CHAPTER 1

INTRODUCTION

Fraud is commonly defined as the crime of gaining money or financial benefits by a trick or by lying or by cheating. In law fraud is intentional deception to secure unfair or unlawful gain. Fraud can violate civil law, criminal law or it may cause no loss of money, or legal right but still be an element of another civil or criminal wrong. [12]

Fish fraud is committed when fish is deliberately placed on the market, for financial gain, with the intention of deceiving the consumer. There are many different types of fish fraud that can take place at multiple points along the fish supply chain. This occurs for a number of reasons, from a simple misunderstanding of regulations to deliberate deception of consumers to increase profits, or to laundering illegally harvested fish and the falsification of trade documentation.

Different forms of fish fraud occur in both domestic and international fish marketing chains. There are different estimates of the scale of fish fraud as many countries do not have the capacity to monitor fraudulent activities in the marketing chain and do not have developed official food control programmes to regulate fish business operators.

Some of the most common forms of fish fraud involve:

Species substitution: Once fish is filleted and skinned, its species can be difficult to determine some sellers take advantage of this and low-value species replaces a more expensive variety for economic gain or where a high-value species is presented as a lower-value species for tax evasion purposes.

Mislabelling of fish to conceal the geographical origin of illegally harvested species: This can occur through trans shipping, at sea transfer and falsifying trade documents.

Marketing of counterfeit products, where brand names are fraudulently used.

Undeclared use of food additives such as water-binding agents to deceptively increase the weight of products: When processors mispresent the weight of fish product through practices such as overglazing, soaking and breading.

Illegal use of food additives such as carbon monoxide to enhance the visual quality of fish products.

Regardless of the manner in which the fraud occurs, fish fraud is illegal, it can affect public health, it undermines confidence in the market place, and it can have serious consequences for fishery management and the fish industry, in addition to economic, social and environmental costs.

Fish fraud can be identified by checking the authenticity of the fish meat. [13]

GC with Flame Ionization Detector (GC-FID)

Gas Chromatography – Flame Ionization Detector or GC-FID is a very common analytical technique that is widely used in the petrochemical, pharmaceutical and natural gas markets.

An FID typically uses a Hydrogen/Air flame into which the sample is passed to oxidise organic molecules and produces electrically charged particles (ions). The ions are collected and produce an electrical signal which is then measured.

As common with other GC techniques, a carrier gas is required with low Water and Oxygen impurities since Water and Oxygen can interact with the stationary phase and cause significant problems such as high baseline noise and column bleed in the output gas chromatogram which both reduces the analyser sensitivity and decreases column lifetime. The FID is also extremely sensitive to Hydrocarbon impurities in the Hydrogen and Air supply for the flame. Hydrocarbon impurities can cause increased baseline noise and reduce the detector sensitivity. [14]

FIGURE 1: GC-FID [15]

Flame photometer

Flame photometer more properly called flame atomic emission spectrometry is a relatively old instrumental analysis method. Its origins date back to Bunsen's flame colour tests for the qualitative identification of select metallic elements. Probably the most common example of the atomic emission effect is fireworks. As an analytical method, atomic emission is a fast, simple, and sensitive method for the determination of trace metal ions in solution. Flame photometry is good only for elements that are easily excited and do not require very high temperatures

(Na, K, Li, Ca are the most widely determined atoms by this technique).

FIGURE 2: Flame photometer [16]

SDS-PAGE

SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) is a variant of polyacrylamide gel electrophoresis, an analytical method in biochemistry for the separation of charged molecules in mixtures by their molecular masses in an electric field. It uses sodium dodecyl sulfate (SDS) molecules to help identify and isolate protein molecules.

SDS-PAGE is a discontinuous electrophoretic system developed by Ulrich K. Laemmli which is commonly used as a method to separate proteins with molecular masses between 5 and 250 K Da.

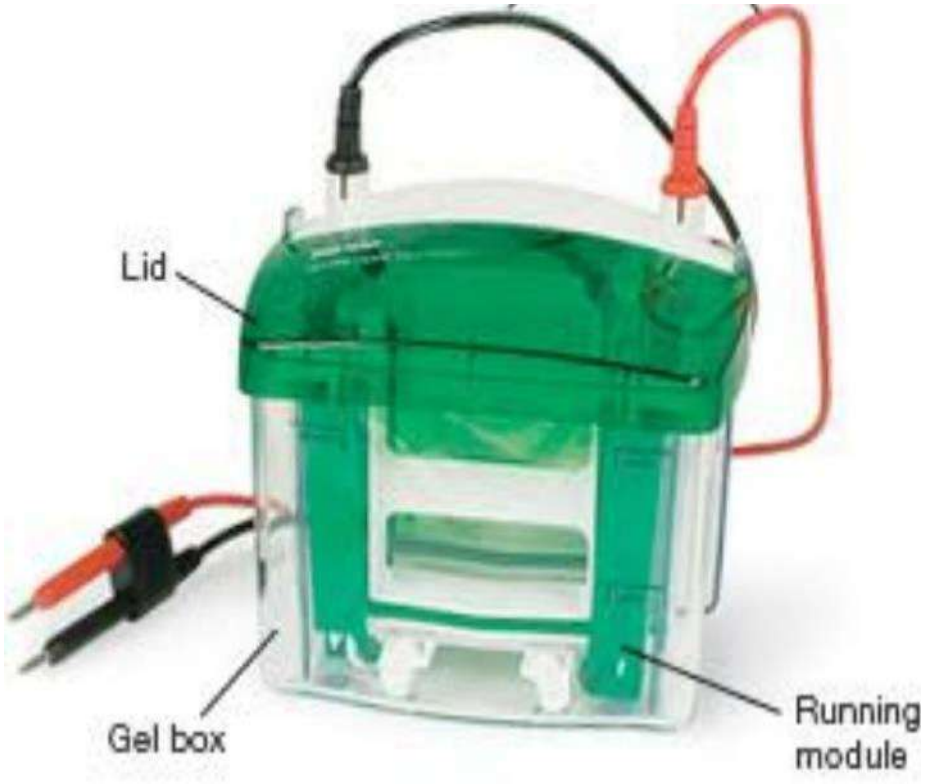


FIGURE 3: SDS-PAGE

CHAPTER 2

LITERATURE REVIEW

Non-invasive analytical technology for the detection of contamination, adulteration, and authenticity of meat, poultry, and fish:
Muhammed Kamruzzaman, Yoshio Makino, Seiichi Oshita, 2015

The requirement of real-time monitoring of food products has encouraged the development of non-destructive measurement systems. Hyperspectral imaging is a rapid, reagentless, non-destructive analytical technique that integrates traditional spectroscopic and imaging techniques into one system to attain both spectral and spatial information from an object that cannot be achieved with either digital imaging or conventional spectroscopic techniques. Recently, this technique has emerged as one of the most powerful and inspiring techniques for assessing different meat species and building chemical images to show the distribution maps of constituents in a direct and easy manner.

Species identification of meat by electrophoretic method:
Magdalena Montowska, Edward Pospiech, 2007

Electrophoretic method can be used to identify meat of various animal species. The protein electrophoresis, especially the IEF of the sarcoplasmic proteins, is a well established technique for species identification of raw fish and is used in the control of seafood authenticity. However, in the case of the analysis of heat-processed fish, the method is applicable only to those species which possess characteristic patterns of the heat-stable parvalbumins. Heat-denatured fish muscle proteins may be solubilised by urea or sodium dodecylsulfate (SDS) and separated by urea-IEF or SDS-PAGE.

Authenticity Determination of Meat and Meat Products on the Protein and DNA Basis:
M Montowska, E Pospiech, 2010

Adulterated food can be defined as food incompatible with the declaration of the seller. In the case of meat and meat articles, adulterations refer not only to the replacement of ingredients but also to inappropriate information concerning the origin of raw materials. Methods aiming at investigating meat and meat product authenticity may be based either on the analysis of protein composition or on the analysis of nucleic acids. At the present time, meat and meat product authenticity investigations based on protein analysis employ electrophoretic, enzymic, and chromatographic methods, sometimes supported by the mass spectrometry technique. On the other hand, species identification is often based on polymerase chain reaction (PCR). Biochips present a promising technology.

7 - Near Infrared Spectroscopy and Food Authenticity:
D.Cozzolino, 2016

Near Infrared (NIR) spectroscopy has been developed as one of the most versatile methods to target authenticity, discrimination, or traceability issues in several food matrices. However, in order to adapt and apply this method to efficiently and consistently monitor authenticity issues in foods, we need to increase our understanding about the chemical and biochemical basis associated with origin/authenticity/traceability derived from the NIR spectra. One of the main advantage of NIR spectroscopy over traditional chemical and chromatographic methods are the speed, minimal sample preparation, and ease to use in an industrial setting or routine operations.

Determination of food authenticity by enzyme-linked immunosorbent assay (ELISA):
Luis Asensio, Isabel Gonzalez, Teresa Garcia, Rosario Martin, 2008

This work intends to provide an updated and extensive overview on the applications of ELISA techniques for meat, fish and milk species discrimination; fruit juice labelling authentication; genetically modified and irradiated food detection; feedstuffs origin and allergen ingredients identification. These methods have been widely used because they reduce the use of costly, sophisticated equipment and time of analysis and are suitable for routine analysis of a large number of samples. Therefore, ELISA could allow, together with other analytical methods such as DNA-based methods, consumer protection and confidence, and an accurate implementation of the traceability for successful regulatory food controls.

Capillary electrophoresis for the analysis of meat authenticity:
Belinda Vallejo-Cordoba, Aaron F. Gonzalez-Cordova, Miguel A. Mazorra-Manzano, Roberto Rodriguez-Ramirez, 2005

The application of capillary electrophoresis (CE) is relatively new in solving meat authentication issues. Several unique CE applications based on meat protein fingerprinting are discussed for the analysis of meat species in unheated meat products. For protein data interpretation, pattern recognition is used to account for the natural variability present within the same meat species. While gel DNA-based methods are widely used for determining meat species in heat processed products, few DNA-based methods utilizing CE have been reported. Moreover, the methods reported are qualitative or semiquantitative. Thus, the need for quantitative competitive PCR CE methods in the determination of meat species is addressed. For the determination of meat extenders, CE methods were either protein-based or based on specific markers. Polyphenols are used as specific markers for soy detection and hydroxyproline is used as a specific marker for collagen determination. Finally, the potential of electrophoretically mediated microanalysis (EMMA) for the detection of meat that may have been previously frozen and retailed as “fresh” is highlighted.

Fish and seafood traceability based on AFLP markers: Elaboration of a species database:

M Maldini, FN Marzano, GG Fortes, R Papa, G Gandolfi, 2006

Several sociological, health and conservation arguments request a correct labelling of seafood products. Nowadays, molecular genetics is a useful tool for food chain traceability, particularly in regards to species identification. Among the variety of PCR-based molecular markers, AFLPs (Amplified Fragment Length Polymorphisms) have recently been used to investigate genomes of different complexities. This paper assesses the potential use of the AFLP technology to determine fish and seafood species in processed commercial products and domestic stocks. In particular a species database of fish, molluscs and crustaceans has been created with the aim to identify species of origin of seafood products by previously defined AFLP patterns. Different EcoRI and TaqI primer combinations were selected from 20 screened combinations in relation to the total number of detected fragments and polymorphic ones. Most informative combinations were E32/T32, E32/T33, E33/T33, E33/T37, E33/T38, E40/T33, E40/T37, E42/T32, E42/T37. The comparison of informative markers between unknown frozen or fresh products and reference samples has enabled the accurate identification of 32 different species. The taxonomic characterization has been performed either at the species or at the population level depending on the number of available individuals. AFLP variation at the population level is particularly helpful for the stock traceability of domestic strains. Size homoplasy was also investigated in one species to assess the rate of non-homologous comigrating fragments and to detect additional polymorphic markers to be used in stock identification. Results of Band Sharing Index (BSI) and percentage of polymorphic fragments are presented and are discussed in relation to the wide applicability of AFLPs both for fish and seafood safety and authenticity testing in such fields as food traceability and restocking management.

Quality evaluation of fish and other seafood by traditional and non-destructive instrumental methods:

Abdo Hassoun, Romdhane Karoui, 2017

Although being one of the most vulnerable and perishable products, fish and other seafoods provide a wide range of health-promoting compounds. Recently, the growing interest of consumers in food quality and safety issues has contributed to the increasing demand for sensitive and rapid analytical technologies. Several traditional physicochemical, textural, sensory, and electrical methods have been used to evaluate freshness and authentication of fish and other seafood products. Despite the importance of these standard methods, they are expensive and time-consuming, and often susceptible to large sources of variation. Recently, spectroscopic methods and other emerging techniques have shown great potential due to speed of analysis, minimal sample preparation, high repeatability, low cost, and, most of all, the fact that these techniques are non-invasive and non-destructive and, therefore, could be applied to any online monitoring system. This review describes firstly and briefly the basic principles of multivariate data analysis, followed by the most commonly traditional methods used for the determination of the freshness and authenticity of fish and other seafood products. A special focus is put on the use of rapid and non-destructive techniques (spectroscopic techniques and instrumental sensors) to address several issues related to the quality of these products. Moreover, the advantages and limitations of each technique are reviewed and some perspectives are also given.

Sea fish fraud? A confirmation of Gadoid species food labelling:

Veronika Kyrova, Pavla Surmanova, Vladimir Ostry, Irena Rehurkova, Jiri Ruprich, Marie Jechova, 2017

Gadoid fish and hake are the species of fishes most frequently imported to the Czech Republic. The purpose of this paper, cross-country hygiene study, is to determine sea fish fraud labelling on the Czech market and catering.
In total, 57 samples of commercial Gadoid fish product from different manufacturers, distributors and catering facilities were gathered. Gadidae family, hake (Merluccius spp. Raf.), saithe (Pollachius virens L.), Atlantic cod (Gadus morhua L.), Alaska pollock (Theragra chalcogramma Pall.), were detected in fish meat, fish products and fish meals by the qualitative PCR method.

The application of biotechnological methods in authenticity testing:
Bert Popping, 2002

By counterfeiting brand names in the food and drink industry as well as fraudulently labelling and selling low quality products as premium products, this sector of the industry has lost significant amounts of money and the consumer has been deceived. While it was difficult to establish certain types of fraud before the advent of modern biotechnology, DNA-based methods make an important contribution to protect high-quality brand names and protect the consumer. Several years ago, DNA technologies were considered as methods used in universities, primarily for research purpose, not so much for 'real-life' applications. However, this has changed and a number of laboratories have specialised in offering such services to the industry. This article will review DNA-based techniques commonly used for authenticity testing.

CHAPTER 3

AIM AND OBJECTIVES

AIM: To determine the authenticity of fish meat- shrimps

OBJECTIVES: To estimate the authenticity of 3 different types of shrimps by

- Mineral Analysis using Flame Photometer
- Fatty acid test using GC-FID
- SDS-PAGE

CHAPTER 4

MATERIALS AND METHODOLOGY

MATERIALS REQUIRED:

1. Fatty acid test: Shrimp meat(15g), measuring cylinder, conical flask, beaker, separating funnel, glass test tube, vials etc.
2. Mineral analysis: shrimp meat(5g), silica dish, test tube, conical flask etc.
3. SDS-PAGE: Shrimp meat(0.3g), test tube, measuring cylinder, beaker, glass rod etc.

REAGENT PREPARATION:

1. Fatty acid test:

2:1 ratio of chloroform and methanol

150% of KOH (9g KOH + 6ml distilled water)

2. SDS- PAGE:

- Sample buffer

TABLE:1

Reagent	Volume (ml) (8ml)
Deionised water	3.8
0.5M tris HCl, pH 6.8	1.0
Glycerol	0.8
10% SDS	1.6
2% mercaptoethanol	0.4
1% bromophenolblue	0.4

- Running buffer

TABLE:2

Reagent	Volume (2l) Weight(g)
Tris buffer	6.056
Glycine	28.82
SDS	2.00

- 10% SDS
- 1.5M Tris HCl, pH 8.8:

  18.15g dissolved in 80 ml deionised water, pH 8.8 with 6N HCl and make up to 150 ml, store at 4 ℃.
- 0.5 M Tris HCl, pH 6.8:

  6g Tris buffer dissolved in 60 ml deionised water, pH 6.8 with 6N HCl and make up to 100 ml, store at 4℃.

- Staining solution (100ml)

TABLE:3

Water	46.5 ml
Methanol	46.5Ml
CH3COOH	7 ml
Coomasie blue	0.2g

3. Mineral analysis:

- 1:1 HCl

INSTRUMENTS

1. GC-FID
2. Flame Photometer
3. SDS-PAGE

METHODS

1. Fatty acid test:

- Lipid extraction: 150ml mixture ($CHCl_3$-CH_3OH, 2:1 ratio) to which 15g of sample is added and homogenised and the residue is again homogenised for 3 times with 150ml of chloroform methanol mixture. Pool the 3 extract. This steps are followed for all the 3 different samples and then transferred the extract to 3 separating funnel. Add 20% water, mix and keep for separation. Transfer $CHCl_3$ layer into a flat bottom flask through anhydrous Na_2SO_4. Flash evaporate to concentrate the solution. Make upto a known volume ml using $CHCl_3$. Transfer ml of the extract into a pre-weight vial. Evaporate the solvent to constant weight and calculate the lipid content.
- Saponification: For 100mg oil10ml of the sample solution is pippeted out and evaporated. Add 25ml methanol and 1.5ml KOH. Reflux for 30 minutes in boiling water bath under N_2, cool slightly. Transfer the solution to a separating funnel and added 25ml distilled water. Extract 3 times with petroleum ether (25ml). acidify the aqueous layer with concentrated HCl and check with indicator paper. Extract 3 times with petroleum ether and combine the extract. Wash it with water 3 times and filter through anhydrous sodium sulphate and collect fatty acid mixture.
- Esterification: Evaporate the solvent. Add 6ml BF_3 methanol and reflux in boiling water bath for 6 minutes. Add 6ml saturated NaCl and transfer to separating funnel then extract 3 times with petroleum ether and combine the

extract. Wash 3 times with water, filter through anhydrous Na_2SO_4, evaporate and make up to 1ml with petroleum ether. Transfer

- the final 1 ml extract to 3 vials and label it and introduce to GC-FID and note the result.

1. Electrophoresis:

Gel production:

- When using different buffers in the gel, the gels are made up to one day prior to electrophoresis, so that the diffusion does not lead to a mixing of the buffers.
- The gel is produced by radical polymerisation in a mold consisting of two sealed glass plates with spacers between the glass plates.
- For pouring the gel solution, the plates are usually clamped in a stand which temporarily seals the otherwise open underside of the glass plates with the two spacers.
- For the gel solution, acrylamide is mixed as gel-former, methylenebisacrylamide as a cross-linker, stacking or separating gel buffer, water and SDS.
- By adding the catalyst TEMED and the radical initiator ammonium persulfate (APS) the polymerisation is started.
- The solution is then poured between the glass plates without creating bubbles. Depending on the amount of catalyst and radical starter and depending on the temperature, the polymerisation lasts between a quarter of an hour and several hours.
- The lower gel (separating gel) is poured first and covered with a few drops of a barely water-soluble alcohol saturated butanol or, which eliminates bubbles from the meniscus and protects the gel solution of the radical scavenger oxygen.
- After the polymerisation of the separating gel, the alcohol is discarded and the residual alcohol is removed with filter paper.

- After addition of APS and TEMED to the stacking gel solution, it is poured on top of the solid separation gel.
- Afterwards, a suitable sample comb is inserted between the glass plates without creating bubbles.
- The sample comb is carefully pulled out after polymerisation, leaving pockets for the sample application.
- Commercial gel systems (so-called pre-cast gels) usually use the buffer substance Bis-tris methane with a pH value between 6.4 and 7.2 both in the stacking gel and in the separating gel. These gels are delivered cast and ready-to-use. Since they use only one buffer (continuous gel electrophoresis) and have a nearly neutral pH, they can be stored for several weeks.

Sample preparation:

- 10% SDS(5ml) and add deionised water(5ml): [10ml]
- From pellet pipetted out 1ml and add 9ml SDS and boil for 30 minutes in boiling water bath, filter the solution.
- From the solution pippet out 1ml and add 1ml sample buffer and keep in boiling water bath for 3 minutes
- Through this steps all the 3 samples are prepared.

FIGURE 4: .3g samples for SDS-PAGE

Electrophoresis

- For separation, the denatured samples are loaded onto a gel of polyacrylamide, which is placed in an electrophoresis buffer with suitable electrolytes.
- Thereafter, a voltage (usually around 100 V, 10-20 V per cm gel length) is applied, which causes a migration of negatively charged molecules through the gel in the direction of the positively charged anode.
- The gel acts like a sieve. Small proteins migrate relatively easily through the mesh of the gel, while larger proteins are more likely to be retained and thereby migrate more slowly through the gel, thereby allowing proteins to be separated by molecular size.
- The electrophoresis lasts between half an hour to several hours depending on the voltage and length of gel used.

- The fastest-migrating proteins (with a molecular weight of less than 5 K Da) form the buffer front together with the anionic components of the electrophoresis buffer, which also migrate through the gel.
- The area of the buffer front is made visible by adding the comparatively small, anionic dye bromophenol blue to the sample buffer. Due to the relatively small molecule size of bromophenol blue, it migrates faster than proteins.
- By optical control of the migrating coloured band, the electrophoresis can be stopped before the dye and also the samples have completely migrated through the gel and leave it.

FIGURE 5: Processing of SDS-PAGE

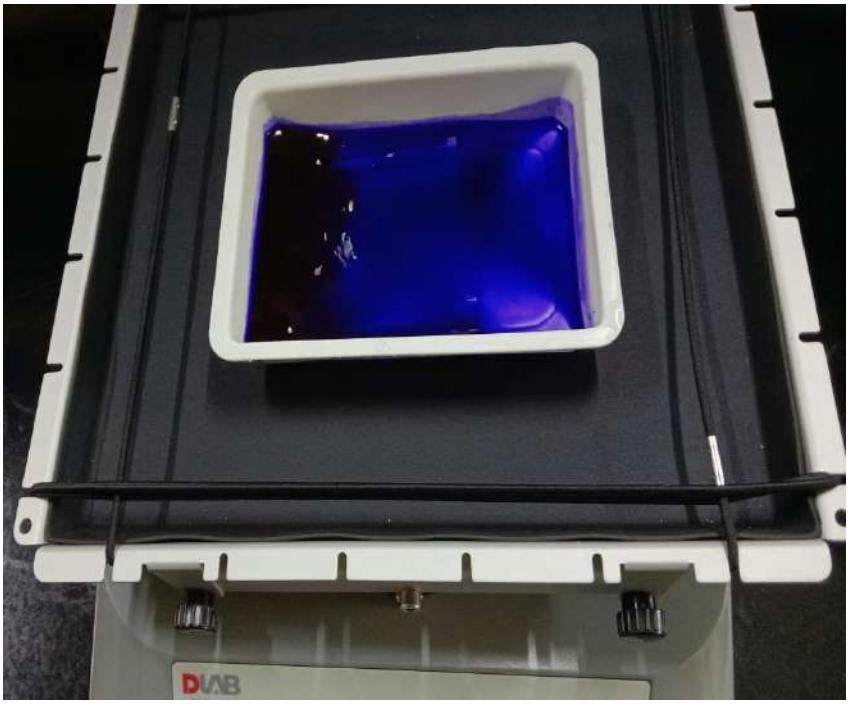


FIGURE 6: Staining

Mineral analysis:

- Preheat the silica dish at 70^0c in oven, place it in desiccator and weigh the silica dish.
- Add 5g of the sample to the silica dish, heat the silica dish along with the sample in oven at 90^0c.
- Dry the portion by heating carefully in fume hood.
- Then heat at 550 – 600^0c in the muffle furnace for 5 hours for ashing.
- Weigh the sample after ashing .
- Using 1:1 HCl, dilute the ash and make up a solution of 100ml using distilled water and filter the solution.
- Make up a solution with 10ml distilled water and 10ml sample solution and introduce it to flame photometer for further results.

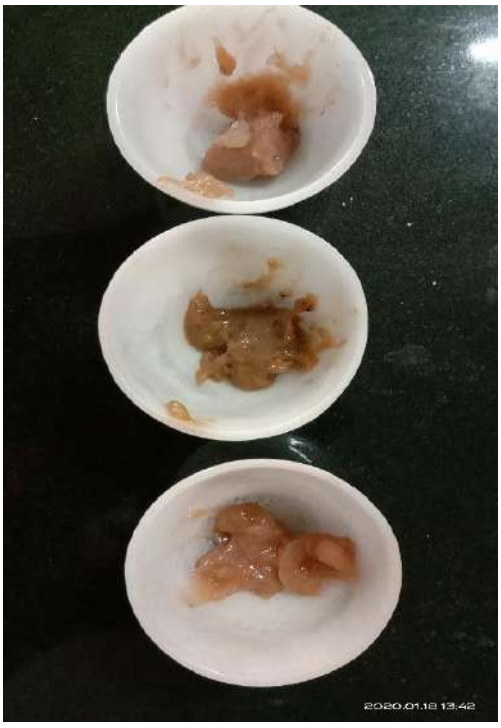


FIGURE 7: Samples for mineral analysis

CHAPTER 5

CALCULATION AND OBSERVATION TABLE

1. Mineral analysis

TABLE:4

Sample	Sodium (Na) (ppm)	Potassium (k) ppm	Calcium (Ca) ppm
Indian white shrimp	33*2*2	36.28*2*2*2	13.3*2
Brown shrimp (small)	27*6	27.2*12	17.8*2
Karikkadi shrimp	14*5*2	25*5*2	11.7*2

Indian white shrimp:

Na=132*100/5 = 2640

K= 294.4*100/5 = 5888

Ca = 26.4*100/5 = 528

Brown shrimp(small):

Na = 162*100/5 = 3240

K = 326.4*100/5 = 6528

Ca = 35.6*100/5 = 712

Karikkadi shrimp:

Na = 140*100/5 = 2800

K = 250*100/5 = 5000

Ca = 324*100/5 = 468

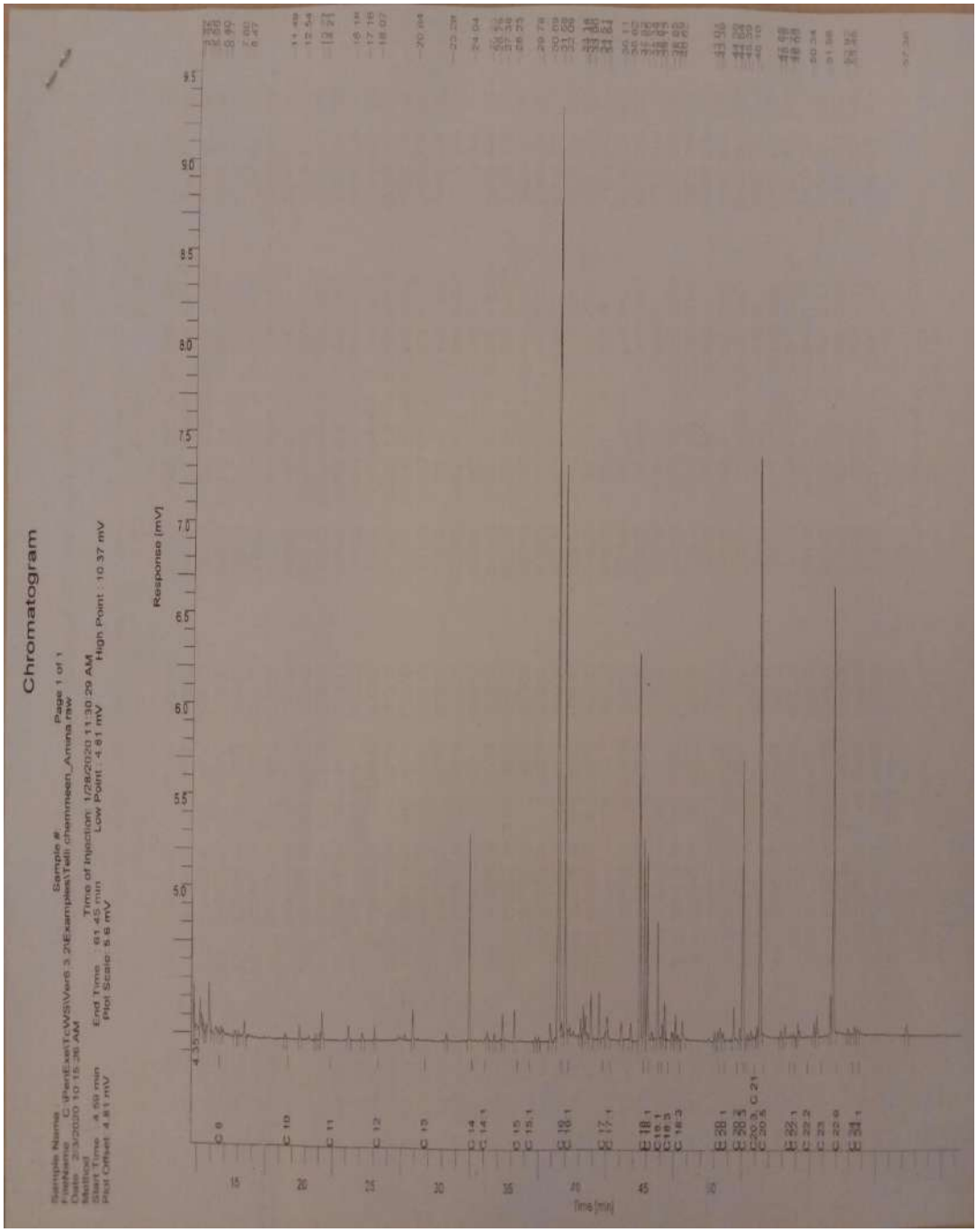
TABLE:5

Miner als	Indian white shrimp	Brown shrimp(small)	Karik-kadi shrimp
Na	2640	3240	2800
K	5888	6528	5000
Ca	528	712	468

1. Fatty Acid Test:
    - Brown shrimp (small)

      15g – 20ml

      10ml-1ml



GRAPH 1: Chromatogram of brown shrimp(small)

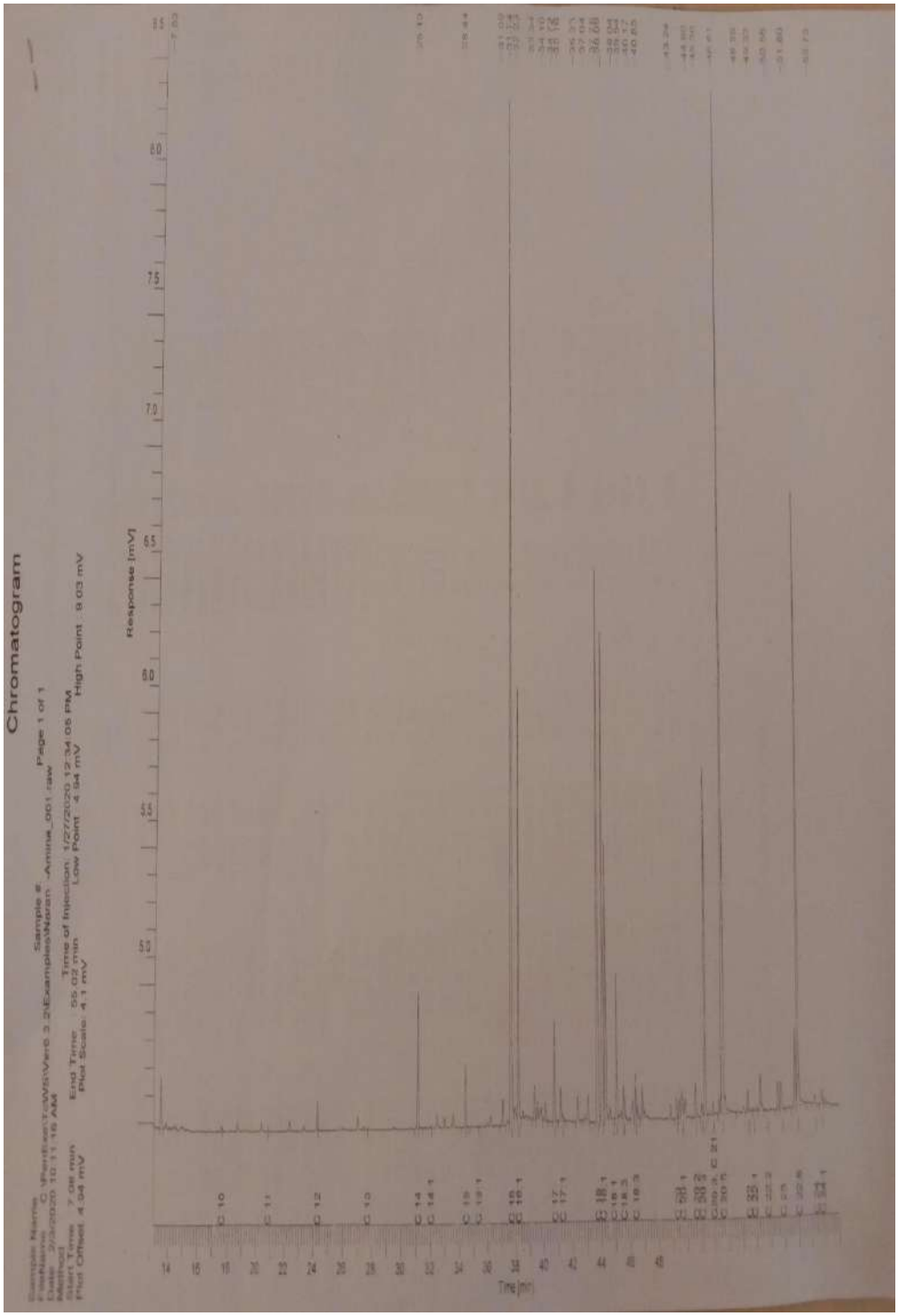
TABLE:6

FAME	**% of FAME**
C12	0.30
C14	4.23
C15	0.64
C16	27.64
C16:1	12.90
C17	1.03
C17:1	0.53
C18	9.33
C18:1	8.19
C18:2	0.30
C18:2	0.28
C18:3	0.89
C18:3	0.12
C20	0.22
C20:1	0.21
C20:4	6.01
C20:5	14.67
C23	0.61
C22:6	11.78
C24:1	0.13

- Indian white shrimp:

  15g – 20ml

  10ml – 1ml



GRAPH 2: Chromatogram of Indian white shrimp

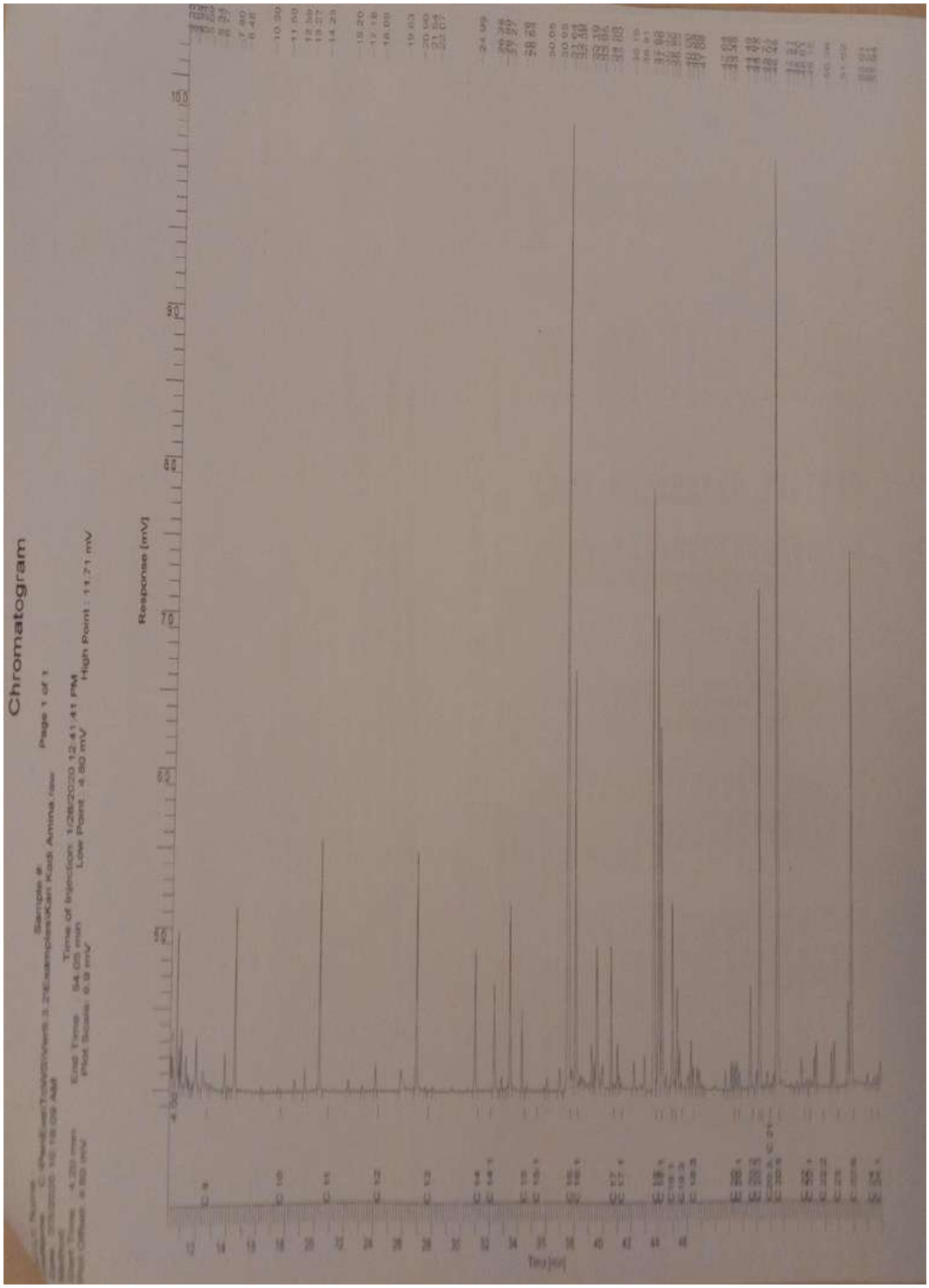
TABLE:7

FAME	% of FAME
C14	2.15
C15	0.98
C16	20.09
C16:1	6.48
C17	1.61
C18	10.54
C18:1	12.17
C18:2	3.06
C18:3	0.67
C18:3	0.96
C20	0.35
C20:4	6.05
C20:5	20.44
C22	0.44
C23	0.65
C22:6	13.03
C24:1	0.31

- Karikkadi shrimp:

  15g – 20ml

  10ml – 1ml



GRAPH 3: Chromatogram of karikkadi shrimp

TABLE:8

FAME	% of FAME
C15	0.34
C14	1.79
C15	0.24
C16	21.65
C16:1	5.91
C17	2.03
C18	12.32
C18:1	12.59
C18:2	3.65
C18:3	0.35
C20	0.39
C20:1	0.34
C20:4	8.11
C20:5	18.87
C23	0.90
C22:6	10.34
C24:1	0.18

SDS PAGE:

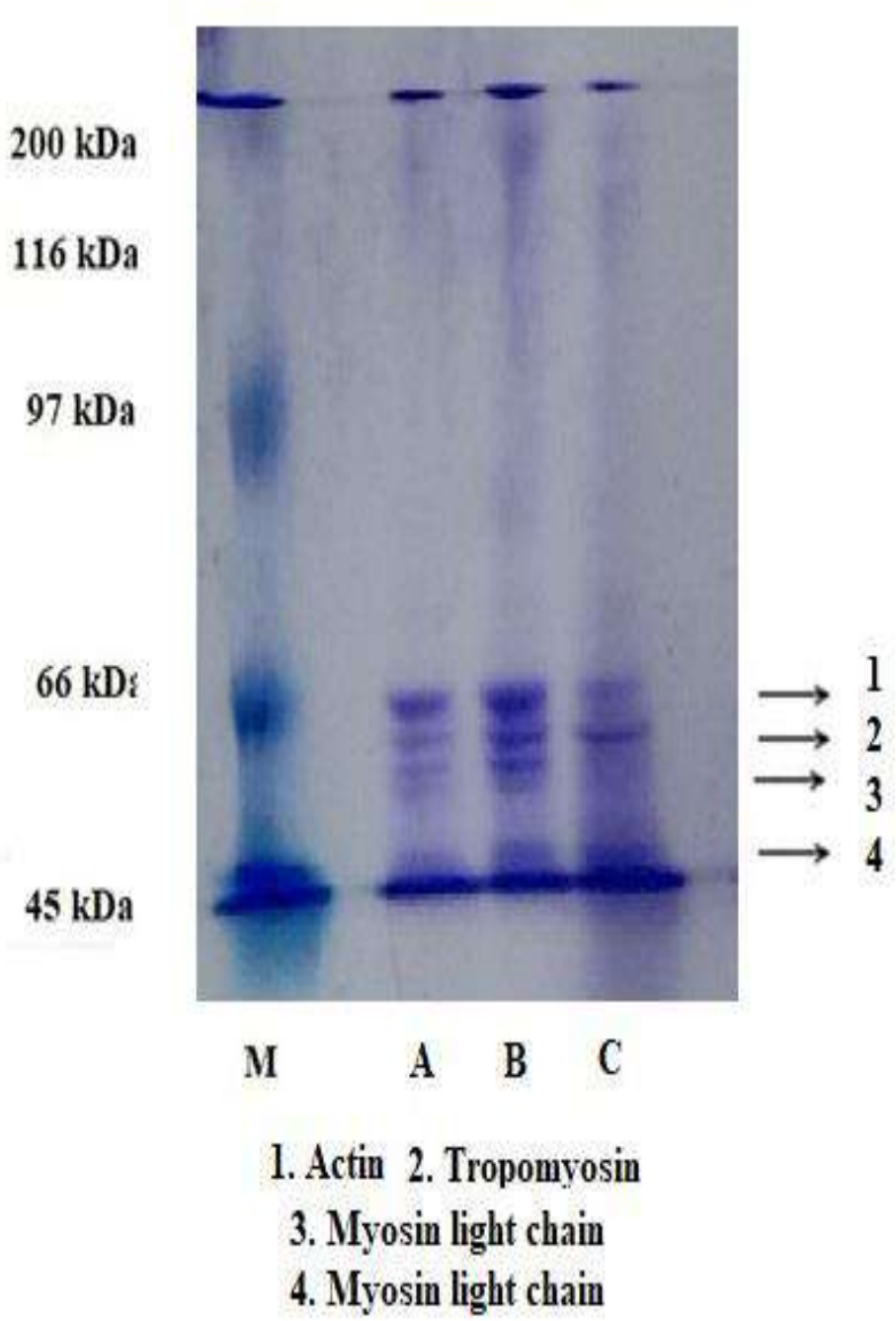


FIGURE 8: SDS PAGE of shrimps

CHAPTER-6

RESULT AND CONCLUSION

RESULT:

- Narrow variations are seen in mineral analysis in the meat of Indian white shrimp, brown shrimp(small), karikkadi shrimp.
- The peak of FAME test shows major variation within the three different shrimp meat.
- The bandwidth of three shrimp meats are different through SDS-PAGE.

CONCLUSION:

- According to my perception, the identification of fish meat authenticity through mineral analysis gives narrow variation.
- The fatty acid test and electrophoresis show much variations compared to mineral analysis, so this method can be used to identify the authenticity of fish meat(shrimps).

CHAPTER 7

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