cannot be reliably reported by this method [6].

$FeSCN^{2+}$ and NO_2^- reaction: Iodide ions can also catalyze the reaction between ferric thiocyanate and nitrite ions in an acid medium. The rate of disappearance of the intense $FeSCN^{2+}$ orange-red color at 460 nm is used for quantifying the amount of iodide ions in the sample. One of the first researchers to use this method was R. V. Sveikina [14] and that is why this method is sometimes called the Sveikina's method. The main interference is caused by Hg(II) ions which form insoluble iodides. Chloride, bromide, and iodate ions do not cause any interference [15]. It has been reported [9], that this method provides better sensitivity and precision than the Sandell-Kolthoff method. Recently, a detection limit of 0.01 ng per 100 g sample has been reported [16]. The method is also being used in conjunction with flow injection analysis [17].

Tetrabase and chloramine T reaction: In 1975 Joerin [18] used the catalytic effect of iodide ions on the oxidation of tetrabase by chloramine T in a weakly acidic solution as the basis for its determination. Free iodine shows a lower catalytic effect, and iodate ion has no effect. The method has been reported to be highly specific, but chloramine T solution is very unstable. The measurements are carried out at 600 nm. This method has a sensitivity of 0.001 $\mu g\ mL^{-1}$ and it was used for iodide determination in milk at concentrations less than 0.04 $\mu g\ mL^{-1}$.

2.1.1.2 Methods based on decomposition of palladium complexes

It is well known that iodide ions form a very insoluble compound with Pd(II) ions in aqueous solutions. Also it is known that Pd(II) ions form soluble complexes with many other ions and ligands. Therefore, a palladium-ligand complex is added to the sample containing iodide ions resulting in the formation PdI_2 [19]. The amount of iodide ions present in the solution is quantified indirectly by the extent of decomposition of the palladium-complex. These methods have three potential sources of error: the ions that could react with iodide, the ions that could react with Pd(II), and the ions that could react with the ligand, producing a complex with a similar color [19]. Different ligands have been used to determine iodine in different matrices.

The Pd(II) ions catalyze the reduction of the Co(III)-EDTA complex by hypophosphite ($H_2PO_2^{2-}$) ions. Iodide ions inhibit this reaction by reacting with Pd(II). Based on this, in 1991 Garcia *et al.* [20] reported an indirect method to determine iodide ions. The inhibition rate was calculated by measuring the absorbance of Co(III)-EDTA complex at 540 nm at different time intervals. Using this method it is possible to determine iodide ions in the range of 2–28 ng mL^{-1}. In general, the method is selective and it has been applied successfully to various types of milk, such as cow's milk 1.59 μg g^{-1}, skimmed milk 1.13 μg g^{-1}, and infant milk powder 3.3–4.0 μg g^{-1} [20].

2.1.1.3 Atomic absorption

Following the same idea as the above methods, AAS has also been used for the indirect determination of iodide ions using reactions of Pd(II), Ag(I) or Hg(I) with iodide ions to form highly insoluble compounds. The readings of the metal ion levels in solution are taken before and after placing the solution in the sample containing iodide ions. The reduction in concentration of the metal ion in solution is proportional to the amount of iodide ions present in the sample. Another approach is to re-dissolve the precipitate formed and to measure the metal ion present in the precipitate. This method was applied to milk and it was possible to detect as low as 5 μmol L^{-1} of iodide ions [20, 21].

2.1.1.4 Other indirect determinations

Nitrate ion, sulfanilic acid, and α-naphthylamine reaction: In 1956, Hanni [22] described a method where a milk sample was treated with sodium carbonate and potassium permanganate. Free iodine was liberated by means of phosphoric acid. The free iodine reacted with hydroxylamine producing nitrite. The nitrite was oxidized to nitrate and finally nitrate was determined colorimetrically by the reaction with sulfanilic acid and α-naphthylamine.

Leuco-crystal violet to crystal violet reaction: In 1977, Ahmad [23] reported trapping of free iodine released during the digestion of milk in ammonia. Hypoiodous acid was then formed by means of N-chlorosuccinimide /succinimide reacting with ammonium iodide. The iodine present in the sample was indirectly determined at 592 nm by the formation of crystal violet from leuco-crystal violet when hypoiodous acid is present. Some species such as Mn(II), Fe(III), nitrite, bromide, cyanide, sulfide and phenol were reported to cause interferences but not at low concentrations [24, 25]. Iodate ions cannot be determined directly using this method [24, 25].

2.1.2 Direct methods

2.1.2.1 Spectrophotometric methods

Solvent Extraction of Iodine: There are several methods for measuring micro-quantities of iodine by solvent extraction coupled to spectrophotometry. These methods use either oxidation or reduction of iodine species to produce free iodine (I_2). This free iodine is then extracted into an organic solvent, and determined spectrophotometrically. Another possibility is the formation of a soluble complex with iodine and its extraction into an organic solvent followed by spectrophotometric determination.

In 1929 Reith [26] developed a procedure for the digestion of milk along with concentration of iodine in iodide form in the aqueous phase. The addition of nitrate ions and sulfuric acid liberated the iodide as free iodine which was then extracted into carbon disulfide. The resultant color was compared against standards in the range of 0-0.5 μg prepared in the same fashion.

Similarly in 1930 McClendon *et al.* [27] used a method similar to that of Reith. After digestion of a milk sample with nitrite ions free iodine was liberated from the aqueous solution. It was extracted into carbon tetrachloride and the color was measured in

a colorimeter. In the same year von Fellenberg [28] applied a similar method where the free iodine was extracted into chloroform.

Spectrophotometric methods have been used for the determination of iodine as complexes in waters. Sometimes the iodine complex is extracted into organic solvents prior to the measurement. Typical examples of iodine complexes include that formed with o-tolidine [29], the well-known iodine-starch complex, and the brilliant green-iodide pair extracted into methylene blue chloride [30]. Because these methods have not been used in milk, they are not discussed here in detail.

2.1.2.2 Electrochemical methods

Different electrochemical techniques such as polarography, ion selective electrode potentiometry, and inverse voltammetry have been used for the determination of iodine in milk. In general terms these are very fast, easy, sensitive and inexpensive techniques.

Polarography is a simple, rapid, specific, free of interferences, and reliable method for iodine determination in milk [31, 32]. Iodine is first oxidized before the measurement because it must be in the iodate form. In differential pulse polarography, the iodate ions are measured at -0.90 V against a saturated calomel electrode [31]. The ideal oxidizing agent is sodium hypochlorite because it oxidizes iodide to iodate quantitatively and no other oxidation product is formed in the solution. Its excess does not interfere with the measurement of the iodate peak. This method has been used for the determination of 0.05-3.5 $\mu g.g^{-1}$ of total iodine in milk with a RSD of 2.9% and a detection limit of 0.05 $\mu g.g^{-1}$ [31].

Ion-selective electrode potentiometry (ISE) is perhaps the most used electrochemical technique for iodine determination. It is advantageous because no sample digestion is needed here. Milk is mixed with potassium chloride and the ISE is dipped into

the sample [33]. It is a very fast technique. Although only iodine in the iodide form is detected, milk is analyzed without any previous separation. Some papers reported the need for skimming the milk before analysis [34]. It has been reported in the literature that such electrodes have a linear response over a wide range of concentration although some variability in the response is shown [33-37]. Recently this method was used to study the effect of pre-dipping cow teats in an iodophor disinfectant on the iodine content of milk; concentrations as low as 213 ng iodine mL^{-1} of milk were detected with an RSD of 17% [35]. It has also been reported that this method might give erroneous results for milk samples preserved with formaldehyde [33].

In 2003, an inverse voltammetric method was used for the determination of iodine in milk [38]. Sample digestion is required prior to voltammetric analysis. Once the iodine is converted to the iodide form, it is continually electrodeposited on the surface of a working electrode. The electrolyte generally contains mercury (II) nitrate, potassium chloride and hydrochloric acid making the pH of the sample between 1 and 3. The working electrode can be graphite, glassy carbon, gold, silver, platinum, copper or nickel.

2.1.2.3 Chromatographic methods

Among the chromatographic techniques, gas chromatography and HPLC have been used to some extent for the determination of iodine in milk. Both techniques require a digestion step and gas chromatography also requires a derivatization before quantifying the iodine.

Gas chromatography: In 1971, Hasty reported micro-determination of iodine using GC and detection by an electron capture detector [39]. The method is based on a derivatization step where iodine is converted into iodoacetone by placing the iodine solution into propanone acidified with sulfuric acid forming monoiodoacetone. After 30 min the iodine derivative is extracted into hexane and injected into a chromatograph. This is the most used GC method for iodine determination. Chlorides and bromides do not interfere. The main disadvantage of this method is that more than one iodine derivative

can be produced. Although the main product is monoiodoacetone, 1,1 diiodoacetone could also be formed at low iodine concentrations [39].

Other reported GC methods are basically a modification of Hasty's. For instance, Grys in 1974 determined iodine in milk using 3-pentanone instead of propanone as the derivatizing agent [40]. In this case the iodine derivative was 2-iodo-3 pentanone. Another iodine derivative reported is the formation of iodobutanone [41]. All these GC-ECD methods have the capability of determining iodide at nanogram levels with a RSD of 1.9%.

HPLC: HPLC has also been used for iodine determination with anion exchange and reverse phase columns. Methods with pre-column derivatization, post-column derivatization and also without derivatization have been reported. The decision of whether or not to use any kind of derivatization depends largely on the availability of columns and detector systems.

In 1983 Hurst *et al.* [42] reported an HPLC method for determining iodine in milk and milk chocolate. The method consisted of digestion of the sample and trapping the free iodine in a reducing mixture to convert it to iodide. HPLC was carried out in a C_{18} column and the detection was performed at 226 nm. The method showed good precision and accuracy and did not require derivatization.

In 1988 Buchberger [43] reported a very sensitive method for iodide and bromide ions using ion chromatography with post-column reaction detection. The method used a Vydac 302-IC anion-exchange column with methanesulphonic acid as the mobile phase for the ion chromatographic separation of iodide and bromide ions. The reaction between iodide or bromide with chloramine-T and 4,4'-bis (dimethylamino) dimethylmethane was developed as post column reaction detection. The detection limit was calculated as 20 pg iodide and 15 ng bromide ions.

In 1992, Verma *et al.* [44] reported a method for iodide determination using pre-column derivatization of iodide into 4-iodo-2,6-dimethylphenol and HPLC of iodophenol with UV detection. The pre-column derivatization involved oxidation of iodide with 2-iodobenzoate at pH 6.4 in presence of 2,6-dimethylphenol. The reaction mixture was separated on an octadecylsilane column using a mobile phase of acetonitrile-water 60:40 (v/v), and detection at 220 nm. For an injection volume of 100 μL, the detection limit was 0.5 ng of iodide ions.

In 1993, Serti and Malone [45] reported a method for determining iodine in milk using LC with electrochemical detection. It was based on removing all insoluble materials and proteins from milk with a 25 000 MW membrane filter, followed by the separation of iodide from the clear filtrate by reversed-phase ion-pair-LC. The mobile phase was a solution of dibasic sodium phosphate, cetyltrimethylammonium chloride in acetonitrile. Detection limits were 0.2 $\mu g\ g^{-1}$ for powdered milk and 10 $\mu g\ L^{-1}$ in liquid milk.

In 1994, Kuwahira and Asai [46] reported a method where milk samples were digested by incineration in an electrical furnace. Iodine was ultrasonically extracted with DDW, and injected into HPLC. They used a Shodex IC-524A column in combination with a electrochemical detector. Iodine was determined in milk with a range of 0.005-0.1 $\mu g.g^{-1}$ with a RSD of 2-3%.

2.1.2.4 Hyphenated methods

Hyphenated techniques are those where two or more individual techniques are coupled. The most common examples for the analysis of iodine in milk are GC-MS, ICP-MS, and ICP-IDMS, where ICP-MS perhaps is most extensively used.

ICP-MS: The application of ICP-MS to iodine analysis has undoubtedly marked the beginning of a new era. Before ICP-MS became available, Grys made the first attempt to achieve iodine speciation analysis in 1970 using GC-ECD [40]. After ICP-MS,

some other iodine speciation work including the determination of inorganic iodine species as well as some organic species like T3 and T4 hormones [47] have been published. ICP-MS is particularly suitable for iodine speciation analysis due to its high sensitivity although it requires a careful selection of preconcentration separation methods to achieve accurate results [48].

Iodine determination using classical ICP-MS requires the digestion of a sample using either an acid or a base [48-52]. The use of an internal standard is also common; tellurium or indium is generally used because their masses and ionization energies are comparable [48-54]. In 1990, Baumann [50] determined iodide levels as low as 50 ng mL^{-1} in fresh milk and milk powder by ICP-MS using tellurium as an internal standard. He claimed that ICP-MS was the only method for the determination of iodine in milk down to the lower $ng.mL^{-1}$ range. This statement might be overlooking the fact that less expensive methods, such as colorimetry, have reported iodine determination in this range. A similar conclusion will be especially true for NAA which is well known for its excellent sensitivity for iodine. Nevertheless, the capabilities of ICP-MS for such determinations have been established.

In 1993, Vanhoe *et al.* [49] also determined iodine in milk and human serum by ICP-MS. They found that the destruction of the sample with nitric acid was not suitable because the ion signal was not stable and samples spiked with iodide yielded erroneous recoveries. For these reasons, they used an ammonia solution instead of nitric acid. Tellurium was used as an internal standard in milk samples but indium was selected for human serum samples. The detection limit was 10 ng g^{-1}. In 1994, Schramel and Hasse [51] determined total iodine in biological materials such as serum, milk, plants, tissue, *etc.* using ICP-MS. Samples were analyzed directly in liquid form after oxidation and dilution in 0.1 M sodium hydroxide solution. The detection limit was in the range of 0.01 $\mu g\ L^{-1}$.

In 1996, Sturup and Buchert [53] determined total iodine in milk and milk powder using flow injection ICP-MS. They did a total iodine determination using an

alkaline digestion. The detection limit was 0.45 μg L^{-1}, and the reproducibility and accuracy were found to be acceptable. In 1998, Fecher *et al.* [54] combined the digestion of milk samples with the alkaline extraction using tetramethylammonium hydroxide (TMAH) and ICP-MS for the determination of iodine. The extraction of iodine using TMAH was considered 100% efficient according to the authors; however, other researchers questioned this efficiency claim. The detection limit based on 9 std. dev of the blank was 30 $ng.g^{-1}$ when a sample size of 100 mg is used. Special care must be taken to avoid sample contamination. This method is the German reference method for the determination of iodine in dietetic foodstuffs.

In 1999, Fernandez-Sanchez and Szpunar carried out an iodine speciation analysis in milk samples using size exclusion chromatography and ICP-MS (SEC-ICP-MS) [47]. They centrifuged the sample in an ultracentrifuge and the whey fraction was analyzed. The whey was injected onto a SEC column, fractions collected and analyzed by ICP-MS. They found iodide, T3, T4 and other small organic molecules related to these thyroid hormones. They reported a detection limit of 1 mg L^{-1}. A very similar work was reported in 2002 [55] where not only iodine but also many other essential trace elements were identified as being associated with whey proteins in milk. In 2001, Leiterer *et al.* [56] determined iodide and iodate ions in milk samples using ion chromatography coupled to ICP-MS. Milk samples where passed through an anion exchange column where iodide and iodate were retained and after elution they were analyzed by ICP-MS. They reported a detection limit of 2 $\mu g.L^{-1}$.

ICP-IDMS: ICP-IDMS is fairly independent of matrix effects unlike ICP-MS making it a suitable method for the determination of iodine in milk at low levels. One disadvantage of this technique is the accessibility to ^{129}I isotope which is commonly used here. This isotope is not a naturally occurring iodine isotope. Another disadvantage is the possible interference of the ^{129}I signal by ^{129}Xe or the possible formation in the plasma of $H_2{}^{127}I^+$.

In 1998, Radlinger and Heumann [57] determined total iodine in food samples using ICP-IDMS after separating iodine using two methods. One method was the extraction of iodine using tetramethylammonium hydroxide (TMAH) at high temperatures and the other involved a complete destruction of the sample with a mixture of perchloric and nitric acids in a microwave oven. The accuracy of ICP-IDMS with both sample treatment methods was high. The RSD was in the range of 0.6-2.8% for iodine concentrations between 0.1 $\mu g.g^{-1}$ and 5 $\mu g.g^{-1}$. The detection limit was 8 $ng.g^{-1}$ for a sample weight of 0.8 g. The ICP-IDMS method is fast, precise, accurate and fairly independent of matrix effects. In 2000, Haldimann *et al.* [58] reported an ICP-IDMS method for iodine determination after digestion of samples with nitric acid. The method was tested on SRMs and then compared statistically to NAA which was considered a reference method and no significant differences were found.

GC-MS: In 1996, Shin *et al.* [59] determined iodine using GC-MS. They basically used the same derivatization method as Verma [44] but GC-MS for detection instead of LC. They reported a detection limit of 0.5 ng mL^{-1} with a RSD of less than ±5% using 20 ng of iodine mL^{-1} and 6 replicates. They found less than 5 ng mL^{-1} of iodide in drinking water; although iodate and free iodine were not detected.

Das *et al.* [60] in 2004 reported a method in which a solid phase micro-extraction (SPME) cartridge was combined with gas chromatography for the determination of iodine in milk. The method was based on a sequence of oxidation reactions using 2-iodosobenzoate followed by the iodination reactions of N,N-dimethylaniline at pH 6.4 resulting in formation of 4-iodo-N,N-dimethylaniline. Once the derivative was formed, it was trapped in the SPME cartridge, followed by the determination of iodine using GC-MS. Once the iodide was determined in the sample, iodate was reduced to iodide by ascorbic acid and it was determined similarly. The rest of the sample was then digested and organic iodine was determined. The detection limit was 25 $ng.L^{-1}$ with an RSD of 3.1%.

2.1.2.5 X-ray Fluorescence Spectrometry

XRF spectrometry has been used to determine iodine in milk since 1975 [33, 61]. Bromine does not cause any interference in this technique. Purdham and Strausz [61] reported a detection limit of 0.8 mg iodine 100 mL^{-1} of milk using XRF. This detection limit could be decreased with longer counting times. Crecelius [33] reported that the standard curve for XRF was linear over the range 0.1-4.0 $\mu g.g^{-1}$ with a RSD of 10%. The detection limit reported, calculated as twice the noise level, was 0.1 $\mu g.g^{-1}$. Crecelius as well as Strausz both found 0.36-1.3 $\mu g.g^{-1}$ iodine in milk. The XRF technique requires no chemical treatment of milk samples. Liquid milk samples were simply freeze-dried and a solid pellet was prepared for analysis. Although XRF is a particularly attractive technique due to its speed and simple sample preparation, Pavel *et al.* [62] found it to have a relatively poor detection limit of 3 mg kg^{-1} in organic samples. For this reason, preconcentration of iodine prior to the measurement by XRF would be advisable.

2.1.2.6 Other techniques

Quartz crystal microbalance (QCM) method: In 1999, Yao *et al.* [63] determined iodine in milk using a QCM method. This method is based on the sensitive response to mass change at the electrodes of a piezoelectric quartz crystal. After digestion the ionic iodine was converted to free iodine which was adsorbed at gold electrodes of QCM and the iodine content in the sample was determined through the decrease of QCM frequency. The only element that interferes is bromine. The method had a good reproducibility and a detection limit of 0.0005 $mg.L^{-1}$.

Fluorescence: In 2003, Zhao *et al.* [64] reported a method for the determination of iodine based on a fluorescence-iodine sensor. A functionalized oligophenylvinylene derivative was synthesized and applied as the fluorescent sensory material for the fabrication of the sensor. Most common anions did not interfere. The sensor was more sensitive for triiodide ion than for iodine. The detection limit was 10^{-8} mol L^{-1} for triiodide ions.

2.1.2.7 Neutron activation analysis methods

The NAA technique is particularly attractive for iodine determination because of its inherent high sensitivity [65-69]. NAA is definitely the preferred technique when low level iodine measurements are required [67]. It has some advantages over ICP-MS. For example, no sample preparation is required in the case of total iodine determination by NAA and it is fairly free from interferences. In samples like milk, the total iodine determination is as easy as taking 1 mL from a carton of milk and irradiating it for a few min in a reactor and counting. In the case of speciation analysis, NAA has another unquestionable advantage because its results are independent of the matrix. That means it is possible to perform irradiation of precipitated protein, extracted oil lipids, aqueous inorganic solutions and obtain reliable results for iodine. For these reasons it is advantageous to use NAA in iodine speciation analysis. It has been widely used for iodine speciation in marine algae and for the determination of inorganic iodine species in milk and urine [70]. It is highly accurate and sensitive. All these characteristics make NAA an invaluable technique. Perhaps its major disadvantage lies in the limited number of reactor facilities available in the world.

Iodine has been determined extensively using NAA because of its high sensitivity. The thermal neutron cross-section for iodine is 6.2×10^{-24} cm^{-2} which is quite high. The interesting fact is that the epithermal neutron cross section is much higher, *i.e.* 147×10^{-24} cm^{-2} and not many nuclides have this characteristic. That is why iodine determination is especially attractive by NAA when the presence of other elements does not cause significant interference when the irradiation is performed using epithermal neutrons [65-69]. Food samples, especially milk, have high amounts of Ca and Mg and smaller amounts of other elements such as Na, Cl and Mn. Except for Ca, which is not very sensitive, the other elements are also quite sensitive by NAA. However, the use of epithermal neutron irradiation significantly reduces their sensitivity. This means that iodine can be detected in the presence of the above elements without any chemical separation [65, 66].

Each radionuclide decays by emitting its characteristic gamma-ray(s). In the detector, not only the photoelectric absorption of the gamma ray occurs but also the undesirable Compton scattering takes place. For this reason, the radionuclides of the above elements contribute to the background in a gamma-ray spectrum. All of them have gamma energies above 600 keV whereas ^{128}I emits a gamma-ray at 443 keV. One of the ways the signals from these interfering nuclides can be reduced is through a special detection system designed for minimizing the Compton effect [68-69]. It comprises a central high- purity germanium detector (HPGe) surrounded by a NaI(Tl) detector whereby the contribution from scattered gamma-rays is drastically reduced. A combination of epithermal irradiation and Compton suppression gamma-ray spectrometry can easily reduce the detection limit of iodine to 20 $ng.g^{-1}$ [67-69] without any chemical treatment of the sample. This approach has been followed in this thesis.

Iodine has also been determined *via* radiochemical neutron activation analysis (RNAA), preconcentration neutron activation analysis (PNAA), and photon activation analysis (PAA) [65-67]. In general, RNAA gives the best detection limit, according to Chatt and recently confirmed by Kucera [65-67]. However, PNAA can also give similar results when extreme care is taken to avoid possible contamination [67]. PAA is an option in the case of laboratories with no access to nuclear reactors facilities because the irradiation source is relatively inexpensive compared to a reactor although it is not applicable to as many nuclides as NAA [67]. In PNAA, the irradiation is carried out after iodine separation, commonly by coprecipitation with Bi_2S_3, PdI_2, or AgI. On the contrary, the separation in RNAA is done, *e.g.*, by coprecipitation after irradiation of the sample [65-67].

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CHAPTER 3

IODINE DETERMINATION IN MILK BY NEUTRON ACTIVATION ANALYSIS

A short history of NAA along with various types of NAA is described in this chapter. The development of INAA and EINAA methods using conventional and Compton suppression gamma-ray spectrometry for the determination of iodine by relative as well as k_0-NAA methods are discussed in detail. The precision and accuracy of the methods have also been evaluated.

3.1 Introduction

3.1.1 Discovery of NAA

In 1936 George de Hevesy and Hilde Levi published the first paper on the nuclear activation analysis method [1]. Hilde Levi herself told the story of discovery of activation analysis at the seventh international conference on modern trends in activation analysis (MTAA-7) held in Copenhagen, Denmark in 1986 [2]. The conference was especially dedicated to celebrate the 50^{th} anniversary of this important event. She explained how they were irradiating rare earth elements using neutrons from a radon-beryllium source with further counting of β-particles in a Geiger counter. The radionuclide identification was performed by the determination of half-life. They noted that among the rare earth elements, dysprosium especially became highly radioactive when it was bombarded with neutrons. They recognized the qualitative and quantitative potentialities of bombarding elements with neutrons for chemical analysis through the determination of "neutron absorbing power" and the determination of their half-lives [2, 3].

However, it was not until 1944 that this technique started to be used to its full potential. In this year, operation of the first research-type nuclear reactor

commenced with the X-10 reactor at the Oak Ridge National Laboratory. Up to that time the highest neutron flux available was 5 x 10^5 cm^{-2} s^{-1}, but the X-10 reactor had a neutron flux of 5 x 10^{11} cm^{-2} s^{-1}. The increase in neutron flux was a major step in increasing the sensitivity of the method immensely [2].

3.1.2 Types of NAA

Instrumental neutron activation analysis (INAA): This is the technique where samples are activated by neutrons and where no chemical treatment of the sample is performed before irradiation. The main advantages of this technique are sensitivity, the elimination of contamination and loss of volatile species in a sample. It is a non-destructive method from the chemical dissolution point of view. Therefore the sample can be re-used for other analysis and also can be preserved as a piece of evidence in a legal case for example. However, the sample is irradiated without separation of either the interfering elements in the matrix or the matrix itself. This could affect the detection limits depending on the radionuclide of interest and the extent of interference by other activities in the sample. In this thesis, the levels of major elements in milk have been determined by INAA.

Epithermal neutron activation analysis (ENAA): The distribution of neutron energy in a reactor neutron flux spectrum varies from a few eV to several MeV. In INAA, the sample is irradiated with neutrons covering the entire neutron energy spectrum. Thermal neutrons, defined as neutrons, which are at equilibrium at room temperature having energy of 0.025 eV, are the major component of reactor neutron flux. When neutrons with energies less than 0.1 eV are "filtered" and the sample is selectively irradiated with the intermediate neutrons, the technique is then called epithermal neutron activation analysis (ENAA). This cut-off of low energy neutrons is generally carried out by absorption of the thermal neutrons onto suitable materials such as cadmium or boron. These intermediate energy neutrons are often referred to as epi-cadmium and epi-boron neutrons, respectively. This means that the nuclei with a high capture cross-section for epi-thermal neutrons will be activated [3].

The main use of ENAA lies in the suppression of interfering activities from nuclides produced from elements with high thermal neutron absorption cross sections. For example, geological and biological materials generally contain significant amounts of elements such as Na, Al, Cl, Mn, V and La, all of which have high thermal neutron cross sections (σ_{th}). In the presence of these elements, the activities of trace elements of interest could very well be masked. If a desired low-level analyte has no interfering elements and has a large resonance integral (*i.e.* neutron cross section in the epithermal region), the irradiation of the sample with epithermal neutrons will result in a better detection limit and improved precision [4-9]. This technique is generally applied in the instrumental mode, *i.e.* without any chemical treatment of the sample, and commonly called epithermal instrumental neutron activation analysis (EINAA). In this thesis, the concentration of iodine in milk has been measured using EINAA. A combination of INAA and EINAA methods has been applied to the determination of aluminum and phosphorus in milk.

Preconcentration neutron activation analysis (PNAA): In this technique a sample undergoes chemical separation whereby the element of interest is preconcentrated and/or interfering elements minimized prior to irradiation. The main advantage lies in the improvement of detection limits in comparison to INAA. This technique can also be advantageously used to reduce a large volume of a sample to a few mL. The disadvantages include the possibility of contamination *via* chemical reagents and loss of some chemical species during separation [3, 10]. Once the analyte is separated, it can be analyzed by either NAA or ENAA.

Radiochemical neutron activation analysis (RNAA): In this technique the sample is irradiated before any chemical treatment. The main advantages of RNAA are the possibility of achieving very low detection limits, high precision and accuracy, and no reagent blanks since the separation is done after the irradiation. The disadvantages are the requirement of specialized laboratory facilities and personnel capable of handling open radioactive sources. Also the technique cannot be applied to all elements [3, 10] since the radionuclide of interest must have an adequately long half-life.

The RNAA technique cannot be used to discriminate between nuclides which are produced by competing reactions.

Cyclic neutron activation analysis (CNAA): When the radionuclide of interest is short-lived, the sample must be irradiated, allowed to decay and counted for short periods usually of the order of a few seconds. Under these conditions, the photopeak of interest may not have a sufficient number of counts above background for reliable measurement. One way to improve the detection limit as well as precision of measurements is to repeat the irradiation-decay-counting process for an optimum number of cycles within a short time. When this process is repeated n times, the peak of the radionuclide of interest can grow n times while the background grows only by $(n)^{0.5}$, leading to a significant improvement of detection limit. This approach has been used for the determination of low levels of elements which produce short-lived radionuclides in a variety of sample matrices [3, 10]. This technique can be employed with reactor flux as well as epithermal neutrons for instrumental measurement and also after preconcentration of elements.

Pseudo-cyclic neutron activation analysis (PCNAA): Sometimes the activity after the first irradiation-decay-counting cycle is so high that the second cycle cannot be immediately repeated. In such cases a longer decay time, for example a few hours to days, is needed before reliable measurement through a second cycle can be performed. Then the technique is called pseudo-cyclic NAA [3, 10]. It has the same advantages as CNAA but the analysis time is longer. In this thesis, a PCINAA method for the determination of very low levels of iodine in milk has been developed.

Cumulative neutron activation analysis (CumNAA): This technique was developed to overcome one disadvantage of PCNAA. If the same sample cannot be irradiated after the first cycle because of elevated activity, then the second cycle is performed with a replicate of the original sample. After n cycles, the total sample mass will be the sum of individual masses of the replicates. This approach reduces analysis

time compared to that in PCNAA but requires an adequate amount of sample available for the desired number of replicates [10].

3.1.3 Dalhousie University SLOWPOKE-2 Reactor (DUSR) Facility

SLOWPOKE is an acronym for Safe LOW POwer Kritical Experiment. The SLOWPOKE-2 is "a compact, inherently safe, swimming-pool type reactor designed by the Atomic Energy of Canada Limited primarily for neutron activation analysis and isotope production" [11]. The Dalhousie University SLOWPOKE-2 Reactor (DUSR) facility was installed in 1976 June and it has been in continued operation since then. At full power of 16 kW, DSUR has a maximum thermal neutron flux of $1x10^{12}$ cm^{-2} s^{-1} in the inner sites and that of $5.4x10^{11}$ in the outer sites. The neutron flux can be varied 3 orders of magnitude, *i.e.* $1x10^{9}$ to $1x10^{12}$ cm^{-2} s^{-1}.

The DUSR has 5 inner pneumatic irradiation sites and 3 outer sites. One of the outer sites is shielded with a 1-mm-thick cadmium layer for absorbing thermal neutrons while allowing the sample to be irradiated with higher energy neutrons. The neutron flux at the inner sites is highly homogeneous both vertically and radially, *viz.* the variation being less than 1% cm^{-1}. The vertical inhomogeneity is less than 5% cm^{-1} in the outer sites. The homogeneity and stability of the neutron flux in the DSUR have been evaluated several times and found to be less than 2% from day to day operation over a period of about three decades [11, 12]. The exceptional homogeneity and stability of the DUSR neutron flux allow one to irradiate samples and standards in a given site at different times without the need of flux monitors. Simultaneous irradiations of samples and standards stacked vertically in the same vial can also be performed at DUSR inner sites under essentially the same neutron flux. Irradiations in the present work were done in inner and Cd-shielded sites of the DUSR.

3.1.4 Counting systems at DUSR facility

Two detection systems were mainly used in this work. One was a Compton suppression gamma-ray spectrometry system. It consisted of an EG&G Ortec HPGe p-type coaxial detector with a resolution (FWHM) of 1.72 keV at the 1332.5 keV photopeak of ^{60}Co and a relative efficiency of 25% with respect to a standard NaI(Tl) detector in conjunction with an Ortec D-SPEC plus pulse height analyzer. The guard detector used in this system was a 10" x 10'' NaI(Tl) annulus with 5 photomultiplier tubes (PMTs) supplied by Harshaw and a 3''x 3'' NaI(Tl) plug with one PMT supplied by Teledyne. The peak-to-Compton ratio of this system was 582:1 at the 662-keV photopeak of ^{137}Cs. The second counting system consisted of a D-SPEC plus multi-channel analyzer in conjunction with a Canberra 60 cm^3 Ge(Li) semiconductor detector with a resolution of 1.88 keV at the 1332.5 keV photopeak of ^{60}Co, a peak-to-Compton ratio of 35:1 and an efficiency of 9.5%. All measurements performed on the first counting system were done at a distance of 2.6 cm in the anticoincidence mode for Compton suppression while in the second system the measurements were done on top of the detector (covered with a plastic dish) in a conventional counting mode.

3.1.5 Quantification methods in NAA

The principal equation in neutron activation analysis can be expressed as:

$$A_0 = \frac{6.023.10^{23}.\Theta.m.R.S.D.C.t_c}{W} = \frac{P_A}{\varepsilon_p \gamma} \qquad (1)$$

where:

A_0: the activity obtained after irradiation of a sample for a time t_i after a decay time of t_d and counting time t_c (expressed in disintegrations per unit time);

Θ: the natural abundance of the target nuclide expressed in (%);

m: the mass of the target element expressed in (g);

W: the atomic mass of the target nuclide expressed in (amu);

S: the saturation factor $\left[1-e^{-\lambda t_i}\right]$ where λ is the decay constant of the radioactive nuclide formed;

D: the decay factor $\left[e^{-\lambda t_d}\right]$;

C: the counting factor $\left[\frac{1-e^{-\lambda t_c}}{\lambda}\right]$;

R: The reaction rate per target nucleus, this parameter is characteristic of each reaction and depends of both the neutron flux $\Phi(E)$ (cm^{-2} s^{-1}) and the cross section $\sigma(E)$ (barns; 1 barn = 10^{-24} cm^2) for the (n,γ) reaction, expressed in (s^{-1});

$6.023x10^{23}$: the Avogadro's number;

P_A: The net area of the photo-peak produced by the radionuclide undergoing the (n,γ) reaction, and corrected for dead time, interferences of first and second order, self-absorption, self-shielding (expressed in disintegrations per unit time);

ε_p: the efficiency of the detection system gamma-ray energy emitted by the radionuclide;

γ: The branching ratio, *i.e.* the emission probability of a γ particle by the radionuclide of interest.

Three different approaches can be used for the quantitative measurement of the concentration of an element using the basic Equation (1) given above. The methods are described below.

3.1.5.1 Absolute method

If the mass of an element was unknown, it would be possible to determine it directly from the equation:

$$m = \frac{A_{nc}.W}{0.6023.\Theta.\gamma.\varepsilon_p.S.D.C}.f(\sigma(E),\phi(E)) \qquad (2)$$

However, this method is not used for high-accuracy work because it can introduce errors from those in the nuclear data and neutron flux. However, in samples where errors of about 20% are acceptable the absolute method can be easily applied. This method has not been used in the present work. The accuracy of the data can be significantly improved if the relative method is introduced.

3.1.5.2 Relative method

The relative NAA method is based on the use of elemental standards of known mass of the element of interest. The elemental standard and the sample are subjected to the same irradiation, decay and counting procedure using the same detector system. Briefly, all experimental conditions are kept the same. The actual values of nuclear data and other constants are not needed since they cancel out. As a result the unknown mass can be determined by:

$$\frac{m_u}{m_s} = \frac{A_{nc,u}}{A_{nc,s}} \tag{3}$$

where, m_u and m_s are the unknown and standard sample masses respectively, and $A_{nc,u}$ and $A_{nc,s}$ are the activities in number of counts of the unknown sample and standard, respectively. The relative method has been extensively used in this thesis.

This method is simpler than the absolute method, but it requires pure elemental standards. The elemental standard preparation may introduce systematic errors in the final results due to contamination and/or loss. To overcome this disadvantage, in 1975 Simonits, De Corte and Hoste introduced the k_o standardization method as a procedure that is expected to combine the simplicity of the absolute method with the accuracy of the relative method [13].

3.1.5.3 k_o standardization method

It is based on co-irradiation of a sample and a neutron flux monitor together with the use of a composite nuclear constant called k_0-factor [13]. This means

that only one elemental standard is used in this method, which is called a monitor. In this case the concentration ($\mu g.g^{-1}$) of an element can be expressed by:

$$m = \frac{A_{nc,u}.S_m.D_m.C_m}{S_u.D_u.C_u}.\frac{\varepsilon_{p,m}}{\varepsilon_{p.m}}.\frac{m_m}{k_{0,m}}.f(\sigma(E),\phi(E)) \quad (4)$$

The subscript "m" refers to the monitor and the k_0 value is defined as:

$$k_{0,m} = \frac{W_m.\Theta.\sigma_0.\gamma}{W.\Theta_m.\sigma_{0,m}.\gamma_m} \quad (5)$$

where σ_0 and $\sigma_{0,m}$ are the thermal cross sections of the target nucleus and the monitor respectively. The k_o coefficients have been experimentally measured with errors less than 4%. The k_0 method has been used by several groups for over 20 years [14].

Equation (4) indicates that the analytical determination by this method depends on the neutron energy spectrum at the irradiation position. In other words, it depends on the function $f(\sigma(E),\phi(E))$, which characterizes the reaction ratio R. The neutron flux spectrum consists of different neutron velocities; therefore, mathematically the reaction ratio would be given by:

$$R = \int_0^\infty \sigma(E).\phi(E).dE \quad (6)$$

where E is the neutron kinetic energy.

A solution of R using Equation (6) is not an easy task because there are no analytical expressions for the functions under the integral. Therefore, some considerations regarding these functions have been done in order to solve R. These considerations have been called conventions, and are named after the people who

developed them. There have been 4 such conventions so far, namely Westcott [15, 16], Stoughton and Halpering [17], Hϕgdahl [18] and Blaauw [19]. They solved Equation (6) assuming different initial conditions but all of them agree on the following aspects [20].

1. The neutron flux of any research nuclear reactor can be divided into three parts:
    - A thermal region below 0.35 eV characterized by the Maxwell-Boltzmann distribution.
    - An epithermal region above 0.35 eV where the neutron flux is proportional to 1/E (E is the neutron energy) in the case of an ideal moderator.
    - A fast neutron region also called fission neutron spectrum with a maximum neutron energy around 0.7 MeV. This is usually described by the semi-empirical Watt expression.
2. In the thermal and low epithermal regions most of the nuclides have neutron capture cross section σ (E) proportional to 1/v (v is the neutron velocity). However, in the region 0.1-10 keV strong resonance spikes characterize the absorption spectrum.
3. The flux of any reactor has a very small component of fission (or fast) neutron density; thus this component is negligible in comparison to the thermal and the epithermal flux density.
4. It is possible to reduce the thermal component of the flux in a research reactor by using a 1-mm-thick cadmium sheet in one of the reactor irradiation positions. Cadmium has a very large thermal neutron cross section and in theory any neutron with energy less than 0.55 eV at 20^0C is absorbed. This energy value is known as cadmium cut-off energy.

Westcott convention (1955): It is applied to any isotope no matter if it follows the 1/v law or not and it assumes that the neutron spectrum is well thermalized. However, it also assumes that the epithermal spectrum is ideal and has a distribution of 1/E [15].

Westcott modified convention (1968): Ryves and Paul modified Westcott's convention introducing the correction factor (α) for non-ideal epithermal spectra distribution $1/E^{(1+\alpha)}$. Westcott's expression is obtained when the α factor is reduced to zero [16].

Stoughton and Halpering convention (1959): They assumed 4 zones in the reactor neutron spectrum. These zones are: thermal, sub-cadmium, epithermal and epi-cadmium zones. They also assumed ideal epithermal flux distribution of 1/E and extended the Maxwell distribution to the whole neutron spectrum. Their final expression for the reaction rate is that obtained by De Corte multiplied by a constant [17].

Hϕgdahl convention (1962): The k_0 method was initially developed based on this convention. It assumes that the reactor neutron spectrum is divided in two zones. The boundary between zones is the cadmium cut-off energy. Its application is restricted to neutron capture cross sections that follow 1/v law in the thermal neutron energy region [18].

Some rare earth isotopes do not follow the 1/v law [14, 20]. To make the k_0-method general and applicable to all nuclides, in 1993 De Corte proposed to use a combination of Westcott and Hϕgdahl conventions for solving the reaction rate equation for those isotopes which do not fit under the Hϕgdahl convention [20, 21].

Blaauw convention (1991): It was developed specifically for working with the k_0-NAA method. It can be used for nuclides that do not follow the 1/v law when they have no resonance peaks above 0.35 eV. In fact, it is a variant of Hϕgdahl convention. The expression obtained for the reaction rate is equivalent to that obtained by Hϕgdahl. The difference is the mathematical way that the function under the integral sign was separated [19].

The Dalhousie University SLOWPOKE-2 reactor (DUSR) was standardized for k_0-NAA method by Acharya in 2001 [22, 23]. The combination of the

Hϕgdahl and Westcott conventions proposed by De Corte was used for solving the reaction rate equation and the input parameter in the method. They were determined for both inner and outer irradiation positions of the DUSR facility [22, 23]. The input parameters are [22-24]:

- The sub-cadmium to epithermal neutron flux ratio (f): it is a measure of the degree of thermalization (f>>1, it is a high thermal neutron flux reactor).
- Epithermal neutron flux shape factor (α): it expresses the degree of the non-ideal epithermal flux deviation. It is a small number usually between –0.05 and + 0.15. The positive α values correspond to a "softened" and a negative to a "hardened" epithermal spectrum compared to an ideal one (α = 0).
- Modified spectral index (MSI): it is, according to De Corte, the ratio of the epithermal flux to the total neutron density.
- Neutron temperature (T_n): it gives the absolute neutron temperature at the irradiation site.

Acharya has described the determination of the above parameters [22, 23]. His results for the DUSR facility are presented in Table 3.1.

Table 3.1. Input parameters for k_0-NAA method at Dalhousie facility

Parameter	Inner positions	Outer position	Convention
f	18.9 ± 0.5	57.1 ± 2.2	Hϕgdahl
α	-0.0427 ± 0.0057	-0.0098 ± 0.0045	Hϕgdahl
MSI	0.0458 ± 0.0027	0.0157 ± 0.0014	Westcott
T_n (^{0}C)	38.8 ± 8.6	33.0 ± 7.4	Westcott

The k_0-NAA method has been used in this thesis. For this reason its rationale is described below. The Equation (1) can be re-written as:

$$\frac{A_0}{t_c} = \frac{0.6023.\Theta.m.R.S.D.C}{W} = \frac{P_A}{\varepsilon_p \gamma} \tag{7}$$

$$A_{sp} = \frac{P_A}{S.D.C.m} = \frac{0.6023.\Theta.R.\varepsilon_p.\gamma}{W} \tag{8}$$

Equation (8) represents the specific count rate ratio of the activation products of an element of interest. The ratio of the specific activity of the element and the monitor can be expressed as:

$$k_{anal} = \frac{A_{sp}}{A_{sp,m}} = \frac{\dfrac{0.6023.\Theta.R.\varepsilon_p.\gamma}{W}}{\dfrac{0.6023.\Theta_m.R_m.\varepsilon_{P,m}.\gamma_m}{W_m}} = \frac{W_m.\Theta.R.\varepsilon_P.\gamma}{W.\Theta_m.R_m.\varepsilon_{P,m}.\gamma_m} \qquad (9)$$

Applying the Hϕgdahl convention, the expression for the reaction rate (R) becomes:

$$R = \int_0^{0.55} \sigma(E).\phi(E).dE + \int_{0.55}^{\infty} \sigma(E).\phi(E).dE \qquad (10)$$

$$R = G_{th}\sigma_{th}\phi_{th} + G_e I_o \phi_e \qquad (11)$$

where G_{th} and G_e are the correction factors for thermal and epithermal neutron self-shielding, respectively. For small samples, these factors are ≈ 1. The other parameters are σ_{th} = thermal neutron cross section and I_0 = epithermal neutron flux cross section, both in barn (10^{-24} cm^{-2}), ϕ_{th} = thermal neutron flux and ϕ_e = epithermal neutron flux both in $cm^{-2}.s^{-1}$. The $\phi_{th} = n_0 v_0$ where n_0 is the thermal neutron density ($n.cm^{-3}$) for energies below 0.55 eV, and v_0 is the neutron velocity =2200 $m.s^{-1}$ which corresponds to $E_0 = kT_0 = 0.0253$ eV, $T_0 = 293.6^0K$ (20^0C) where k is the Boltzmann constant. The ϕ_e is the same as ϕ_{th} for energies above 0.55 eV. Equation (9) can be re-written as:

$$k_{anal} = \frac{W_m.\Theta.\gamma.(\sigma_{th}\phi_{th} + I_0\phi_e).\varepsilon_P}{W.\Theta_m\gamma_m(\sigma_{th,m}\phi_{th} + I_{0,m}\phi_e).\varepsilon_{P,m}} = \frac{W_m.\Theta.\gamma}{W.\Theta_m.\gamma_m} \cdot \frac{\sigma_{th}\phi_e\left(\dfrac{\phi_{th}}{\phi_e} + \dfrac{I_0}{\sigma_{th}}\right).\varepsilon_P}{\sigma_{th,m}\phi_e\left(\dfrac{\phi_{th}}{\phi_e} + \dfrac{I_{0,m}}{\sigma_{th,m}}\right).\varepsilon_{P,m}} \qquad (12)$$

Substituting in equation (12)

$$f = \frac{\phi_{th}}{\phi_e} \tag{13}$$

and

$$Q = \frac{I_0}{\sigma_{th}} \tag{14}$$

$$k_{anal} = \frac{W_m.\Theta.\gamma.\sigma_{th}}{W.\Theta_m.\gamma_m.\sigma_{th}} \cdot \frac{f + Q_0}{f + Q_{0,m}} \cdot \frac{\varepsilon_p}{\varepsilon_{P,m}} \tag{15}$$

The first fraction of equation (15) is the k_0 value defined in equation (5). Therefore:

$$k_{anal} = k_0 \cdot \frac{[f + Q_0]}{[f + Q_{0,m}]} \cdot \frac{\varepsilon_P}{\varepsilon_{P,m}} \tag{16}$$

The α factor is the epithermal neutron flux shape factor, which describes the non-ideality of the epithermal flux. It is introduced in the resonance integral I_0 (also known as epithermal neutron flux cross section).

$$I_0(\alpha) = \int_{0.55}^{\infty} \frac{\sigma(E).dE}{E^{1+\alpha}} \tag{17}$$

The ratio of the resonance integral corrected for α and the thermal cross section σ_{th} is called $Q_0(\alpha)$ and its relation with Q (Equation 14) is given by:

$$Q_0(\alpha) = \frac{Q_0 - 0.429}{E_r^{\alpha}} + \frac{0.429}{(2\alpha + 1)(0.55)^{\alpha}} \tag{18}$$

where E_r is the effective resonance energy in eV, 0.429 is obtained from $2(E_0/E_{Cd})^{1/2}$, E_0=0.0252 eV and E_{Cd}=0.55 eV. The equation (16) can be re-written as:

$$k_{anal} = k_0 \cdot \frac{[f + Q_0(\alpha)]}{[f + Q_{0,m}(\alpha)]} \cdot \frac{\varepsilon_P}{\varepsilon_{P,m}} \tag{19}$$

The mass of the element of interest can be found by rearranging equation (9) as:

$$m(\mu g) = \frac{P_A}{S.D.C.A_{sp,m}.k_{anal}} \tag{20}$$

Multiplying both sides of the equation by $1/M_s$, where M_s is the mass of the sample, the concentration of the element of interest is found by:

$$C(\mu g.g^{-1}) = \frac{A_{sp}}{A_{sp,m}} \cdot \frac{1}{k_{0,\exp}} \cdot \frac{f + Q_{0,m}(\alpha)}{f + Q_0(\alpha)} \cdot \frac{\varepsilon_{P,m}}{\varepsilon_P} \tag{21}$$

or more generally:

$$C(\mu g.g^{-1}) = \frac{\frac{P_A}{S.D.C.M_s}}{\frac{P_{A,m}}{S_m.D_m.C_m.m_m}} \cdot \frac{1}{k_{0,\exp}} \cdot \frac{f + Q_{0,m}(\alpha)}{f + Q_0(\alpha)} \cdot \frac{\varepsilon_{P,m}}{\varepsilon_P} \tag{22}$$

The last equation is the most general equation for the determination of the concentration of an element of interest in a sample using the k_0-NAA method.

3.2 Experimental

3.2.1 Irradiation conditions

All irradiations of samples, elemental standards, and reference materials in this thesis were carried out at the Dalhousie University SLOWPOKE-2 Reactor (DUSR) facility. Some of the irradiations were performed in the outer epithermal site and others in the inner site. The epithermal site is shielded with a 1-mm-thick cadmium sheet for

absorbing thermal neutrons while allowing the epithermal neutrons to activate the samples [25].

The selection of irradiation, decay and counting times was largely based on half-lives, allowing for adequate decay of interfering radionuclides while leaving enough activity of the radionuclide of interest. The nuclear data of iodine are shown in Table 3.2.

Table 3.2. Nuclear data of iodine.

Nuclear reaction : $^{127}I(n,\gamma)^{128}I$			
Target nuclide: ^{127}I		**Product nuclide: ^{128}I**	
Isotopic abundance	100 %	**Half-life**	25.0 min
Thermal cross-section	(6.2 ± 0.2) b	**γ Energy**	443 keV
Resonance Integral	(147 ± 6) b	**γ Population**	0.16

The half-life of iodine is 25 min; an irradiation time of 25 min is then sufficient to reach 50% of the maximum possible activity (saturation factor). This was the irradiation time used for all ENAA performed in this work. In the case of inner sites, the irradiation times were varied from 5 to 10 min depending on the overall activity of the samples. The 443-keV photopeak of ^{128}I suffered from a second order interference (there is no first order interference in this case) by the 439-keV photopeak of ^{23}Ne produced by the $^{24}Na(n,p)^{23}Ne$ reaction under the same experimental conditions used for NAA of milk. The ^{23}Ne isotope has a half-life of 37.6 s. A decay time of 2-5 min was sufficient for eliminating this interference. For ^{128}I, one half-life (25 min) was selected as the maximum counting time. However, depending on the iodine content of the sample shorter counting times were possible.

3.2.2 Preparation and accuracy of elemental standards

Three different stock solutions of 4 μg mL^{-1} of iodine using ammonium iodide (Spex, ultrapure), potassium iodide (BDH, analytical reagent) and potassium iodate (Merck, analytical reagent) were prepared as the iodine elemental standards for use in the relative method of calculating concentrations. Three secondary stock solutions of

1.5 μg mL^{-1} were prepared from the above solutions. Different volumes of these solutions were pipetted out to cover a mass range of 0.2-1.0 μg. Distilled deionized water was used for making up the volume to 0.75 mL. Three calibration curves were constructed using these points (mass range). Each point on the curve is the average of three replicates. The slope of each curve represents the iodine sensitivity; in theory, these three slopes should be identical because the iodine sensitivity is independent of the physico-chemical form of iodine. The three calibration curves constructed using an analysis scheme 25-5-25 min and an EINAA-CS method are shown in Fig. 3.1.

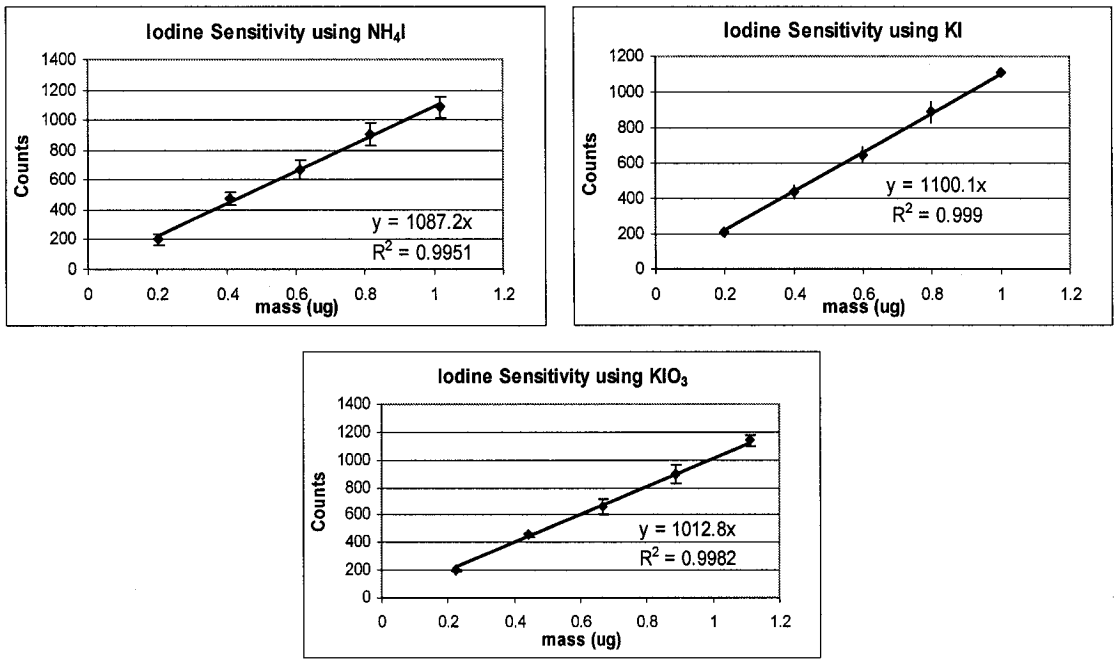

Fig. 3.1. Sensitivity of iodine using three different iodine salts.

An average sensitivity value of 1067 counts.μg^{-1} was obtained from the three standards. However, the sensitivity value obtained from the KIO_3 calibration curve alone (1013 counts.μg^{-1}) was a little bit lower than the average. For this reason one single ANOVA test was performed using the NIST Non-fat Milk Powder (SRM-1549). The iodine concentrations obtained using the three elemental standards are shown in Table 3.3.

Table 3.3. Iodine content in ppm of NIST-SRM-1549 using various standards.

1087 counts.μg $^{-1}$ of NH_4I standard	**1100 counts.μg $^{-1}$ of KI standard**	**1013 counts.μg $^{-1}$ of KIO_3 standard**
3.3011	3.2621	3.5422
3.3229	3.2836	3.5657
3.3617	3.3220	3.6073
Average value in this work (Certified value : 3.38 ± 0.03)		
3.33 ± 0.03	3.29 ± 0.03	3.57 ± 0.03

The ANOVA test performed showed that there are significant differences among the three iodine values obtained for NIST SRM-1549 at 95% of confidence level. However, this difference disappears when the value obtained using the KIO_3 standard was removed. It was then concluded that the average sensitivity value of 1094 counts.μg^{-1} obtained from the KI and NH_4I calibration curves should be used for calculating iodine concentrations in milk.

To confirm this sensitivity value, one last experiment was set up. A standard addition curve was constructed using the NH_4I standard solution. Pre-determined amounts of solution were added to irradiation vials containing NIST SRM-1549. Some samples required a reduction in volume by evaporation in air. The curve was constructed using three replicates at each point. The results are presented in Fig. 3.2. The standard addition curve gave a slope of 1088 counts.μg^{-1} which is almost identical to the slope of 1087 counts.μg^{-1} of the NH_4I standard solution reported in Table 3.2. The intercept of 3498.3 counts per g of SRM corresponded to an iodine concentration of 3.22 $\mu g.g^{-1}$.

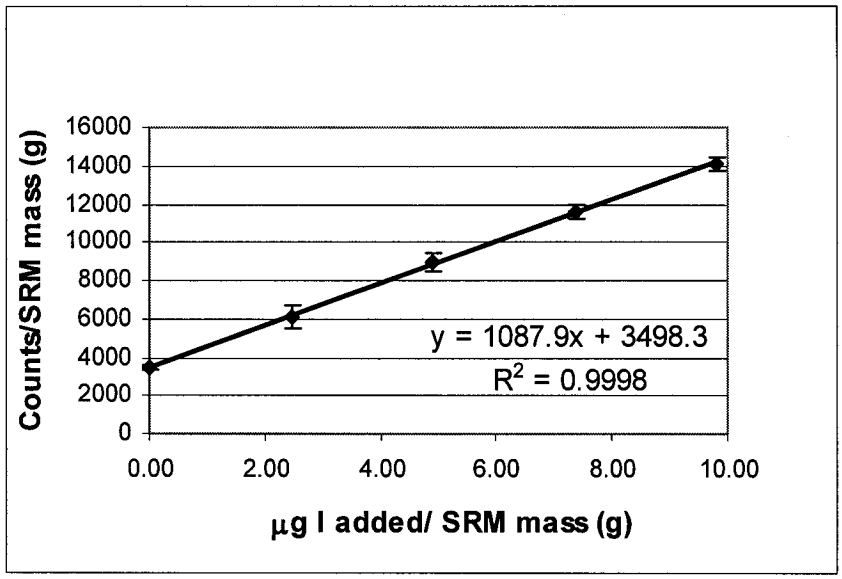

Fig. 3.2. Standard addition curve for NIST Non-fat Milk Powder SRM-1549.

This experiment illustrates the well-known fact that NAA is independent of the matrix and also confirms the reliability of the sensitivity value. Table 3.2 lists the sensitivities obtained just from the pure elemental standard solution while Fig. 3.2 shows the sensitivity obtained when a matrix is present. The sensitivity value of 1094 counts.μg^{-1}, *i.e.* the average of KI and NH_4I standards, was used under the experimental conditions of 25-5-25 min and EINAA-CS.

The same primary stock solution of NH_4I was then used to determine the sensitivities under different experimental conditions listed in Table 3.4. In these cases, no calibration curves were constructed. Instead three replicates were used to obtain average sensitivities which in turn were employed to determine the iodine concentrations in NIST Non-fat Milk Powder SRM-1549 with a certified value of 3.38 ± 0.03 $\mu g.g^{-1}$. Table 3.4 shows good agreement between the calculated iodine concentrations and the certified value. These results also indicate the reliability of various methods used in this work for the determination of low levels of iodine in milk and its fractions.

Table 3.4. Sensitivities for iodine under different experimental conditions.

Scheme t_i-t_d-t_c (min)	Reactor position / Counting system	Sensitivity Counts.μg^{-1}	Iodine $\mu g.g^{-1}$ in NIST-SRM 1549
5-2-10	INAA-CS	1686 ± 4	3.22 ± 0.03
5-2-10	INAA-GeLi#1	3486 ± 40	3.20 ± 0.10
5-2-10	EINAA-GeLi#1	274 ± 7	3.40 ± 0.20
10-2-10	INAA-GeLi#1	6626 ± 143	3.21 ± 0.05
5-6-15	INAA-CS	2067 ± 29	3.30 ± 0.10
10-3-10	EINAA-CS	254 ± 3	3.30 ± 0.40

A gold standard stock solution was also prepared for use in the k_0-NAA method. A volume containing 50 μg of gold was pipetted from an ultra-pure gold ICP-standard solution (SPEX) and the volume was made up to 0.75 mL with DDW. The gold standard was irradiated and counted using the same reactor position and counting system as the samples. The activity obtained was used in Equation (22).

3.2.3 Validation of NAA methods

In order to validate the accuracy of both relative and k_0-NAA methods used in this work, five NIST reference materials (RM) and SRM were analyzed. These materials were: Non-fat Milk Powder (SRM-1549), Whole milk powder (RM-8435), Oyster tissue (SRM-1566a), Whole egg powder (RM-8415) and Wheat gluten (RM-8418). Four portions of each material were weighed out into polyethylene irradiation vials. The geometry of the sample and the elemental standard was essentially the same. The SRM and RM were irradiated under the same conditions as the elemental standards using the 25-5-25 min scheme and EINAA-CS. Each material was analyzed by relative and k_0-NAA methods.

The quantification of iodine content by the relative method was done using Equation (3) while Equation (22) was used for the k_o-NAA method. The input

parameters for the k_o-NAA method are summarized in Table 3.5, where the first four parameters are taken from tables of nuclear data [26].

Table 3.5. Input parameters for k_o-NAA method.

Input parameter	Interested element	Monitor
Target isotope	^{127}I	^{197}Au
Product nuclide	^{128}I	^{198}Au
Half-life	25 min	2.7 d
γ energy (keV)	442.9	411.8
Er	57.6	5.65
Q_0	24.8	15.7
α	-0.0098	-0.0098
$Q_0(\alpha)$	25.794	15.967
f	57.1	57.1
Absolute γ energy efficiency	0.004782	0.005241
$k_{0,exp}$	0.0113	1

The half-life, gamma-ray energy, Er, and Q_0 data were taken from De Corte [21, 27, 28]. The α, $Q_0(\alpha)$ and f values are characteristic of the DUSR facility [22, 23]. The γ energy efficiency is characteristic of the counting system used and the k_0 value is characteristic of each isotope. The $k_{0,exp}$ value was obtained at the Dalhousie facility while the theoretical value is 0.0172 and the recommended value is 0.0174 [21], although other k_0 values for iodine such as 0.0112 and 0.0158 have been reported [14, 24]. The absolute γ energy efficiency was found by Acharya using a set of standard γ sources of known activity and fitting the points to a polynomial curve efficiency *vs.* energy of the third order [22, 23]. The iodine content of RM and SRM using both NAA methods are shown in Table 3.6.

Table 3.6. Iodine content ($\mu g.g^{-1}$) of SRM and RM by relative and k_0-NAA methods.

SRM / RM	Relative method	k_0-method	Certified Value
Non-fat Milk Powder	3.33 ± 0.03	3.37 ± 0.03	3.38 ± 0.03
Whole Milk Powder	2.35 ± 0.08	2.38 ± 0.08	2.3 ± 0.4
Oyster Tissue	4.44 ± 0.08	4.49 ± 0.08	4.46 ± 0.40
Whole Egg Powder	1.92 ± 0.19	1.95 ± 0.19	1.97 ± 0.45
Wheat Gluten	0.064 ± 0.009	0.067 ± 0.009	0.06 ± 0.01

The values in Table 3.6 show the excellent agreement in the results obtained by the two different NAA methods as well as between our and certified values. One method can then be used for validating the other. However, the advantage of the k_0-NAA method is that it allows the quantitative determination of unexpected elements. The concentrations of some elements in SRM-1549 using only k_0-NAA method are shown in Table 3.7.

Table 3.7. Multielement content ($\mu g.g^{-1}$) of NIST SRM-1549 by k_0-NAA method.

Element	This work	Certified (Info) value
Bromine	9 ± 1	(12)
Magnesium	1176 ± 226	1200 ± 36
Sodium	4874 ± 217	4970 ± 99
Chlorine	11949 ± 655	10900 ± 200
Calcium	13370 ± 1494	13000 ± 500

It is important to point out that the irradiation conditions chosen are not actually optimized for the determination of the elements listed in Table 3.7. In fact these conditions were particularly selected to minimize the activity of those elements so that the iodine levels could be reliably determined. For this reason the RSD does not look good in all cases.

3.2.4 Determination of total iodine in milk by NAA methods

Experiments were carried out to optimize the best experimental conditions for the determination of total iodine in milk by the relative INAA method prior to actual measurements. About 0.75 mL of milk was placed in polyethylene irradiation vials and the samples were irradiated and counted under the conditions shown in Table 3.8. The detection limits as well as precision of each method were evaluated. The average of three measurements and their percent relative standard deviation (RSD) in parenthesis are shown in Table 3.8. The detection limits were calculated using Currie's method [29]:

$$D.L = 2.71 + 3.29 * (\text{Gross Counts-Net counts})^{0.5}$$

Table 3.8. Precision and detection limits of INAA methods for iodine in milk.

Scheme t_i-t_d-t_c (min)	Reactor position / Detection system	Iodine content $\mu g.mL^{-1}$, (%RSD)	Detection Limit ($\mu g.mL^{-1}$)
5-2-10	INAA-GeLi#1	0.34 ± 0.2 (6)	0.08
5-2-10	INAA-CS	0.378 ± 0.008 (2)	0.06
5-6-15	PCINAA-CS	0.341 ± 0.008 (2)	0.02
25-5-25	EINAA-CS	0.463 ± 0.006 (1)	0.06
10-3-10	EINAA-CS	0.34 ± 0.2 (6)	0.1

It is evident from Table 3.8 that the worst detection limit of 0.1 $\mu g.mL^{-1}$ with a RSD of 6% was obtained by EINAA-CS using a 10-3-10 min scheme. However, the detection limit was lowered to 0.02 $\mu g.mL^{-1}$ using a PCINAA-CS method and 6 cycles of irradiation-decay-counting. Although the experiments were not done, detection limits lower than 0.02 $\mu g.mL^{-1}$ using a fewer number of cycles but a 25-5-25 min scheme could be expected. The improvement in detection limits with increasing number of cycles (N) using the 5-6-15 min scheme in PCINAA-CS is shown in Fig. 3.3.

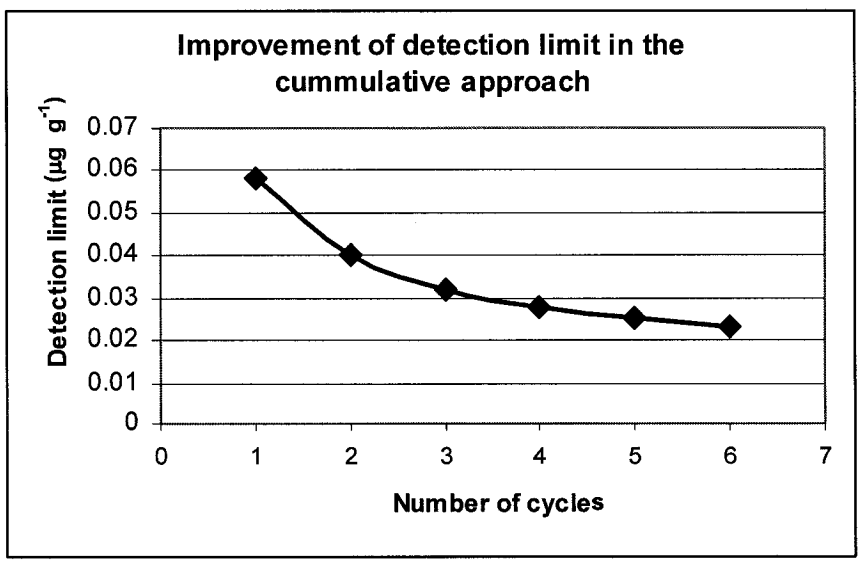

Fig 3.3. Improvement of detection limits for iodine by PCINAA-CS.

Although the PCINAA-CS method provided the best detection limits and second lowest RSD, it was generally not used in this work because of the longer experimental time involved in doing 6 or so cycles. Instead, the INAA-CS method using 5-2-10 min scheme was applied to 5 milk samples of the same brand name but with different fat content purchased at the local supermarket market in Halifax on the same day. The results are presented in Table 3.9. It appears that there is no appreciable difference in iodine content among these samples, except for the 1% MF calcium plus sample. When an ANOVA test was performed using all milk samples, remarkable differences appeared as expected due to the 1% MF calcium plus sample. However, when this sample was taken out of the ANOVA test, there were still significant differences at the 95% confidence level.

Table 3.9. Iodine levels of milk samples with different fat content.

Milk	**Iodine concentration ($\mu g.mL^{-1}$)**
Homogenized, 3.25% MF	0.40 ± 0.01
Partially skimmed, 2% MF	0.40 ± 0.01
Partially skimmed, 1% MF	0.42 ± 0.01
Skimmed < 0.5% MF	0.42 ± 0.01
Partially skimmed 1% MF calcium plus	0.96 ± 0.01

The slightly higher iodine levels (Table 3.9) of the partially skimmed (both 1% and <0.5 MF) samples compared to the homogenized and partially skimmed 2% MF were somehow expected. Since the highest fraction of iodine in milk is reported to be of inorganic form, it is expected that the skimming process leading to a reduction of fat content will slightly "concentrate" this iodine species. There is no obvious explanation for the high level of iodine in the skimmed milk 1% fortified with calcium especially when all the milk samples were of the same brand name and purchased from the same supermarket at the same time. It might be possible that the compound used to add extra calcium in milk contained iodine.

The seasonal variation of iodine levels in milk was studied. Homogenized milk samples of the same brand name were purchased from the same supermarket over a period of almost three years. The results are presented in Fig. 3.4.

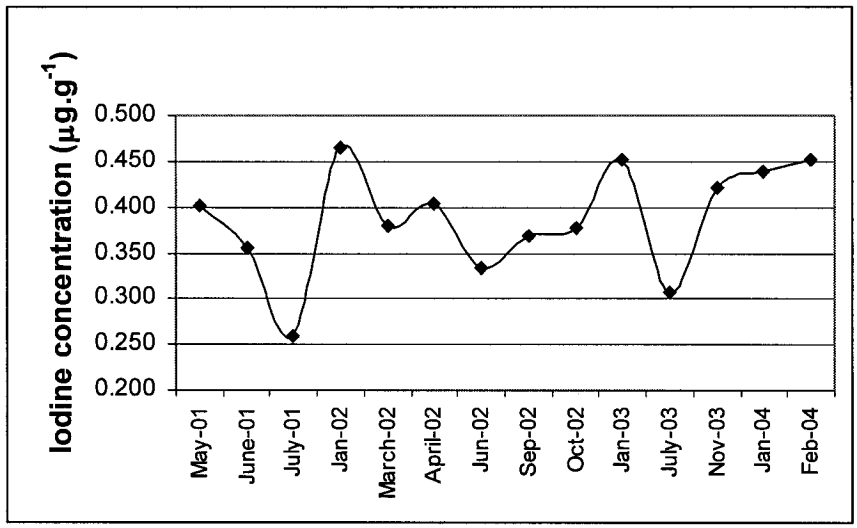

Fig. 3.4. Seasonal variation of iodine in homogenized milk.

The iodine content was found to vary from 0.25 to 0.45 $\mu g.g^{-1}$ during 2001 May - 2004 Feb. A lower limit of 0.2 $\mu g.g^{-1}$ is considered an acceptable value for iodine concentration in other regions of the world. This implies that the intake of iodine via milk in Nova Scotia is not particularly low. A daily consumption of 250 mL milk translates to an intake of about 51 µg of iodine (0.2 $\mu g.g^{-1}$ x 1.029 g. mL^{-1} x 250 mL) which is about one third of the daily recommended nutrient intake (RNI) of iodine from all sources of food and drinks.

3.3 Summary

Two different NAA quantification methods, namely the relative and the k_0 methods, were evaluated for the determination of iodine in milk. The agreement in results between these methods was considered good. In terms of suitability the k_0-NAA might be attractive to some researchers because multielement determinations can be done without the prior knowledge and preparation of comparator standards. Experiments were

carried out in depth to optimize the irradiation-decay-counting schemes along with the detection systems. The worst detection limit of 0.1 $\mu g.mL^{-1}$ with a RSD of 6% was obtained by EINAA-CS using a 10-3-10 min scheme while the detection limit was lowered to 0.02 $\mu g.mL^{-1}$ using a PCINAA-CS method and 6 cycles of irradiation-decay-counting. This value could be further improved if the same pseudo-cyclic approach is used in combination with EINAA-CS. The accuracy of all methods developed was evaluated using the NIST Non-fat Milk Powder SRM-1549 and was found to be good. Not much difference was found in the iodine concentration among milk samples with varying milk fat content. However, the iodine levels were observed to vary between 0.25 and 0.45 $\mu g.g^{-1}$ with the lowest during the summer months and the highest through the winter months.

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CHAPTER 4

COMPOSITION OF MILK

Before irradiating the milk fraction samples to investigate iodine content, it was necessary to know the composition of the milk and to properly separate the components of interest. In this chapter, the analytical determination of the major milk components such as proteins, carbohydrates, water, some minerals and lipids is reported. The content of vitamins, gases, and organic anions of acids is considered negligible, and is not covered in this work. Determination methods, as well as the separation methods, where applicable, are described below.

4.1 Introduction

Milk is produced by female mammary glands; it is meant for the purpose of feeding their offsprings and providing, among other things, immunological protection. Every female mammal produces milk with the essential nutrients required by the newborns of that species. For example, seal milk is rich in fat which is needed to develop a thick layer of insulating fat in seal pups while bovine milk has less fat but more proteins and minerals which are needed by their rapidly growing calves whose birth weight double in merely 50 days [1, 2].

As early as 6 000-8 000 BC humans learned how to domesticate animals such as cows, goats, sheep, camels, reindeer, buffaloes and llamas for reasons such as their milk [3]. Cow's milk is the most widely used type of milk in the world. The research work presented in this thesis involves only cow's milk, which is commonly referred to as milk.

The composition of milk is not the same throughout the world. There are many variables such as geographical location, climate, and feeding habits of the cow that can affect the composition of milk. The average composition of milk is shown in Table

4.1 and the average composition of non-fat soluble fractions in milk is given in Table 4.2 [3].

Table 4.1. Average composition of milk.

Fraction	Range (%)	Average composition (%)
Water	85.5 - 88.7	83.7
Milk-fat	2.4 – 5.5	3.9
Non-fat soluble	7.9 – 10.0	8.8

Table 4.2. Average composition of Non-fat soluble fraction of milk.

Component	Average composition (%)	Main component
Proteins	3.25	Mostly casein
Carbohydrates	4.6	Mostly lactose
Minerals	0.65	Mostly Ca and P
Anions of acids	0.18	Citrate, formate, acetate
Vitamins and gases	0.12	B1, B6, B12, A, and O_2, N_2

4.1.1 Vitamins in Milk

The word vitamin comes from the combination of *vital* and *amine.* The vitamins act as co-factors. In their absence, normal functions of the human body break down making it more susceptible to disease. Living organisms generally have the ability to produce their own vitamins. However, human beings and their primate relatives such as apes and chimps have lost this ability. For this reason, vitamin intake is essential for these species for maintaining good health [1-4].

Vitamins can be either water-soluble or fat-soluble [1, 4, 5]. Milk has three fat-soluble vitamins, namely vitamins A, E and K. During the commercial process of lowering milk-fat, the amounts of these vitamins are also reduced. Therefore, they are added again as supplements after the skimming process. Vitamin D is also added to commercially available milk [1, 4].

Milk is also an important source of water-soluble vitamins such as B1 (thiamine), B2 (riboflavin), B6 (pyridoxine), B12 (cyanocobalamin), niacin and

pantothenic acid. Raw milk contains vitamin C (ascorbic acid) which is also a water-soluble vitamin; but this vitamin is destroyed during the pasteurization process [1, 4].

4.1.2 Minerals in Milk

There are 22 chemical elements (minerals) generally considered to be essential for human nutrition. All of them are reported to be present in milk. These include three families of ions [1, 3]: (i) sodium, potassium and chloride - these ions have a negative correlation to lactose for maintaining osmotic equilibrium of milk with blood; (ii) calcium, magnesium, inorganic phosphate, and citrate - this group is present in the casein micelle and consists of two thirds of calcium, one third of magnesium, one-half of inorganic phosphorous, and one-tenth of the citrate present in milk; (iii) diffusible calcium, magnesium, citrate and phosphate - these ions contribute to the overall acid-base equilibrium of milk and they are very dependent on pH. Milk also contains iron, zinc, copper, manganese, iodine, fluorine, selenium, cobalt, chromium, molybdenum, nickel, silicon, vanadium, tin, arsenic and sulfur in microgram quantities [1-3].

4.1.3 Carbohydrates in Milk

Lactose is the main carbohydrate in milk. It accounts for 4.8-5.2% of the milk mass although small amounts of glucose, galactose, and oligosaccharides are also present [1-3]. Lactose is also called milk sugar (it can only be found in milk) although it is not as sweet as sucrose (sugar). It is a disaccharide compound of two monosaccharide molecules, glucose and galactose. Lactose is very important because it is used as a fermentation substrate. Bacteria that have the ability to metabolize lactose produce lactic acid and this process is the starting point of many dairy products [1-3]. The human body breaks down the lactose molecule through the lactase enzyme into the monosaccharides units and in this process the body acquires energy. Infants digest lactose easily; however, many adults especially Asians and Africans have lost this ability because they lack the lactase enzyme. Thus, if they consume milk or dairy products, they could suffer from gastric distress and diarrhea. These persons are known to suffer from lactose intolerance [1-3].

4.1.4 Proteins in Milk

Proteins found in milk contain all the essential amino-acids that human beings need [2]. This fact indicates the importance of milk in human health. The major proteins in milk can be divided into two classes on the basis of their structure and physicochemical behavior. These classes are known as casein and whey. "Whole casein" is the name for the phosphoproteins precipitated from milk at pH 4.6 and 20°C [1, 4-7]. This protein represents as much as 80% of the total milk protein and between 2.5 and 3.2% of milk mass. Casein by itself is not an isolated protein; it comprises four major and one minor groups as shown in Table 4.3 [3, 6-9].

Table 4.3. Distribution and properties of caseins in milk.

Protein	Fraction of total casein (%)	P atom (per mol)	SH group (per mol)	S-S group (per mol)	Mol. Wt. (Dalton)
α_{s1}-Casein	44	8	0	0	23 500
α_{s2}-Casein	11	10	0	2	25 100
β–Casein	25	5	0	0	24 000
κ-Casein	14	1	0	1	19 000
γ-Casein	5	1	0	0	20 000

Whey proteins are the proteins remaining in solution after the separation of casein. They represent about 20% of total milk proteins [1, 7, 9]. The distribution and composition of some major whey proteins are given in Table 4.4. Immunoglobulins are also whey proteins. There are four classes of immunoglobulins in milk, namely IgG1, IgG2, IgA, and IgM. All these molecules have similar structures composed of two light chains with molecular weights of 20-50 kDa and two heavy chains of 50-70 kDa [1, 7].

Table 4.4. Distribution and properties of some whey proteins in milk.

Protein	Fraction of whey (%)	P atom (per mol)	SH group (per mol)	S-S group (per mol)	Mol. Wt. (Dalton)
β–lactoglobulin	58	0	1	2	18300
α-lactalbumin	13	0	0	4	14146
Serum Albumin	6	0	1	17	69000

When milk is heated at 95°C for 20 min and then acidified to pH 4.7, most proteins precipitate although some proteins still remain in solution. The soluble proteins are called minor proteins or proteose-peptones [1, 7]. These proteins can be precipitated with 12% trichloroacetic acid. Minor proteins include 4 different proteins, namely Proteose-peptone component 3, proteose-peptone component 5, proteose-peptone component 8 fast, and proteose peptone component 8 slow. Some of their characteristics are shown in Table 4.5 [1, 7].

Table 4.5. Properties of some minor proteins in milk.

Protein	Molecular Wt. (Da)	Characteristics
Proteose-peptone component 3	20 000	Only associated with whey fraction; contains over 17% of carbohydrates.
Proteose-peptone component 5	13 000	Associated with whey and casein fraction; derived from d β-casein.
Proteose-peptone component 8 fast	3 900	Associated with whey and casein fraction; derived from d β-casein.
Proteose-peptone component 8 slow	9 900	Associated with whey and casein fraction.

4.1.5 Lipids in Milk

Milk fat provides energy and nutrients such as essential fatty acids and fat-soluble vitamins. In addition, fat content of milk has economic value because milk is sold based on the amount of fat present [1, 2]. The main milk lipids are triglycerides which account for 98.3% of milk fat, although smaller amounts of mono- and di-glycerides, and free fatty acids can be also found. Other classes of lipids include phospholipids which represent 0.8% and cholesterol accounts for 0.3% [3].

The major long chain fatty acids found in milk are myristic acid (C_{14}), palmitic acid (C_{16}), stearic acid (C_{18}), and oleic acid (C_{18}: 1) accounting for 11%, 26%, 10%, and 20% of the milk lipids, respectively. Among the short chain fatty acids, milk contains butyric (C_4), caproic (C_6), caprylic (C_8), and capric (C_{10}) acids [3].

Milk in its natural form and without any industrial processing is called raw milk [1, 2]. The raw milk undergoes pasteurization and homogenization processes and a decrease in the amounts of fat in some instances before being marketed as "milk". If the fat content is reduced to 3.25%, the milk is sold as whole (sometimes called homogenized) milk. Partially skimmed milk has typically 2% or 1% fat while skim milk or non-fat milk contains about 0.5% milk fat. Once the fat has been reduced to a desired level, the milk undergoes pasteurization and homogenization processes, which in Canada and Europe is actually ultra pasteurization [2].

Partially skimmed milk (2%) has been the most consumed milk in Canada in the last decade [9]. However, a significant increase in the consumption of partially skimmed milk (1%) has also occurred (Fig. 4.1). Overall, the consumption of milk has decreased over the time although the dairy industry is the fourth largest sector of the Canadian agri-food economy, preceded by grains, red meat and horticulture [10].

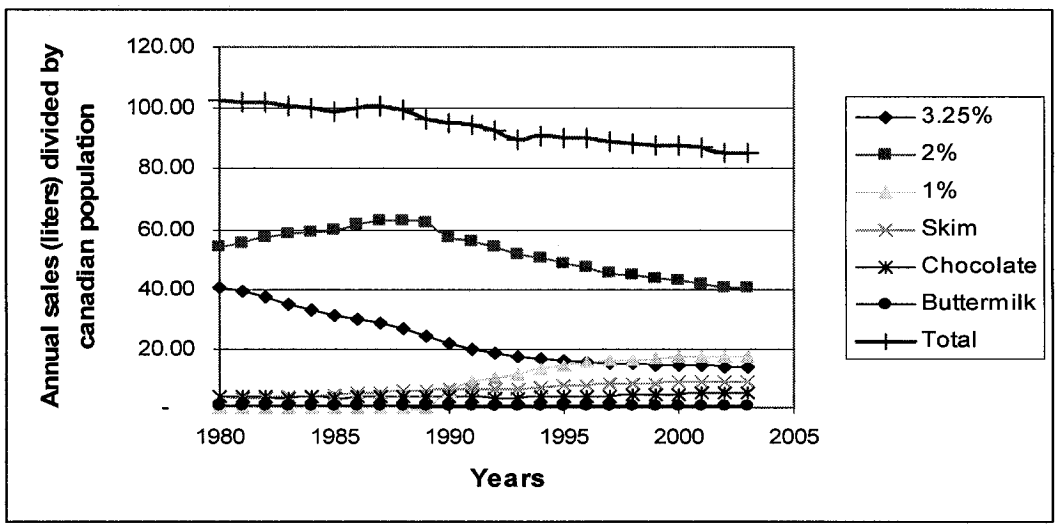

Fig. 4.1. Per capita consumption of dairy products in Canada [10].

4.2 Determination and Separation of Protein

4.2.1 Literature review

Protein determination may be carried out using different techniques such as spectrophotometry, infrared spectroscopy, and fluorimetry. Among them, spectrophotometry perhaps plays the most important role [11]. Spectrophotometric determinations may be performed either in the ultraviolet or visible region [12]. The visible region is generally used for quantitative measurement while the ultraviolet region is employed for qualitative detection. The protein determination in the ultraviolet region is usually done at 280 nm, although 205 nm could be used as well. Absorbance at 280 nm is primarily due to the presence of tyrosine and tryptophan amino acids; in the case of absorbance at 205 nm it is due to the presence of peptide bonds. Estimation of the absorbance ratio at 280 nm to that at 260 nm allows for the detection of interfering nucleic acids whose strong absorbance is at 260 nm. When their presence is confirmed, the correction factor developed by Warburg and Christian can be applied [11]. If contamination with nucleic acid is found then protein determination should be carried out at 205 nm given the poor absorbency of nucleic acids at this wavelength [12].

The main disadvantage of using the ultraviolet region for analytical quantification lies in the determination of the extinction coefficient. With a pure protein it is possible to determine its extinction coefficient value and use it in the determination of the absolute amount of protein. This task is not that easy in the case of a mixture of proteins. The main advantages are its non-destructiveness and speed, since no additional reagents or incubation are required [12].

Protein determinations performed in the visible region, also known as colorimetric determinations, are perhaps the most widely used methods today for protein assays [11]. They can easily be set up based on calibration curves. However, all colorimetric methods, except for the Biuret method, are dependent on protein composition [11]. This means that the method response is based on interactions with some amino acids. Therefore, it is crucial that the protein standard used in the

construction of the calibration curve represent sample composition as closely as possible. If this cannot be achieved, then it is advisable to use a method independent of protein composition, such as the cumbersome Kjeldahl technique, or a less sensitive one like the Biuret method [11]. The colorimetric methods can be divided into two groups based on either chemical reaction such as Biuret and Lowry methods or on dye-binding capacity like the Bradford method [11, 12].

The Biuret protein assay method is based on the formation of complex substances containing two or more peptides bonds with copper salts under alkaline conditions. The color of the complex is purple and it is determined at 550 nm. This assay works in the range of 1-20 mg of protein; therefore, it is considered an insensitive assay. However, it is completely independent of protein structure because the number of peptide bonds per given unit weight is about the same. This method suffers from some interference, such as the presence of the ammonium salts, Tris buffer, sucrose, primary amines, and glycerol. The Biuret method for the determination of milk proteins has been used in the present work.

The Lowry method is based on both the Biuret reaction, where the copper (II) ions form a complex with the peptide bonds in proteins under alkaline conditions, and the further enhancement of the color intensity by reduction of the Folin-Ciocalteau reagent by tyrosine and tryptophan residues. The absorbance measurements are taken at 500 nm. The method suffers from much interference, such as, Tris buffer and nitrogen containing buffers in general. Several modifications to this method have been proposed in order to reduce protein variability and also to produce more stable color complexes [11].

The Bradford assay method is probably one of the most widely used assays for protein determination. The advantages are speed, simplicity, reproducibility, high sensitivity and lack of interferences. The method is based on the protein behavior in presence of the Coomassie Brilliant Blue G-250 dye. The assay is performed at acidic pH where the protonated dye species absorbs at 465 nm in solution. When the dye is in

the presence of proteins, there is a shift in the absorbance maximum to 595 nm, where the de-protonated dye species absorbs. The reason for this shift in the absorbance maximum is still under debate. This method is not independent of protein composition, because the dye responds in decreasing order to arginine, histidine, lysine, tyrosine, tryptophan, and phenylalanine residues. The Bradford method for the determination of milk proteins has been used in the present work.

The bicinchoninic acid (BCA) method of protein determination is a variation of the Lowry method; therefore, it is also based on the Biuret method followed by a color enhancement reaction. After the formation of a copper (II)-protein complex under alkaline conditions (Biuret reaction) a reduction of copper (II) to copper (I) occurs by cysteine, cystine, tryptophan, tyrosine and the peptide bond. The BCA forms a purple complex with copper (I) ions in alkaline conditions; the intensity of this purple color is proportional to the amount of protein. The measurements are done at 562 nm. The main advantage of this method over the previous methods is the stability of the color complex. It is interfered with by chelating agents such as EDTA, reducing sugars, phospholipids, and some detergents. The BCA method for the determination of milk proteins has been used in the present work.

There are other colorimetric assays for protein determination, such as the amido black, ponceau S, and erythrosine B methods [11], which were not considered in this work because currently they are not widely used, and they do not differ significantly from the ones described above.

Although, colorimetric methods have a common disadvantage of high dependence on protein composition, they are still widely used due to their sensitivity, accuracy, reproducibility, and ease of performance. When the exact protein concentration is required, or when a colorimetric method needs to be validated, the Kjeldahl method is used as a reference method although it is time-consuming and requires a large amount of sample [11]. The Kjeldahl method is not used now-a-days as a routine method, but is still one of the better reference methods for protein determination.

The methods for protein separation include precipitation, chromatography, electrophoresis, centrifugation, *etc.* [13, 14]. All of them can be applied to the separation of proteins in bovine milk [1, 13]. Some chromatographic methods as well as precipitation methods used in this work are described below.

It is well known that proteins are easily dissolved at low salt concentrations (salting-in process) and precipitated at very high salt concentrations (salting-out process). The latter principle is used in the protein precipitation method. Not only salts like ammonium sulfate at high concentration (4 mol. L^{-1}) produce the protein precipitation, but also other reagents such as trichloroacetic acid and alcohols also induce protein precipitation [1, 13, 14].

In the case of milk, whole casein can be separated by precipitation using a different principle. Casein is the protein that precipitates from solution at pH 4.6 and 20°C when its isoelectric point is reached. This property is used for separating the major milk proteins (casein) from the minor proteins (whey) [1].

Chromatographic techniques have been applied extensively to the separation of milk proteins. Either preparative or analytical separation can be successfully performed. Preparative separations are most effective using ion-exchange chromatography (DEAE- cellulose) or size exclusion chromatography (Sephadex gels). For analytical separations HPLC based on adsorption columns is also commonly used [7].

4.2.2 Experimental and Results

4.2.2.1 Protein assays

The Biuret, Bradford and Lowry variant (BCA) methods were studied for the determination of protein in homogenized bovine milk. Calibration curves using bovine serum albumin (BSA), casein, and β-lactoglobulin standard proteins were constructed. The details of each procedure are described below.

Biuret method: A stock solution of 10 mg mL^{-1} BSA was prepared as the standard solution. Various aliquots of this solution were used in order to cover the range of 1-10 mg protein and the volume was made up to 1 mL with water. Three mL of Biuret reagent were added and the sample was shaken in a vortex mixer. Readings at 550 nm were taken after 20 min of reaction at room temperature. The same procedure was followed using 10 mg mL^{-1} casein as the standard protein, 2 mg mL^{-1} of BSA, casein, and β-lactoglobulin. Three replicates were used to generate each point on the calibration curves.

BCA method: Different volumes of a standard solution of 2 mg BSA mL^{-1} were taken to cover a range of 4-100 μg of protein. Ten μL of 4.8 mg mL^{-1} of lactose were added followed by 1 mL of 2% SDS solution. Then water was added to make up the volume to 1 mL. Finally, 1 mL of the BCA solution was added and the solution was stirred in a vortex mixer. Absorbance at 562 nm was recorded after 30 min of heating at 60°C. The same procedure was followed using casein, as the standard protein, stock solution of 1 mg mL^{-1}. Three replicates were used for each point on the calibration curves.

Bradford method: Different volumes of a standard solution of 1 mg BSA mL^{-1} were taken to cover a range of 4-100 μg of protein. The total volume was made up to 1 mL with water. Three mL of Coomassie brilliant blue G-25 reagent were added, and the solution was shaken in a vortex mixer. Absorbance at 596 nm was measured immediately and without any heating. The same procedure was followed using 1 mg mL^{-1} solutions of casein and β-lactoglobulin as standard proteins. Three replicates were used to generate each point on the calibration curves.

Since BSA is perhaps the most widely used standard protein for constructing calibration curves, it was used as the first option in this work. Three calibration curves shown in Figs. 4.2, 4.3 and 4.4 corresponding to Biuret, Bradford and BCA methods were constructed where BSA was the standard protein. The methods were

applied to aliquots of a milk sample. The protein content of the milk is shown in Table 4.6 along with a comparison of the methods.

It is known that whey protein accounts for 19-21% of the total proteins in milk. Table 4.6 shows that only the Biuret method agrees with the literature values. It appears that the Bradford and BCA methods are not suitable for the quantitative assessment of either total or whey proteins in milk using BSA as the standard protein since the results differ significantly from the Biuret method.

It was expected that the Biuret method would agree with the literature values since it is independent of the protein standard. It could always be used as "the method" for protein determination but its high detection limit was the biggest disadvantage. It was then concluded that perhaps more sensitive methods such as those of Bradford and BCA should be used in this thesis.

Table 4.6. Determination of protein in milk using three different methods and BSA as the protein standard

	Biuret	**Bradford**	**BCA**
Total protein (mg mL^{-1})	38 ± 2	24 ± 2	27 ± 2
Whey protein (mg mL^{-1})	8.0 ± 0.3	1.11 ± 0.02	1.83 ± 0.06
Whey as % of total protein	21	4.6	6.8
Detection limit (μg)	100	3	3

It was then decided to make attempts to improve the results of the Bradford and BCA methods. Instead of using BSA, casein was tested as a protein standard in all three methods. The Biuret was considered to be the reference method. Casein is the major protein in milk and it accounts for 78-80% of total milk proteins. Since this protein is more representative of the sample matrix, an improvement in the results by the Bradford and BCA methods was expected using the casein standard. The results are given in Table 4.7 and the calibration curves are shown in Figs. 4.5 to 4.7.

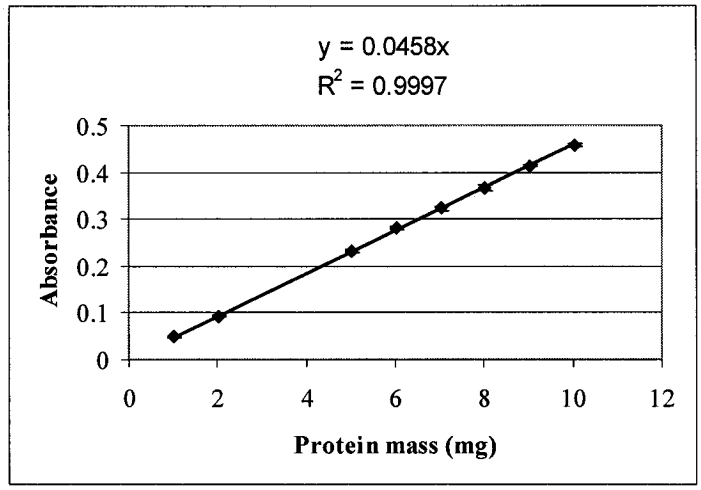

Fig. 4.2. Calibration curve for the Biuret method using BSA as standard.

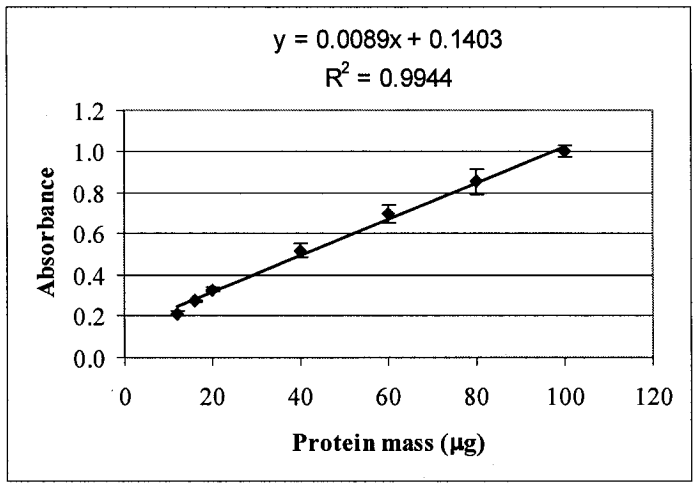

Fig. 4.3. Calibration curve for the Bradford method using BSA as standard.

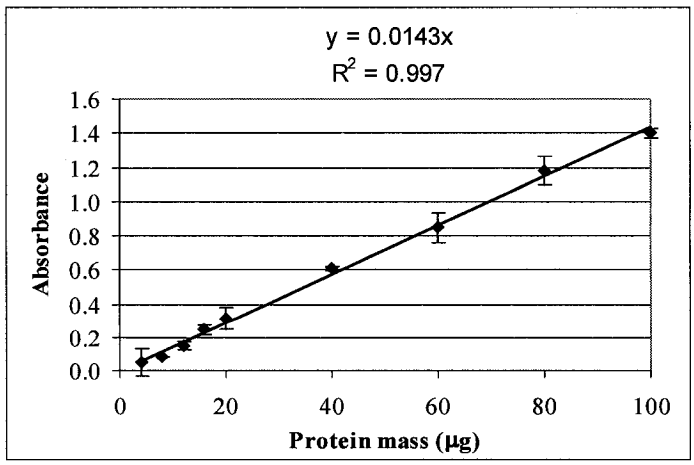

Fig. 4.4. Calibration curve for the BCA method using BSA as standard.

Table 4.7. Determination of protein in milk using three different methods and casein as the protein standard.

	Biuret	**Bradford**	**BCA**
Total protein (mg mL^{-1})	39 ± 2	42 ± 2	40 ± 2
Whey protein (mg mL^{-1})	8.2 ± 0.3	1.23 ± 0.02	2.73 ± 0.09
Whey as % of total protein	21	2.9	6.8

It is evident from Table 4.7 that there is no significant difference in the total protein content of the milk by the three methods. This observation is important because it allows the use of either the Bradford or the BCA method for the determination of total protein using casein as the standard. The possibility of using one of these methods improves the reliability of the measurement. Due to the simplicity of the Bradford method, it was chosen for subsequent total protein determinations in milk.

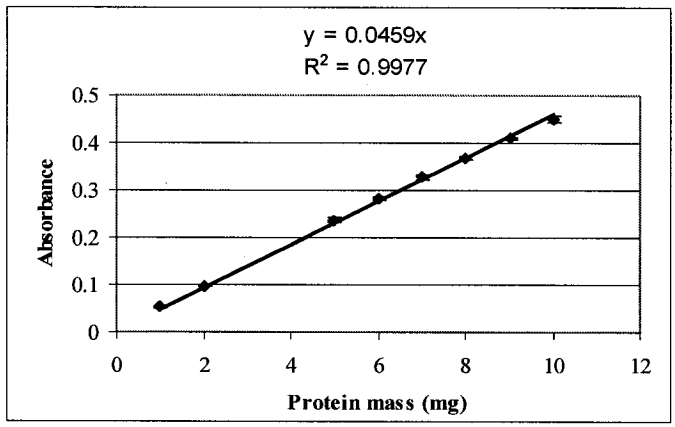

Fig. 4.5. Calibration curve for the Biuret method using Casein as standard.

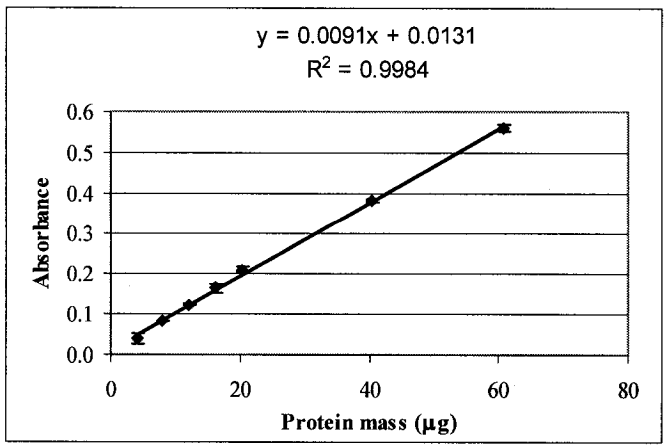

Fig. 4.6. Calibration curve for the Bradford method using casein as standard.

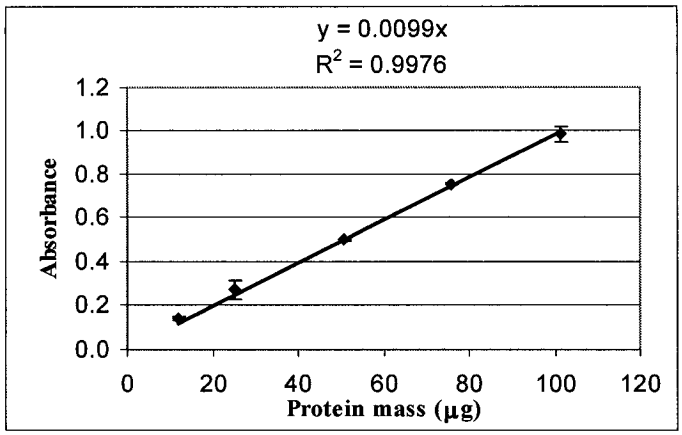

Fig. 4.7. Calibration curve for the BCA method using casein as standard.

However, the Bradford and BCA methods were still not satisfactory for the determination of whey proteins. Following reasoning similar to that used for selecting casein as the standard protein, β-lactoglobulin was tested as the standard protein for the determination of whey proteins. Since the major component of whey proteins is β-lactoglobulin, it should be more representative of the sample matrix. The results for the Biuret and Bradford methods are shown in Table 4.8 and the calibration curves are shown in Figs. 4.8 and 4.9.

Unfortunately, β-lactoglobulin did not work well enough for the determination of whey protein in milk as evident from Table 4.8. It worked better than BSA and casein protein standards (Tables 4.6 and 4.7) but was still not satisfactory. The total and whey protein concentrations given in Table 4.8 by the Biuret method differ from those in Tables 4.6 and 4.7 because two different homogenized milk samples were used. However, whey protein as the percentage of the total protein is the same in both milk samples, as expected.

Table 4.8. Determination of protein in milk using two different methods and β-lactoglobulin as the protein standard.

	Biuret	Bradford
Total protein (mg mL^{-1})	32 ± 2	88 ± 6
Whey protein (mg mL^{-1})	6.7 ± 0.3	3.15 ±0.06
Whey as % total protein	21	3.6

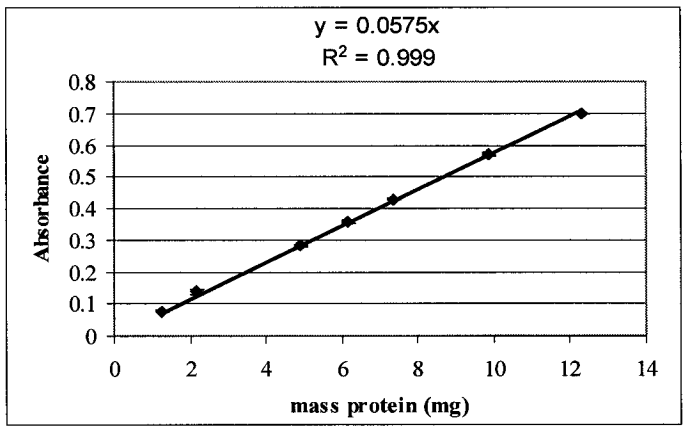

Fig. 4.8. Calibration curve for the Biuret method using β-lactoglobulin as standard.

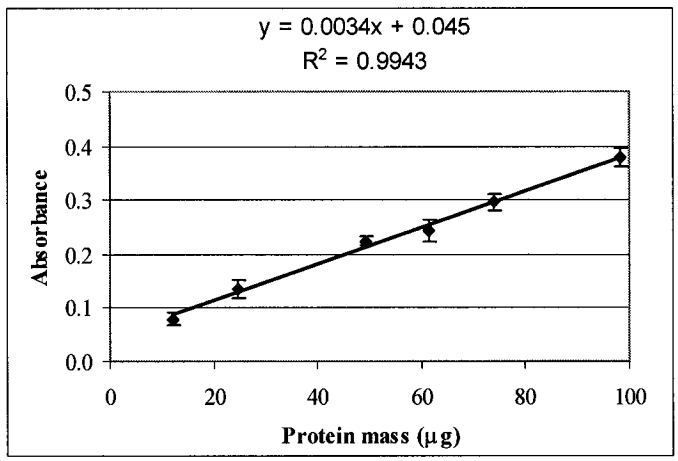

Fig. 4.9. Calibration curve for the Bradford method using β-lactoglobulin as standard.

Although an appropriate protein standard for the determination of whey protein using the Bradford method was not found, the determination of total protein can be carried out by this method. It was noted earlier that the total protein determination

using the Biuret method was very close to the detection limit; however, this is not the same for whey protein. It was concluded that the Biuret method is reliable for the determination of whey proteins in milk.

The reasons for the reliability of the Biuret method for whey proteins but not for the total proteins can perhaps be explained. Spectrophotometry requires the measurement of absorbance in a clear solution while milk is a white suspension. For this reason milk is diluted 100 fold before any spectrophotometric readings are taken. About 100 μL of the samples are used in the Bradford and BCA methods while the Biuret method needs at least 1 mL of the sample. Even then the values are close to the detection limit of the Biuret method. A reduction of the dilution factor of milk does not work because the solution is not clear. On the other hand, once the casein is removed the remaining solution containing whey proteins is clear and can be used directly for its reliable determination by the Biuret method. Dilution is not needed, although it can be done if desired.

4.2.2.2 Determination of proteins in milk

As was explained in the previous section, the protein in milk was determined by the Bradford method using casein as the standard protein. After removing casein, whey protein was determined by the Biuret method either directly with 100 μL of whey solution or using 500 μL of whey mixed with 500 μL of water. The amount of casein was determined by difference between the total and whey protein. The results are shown in Table 4.9. The numbers in parenthesis represent each protein as the percentage of the total protein.

Table 4.9. Determination of total, whey and casein concentrations in milk.

Concentration of total proteins in milk (mg mL^{-1}) by Bradford method	**Concentration of whey proteins in milk (mg mL^{-1}) by Biuret method**	**Concentration of casein in milk (mg mL^{-1}) by subtraction**
33 ± 2	6.8 ± 0.3 (21)	26 ± 2 (79)

The protein content of milk, in particular casein, is routinely determined in the dairy industry by either infrared spectroscopy or the Kjeldahl method. Despite the simplicity and rapidity of spectrophotometric methods, they are not routinely used for this purpose. However, Table 4.9 shows that these methods could offer a very good alternative for the routine determination of protein in milk if appropriate protein standards are employed.

4.2.2.3 Precipitation of total protein and casein

It is known that compounds such as alcohols and salts can precipitate proteins (salting-out process). In this work ammonium sulfate was chosen as the precipitating agent not only because it is widely used but also because it produces minimal interference in NAA. A saturated ammonium sulfate solution at room temperature was prepared. Various amounts of this solution were added to 400-500 μL of skim milk placed in micro-centrifuge tubes (1.8-mL Eppendorf tubes) to obtain different concentrations of the salt as shown in Table 4.10. The samples were centrifuged at 15 000 rpm for 90 min at 4°C and then the mass of protein in solution was determined by the Bradford method using casein as the standard protein. The concentration of ammonium sulfate solution required to produce quantitative precipitation of proteins is shown in Fig. 4.10. It is not advisable to use the Biuret method here because of the high concentration of ammonium sulfate solution required, which interferes with the determination of protein.

Table 4.10. Protein precipitation conditions.

Volume of milk (μL)	**Volume of $(NH_4)_2SO_4$ (μL)**	**Final concentration of $(NH_4)_2SO_4$ (mol L^{-1})**
500	0	0.00
500	34	0.25
500	118	0.75
500	309	1.50
500	518	2.00
500	875	2.50
400	1290	3.00

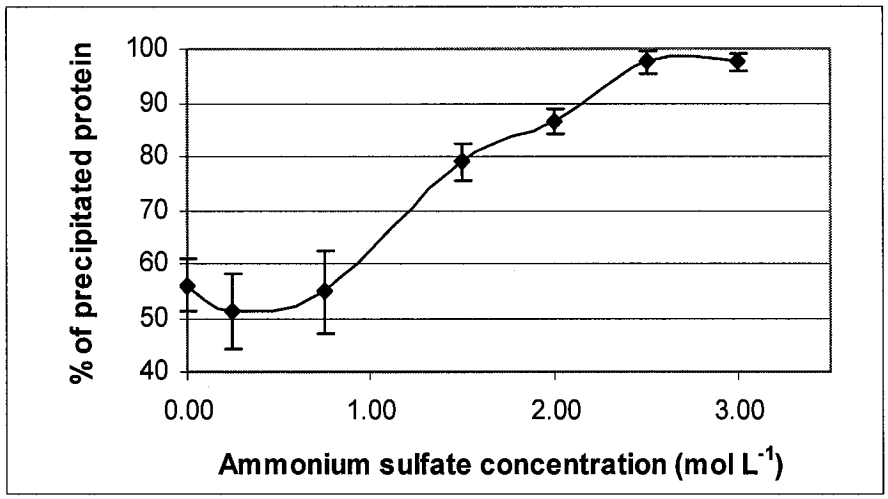

Fig. 4.10. Protein precipitation as a function of ammonium sulfate concentration.

Fig. 4.10 shows that a minimum of 2.5 mol L^{-1} ammonium sulfate solution was needed for the quantitative precipitation of proteins in milk. In practice, a saturated solution at room temperature (about 4 mol L^{-1}) was prepared and an appropriate volume was added to the sample. This protocol was followed for both the precipitation of total proteins and the precipitation of whey proteins once casein was removed.

Casein was separated from milk according to the following procedure. A sample of milk was heated at 60°C for 15 min. Glacial acetic acid was added to decrease the pH to 4.6. Approximately 8 drops of the acid were needed for 25 mL of milk. Casein precipitated under this condition because the isoelectric point of the protein was reached. The mixture was heated at 60°C for 10 more min, and it was allowed to stand for about 30 min. Then the mixture was filtered through a 0.45 μm pore size Millipore filter before assaying whey protein. The precipitated casein was suspended in ethanol for about 24 h, filtered, and allowed to dry completely in a desiccator prior to NAA. The use of acetic acid for lowering the pH was found to be advantageous in this work because it did not introduce any additional interference in NAA. The filtrate contained whey proteins that were precipitated using ammonium sulfate. The solid whey proteins were also suspended in ethanol, filtered, dried and saved for NAA as well.

4.2.2.4 Summary

The protein content of milk can be determined using spectrophotometric methods. It has been shown (Tables 4.6 to 4.8) that the Biuret method can be applied to both total proteins and whey proteins regardless of the standard protein used. The whey proteins can be reliably determined using this method. However, the detection limit of this method for total protein is poor and close to the actual content (Tables 4.6 to 4.8). The Bradford method using casein as the standard protein is a better option for the determination of total protein (Table 4.7). It has been found that the total milk protein content is about 0.03 g mL^{-1} where casein accounts for about 79% and whey for about 21% of the milk proteins. The precipitation methods used for separating total and casein proteins have proven to be adequate for this study. These methods were used in various separation schemes described in Chapter 6 for the determination of total and whey proteins, and casein.

4.3 Separation and Determination of Lipids

4.3.1 Literature review

Lipids have been the subject of countless research projects. These include lipid determination for nutritional and environmental monitoring purposes. Despite the fact of being investigated for more than 50 years, lipids still remain an important research area.

The term lipid encompasses a wide variety of compounds. They have been defined as natural substances containing one chain of at least 10 carbon atoms in their structure [15]. Therefore, any compound which contains long fatty acid chains, saturated or unsaturated, or any natural material soluble in organic solvents and not soluble in water is considered a lipid. For the sake of simplicity, lipids have been classified as either simple, complex, or derived [15, 16]. Simple lipids include acylglycerols (also referred to as triglycerides) and waxes with apolar properties. Complex lipids are phospholipids, lipoamino-acids, and glycolipids. Within this group

are the phosphoacylglycerols, sphyngomyelins, cerebrosides and gangliosides. Complex lipids generally have polar properties. Derived lipids include carotenoids, steroids and fat-soluble vitamins [16].

The fact that lipids have not been clustered under a common functional group (such as alcohols, ketones, proteins, *etc.*) makes their determination a rather complex task. Gravimetry is the most commonly used technique for total lipid determination. This fact makes the method for the extraction of lipids a key step in achieving good results [17].

The extraction method faces the dilemma of dealing with different types of compound. The solvent system used should be as non-specific as possible in order to extract as many lipid classes as possible from the sample [18]. In 1957 Folch introduced the chloroform-methanol (2:1) solvent system for the separation of lipids while he was working with brain tissue [15, 19]. Although this system has been extensively used since then, it requires large amounts of solvent and long experimental time [19].

Bligh and Dyer modified Folch's method by introducing water as part of the solvent system [20]. They explained this modification: "...an optimum lipid extraction should result when the tissue is homogenized with a mixture of chloroform and methanol which when mixed with the water in the tissue would yield a monophasic solution. The resulting homogenate could then be diluted with water and/or chloroform to produce a biphasic system...". They found that the proportions of chloroform-methanol-water for forming the monophasic solution were 1:2:0.8 and for forming the biphasic solution 2:2:1.8. This means that if 100 g of sample containing 80 g of water is taken, 100 mL of chloroform followed by 200 mL of methanol should be added. After shaking the system with another 100 mL of chloroform, 100 mL of water are added for producing the biphasic system.

The Bligh and Dyer method was the most widely used method for lipid extraction until 1987 when the Montreal Protocol was reached [18]. According to this

protocol, the use of chlorinated compounds (such as chloroform) should be avoided or used sparingly due to environmental problems they cause. As a result, the scientific community started to explore other options for lipid separation. Smedes studied the Bligh and Dyer method in detail to understand the effect of methanol on the yield of lipids, phospholipids in particular [18]. It was then concluded that the new systems must contain a polar solvent which can also separate the aqueous phase from the organic phases [18]. Thus, many systems such as methanol-ethanol-diethyl ether, hexane-isopropanol (3:2), hexane-acetone (2:1), isopropanol-cyclohexane-water (8:10:11), ethyl acetate-methanol-hexane (4:4:1), ethanol-petroleum ether-ethyl ether (2:1:1) and many others have been used successfully as alternative systems for lipid extraction [16, 18, 21-24].

After the extraction, the total lipid needs to be fractionated into major lipid classes. Different techniques such as solvent fractionation, counter-current distribution, column chromatography (adsorption and ion exchange), thin layer chromatography, and solid phase extraction have been used for this purpose either on an analytical or a semi preparative scale [16]. Among them, adsorption chromatography using silica gel and thin layer chromatography have been widely used. The appropriate technique of course depends on the type of lipid being investigated.

Once the lipids have been separated into major classes, individual lipid separation may be required. Techniques such as HPLC, GC, SFC are commonly used for this purpose [25-29]. Reversed-phase HPLC is routinely used for triacylglycerol separation and also for phospholipids, while HPLC-SEC is used for the separation of polymerized triacylglycerols. Fatty acid methyl esters can be analyzed by GC with good results [25-29]. The SFC technique is being increasingly used for major class lipid separation, specifically for triglycerols [25-29]. This type of chromatography uses the same columns as GC, and in many cases the same type of detector; the main difference lies in the mobile phase, which in this case is a supercritical fluid, usually carbon dioxide. The use of SFC offers many advantages such as low cost and non-toxic properties of the

mobile phase. Like GC, SFC can be coupled to mass spectrometry to form a powerful technique for lipid analysis [27, 28].

4.3.2 Experimental and Results

4.3.2.1 Extraction of total lipids

As mentioned earlier, the classical method for the determination of total lipid is gravimetry which involves an extraction step. In order to identify a suitable method for the extraction of lipids in milk, four methods were assessed. The well known Bligh and Dyer chloroform-methanol-water (1:2:0.8) system [20] was designated in this study as the reference method. The second method was originally proposed by Hara [24] using isopropanol-hexane (3:2) but modified by Indrasena of our laboratory using a 1:1 ratio of the same solvent system [30]. The AOAC reference method for lipid extraction in milk [21] using ethanol-petroleum ether-ethyl ether (2:1:1) was the third method investigated. The fourth method was proposed by Indrasena *et al.* [22] where the solvent system consisted of ethyl acetate-methanol-hexane (4:4:1). The extraction procedure for each method is given below in detail.

Bligh and Dyer method: About 25 mL of a milk sample were placed in a separatory funnel and 2 mL of conc. ammonia solution were added to it. After shaking for 1 min, 32 mL of chloroform and 64 mL of methanol were added, and the sample was mixed for an additional 2 min. Then, 32 mL of chloroform and 32 mL of water were added and mixed for another 2 min. The mixture was centrifuged at 5 000 rpm at room temperature for 10 min. The lower phase (chloroform phase) was separated and kept. About 32 mL of 10% (v/v) methanol in chloroform were added to the upper phase and mixed for 2 min. After centrifugation, the lower phase was added to that from the first extraction.

Hara's method modified by Indrasena: About 25 mL of a milk sample were placed in a separatory funnel and 2 mL of ammonia solution were added to it. After shaking for 1 min, 15 mL of hexane and 25 mL of isopropanol were added, and the sample was mixed for 2 more min. Then 10 mL of hexane were added and mixed for 1

min. After the separation of phases (no centrifugation was required), the upper phase (hexane) was removed and stored. The lower phase was taken through a second extraction similar to the first one except using half of the initial volumes. After separating the upper phase in the second extraction, 10 mL of hexane were added to the bottom phase and a third extraction was carried out. The three hexane phases from each extraction were combined. The AOAC Reference method recommends that three extractions should be sufficient for extracting the lipids from milk. Therefore, three extractions were assumed to be adequate in each case, except for Bligh and Dyer where only 2 extractions were made.

AOAC Reference method: About 25 mL of a milk sample were placed in a separatory funnel and 2 mL of ammonia solution were added to it. After shaking for 1 min, 40 mL of ethanol, 10 mL of ethyl ether and 10 mL of petroleum ether were added, and the sample was mixed for 1 min after each addition. Then another 10 mL of ethyl ether and 10 mL of petroleum ether were added and mixed for an additional 1 min. After separation of the phases (no centrifugation was required), the upper phase (ether mixture) was removed and stored. The bottom phase was taken through a second extraction similar to the first one but using half of the initial volumes. After separating the upper phase in the second extraction, 10 mL of ethyl ether and 10 mL of petroleum ether were added to the bottom phase and a third extraction was carried out. The three ether phases separated in each extraction were combined.

Indrasena *et al.* method: About 25 mL of a milk sample were placed in a separatory funnel and 2 mL of ammonia solution were added to it. After shaking for 1 min, 40 mL of methanol, 20 mL of ethyl acetate and 20 mL of methyl acetate were added, and the solution was mixed for 1 min after each addition. Then 10 mL of hexane were added and mixed for another 1 min. After separation of the phases (no centrifugation was required), the upper phase (hexane) was removed and the bottom phase was taken through a second extraction using 10 mL of ethyl acetate and 5 mL of hexane. After separating the upper phase in the second extraction, the bottom phase was

taken through a third extraction identical to the second one. The three organic phases obtained from each extraction were combined.

In the above methods, first the organic phases were combined then the solvents were evaporated almost to dryness. Afterwards a washing step involving just water was applied. Once the water (about 20 mL) was added to the organic phase in a separatory funnel, shaken and allowed to stand, the two phases separated and the organic phase was removed. Ten mL of the extracting solvent mixture were then added to the aqueous phase, allowed to stand, and the organic phase removed. This step was repeated one more time with the aqueous phase. The aqueous phases were combined and added to the rest of the milk sample. All organic phases were combined, and evaporated to dryness to a constant weight. The results obtained using these four methods are shown in Table 4.11.

Table 4.11. Total lipid concentration ($mg.mL^{-1}$) in milk by different methods.

Bligh and Dyer method	**Hara's method modified by Indrasena**	**AOAC Reference method**	**Indrasena method**
0.0319 ± 0.0002	0.0329 ± 0.0002	0.0316 ± 0.0004	0.0325 ± 0.0003

Apparently, the results in Table 4.11 show no difference among the four methods. However, a single factor ANOVA using α=0.01 shows highly significant differences ($F= 13.82$, $p<0.01$) among all of them. This observation is very important for the purpose of this thesis because it provides statistical evidence that supports the selection of either Hara's or Indrasena's method for assessing total lipid content in milk over both the AOAC Reference or the well-known Bligh and Dyer methods.

Indrasena *et al.* originally developed [22] the method for the determination of organochlorines in fish tissues containing water and as an alternative to the Bligh and Dyer method. In some cases, the first method gave higher yields for lipids than the Bligh and Dyer method indicating that it was also applicable to milk. In this thesis Hara's

method modified by Indrasena *et al.* [30] was chosen over the method by Indrasena *et al.* [22] for the determination of total lipids in milk because it is faster and uses a smaller number as well as volumes of solvents.

4.3.2.2 Indirect determination of lipids

Since the lipid determination is a key point in any lipid investigation and since there is no unique method available for this task, the efficiency of the solvent system used is of primary importance which is estimated relative to the reference method. However, this implies that the reference method extracts 100% of the lipids. In order to find the efficiency of the extraction system independent of the reference method, an indirect method for lipid determination based on mass balance was carried out.

The mass of a sample of milk is largely the sum of water, proteins, carbohydrates, lipids, minerals, vitamins, gases and anions of acids. If one knows the content of all other fractions except lipids in milk then the lipid mass can be estimated from the mass balance equation. Given the very low contributions from gasses, anions of organic acids and vitamins in the milk (about 0.30%), their masses will be considered negligible. Hence, the masses of water, proteins, carbohydrates and minerals need to be determined. The water content was determined by lyophilization, while protein and carbohydrate contents were determined by spectrophotometric methods (those for protein have already been discussed in section 4.2). The amount of minerals was expressed as the sum of magnesium, sodium, potassium, calcium, chlorine, phosphorous, bromine and iodine determined by NAA. The indirect determination of lipids was based on the following mass balance equation:

Lipid (g) = Total milk (g)–Water (g)–Protein (g)–Carbohydrate (g)–Mineral (g)

4.3.2.2.1 Determination of carbohydrate

One of the methods for the determination of total carbohydrate in a sample is the phenol-sulfuric acid method [31]. This method is based on the stable orange color that simple sugars, oligosaccharides, polysaccharides and their derivatives give when they react with phenol and concentrated sulfuric acid. The intensity of the color is

proportional to the amount of total carbohydrates present, and the absorbance is measured at 492 nm.

As with most spectrophotometric methods, a calibration curve is needed for quantitative measurements. Given the fact that lactose represents most of the carbohydrate content of milk, lactose was used for constructing the calibration curve. Different concentrations of lactose solutions were prepared. One mL of the solution was mixed with 1 mL of phenol solution (5%) and 3 mL of concentrated sulfuric acid. The solutions were allowed to stand at room temperature for 3 h and then the absorbance was measured. Milk samples were diluted 500 fold before analysis. The calibration curve is shown in Fig. 4.11.

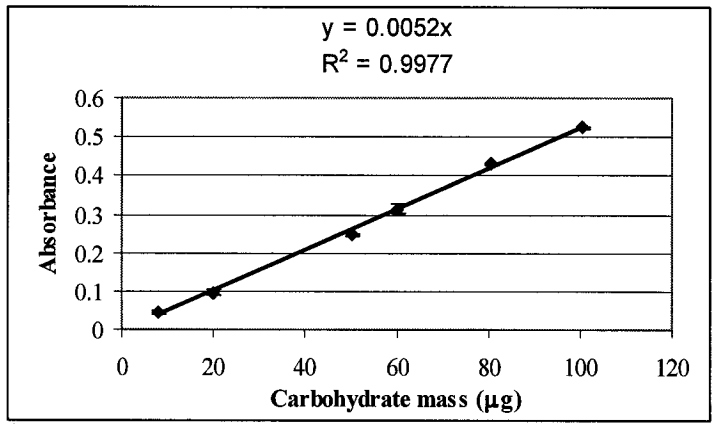

Fig. 4.11. Calibration curve for carbohydrate determination by phenol-sulfuric acid.

The carbohydrate content of milk determined by this method was 48±0.4 mg mL^{-1}.

4.3.2.2.2 Determination of Minerals

The concentrations of the major elements in milk, namely aluminum, bromine, calcium, chlorine, iodine, magnesium, potassium, phosphorous, and sodium, were measured by INAA. With the exceptions of aluminum and phosphorous, all other elements were determined in one irradiation. A standard solution containing all elements of interest was prepared and the relative method for determining their concentrations was

used. The sample was irradiated for 5 min in one of the inner sites of the SLOWPOKE-2 reactor, allowed to decay for 2 min, and then counted for 10 min using the Compton suppression detector system. These timing parameters were identical to those used for the determination of iodine in milk samples, as described in Chapter 3.

The determination of aluminum and phosphorous was slightly different from the rest of the elements. The ^{28}Al isotope is produced in the reactor mainly by the nuclear reaction $^{27}Al(n,\gamma)^{28}Al$. The same nuclide ^{28}Al is also produced by the $^{31}P(n,\alpha)^{28}Al$ reaction. In the presence of thermal neutrons both reactions occur. However, in the presence of epithermal neutrons only the second reaction takes place. The DUSR facility has two types of irradiation site, namely inner (#1 to 5) and outer (#9, 10). Both outer sites are at the same distance from the reactor core and thus have same neutron flux. The site #9 has a unique feature in the sense that it is surrounded by 1-mm thick cadmium. The ^{113}Cd isotope has a very high thermal neutron absorption cross section ($2x10^4$ b). The net effect of the cadmium shield is a suppression of thermal neutrons allowing just epithermal neutrons to reach the sample.

Given the fact that the $^{31}P(n,\alpha)^{28}Al$ reaction is produced only by epithermal neutrons while thermal neutrons produce both reactions, a sample of milk was irradiated in both inner and cadmium-shielded sites, and the aluminum concentration was determined by difference. The milk sample and a phosphorous standard were irradiated with epi-cadmium neutrons in site #9, and the 1779-keV photopeak of ^{28}Al formed was used to assay its phosphorous content. Afterwards the milk sample and an aluminum standard were irradiated in site #10 (mostly thermal neutrons), and the aluminum concentration was determined by difference. The irradiation-decay-counting scheme was the same as that for the determination of total iodine in milk sample, *i.e.* 5-2-10 min. The Compton suppression detector system was used in this work. The levels of the major minerals in milk determined by NAA using the above conditions are summarized in Table 4.12 along with some literature values [32].

Table 4.12. Concentration of major minerals in milk.

Element	Concentration (μg mL^{-1}) (this work)	Concentration (μg mL^{-1}) (literature)
Aluminum	140 ± 38	
Bromine	3.49 ± 0.04	
Calcium	1085 ± 26	1110 - 1300
Chlorine	965 ± 9	900 - 1100
Iodine	0.42 ± 0.02	0.02 - 0.06
Magnesium	99 ± 2	90 - 140
Phosphorous	696 ± 18	900 - 1000
Potassium	1614 ± 27	1100 - 1700
Sodium	393 ± 11	350 - 900
Sum	4996 ± 50	

4.3.2.2.3 Determination of water

The water content of milk was determined by the freeze-drying process. Milk samples between 100-500 mL were weighed, placed in an adequate container, and freeze-dried. Once all the water evaporated, samples were re-weighed and the water content determined by difference. About 87.3±0.3 % (an average of 3 measurements) of the milk mass was found to be water.

4.3.2.2.4 Mass balance

Starting from an initial volume of 10 mL of milk corresponding to a mass of 10.2931 g, and measurement of the concentrations of protein (38 mg L^{-1}), carbohydrate (48 mg mL^{-1}), minerals (4995.91 μg mL^{-1}), and water (87.3%), one can calculate the lipid mass as shown in Table 4.13.

Table 4.13. Composition of milk determined by mass balance.

Milk fraction	Mass (g)	% of total mass (this work)	Average % (literature)
Total mass (g)	10.2931	-	-
Proteins (g): 38 mg mL^{-1} x 10 mL /1000 mg g^{-1}	0.38	3.2	3.25
Carbohydrates (g): 48 mg mL^{-1} x 10 mL /1000 mg g^{-1}	0.48	4.5	4.6
Water (g): 10.2931 g x 0.873	8.9859	87.3	85.5-88.7
Minerals (g): 4995.91 μg mL^{-1} x 10mL/10^6 μg g^{-1}	0.0496	0.48	0.65
Lipid (g): Mass balance	**0.3976**	5.0	2.4-5.5

Table 4.13 shows that the masses calculated in this work for the milk components are in good agreement with the values reported in the literature, indicating that the indirect method of lipid determination is reliable. Assuming that the lipid mass calculated by the indirect method is the total possible mass to be extracted, then the percentage of recovery by the solvent extraction system of Hara's method modified by Indrasena (Table 4.11) is about 92%. In fact, the recovery value is slightly higher than the reported value since the indirect method did not take into account about 0.3% of the mass corresponding to dissolved gasses and anions of organic acids.

4.3.2.3 Fractionation of lipids

It was noted earlier that lipids in general consist of a variety of compounds. It is also known that lipids in milk, commonly referred to as milk fat, are mainly simple lipids. About 98% of milk fat is triglycerides which are classified as simple lipids, and the remaining are complex lipids, mainly phospholipids [1].

The total lipid extract was fractionated in this work into three classes differentiated by their polarities using a silica solid-phase extraction cartridge. This SPE method, developed in our laboratory by Gonzalez-Labrada [33], is a modification of the original method developed for an open column [34]. The total lipid, after the gravimetric

determination, was dissolved in an appropriate volume of hexane. About 1 mL of the solution was placed on a 6-mL LC-Si solid-phase extraction (SPE) cartridge previously conditioned with 2 mL hexane. Most apolar compounds were eluted by 10 mL hexane, followed by those of intermediate polarity using 15 mL acetone, and finally the more polar compounds by 10 mL methanol. These separated fractions were then dried in air and re-dissolved in 1 mL of the same solvent used for their elutions. Then they were passed through another LC-Si SPE cartridge for the purpose of purification followed by the same elution scheme described above. The mass of lipid in each of the purified fractions was determined by gravimetry.

The apolar class was then re-dissolved in hexane, and 1 mL of the solution was placed on a 6-mL florosil SPE cartridge previously conditioned with 2 mL of hexane. This method was a modification of an open column method [34] by Gonzalez-Labrada [33]. Hydrocarbons were then eluted using 15 mL hexane. Triacylglycerols were eluted using a mixture of hexane:diethylether (85:15). Diacyl and monoacylglycerols were eluted using 15 mL of a mixture of diethylether:methanol (98:2), while free fatty acids were eluted using 15 mL of diethylether:acetic acid (96:4). Masses of all fractions were determined gravimetrically.

The results of the above lipid fractionation process applied to the total lipid extract are shown in Table 4.14. The total extract was fractionated into three different groups differentiated by their polarities. The recovery value in the lipid fractionation is 99.8%, which indicates the reliability of the SPE method.

Table 4.14. Fractionation of the total lipid extract

Fraction	% of total lipid mass
Apolar (hexane)	96 ± 1
Medium polarity (acetone)	3.6 ± 0.6
Polar (methanol)	0.18 ± 0.07

It is expected that the apolar class would include simple lipids, the medium polar class glycolipids, and the polar class phospholipids. The identification of the individual lipids present in each fraction was not carried out due to lack of time. However, the data in Table 4.14 suggest that fractionation provided the expected results. The apolar fraction represents 96% of the total lipid extract. This fraction contains the simple and neutral lipids. It is well known that triacylglycerides (which are neutral lipids) represent about 98% of the total milk lipids [1]. It is also known that about 0.2% of milk lipids correspond to phospholipids and they are expected to elute in the most polar fraction [1, 34].

The apolar fraction was taken through a second fractionation process where four different groups were separated. The expected compounds as well as their distributions in the apolar class are shown in Table 4.15. The recovery of the second fractionation process was >99%. Further characterization of these fractions was not carried out due to the lack of time. It has been reported in the literature [1] that about 98% of milk fat consists of triacylglycerols. Table 4.15 shows that only 2.5% is triacylglycerols. There could be two explanations for this discrepancy. It is possible that what is referred to in the literature as 98% triacylglycerols is in fact the whole (total) apolar fraction as shown in Table 4.14. The other possibility is that the fractionation process carried out in this work consistently eluted triacylglycerols in the hexane fraction, which should only contain hydrocarbons.

Table 4.15. Fractionation of the apolar lipid extract.

Fraction	% of total apolar lipid mass
Hexane: Hydrocarbons	95 ± 0.3
Hexane : diethylether (85:15) Triacylglycerols	2.4 ± 0.2
Diethylether : methanol (98:2) Di- and mono-acylglycerols	0.32 ± 0.03
Diethylether : acetic acid (96:4) Free fatty acids	0.53 ± 0.06

Table 4.15 indicates that hydrocarbons are the major components of the apolar fraction. If milk contains any of the toxic anthropogenic organohalogen compounds, they should be separated in this hexane fraction. Therefore, any study related to PCB, DDT and their derivatives in milk should be done in the hexane fraction.

4.3.2.4 Separation of lipids and proteins from milk

The extraction process used in Section 4.3.2.1 separated the milk into an organic phase containing lipids and an aqueous phase containing the proteins and other compounds. Another approach would be to precipitate the proteins using ammonium sulfate followed by the lipid extraction. The extraction of lipids using both methods was studied.

In the case where the lipids were extracted first from the milk sample, proteins remained in the aqueous phase. When the sample treatment began with the addition of ammonium sulfate, proteins were found in the precipitate but the filtrate was lipid-free. This meant that the lipids coprecipitated with proteins when ammonium sulfate was added. This observation was taken into account in developing separation schemes described in Chapter 6. To confirm that lipids coprecipitate along with proteins, the precipitate obtained after treating the milk sample with ammonium sulfate was re-dissolved in a volume of water identical to the initial sample volume. Two mL of concentrated ammonia solution were added and the lipid extraction was carried out as described in section 4.3.2.1. The results are shown in Table 4.16.

Table 4.16. Lipid extraction before and after protein precipitation.

Lipid concentration (%), performed directly from milk	**Lipid concentration (%), performed after protein precipitation**
3.13 ± 0.06	2.52 ± 0.07

The difference in yield between the two methods is mainly due to recovery. There are losses in the filtration step. Despite the differences in results, it is

apparent that lipids coprecipitated with proteins. This could be advantageous if fractionation of organic and inorganic components is desired. The fact that all lipids cannot be recovered when protein precipitation is carried out first is not a problem, since the lipid concentration can be determined by the indirect method.

4.3.2.5 Summary

Experiments showed that at least two methods based on solvent extraction are suitable for extracting milk lipids more efficiently than the AOAC reference and the well-known Bligh and Dyer methods. Hara's method modified by Indrasena was chosen as the working method in this thesis because it uses fewer solvents than Indrasena's own method and it is faster. Assuming that the indirect method for determining lipid content in milk represents 100% recovery then Hara's modified method yielded an extraction efficiency of at least of 82%. Total milk lipids were then fractionated into three groups with different polarities, namely neutral lipids, glycolipids and phospholipids. A second fractionation process separated the neutral lipids into another 4 groups corresponding to hydrocarbons, triacylglycerides, di- and mono-acylglycerides, and free fatty acids. Both fractionation processes gave good recoveries indicating the suitability of SPE for this purpose. Lipids were found to coprecipitate with proteins if the protein precipitation process was performed before the lipid extraction. This observation would be advantageously used in developing various separation schemes described in Chapter 6.

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CHAPTER 5

SEPARATION OF INORGANIC IODINE SPECIES

5.1 Literature review

The various inorganic species of iodine found in biological materials include iodide (I^-), iodate (IO_3^-), and periodate (IO_4^-) ions and free iodine (I_2). In food items, such as seafood, milk and seaweeds which are the major sources of iodine for humans, the main forms of iodine are iodide and iodate [1-2].

The determination of individual inorganic iodine species in food basically involves the separation of iodide and iodate ions. Chromatographic techniques have gained greatest acceptance for this task [3-9]. Among these techniques, anion exchange chromatography using either open column or HPLC has perhaps been used most extensively.

In 1990, Reinfenhauser and Heumann reported the separation of iodide and iodate ions using Dowex 1X8 resin and detection by isotope dilution mass spectrometry (IDMS) employing ^{129}I as the tracer [6]. A column (12 cm high, 0.7 cm wide) containing a chloride form of the resin was used for the separation. Iodate and iodide ions were eluted using 0.1 and 1.5 M sodium nitrate solution, respectively. However, the elution volumes were typically large, especially for iodide ion because of its high affinity for this resin. The order of affinity of a number of anions for the Dowex 1X8 resin are as follows: $I^->HSO_4^->ClO_3^->NO_3^->Br^->CN^->HSO_3^->NO_2^->Cl^->HCO_3^->IO_3^->HCOO^->Ac^->OH^->F$ [4].

In 1999, Hou *et al.* [9] applied the same method but used AG 1X4 (50-100 mesh) resin in chloride form which is similar to Dowex 1X8. They used potassium nitrate solution instead of sodium nitrate solution to elute the inorganic iodine species. The column height was shorter (4 cm) but the diameter was the same (0.7 cm). The

inorganic iodide ions were separated after irradiation of the samples, *i.e.*, using radiochemical neutron activation analysis (RNAA) [9]. In 2000, the same authors reported that this method produces very high radiation doses using only 1 mL of seawater sample [4]. This observation makes the method unsuitable because the dosage received by a worker can exceed the maximum recommended dose within a short time. The elution volumes were very large as well [4].

In order to avoid the above disadvantages Hou *et. al.* [4, 9]) proposed to eliminate the elution step of iodide ions and to irradiate the resin directly where the ions were separated [4]. In the new method, the sample was passed through the resin and iodide ions were concentrated and retained on the resin, while iodate ions were eluted. Iodate ions were then reduced to iodide before being passed through a fresh resin. This method has the advantage of its rapidity because no elution step for iodide ions is involved. A combination of water and 0.5 M potassium nitrate solution was employed for the elution of iodate ions, while a 0.3 M potassium hydrogen sulphate solution was used for the reduction of iodate to iodide ions [4]. The disadvantage of this method lies in the time-consuming pre-cleaning process of the resin.

Adsorption chromatography has also been used for the separation of iodide and iodate ions. In 2001, Machado *et al.* [7] published a short communication on the separation of iodide and iodate using alumina and silica as sorption materials. The elution solutions were 0.1 M sodium nitrate and 0.5 M sodium hydrogen carbonate for iodide and iodate ions, respectively. No real advantages over the anion exchange methods were evident because large elution volumes were still required. In addition, the use of sodium salt is not particularly desirable in NAA.

HPLC methods have also been used for the separation of inorganic iodine species [12-18]. In fact, there are several reports where iodide ion was separated from a mixture of anions but not necessarily containing iodate ions. Conductometric and spectrophotometric detectors were mainly used although the former ones were more suitable. The columns were generally anionic exchangers. The eluting solutions usually

consisted of buffer systems [12-18] but they required special care to avoid any salt precipitation inside the HPLC instrument. One of the typical eluting systems was 0.004 M 4-amino-2-hydroxybenzoic acid at pH 5.85 [18]. This system was used for eluting iodate and iodide ions from a PRP-1 column (150x4.1 mm) using a flow rate of 2.0 $mL.min^{-1}$. Iodide and iodate ions were eluted in less than 10 mL of the eluent and detected using UV at 226 nm. This small elution volume makes this procedure preferable to most reported HPLC or open-column chromatographic methods.

5.2 Experimental

5.2.1 Cleaning of resin

The general procedure for cleaning the resin was as follows. About 50 g of Dowex 1X8 (100-200) mesh resin in chloride form was soaked in 5% of sodium hypochlorite solution at pH 1-2. The resin was stirred for about 30 min, followed by washing with DDW until no more chloride ions were detected by the silver nitrate test. The resin was split in two portions; one half was soaked in 25% v/v ammonia solution and the other half in 25% v/v nitric acid. In each case, the resin mixture was stirred overnight, washed with water until neutral pH was attained, and dried in air. A portion of the dried resin was irradiated for 1 min in the inner position of the DUSR facility, allowed to decay for 1 min, and counted for 5 min. If the 443-keV photopeak of ^{128}I was detected in the spectrum then the cleaning procedure was repeated again until there was no iodine peak. In general, three washings were required to make the resin iodine-free and to attain a constant ratio of chlorine counts/mass. At the end, about 25 g of resin in the OH^- form and an equal amount in the NO_3^- form were obtained. Both of these forms were suitable for irradiation since they introduce no interference in NAA. About 25 g of Dowex 50X8 (50-100) mesh resins in H^+ form was also cleaned by a procedure similar to the one described above.

5.2.2 Batch separation of inorganic iodine species

Based on the affinity of iodide and iodate ions for the Dowex (1X8) resin it was hypothesized that these two ions could be quantitatively separated if the resin was in the OH^- form. To corroborate this hypothesis, a standard solution of 0.5 $\mu g.mL^{-1}$ of iodine containing 0.25 $\mu g.mL^{-1}$ of each of iodide and iodate ions was prepared. Exactly 0.75 mL of it was irradiated for 5 min in the inner site of DUSR facility, allowed to decay for 2 min, and counted for 10 min.

Approximately 50 mL of a solution containing iodide and iodate ions were stirred with about 0.5 g of Dowex (1X8) resin in OH^- form for 5 min and a batch separation was done. The sample was filtered and 0.75 mL of the filtrate was irradiated under the same conditions as described above. The batch separation was repeated 2 more times. The iodine concentration remaining in the solution after each batch separation is shown in Fig. 5.1. Only one extraction was required to quantitatively retain the iodide and iodate ions present in the standard sample.

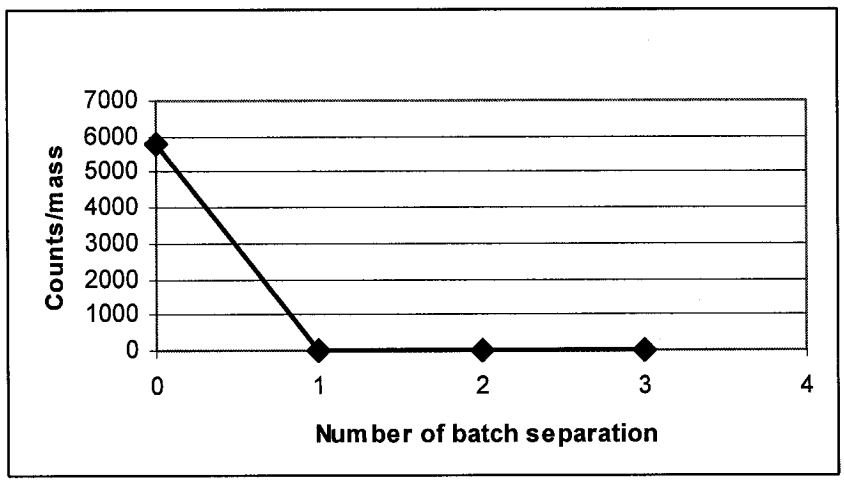

Fig. 5.1. Batch separation of inorganic iodine species in a standard solution.

The same experiment was then performed using milk samples. However, a pseudo-cyclic INAA (PCINAA) method was used for measuring the iodine content. Three spectra were collected, using the same t_i-t_d-t_c scheme used for the standards, and

compiled for the quantification of the iodine remaining in solution after each batch separation. The results of the batch separations are presented in Fig. 5.2.

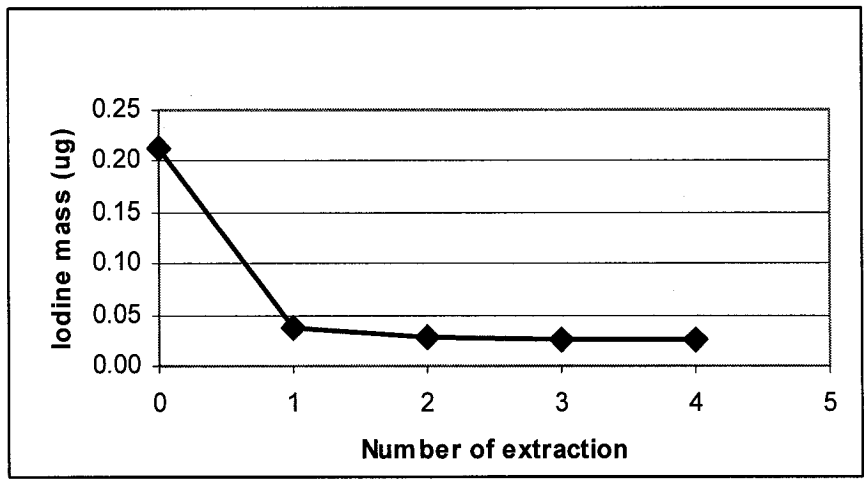

Fig. 5.2. Batch separation of inorganic iodine species in milk.

Two batch separations are sufficient for the extraction of all the inorganic iodine species from the milk sample as shown in Fig. 5.2. Nevertheless, three extractions were taken as the minimum required number for assuring quantitative separation. The difference between Figs. 5.1 and 5.2 is that the second one does not reach a plateau at zero. The reason for this is that Fig. 5.1 was obtained using an iodine standard solution where no organic iodine species were present while Fig. 5.2 was a real milk sample where organically bound iodine species are present which are not retained on the resin. In fact, the organic iodine species, according to Fig. 5.2, are about 10% of the total iodine present in milk, which agrees with the literature values. An alternative approach would be to irradiate the resin after batch separation. This second approach would be followed through in Chapter 6 for the quantification of the iodide and iodate species. It was possible to conclude that a batch separation using Dowex 1X8 (in OH^- form) was suitable for the separation of iodide and iodate species at once from milk.

To study the possible presence of cationic inorganic species of iodine in milk, the sample was passed through a cationic resin Dowex 50X8 (in NH_4^+ form) after the batch separation on Dowex 1X8. The experiment was also carried out in reverse, *i.e.*

Dowex 50X8 first and Dowex 1X8 later on. In all cases PCINAA (N=3 cycles) was used for the quantification of iodine. Table 5.1 shows the results of 3 different determinations each one with 3 cycles. It is clear that there were no significant differences between the two methods. This also means that there were no detectable cationic inorganic iodine species in milk and also that there was no interconversion of one species into another during the batch separation.

Table 5.1. Comparison between two batch separation methods.

Ion exchange separation of inorganic iodine in milk	**Organically bound-iodine concentration ($\mu g.mL^{-1}$)**
Dowex (1X8) – Dowex (50X8)	0.041 ± 0.04
Dowex (50X8) – Dowex (1X8)	0.044 ± 0.03

The batch treatment using Dowex resin in OH^- form facilitates the extraction of both iodide and iodate ions simultaneously from the milk sample. In order to study the selective separation of iodide ion from the sample, the same experiment was performed using Dowex 1X8 in NO_3^- form. Given that the affinity of the NO_3^- form of the resin is higher for iodide than for iodate, it was expected to retain the iodide in the resin while the iodate remains in the solution. To test this hypothesis, a 0.25 $\mu g.mL^{-1}$ of iodate solution was prepared and approximately 50 mL of the solution were placed in contact with about 0.5 g of the Dowex 1X8 resin in NO_3^- form. The batch extraction was performed as described above. Although it was not expected, iodate ions were partially retained on the resin. Therefore, this method was not applied to milk samples. Instead, a column separation method was followed.

5.2.3 Column separation of inorganic iodine species

The separation method reported by Hou [20] was slightly modified and used in this work. In this method 10 mL of milk were passed through a column loaded with an anionic exchange resin in NO_3^- form; the iodide ions were retained while the

iodate ions were eluted and further reduced to iodide ions. A slight modification was made in our method in which hydrazine sulfate was used as the reducing reagent instead of potassium bisulfite solution. Hydrazine sulfate was used in 1999 by Rao and Chatt in our laboratory for reducing iodate into iodide ions in NIST SRM-1549 Non-fat Milk Powder with very good results [15, 16]. The purified Dowex 1X8 resin (in NO_3^- form) was packed in a polyethylene column of 4 cm length and 0.7 cm width.

Ten mL of a 0.5 $\mu g.mL^{-1}$ of an iodide, iodate, and iodide/iodate standard solution was passed by gravity through three different columns. The effluents and washings of each column were collected in portions of 10 mL. In each case, fraction 1 was the effluent from the initially loaded sample. The columns were then washed with 50 mL of DDW (fractions 2-6). An additional 40 mL of 0.1 M potassium nitrate solution was used to wash them (fractions 7-10), followed by a final wash with 20 mL DDW (fraction 11-12). Under these conditions no iodate should be present on the columns. The collected fractions were evaporated almost to dryness by placing them under an infra red lamp. A final sample volume of 0.75 mL was encapsulated and irradiated using a t_i-t_d-t_c scheme 5-2-10 min.

In the first column, where only the iodide solution was passed through, no iodine was found in any of the collected fractions indicating that all of the iodide ions were retained on the column. In the second column containing the iodate solution, all of the iodate (~99%) ions were found in the first 5 fractions indicating that further washings were not required. The elution profile in the case of the iodate standard is shown in Fig. 5.3. In the trial run containing the iodide/iodate solution, the elution profile was identical to that in Fig. 5.3 indicating that the iodate was eluted from the column while the iodide was retained. The procedure proposed by Hou [20] was suitable for iodide/iodate separation. It was modified for our purpose so that after washing with 50 mL of DDW no more washing was required to elute the iodate ions from the resin.

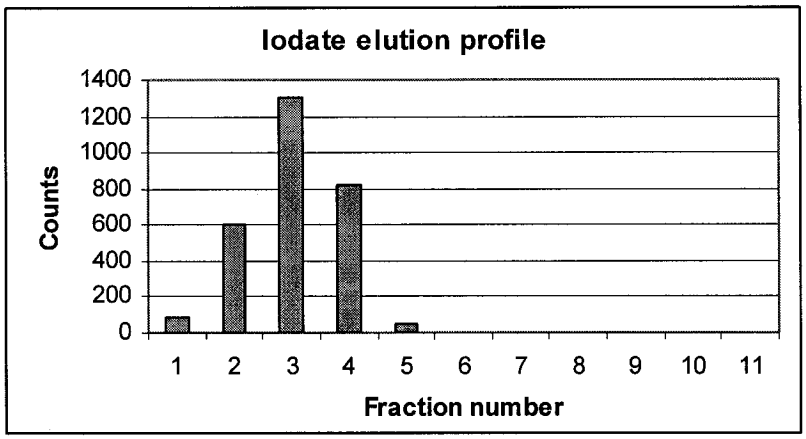

Fig. 5.3. Elution profile of iodate from Dowex 1X8 (NO_3^-) resin.

Once the iodate ion was eluted, all fractions from the column were combined and 5 g of hydrazine sulfate were added to reduce iodate to iodide ions. This solution was then passed through a fresh ion exchange column. The column was then washed with 50 mL of DDW as described above, and all collected fractions were irradiated to determine their iodine content. No iodine signal was found in any fraction indicating that all the iodate ions were reduced to iodide ions and they were quantitatively retained on the resin. In this fashion the separation of iodide/iodate was easily accomplished.

Although the separation of iodide/iodate ions can be performed in one step using a batch separation method as explained earlier, a column method was tested nevertheless. Dowex 1X8 resin in OH^- form was packed in a polyethylene column of same dimensions as described above, and 10 mL of the 0.5 $\mu g.mL^{-1}$ iodide/iodate standard solution were passed through it. Five mL of DDW were passed through the column and the effluent was tested for iodine. No iodine signal was found indicating that both iodine species were completely retained on the resin.

This method was then applied to real milk samples (Table 5.2). Initially 10 mL of a milk sample were passed through a column containing the Dowex 1X8 resin in OH^- form. Ten mL of a milk sample were passed through another column containing

the same resin in the NO_3^- form. The first column was washed with 5 mL of DDW and the second column was washed out with 50 mL of DDW. From the second column all effluents were combined and 5 g of hydrazine sulfate were added to it. The sample was then passed through a second column containing the Dowex 1X8 resin in NO_3^- form. All three resins from the three columns were carefully collected and irradiated in the epithermal site of the DUSR facility for 5 min, allowed to decay for 2 min, and counted for 10 min.

Table 5.2. Application of the iodide/iodate column separation method to milk samples.

Milk (n=3)	Concentration $\mu g.mL^{-1}$	Column method used
Total inorganic species	0.403 ± 0.003	Dowex 1X8 (OH^-)
Iodide	0.335 ± 0.003	Dowex 1X8 (NO_3^-)
Iodate	0.061 ± 0.006	Dowex 1X8 (NO_3^-) after iodate reduction
Recovery	98.2	

This method is reproducible, relatively simple and fast. The NAA method is highly suitable because the resin can be directly irradiated. This separation method could be applied to other analytical techniques but an elution or extraction step would be necessary which will, in turn, make the method cumbersome.

5.3 Summary

Batch separation using Dowex 1X8 column in OH^- form is convenient, fast and reproducible for the extraction of inorganic anionic iodine species from milk in one step. Although, it was expected that no extraction of iodate would occur when the batch method was applied using the same resin in the NO_3^- form, a partial retention in fact did occur. Therefore, the column method described by Hou [20] was set up. The column method was modified since washing with 50 mL of water was enough for the reduction of iodate to iodide by means of hydrazine sulfate instead of potassium bisulfite.

The separation of iodide and iodate ions was quantitative proving the suitability of the method. There were no detectable cationic inorganic iodine species in milk and there was no evidence of interconversion of inorganic species during the separation method. As expected, about 10% of the iodine species in milk are organically bound, while inorganic anionic species account for the remaining 90%. Iodide is the major species in the inorganic fraction and as such is the major iodine species in milk.

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CHAPTER 6

SEPARATION SCHEMES FOR IODINE SPECIES

Various separation methods used in Chapters 4 and 5 are combined in this chapter to give integrated speciation schemes for iodine in milk. The advantages and disadvantages of each scheme are discussed here. Although all of the schemes were applied to real milk samples, only the results obtained from a few selected schemes, which were considered more reliable, are presented below.

6.1 Experimental conditions for iodine determination by NAA

As stated before, NAA has been exclusively used for the determination of iodine in milk samples and in various fractions obtained after the separation of iodine-containing species of interest. The experimental conditions used for NAA varied depending upon the levels of iodine and the interfering elements present in a given fraction. A simplified version of the irradiation, decay and counting times, type of neutrons used for irradiation, and the type of gamma-ray spectrometry employed is presented in Table 6.1.

Most of the samples were analyzed by either reactor flux instrumental neutron activation analysis (INAA) or epithermal instrumental neutron activation analysis (EINAA). In some cases, the iodine levels were so low that a pseudo-cyclic INAA (PCINAA) method involving 3 cycles was needed. Either conventional Ge(Li) or Compton suppression gamma-ray spectrometry was used for detection purposes. These conditions were applied to all fractions regardless of the separation scheme. It will be evident below that not all fractions were separated in all the schemes.

Table 6.1. Experimental conditions used for iodine determination by NAA

Milk / Milk fraction	NAA technique	t_i-t_d-t_c (min)	Reactor site	Detection system
Total milk	INAA	5-2-10	Inner	Compton suppression
Organic and/or anionic	INAA	5-2-10	Inner	Compton suppression
Lipids and Proteins (solution)	PCINAA (n=3)	5-2-10	Inner	Compton suppression
Lipids and Proteins (solid)	INAA	10-2-10	Inner	Conventional
Resins (iodide and iodate)	EINAA	5-2-10	Cd-shielded	Conventional

6.2 Sequential separation of organic and inorganic iodine species

Two schemes were developed, one after the other, for the separation of fractions containing organically bound iodine from the inorganically bound species in homogenized milk samples. Both schemes are presented below.

The first approach (Scheme #1) was meant to develop a rapid scheme to precipitate the organic species of iodine while leaving the inorganic species in solution. About 7 g of solid ammonium sulfate was added to about 10 mL of milk, and the mixture was filtered using a Whatman No. 41 filter paper under vacuum suction. The organic iodine species containing proteins and lipids was precipitated while the inorganic species remained in the solution (filtrate). The solution was then passed through a solid-phase extraction (SPE) cartridge RP-18 for removing any remaining proteins and lipids that might not have been removed during the precipitation with ammonium sulfate.

All fractions obtained in this scheme were analyzed by NAA. In Fig. 6.1, $NAA_{1\text{-}1}$ represents the analysis of the homogenized milk sample, $NAA_{1\text{-}2}$ is that of the

precipitate containing the organic species, and NAA_{1-3} is that of the solution containing the inorganic species.

When ammonium sulfate was added in solid form, the volume of the sample was not markedly increased. However, the precipitate containing the organic iodine fraction did not appear to be homogeneous due to the presence of the undissolved salt.

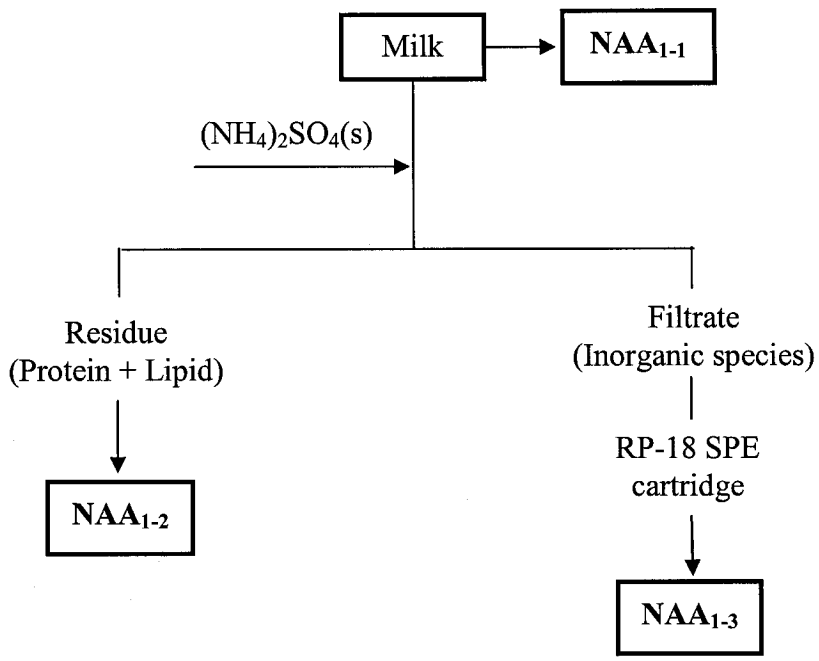

Fig. 6.1. Scheme #1 for the separation of organic and inorganic iodine species.

Although the addition of a saturated solution of ammonium sulfate solved the problem of solid salt in the precipitate, it increased the volume of the sample thereby decreasing the sensitivity for analysis of the NAA_{1-3} fraction. It is well known that most of the iodine in milk is present as inorganic species. It may not then be wise to first precipitate the organic fraction, which might initiate partial co-precipitation of the inorganic species with it and also lead to a lower recovery. For these reasons, the Scheme #1 was modified as described below.

The strategy followed in Scheme #2 was to separate the inorganic iodine species first using ion-exchange chromatography. In order to remove any possible cationic iodine species, about 0.5 g of pre-cleaned Dowex 50x8 (50-100 mesh) resin in NH_4^+ form was placed in about 50 mL of milk and batch extracted. After 10 min of stirring the mixture was filtered. The process was repeated 2 more times. Any possible cationic species of iodine would have been retained on the resin. The extractant was then placed in about 0.5 g of a pre-cleaned Dowex 1x8 (100-200 mesh) resin in OH^- form. Three batch separations were again carried out.

The Scheme #2 is presented in Fig. 6.2. In this diagram, $NAA_{2\text{-}1}$ represents the analysis of the homogenized milk sample, $NAA_{2\text{-}2}$ is that of the cationic species, $NAA_{2\text{-}3}$ is that of the extractant from the cationic resin, $NAA_{2\text{-}4}$ is that of the extractant from the anionic resin, and $NAA_{2\text{-}5}$ is that of the species retained on the anionic resin.

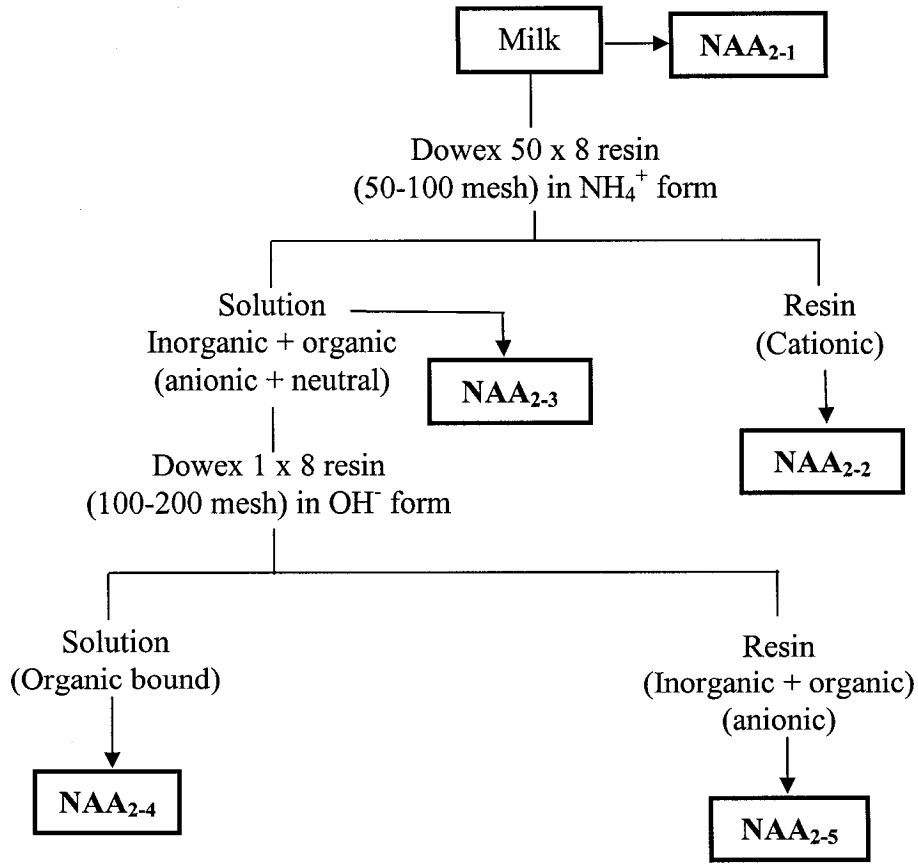

Fig. 6.2. Scheme #2 for the separation of organic and inorganic iodine species.

Unlike in Scheme #1, the separation of the cationic species was followed by the retention of the anionic iodine species on the resin in Scheme #2. However, the mass of the resin recovered from batch extractions was incomplete because of loss due to adherence of the resin particles to the walls of the container. The scheme was then slightly modified from batch to column extraction for the separation of the anionic inorganic species. A column of 4 cm long and 0.7 cm i.d. was used. A sample size of 10 mL was used in the column separation instead of the 50 mL in the batch mode.

One of the main advantages of this scheme (Fig. 6.2) was that anionic iodine species of both inorganic and organic origin were separated first using a simple and rapid method. Then the inorganic iodine species separated from the organic ones. If any positive iodine species is expected to be present in milk then they should be retained on the cationic resin ($NAA_{2\text{-}2}$). As noted before, all fractions were analyzed for iodine by NAA.

The above methods were applied to various fractions obtained using the Scheme #2. A spectrum of the anionic inorganic fraction separated using Dowex 1X8 in OH- form resin is shown in Fig. 6.3. The PCINAA approach was used for quantifying the organic iodine as shown in Fig. 6.4. The results are summarized in Table 6.2. It is evident that most of the iodine present in milk is in inorganic form, as expected.

Table 6.2. Levels of iodine species in milk by Scheme #2 (Fig. 6.2)

Milk fraction	**[I] $\mu g.mL^{-1}$**	**% of total iodine**
Total milk ($NAA_{2\text{-}1}$)	0.457 ± 0.008	-
Cationic Iodine ($NAA_{2\text{-}2}$)	N.D.	-
Anionic inorganic ($NAA_{2\text{-}5}$)	0.405 ± 0.003	88.6
Organic Iodine ($NAA_{2\text{-}4}$)	0.049 ± 0.004	10.7
Inorganic + Organic ($NAA_{2\text{-}3}$)	0.452 ± 0.004	99.3

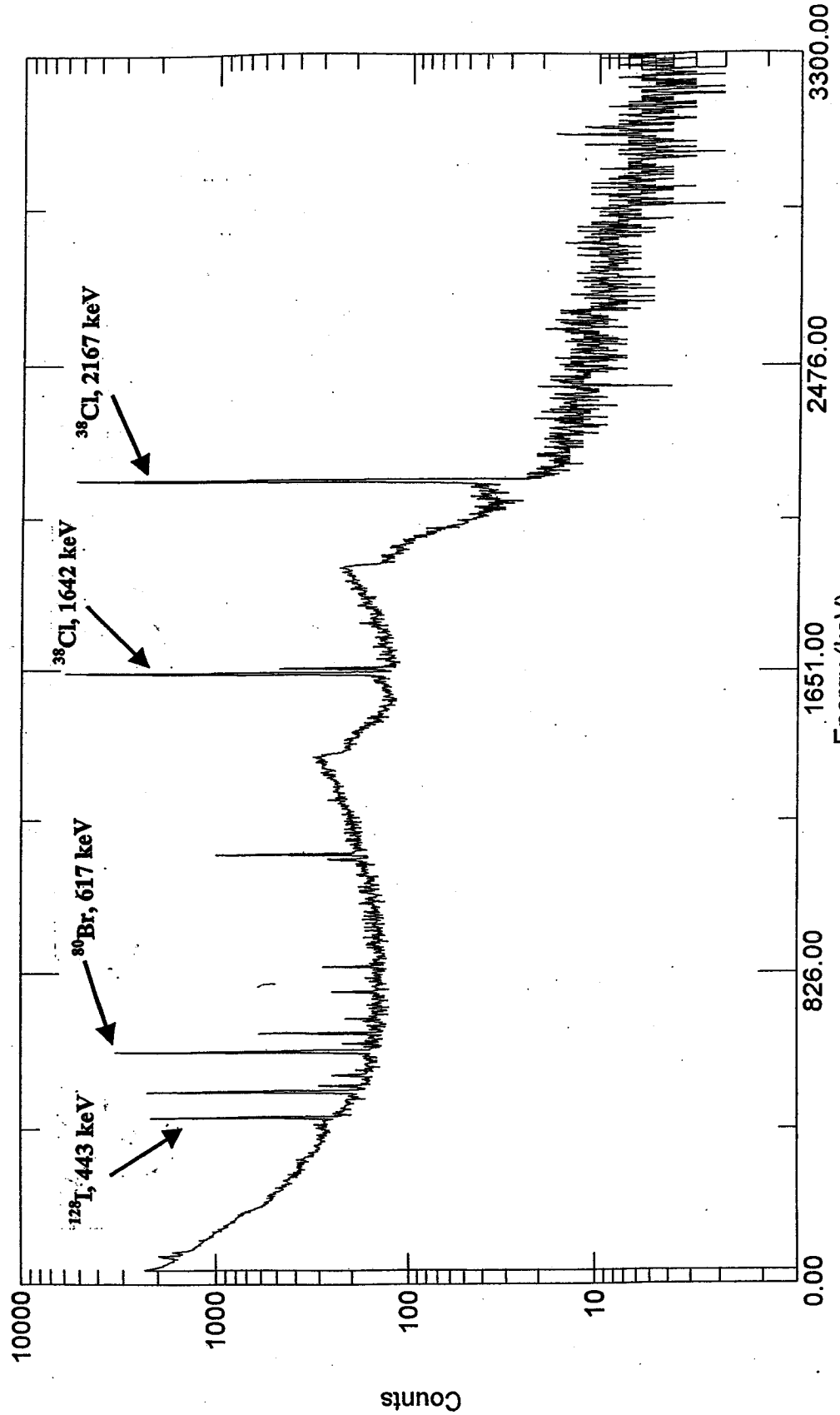

Fig. 6.3. Spectrum of anionic inorganic fraction, ENAA-GeLi t_i-t_d-t_c: 5-2-10 min.

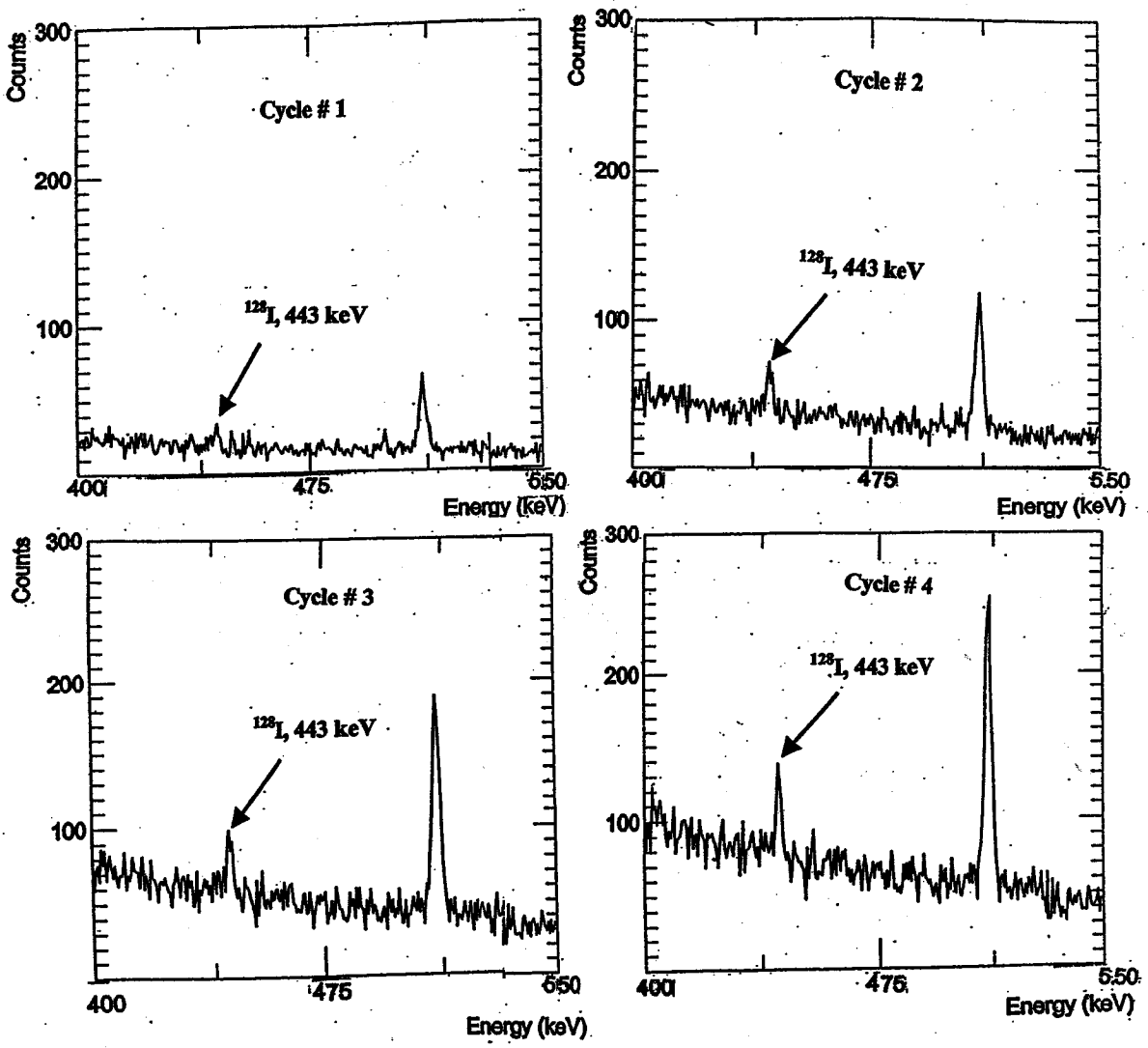

Fig. 6.4. Iodine peak in the organic fraction after 4 cycles using PCINAA-CS, t_i-t_d-t_c: 5-2-10 min.

6.3 Sequential separation of lipid- and protein-bound iodine, iodide and iodate species

It is evident from above that the iodine species present in milk were very well separated into total organic and inorganic fractions. The next task was to separate various iodine species to either smaller groups or individual components. A total of 5 schemes was developed one after the other. These schemes are discussed below in chronological order.

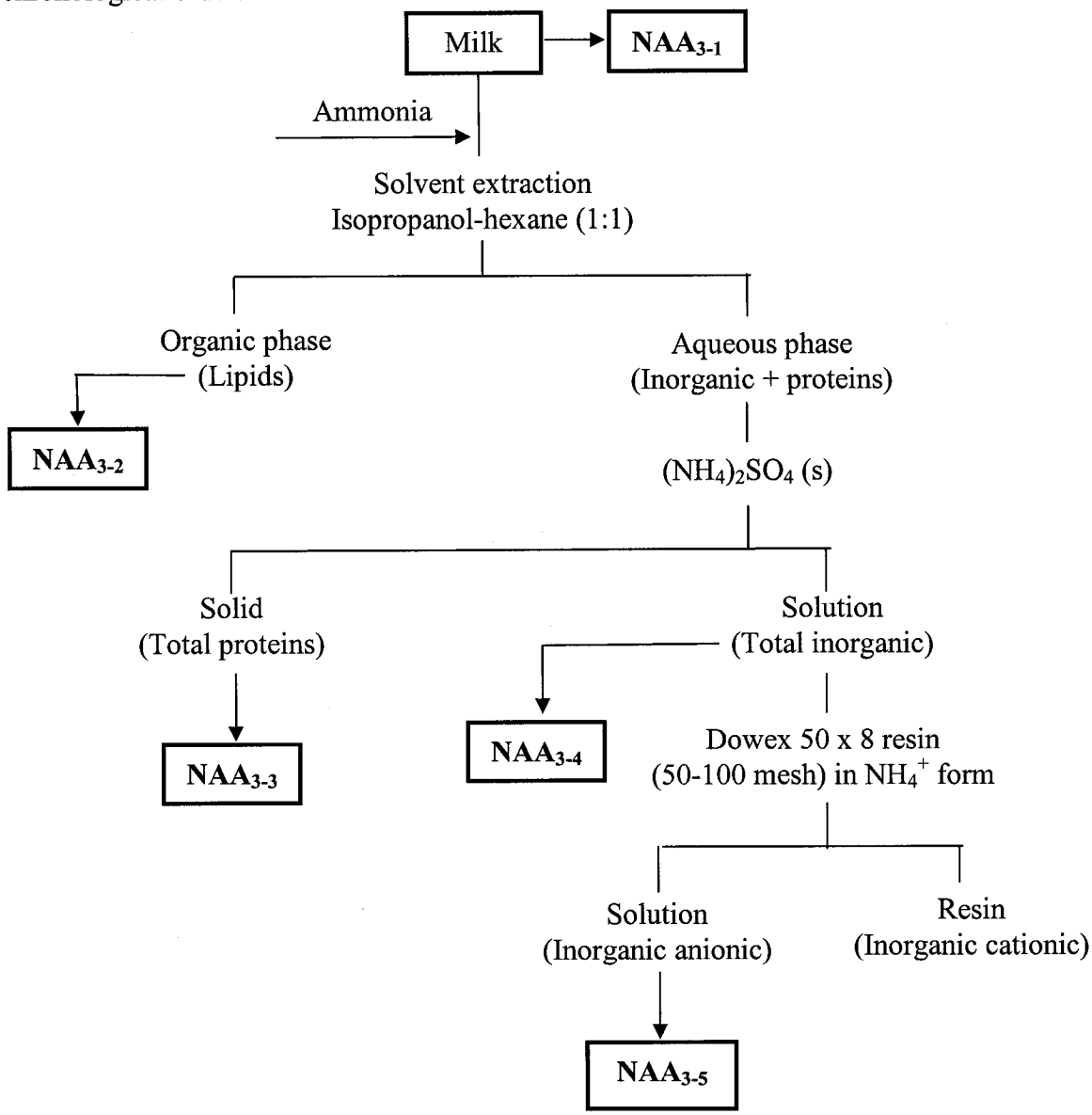

Fig. 6.5. Scheme #3 for the separation of organic and inorganic iodine species.

The first step in scheme #3 shown in Fig. 6.5 involved the addition of ammonia to milk followed by the separation of extractable iodine species in an organic solvent mixture, namely isopropanol-hexane (1:1). The organic phase contained mostly lipids (designated as the NAA_{3-2} fraction). The aqueous phase containing mostly proteins and inorganic iodine species was treated with solid ammonium sulfate to precipitate the proteins (NAA_{3-3} fraction). The supernatant contained the inorganic iodine species. It was then passed through a SPE cartridge RP-18 to remove any remaining lipids and/or proteins (NAA_{3-4} fraction). The purified supernatant was batch extracted using a cationic resin. The solution phase contained mostly the anionic inorganic iodine species (NAA_{3-5} fraction).

The various fractions obtained above (NAA_{3-1} to NAA_{3-5}) using Scheme #3 were analyzed by NAA. The results are given below. The organic iodine species (Table 6.2) was further separated into lipid- and protein-bound iodine (Table 6.3) using the Scheme #3. A comparison of the data in these two tables shows that the sum of these lipid- and protein-bound iodine species is comparable to the total organic iodine.

Table 6.3: Total iodine, lipid-bound, protein-bound and anionic iodine species

	[I] $\mu g.mL^{-1}$	**% of total iodine**
Total milk (NAA_{3-1})	0.48 ± 0.02	-
Lipid-bound iodine (NAA_{3-2})	0.020 ± 0.003	4.2
Protein-bound iodine (NAA_{3-3})	0.039 ± 0.002	8.1
Anionic inorganic (NAA_{3-5})	0.45 ± 0.04	93.7
Protein + Lipids + Inorganic	0.509	106

Scheme #3 is fairly rapid. It separates and concentrates lipid and protein fractions of milk. However, the method has a couple of limitations. Firstly, since the scheme starts with solvent extraction, it increases the sample volume. Secondly, the solvent system used here can partially precipitate proteins. For this reason, ammonia had to be added to the milk sample to keep the proteins in solution before the lipid extraction step. The next modification of the scheme involved the separation of iodide and iodate

ions from the inorganic anion fraction (NAA_{3-5}) shown in Fig. 6.5. It was accomplished by ion-exchange chromatography using the Dowex 1x8 resin in nitrate form (NO_3^-) as shown in Scheme #4 (Fig. 6.6) and explained in Chapter 5.

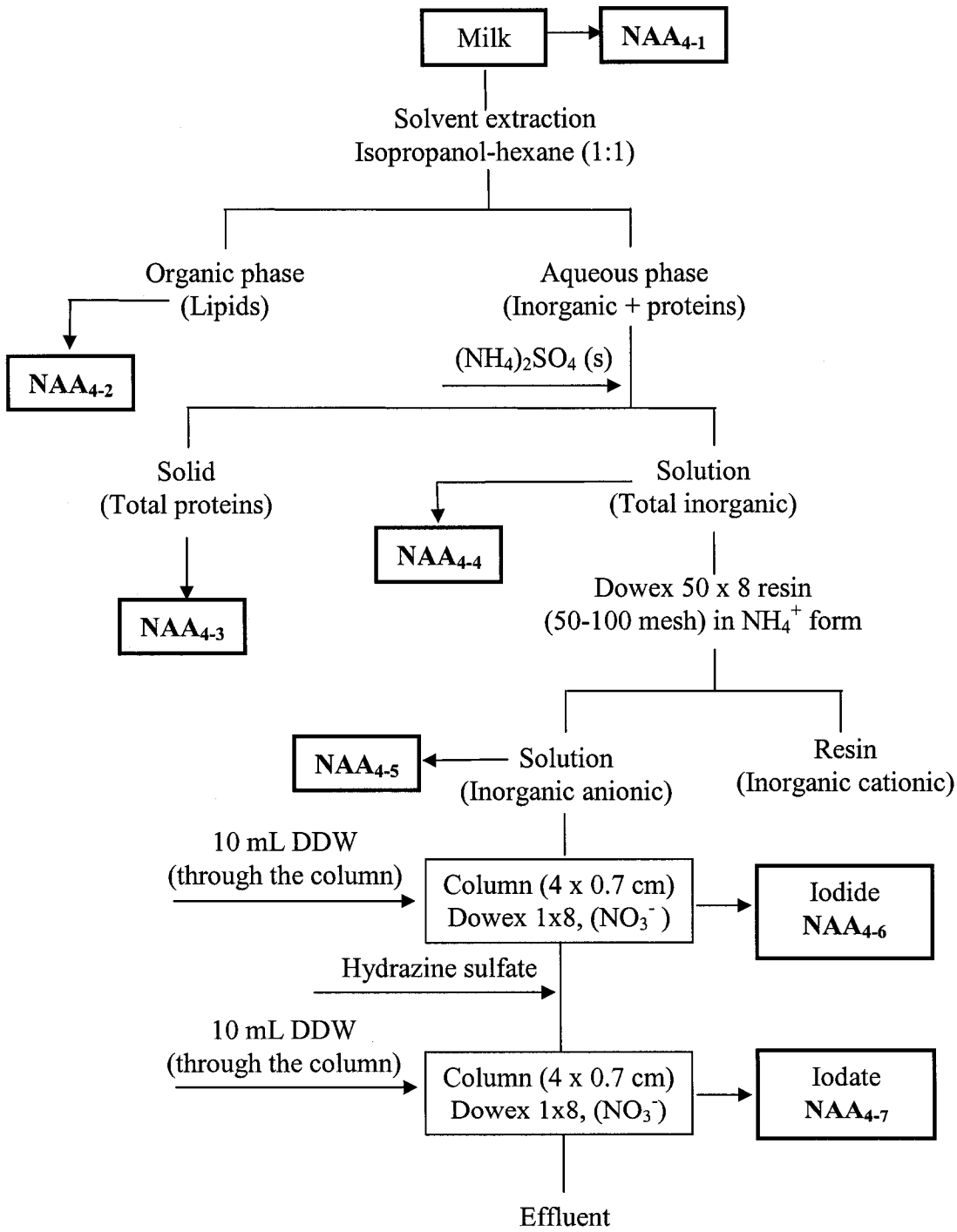

Fig. 6.6. Scheme #4 for the separation of organic and inorganic iodine species.

The levels of iodide (NAA_{4-6}) and iodate (NAA_{4-7}) ions in Scheme #4 could be detected but not reliably determined, as shown in Table 6.4. The reproducibility of the iodide and iodate values in three different experiments was very poor at the best. There could be two possible explanations for this observation. One of them could be the partial elution of the ions from the column and the other could be due to the bleeding of the column. It was therefore decided to modify Scheme #4.

Table 6.4. Reproducibility of iodide and iodate determinations by Scheme # 4.

Iodide (μg)	**Iodate (μg)**
5.68	0.21
6.07	0.12
4.23	0.04

It was decided to take the best features of Schemes #1, #3 and #4 to develop a new scheme (Scheme #5, Fig. 6.7) in which iodide and iodate ions could be reliably determined. In Scheme #1, the inhomogeneity of the residue after precipitation with ammonium sulfate was an issue. For this reason, the initial precipitate in Scheme #5 was re-dissolved in ammonia solution prior to proceeding further. It was then followed by the lipid and protein separation procedure developed in Scheme #3. The supernatant after the ammonium sulfate precipitation in Scheme #5 is the same as that described in Scheme #4.

In Scheme #5, NAA_{5-1} represents the total iodine in milk. The NAA_{5-2} is the lipid-bound iodine fraction. The NAA_{5-3} represents protein-bound iodine. The NAA_{5-4} is the supernatant after the separation of lipids and protein. Anionic inorganic iodine species are represented by NAA_{5-5} while NAA_{5-6} is iodide and NAA_{5-7} iodate ions. The Scheme #5 indeed does not increase the volume of the aqueous phase that contains the inorganic species making their reliable determinations possible. The concentration of sulfate ions (SO_4^{2-}) in the solution from ammonium sulfate is quite high, although it did not appear to affect the separation of iodide and iodate ions. Table 6.5 shows the results obtained when Scheme #5 was applied.

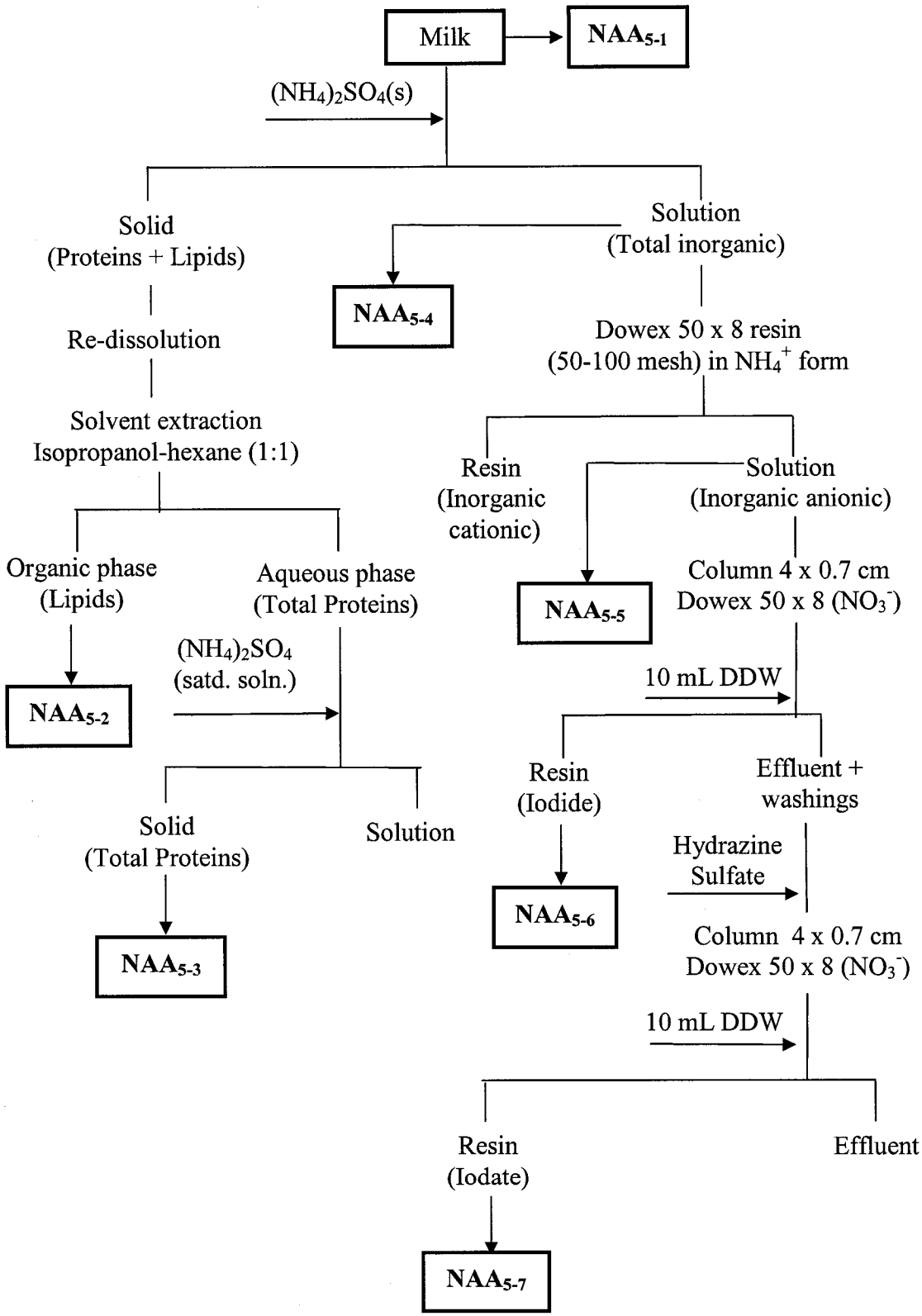

Fig. 6.7. Scheme #5 for the separation of organic and inorganic iodine species.

Table 6.5. Lipid yield and lipid-bound iodine in Schemes #4 and #5.

	Scheme #4	**Scheme #5**
Lipid yield (% w/w)	3.19 ± 0.04	2.57 ± 0.07
Lipid-bound iodine ($\mu g.g^{-1}$)	0.28 ± 0.03	0.62 ± 0.06

Table 6.5 shows that the lipid-bound iodine obtained by Scheme #5 was quite high and a mass balance could not be achieved using this value. Also the lipid yield was quite low. In order to avoid this problem, Scheme #2 was further modified and presented below as Scheme #6 (Fig. 6.8). In this case the strategy was to start with the separation of the inorganic iodine species followed by that of lipids and finally proteins. Here, $NAA_{6\text{-}1}$ represents the total iodine in milk; $NAA_{6\text{-}2}$ is the resin containing anionic inorganic iodine species; $NAA_{6\text{-}3}$ represents the lipid-bound iodine fraction; and $NAA_{6\text{-}4}$ is protein-bound iodine.

Homogenized milk was used in all experiments described previously. The lipid ($NAA_{6\text{-}3}$) and protein ($NAA_{6\text{-}4}$) fractions in various types of milk were determined using Scheme #6. The results are presented in Table 6.6. It is evident that the lipid content varied directly with the fat content while protein content remained constant. The iodine levels in the separated fractions are shown in Table 6.7.

Table 6.6. Lipid and protein content of various types of milk by Scheme #6.

Milk	**Lipid Content (%)**	**Protein Content ($mg.g^{-1}$)**
Homogenized (3.25% MF)	3.16 ± 0.07	32 ± 2
Partially skimmed (2% MF)	1.81 ± 0.06	34 ± 3
Partially skimmed (1% MF)	0.8 ± 0.1	35 ± 3
Partially skimmed (1% MF + Ca)	0.79 ± 0.08	35 ± 3
Skimmed (<0.05% MF)	0.06 ± 0.01	35 ± 2

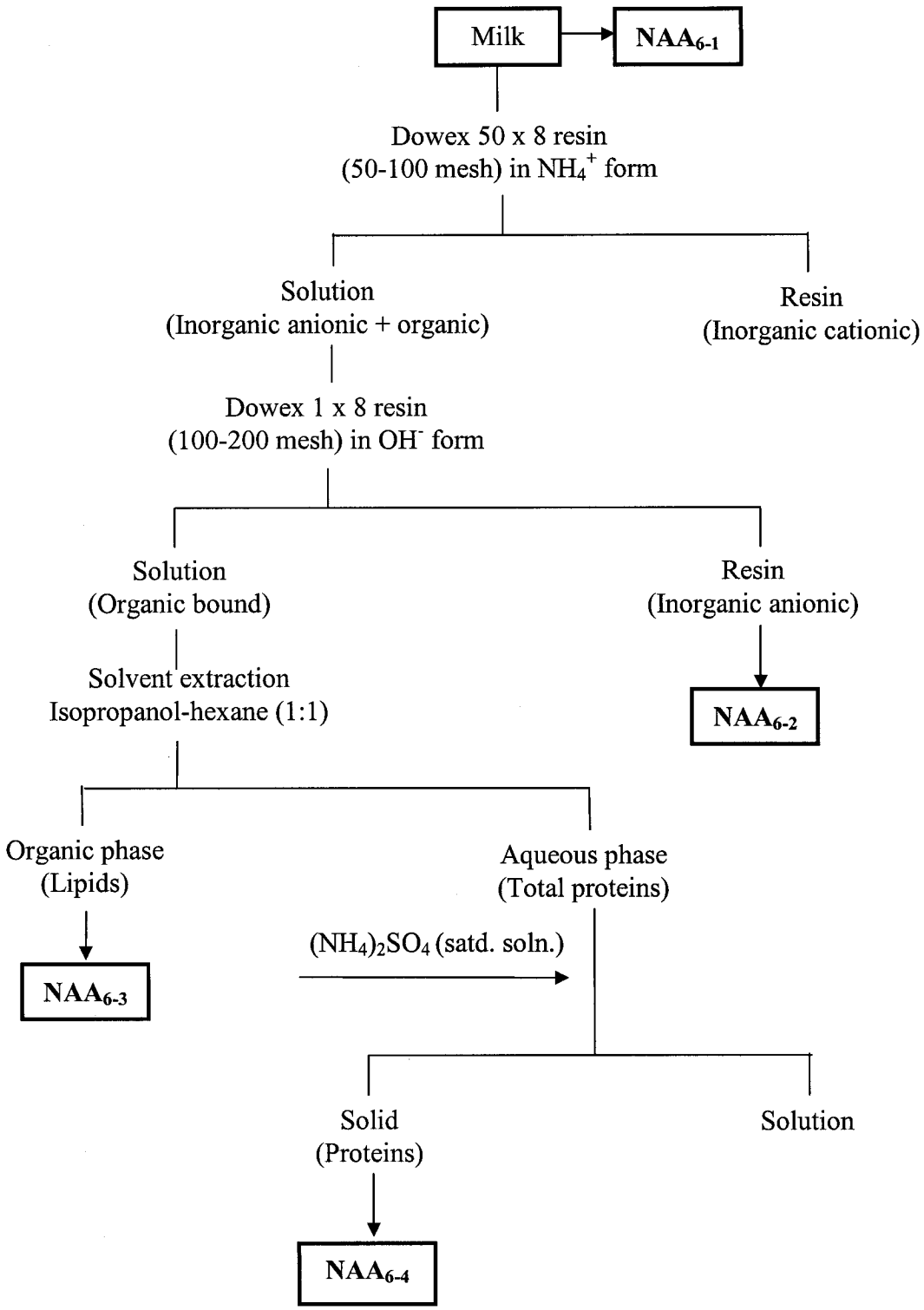

Fig. 6.8. Scheme #6 for the separation of organic and inorganic iodine species.

Table 6.8. Iodine levels in different types of milk and in their fractions by Scheme #6.

Milk (MF) / Fraction	**[I] μg.mL^{-1} of milk**	**% from total**
Total milk (3.25% MF)	0.40 ± 0.01	
Lipids	0.0073 ± 0.0004	1.8
Proteins	0.020 ± 0.005	5.0
Anionic inorganic	0.359 ± 0.008	90
Recovery	0.386	96
Total milk (2% MF)	0.40 ± 0.01	
Lipids	0.0044 ± 0.0006	1.1
Proteins	0.019 ± 0.003	4.8
Anionic inorganic	0.364 ± 0.005	91
Recovery	0.387	97
Total milk (1% MF)	0.42 ± 0.01	
Lipids	0.0021 ± 0.0007	0.5
Proteins	0.021 ± 0.004	5
Anionic inorganic	0.386 ± 0.006	92
Recovery	0.409	97
Total milk (1%+Ca)	0.96 ± 0.01	
Lipids	0.0046 ± 0.0005	0.48
Proteins	0.018 ± 0.003	1.9
Anionic inorganic	0.913 ± 0.008	95
Recovery	0.936	97
Total milk (<0.05%)	0.42 ± 0.01	
Lipids	0.0002	0.05
Proteins	0.020 ± 0.005	4.8
Anionic inorganic	0.391 ± 0.006	93
Recovery	0.411	98

It is evident that the Scheme #6 is appropriate for the determination of total inorganic iodine, lipid-bound and protein-bound iodine. This scheme was then slightly modified to include the separation of iodide and iodate ions as shown in Fig. 6.9.

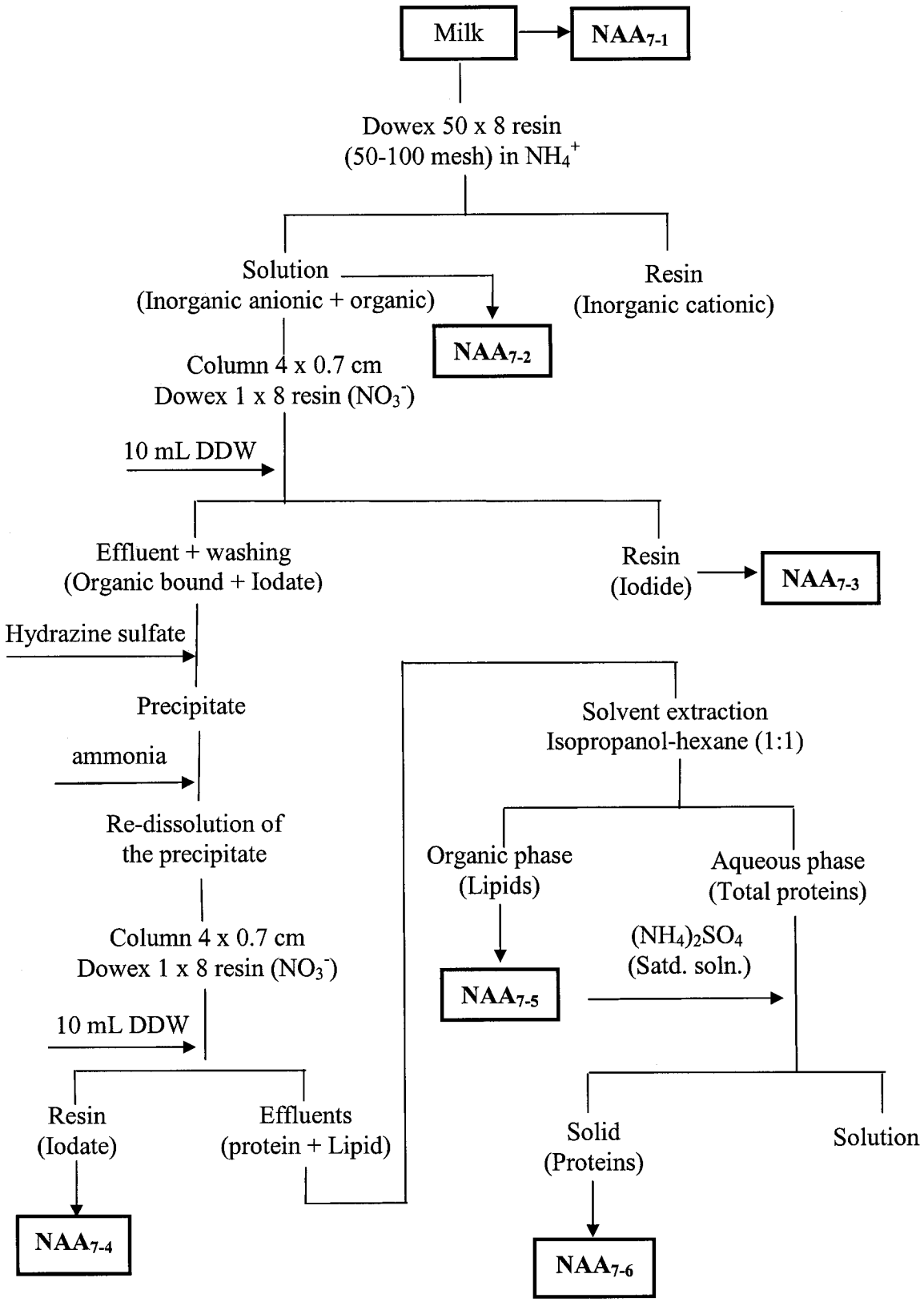

Fig. 6.9. Scheme #7 for the separation of organic and inorganic iodine species.

In Scheme #7, after passing the solution through a second column loaded with Dowex 1X8 in NO_3^- form, the organic-bound iodine compounds and iodate (NAA_{7-2}) were in the effluent fraction while the iodide ion was retained in the resin (NAA_{7-3}). Anion exchange chromatography was used again to separate the iodate ions (NAA_{7-4}) from the lipids (NAA_{7-5}) and proteins (NAA_{7-6}), which were further, separated using solvent extraction and coprecipitation as shown in Scheme #7. Spectra of total milk and the separated fractions are shown in Fig. 6.10 -6.14. Table 6.8 shows the results obtained by applying this scheme to homogenized milk samples. Both relative and k_0 methods of quantification were used for calculating the iodine concentrations.

Table 6.8. Iodine concentration in different milk fractions by Scheme #7.

Fraction	[I] μg.mL^{-1} of milk by relative method	% of total iodine	[I] μg.mL^{-1} of milk by k_0 method	% of total iodine
Total milk	0.457 ± 0.008	-	0.452 ± 0.009	-
Lipid-bound	0.013 ± 0.003	2.8	0.015 ± 0.004	3.3
Protein-bound	0.034 ± 0.003	7.4	0.034 ± 0.003	7.5
Iodide	0.333 ± 0.009	72.9	0.341 ± 0.003	75.4
Iodate	0.064 ± 0.003	14.0	0.065 ± 0.002	14.4
Mass balance	0.444	97.1	0.455	101

Table 6.8 shows no difference in iodine values between the relative and k_0 methods. The values in the table are reported in the form of average ± standard deviation of at least three determinations. Therefore, these values only represent the reproducibility of the method and not the overall uncertainty, which is described in Chapter 7. In order to validate the iodate separation method, a milk sample was spiked with a known amount of iodate ions and the separation Scheme #7 was applied. The recovery was 101±3% for three replicates.

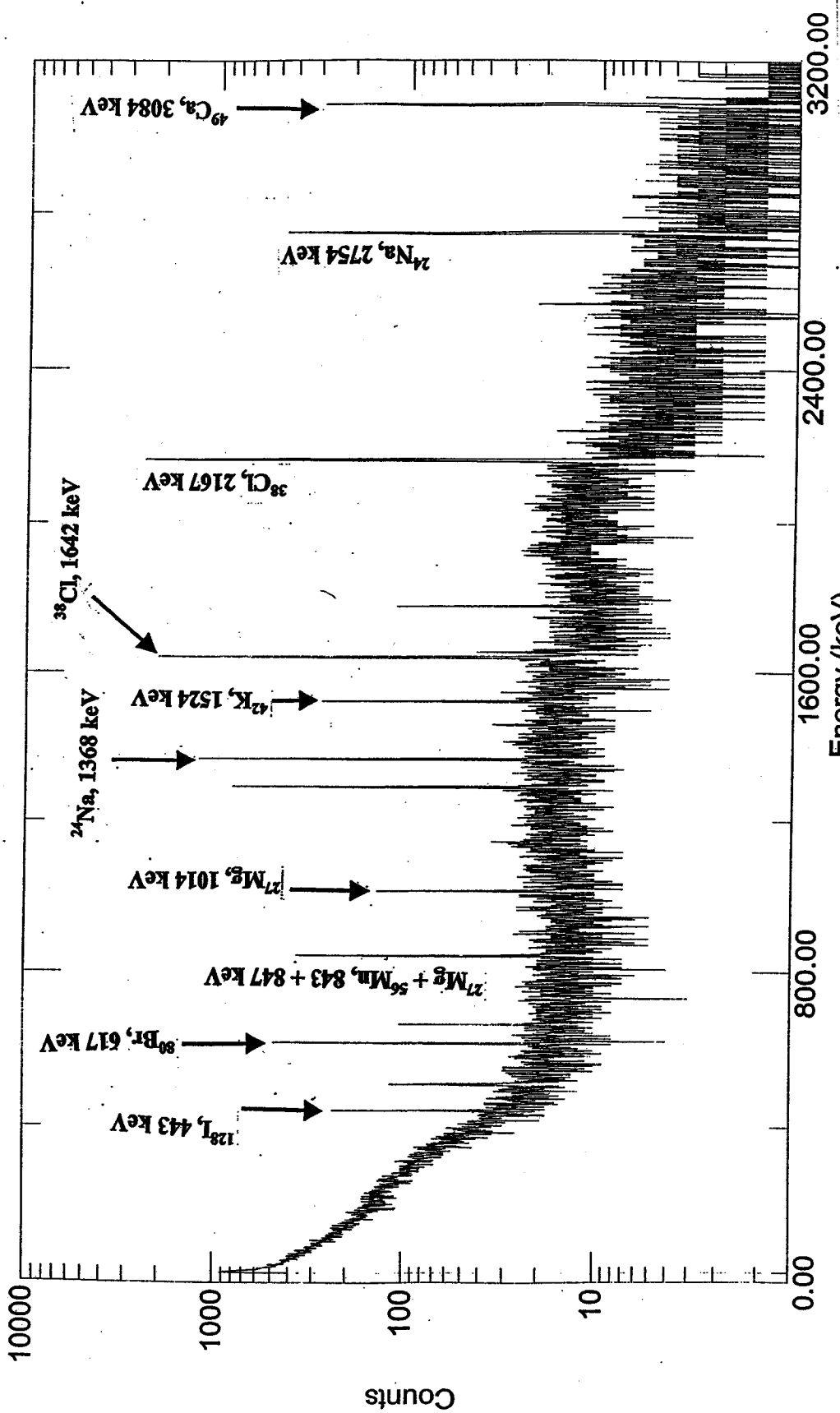

Fig. 6.10. Spectrum of total milk, INAA-CSS t_i-t_d-t_c : 5-2-10 min.

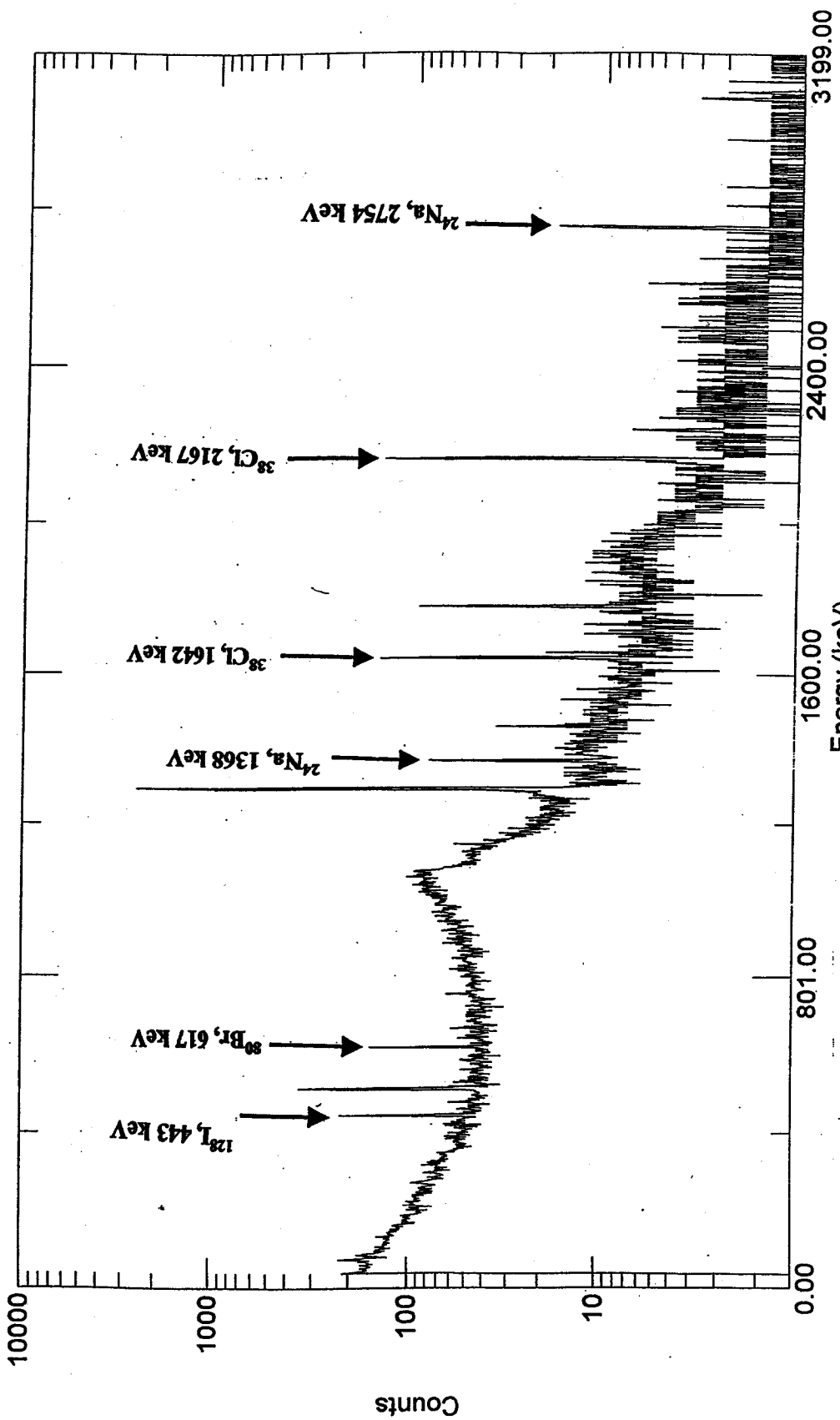

Fig. 6.11. Spectrum of lipid fraction, INAA-GeLi t_i-t_d-t_c : 10-2-10 min.

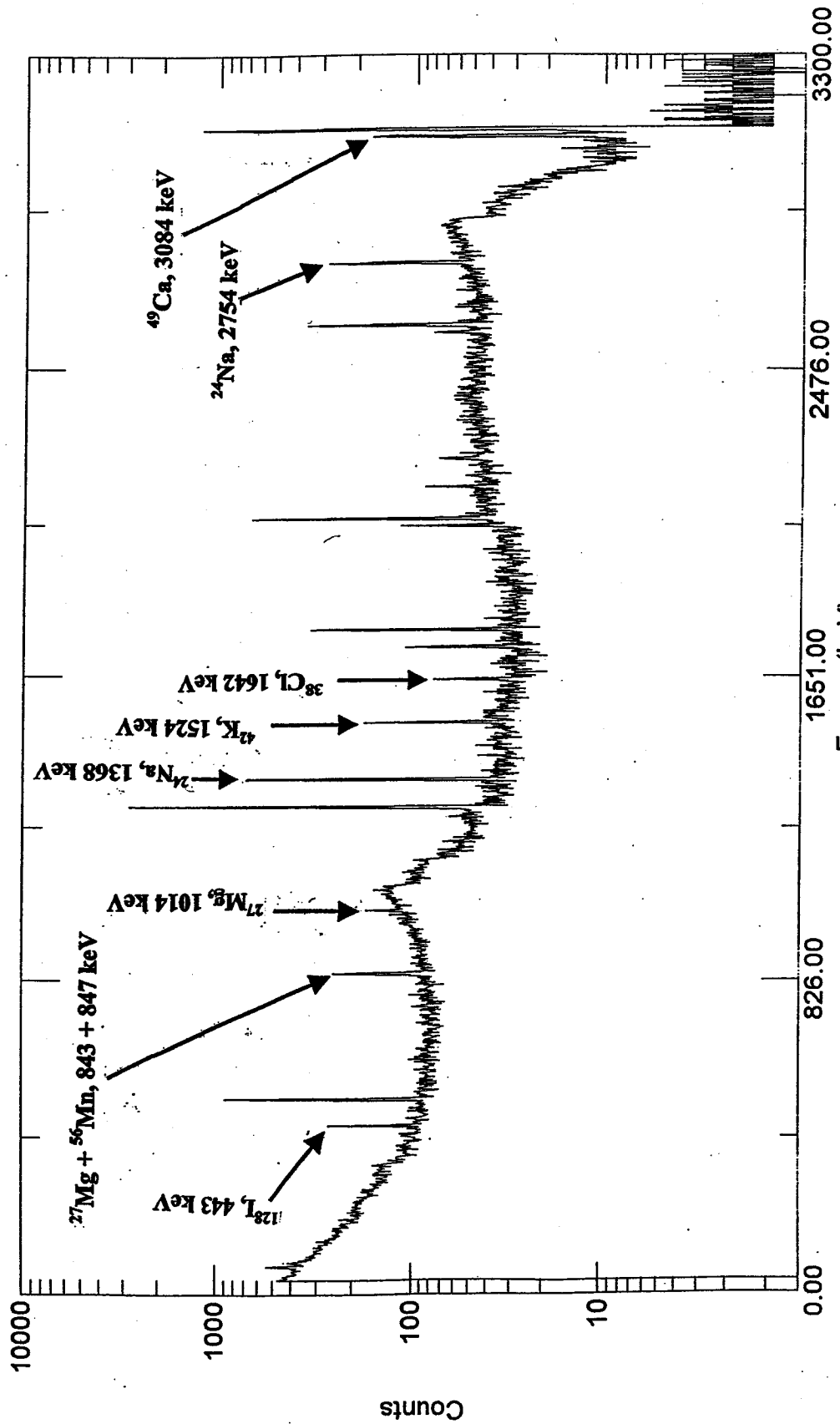

Fig. 6.12. Spectrum of protein fraction, INAA-GeLi t_i-t_d-t_c : 10-2-10 min.

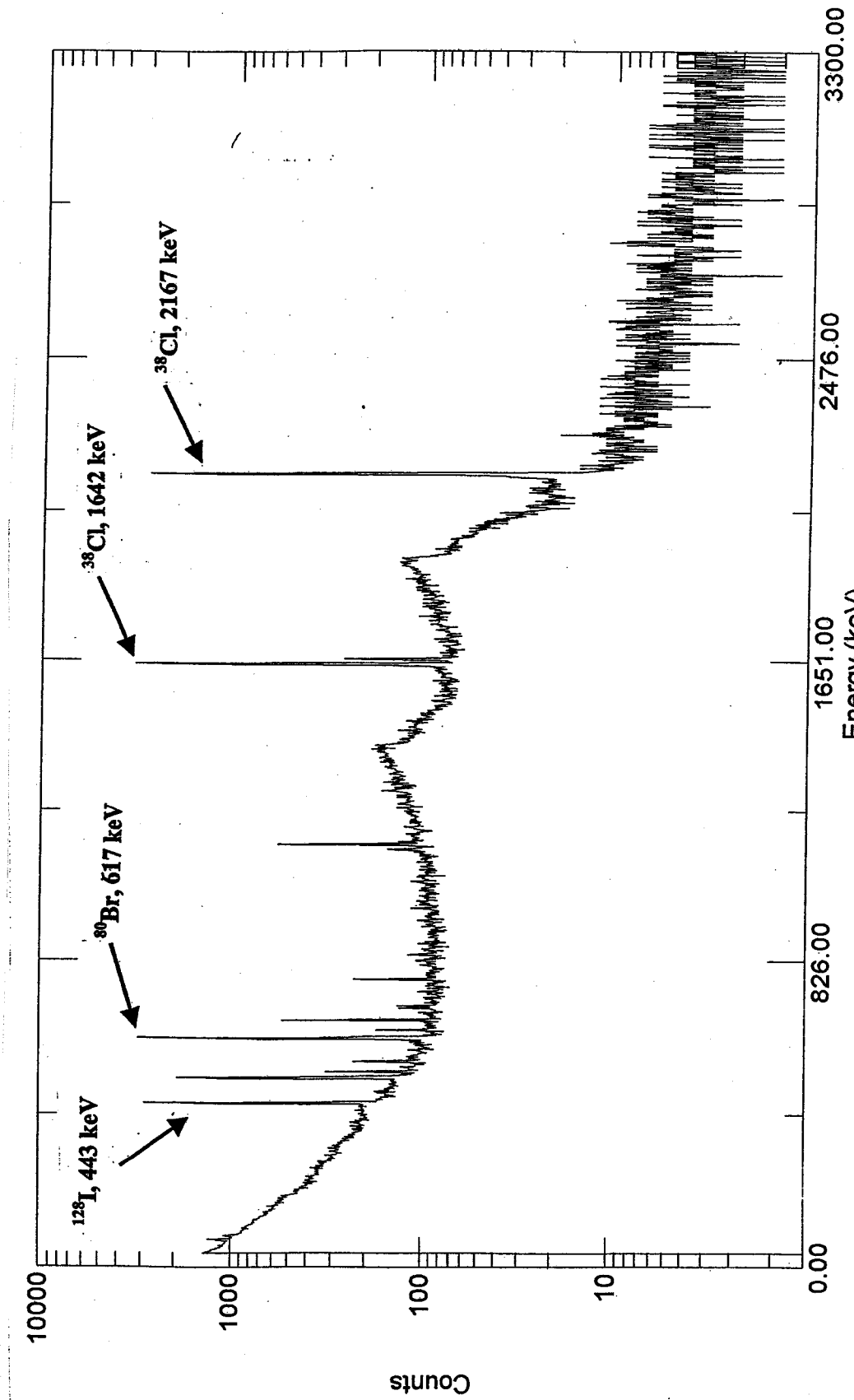

Fig. 6.13. Spectrum of iodide fraction, ENAA-GeLi t_i-t_d-t_c : 5-2-10 min.

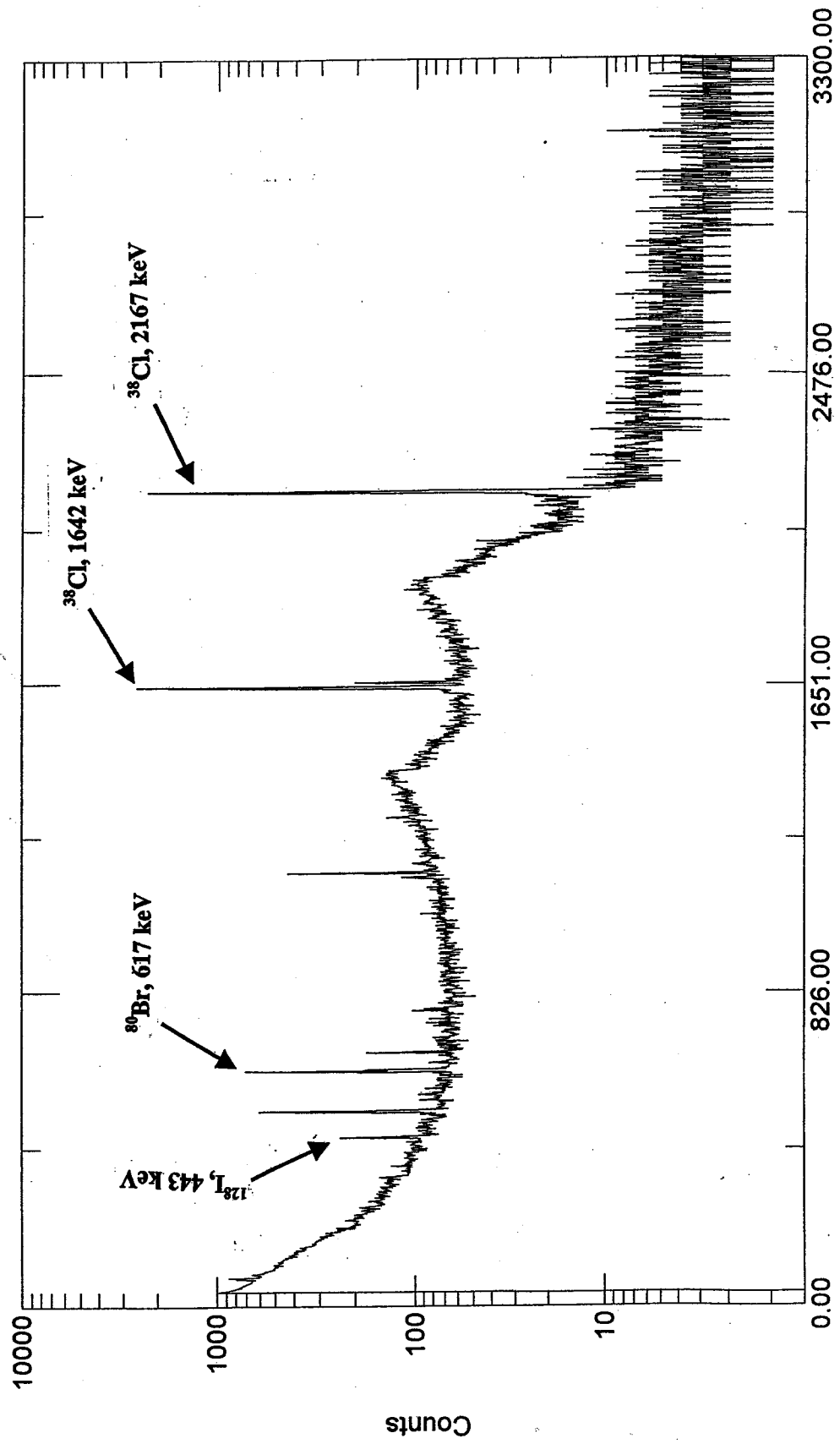

Fig. 6.14. Spectrum of iodate fraction, ENAA-GeLi t_i-t_d-t_c : 5-2-10 min.

6.4 Simultaneous Separation of lipid- and protein-bound iodine, iodide and iodate species

All schemes described above for the speciation of iodine in milk involve sequential separations. This means that all components were separated from the same milk sample. It is often argued in speciation analysis that there exits a possibility of transformation of species during the separation procedure.

In an attempt to examine the possibility of one iodine species in milk being converted to another, for example iodide to iodate, during the sequential separation Scheme #7, a simultaneous separation scheme, shown as Scheme #8 (Fig. 6.15), was designed. This scheme contained the same separation methods described in Scheme #7 except that the methods were applied simultaneously to 4 different aliquots of the same milk sample.

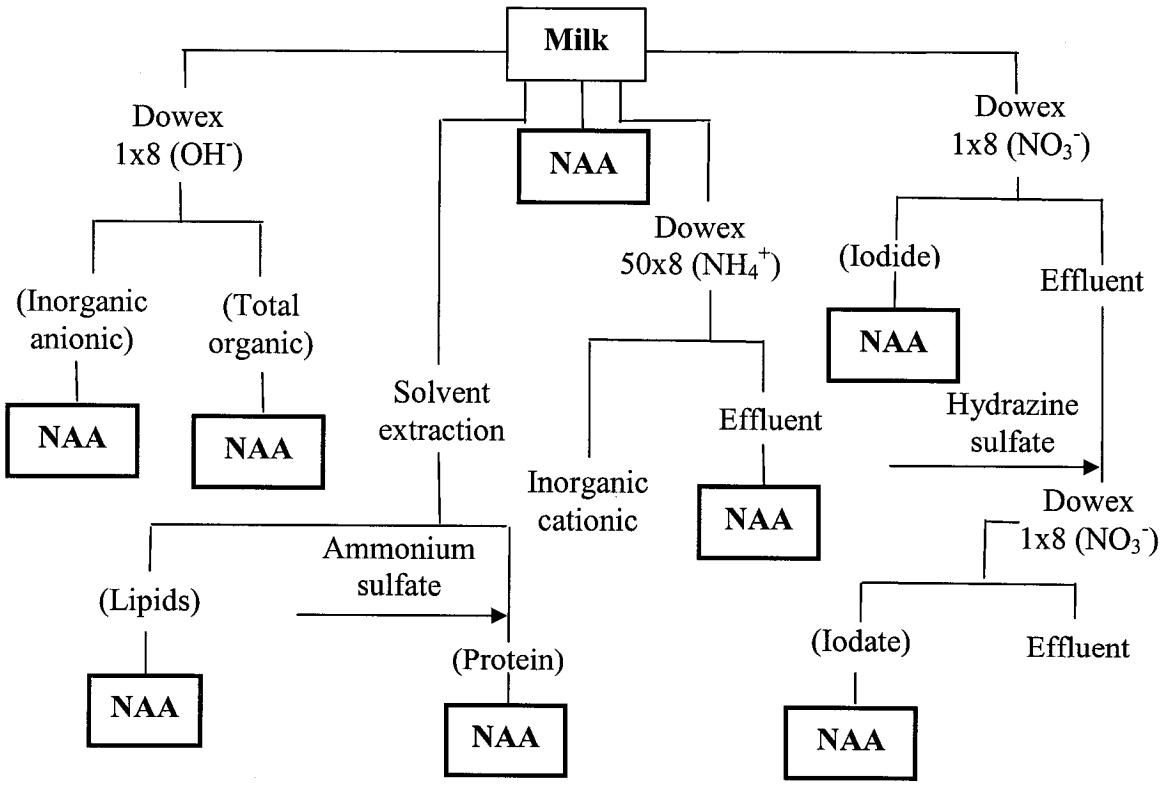

Fig. 6.15. Scheme #8 for the simultaneous separation of organic and inorganic iodine species.

The results obtained using Scheme #8 by the relative method are given in Table 6.9. There are no significant differences between the results obtained by the sequential (Table 6.8) and simultaneous methods. This proves the reliability of the sequential separation scheme developed in this thesis.

Table 6.9. Iodine concentrations in different milk fractions by Scheme #8.

Fraction	[I] $\mu g.mL^{-1}$ of milk by relative method	% of total iodine
Total milk	0.457 ± 0.008	-
Organic (protein + lipids)	0.049 ± 0.003	11
Protein-bound	0.034 ± 0.003	7.4
Lipid-bound	0.016 ± 0.004	3.5
Organic mass balance		7.4 +3.5 = 10.9
Inorganic anionic	0.405 ± 0.003	89
Iodide	0.337 ± 0.008	74
Iodate	0.066 ± 0.005	14
Inorganic mass balance		74 +14 = 88
Total mass balance		88 + 10.9 = 98.9

6.5 Fractionation of protein into casein and whey proteins

Regardless of the separation scheme used, once the total protein was separated from the milk sample it was further fractionated to casein and whey proteins by the method developed in this study (Scheme #9 given in Fig. 6.16). The details of each step of this scheme are given in Chapter 4.

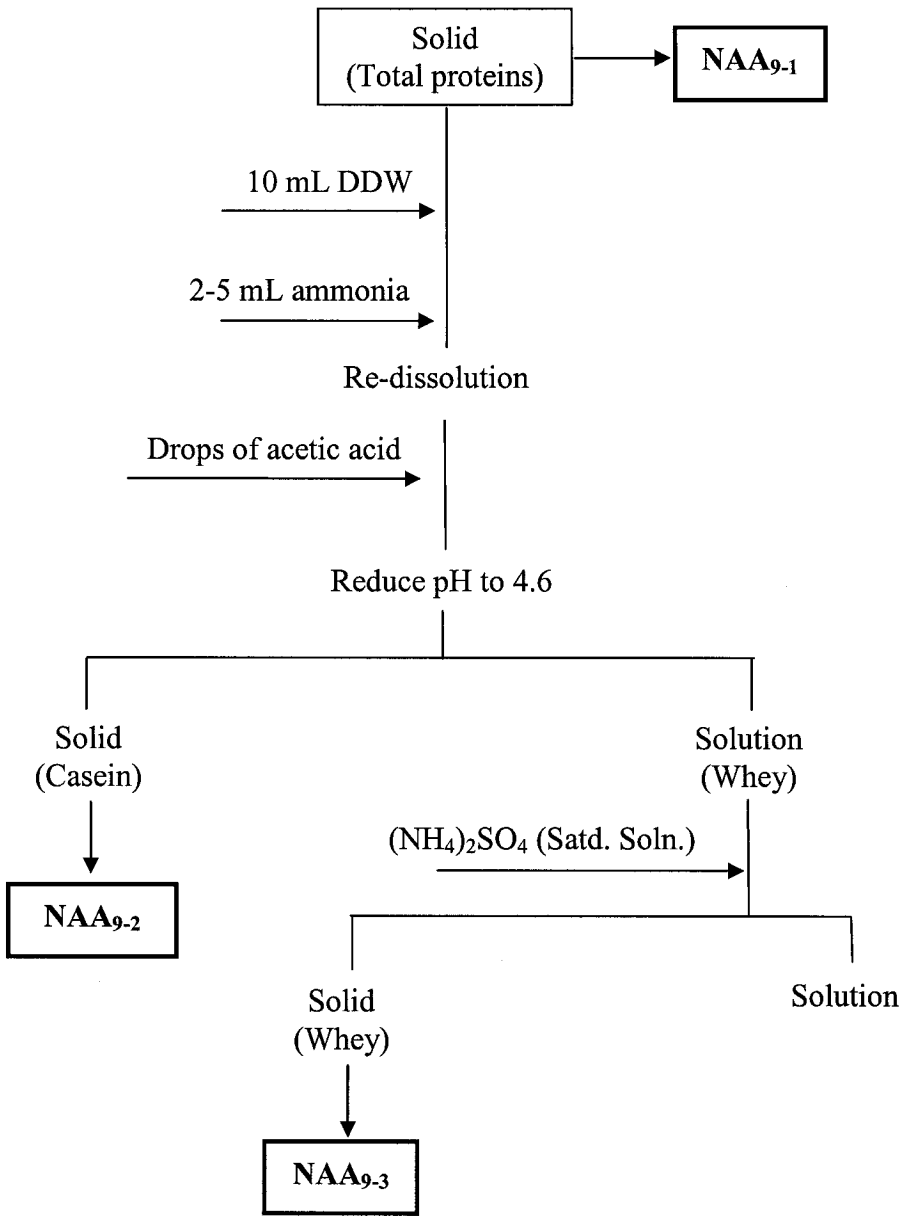

Fig. 6.16. Separation of proteins into casein and whey proteins (Scheme #9)

Sequential Scheme #7 was applied to 3 aliquots of a homogenous milk (3.25% MF) sample. Three protein fractions (NAA_{7-6}) were combined to a single sample. These steps were necessary due to the low levels of whey proteins in milk. Casein (NAA_{9-2}) and whey (NAA_{9-3}) protein fractions were then precipitated from the combined sample using Scheme #9. The details of this scheme have already been described in Chapter 4. All fractions were irradiated in the inner reactor position for 10 min and

counted for 10 min after a 2-min of decay as shown in Table 6.1. Spectra of the separated fractions are shown in Fig. 6.17 - 6.18. The results of the protein fractionation Scheme #9 are shown in Table 6.10. It is evident that about 76% of protein-bound iodine in milk is in the casein fraction and about 32% is with the whey proteins.

Table 6.10. Total protein-, casein- and whey protein-bound iodine.

Fraction	Iodine concentration ($\mu g.mL^{-1}$)
Total protein (NAA_{9-1})	0.034 ± 0.003
Casein (NAA_{9-2})	0.026 ± 0.001
Whey proteins (NAA_{9-3})	0.011 ± 0.006

6.6 Lipid fractionation and determination of lipid-bound iodine

The presence of organohalogen compounds as persistent organic pollutants (POPs) in food has become of much concern lately. Any anthropogenic organohalogen compound present in milk would have been extracted in the organic phase (NAA_{7-5}), that is the lipid fraction, of Scheme #7.

Since iodine levels in the lipids were very low, 5 organic phases (NAA_{7-5}) were combined, and allowed to dry by evaporating in air for about 16 h. The dried lipids were dissolved in hexane to give a solution of about 1 g of lipid per mL. This lipid solution was then separated to apolar (neutral), moderate (mostly glycolipids and pigments) and polar (mostly phospholipids) fractions using a solid phase extraction procedure described in Chapter 4.

These fractions were analyzed for iodine, bromine and chlorine by NAA. The fractions were irradiated in the inner position of the reactor for 10 min, allowed to decay for 2 min, and counted for 10 min using a conventional gamma-ray spectrometry system. Spectra of the separated fractions are shown in Fig. 6.19 - 6.21. The results are presented in Table 6.11.

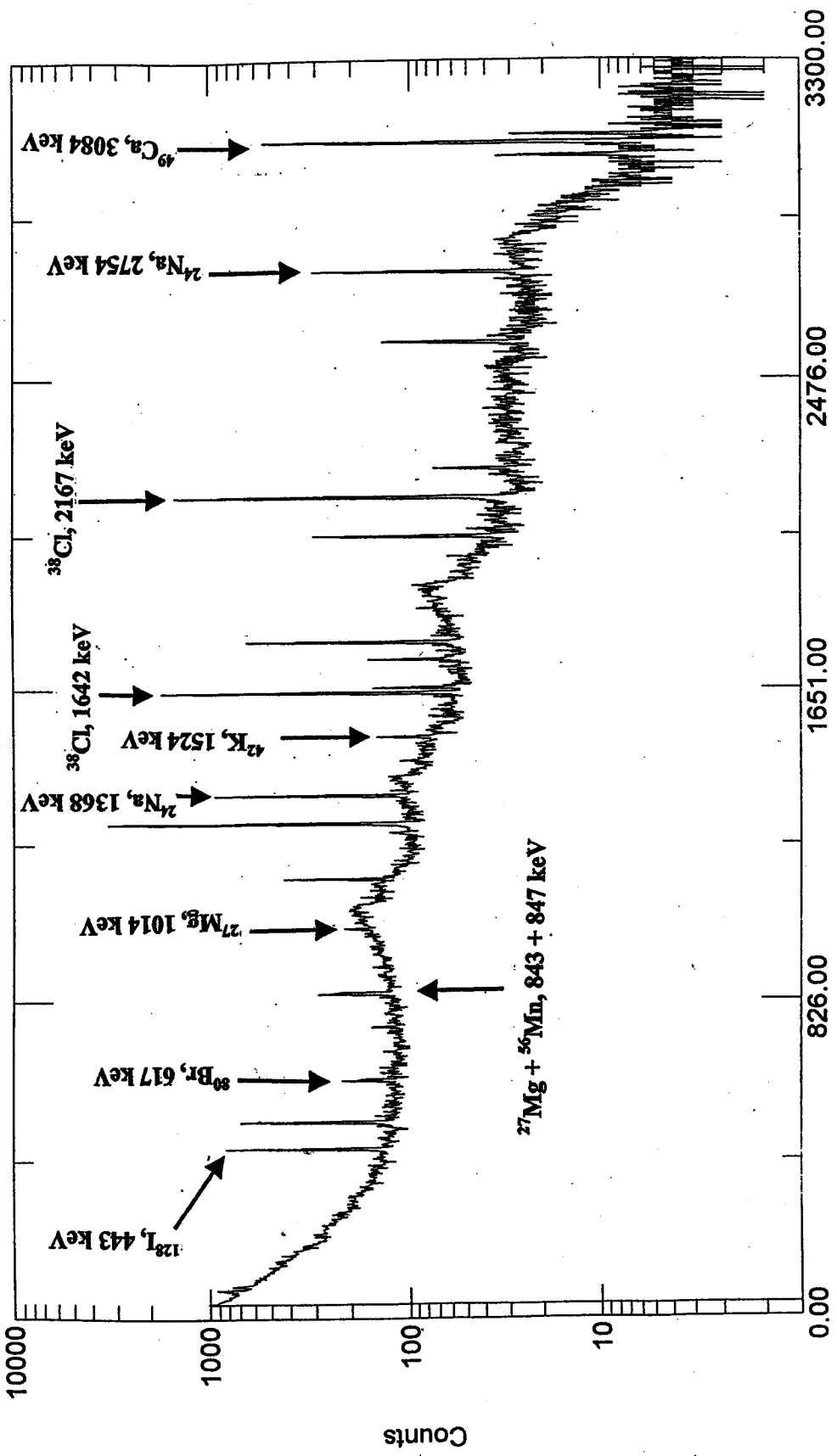

Fig. 6.17. Spectrum of casein fraction, INAA-GeLi t_i-t_d-t_c : 10-2-10 min.

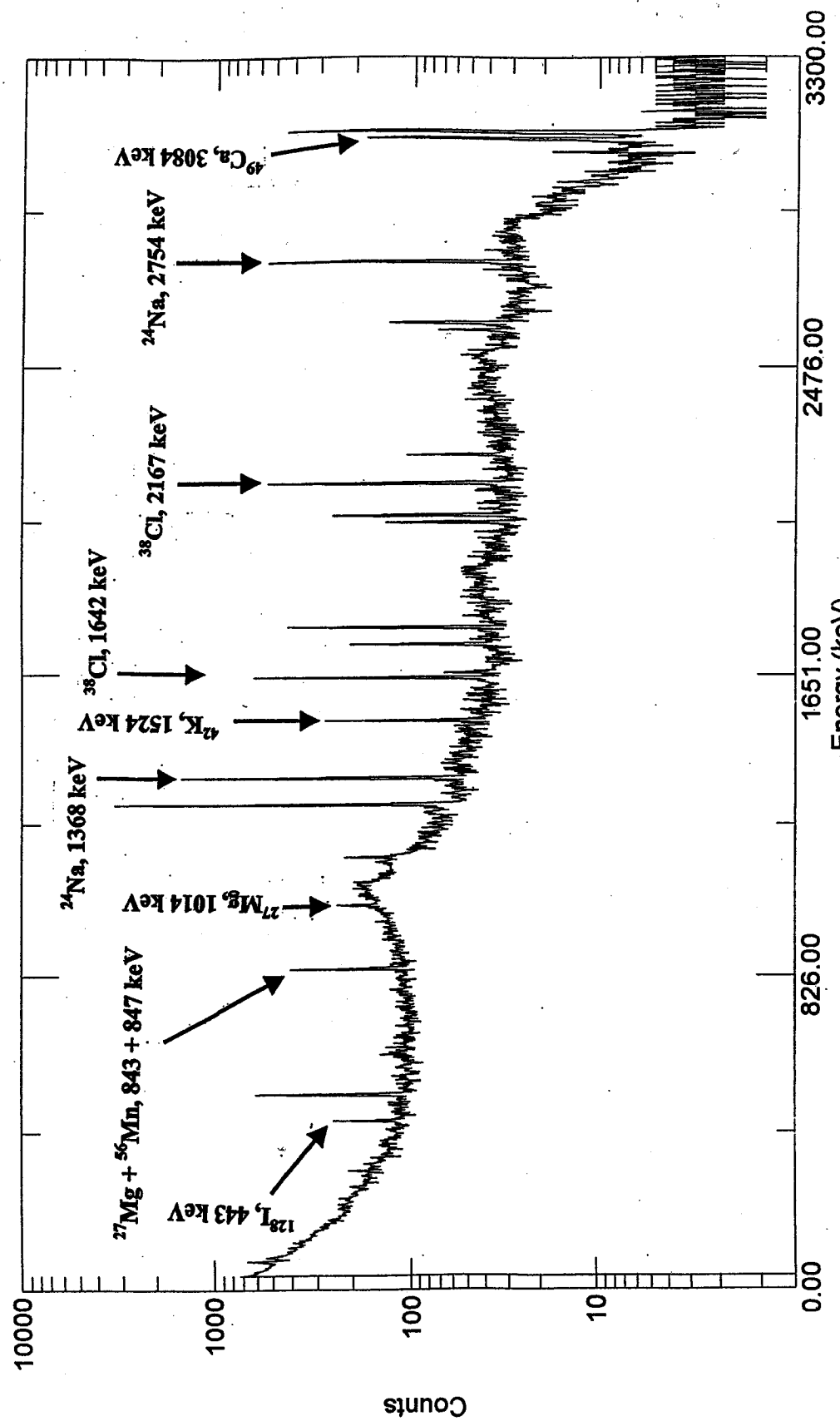

Fig. 6.18. Spectrum of whey fraction, INAA-GeLi t_i-t_d-t_c : 10-2-10 min.

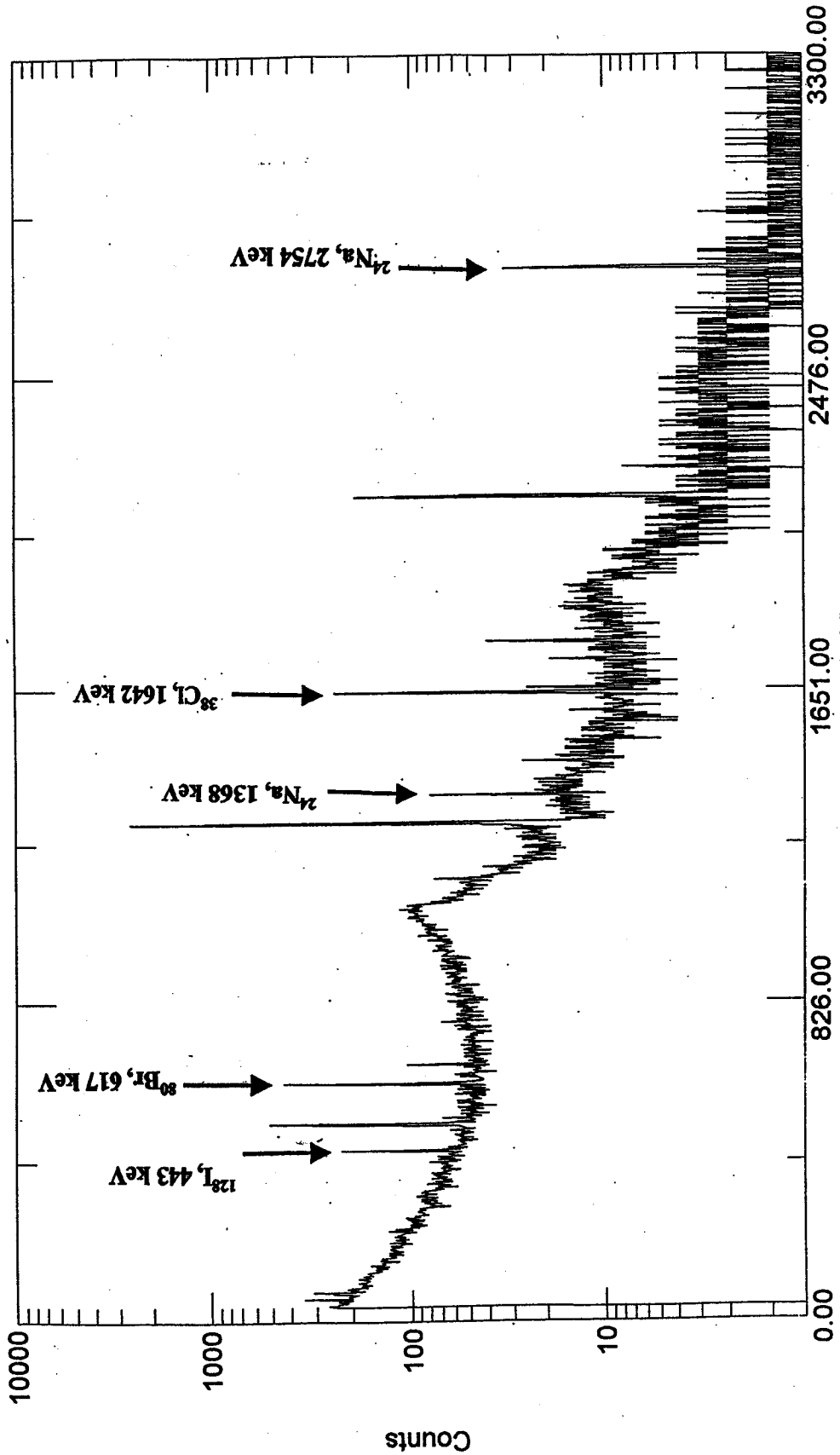

Fig. 6.19. Spectrum of apolar lipid fraction, INAA-GeLi t_i-t_d-t_c : 10-2-10 min.

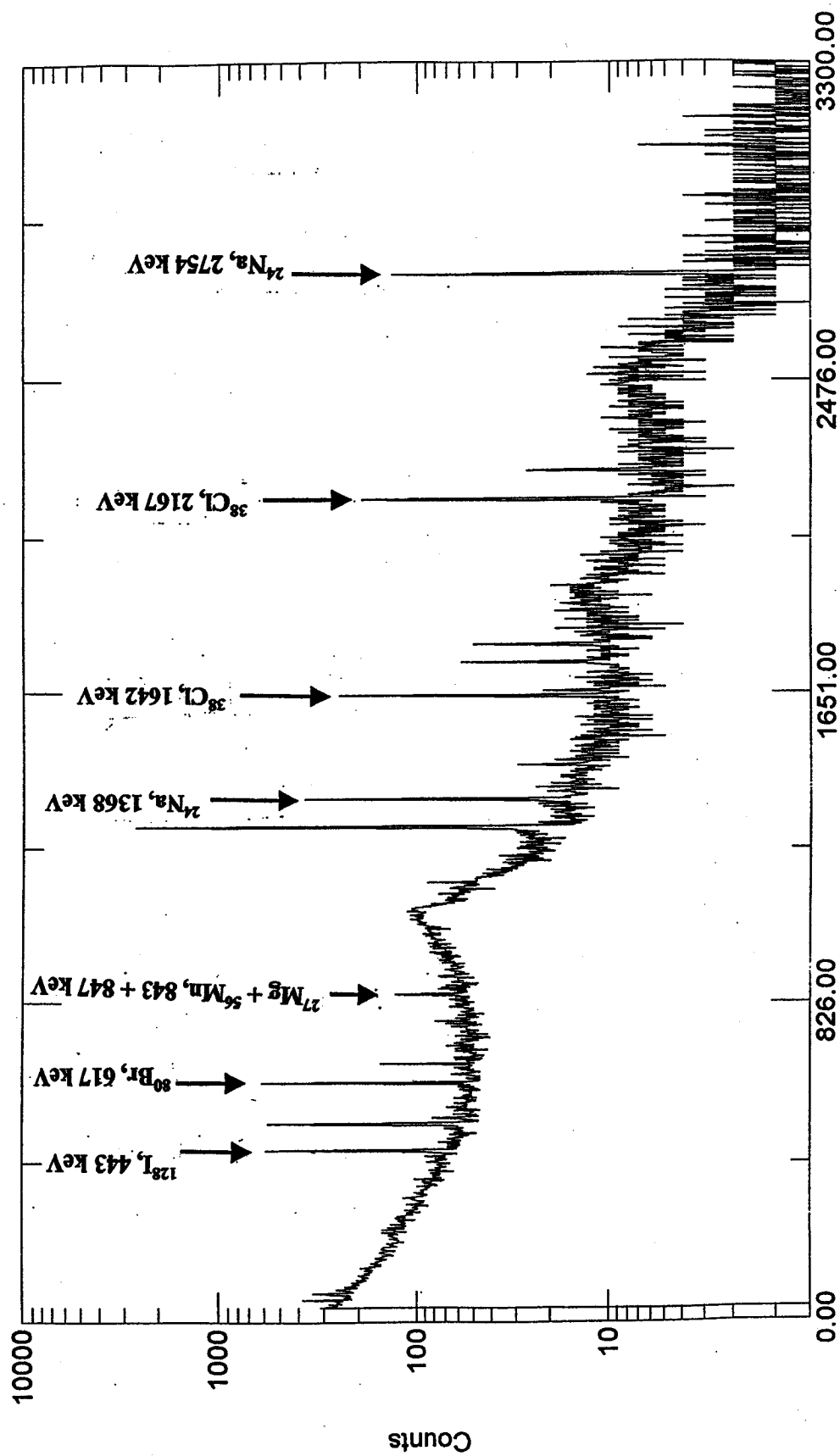

Fig. 6.20. Spectrum of less polar lipid fraction, INAA-GeLi t_i-t_d-t_c : 10-2-10 min.

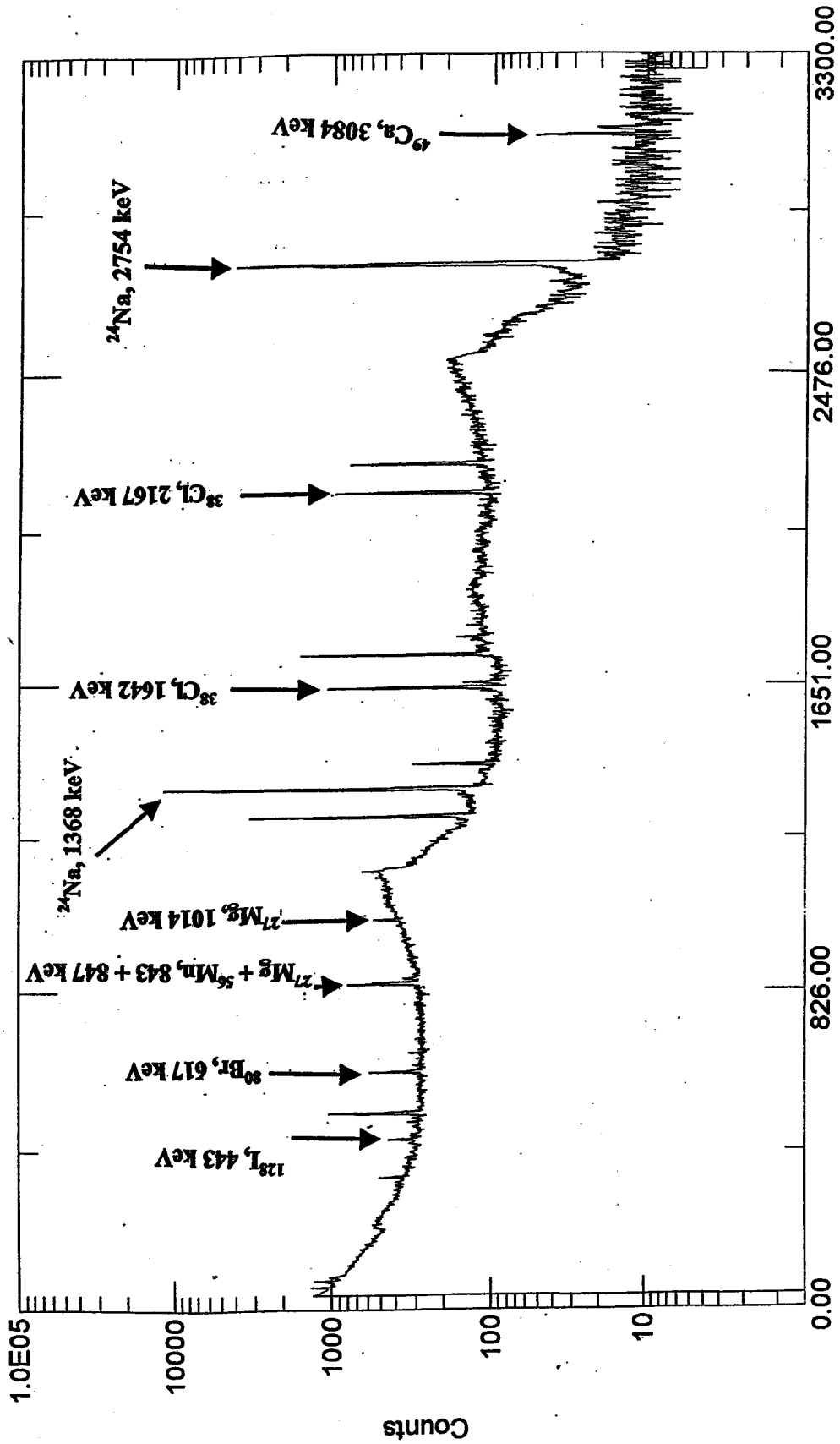

Fig. 6.21. Spectrum of polar lipid fraction, INAA-GeLi t_i-t_d-t_c : 10-2-10 min.

Table 6.11. Halogen concentrations ($\mu g.g^{-1}$) in various lipid fractions.

Element	Total lipid extract	Hexane (apolar) fraction	Acetone (less polar) fraction	Methanol (polar) fraction
Iodine	0.21 ± 0.02	0.206 ± 0.002	0.64 ± 0.03	0.71 ± 0.05
Bromine	2.65 ± 0.04	2.54 ± 0.09	6.795 ± 0.004	9.71 ± 0.04
Chlorine	32 ± 1	30 ± 1	40 ± 1	652 ± 7

It has been shown in Chapter 4 that 96% of the lipid is apolar, 3.6% less polar, and 0.18% polar for a lipid of mass 1.037 ± 0.005 g. Using these values and the above table, a halogen mass balance in the lipid extract can be calculated as shown in Table 6.12. It is evident that the apolar fraction contained 89.1% of total iodine, 49.8% of total bromine, and 65.9% of total chlorine.

Table 6.12. Halogen mass distribution (μg) in various lipid fractions.

Element	Total lipid mass (μg)	mass in apolar fraction	mass in less polar fraction	mass in polar fraction	Recovery %
Iodine	0.218 ± 0.001	0.198 ± 0.03	0.023 ± 0.004	0.0013 ± 0.0005	102
Bromine	2.75 ± 0.04	2.44 ± 0.09	2.4 ± 0.4	0.017 ± 0.007	106
Chlorine	33 ± 1	29 ± 1	14± 2	1.2 ± 0.4	99

Since the apolar fraction contained the highest amounts of the organohalogens, it was decided to further study this fraction. Ten apolar fractions were combined, and allowed to dry by evaporating in air for about 16 h. The dried lipids were dissolved in hexane to give a solution of about 1 g of lipid per mL. Then the solution was separated into hydrocarbons, tri-acylglycerides, mono- and di-acylglycerides, and free fatty acids using solid phase extraction as described in Chapter #4. These fractions were analyzed for halogens by NAA using the same conditions as given above. Spectra of the separated fractions are shown in Fig. 6.22 - 6.25. The results are presented in Table 6.13.

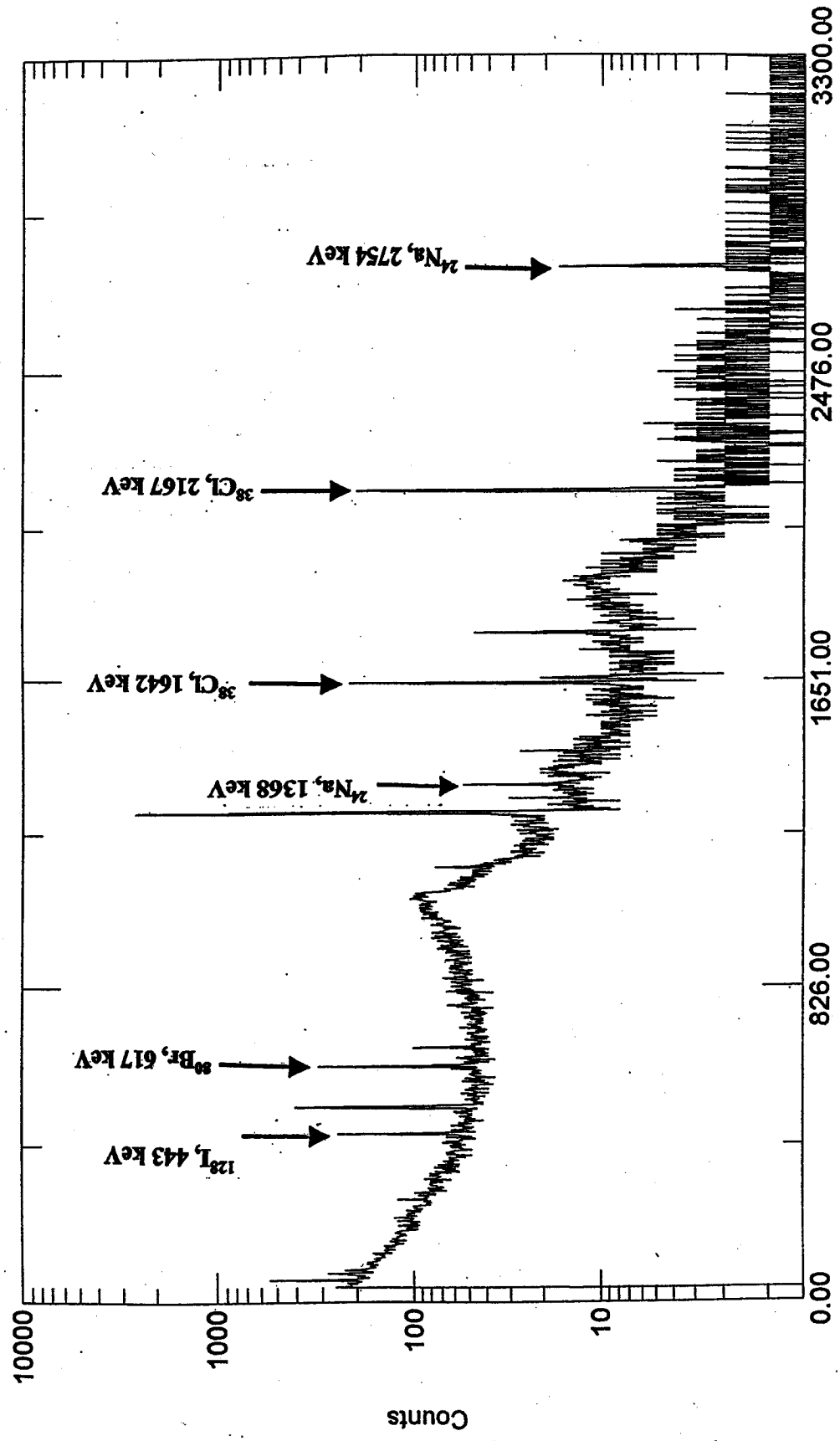

Fig. 6.22: Spectrum of hydrocarbon fraction, INAA-GeLi t_i-t_d-t_c : 10-2-10 min.

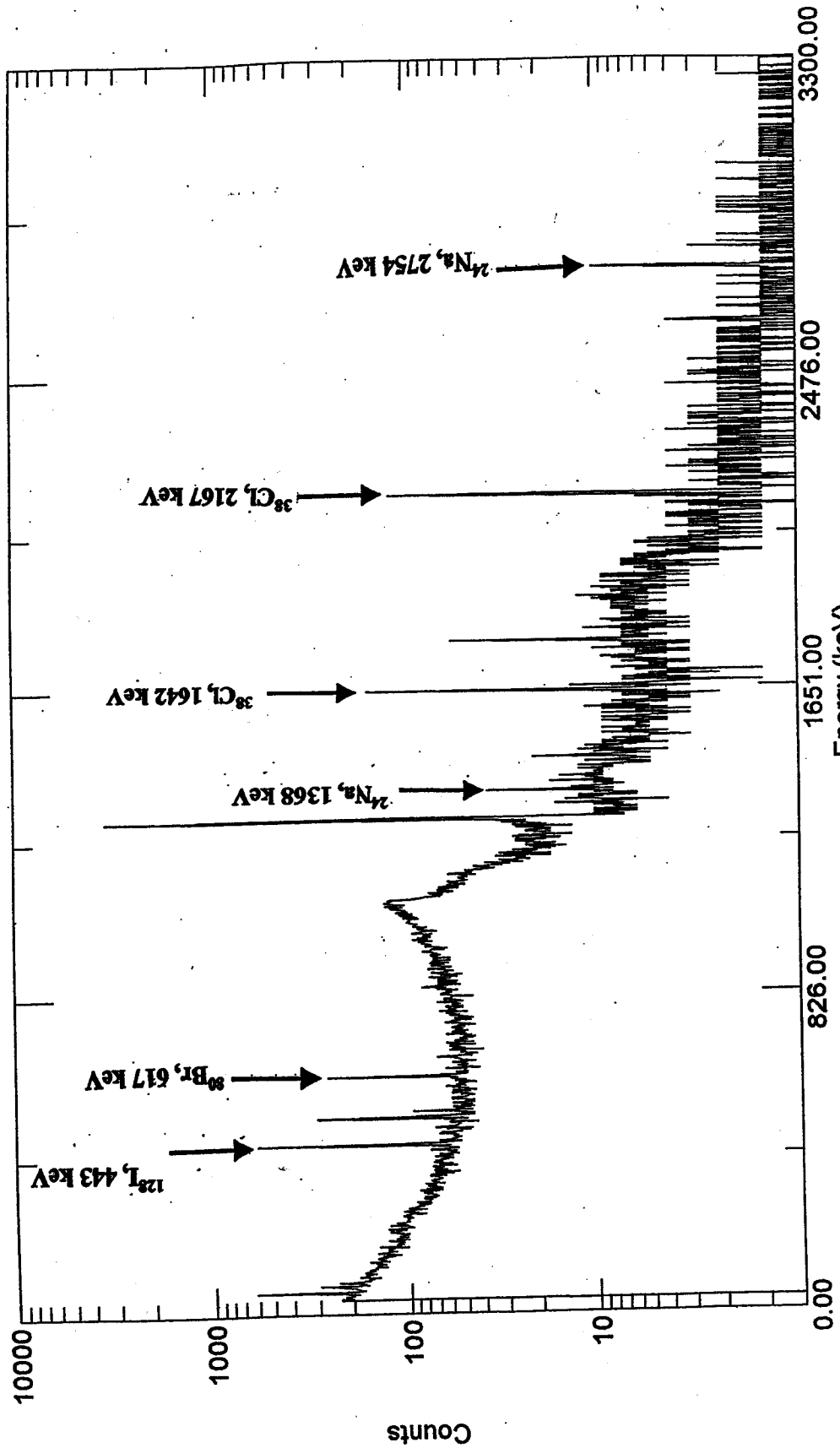

Fig. 6.23. Spectrum of triacylglycerols fraction, INAA-GeLi t_i-t_d-t_c : 10-2-10 min.

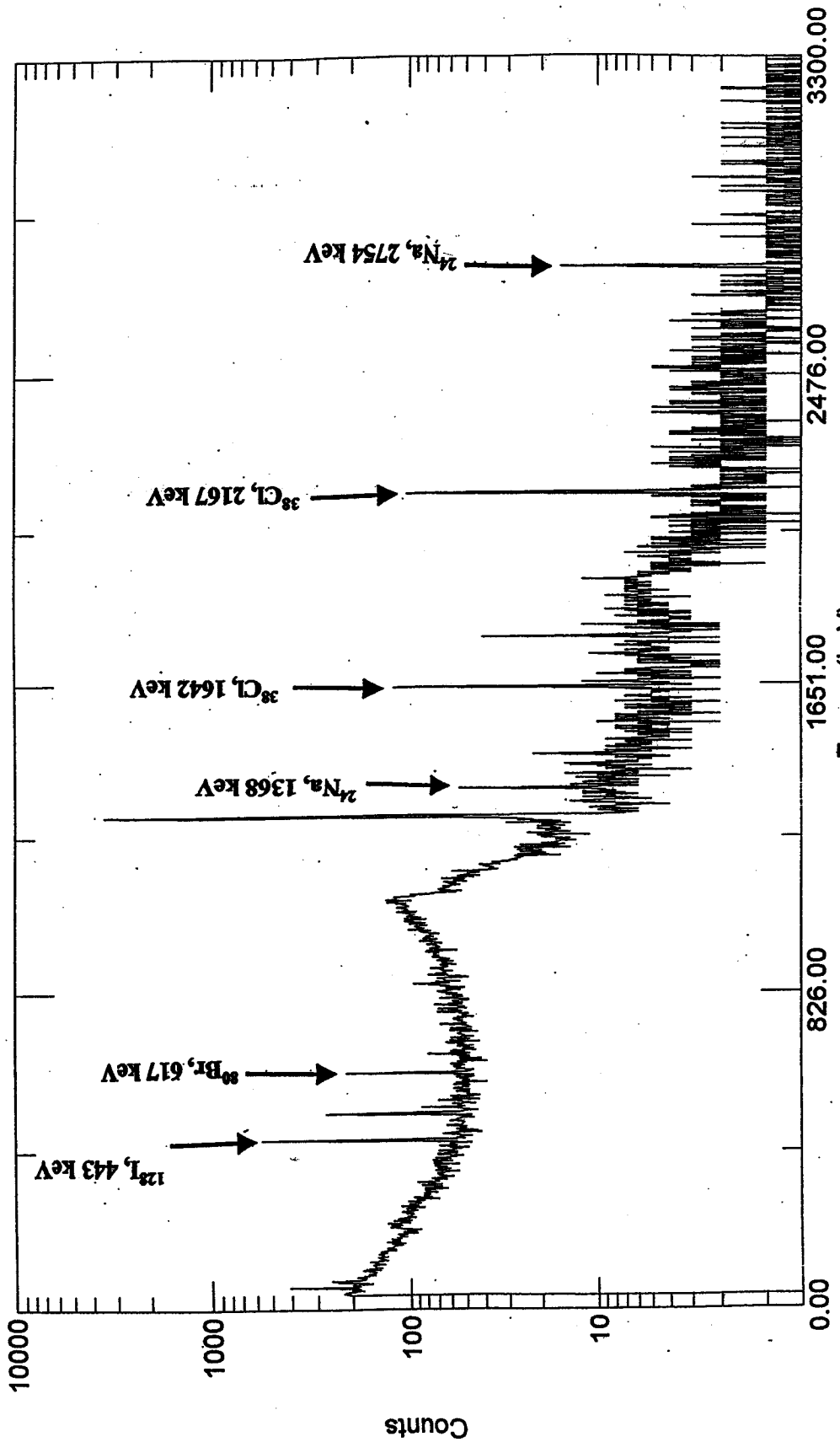

Fig. 6.24. Spectrum of mono- and di-acylglycerols fraction, INAA-GeLi t_i-t_d-t_c : 10-2-10 min.

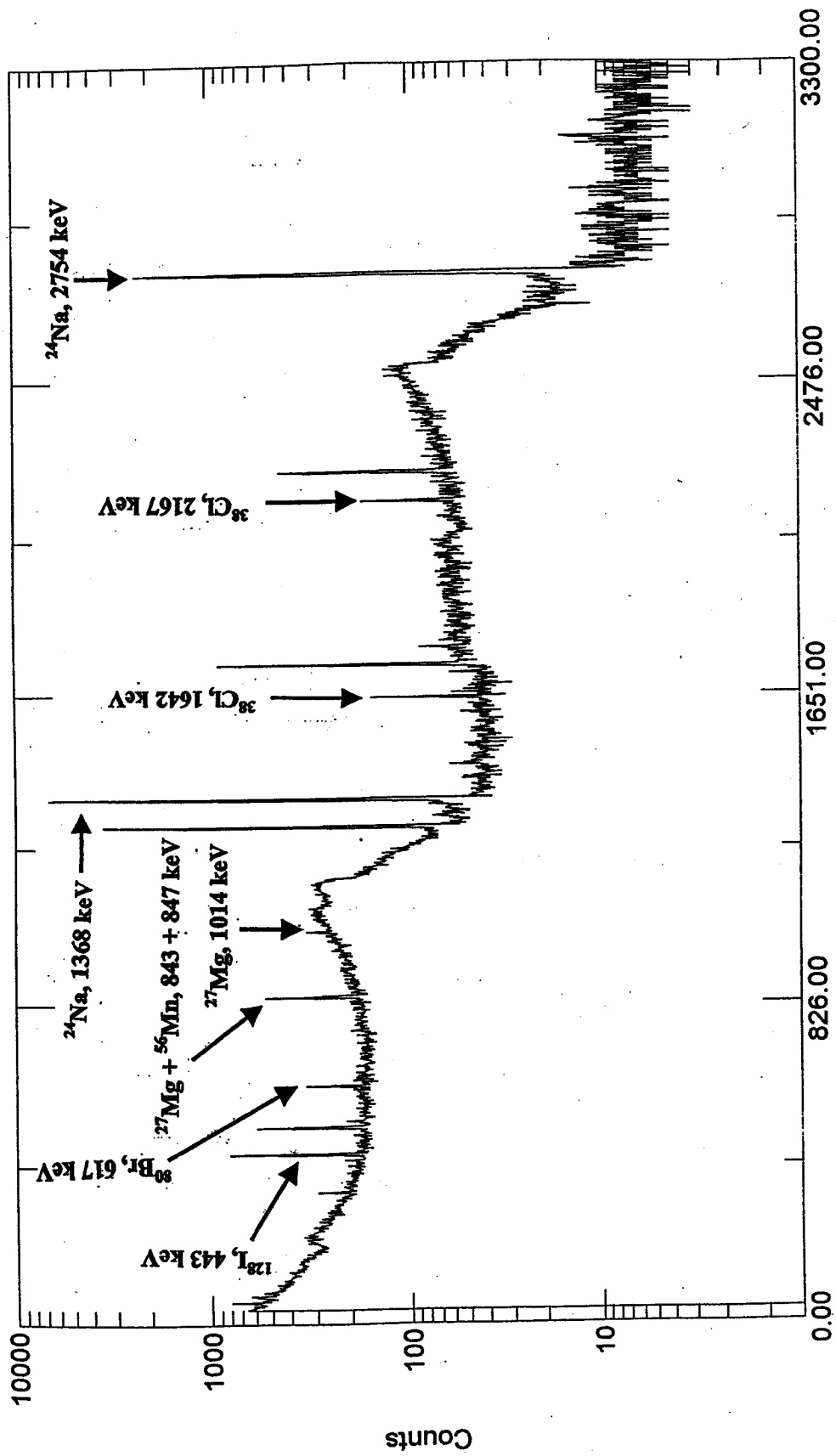

Fig. 6.25. Spectrum of free fatty acid fraction, INAA-GeLi t_i-t_d-t_c : 10-2-10 min.

Table 6.13. Halogen concentrations ($\mu g.g^{-1}$) in the apolar lipid fractions.

Element	Hydrocarbons	Tri-acylglycerols	Mono- and Di-acylglycerols	Free fatty acids
Iodine	0.065 ± 0.007	2.2 ± 0.2	11.1 ± 0.8	10.7 ± 0.7
Bromine	1.5 ± 0.2	13.2 ± 0.2	52 ± 5	46.4 ± 0.4
Chlorine	26 ± 1	16.2 ± 0.4	757 ± 2	408 ± 16

It has again been shown in Chapter 4 that 95% of the apolar lipid is hydrocarbons, 2.4% tri-acylglycerides, 0.32% mono- and di-acylglycerides, and 0.53% free fatty acids for a lipid of mass 1.022 ± 0.008 g. Using these values and Table 6.13, a halogen mass balance in the apolar lipid extract can be calculated as shown in Table 6.14.

Table 6.14. Halogen mass balance (μg) in the apolar lipid fraction.

	Iodine	Bromine	Chlorine
Total apolar mass	0.211 ± 0.007	2.60 ± 0.02	31 ± 0.2
Hydrocarbons	0.063 ± 0.007	1.4 ± 0.2	25.2 ± 0.9
Tri-acylglycerides	0.054 ± 0.005	0.32 ± 0.03	0.39 ± 0.03
Mono- and Di-acylglycerides	0.036 ± 0.004	0.17 ± 0.2	2.4 ± 0.2
Free fatty acids	0.057 ± 0.007	0.25 ± 0.03	2.16 ± 0.08
Recovery (%)	98	82	96

The facts that the hydrocarbons are the major components of the apolar fraction and that there are halogens present in this fraction indicate the possible presence of anthropogenic organohalogen compounds in milk. The amounts of halogen associated with hydrocarbons in the apolar fraction are 29%, 54% and 80% for iodine, bromine and chlorine, respectively.

6.7 Summary

A series of schemes was developed for the separation of different iodine species in milk. Scheme #2 is appropriate for just the separation of the organic and inorganic fractions. Scheme #6 is recommended for the separation of inorganic iodine, protein-bound and lipid-bound iodine. When the separation of iodide and iodate from the inorganic fraction is needed, as well as the determination of the protein-bound and lipid-bound iodine then Scheme #7 is most suitable.

As expected, inorganic iodine accounted for 90% of the total iodine content of milk. Iodide ion is in fact the major contributor species, followed by iodate ions. The remaining 10% corresponded to protein-bound and lipid-bound iodine. The protein fraction was fractionated into casein and whey proteins. Similarly the lipid fraction was fractionated into three lipid groups: apolar, moderate polar and polar. The apolar fraction was further fractionated into hydrocarbons, triacylglycerides, mono and di-acylglycerides and free fatty acids. The iodine bound to each separated protein and lipid fraction was determined.

CHAPTER 7

CALCULATION OF EXPANDED UNCERTAINTY

The result of an analytical determination is usually the concentration of the analyte of interest. This concentration is reported customarily as the average of certain number of replicates plus/minus its standard deviation. However, this standard deviation value only represents the reproducibility of the analytical method used. Nothing is said about all the sources of uncertainty involved in such a method. In order to improve an analytical result it is important to know as much as possible the origin of all sources of error. An analytical determination without the calculation of the expanded uncertainty associated with it is often considered an unreliable result in today's world. The detailed calculation of expanded uncertainty is described in this chapter.

7.1 Introduction

According to the EURACHEM guide, uncertainty is: "A parameter associated with the result of a measurement, that characterises the dispersion of the values that could reasonably be attributed to the measurand" [1]. Strictly speaking the word uncertainty refers to doubt; however, in the analytical chemistry world the term uncertainty refers to either the EURACHEM definition given above or "to a limited knowledge about a particular value" [1]. "Uncertainty of measurement does not imply doubt about the validity of a measurement; on the contrary, knowledge of the uncertainty implies increased confidence in the validity of a measurement result" [1].

It is also important to distinguish between error and uncertainty. Error by definition is "the difference between an individual result and a true value of the measurand. As such error is a single value" [1]. On the other hand uncertainty "takes the form of a range, and, if estimated for an analytical procedure and defined sample

type, may apply to all determination so described" [1]. In principle, a result can be corrected if the errors with it are known. This does not apply to the uncertainty value.

The calculation of the uncertainty of a measurement (from now on called combined standard uncertainty) involves the identification of all possible sources of error (*e.g.* purity of starting materials, errors of all glassware, balance and any other instrument used, environmental conditions, random variations, *etc*). These sources of error are converted into standard deviation and this standard deviation (hereafter called standard uncertainty of each component) is used in the calculation of the combined standard uncertainty by applying a propagation error calculation method. Each source of error contributes as an independent component, although some of them could be correlated, and the determination of the covariance is then needed. Sometimes however, it is possible to evaluate the combined effect of various components and to treat the combined effect as a new independent error. This eliminates the necessity of taking into account the correlation and simplifies the mathematical work.

The standard uncertainty of each source of error involved in the combined standard uncertainty can be evaluated either from the statistical distribution of the results of many measurements and then characterized by the standard deviation (type A evaluation) or evaluated after assuming a probability distribution based on experience or other information (type B evaluation). Once the combined standard uncertainty is calculated, the expanded uncertainty can be determined. This value represents "the interval within which the value of the measurand is believed to lie with a higher level of confidence" [1]. The expanded uncertainty is calculated by multiplying the combined standard uncertainty by a coverage factor. The coverage factor is determined based on the level of confidence desired in the final expression of the expanded uncertainty value. For an approximate level of confidence of 95%, the value of the coverage factor is 2.

The standard uncertainty of a parameter is calculated as the limit value divided by the statistical distribution that best fit such error. There are three distributions that can be used in the following cases:

Rectangular distribution: A certificate or other specifications about the source gives limits without reporting a confidence level and there is reason to expect that extreme values are likely. A maximum range has been estimated for the source (*e.g.* ± a) without knowing the shape of the distribution. The standard uncertainty is calculated dividing the limit value (a) by the square root of 3.

Triangular distribution: Information about the source is less limited than in the case of the rectangular distribution. It is expected that values were nearer to the average reported value than to the bounds. A maximum range is estimated for the source described by a symmetric distribution. The standard uncertainty is calculated dividing the limit value by the square root of 6.

Normal distribution: An estimate of it is made by repeating observations of a randomly varying process. In this case the standard uncertainty is the standard deviation. The standard uncertainty is given in the form of a standard deviation, relative standard deviation or coefficient of variance without specifying the distribution. An uncertainty is given specifying the confidence of interval (x ± c) but nothing is said about the distribution. In this case the standard uncertainty is calculated as the limit value divided by the percentage points on a normal distribution. For instance, standard uncertainty u(x) = c/2 for a c at 95%, and u(x)=c/3 for c at 99.7% of confidence level respectively.

In this chapter the uncertainty associated to the NAA method is calculated following the method described by Greenberg and Bode, which was developed following the EURACHEM guide among others. The whole process is divided into three steps: pre-irradiation step, the irradiation step and the counting step. The uncertainty involved in the preparation of the comparator standard was also calculated following the EURACHEM guide. In all cases the process is as follows: identification of the source or error, estimation of the standard uncertainty associated to the source of error, and finally application of the propagation error formula accordingly.

7.2 Experimental and results

7.2.1 Pre-irradiation step

This step has mainly 4 sources of uncertainty:

- The concentration of the working comparator solution which involved a prior preparation of a stock solution (Evaluation of Type B error);
- The sample-size-precision for samples and standards (Evaluation of Type A error);
- The sample-to-sample variation for samples and standards (Evaluation of Type A error); and
- The isotopic variability in the radionuclide of interest (Negligible).

The last step is negligible in this case since ^{127}I is 100% abundant. The second and the third ones depend only on the balance used and the first one involves more than one sub-step.

7.2.1.1 Preparation of the stock solution

Three major sources of uncertainty are involved in the preparation of the stock solution for comparator standards:

- Purity of the initial material: ammonium iodide SPEX, ultrapure quality, 100.04% (Evaluation of Type A error);
- Volume of the prepared solution (Evaluation of Type B error); and
- Uncertainty associated with the balance (Evaluation of Type A error).

Purity of the initial material: Since the purity of the starting material reported by the manufacturer is 100.04%, a value of 1.0004-1.0000 = 0.0004 was used as uncertainty value. The standard uncertainty of the purity was calculated then as the uncertainty value divided by square root of 3 (assuming rectangular distribution) given a value of 2.31×10^{-4}.

Volume of the prepared solution: The standard uncertainty associated with the volume of the stock solution prepared has three sources of error:

- Uncertainty of the volumetric flask (Evaluation of Type A error);
- Repeatability of that value (Evaluation of Type A error); and
- Uncertainty associated to the dilatation or compression of the volume in the flask depending on the temperature (Evaluation of Type B error).

The volumetric flask used was Pyrex labeled as (1000±0.6) mL where 0.6 was the uncertainty value. Assuming a triangular distribution, the standard uncertainty associated with the volumetric flask is 0.6 divided by square root of 6 given a value of 0.2449 mL.

The repeatability of the volumetric flask was found experimentally and a normal distribution was used. In this case the same volumetric flask was filled up with DDW and it was weighed. Using the density of the water the mass values were converted into volume. This process was repeated 10 times and the standard deviation of the mean was used as standard uncertainty of this parameter.

The uncertainty associated with the dilatation or compression of the liquid in the volumetric flask was calculated assuming a maximum difference in temperature (ΔT) of 4 degree Celsius between the volumetric flask and the liquid. Knowing that the coefficient of expansion for water (α) is $0.00021^{\circ}C^{-1}$ and the total volume (v) is 1 000 mL, then the uncertainty associated with the volume variation was $\Delta V=\alpha\Delta Tv = 0.84$ mL. The standard uncertainty for this parameter assuming a rectangular distribution was 0.4850 mL.

Uncertainty associated with the mass: This uncertainty is calculated directly from the balance. Strictly speaking, there are various sources of uncertainty in the mass determination using an analytical balance such as:

- Repeatability (Evaluation of Type B error);
- Balance calibration uncertainty;

- Linearity;
- Readability (digital resolution);
- Daily drift; and
- Density effect

Typically, repeatability is the major source of uncertainty which is usually the only one taken into account. The second and the third factors can be estimated from the manufacturer's certificates. The fifth parameter depends on temperature variation; it can be considered negligible if the temperature is kept quite constant. The density effect is always considered negligible.

The minimum resolution of the balance is 0.1 mg; assuming a rectangular distribution the standard uncertainty would be 0.0577 mg. However, taking into account that in every mass determination the balance is used twice, the standard uncertainty for this operation is 0.0816 mg.

The sources of error and the calculation of the relative standard uncertainty for the stock solution are summarized in Tables 7.1 and 7.2. The major contributor for the relative standard uncertainty in the preparation of the stock solution was the uncertainty associated with the volumetric flask. The prepared stock solution had a concentration of 4 000 $\mu g.mL^{-1}$ with a relative standard uncertainty of 0.06%.

The standard uncertainty of the stock solution was then $4000 \times 6.51 \times 10^{-4} =$ 2.6 $\mu g.mL^{-1}$ leading to the concentration of the stock solution as 4000 ± 3 $\mu g.mL^{-1}$.

Table 7.1. Sources of error and calculation of standard uncertainty in the preparation of the stock solution

Step	Uncertainty	Distribution used	Standard uncertainty
a. Purity of the starting material	0.0006 g	Rectangular	**2.31×10^{-4}**
b. Volume			
b1. Calibration of volumetric flask	0.6 mL	Triangular	0.2449 mL
b2. Repeatability of volumetric flask			0.1 mL
b3. Calibration of volume			0.4850 mL
Combination of b1 + b2 + b3			**0.5524 mL**
c. Balance	0.1 mg	Rectangular	**0.0816 mg**

Table 7.2. Calculation of standard uncertainty in the preparation of the stock solution

Step	Value	Standard uncertainty	Rel. std. uncertainty
Purity	1.0004	3.46×10^{-4}	3.45×10^{-4}
Volume	1 000 mL	0.5524 mL	5.524×10^{-4}
Mass	4.5686 g	0.0816×10^{-3} g	1.78×10^{-5}
Combined relative standard uncertainty			**6.51×10^{-4}**

7.2.1.2 Preparation of comparator standard

The working comparator standard was prepared by diluting the stock solution 1000 times in a volumetric flask; it was then transferred to a bottle. One mL of the stock solution was pipetted out from the bottle and placed in the same 1000-mL volumetric flask previously used for preparing the stock solution. The same volumetric flask was used twice so that the uncertainty associated with the volumetric flask remained the same. However, a new source of uncertainty associated with the use of the pipette

now has to be taken into consideration. The standard uncertainty associated with the pipette was found using the same method employed for the volumetric flak.

Volume taken with the pipette: The standard uncertainty associated with the volume taken using the pipette has three sources of error:

- Uncertainty associated with the calibration of the pipette (Evaluation of Type A error);
- Repeatability of that value (Evaluation of Type A error); and
- Uncertainty associated with the dilatation or compression of the volume in the pipette tip depending on the temperature (Evaluation of Type B error).

The standard uncertainty associated with the calibration of the pipette was calculated from the data reported by the manufacturer. The maximum relative error for the pipette was used for finding the error at a specific volume. In this case the manufacturer reported that the pipette had the maximum error at 0.5 mL and the error was 1%. Therefore, the pipette had a maximum error of 0.005 mL, and from this value the standard uncertainty was calculated assuming a triangular distribution.

The repeatability of the pipette was determined in a fashion similar to that of the volumetric flask (already explained above).

The variation due to dilatation or compression of the liquid in the pipette was calculated as above but in this case the maximum volume was 1 mL.

All calculations involved in the determination of the standard uncertainty of the working solution are summarized in Tables 7.3 and 7.4. The working solution had a concentration of 4 $\mu g.mL^{-1}$ and a relative standard uncertainty of 0.23%. The major contributor to this relative standard uncertainty was the uncertainty associated with the pipette.

Table 7.3. Sources of error and calculation of standard uncertainty in the preparation of the working solution

Step	Uncertainty	Distribution	Standard uncertainty
c. Volume from Pipette			
c1. Calibration of pipette	0.005 mL	Triangular	0.2041×10^{-3} mL
c2. Repeatability of pipette			4.082×10^{-4} mL
c3. Calibration of volume of pipette			4.85×10^{-4} mL
Combination of c1 + c2 + c3			**0.0021 mL**

Table 7.4. Calculation of the standard uncertainty in the working solution

Step	Value	Standard uncertainty	Rel. std uncertainty
a. Concentration of the stock solution	4000 $\mu g\ mL^{-1}$	3	7.5×10^{-4}
b. Volume from the stock	1 mL	0.0021 mL	2.14×10^{-3}
c. Total volume	1000 mL	0.5524 mL	5.524×10^{-4}
Combined relative standard uncertainty			**2.33×10^{-3}**

7.2.1.3 Sample to sample precision:

This step depends only on the uncertainty associated with the balance. The standard uncertainty of the balance was 0.0816 mg as it was explained above and the standard uncertainty due to sample-to-sample precision was 0.0816 divided by average sample mass and divided by the square root of the number of replicates analyzed by sample.

7.2.1.4 Sample to sample variation:

This value comes from the repeatability of the balance and it was calculated experimentally as explained by Greenberg [2]. Nine samples of 0.5 mL of DDW were weighed three times each and the relative standard deviation was calculated

for each set of 3 values. The measurements are shown in Table 7.5. The higher relative standard deviation calculated value was taken as the maximal variability of the balance. The standard uncertainty associated with the repeatability of the balance was calculated as the maximal variability (1.15×10^{-4}) divided by square root of 27 giving 2.21×10^{-5}. This number was used as the standard uncertainty for this source of error.

Table 7.5. Experiments designed for calculating the standard uncertainty associated with the repeatability of the balance.

Sample #	Mass 1 (mg)	Mass 2 (mg)	Mass 3 (mg)	Average mass (mg)	Std. Dev	RSD
1	0.4993	0.4993	0.4993	0.4993	0	0
2	0.5061	0.5061	0.5060	0.5061	5.8×10^{-5}	1.14×10^{-4}
3	0.5018	0.5018	0.5018	0.5018	0	0
4	0.5074	0.5073	0.5073	0.5073	5.8×10^{-5}	1.14×10^{-4}
5	0.5071	0.5072	0.5071	0.5071	5.8×10^{-5}	1.14×10^{-4}
6	0.5078	0.5079	0.5079	0.5079	5.8×10^{-5}	1.14×10^{-4}
7	0.5069	0.5068	0.5069	0.5069	5.8×10^{-5}	1.14×10^{-4}
8	0.5067	0.5067	0.5066	0.5067	5.8×10^{-5}	1.14×10^{-4}
9	0.5033	0.5034	0.5034	0.5034	5.8×10^{-5}	1.15×10^{-4}

7.2.1.5 Uncertainty calculation of the pre-irradiation step

The relative standard uncertainty calculation of the pre-irradiation step for all irradiated fractions is summarized in Tables 7.6 and 7.7. The major source of error was still the one associated with the concentration of the comparator standard solution.

Table 7.6. Sources of error and calculation of standard uncertainty in the pre-irradiation step

Samples (n=5)	Average mass value (g)	Standard uncertainty	Relative standard uncertainty
Sample to sample precision			
Total milk	0.75	0.0816×10^{-3}	4.866×10^{-5}
Lipids	0.45	0.0816×10^{-3}	8.109×10^{-5}
Total Proteins	0.35	0.0816×10^{-3}	1.042×10^{-5}
Casein	0.35	0.0816×10^{-3}	1.042×10^{-5}
Iodide	0.45	0.0816×10^{-3}	8.109×10^{-5}
Iodate	0.45	0.0816×10^{-3}	8.109×10^{-5}
Total inorganic	0.45	0.0816×10^{-3}	8.109×10^{-5}
Standards	0.75	0.0816×10^{-3}	4.866×10^{-5}
Sample to sample variation			
All samples and standards			2.21×10^{-5}
Concentration of the comparator standard			
All samples			2.33×10^{-3}
Isotopic variability			
All samples and standards	None		

Table 7.7. Combined relative standard uncertainty in the pre-irradiation step

Sample	Combined relative standard uncertainty
Total milk	2.331×10^{-3}
Lipids	2.330×10^{-3}
Total Proteins	2.330×10^{-3}
Casein	2.330×10^{-3}
Iodide	2.331×10^{-3}
Iodate	2.331×10^{-3}
Total inorganic	2.331×10^{-3}

7.2.2 Irradiation step

This step has 5 major sources or error:

- Irradiation geometry (Evaluation of Type A error);
- Neutron scattering and/or neutron absorption (Evaluation of Type B error);
- Irradiation time differences between samples and standards (Evaluation of Type A error);
- Errors associated with timers for irradiation, decay and counting (Evaluation of Type A error); and
- Interfering reactions

The same irradiation-decay-counting scheme was used for both samples and standards; therefore it was not necessary to find the standard uncertainty associated with the timing differences. The last factor can be neglected since no other nuclear reaction produces ^{127}I.

7.2.2.1 Irradiation geometry differences

This value was experimentally measured as prescribed by Greenberg [2]. A set of 7 samples of SRM-NIST 1566b (Oyster Tissue-b) were analyzed using t_i-t_d-t_c of 30-5-30 min. The samples were placed in such a way so that 1.5 cm of the 2-cm-long polyethylene irradiation vials was filled. The 666-keV photopeak of ^{80}Br was assayed. The minimum variation in the concentrations of bromine among the 7 samples was found to be 0.4%. This value was used for calculating the standard uncertainty due to geometry differences in irradiations of milk samples and standards in similar vials but filled up to 1.1 cm: (0.004x0.55)/0.75=0.0029. The number of milk samples and standards analyzed was 5 each; therefore the relative standard uncertainty was 1.311×10^{-3}.

7.2.2.2 Neutron scattering and/or absorption

Calculation of the uncertainty associated with the neutron scattering and/or absorption during the irradiation of the sample was pursued through an experiment

as described by Greenberg and Bode [3]. Different sample masses were irradiated and Table 7.8 shows the $\log_e$(counts/mass) ratio used for finding the parameter. No neutron scattering was found at all in standards and lipids samples. Some effect was found in milk samples with masses higher than 1 g. Since the average mass of milk samples irradiated was 0.75 g, this effect was considered negligible in this particular case. In the case of proteins and resins, there were effects of neutron scattering even in the working range of masses; therefore this parameter had to be taken into account.

Table 7.8. Neutron scattering in samples and standards.

Standards		**Milk**		**Lipids**	
Mass (g)	**$\log_e$ (counts/g)**	**Mass (g)**	**$\log_e$ (counts/g)**	**Mass (g)**	**$\log_e$ (counts/g)**
0.2495	5.41	0.2566	7.85	0.2574	7.83
0.4967	5.44	0.5120	7.85	0.4276	7.84
0.7491	5.41	0.7716	7.84	0.5967	7.83
0.9967	5.46	1.0205	7.81	0.7563	7.85
1.4963	5.42	1.5394	7.71	1.1496	7.80
1.6961	5.40	1.7149	7.64	0.2574	7.83
Proteins				**Resin**	
Mass (g)	**$\log_e$ (counts/g)**			**Mass (g)**	**$\log_e$ (counts/g)**
0.2555	8.84			0.2570	9.06
0.4140	8.67			0.4207	8.95
0.5309	8.63			0.5582	8.90
0.6828	8.43			0.6746	8.85
0.7603	8.41			0.7596	8.82

The slopes of the neutron scattering attenuation coefficients for proteins and resin, are shown in Figs. 7.1 and 7.2, respectively. The relative standard uncertainty was calculated as 10% of the attenuated average mass of the sample divided by the

square root of the number of samples. Therefore, for an average protein mass of 0.35 g, there was an effective mass of 0.35x0.872=0.31 giving a correction factor of 0.04 leading to a neutron scattering attenuation coefficient of 0.04/0.35=0.1142. The relative uncertainty was estimated as 10% of the above value which is $1.142x10^{-2}$. In the case of resins, the relative standard uncertainty would be 0.05.

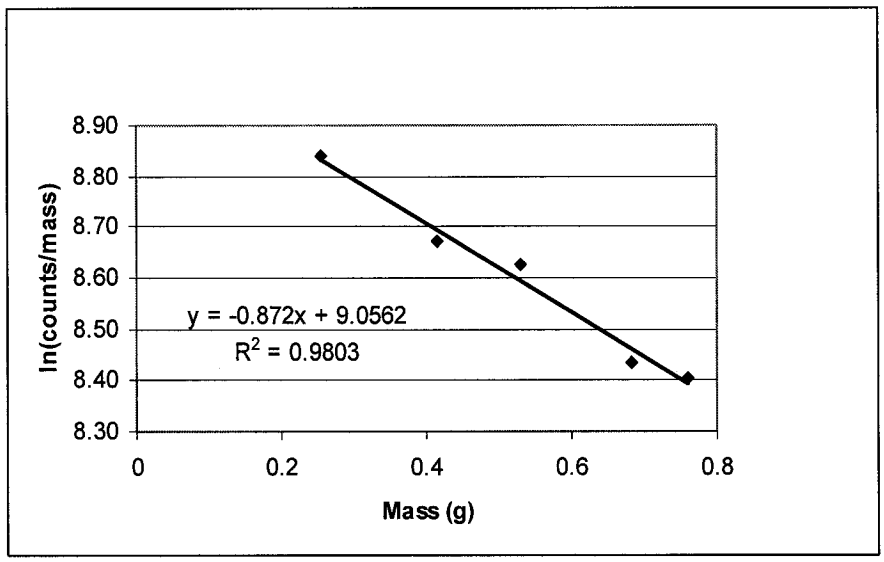

Fig. 7.1. Determination of the neutron scattering attenuation factor in proteins.

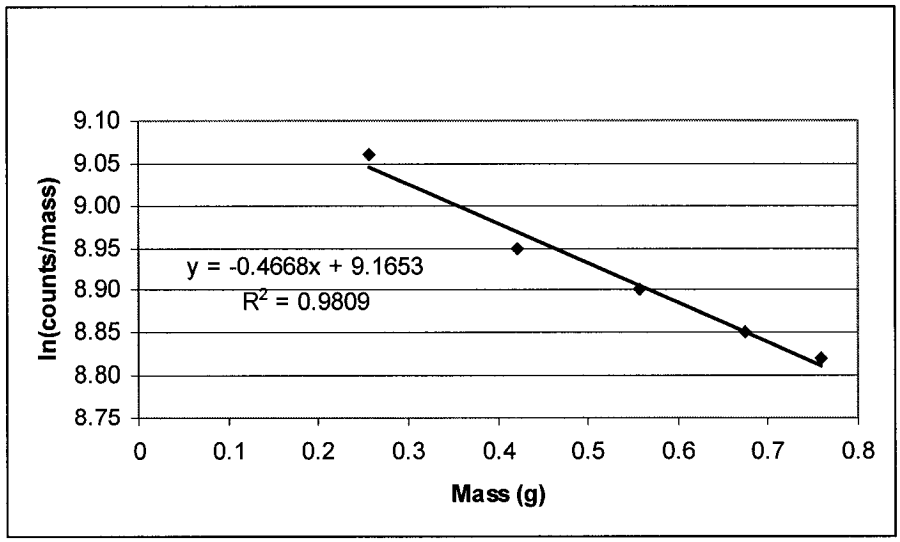

Fig 7.2. Determination of the neutron scattering attenuation factor in resin.

7.2.2.3 Errors associated with timers for irradiation, decay and counting

The minimum resolution of the timer that controlled the irradiation time, the clock used for setting the decay time, and the timer used for counting is 1 s. Assuming a rectangular distribution, the standard uncertainty would be 0.57 s.

The different t_i-t_d-t_c schemes involved are: 5-2-10 min and 10-2-10 min, therefore the relative standard uncertainty associated to each time is: $0.57/300=1.9\text{x}10^{-3}$, $0.57/120=4.75\text{x}10^{-3}$, $0.57/600=9.5\text{x}10^{-4}$. Therefore, the first t_i-t_d-t_c scheme has a relative standard uncertainty of $5.203\text{x}10^{-3}$ divided by the square root of 5 resulting in $2.33\text{x}10^{-3}$. The second scheme has a relative standard uncertainty value of $2.21\text{x}10^{-3}$.

7.2.2.4 Uncertainty calculation of the Irradiation step

The standard uncertainty calculation for this step is summarized in Tables 7.9 and 7.10.

7.2.3 Counting step

Many sources of error are involved in this step such as:

- Blank and blank correction;
- Decay correction;
- Difference in counting geometry between samples and standard;
- Dead time correction;
- Pulse pile up;
- Peak integration method;
- Gamma-ray interferences;
- Gamma-ray self- shielding and/or self-absorption;
- Repeatability of sample and standards; and
- Counting statistics of samples and standards

Table 7.9. Sources of error and calculation of the standard uncertainty in the irradiation step

Samples	**Relative standard uncertainty**
Irradiation geometry	
All samples	1.311×10^{-3}
Standards	1.311×10^{-3}
Neutron Scattering	
All samples and standards except proteins and resins	Negligible
Protein	0.01
Resins	0.05
Timing	
Total milk, iodide, iodate, total inorganic, standards (t_i-t_d-t_c of 5-2-10 min)	2.33×10^{-3}
Lipids, total proteins and casein, standards (t_i-t_d-t_c of 10-2-10)	2.21×10^{-3}
Interfering reactions	
All samples and standards	Negligible

Table 7.10. Combined relative standard uncertainty in the irradiation step

Sample	**Combined relative standard uncertainty**
Total milk	3.78×10^{-3}
Lipids	3.63×10^{-3}
Total Proteins	3.63×10^{-3}
Casein	3.63×10^{-3}
Iodide	0.05
Iodate	0.05
Total inorganic	0.05

The first 5 sources of error in this step are negligible. There was no iodine in the irradiation vials or in the resins irradiated. There were no differences in the decay time for standards and samples since the same irradiation-decay-counting scheme was used in both cases. There was no difference in the counting and counting geometry for standards and samples. The dead time during counting was always less than 5% for samples and standards; therefore there was no pulse pile up. The number of channels used in the integration method was always the same for standards and samples.

7.2.3.1 Repeatability of sample and standards

The relative standard uncertainty of this parameter was calculated by first dividing the standard deviation of the iodine concentration in each sample by the average of the iodine concentration and then by dividing it by the square root of the number of samples. This process was applied to samples and standards.

7.2.3.2 Counting statistics of sample and standards

The relative standard uncertainty in this case was calculated by applying Poison distribution. The square root of the number of counts divided by the number of counts gives the relative standard deviation in the number of counts. This value divided by the number of samples gives the relative standard uncertainty. It was also applied to standards and samples.

7.2.3.3 Gamma self-shielding and absorption

This parameter was experimentally determined following Greenberg's procedure [1]. The absorption of gamma ray by a sample was studied by placing a radioactive source at a fixed distance from the detector, placing samples of varying mass between the source and the detector, and counting them. A solution of ammonium iodide was irradiated and used as the radioactive source. Gamma self-shielding was evaluated by using samples of milk, lipid, protein and resin. The results presented in Table 7.11 show that there was a negligible effect of gamma self-shielding/absorption in standards and all samples with the exception of total milk which exhibited a slight effect

for masses greater than 0.80 g. The mass of milk sample irradiated in this thesis was less than 0.80 g; therefore, this source of error was considered negligible.

Table 7.11. Gamma-ray self absorption in samples and standards.

Standards		**Milk**		**Lipids**	
Mass (g)	**$\log_e$ (counts/g)**	**Mass (g)**	**$\log_e$ (counts/g)**	**Mass (g)**	**$\log_e$ (counts/g)**
0.0000	5.11	0.0000	5.43	0.0000	6.55
0.2495	5.16	0.2566	5.49	0.2574	6.58
0.4967	5.12	0.5120	5.47	0.4276	6.52
0.7491	5.10	0.7716	5.49	0.5967	6.59
0.9967	5.14	1.0205	5.32	0.7563	6.54
1.4963	5.10	1.5394	5.32	1.1496	6.53
1.6961	5.14	1.7149	5.23		
Proteins				**Resin**	
Mass (g)	**$\log_e$ (counts/g)**			**Mass (g)**	**$\log_e$ (counts/g)**
0.0000	9.06			0.0000	9.06
0.2555	9.02			0.2570	9.06
0.4140	9.02			0.4207	9.02
0.5309	9.00			0.5582	9.03
0.6828	9.04			0.6746	9.04
0.7603	9.02			0.7596	9.06

7.2.3.4 Gamma ray-interferences

The half-life of ^{128}I was measured through its 443-keV photopeak in all samples and standards in order to determine if any other gamma ray was interfering with it. The experimental half-lives determined in each case were within 97-102% of the literature half-life of 24.99 min of ^{128}I. In conclusion, there were no interfering gamma

rays. Furthermore, the potential interference by the 439-keV gamma ray of ^{23}Ne (half-life = 37.6 s) in total milk produced by the ^{24}Na(n,p)^{23}Ne reaction was minimized by using a high-resolution Ge detector and by allowing the samples to decay for 2 min. As a result this source of error was also considered negligible.

7.2.3.5 Uncertainty calculation of the counting step

The calculations of the standard uncertainty in this step are summarized in Tables 7.12 and 7.13 shown in the next two pages.

Table 7.12. Sources of error and calculation of the standard uncertainty in the counting step.

Sample (n=5)	Mean value	Standard deviation	Relative standard uncertainty
Repeatability of the sample: mean value refers to iodine concentration ($\mu g.ml^{-1}$) **Repeatability of the Standards: mean value refers to iodine sensitivity (counts/μg)**			
Total milk	0.457	0.005	4.893×10^{-3}
Lipids	0.013	0.003	0.103
Total proteins	0.034	0.003	0.039
Casein	0.026	0.001	0.017
Iodide	0.333	0.003	4.029×10^{-3}
Iodate	0.064	0.005	0.035
Total inorganic	0.397	0.004	4.505×10^{-3}
Standard (INAA-CSS) 5-2-10 min	1686	4	1.061×10^{-3}
Standard (INAA-GeLi) 10-2-10 min	6626	143	9.65×10^{-3}
Standard (ENAA-GeLi) 5-2-10	274	7	0.011
Counting statistics			
Total milk			0.021
Lipids			0.019
Total proteins			0.026
Casein			0.024
Iodide			9.21×10^{-3}
Iodate			0.034
Total inorganic			3.34×10^{-3}
Standard (INAA-CSS) 5-2-10 min			6.29×10^{-3}
Standard (INAA-GeLi) 10-2-10 min			3.17×10^{-3}
Standard (ENAA-GeLi) 5-2-10			0.016
All samples and standards			
Gamma-ray shielding	Negligible		
Decay correction	Negligible		
Counting geometry	Negligible		
Dead time correction	Negligible		
Pulse pile up	Negligible		
Gamma-ray interferences	Negligible		
Peak integration method	Negligible		
Blank and blank correction	Negligible		

Table 7.13. Combined relative standard uncertainty in the post-irradiation step.

Sample	Combined rel. std. uncertainty
Total milk	0.0220
Lipids	0.1052
Total Proteins	0.0470
Casein	0.0310
Iodide	0.0219
Iodate	0.0532
Total inorganic	0.0202

7.2.4 Calculation of expanded uncertainty

Finally, the calculations of the expanded uncertainty for the determination of iodine in total milk and its various fractions are shown in Table 7.14.

Table 7.14. Calculation of the expanded uncertainty in iodine milk species.

	Pre-irradiation	Irradiation	Counting	Combined relative standard uncertainty	Expanded uncertainty, % (K=2)
Total milk	2.33×10^{-3}	3.78×10^{-3}	0.0220	0.0224	4.5
Lipids	2.33×10^{-3}	3.63×10^{-3}	0.1052	0.1052	21.0
Proteins	2.33×10^{-3}	3.63×10^{-3}	0.0470	0.047	9.4
Casein	2.33×10^{-3}	3.63×10^{-3}	0.0310	0.0313	6.2
Iodide	2.33×10^{-3}	0.05	0.0219	0.0546	11.0
Iodate	2.33×10^{-3}	0.05	0.0532	0.0730	14.6
Total Inorganic	2.33×10^{-3}	0.05	0.0202	0.0540	11.0

Table 7.14 shows that the counting statistics step contributed most to the expanded uncertainty values of total milk, lipids and proteins. The expanded uncertainty for these samples could be improved significantly if higher numbers of count are

accumulated. For instance, if the number of counts in the total milk determination was doubled then the percent expanded uncertainty would be reduced from 4.5 to 3.5. The increased number of counts can be easily obtained by PCINAA-CS, as explained in Chapter 3. For an expanded uncertainty of 1%, one needs 10 000 counts which can be reached by either running the sample in the DUSR facility for 25 cycles by PCINAA-CS or by using higher flux in another reactor facility.

In the case of iodide and iodate ions, and total inorganic iodine, the irradiation step contributed most to the expanded uncertainty. This observation was unexpected. Perhaps the resin was a major source of uncertainty due to the error in neutron scattering effect. This aspect needs to be studied in more detail.

In this work the k_o-NAA method for quantification was also used. An analysis of the uncertainty in the irradiation step of this method needed to be considered. The uncertainty associated with the pre-irradiation step in the k_o-NAA method was assumed to be the same as that in the relative method. Although in k_o-NAA there is no elemental comparator standard as such, there is a gold flux monitor. It was assumed that the uncertainty associated with the preparation of the gold monitor was similar to that for the preparation of the elemental comparator standard in the relative method.

However, in the irradiation step the situation is different because the k_o-NAA method not only includes the parameters already taken into account in the relative method but also other parameters, such as reactor neutron flux and the resonance neutron energies, thermal and resonance integral cross sections, half-lives of iodine and gold, which are beyond the control of this experimenter. Moreover, some of these parameters have high uncertainty values. For example, some of the parameters involved in the k_o-NAA method are shown in Tables 7.14 and 7.15. Therefore, the combined standard uncertainty in this step would be higher than that in the relative method.

Table 7.15. Thermal and integral cross sections, and half-lives of ^{128}I and ^{198}Au.

Parameters	**^{198}Au**	**^{128}I**
Thermal cross section (b)	98.8 ± 0.3	6.2 ± 0.2
Resonance integral cross section (b)	1560 ± 40	147 ± 6
Half-life	2.69571 ± 0.00021 d	24.99 ± 0.02 min

Table 7.16. Reactor flux parameters used in the k_o-NAA method.

Reactor parameter	**Inner positions**	**Outer positions**
α	-0.0427 ± 0.0057	-0.0098 ± 0.0045
f	18.9 ± 0.5	57.1 ± 2.2

The following equation was used for the calculation of the concentrations in the k_o-NAA method (equation 22, Chapter 3).

$$C(\mu g.g^{-1}) = \frac{\dfrac{P_A}{S.D.C.M_s}}{\dfrac{P_{A,m}}{S_m.D_m.C_m.m_m}} \cdot \frac{1}{k_{0,\exp}} \cdot \frac{f + Q_{0,m}(\alpha)}{f + Q_0(\alpha)} \cdot \frac{\varepsilon_{P,m}}{\varepsilon_P}$$

The error associated with the parameter P was taken into account in the counting step. The error associated with the efficiency *vs.* energy polynomial was deemed negligible. The remaining parameters were then considered. The error associated with the parameters S, D, and C was actually the error associated with the half-life of the isotope and the relative standard uncertainty value used for the k_o parameter is 4%. Table 7.17 shows the standard uncertainty associated with this method that was not part of the relative method.

Table 7.17. Calculation of the standard uncertainty in k_o-NAA method.

Parameter	**Relative standard uncertainty**
S (^{128}I), D(^{128}I), C(^{128}I)	8.00×10^{-4} (for each)
S (^{198}Au), D(^{198}Au), C(^{198}Au)	7.79×10^{-5} (for each)
f (both isotopes)	0.026
Q_o(^{128}I)	0.143
Q_o(^{198}Au)	0.136
Combined relative standard uncertainty for k_o value	0.04
Combined relative standard uncertainty for ^{128}I	0.145
Combined relative standard uncertainty for ^{198}Au	0.138
Combined relative standard uncertainty (n=5)	0.09
Expanded uncertainty	**0.18**

The expanded uncertainty associated with the k_o method in comparison to the relative method introduced at least an extra 18% error in the irradiation step.

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CHAPTER 8

CONCLUSIONS AND FUTURE WORK

The primary objective of this thesis was to do speciation analysis of iodine in bovine milk. In order to achieve this objective several other objectives had to be satisfied first by developing appropriate methods. These involved: (1) development of NAA methods for the determination of total as well as species of iodine in milk with high precision, accuracy and sensitivity, low detection limits, and freedom from interference; (2) separation and determination of various components of milk, such as vitamins, carbohydrates, water, minerals, proteins, and lipids; (3) separation of inorganic iodine species, namely iodide and iodate ions; (4) development of simultaneous and sequential schemes for the separation and determination of total organic, lipid-bound, protein-bound, and inorganic anionic iodine species as well as iodide and iodate ions, casein and whey proteins containing iodine, and fractionation of apolar, less polar and polar lipid-bound iodine; and (5) detailed calculation of expanded uncertainty of the methods developed.

A thorough literature survey was done to understand the problem of speciation analysis and the scope of the present work. The survey was presented in Chapter 2. Chapter 3 was devoted to the total iodine determination in milk by neutron activation analysis (NAA). Three different types of NAA were used, namely instrumental neutron activation analysis (INAA), epithermal INAA (EINAA), and pseudo-cyclic INAA (PCINAA). In general the iodine determination could be reliably performed with any of them but the detection limit was improved accordingly depending on the irradiation-decay-counting scheme. Using the PCINAA approach a detection limit of 20 $ng.g^{-1}$ was achieved after 6 cycles using a scheme of 5-6-15 min. This value could be improved if PCINAA was done with epithermal neutrons and longer irradiation and counting times. In this chapter, a detailed rationale about the quantification method

known as k_o-NAA was given. Results obtained from this method did not differ from those using the classical relative method.

Chapter 4 included the determination and in some cases the separation of the major milk components such as lipids, proteins, carbohydrates and water. The lipid determination was a rather difficult task due to the lack of a general method for quantification. Gravimetry is one of the most common methods and it was the chosen in this work. The main handicap of this method was the assumption that all lipids were extracted in the solvent system used. In order to choose a convenient method for the gravimetric determination of lipids, 4 organic solvent systems were tested and the isopropanol-hexane solvent system using a ratio of 1:1 proposed by Indrasena of our group was selected. The efficiency of extraction of the lipids was 100% assuming that the reference method actually extracted all the lipids present in the sample. In order to estimate the extraction efficiency of the selected solvent system in a different way, an indirect method for lipid quantification was implemented. The contents of proteins, carbohydrate, major minerals and water were determined and the lipid content was estimated using a mass balance equation. The extraction efficiency of the lipids was around 80% using this indirect method.

The determination of proteins was also described in Chapter 4. It is customary to determine proteins using spectrophotometric methods. In this work different colorimetric methods were tested using different protein standards. The use of casein as standard protein allowed the total protein determination regardless of the colorimetric method. In the case of whey proteins, only the Biuret method could be used to reliably assess the protein content. Casein was then estimated by subtraction of whey protein from the total protein content. As a result, the typical range of total protein was 32-34 $mg.g^{-1}$ in homogenized milk where casein content was 79% of the total protein and whey proteins accounted for the remaining 21%. Carbohydrates were also determined by a spectrophotometric method and the typical range was 40-46 $mg.g^{-1}$. The water content was determined by lyophylization and found to be in the range of 83-85%.

The total protein separation was achieved using a saturated ammonium sulfate solution. Casein was separated by precipitation as well, but in this case the precipitation occurred when the pH of the solution was reduced to 4.6.

The method for the fractionation of lipids to different classes using solid phase extraction cartridges was discussed in Chapter 4. The first fractionation method used a silica SPE-Cartridge and it divided the total lipid extracts into three classes, namely apolar, less polar and polar, with mass distributions of 96, 3.6 and 0.18%, respectively. The second fractionation method used a Florosil SPE-cartridge and sub-classified the apolar lipids into 4 groups: hydrocarbons, triacylglycerides, mono and di-acylglycerides, and free fatty acids where the mass distributions were 95, 2.4, 0.32 and 0.53 %, respectively.

Chapter 5 was dedicated to study the separation of inorganic iodine species in milk. Ion exchange separation methods were implemented for this purpose. In general, column methods worked better than batch separation methods. Dowex anion and cation exchange resins were carefully cleaned and converted to OH^- and NH_4^+ forms, respectively. Iodide and iodate ions were retained onto the anionic resin while any possible cationic iodine species was expected to be retained on the cationic resin. The anionic resin was directly irradiated for quantification of iodine. The estimation of the cationic iodine species was performed by subtracting the iodine concentration of the solution after being in contact with the cationic resin from the iodine concentration of the original milk. Inorganic anionic iodine species were about 85-90% of the total iodine. There was no indication of any cationic iodine species in milk. The iodide and iodate were separated using the same kind of resin but converted to NO_3^- form. Under this condition, iodide ion was completely retained on the resin while iodate ion was not. Iodate ion was then reduced to iodide ions by means of hydrazine sulfate and retained on a fresh resin.

The development of a number of schemes for the separation of milk components using a sequential approach was described in Chapter 6. Their advantages

and disadvantages were evaluated in each case. Depending on the component, different INAA methods were used for iodine quantification. Although EINAA could be used for the determination of the total iodine in milk, an INAA scheme of t_i-t_d-t_c of 5-2-10 min was used to save time. The INAA scheme of 10-2-10 min was used for lipid extracts, all lipid fractions, total protein and casein. All anionic inorganic separations were performed using an EINAA scheme of 5-2-10 min. The iodine content of the sample after being passed through the Dowex anionic resin was performed by PCINAA scheme of 5-2-10. The use of epithermal neutron combined with PCINAA would have improved the detection limits but longer irradiation and counting times were required. In general about 10% of iodine in homogenized milk was bound to organic compounds such as lipids and proteins, where most of it was associated with proteins. The rest of it was anionic inorganic species where iodide ion was the major component.

Chapter 7 was devoted to the calculation of the expanded uncertainty of the relative NAA method developed in this thesis. The major contributor to the expanded uncertainty was in general the post-irradiation step where the counting statistics accounted for most of the uncertainty. However, in the case of the species determined by irradiation of the resin, the decisive step in the uncertainty calculation was the irradiation step due to the scattering process. This result was unexpected.

Improvement of the counting statistics in a sample implies either increases of sample mass, irradiation and/or counting time, or neutron flux. These processes could also produce higher dead times and longer analysis times. Improving the uncertainty due to neutron scattering could be done by either reducing the mass of resin irradiated or extracting iodine from the resin prior irradiation. This second option would need a careful handling of the sample. Although the expanded uncertainty calculation using k_o-NAA as a quantification method was not done, a qualitative analysis concluded that in fact this method had a bigger uncertainty.

In order to complete this work, a more detailed study on the lipid fractions should be undertaken. The possible presence of non-naturally occurring organohalogen

compounds should be analyzed, and if present their identification should be almost mandatory. A better insight into the protein fraction should also be carried out. The expanded uncertainty can be improved especially if the uncertainty associated with neutron scattering in the resin is re-evaluated.

CHAPTER 9
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