PROXIMATE ANALYSIS OF FISH TISSUE BY MID-INFRARED TRANSMISSION SPECTROSCOPY

M.Sc. Thesis

by

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ABSTRACT

PROXIMATE ANALYSIS OF FISH TISSUE BY MID-INFRARED TRANSMISSION SPECTROSCOPY

Mid-infrared spectroscopy was assessed as a means for rapidly determining the fat, protein and moisture content of fish tissues. For fat and protein, a sample preparation protocol was developed for the conversion of fish tissue into a milk-like emulsion for analysis by a Multispec MK1 infrared milk analyser. Fish (cod, tuna and mackerel) were minced, dissolved and emulsified in 0.2N NaOH using a Brinkmann Polytron and analysed for fat and protein using the conventional fat and protein wavelengths used for milk analysis. Instrument calibration for fat and protein was based on the Mojonnier and Kjeldahl procedures respectively. Moisture was determined separately by extracting the water from the minced tissue using absolute methanol and measuring the absorbance of the resulting solution using a 5.56/6.02 um filter pair. The instrument was calibrated using standardized water/methanol mixtures and the results compared to the standard vacuum oven method. Rapid analyses of fat and protein were possible giving individual results to within 1% of the standard chemical determinations. Moisture analysis also worked well, being more precise and rapid than the vacuum oven procedure. The limiting factor in the use of Mid-IR spectroscopy for proximate analysis appears to be a combination of sample compositional variation and/or the general reproducibility of the commonly employed standard methods. It was concluded that the analysis of fish by mid-infrared transmission spectroscopy could serve as a means for rapidly carrying out the proximate analysis of fish and fish products for quality control purposes.

RESUME

ANALYSE APPROXIMATIVE DES TISSUS DE POISSONS

PAR LA SPECTROSCOPIE DE TRANSMISSION PAR MID-INFRAROUGE

La Spectroscopie par Mid-infrarouge a été evaluée comme moyen rapide d'évaluation du contenu en gras, en protéines et en humidité des tissus de poissons. Pour le gras et les protéines, un protocole pour la préparation d'échantillons a été développé pour la conversion des tissus de poissons en une émulsion crémeuse à être analysée par un analyseur de lait par infrarouge, le Multispec MK1. Les poissons (morue, thon et maquereau) ont été hachés, dissouts et emulsifiés avec du 0.2N NaOH utilisant le Polytron Brinkmann et analysés pour le gras et les protéines selon les longueurs d'ondes conventionnelles utilisees pour l'analyse du lait. La calibration de l'appareil est basée sur les méthodes Mojonnier et Kjeldahl respectivement. L'humidite a ete determinee separement, extrayant l'eau des tissus de poissons utilisant du methanol pur et mesurant l'absorption des solutions obtenues par l'utilisation d'une paire de filtres 5.56/6.02 µm. La calibration de l'appareil a été faite selon les mélanges standards eau/methanol et les resultats comparés a ceux de la methode standard du four sous vide. L'analyse rapide du gras et des protéines a été possible donnant des resultats en deca de 1% des méthodes chimiques standards. L'analyse de l'humidité a aussi bien réussi, étant plus precise et rapide que la méthode du four sous vide. Le facteur limitant son utilisation pour les analyses approximatives semble être une combinaison d'échantillons de composition différente et/ou la reproducibilité générale des méthodes conventionnelles. Il a été conclu que l'analyse des poissons par spectroscopie de transmission Mid-IR pourrait servir comme moyen d'évaluation approximative rapide pour les poissons et leurs sous-produits aux fins du controle de la qualité.

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CHAPTER 1

INTRODUCTION

The successful application of mid-infrared transmission spectroscopy to the rapid and quantitative analysis of milk has had a tremendous impact on the dairy industry in the industrialized world (Biggs 1967, Grappin and Jeunet 1976). The speed of multicomponent analysis for fat, protein and lactose along with its sound correlation with the more time consuming standard chemical methods has led to its world-wide adoption for producer payment (van de Voort 1980) and dairy herd improvement work (van de Voort et al. 1987). Aqueous infrared transmission spectroscopy is based on the absorption of infrared energy at specific wavelengths by functional groups such as the carbonyl group in the ester linkage (5.73/5.58 μm) of fat or the peptide bond (6.46/6.68 μm) of proteins. Although water interferes significantly in the mid-infrared region of the spectrum, modern interference filter instrumentation, using a narrow (~40 μm) calcium fluoride cell, a short optical path and sensitive infrared detectors are capable of overcoming this formerly formidable limitation. To date, these instruments have been specifically designed for milk analysis; however, the potential exists to expand the use of this methodology to other food systems. Some of the applications investigated include the analysis of fat and protein in meat products (Mills et al. 1983, 1984), the study of sugars (Mills et al. 1986), ammonia in Kjeldahl distillates (van de Voort et al. 1986) and the development of a method of moisture analysis (van de Voort et al.1988). Canada is a leading world exporter of fish and is in the

process of diversifying its processing operations to produce more value added products rather than simply exporting the raw material. The success of surimi and related products has led to the concept of using fish tissue as a base component for producing new value added products and offers new ways to make use of underutilized species. Mechanical deboners and more efficient processing techniques have also reduced waste and allowed more fish flesh to be recovered, which in turn can be formulated into products such as fish patties, fish balls and other reformed products. The moisture content of fish affects it stability, processing characteristics and retail value and can vary substantially with season and reproductive cycle (Love 1976). The fat portion is a complex mixture of triglycerides, phospholipids and other minor lipid constituents, the total content of which is also highly variable and its determination is crucial for the assessment of fish stability (susceptibility to autoxidation) during frozen storage, adjustments for processing requirements (canning) and nutritional labelling (Anon 1979). Protein is the main functional and nutritional component of fish tissue and also varies considerably within and between species (Konosu et al. 1974). The manufacture of formulated processed products requires more stringent raw material quality control assessment and most commonly involves the analysis for fat, protein and moisture. Conventional methods for the determination of these components, i.e., vacuum oven for moisture, the Bligh and Dyer method (1959) for fat and the Kjeldahl (Concon et al. 1973) for protein are slow and time consuming.

The proximate analysis of food products, including fish, is an ongoing concern and expense in the food industry. Previous work had shown that mid-infrared transmission analysis could be used to determine fat and protein in raw and processed meat (Bjarno, 1981, 1982, Mills et al. 1984). The success of surimi, its related products, and the more efficient use of mechanically deboned fish tissue in formulated products requires that more consideration be given to ensuring and/or adjusting the uniformity or composition of the raw material. Substantial research is being done to simplify or automate proximate analyses of food to minimize the labour and time factors associated with the traditional standard methods. Near infrared spectroscopy has made the greatest strides in this regard (Williams and Norris 1987), while mid-infrared spectroscopy has dominated milk analysis, but has not seen widespread development for other products. The mid-infrared portion of the spectrum has the advantage of being more specific in its measurement, since it is based on the measurement of fundamental wavelengths rather than overtones (van de Voort, 1980). Clearly sample preparation is a potential limitation, however the conversion of samples to a milk-like emulsion is not complicated and is possible for many food systems. This thesis reports on the development, assessment and application of mid-infrared transmission spectroscopy as a means of providing proximate analysis (fat, protein and moisture) data for fish tissue.

CHAPTER II

LITERATURE REVIEW

2.1 IR SPECTROSCOPY OF AQUEOUS SYSTEMS

Historically, IR spectroscopy originated in 1800 with Sir William Herschel's work and his recognition of considerable heating effect beyond the red area of the visible spectrum. Using thermometry, he was the first to prepare an infrared spectrum, by plotting temperature of IR radiation passing through a sample as a function of wavelengths, and the term "infrared" is a product of his observations. Today, the infrared region is considered to lie between the visible and microwave region, taking into consideration the wavelengths from 0.7 microns to 100 microns. Some 100 years later, William W. Coblentz (1908) carried out systematic studies of infrared absorption of various compounds and prepared a collection of IR spectra over the 2.5 and 15 um range and established the link between molecular structure and the infrared spectra. Infrared analysis languished for many years because of instrumental limitations, until the development of double-beamed spectrophotometer equipped with choppers which compensated for the solvent spectra and detector response to environmental temperature changes (Lacomte 1948).

Instrumental advances allowed extensive headway to be made in relating functional groups to specific frequencies and the elucidation of molecular structures. Most IR spectroscopic work was restricted to mulls and compounds which could be dissolved in IR transparent solvents such as CCl_4 and H_2S.

Water was not considered as a solvent for infrared analysis due to its strong, broad absorption bands which obscured the absorption bands of the components to be analysed and due to the limitations of salt windows. Some preliminary work was however attempted and Gore (1949) published aqueous infrared spectra of a variety of amino acids. In 1953, Lenormant et al. obtained spectra of plasma protein solutions using a double-beamed spectrophotometer which compensated for solvent absorption by the placement of the same solvent in the reference beam. The same group published a series of IR spectra of biological polymers in water and deuterium oxide solutions. This work proved that with proper instrumentation, aqueous IR spectra could be obtained from aqueous solutions in the region of 6.5 to 10μm.

In 1954, Pyler and Acquista published spectra of water, which revealed that the area between 6.5μm-10μm was capable of sufficient transmission for quantitative analysis. In 1956, Potts et al. developed one of the first permanent barium fluoride cells with a pathlength of 0.027mm which was durable, could be polished, was insoluble in water and IR transparent up to 12.5μm. Scientists at Dow

Chemical Co. utilized these cells in a double-beamed spectrophotometer that they designed and obtained quantitative spectra of organic acids, glycols, and other compounds which could only be analyzed in aqueous solution. Due to the energy lost via water absorption, both the entrance and exit slits were widened by $(I/T)^{0.5}$, where T is the percent transmission of water. In 1955, Kaye published details pertaining to infrared transmission, water solubility and refractive index of a series of useful window materials. Another further advance was the development of diffraction grating initially incorporated into the Grubb Parsons GS2 grating specrophotometer, designed by Martin (1956).

A major contributor to aqueous IR spectroscopy was Goulden (1959a) who made substantial headway using barium fluoride cells to obtain spectra of carbohydrates, amino acids, and other biological compounds, including lactose and inorganic salts. In 1965, Thompson developed techniques for preparing cells having a pathlength of 0.4-1.7μm to reduce the effect of water absorption.

The use of IR spectroscopy in the field of food analysis was limited as a result of difficulties involved in sample preparation as well as the fact that water is a constituent of most food systems. In 1959, Goulden, an innovator in the application of quantitative analysis, measured lactose in milk within an accuracy of 1.5%. In 1961, he noted that under favourable conditions, infrared spectra of homogenized milk revealed major absorption peaks at 5.8, 6.5 and

9.6μm. These peaks were related to fat (carbonyl ester or C=O linkage), protein (peptide or CONH linkage) and lactose (hydroxyl or OH group) absorbance bands respectively. Goulden noted that, with the exception of the fat absorbance affecting the 6.5μm protein band, the absorbancies at 5.8 and 9.6μm were directly proportional to the fat and lactose concentrations. Improved protein results could be obtained by using a correction factor based on fat. He also demonstrated that quantitative IR measurements of milk samples would only be accurate if the effects of light scattering were minimized via proper homogenization of the fat. Goulden utilized his research results to design a commercial infrared milk analyser (IRMA). This instrument was commercially produced by Grubb-Parsons of (England) in 1964 and capable of determining fat, protein, lactose and solid non-fat (SNF) in less than 1 minute per sample.

In 1967, one of the first prototypes was sent to Guelph, Ontario, Canada, where Biggs (1967) was involved in evaluating the instrumentation. This situation came about due to the fact that dairy plants were paying farmers on the basis of the Babcock and there were complaints that the test results were biased in favour of the processor plants. Biggs determined a number of variables to be important in terms of accuracy and reproducibility of the instrument, including homogenization efficiency, wavelength accuracy, adequacy of cell purging and instrument temperature control. Methods were developed to control these factors and the precision of measurement was improved to +/- 0.03%, and standard deviation between infrared and

the chemical methods were +/- 0.06%, +/- 0.07% and +/- 0.06% for fat, protein and lactose respectively. In 1967, the first production line instruments (called IRMA's), modified as per Biggs's work, purchased and installed in Ontario and the CMTL (The Central Milk Testing Laboratory) became the first central milk testing laboratory in the world.

Shields (1968), the designer of the IRMA, joined Foss Electric Ltd in 1968 and designed a prototype infrared analyser based on multilayer dielectric interference filters rather than a grating. The instrument made use of a single cell, dual wavelength system using optical filters to isolate the specific wavelengths absorbed by the fat, protein, lactose and total solids. Reference filters were based on wavelengths not absorbed by these components. This approach eliminated the need for a diffraction grating, a reference cell and led to the development of the Milkoscan 300 and 203 single cell dual wavelength IR milk analysers (Foss Electric Ltd, Denmark).

Biggs (1972) continued to study the IR method for milk analysis and showed that the IRMA methodology was as efficient and precise relative to the standard methods; the Babcock for fat, Kjeldahl for protein and polarimetry for lactose. In 1979a, Biggs developed performance specifications for the infrared milk analysers including homogenizing efficiency, the effects of moisture in the instrument and the quality of the milks used for calibration.

In 1980, van de Voort evaluated the Milkoscan 104 using selected herd milk and found that the instrument met the AOAC specifications for accuracy and reproducibility for fat, protein and lactose. Later, as founder of Multispec Instruments (Berwind Instruments Group, Birmingham), Shields introduced the Multispec MK1. In this instrument, pairs of filters (sample and reference) on a disk alternately intercepted a beam from the chopper and passed it through the sample to be analysed. The transmission wavelength of the sample filter was made to coincide with the major absorption band of the component of interest and the reference filter was set to a neighboring wavelength where the absorption was minimal. By alternating the sample and reference beams via the chopper, two levels of energy reaching the detector could be compared. The logarithm of the difference in signal was directly proportional to the percent component in the sample which was transformed logarithmically and the results displayed directly in percent on a digital display. The advantages of this approach were the increased speed of analysis, the constant water concentration with the use of only one cell and the higher energy levels obtained with the wide band pass filters.

In 1982, Mills et al. expanded on the IR spectroscopic applications to food systems. They studied the CH stretch (3.4μm) filter to gauge the IR absorption of fat in aqueous solutions. They cited that the use of a CH filter, a CO filter or a mixture of both could compensate for fats having differing molecular weights and degrees of saturation.

Bjarno (1982) attempted to utilize the IR analysis methodology for meat products and based his calibration on milk samples, obtaining reasonable results for low resolution, quality control work. This work was expanded upon by Mills et al. (1984) who demonstrated that the IR instrumental method could produce quality analytical results for fat and protein in meat with a precision and an accuracy comparable to that of milk, as long as the calibration was based on the meat samples to be analysed. In terms of fat analyses, it was shown that the Mojonnier method was required as a calibration vehicle (Mills et al., 1983). Mills et al. (1986) also investigated the mid-infrared transmission spectroscopy of sugar solutions, using a specially designed IR spectrophotometer (Spectro-processor IV) to search for an isobestic point for multicomponent sugar analyses.

Van de Voort et al. (1986) carried out a study to determine if the ammonium ion could be quantitated in aqueous solution by utilizing commercial infrared filter equipment such as the Multispec MKI analyser. Based on this work, it was concluded that the IR method could obtain results with greater precision than the standard Kjeldahl distillation/titration procedure.

Van de Voort et al. (1987) studied the stability of record of performance milk samples for infrared milk analysis and showed that lipolysis affected the carbonyl fat (5.73/5.58μm) and protein (6.46/6.68μm) signals when lipolysis was the sole variable affecting

the milk system. As part of the same study, Ng-Kwai-Hang et al. (1988) carried out a collaborative study assessing the differences between IR analyses and the Babcock, Rose-Gottlieb and Kjeldahl as a function of milk quality.

In 1988, van de Voort et al. investigated the utility of the Multispec MK1 analyser as a means to quantitate moisture in selected food products using a new 6.02μm/5.56μm wavelength pair. The results indicated that commercial filter based mid-infrared instruments such as the Dairy Lab, Foss 104 and 605 milk analysers could be used for moisture analysis if such instruments were equipped with appropriate interference filters to carry out the determination.

The potential for the application of infrared analysis to a variety of analytical situations has been demonstrated and other applications are worth exploring. In Europe and Japan, many processed fish products (i.e. fish loaf, fish pudding, fish nuggets and fish pastes) are being marketed successfully. Similar potential exists in the Canadian fish processing industry, which requires product development technology and the corresponding development of more adequate quality control measures. Substantial research is being done to simplify or automate proximate analyses of food to minimize the labour and time factors associated with the traditional standard methods. Near infrared spectroscopy has made the greatest strides in this regard (Williams and Norris 1987), while mid-infrared spectroscopy has dominated milk analysis, but has not seen widespread

development for other products. The mid-infrared portion of the spectrum has the advantage of being more specific in its measurement, since it is based on the measurement of fundamental wavelengths rather than overtones (van de Voort 1980). The conversion of samples to a milk-like emulsion is not complicated and is possible for many food systems. This thesis reports on the development, assessment and application of mid-infrared transmission spectroscopy as a means of providing proximate analysis (fat, protein and moisture) data for fish tissue.

CHAPTER III

MATERIAL AND METHODS

3.1 Instrumentation

A Multispec MK1 infrared milk analyser, manufactured by Shields Instruments Ltd, York, England (Biggs 1979b) was used for this work (Figure 1), modified only by placing a water wavelength filter pair (van de Voort et al. 1988) in the CH stretch wavelength slot on the filter wheel (Mills et al. 1982). Schematic diagrams of the major components of the instrument are presented in Figures 2 and 3. The instrument was connected to a HP-85 micro-computer to process the uncorrected instrumental signals via a multi-component calibration equation into its final corrected form. After zeroing the instrument against water, the fat and protein channels were linearized using standard solutions of 1,4-butyrolactone and calcium propionate while for moisture, the instrument was zeroed against dry methanol and linearized using water/methanol mixtures.

3.2 Fat and Protein

Three types of fresh fish, tuna, mackerel and cod were purchased locally, minced twice at room temperature through a 3.2 mm plate, mixed thoroughly, subdivided into homogeneous packets of approximately 50g each and individually placed in zip-lock polyethylene bags (Figure 4). The sample bags were frozen in liquid air and stored at -18°C for

Figure 1. The Multispec MK1 with HP-85 microprocessor used for the proximate analysis of fish.

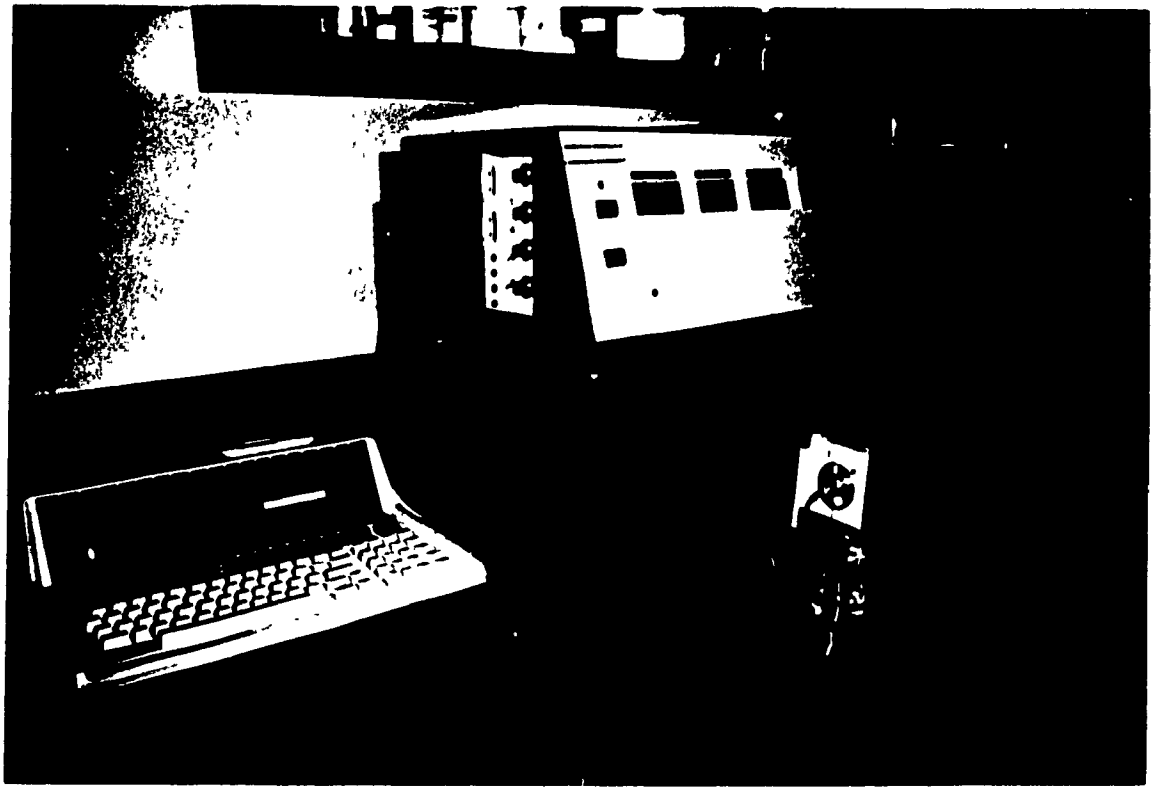


Figure 2. A schematic diagram of the major external components of the Multispec MK1, (Anonymous, 1985).

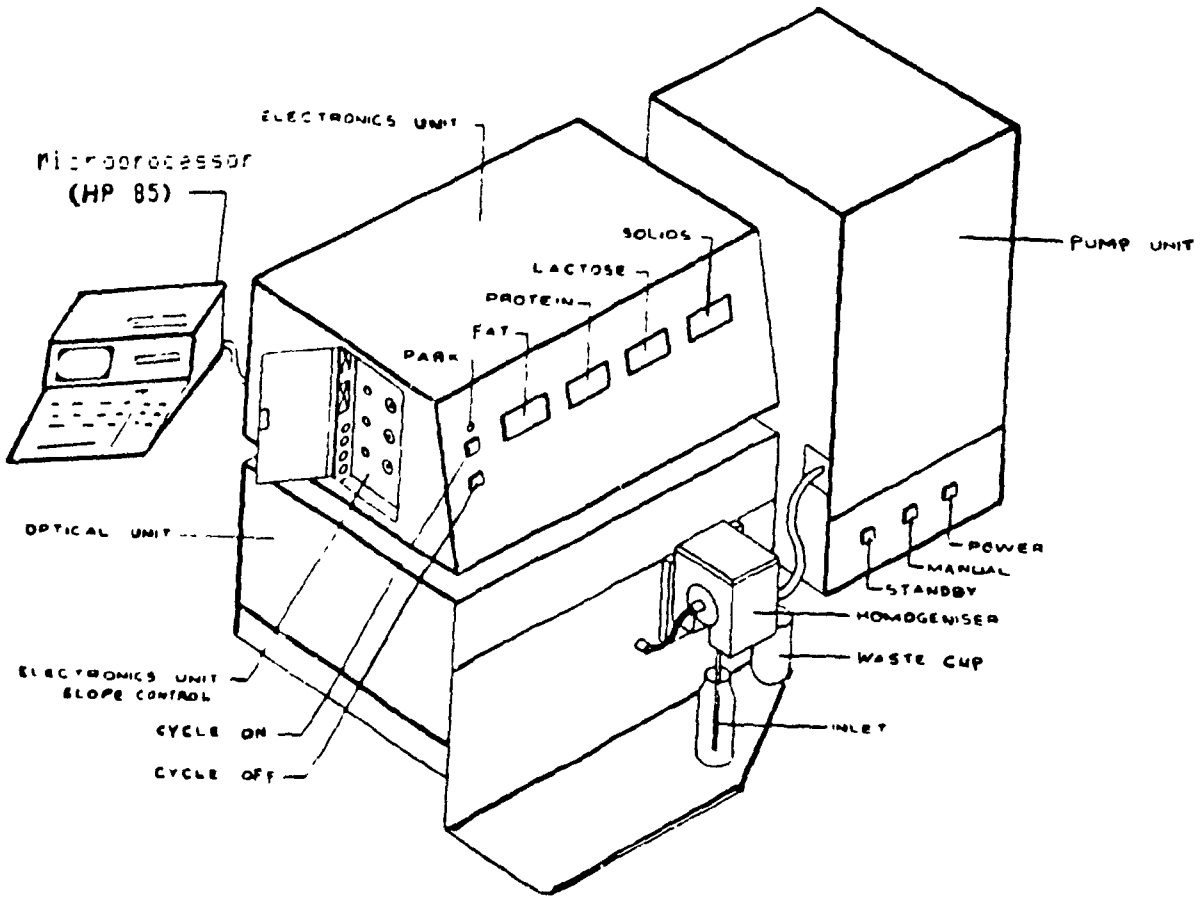


Figure 3. A schematic diagram of the optical, mechanical and logic components of the Multispec MK1, (Anonymous 1985).

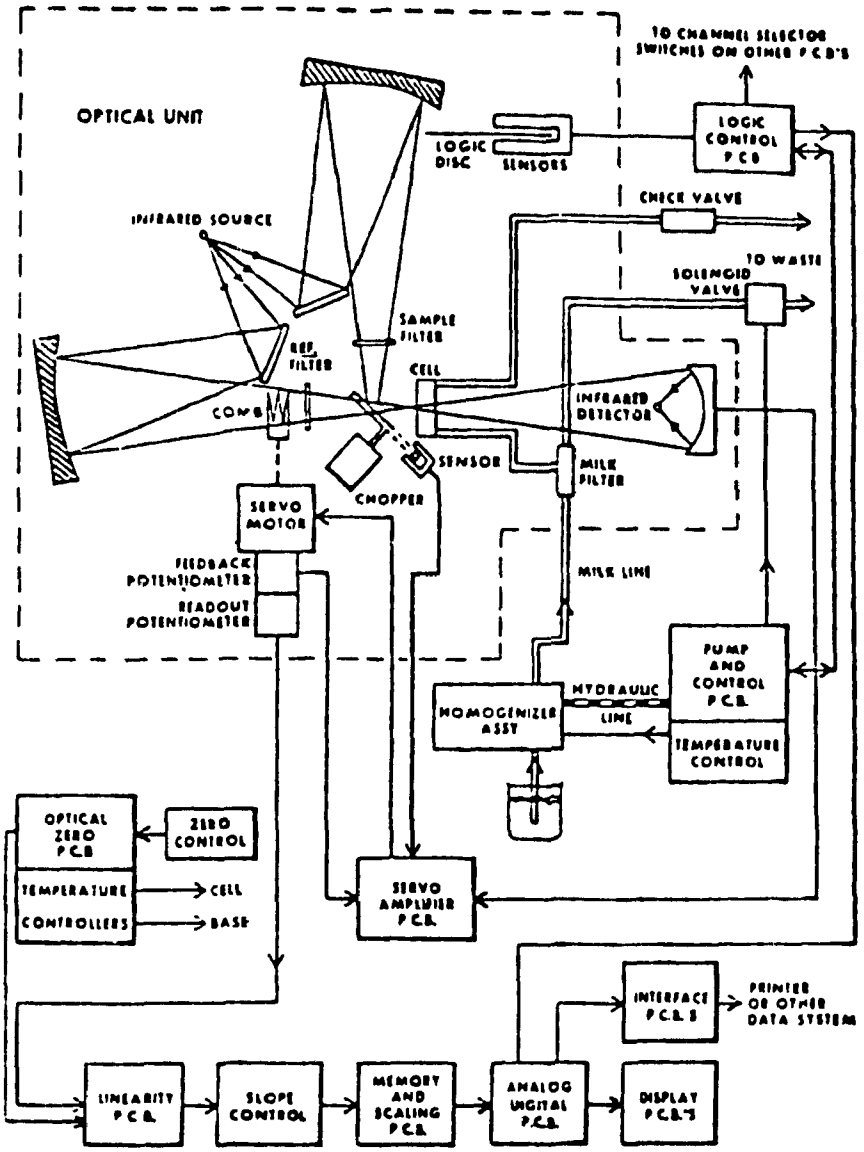
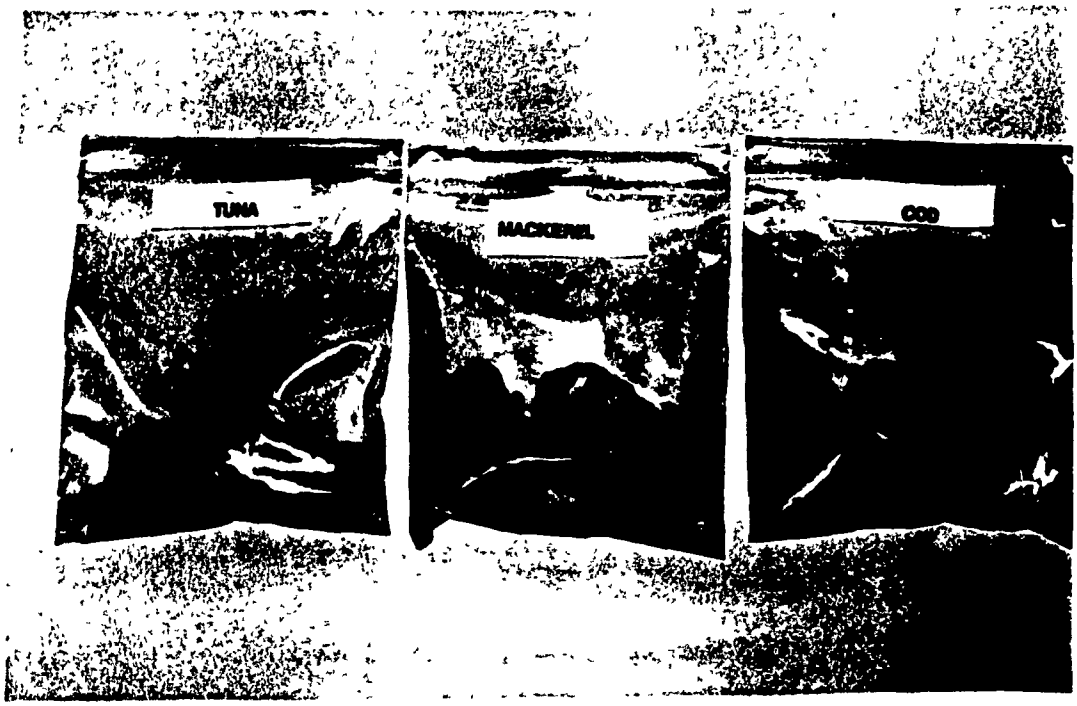


Figure 4. Samples of tuna, mackerel and cod frozen in liquid air and stored in zip-lock bags for future analysis.



use as required. For fat and protein analysis, the sample preparation protocol previously used for meat products (Mills et al. 1984) was modified slightly (0.2N NaOH and 85°C) to convert the minced fish tissue into a stable milk-like emulsion. The basic process involved the weighing of 20g of minced fish into a tared 500g beaker, the addition of one drop of a Dow Corning antifoaming agent, followed by ~150g of preheated (85°C) 0.2N NaOH, after which the tissue was disrupted and emulsified for four minutes at high speed using a Polytron (Brinkmann Instruments Ltd). After blending, the Polytron stem was rinsed with warm 0.2N NaOH using a squeeze bottle and the weight brought to 200g with the same solution. All operations were carried out on an accurate weight/weight basis (2 place top loading balance) to ensure simplicity of preparation and to maintain quantification. As a precaution to prevent any undissolved tissue from clogging the 40 um calcium fluoride cell, the sample was strained through a small (8cm diameter) stacked triple sieve set (sieve order 100, 150, 325 mesh respectively) held in a Buchner funnel to allow passage of the solution into a 300mL screw capped container. Figures 5 and 6 illustrate preparation set-up and sample preparation protocol respectively. Prior to analysis, the sample container was placed in a 40°C water bath to match the operating temperature of the infrared instrument, inverted several times to mix the sample and pumped through the instrument. The resulting emulsion was generally grey-brown to milk white in appearance depending on the fish used and was subsequently analysed in a manner similar to milk.

Figure 5. Sample preparation protocol for the analysis of fat and protein content of fish

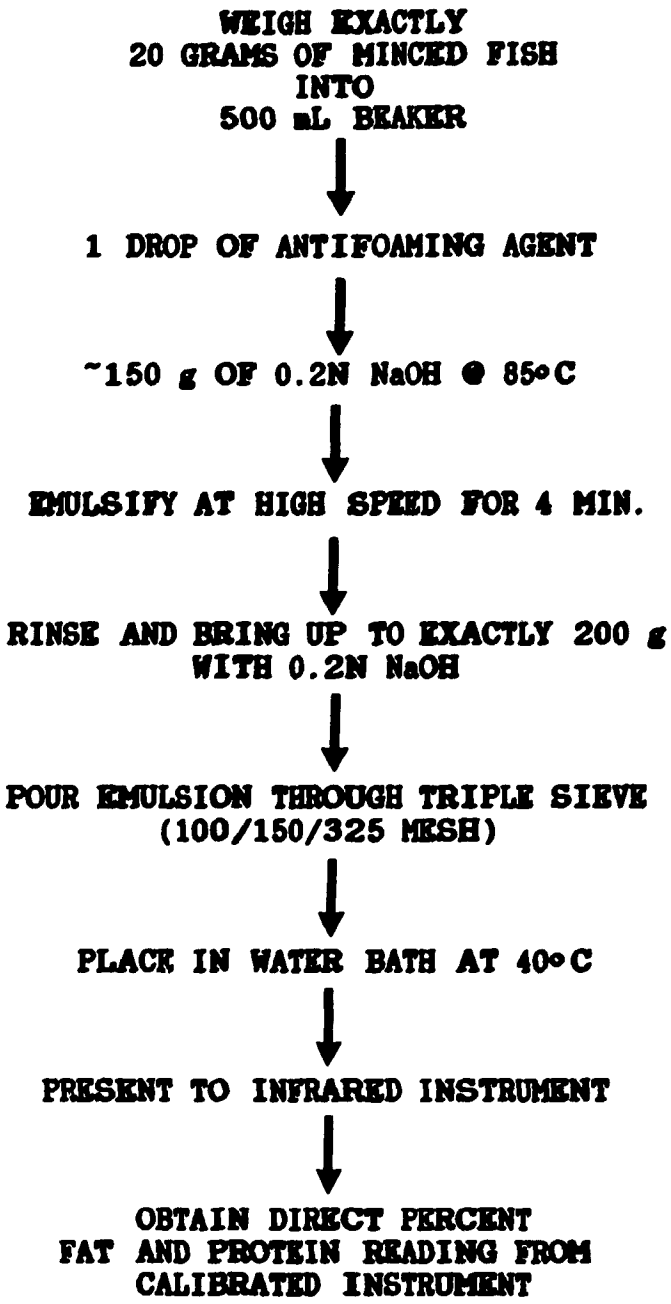
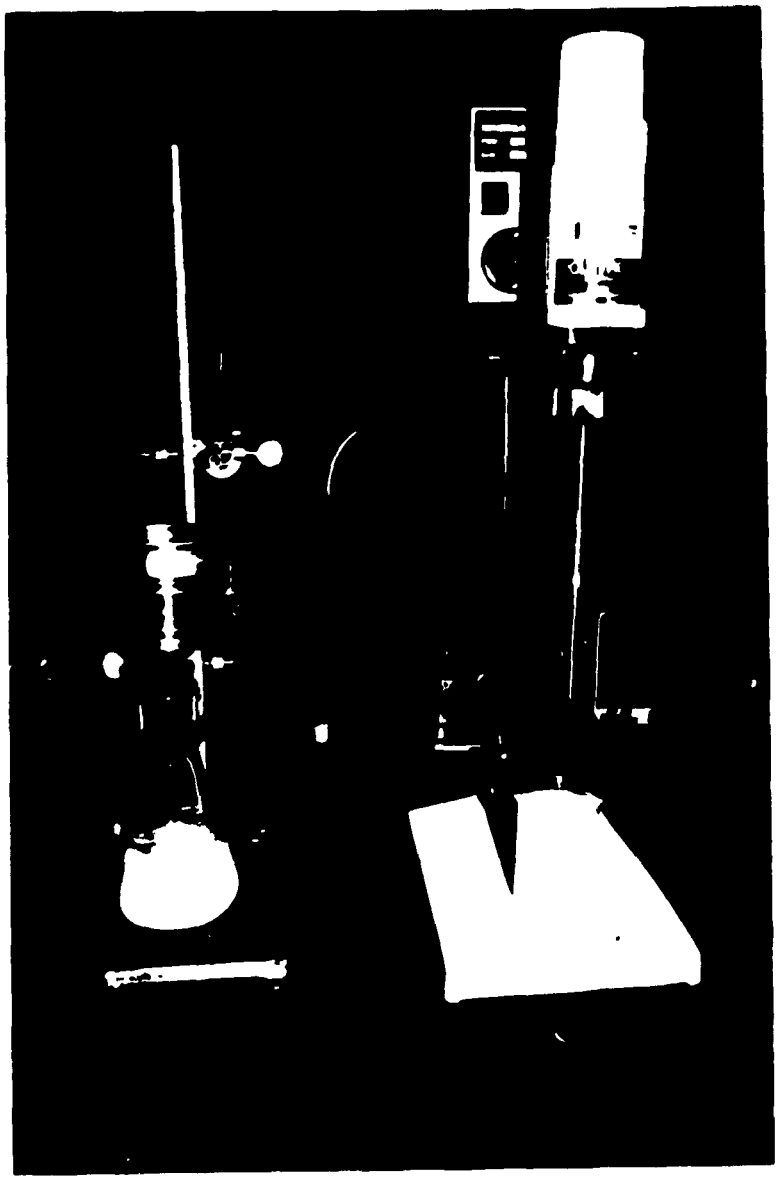
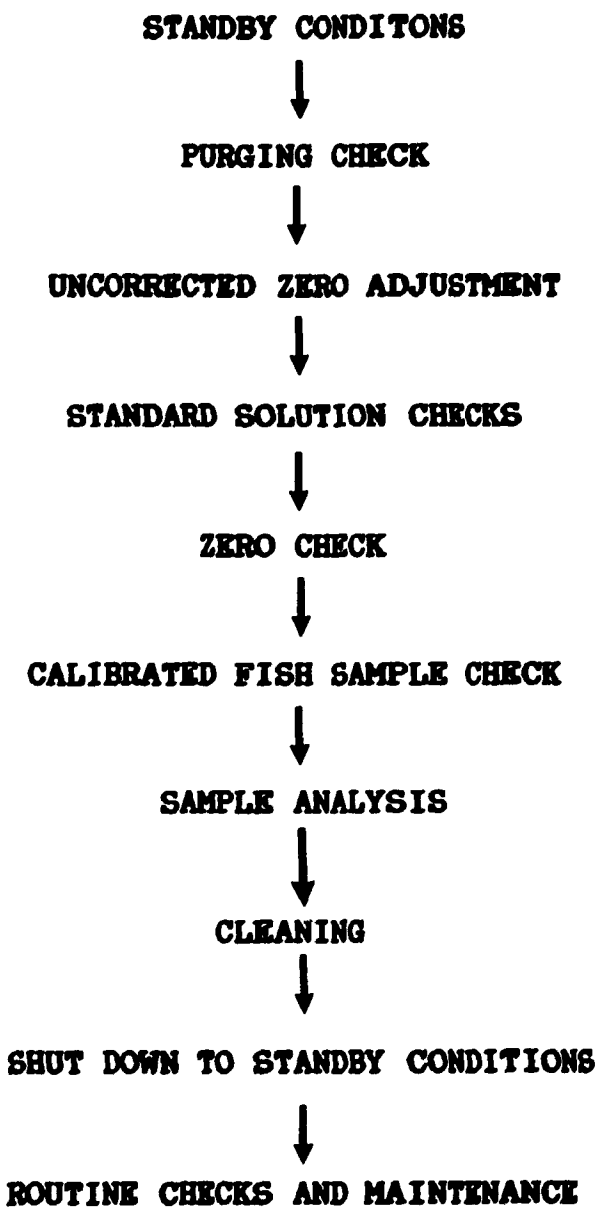


Figure 6. Polytron and stacked sieve set used for preparation of fish emulsions.



Because the infrared milk analyser is a secondary method of analysis, it requires calibration against primary standard methods (van de Voort 1980). The general protocol for standardization of the Multispec for routine operation is presented in Figure 7. To prepare appropriate material for calibration purposes, minced fish tissue was chemically analysed to determine its fat and protein content. Based on this information, the total solids content of the calibration solutions were adjusted and/or samples mixed to obtain a range of values for fat and protein. The chemical analyses for the calibration were carried out on the prepared emulsions rather than the tissue because previous work (Mills et al. 1984) indicated that direct tissue analyses were not sufficiently representative of the material presented to the instrument. Twelve chemically pre-analysed samples of each species (cod, tuna and mackerel) were used for the calibration (Biggs 1979a) which were split, with half the sample being assessed instrumentally, while the other half was analysed chemically. Protein determinations were done by micro-Kjeldahl (AOAC 1980) using a 6.25 conversion factor for nitrogen to protein while the fat content was determined by Mojonnier (Mills et al. 1982). The chemical data were processed in relation to the uncorrected instrument signals for fat and protein using multiple regression (Statgraphics 1985) to derive two multicomponent calibration equations, which in turn were used to calculate chemical estimates for the solutions analysed by the instrument. Comparative data analysis was carried out using the techniques outlined in the Statistical Manual of the AOAC (Youden and Steiner 1975).

Figure 7. Outline of the operational procedure for standardisation of the Multispec MK1 for analysis

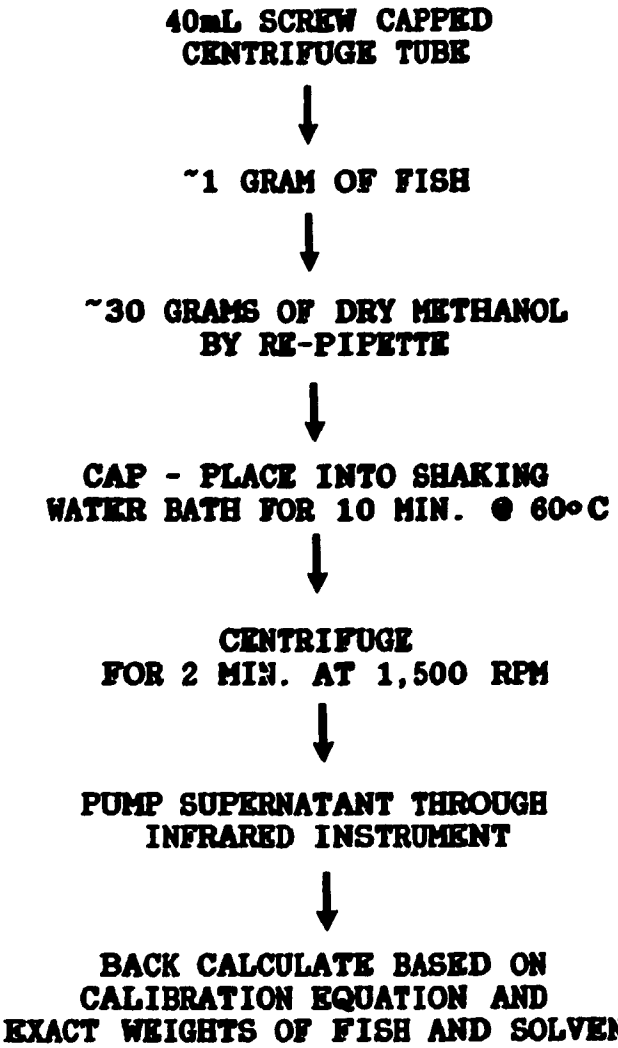


3.3 Moisture

For moisture analysis similar fish samples prepared for fat and protein analysis were used. After thawing, the well homogenized samples were split, four portions taken for moisture analysis by the vacuum oven method (AOAC 1980) and the remainder used for the methanol extraction procedure and subsequent infrared analysis. To produce fish samples which varied significantly from the original moisture content, selected samples were placed above saturated salt solutions in dessicators which generated specific relative humidities (van de Voort et al. 1988), which gradually adjusted the moisture content of the minced fish to new values. By using this technique, a wide range of moisture contents could be generated. To calibrate the infrared milk analyser for moisture measurements, calibration standards were prepared by adding distilled deionized water to absolute methanol (kept dry over molecular sieve) on a weight/weight basis (~0-10%), which in turn were stored in screw capped glass bottles for use as needed. The water channel was zeroed against absolute methanol, the standards pumped through the instrument and the resulting water signals recorded. The signals produced by the solutions were plotted against percent moisture to produce a standard curve.

For the extraction of moisture using dry methanol (Figure 8), a series of large (40mL) screw capped centrifuge tubes were prepared. To each, approximately one gram of fish was added, followed by ~30mL of dry methanol delivered by re-pipette, the test tube capped and

Figure 8. Sample preparation protocol for the extraction of moisture from fish samples and its subsequent analysis by Mid-IR spectroscopy.



fat placed for 10 minutes in a vigorously shaking water bath held at 60°C. The tubes were then removed from the water bath, centrifuged using a clinical centrifuge for 2 minutes at maximum speed (~1,500 rpm) to remove any suspended matter and the clear supernatant pumped through the infrared milk analyser (~4 pumpings). To ensure good quantification, all operations required the accurate recording of the weights of fish and methanol. By writing a short programm in APL (A Programming Language) which made use of the calibration equation derived and the weights of methanol and fish, the moisture content of the original sample was readily calculated.

CHAPTER IV

RESULTS AND DISCUSSION

4.1 Fat and Protein

The sample preparation protocol developed for meat products (Mills et al. 1984), modified only in terms of the concentration of base (0.2N NaOH vs. 0.1N) and an increase in solution temperature (85°C vs. 70°C) worked well in producing a homogeneous emulsion for the fish investigated. Some foaming was encountered; however, this was minimized by the use of a small amount of an antifoaming agent which was not found to interfere with the instrument signals at the levels used. Using multiple regression, the chemical data from the pre-analysed calibration samples were related to the uncorrected instrumental signals for fat and protein to derive two calibration equations used to calculate the instrumental chemical estimates, in terms of percent (weight/weight).

[1] $F_{ce} = 11.7950\ F_i + 1.9370\ P_i$

$r^2 = 0.994$
SEE = 0.878
MD = 0.038
SDD = 0.810

[2] $P_{ce} = 8.0435\ P_i + 0.5193\ F_i$

$r^2 = 0.999$
SEE = 0.674
MD = -0.026
SDD = 0.694

Where
F_{ce} = Fat instrumental chemical estimate
P_{ce} = Protein instrumental chemical estimate
F_i = Fat instrumental signal
P_i = Protein instrumental signal

r^2 = Correlation coefficient
SEE = Standard error of the estimate
MD = Mean difference between chemical data and instrumental estimate
SDD = Standard deviation of the difference between chemical and instrumental estimate

Figures 9 and 10 illustrate the relationship of the chemically estimated values (least squares regression line) obtained using equations [1] and [2] in relation to the chemical data for fat and protein respectively. This type of data has traditionally been assessed using the method proposed by Youden and Steiner (1975) using MDs and SDDs in terms of accuracy (instrumental data in relation to chemical data) and reproducibility (in terms of duplicate instrumental values). Equations [1] and [2] do not fully meet the standards suggested for milk calibrations (Biggs 1979a) which are specified in terms of accuracy as having MD<= 0.05% and SDD <= 0.06%. The MDs of 0.038 and -0.026% for fat and protein indicates that the calibration was good on average and met the milk analysis specifications; however, the SDD, or the spread around the difference is tenfold higher than milk specifications, indicating a greater variability in the individual results. Subsequent instrumental analysis for fat and protein of 50 duplicate samples, without reference to chemical data,

Figure 9. Predicted chemical estimates for fat vs. the respective chemical analysis as determined by the multicomponent calibration equation [1].

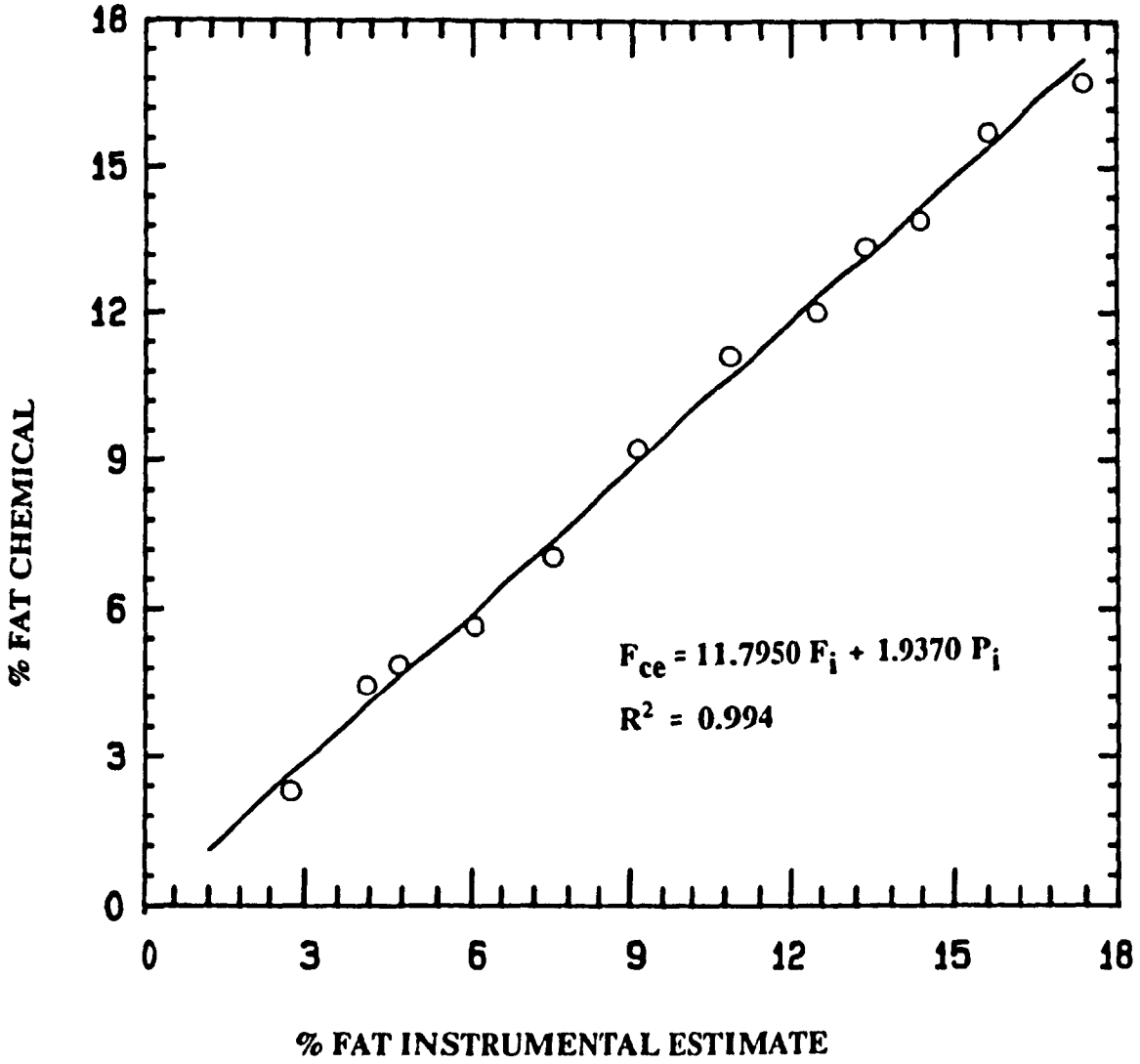
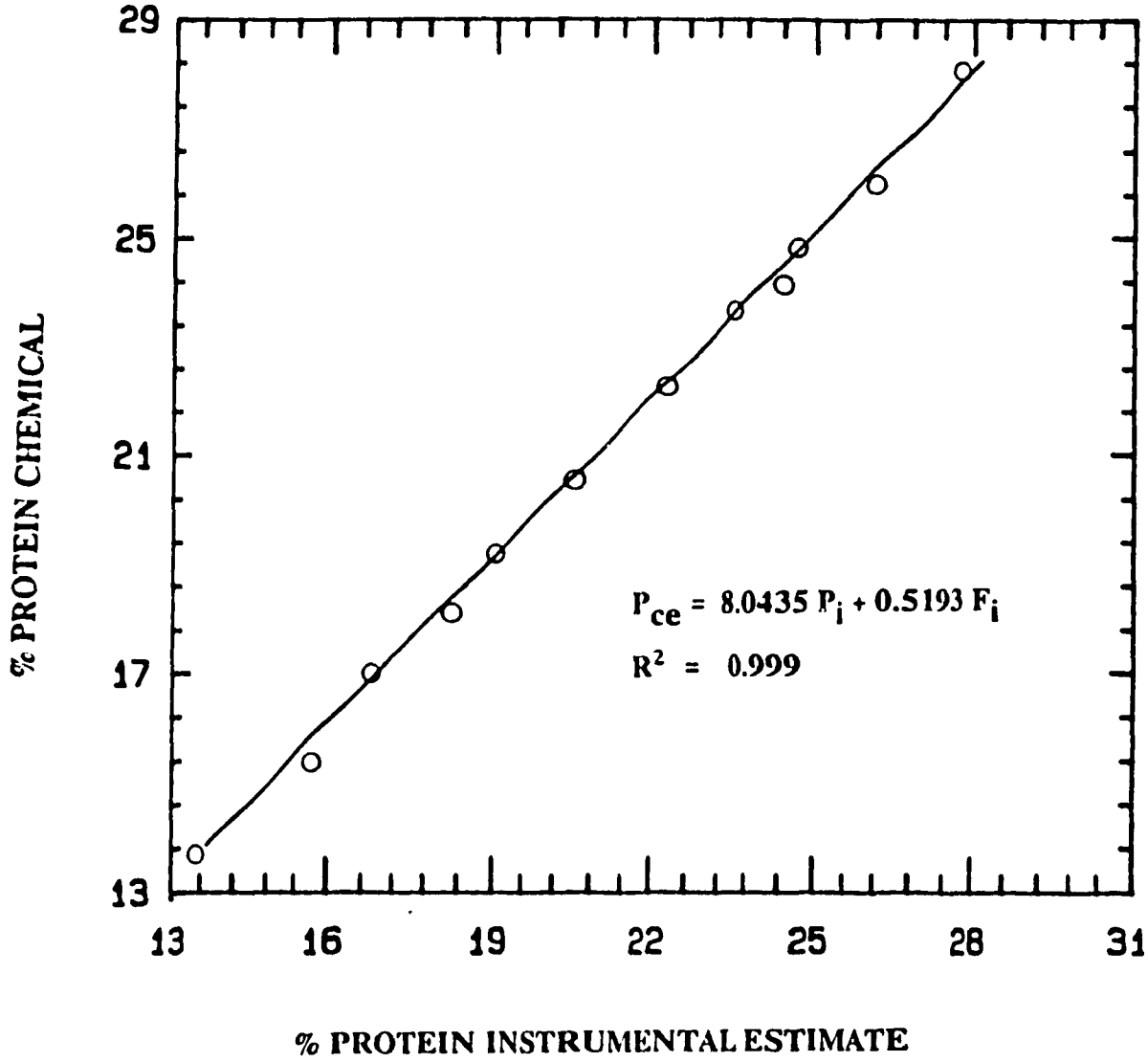


Figure 10. Predicted chemical estimates for protein vs. the respective chemical analysis as determined by the multicomponent calibration equation [2].



to determine the general reproducibility of instrumental results, produced a MD of -0.003% and -0.04%, and a SDD = 0.19% and 0.21% for fat and protein respectively. Once again, on average the mean differences were close to specifications overall, the SDD was reduced by about one third, but were still about one magnitude greater than milk specifications.

Forty five samples were taken from the same lot of fish, emulsions prepared therefrom and subsequently analysed chemically for fat and protein (Table 1). By averaging the fat and protein standard deviations, the chemical results varied +/- 0.73 and +/- 0.83% respectively and the individual range values were an indication of maximum spread encountered in the values from the lot. Instrumental data from a second lot of fish samples had an averaged standard deviation of +/- 0.42 and +/- 0.52% for fat and protein respectively (Table 2). The data obtained for the two lots are generally comparable, however, the SD of the instrumental results was lower overall, indicating that the instrumental data was less variable relative to the chemical methods. With the exception of the data for cod, which are slightly higher, the chemical and instrumental results were in line with the ranges found in the literature (Sohn et al. 1961). Thus the instrument may not be a limiting factor, rather sample preparation, chemical analysis, the inherent sample variability and/or a combination of these variables may limit the accuracy and reproducibility of the infrared method.

Table 1. Comparative data[1] for chemical results for fat and protein (%) obtained by repeated preparation of emulsions from a selected lot of fish (n=45).

	COD		MACKEREL		TUNA	
	Fat	Protein	Fat	Protein	Fat	Protein
M	3.05	16.02	11.87	18.22	12.80	22.30
SD	0.43	0.86	0.92	0.66	0.88	0.98
R	0.89	2.23	2.13	1.90	1.63	2.30

[1]M = Mean; SD = Standard deviation; R = Range

Table 2. Comparative data[1] for instrumental results for fat and protein (%) obtained by repeated preparation of emulsions from a second selected lot of fish (n=45)

	COD		MACKEREL		TUNA	
	Fat	Protein	Fat	Protein	Fat	Protein
M	3.14	15.27	12.59	17.29	10.48	21.08
SD	0.20	0.43	0.64	0.55	0.42	0.57
R	0.55	1.14	2.27	2.05	1.42	1.87

[1]M = Mean; SD = Standard deviation; R = Range

These limiting factors are superimposed on the resultant instrumental estimates of the chemical values and as a consequence the standard deviation became significant when the fat or protein content at the lower end of the scale were assessed. The means of the instrumental chemical estimates were compared to means of samples checked chemically immediately after instrumental analysis (Table 3). This assessment included all the cumulative sources of variability: chemical, sample and calibration. On average the protein data was similar while the fat results were slightly low (0.39%), with the SD around the differences being in the order of 1%. These values can be compared to the previous meat study (Mills et al. 1984), where MD and SDD values for fat and protein were 0.21; 0.13% and -0.10%; 0.45% respectively. Averaging out all the chemical SDs from Table 1 (~0.72%) and those in Table 3 for accuracy (0.91%) indicated that the infrared method could be a reasonable substitute for the chemical procedures for quality control purposes, but not for analysis requiring a high degree of accuracy.

4.2 Moisture

The basic principles and development of using mid-infrared transmission spectroscopy to determine moisture has been discussed in a previous publication (van de Voort et al. 1988) and was based on the initial near infrared work by Goulden et al. (1967). The basic premise was to take advantage of the high sensitivity of the mid-infrared portion of the spectrum to moisture by extracting it into a

Table 3. Comparison of mean instrumental estimates of mixed fish samples to the mean chemical values of the same samples for fat and protein (n=33).

FAT		PROTEIN	
Chemical Mean	10.95	Chemical Mean	20.46
Instrumental Mean	10.54	Instrumental Mean	20.43
MD[1]	0.41	MD	0.03
SDD[2]	0.97	SDD	0.84

[1]MD = Mean Difference
[2]SDD = Standard Deviation of the Difference

hygroscopoic solvent such as methanol which does not interfere with the strong absorption band at 6.02 um. Previous work had concentrated on low moisture products such as skimmilk powder, bread crumbs and flour, which are difficult to analyse, in relation to the vacuum oven method, the AOAC near infrared method and the Karl Fischer titration procedure (van de Voort et al. 1988).

A typical calibration curve (Figure 11) derived using 21 water/methanol mixtures is described by the following equation:

[3] $W = 0.1271 + 0.5678\ I$

$r^2 = 0.999$
SE = 0.131
MD = -0.002
SDD = 0.174

Where: W = Moisture content (%)
I = Instrument signal

Fish samples including those adjusted in moisture content using equilibrium relative humidity chambers were extracted with methanol for infrared analysis and also subjected to moisture analysis by vacuum oven. The methods were first assessed for their relative reproducibility (Table 4). The MD and SDD for the instrumental method for the same sample was quite good (-0.03; +/- 0.49% respectively) with the SDD being about half of that obtained for the fat and protein results. When individual samples were prepared and extracted, the SDD doubled (1.19%), indicating that inherent sample variability contributed to the overall variability in the results. The vacuum

Figure 11. A calibration curve of percent moisture vs. instrument signals obtained for the water channel for water/methanol mixtures.

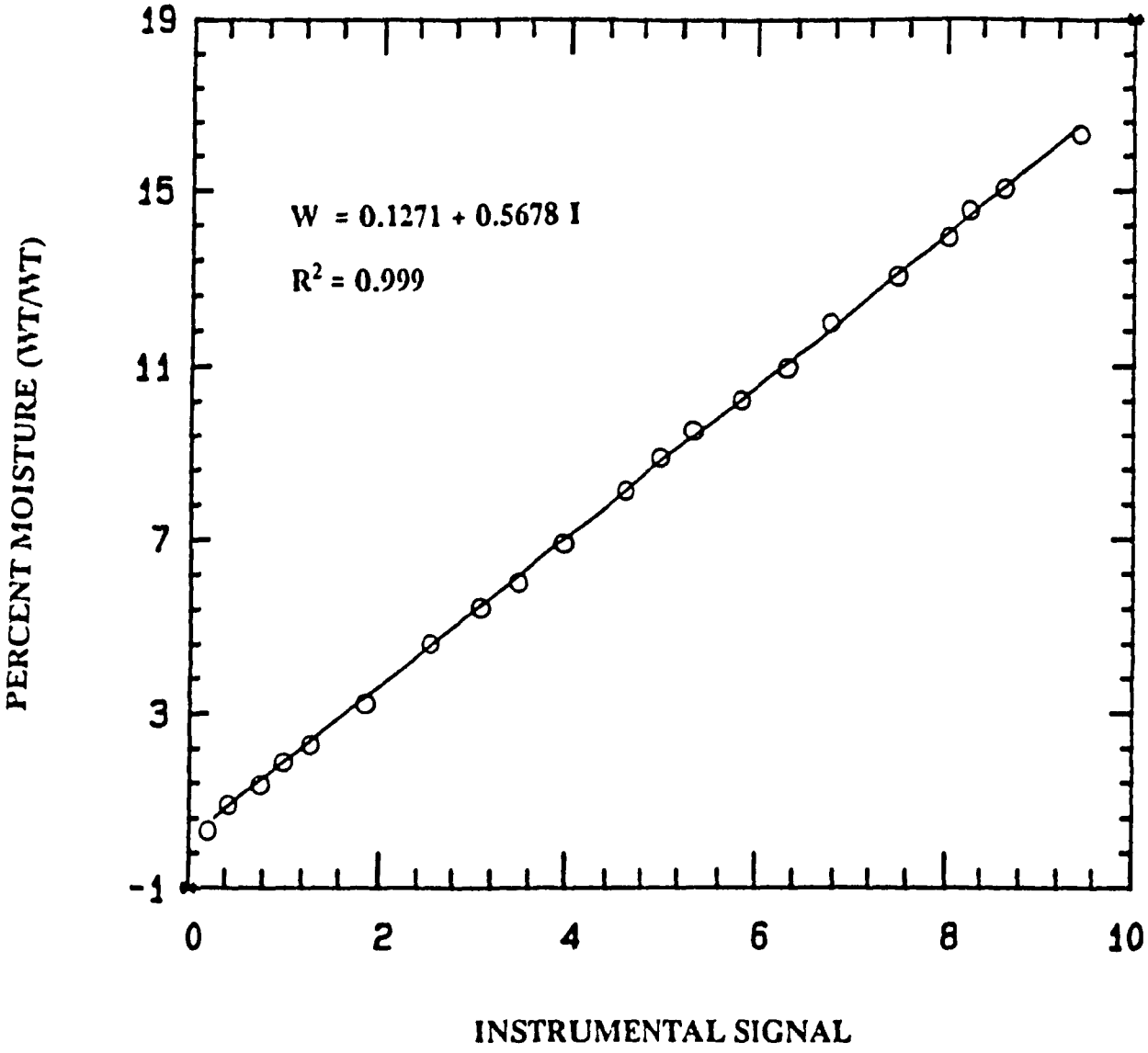


Table 4. Analysis for reproducibility of pairs (means 1 and 2) of chemical and instrumental moisture determinations (n=50).

Analysis	Means 1	Means 2	MD	SDD
Instrumental[1]	67.66	67.63	-0.03	0.49
Instrumental[2]	67.70	67.58	-0.12	1.19
Vacuum Oven[3]	67.75	67.07	-0.68	1.52

1 Duplicate readings of same sample
2 Duplicate readings of different samples
3 Duplicate vacuum oven results

oven method had even greater variability, deviating substantially from the expected MD of zero (by -0.68%) and having a higher SDD (1.52%). A similar analysis was carried out comparing the instrumental data obtained for moisture to the vacuum oven method, as a function of the type of Fish (Table 5). Overall, the vacuum oven method gave higher results, in the order of 1% and it would appear the infrared procedure is more reproducible and may even be a a better indicator of moisture than the reference method. A similar conclusion was reached when the infrared method was compared to the vacuum oven method for low moisture materials (van de Voort et al. 1988).

There are a number of limitations to the assessment of the infrared protocol developed. It is well known that the lipids of each fish species differ in their fatty acid composition and that significant levels of phospholipids may be present. The response of the fat wavelength is a function of the number of ester linkages per unit weight of lipid and will vary depending on the average molecular weight. Better results can be obtained using a separate calibration for each fish species to account for this effect; however, the development of multiple calibrations would be an onerous task. The substitution of the Mojonnier procedure for the Bligh and Dyer fat analysis may have produced a small bias due to the selectivity of the lipids extracted by each method; however, preliminary work indicated that similar results could be obtained by either method. In the case of protein, fish may contain significant levels of non protein nitrogen in the form of trimethylamine oxide, taurine, peptides, amino

Table 5. Comparative data for moisture analysis by the vacuum oven and infrared methods for fish (n=100).

Analysis	Vac[1]	IR	MD	SDD
Cod	72.92	72.04	0.88	2.03
Mackerel	66.47	65.12	1.35	2.12
Tuna	61.28	60.37	0.92	2.83

[1] Vac = Mean vacuum oven; IR = Mean Infrared; MD = Mean Difference; SDD = Standard deviation of the difference

acids and related purine based compounds. The Kjeldahl method includes these as nitrogen (converted to protein), while the infrared method does not account for these compounds. Considering the limitations indicated above, the inherent variability of the sample itself, the effects associated with the preparation procedure and the calibration, the results are quite reasonable. Overall, the method has a significant advantage in terms of analytical time even though some sample preparation is required and ten protein determinations can be performed in one hour while a similar number of moisture analyses can be done in twenty minutes. The major drawback to the more extensive utilization of mid-infrared technology is to a large extent the general lack of awareness of the technology, its singular association with milk analysis and the lack of research into new applications.

CHAPTER V

CONCLUSION

Infrared analysis of fish as a means of obtaining proximate analysis data for fat, protein and moisture appears to be feasible with a single instrument being capable of substituting for three common analytical methods with substantial time and labour savings. The reproducibility of instrumental results were generally better than those of the chemical methods, while in terms of accuracy, it appeared that the limitation hinged mainly on the variability of the raw material and the reproducibility of the chemical methods. Combining all sources of error, it can be stated that the use of infrared transmission analysis is capable of providing results of similar accuracy to the chemical methods, but that individual analyses would provide results of +/- 1.0%. The level of accuracy obtainable for fat and protein is dependent on deriving a sound calibration curve through careful chemical analysis of prepared solutions based on having a homogeneous and representative sample. In the case of moisture, the instrumental results were more consistent than the vacuum oven results and this procedure has the advantage of being calibrated directly without recourse to a reference method. Although infrared milk analysers are not presently being used for proximate analysis of fish, meat or other food systems, this study indicates that its application to fish tissue is potentially feasible for quality control purposes.

CHAPTER VI

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