ANALYTICAL METHODOLOGIES FOR THE ESTIMATION OF POLYMER PRECURSORS

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DECLARATION

I hereby declare that the thesis entitled **"Analytical Methodologies for the Estimation of Polymer Precursors"** submitted for Ph.D. degree to the University of Pune has been carried out at National Chemical Laboratory, Pune, India, under the supervision of **Dr. S. Ponrathnam,** Division of Polymer Science and Engineering, National Chemical Laboratory, Pune - 411008. The work is original and has not been submitted in part or full by me for any degree or diploma to this or any other University.

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Dedicated to,

My Eternal Guru Shri Brahmachaitanya
&
My Dear Parents, Anna and Akka

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ESTIMATION OF CHEMICAL PURITY OF LACTIC ACID MONOMER

LIST OF ABBREVIATIONS

AAS	Atomic Absorption Spectroscopy
AES	Atomic Emission Spectroscopy
ANOVA	Analysis of Variance
Arquad 2HT-75	di(hydrogenated tallow alkyl) dimethyl ammonium chloride
BA	Butyl acrylate
BMA	Butyl methacrylate
CE	Capillary Electrophoresis
CLEC	Chiral Ligand-Exchange Chromatography
DF	Degrees of Freedom
DVB	Divinylbenzene
ECD	Electron Capture Detector
EGDMA	Ethylene dimethacrylate
EHA	2-Ethylhexyl acrylate
EHMA	2-Ethylhexyl methacrylate
ELSD	Evaporative Light Scattering Detector
EVB	Ethylvinylbenzene
FAM	Functional Absorbent Material
FID	Flame Ionisation Detector
GC	Gas Chromatography
GC-MS	Gas Chromatography – Mass spectrometry

HETP	Height Equivalent to Theoretical Plate
HIC	Hydrophobic Interaction Chromatography
HIPE	High Internal Phase Emulsion
HPLC	High-Performance Liquid Chromatography
HPTLC	High-Performance Thin Layer Chromatography
IEC	Ion-Exchange Chromatography
IPA	Isopropanol
IR	Infrared Spectroscopy
IS	Internal Standard
LA	Lactic acid
LC	Liquid Chromatography
LC-MS/MS	Liquid Chromatography-Tandem Mass Spectrometry
LOD	Limit of Detection
LOQ	Limit of Quantification
MEHQ	Monomethyl ether hydroquinone
MMA	Methyl methacrylate
NMR	Nuclear Magnetic Resonance
NoDC	Number of Distinct Categories
NPD	Nitrogen Phosphorus Detector
OPLC	Over pressure Layer Chromatography
PAD	Photo Diode Array Detector
PID	Photo Ionisation Detector

PLA	Poly(lactic acid)
PTZ	Phenothiazine
RI	Refractive Index
ROP	Ring-Opening Polymerisation
RSD	Relative Standard Deviation
RP	Reverse Phase
R&R	Repeatability & Reproducibility
SD	Standard Deviation
SEC	Size Exclusion Chromatography
SFC	Supercritical Fluid Chromatography
SFE	Supercritical Fluid Extraction
SS	Sum of Squares
SP	Sample Preparation
Span 80	Sorbitan monooleate
St	Styrene
TCD	Thermal Conductivity Detector
TLC	Thin Layer Chromatography
UV-VIS	Ultraviolet–Visible Spectroscopy
W/O	Water-in-Oil

Analytical Methodologies for the Estimation of Polymer Precursors

Abstract

The thesis has been divided into seven chapters which comprise of introduction, aims and objectives, estimation of chemical purity of lactic acid monomer, estimation of optical purity of lactic acid monomer, estimation of monomers (EHA, EGDMA and EHMA) in acrylic high internal phase emulsions (HIPEs), estimation of monomers (styrene, DVB and EHA) in styrenic HIPEs and summary and conclusions.

In this work, new analytical methodologies were developed for estimation of monomers which are useful to address the important issues related to industrial production of poly(lactic) acid (PLA) and polymerisation processes that include acrylic and styrenic HIPEs.

Lactic acid produced by fermentation process contains carboxylic acid impurities which are detrimental to the polymerisation ability of lactic acid resulting in low molar mass PLA, which is not useful for most applications. PLA of high molecular weight is needed to produce devices of high mechanical strength. Evaluation of chemical purity of lactic acid monomer (in terms of carboxylic acid impurities) is therefore important and crucial. The analytical method based on HPLC-UV technique was developed for determining chemical purity of lactic acid by impurity profiling. The commercial and laboratory synthesised lactic acid samples were investigated for their chemical purity in terms of impurities of carboxylic acids, methanol and ethanol. A method based on polar-embedded reverse phase was established for executing separation of carboxylic acids in lactic acid matrix. A gas chromatographic method was developed for determination of ethanol and methanol, the byproducts of hydrolysis of lactic acid

ester, which may appear as impurities in final lactic acid product, if not completely removed in reactive distillation step of its downstream purification. Method validation was carried out by investigating method specificity, system precision, linearity, limit of detection (LOD), limit of quantification (LOQ), method accuracy and ruggedness. Gauge R&R study was conducted to assess the performance of method.

L (+) lactic acid is required for synthesis of PLA suitable for most of the applications. The small amounts of enantiomeric impurities drastically change properties such as crystallinity or biodegradation rate of the polymer. Therefore, besides evaluation of chemical purity, the method for estimation of optical purity of lactic acid monomer is also essential. HPLC method based on chiral ligand-exchange chromatography (CLEC) was developed for determining optical purity of lactic acid. Lactic acid samples, commercial and laboratory prepared, were investigated for their optical purity. Enantiomeric separation of lactic acid was accomplished by employing stationary phase ligand, which used L-hydroxy proline as the 'immobilised chiral selector'. Method was validated by assessment of system precision.

Studying rate of monomer conversion (reaction kinetics) is important in polymerisation reactions for process optimisation. Optimising the process parameters is essential since it has a significant impact on plant operability and economics. An analytical method for estimating monomer conversions in acrylic HIPE polymerisation was developed. A protocol for extraction of monomers (EHA, EGDMA and EHMA) from HIPE matrix was developed. Method validation was carried out by investigating system precision, method specificity, linearity, LOD, LOQ and 'method precision and accuracy'. Gauge R & R study was conducted to assess the performance of method. The

usefulness of method was demonstrated by estimating monomer conversions in HIPE polymerisation reactions conducted with certain selected reaction parameters.

A fast and sensitive HPLC-UV method was developed for estimation of EHA, EGDMA and EHMA present at low ppm levels in HIPE process water. The HIPE process water samples were generated in laboratory and investigated for contents of residual monomers at trace levels. Method validation was performed by evaluating system precision, method specificity, linearity, response factors, LOD, LOQ and 'method precision and accuracy'. This method is useful to assess the quality of process water (in terms of residual monomers) before being recycled within industrial process pertaining to production of Functional Absorbent Materials (FAM) obtained by HIPE methodology.

The analytical method for estimating monomer conversions in styrenic HIPE system was developed. A protocol for extraction of monomers (styrene, DVB and EHA) was established. Method validation was carried out by investigating system precision, method specificity, linearity, LOD, LOQ and 'method precision and accuracy'. Gauge R & R study was conducted to assess the performance of method. The usefulness of method was demonstrated by estimating monomer conversions in HIPE polymerisation reactions conducted using thermal and redox initiator systems.

Chapter 1

INTRODUCTION

1.1 Analytical Chemistry

Analytical chemistry is an important branch of chemistry that deals with the qualitative and quantitative analysis of natural and synthetic materials. Many problems in analytical chemistry begin with the need to identify what is present (qualitative analysis) and how much of it is present (quantitative analysis) in a sample. Analytical chemist works to improve the ability of other chemists to make meaningful measurements. Analytical chemistry has applications in forensics, toxicology, bioanalysis, clinical analysis, polymers, environmental analysis, geochemistry and materials analysis. Chemists working in various fields of chemistry need better tools for analysing their materials. The role of analytical chemist has thus become very important and crucial in today's fast growing world. Analytical chemists are required in all aspects of chemical research in industry, academia, and government. They have to apply their knowledge of chemistry, instrumentation, computers, and statistics to develop methods of identification and measurements to solve problems in various fields of chemistry. Some of the measurements are important to assure compliance with environmental and other regulations and to assure the safety and quality of food, pharmaceutical products, water, etc. which are directly related to quality of life. Analytical chemistry is also focused on improvements in experimental design, chemometrics, and the creation of new measurement tools to provide valuable information.

In recent years, research in analytical chemistry has mainly focused on the development and application of physical and physicochemical analytical methods and instrumental analysis. The tremendous developments in instrumentation have surpassed the classic methods of gravimetric and volumetric analysis. With the advantage of

development in electronics, the chromatographic techniques like gas chromatography (GC) and high performance liquid chromatography (HPLC) have become very powerful analytical tools which can separate and analyse complex materials with greater speed and sensitivity of analysis. These techniques are widely used in quality control and process control in chemical, polymer and drug industries, in environmental pollution investigations and in clinical analysis.

1.2 Analytical Chemistry: History

Analytical chemistry has been important since the early days of chemistry, providing methods for determining which elements and chemicals are present in the world around us. During this period significant analytical contributions to chemistry include the development of systematic elemental analysis by Justus Von Liebig and systematized organic analysis based on the specific reactions of functional groups. The first instrumental analysis was flame emissive spectrometry developed by Robert Bunsen and Gustav Kirchhoff, who discovered rubidium (Rb) and caesium (Cs) in 1860.[1] Most of the major developments in analytical chemistry took place after 1900. During this period, instrumental analysis became progressively dominant in the field. In particular, many of the basic spectroscopic and spectrometric techniques were discovered in the early 20th century and refined in the late 20th century.[2] The separation sciences follow a similar time line of development and also became increasingly transformed into high performance instruments.[3] In the 1970s many of these techniques began to be used together to achieve a complete characterisation of samples.

Starting in approximately the 1970s to the present day analytical chemistry has progressively become more inclusive of biological questions (bioanalytical chemistry),

whereas it had previously been largely focused on inorganic or small organic molecules. Lasers have been increasingly used in chemistry as probes and even to start and influence a wide variety of reactions. The late 20th century also saw an expansion of the application of analytical chemistry from academic chemical questions to forensic, environmental, industrial and medical questions, such as in histology.[4]

Chromatography has evolved to become one of the most widely used analytical techniques to separate variety of compounds. The history of chromatography spans from the mid-19th to the 21st century. Chromatography (meaning "colour writing") was used and named so in the first decade of the 20th century, primarily for the separation of plant pigments such as chlorophyll (which is green) and carotenoids (which are orange and yellow). New forms of chromatography developed in the 1930s and 1940s made the technique useful for a wide range of separation processes and chemical analysis tasks. Chromatography of many kinds is widely used throughout the chemical industry. For example, the Environmental Protection Agency uses chromatography to test drinking water and to monitor air quality. Apart from analysing purified compounds, the growing use of chromatography in the pharmaceutical industry is for the separation of chiral compounds.

1.3 The Analytical Perspective

Analytical chemists typically operate at the extreme edges of analysis, extending and improving the ability of all chemists to make meaningful measurements on smaller samples, on more complex samples, on shorter time scales, and on species present at lower concentrations. Throughout its history, analytical chemistry has provided many of the tools and methods necessary for research in the other four traditional areas of

chemistry, as well as fostering multidisciplinary research in various fields of chemical sciences. Each field within chemistry brings an unique perspective to the study of chemistry. The analytical perspective has been described by many analytical chemists, with several viewpoints, as an analytical approach to solving problems.[5-11] Figure 1.3 shows an outline of the analytical approach along with some important considerations at each step. Although there are many descriptions of the analytical approach, it is convenient to treat it as a five-step process as given below:

1. Identify and define the problem.

2. Design the experimental procedure.

3. Conduct an experiment, and gather data.

4. Analyse the experimental data.

5. Propose a solution to the problem.

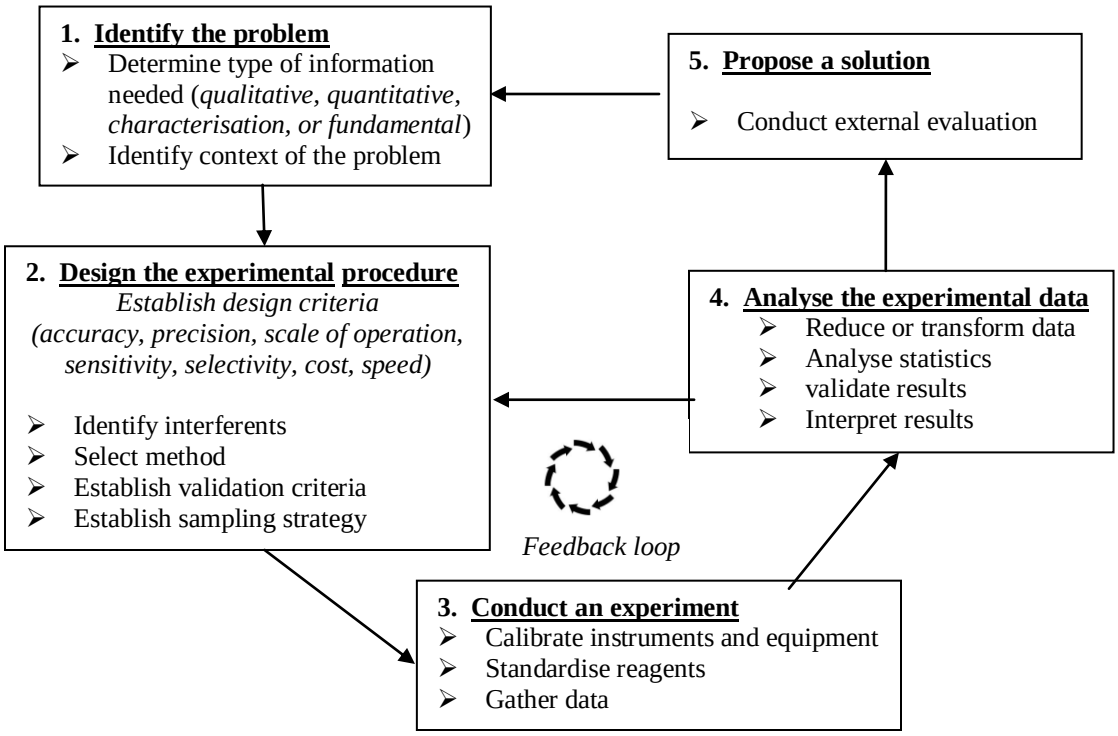

Figure 1.1: Flow diagram for the analytical approach to solving problems

Three general features of this approach deserve attention. First, steps 1 and 5 provide opportunities for analytical chemists to collaborate with individuals outside the realm of analytical chemistry. In fact, many problems on which analytical chemists work originate in other fields. Second, the analytical approach is not linear, but incorporates a "feedback loop" consisting of steps 2, 3, and 4, in which the outcome of one step may cause a re-evaluation of the other two steps. Finally, the solution to one problem often suggests a new problem. Analytical chemistry begins with a problem and analytical approach involves collaboration between the analytical chemist and the individuals responsible for the problem. Together they decide what information is needed. It is also necessary for the analytical chemist to understand how the problem relates to broader research goals. The type of information needed and the problem's context are essential to designing an appropriate experimental procedure.

Designing an experimental procedure involves selecting an appropriate method of analysis based on established criteria, such as accuracy, precision, sensitivity, and detection limit; the urgency with which results are needed; the cost of a single analysis; the number of samples to be analysed; and the amount of sample available for analysis. Finding an appropriate balance between these parameters is frequently complicated by their interdependence. For example, improving the precision of an analysis may require a larger sample. Consideration is also given to collecting, storing, and preparing samples, and to whether chemical or physical interferences will affect the analysis. Finally, a good experimental procedure may still yield useless information if there is no method for validating the results.

The most visible part of the analytical approach occurs in the laboratory. As part of the validation process, appropriate chemical or physical standards are used to calibrate any equipment being used and any solutions whose concentrations must be known. The selected samples are then analysed and the raw data is recorded and further analysed. The raw data collected during the experiment are then analyzed. Frequently the data must be reduced or transformed to a more readily analysable form. A statistical treatment of the data is used to evaluate the accuracy and precision of the analysis and to validate the procedure. These results are compared with the criteria established during the design of the experiment, and then the design is reconsidered, additional experimental trials are run, or a solution to the problem is proposed. When a solution is proposed, the results are subject to an external evaluation.

1.4 Selection of Analytical Method

1.4.1 Analytical methods

The recently developed analytical methods have the advantage of not only using small amounts of sample, reagents and less time, but also produce accurate results. These analytical techniques are based on following methods:

- **Physico-chemical methods:** Spectroscopy, including colorimetry, spectrophotometry covering ultra-violet and visible region or fluorimetry, nephelometry or turbidimetry, nuclear magnetic resonance (NMR) and mass spectrometry (MS).
- **Electro-analytical methods:** The electro-analytical methods cover potentiometry, amperometry, voltammetry, electrophoresis and polarography.

- **Separation-based methods**: HPLC, thin-layer chromatography (TLC), high-performance thin layer chromatography (HPTLC), capillary electrochromatography (CEC), supercritical-fluid chromatography (SFC) and gas chromatography (GC).

1.4.2 Parameters for selection of analytical technique

The necessary sampling and sample preparation (SP) steps for the determination of analytes in complex and dirty matrices is one of the most troublesome to perform, and therefore, the degree of SP depends on quite a number of parameters. The most important ones are the (i) concentration of the analyte, (ii) composition of the matrix, (iii) number of samples to be analysed, and (iv) chosen separation/detection system. Tables 1.1, 1.2 and 1.3 describe guidelines for selection of analytical technique/procedure.

Table 1.1: Questions related to the matrix

Complexity of the matrix:		**Degree of automation, amount of effort:**
Analyte - matrix binding:	None	No special precautions
	Yes	Denaturation procedures should be used in case of drug - protein binding or other analyte - matrix disrupting techniques
Minimum detectable concentration(s):	1 - 1000 µg/mL	CE, GC, LC, OPTLC, HPTLC, SFC, AMP, CON, ECD, FID, IR, NMR, NPD, PID, POL, RI, SIM, UV-VIS
	1 - 1000 ng/mL	CE, GC, LC, OPTLC, HPTLC, SFC AMP, CL, ECD, LIF, NPD, PID, SIM, UV-VIS
	1 - 1000 pg/mL	CE, GC, LC, SFC, AMP, CIF, CL, ECD, LIF, SIM
	1 - 1000 fg/mL	CE, LC, (SFC), CL, LIF
Stability:	Bad	Stabilising procedures needed
	Good	No stabilising procedures needed

CE: Capillary Electrophoresis, GC: Gas Chromatography, LC: Liquid Chromatography, OPTLC: Overpressurised thin-layer chromatography, HPTLC: High-Performance Thin Layer Chromatography, SFC: Supercritical Fluid Chromatography, AMP: Amperometry, CON: Conductometry, ECD: Electron Capture Detector, FID: Flame ionisation Detector, IR: Infrared spectroscopy, NMR: Nuclear Magnetic Resonance, NPD: Nitrogen Phosphorus Detector , PID: Photo Ionisation Detector, POL: Polarography, RI: Refractive Index Detector, SIM: Single Ion Monitoring, UV-VIS: Ultraviolet–Visible Spectroscopy, LIF: Laser-induced fluorescence

Table 1.2: Questions on the physico-chemical properties of the matrix

Questions to be answered:		**Possible techniques:**
Aggregation phase:	Gas	GC, SFC
	Liquid / solid	CE, GC, IEC, LC, OPTLC, HPTLC, SFC
Charge:	Not present	GC, LC, MECC, OPTLC, HPTLC, SFC
	Present	CE, IEC, LC (IP, IS)
Functional groups:	Not present	Almost no derivatisation possibilities
	Present	Derivatisation potential
Polarity:	Low	Non-polar sorbents in chromatography
	High	Polar sorbents in chromatography
Saturations (aromaticity):	Aliphatic	AMP, CL, CON, ECD, FID, FS, IR, NMR, NPD, PID, POL, RI, SIM
	Conjugated / aromatic	AMP, CL, CON, ECD, FID, FS, IR, LIF, NMR, NPD, PID, POL, RI, SIM, UV-VIS
Solubility:	Polar solvents	CE, IEC, LC, OPTLC, HPTLC
	Non-polar solvents	GC, LC, OPTLC, HPTLC, SFC
Volatility:	Low	CE, IEC, LC, OPTLC, HPTLC, SFC
	High	GC, LC, SFC

GC: Gas chromatography, SFC: Supercritical Fluid Chromatography, CE: Capillary Electrophoresis, IEC: Ion-Exchange Chromatography, LC: Liquid Chromatography, OPTLC: Overpressurised thin-layer chromatography, CE: Capillary Electrophoresis, HPTLC: High -Performance Thin Layer Chromatography, MECC: Micellar Electrokinetic Capillary Chromatography, LC(IP, IS): LC (Ion-Pairing, Ion-Suppression), AMP: Amperometry, CL: Chemiluminescence, CON: Conductometry, ECD: Electron Capture Detector, FID: Flame ionisation Detector, FS: Fluorescence spectroscopy, IR: Infrared spectroscopy, NMR: Nuclear Magnetic Resonance, NPD: Nitrogen Phosphorus Detector, PID: Photo Ionisati- on Detector, POL: Polarography, RI: Refractive Index Detector, SIM: Single Ion Monitoring, UV-VIS: Ultraviolet-Vi- sible Spectroscopy.

Table 1.3: General questions

General questions		
Availability equipment:		Determines choice of SP / ST, separation / detection system
Available expertise:		Determines choice of system components and the degree of automation
Number of solutes to be determined	< 10	CE, GC, LC, OPTLC, SFC
	> 10	CE, GC, LC
No. of samples to be analysed in each series:		Degree of automation
Profiling of analytes:	Yes	LC-MS/MS, GC-MS, LC-FTIR, LC, LC-UV
	No	No restriction in separation / detection mode
Rationale for analysis	Qualitative	CE, GC, LC, OPTLC, SFC, TLC
	Semi-quantitative	CE, GC, LC, OPTLC, SFC, TLC
	Quantitative	CE, GC, LC, SFC
Reason for analysis:	Legal	Reliability most important parameter
	Toxicological	Speed most important parameter
	TDM	Throughput important parameter
	Drug development	Screening and identification of metabolites important parameters
Ruggedness of the method	High	CE-DAD, GC-ECD, GC-FID, GC-NPD, LC-UV, automated reaction / detection systems
	Low	No restrictions

CE: Capillary Electrophoresis, GC: Gas Chromatography, LC: Liquid Chromatography, OPLC: Over-pressurised thin-layer chromatography, SFC: Supercritical Fluid Chromatography, *LC-MS/MS:* Liquid Chromatography-Tandem Mass Spectrometry, GC-MS: Gas Chromatography-Mass Spectrometry, LC-FTIR: Liquid Chromatography-Fourier Transform Infrared Spectrometry, LC-UV: Liquid Chromatography-Ultraviolet-Visible Spectroscopy, CE-UV: Capillary Electrophoresis-Ultraviolet-Visible Spectroscopy

1.4.3 Separation-detection systems

In analytical chemistry most of the time selectivity, sensitivity, reproducibility, speed and costs are the critical parameters when organic compounds are to be determined in complex samples. This means that to develop a qualitative or quantitative method, we will select an optimal combination of the following techniques (as given in Table 1.4):

Table 1.4: Overview of analytical techniques

Sample preparation / treatment	Labeling	Separation	Labeling	Detection
Precipitation	Pre-column	TLC	Post-column	UV-VIS
LLE		HPTLC		FL
SPE		OPLC		AMP
Column switching		SFC		CL
Dialysis		HIC		LIF
Dehydration		AC		MS
Distillation		SEC		IR
Electrophoresis		GC		NMR
Freezing		LC		FID
Hydrolysis		CE		RI
Micelles				NPD
Immunoaffinity				POL
Lyophilization				ECD
Microwaves				AAS
Soxhlet				AES
Ultrafiltration				PID
SFE				CON
Saponification				DAD

TLC: Thin Layer Chromatography, HPTLC: High –Performance Liquid Chromatography, OPLC: Over pressure Layer Chromatography , SFC: Supercritical Fluid Chromatography, HIC: *Hydrophobic Interaction Chromatography* , SEC: Size Exclusion Chromatography, GC: Gas Chromatography, LC: Liquid Chromatography, CE: Capillary Electrophoresis, UV-VIS: Ultraviolet–Visible Spectroscopy, FL: fluorescence detection, AMP: Amperometry, CL: Chemiluminescence detection LIF: Laser-induced fluorescence POL: Polarography , ECD: Electron Capture Detector, AAS: Atomic absorption spectroscopy, AES: Atomic Emission Spectroscopy, PID: Photo Ionisation Detector, SFE: Supercritical fluid extraction, CON: Conductometry, DAD: Diode Array Detector

In order to choose the proper methods and to develop a suitable method first of all, we have to answer the principal questions in the Tables 1.1, 1.2 and 1.3. After answering these questions, the separation and detection technique must be chosen and based on this, the sampling and SP procedure can be selected. From the tables, it can be

seen that numerous combinations of separation and detection techniques can be chosen for a particular problem.

1. The first choice that should be made is which mode of chromatography or electrophoresis should be applied. In general, the analyte(s) should be determined quantitatively, which means that selectivity and sensitivity are the most critical parameters.
2. It can be seen from Table 1.3 that CE, GC and LC (or HPLC) are the most obvious separation techniques.
3. The most likely suitable detection modes are UV-VIS, DAD, FL and MS in combination with LC, FID, ECD, NPD and MS for GC, and UV-VIS, DAD, LIF and MS in combination with CE. Especially, the use of MS or MS/MS approaches is still gaining popularity.
4. The sampling and SP approach are selected on the basis of the chosen separation-detection system. Normally a combination of initial, non-selective, technique in combination with a selective SP procedure is applied.

The physico-chemical properties of the analytes and the origin of the sample determine the separation technique that is aptly suitable. Some of the important features are:

- Using (capillary) GC mainly volatile solutes, compounds that are thermally sufficiently stable, and have a molecular-weight less than 500 can be determined without a derivatisation procedure.

- An important feature is that the separation power of GC is about 100 times higher as of LC. This means that if a problem can be solved with both LC and GC, GC normally is the first option.
- Other differences between GC and LC are that automated sample preparation in LC is more sophisticated than in GC, but in GC a number of selective detection approaches are available, meaning that the requirements for the sample preparation are less critical. Nowadays, both LC-MS//MS and GC-MS/MS techniques can be used for routine analysis.
- Another limitation of GC, the relatively small injection volumes, can be circumvented by using PTV (Programmed Temperature Vapouriser) injectors. The result is that volumes up to about 1 mL can be injected, even in a narrow-bore capillary GC system.
- CE is, in particular, developed for the separation of charged compounds. CE is mainly suitable for the qualitative and semi-quantitative determination of relatively high concentrations of organic and inorganic compounds in relatively simple matrices.

1.5 Chromatographic Techniques

Chromatography is probably the most powerful and versatile technique available to the modern analyst. In a single step process it can separate a mixture into its individual components and simultaneously provide the quantitative estimate of each constituent. Samples may be gaseous, liquid or solid in nature and can range in complexity from a simple blend of two enantiomers to a multi-component mixture containing widely differing chemical species. Furthermore, the analysis can be carried

out, at one extreme, on a very costly and complex instrument, and at the other, on a simple, inexpensive thin layer plate.

1.5.1 Chromatographic separation

Chromatography is a separation process that is achieved by distributing the components of a mixture between two phases, a stationary phase and a mobile phase. Those components held preferentially in the stationary phase are retained longer in the system than those that are distributed selectively in the mobile phase. As a consequence, solutes are eluted from the system as local concentrations in the mobile phase in the order of their increasing distribution coefficients with respect to the stationary phase; *ipso facto* a separation is achieved.

In practice, the distribution system (that part of the chromatographic apparatus where the solutes are distributed between the phases) can take the form of a column such as a tube packed with particulate material on which the stationary phase is bonded or coated. The mobile phase (which may be a gas or a liquid) passes under pressure through the column to elute the sample. The column form may also be a long, small-diameter open tube that has the stationary phase coated or bonded to the internal surface. Alternatively, the chromatographic system may take the form of a plate (usually glass) the surface of which is loaded with particulate material to which the stationary phase is coated or bonded. The mobile phase (a liquid) is arranged such that it travels up the plate (usually by surface tension forces) and sample elution takes place. The sample is injected into the mobile phase stream just before the front of the columns. The column is designed to allow two processes to take place that will produce the separation. First, as a result of different forces between each molecular type and the stationary phase, each solute is

retained to a different extent and, thus, the more weakly held will elute first and the more strongly held elute last. Consequently, each solute will be sequentially eluted from the column in the reverse order of the magnitude of the interacting forces between each solute and the stationary phase. Secondly, the spreading of each solute band (i.e. its dispersion) must be constrained so that each solute is eluted discreetly. The first function of the column is achieved by choosing the appropriate phase system (the optimum stationary phase in GC and the optimum combination of mobile phase and stationary phase in LC) to separate the solutes. The second function is achieved by selecting the optimum physical properties of the column (column dimensions, particle diameter, mobile phase velocity etc.) to ensure that band dispersion is adequately constrained. As all chromatographic separations are carried out using a mobile and a stationary phase, the primary classification of chromatography is based on the physical nature of the mobile phase. The mobile phase can be a gas or a liquid which gives rise to the two basic forms of chromatography, namely, gas chromatography (GC) and liquid chromatography (LC). Table 1.5 gives information on primary classification of chromatography.

Table 1.5: The primary classification of chromatography

Mobile phase	**Stationary phase**	**Chromatographic technique**
Gas *Gas Chromatography (GC)*	Liquid	Gas-Liquid Chromatography (GLC)
	Solid	Gas-Solid Chromatography (GSC)
Liquid *Liquid Chromatography (LC)*	Liquid	Liquid-Liquid Chromatography (LLC)
	Solid	Liquid-Solid Chromatography (LSC)

1.5.2 Theory of chromatography

Chromatographic techniques such as HPLC and GC use columns - narrow tubes packed with stationary phase, through which the mobile phase is forced. The sample is transported through the column by continuous addition of mobile phase. This process is called *elution*. The average rate at which an analyte moves through the column is determined by the time it spends in the mobile phase.

1.5.2.1 Distribution of analytes between phases

The distribution of analytes between phases can often be described quite simply. An analyte is in equilibrium between the two phases as:

$$A_{mobile} \rightleftharpoons A_{stationary}$$

1.5.2.2 Partition coefficient

The equilibrium constant (*K)* is termed as the partition coefficient, which is defined as the molar concentration of analyte in the stationary phase [$C_{A\,(S)}$], divided by the molar concentration of the analyte in the mobile phase [$C_{A\,(m)}$]. It is calculated by equation 1.1:

$$K = \frac{C_{A\,(S)}}{C_{A\,(m)}} \qquad 1.1$$

1.5.2.3 Retention time

The visual output from a chromatograph (chromatographic instrument), termed chromatogram, is shown in Figure 1.2. It is a graphical display of detector signal and elution time. The time between sample injection and an analyte peak reaching a detector at the end of the column is termed as the retention time (t_R). Each analyte in a sample will

have a different retention time. The time taken for the mobile phase to pass through the column is called t_M.

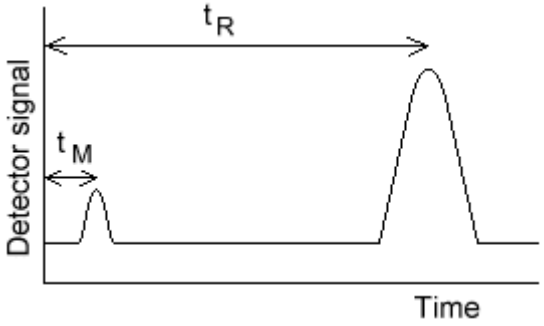

Figure 1.2: A typical chromatogram

1.5.2.4 Capacity factor

A term called the capacity factor, *k'*, is often used to describe the migration rate of an analyte on a column. The capacity factor (also termed as retention factor) for analyte *A* is defined as:

$$k'A = \frac{(t_R - t_M)}{t_M} \qquad 1.2$$

t_R and t_M are easily obtained from a chromatogram. When an analyte's capacity factor is less than one, elution is so fast that accurate determination of the retention time is very difficult. High retention factors (greater than 20) mean that elution takes a very long time. Ideally, the retention factor for an analyte is between 1 and 5.

1.5.2.5 Symmetry factor

The symmetry factor (A_S) for a peak can be calculated using equation 1.3:

$$A_S = \frac{W_x}{2d} \qquad 1.3$$

where, W_x is the peak width at 5% of peak height, measured from the baseline and *d* is the baseline distance between the perpendicular dropped from the peak maximum and the

leading edge of the peak at 5% of the peak height, measured in the same units as W_x. A_S value greater than 2 may lead to an incorrect integration, resulting in erroneous quantitation. The main factors that influence peak symmetry depend upon retention, solvent effects, incompatibility of the solute with the mobile phase, or development of an excessive void at the inlet of the column. In reverse-phase chromatography, adsorption phenomena due to the presence of residual silanol groups in the stationary phase may lead to tailing (poor peak symmetry).

1.5.2.6 Selectivity factor

A quantity called the selectivity factor, α, which describes the separation of two species (*A* and *B*) on the column can be calculated from equation 1.4:

$$\alpha = \frac{k'_A}{k'_B} \qquad 1.4$$

When calculating the selectivity factor, species *A* elutes faster than species *B*. The selectivity factor is always greater than one.

1.5.2.7 Band broadening and column efficiency

In order to achieve optimal separations, sharp, symmetrical (Gaussian) chromatographic peaks must be obtained. This means that band broadening must be limited.

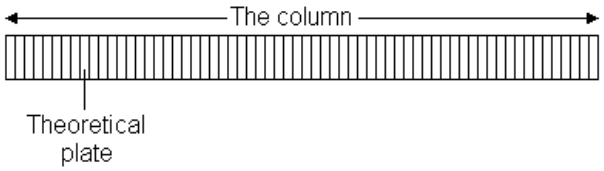

Figure 1.3: Chromatographic column and theoretical plates

It is also beneficial to measure the efficiency of the column. The plate model supposes that the chromatographic column is made up of a large number of separate layers, called *theoretical plates,* as shown in Figure 1.3.

Separate equilibrations of the sample between the stationary and mobile phase occur in these "plates". The analyte moves down the column by transfer of equilibrated mobile phase from one plate to the next. It is important to remember that the plates do not really exist; they are a figment of the imagination that helps us understand the processes at work in the column. They also serve as a way of measuring column efficiency, either by stating the number of theoretical plates in a column, *N* (the more plates the better), or by stating the plate height; the Height Equivalent to a Theoretical Plate (the smaller the better). The Height Equivalent to a Theoretical Plate (HETP) can be given by equation 1.5:

$$\text{HETP} = \frac{L}{N} \qquad 1.5$$

where, *L* is the length of the column and *N* is the number of theoretical plates in a column.

The number of theoretical plates that a real column possesses can be found by examining a chromatographic peak after elution from equation 1.6:

$$N = \frac{(5.55 \text{ x } t_R^2)}{W_{1/2}^2} \qquad 1.6$$

where, $W_{1/2}$ is the peak width at half-height. As can be seen from equation 1.5, columns behave as if they have different numbers of plates for different solutes in a mixture.

1.5.2.8 The rate theory of chromatography

A more realistic description of the processes at work inside a column takes account of the time taken for the solute to equilibrate between the stationary and mobile phase (unlike the plate model, which assumes that equilibration is infinitely fast). The resulting band shape of a chromatographic peak is therefore affected by the rate of elution. It is also affected by the different paths available to solute molecules as they travel between particles of stationary phase. If we consider the various mechanisms which contribute to band broadening, we arrive at the Van Deemter equation (equation 1.6) for plate height as given below:

$$HETP = A + \frac{B}{u} + C * u \qquad 1.7$$

where, u is the average velocity of the mobile phase, and A, B, C are the factors which contribute to band broadening. These factors are defined below:

***A*- Eddy diffusion:**

The mobile phase moves through the column which is packed with stationary phase. Solute molecules will take different paths through the stationary phase at random. This will cause broadening of the solute band, because different paths are of different lengths.

***B*- Longitudinal diffusion:**

The concentration of analyte is less at the edges of the band than at the centre. Analyte diffuses out from the centre to the edges. This causes band broadening. If the velocity of the mobile phase is high then the analyte spends less time on the column, which decreases the effects of longitudinal diffusion.

***C*- Resistance to mass transfer:**

The analyte takes a certain amount of time to equilibrate between the stationary and mobile phase. If the velocity of the mobile phase is high, and the analyte has a strong affinity for the stationary phase, then the analyte in the mobile phase will move ahead of the analyte in the stationary phase. The band of analyte is broadened. The higher the velocity of mobile phase, the worse the broadening becomes.

A plot of plate height vs. average linear velocity of mobile phase is called as Van Deemter plot (Figure 1.4). Such plots are of considerable use in determining the optimum mobile phase flow rate.

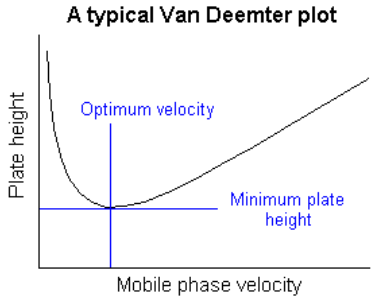

Figure 1.4: Van Deemter plot

1.5.2.9 Resolution

Although the selectivity factor, α, describes the separation of band centres, it does not take into account peak widths. Another measure of how well species have been separated is provided by measurement of the resolution. The resolution (*R*) of two species, *A* and *B*, is defined by equation 1.8:

$$R = \frac{2\,[(t_R)_B - (t_R)_A]}{W_A + W_B} \qquad 1.8$$

Baseline resolution is achieved when R = 1.5. It is useful to relate the resolution to the number of plates in the column, the selectivity factor and the retention factors of the two solutes, as given in equation 1.9:

$$R = \frac{\sqrt{N}}{4}\left(\frac{\alpha - 1}{\alpha}\right)\left(\frac{1 + k'_B}{k'_B}\right) \qquad 1.9$$

In order to achieve high resolution, the three terms must be maximised. An increase in N, the number of theoretical plates, by lengthening the column leads to an increase in retention time and increased band broadening - which may not be desirable. Instead, to increase the number of plates, the height equivalent to a theoretical plate can be reduced by reducing the size of the stationary phase particles. It is often found that by controlling the capacity factor, k', separations can be greatly improved. This can be achieved by changing the temperature (in Gas Chromatography) or the composition of the mobile phase (in Liquid Chromatography).

The selectivity factor, α, can also be manipulated to improve separations. When α is close to unity, optimising k' and increasing N is not sufficient to give good separation in a reasonable time. In these cases, k' is optimised first, and then α is increased by one of the following procedures:

1. Changing mobile phase composition.
2. Changing column temperature.
3. Changing composition of stationary phase.
4. Using special chemical effects (such as incorporating a species which complexes with one of the solutes into the stationary phase.

1.6 Liquid Chromatography (LC)

High-performance liquid chromatography (HPLC) is a form of liquid chromatography to separate compounds that are dissolved in solution. HPLC instruments consist of a reservoir of mobile phase, a pump, an injector, a separation column, and a detector. Compounds are separated by injecting a plug of the sample mixture onto the column. The different components in the mixture pass through the column at different rates due to differences in their partitioning behaviour between the mobile liquid phase and the stationary phase.

1.6.1 HPLC instrumentation

Main components in an HPLC system include the solvent reservoir, or multiple reservoirs, a high-pressure pump, a column, injector system and the detector. The reservoir holds the solvent, which is referred to as the mobile phase. There are usually a minimum of two reservoirs in a system, with each holding up to 1000 mL of solvent and usually fitted with a gas diffuser through which helium can be bubbled. A pump is used to generate a specified flow of the mobile phase. Although manual injection of samples is still possible, most HPLCs are now fully automated and controlled by a computer. The injector, or auto sampler, introduces the solvent into a phase stream that carries the sample into the high pressure (up to 400 bar) column, which contains specific packing material needed to effect separation. The packing material is referred to as the stationary phase because it is held in place by the column hardware. A detector is needed to see the separated compound bands as they elute from the high pressure column. The information is sent from the detector to a computer which generates the chromatogram. The mobile

phase exits the detector and is either sent to waste, or collected, as desired. The schematic of HPLC instrumentation is given in Figure 1.5.

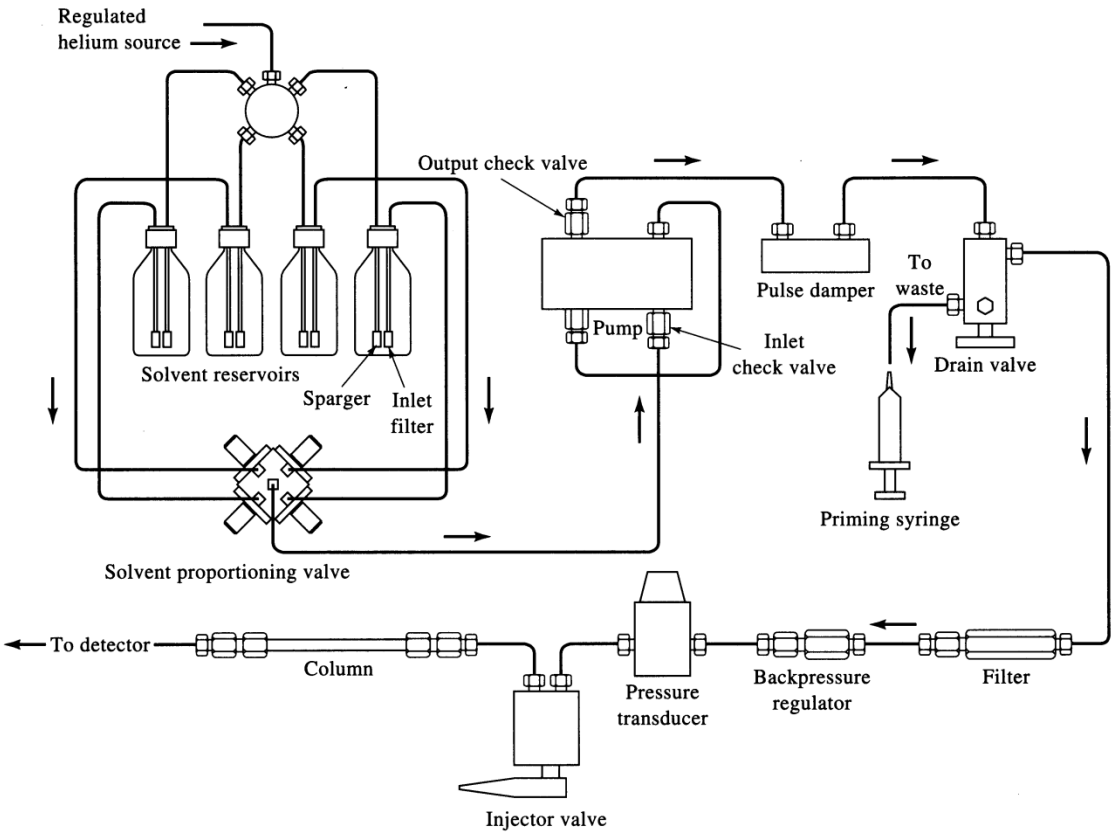

Figure 1.5: Schematic of HPLC instrumentation

1.6.2 Liquid chromatographic modes

1.6.2.1 Normal-Phase Chromatography

Normal-Phase liquid-liquid chromatography uses a polar stationary phase (often hydrophilic) and a less polar mobile phase. Here the mechanism involved in separation is adsorption of solute molecules on the stationary phase. The stationary phases generally used are silica, alumina, nitrile and amino bonded phases, diol-bonded phase; and the mobile phases used are non-polar solvents like pentane, hexane, cyclohexane etc.

1.6.2.2 Reverse-Phase (RP) chromatography

RP chromatography uses hydrophobic bonded packing, usually an octadecyl silane (ODS, or C18) or octal silane (C8) functional groups bonded to silica surface through silanol groups; and a polar mobile phase, which often comprises of partially aqueous mixtures of methanol and acetonitrile.

When a solute dissolves in water, the strong attractive forces between water molecules become distorted. These attractive forces arise from the three-dimensional network of intermolecular hydrogen bonds. Only highly polar or ionic solutes can interact with the network. Non-polar solutes are "squeezed out" of the mobile phase but bind with the hydrocarbon moieties of stationary phase. In reverse phase chromatography the driving force for retention is not the interaction of solute with the stationary phase, but the effect of the mobile phase solvent in forcing the solute into the hydrocarbon chains of bonded layer.

1.6.2.3 Ion-Exchange chromatography

Ion-exchange chromatography is carried out with column packing that have charge-bearing functional groups attached to the polymer matrix. The functional groups are permanently bonded ionic groups associated with counter ions of the opposite charge. The most common mechanism is the simple exchange of sample ions and mobile-phase ions with the charged group of stationary phase. Common functional groups in column packings are sulfonate for cation exchange and quaternary amine group for anion exchange.

1.6.2.4 Size Exclusion chromatography

It is also called *gel permeation chromatography,* which is a non-interactive mode of separation. Essentially a maze for molecules, the particles of column packing have various size pores and pore network, so that solute molecules are retained or excluded on the basis of their hydrodynamic molecular volume. As a sample passes through the column, the solute molecules are stored. Very large molecules cannot enter many of the pores, and they also penetrate less into comparatively open regions of the packing. Thus excluded, they travel mostly around the exterior of the packing and elute at the bed void volume of the mobile phase. Very small molecules diffuse into all or many of the pores accessible to them. A small molecule exits the column last.

1.6.2.5 Chiral chromatography

In chiral chromatography stationary phase contains one form of an enantiomeric compound immobilized on the surface of the support material. Typically, derivatives of optically active polysaccharides that are chemically bonded to silica form the packing material. A chiral separation is based on differing degrees of stereo chemical interaction between the components of an enantiomeric sample mixture and the stationary phase. One of the useful modes of chiral chromatography is chiral ligand exchange chromatography.

1.6.2.6 Chiral Ligand-Exchange Chromatography (CLEC)

Chiral Ligand Exchange Chromatography (CLEC), developed by Davankov[12] separates enantiomers by formation of diastereomeric metal complexes. The method is mainly used for separation of amino acids and also for α-hydroxy acids. A chiral 'acid-copper' complex is bound to silica or a polymeric stationary phase and copper ions are

included in the mobile phase to ensure there is no loss of copper. Acids may then be separated by forming diastereomeric copper complexes. Water stabilises the complex by coordinating in an axial position. Steric factors then determine which of the two complexes is most stable; one of the water molecules is usually sterically hindered from coordinating to the copper. A few other transition metals have been used in ligand exchange chromatography; however copper is most widely used. The complexation process is a comparatively slow one which is helped by running at elevated temperatures. The optimum temperature is around 50 ^{o}C.

1.6.3 HPLC detectors

The HPLC detectors are used to detect the solute present in the eluent comes from the HPLC column. The desired characteristics of HPLC detectors are given below:

- It should give response to all compounds in the mixture (a general detector) or it should give response with known sensitivity (a specific detector).
- It should not give response to mobile phase.
- It should give linear response to solute concentration.
- It should be unaffected by variation in temperature and flow rate.
- It should not contribute to zone spreading.[13]

Suitable detectors can be broadly divided into the following two classes:

1.6.3.1 Bulk property detectors

Bulk property detectors measure the difference in some physical property of solute in the mobile phase compared to the mobile phase alone, e.g. refractive index and conductivity detectors. They are generally universal in their application but tend to have poor sensitivity and limited range. Such detectors are usually affected by even small

changes in the mobile-phase composition which precludes the use of gradient elution.

1.6.3.2 Solute property detectors

These respond to a particular physical or chemical property of the solute, being ideally independent of the mobile phase. In practice, however, complete independence of the mobile phase is rarely achieved, but the signal discrimination is usually sufficient to permit operation with solvent changes, e.g. gradient elution. They generally provide high sensitivity (about 1 in 109 being attainable with UV and fluorescence detectors) and a wide linear response range but, as a consequence of their more selective nature, more than one detector may be required to meet the demands of an analytical problem. The examples of solute property detectors are spectrophotometric, fluorescence and electrochemical detectors.

Some of the important characteristics required of a detector are the following.

(i) Sensitivity, which is often expressed as the noise equivalent concentration, i.e. the solute concentration, C_n, which produces a signal equal to the detector noise level. The lower the value of C_n for a particular solute, the more sensitive is the detector for that solute.

(ii) A linear response, the linear range of a detector is the concentration range over which its response is directly proportional to the concentration of solute. Quantitative analysis is more difficult outside the linear range of concentration.

(iii) Type of response, i.e. whether the detector is universal or selective. A universal detector will sense all the constituents of the sample, whereas a selective one will only respond to certain components. Although the response of the detector will not be independent of the operating conditions, e.g. column temperature or flow rate, it is

advantageous if the response does not change too much when there are small changes of these conditions.[14]

1.6.3.3 UV-visible detector

UV and visible region of the electromagnetic radiation corresponds to the excitation of relatively low energy electrons such as Π-electrons, or non-paired electrons of some functional groups. For example, n-alkanes could absorb in the UV region below 180 nm. s-electrons require high energy radiation to get excited and to show absorption of the radiation. But, any compound which has benzene ring will show absorbance in the range 205-225 and 245-265 nm. The last corresponds to the excitation of conjugated Π - electrons of the benzene ring.

A majority of organic compounds can be analysed by UV/VIS detectors. Almost 70% of published HPLC analyses were performed with UV/VIS detectors. This fact, plus the relative ease of its operation, makes the UV detector the most useful and the most widely used LC detector.[15] A schematic diagram of UV-Visible detector is given in Figure 1.6.

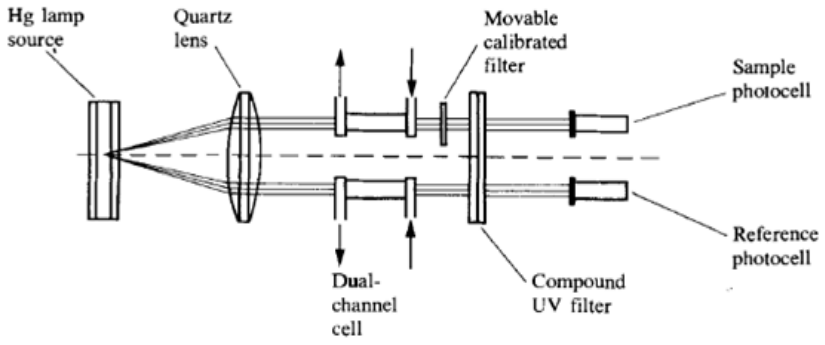

Figure 1.6: Schematic diagram of UV-Visible detector

1.6.3.4 Photodiode array detector (PAD)

This is also a UV detector wherein the light from the broad emission source is collimated by an achromatic lens system so that the total light passes through the detector cell onto a holographic grating. In this way the sample is subjected to light of all wavelengths generated by the lamp. The dispersed light from the grating is allowed to fall onto a diode array. The array may comprise of many hundreds of diodes and the output from each diode is regularly sampled by a computer and stored on a hard disc. At the end of the run, the output from any diode can be selected and a chromatogram produced using the UV wavelength that falls on that particular diode. During chromatographic development, the output of one diode is recorded in real time producing a real time chromatogram. It is seen that by noting the time of a particular peak, a spectrum can be obtained by recalling from memory the output of all the diodes at that particular time.[16] A schematic diagram of photodiode array detector is given in Figure 1.7

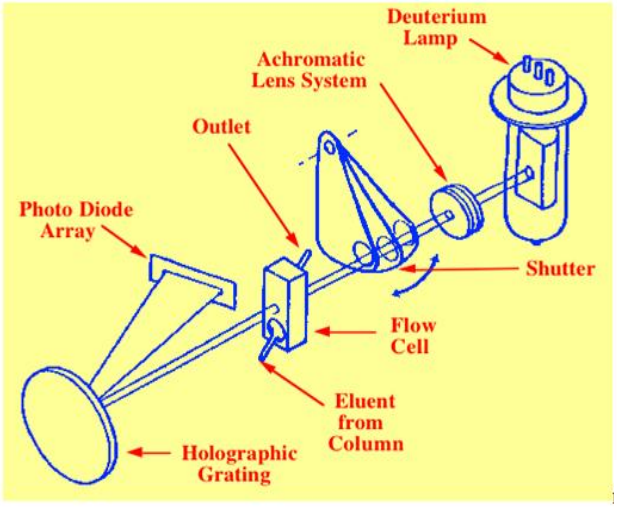

Figure 1.7: Schematic diagram of photodiode array detector

1.6.3.5 Refractive index (RI) detector

The refractive index (RI) detector is the only universal detector in HPLC. The detection principle involves measuring the change in refractive index of the column effluent passing through the flow-cell. The greater the RI difference between sample and mobile phase, the larger is the imbalance. Thus, the sensitivity will be more for the higher difference in RI between sample and mobile phase. On the other hand, in complex mixtures, sample components may cover a wide range of refractive index values and some may closely match with that of the mobile phase, becoming invisible to the detector. RI detector is a pure differential instrument, and any changes in the eluent composition require rebalancing of the detector. This factor severely limits RI detector application in analyses requiring gradient elution, where mobile phase composition is changed during the analysis to effect separation.

This detector is based on the deflection principle of refractometry, where the change in deflection of a light beam is observed when the composition in the sample flow-cell changes in relation to the reference side (as eluting sample moves through the system). When no sample is present in the cell, the light passing through both sides is focused on the photodetector (usually photoresistor). As sample elutes through one side, the changing angle of refraction moves the beam. This results in a change in the photon current falling on the detector which unbalances it. The extent of imbalance (which can be related to the sample concentration) is recorded on a strip chart recorder. The optical schematic of the deflection detector is shown in Figure 1.8.

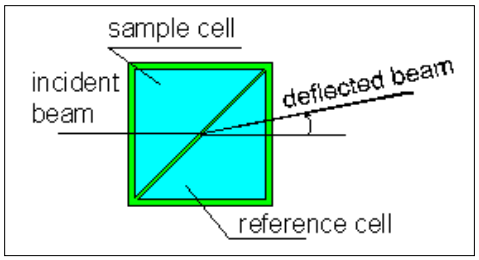

Figure 1.8: The optical schematic of the deflection RI detector

The advantages of this type of detector are: (1) universal response; (2) low sensitivity to dirt and air bubbles in the cells; and (3) the ability to cover the entire refractive index range from 1.000 to 1.750 RI with a single, easily balanced cell. The disadvantages are the relatively low sensitivity and a general inability to easily remove and clean or replace the cell when filming or clogging occurs.

1.6.3.6 Fluorescence detector

Compounds (solutes) present in the mobile-phase on being passed as column effluent through a cell irradiated with Xenon or Deuterium source first absorb UV radiation and subsequently emit radiation of a longer wavelength in two different manner, viz. (*a*) Instantly- termed as Fluorescence and (*b*) After a time-gap- known as Phosphorescence.[17]

When a molecule adsorbs light, a transition to a higher electronic state takes place and this absorption is highly specific for the molecules concerned; radiation of a specific wavelength or energy is only absorbed by a particular molecular structure. If electrons are raised to an upper excited single state, due to absorption of light energy, and the excess energy is not immediately dissipated by collision with other molecules or by other means, light will be emitted at a lower frequency as the electron returns to its ground state and the substance is said to fluoresce.[18] These devices enable fluorescent

compounds (solutes) present in the mobile phase to be detected by passing the column effluent through a cell irradiated with ultraviolet light and measuring any resultant fluorescent radiation. The application of fluorescence detectors has been extended by means of pre- and post-column derivatisation of non-fluorescent or weakly fluorescing compounds.[14] A schematic diagram of fluorescence detector is given in Figure 1.9.

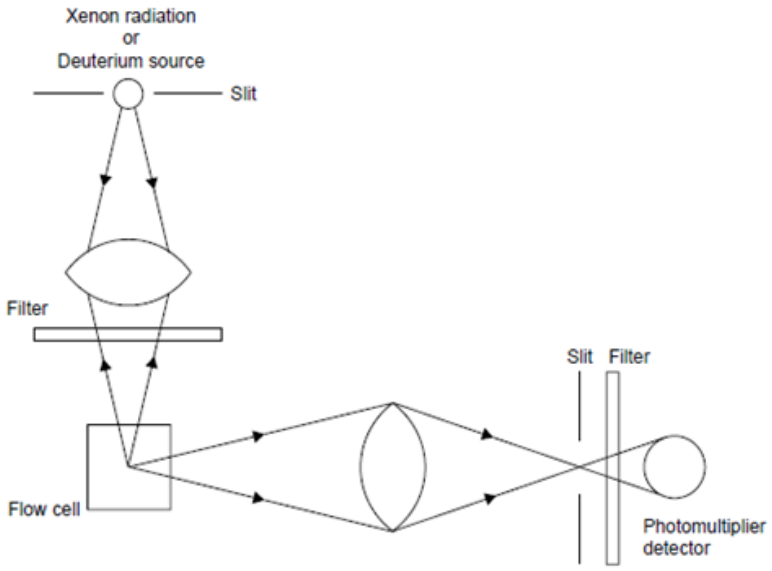

Figure 1.9: Schematic diagram of fluorescence detector

1.6.3.7 Electrochemical detector

The term 'electrochemical detector' in HPLC normally refers to amperometric or coulometric detectors, which measure the current associated with the oxidation or reduction of solutes. In practice it is difficult to use electrochemical reduction as a means of detection in HPLC because of the serious interference (large background current) caused by reduction of oxygen in the mobile phase. Complete removal of oxygen is difficult so that electrochemical detection is usually based on oxidation of the solute. Examples of compounds which can be conveniently detected in this way are phenols, aromatic amines, heterocyclic nitrogen compounds, ketones, and aldehydes.

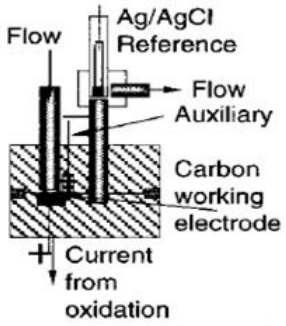

Figure 1.10: Schematic diagram of electrochemical detector

Since not all compounds undergo electrochemical oxidation, such detectors are selective and selectivity may be further increased by adjusting the potential applied to the detector to discriminate between different electro active species. In short, the amperometric detector is presently considered to be the best electrochemical detector having the distinct advantages, such as (*i*) very small internal cell-volume, (*ii*) high degree of sensitivity, (*iii*) more limited range of applications, and (*iv*) excellent for trace analyses as UV-detector lacks adequate sensitivity.[17] A schematic diagram of electrochemical detector is given in Figure 1.10.

1.6.3.8 Evaporative light scattering detector (ELSD)

In ELSD, detection is based on the scattering of a beam of light by particles of compound remaining after evaporation of the mobile phase. This detector is of growing importance and known to be an universal detector, which does not require a compound to have a chromophore for detection. Application includes the analysis of surfactant, lipid, and sugar. Unlike the refractive index detector, which was formerly used for this analysis, it can be used with gradient elution and is robust enough to function under a wide range of operating conditions. However, it can not be used with non-volatile material such as buffer in mobile phase or to detect very volatile analytes. The typical applications include

analysis of chloride and sodium ions in pharmaceuticals, lipids used as component in formulations, sugar and sugar polymers.[19] A schematic diagram of evaporative light scattering detector is given in Figure 1.11.

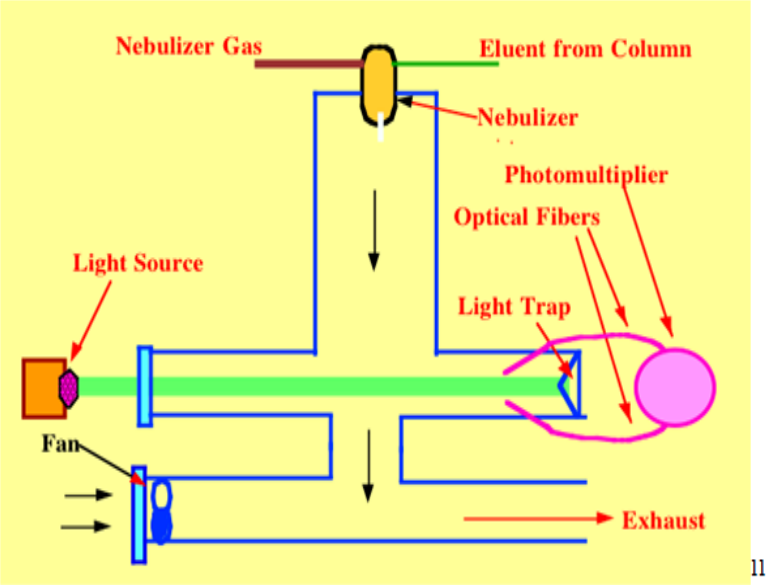

Figure 1.11: Schematic diagram of evaporative light scattering detector

1.6.3.9 Mass spectrometer (MS) detector

Mass spectrometry is a sensitive technique used to detect, identify and quantitate molecules based on their mass and charge (*m/z*). Originally developed almost 100 years ago to measure elemental atomic weights and the natural abundance of specific isotopes[20], MS was first used in the biological sciences to trace heavy isotopes through biological systems and later to sequence oligonucleotides and peptides and analyse nucleotide structure.[21]

All mass spectrometers have an ion source, a mass analyser and an ion detector. The nature of these components varies based on the type of mass spectrometer, the type of data required and the physical properties of the sample. Samples are loaded into the

mass spectrometer in liquid or dried form and then vapourised and ionised by the ion source (e.g., ESI, MALDI). The charge that these molecules receive allows the mass spectrometer to accelerate the ions throughout the remainder of the system. The ions encounter electrical and/or magnetic fields from mass analysers, which deflect the paths of individual ions based on their mass and charge (*m/z*). Commonly used mass analysers include time-of-flight [TOF], quadrupoles and ion traps, and each type has specific characteristics. Mass analysers can be used to separate all analytes in a sample for global analyses, or they can be used essentially like a filter to properly deflect only specific ions towards the detector. Ions that have successfully been deflected by the mass analysers then hit the ion detector. Most often, these detectors are electron multipliers or microchannel plates, which emit a cascade of electrons when each ion hits the detector plate.[21] This cascade results in amplification of each ion hit for improved sensitivity.

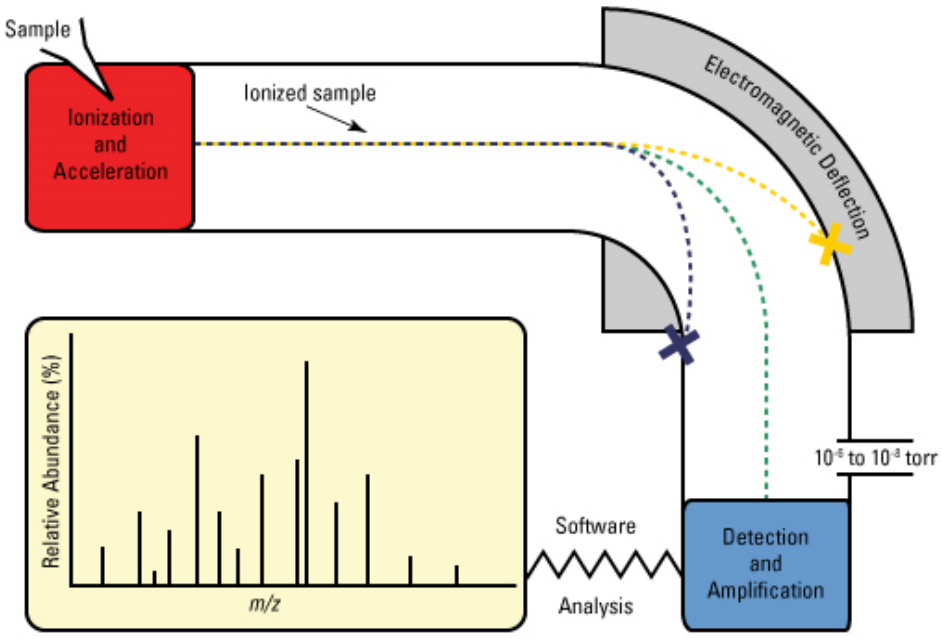

Figure 1.12: Diagram of a sector mass spectrometer

This entire process is performed under an extreme vacuum (10^{-6} to 10^{-8} Torr) to remove contaminating non-sample ions, which can collide with sample ions and alter their paths or produce non-specific reaction products.[22] Mass spectrometers are connected to computers with software that analyses the ion detector data and produces graphs that organise the detected ions by their individual *m/z* and relative abundance. These ions can then be processed through databases to predict the identity of the molecule based on the *m/z*. The diagram of a sector mass spectrometer is given in Figure 1.12.

1.7 Gas Chromatography (GC)

In gas chromatography, the *mobile phase* (or "moving phase") is a carrier gas, usually an inert gas such as helium or nitrogen. The *stationary phase* is a microscopic layer of liquid or polymer on an inert solid support, inside a piece of glass or metal tubing, called a column. The gaseous compounds being analysed interact with the walls of the column, which is coated with a stationary phase. This causes each compound to elute at a different time, known as the *retention time* of the compound. To be suitable for GC analysis, a compound must have sufficient volatility and thermal stability. If a compound is fully or partially in the gas or vapour phase at 400-450 °C, and is stable at these temperatures, the compound can probably be analysed by GC.

1.7.1 GC instrumentation

One or more high purity gases are supplied to the GC. One of the gases (called the carrier gas) flows into the injector, through the column and then into the detector. A sample is introduced into the injector usually with a syringe or an exterior sampling device. The injector is usually heated to 150-250°C which causes the volatile sample

solutes to vapourise. The vapourised solutes are transported into the column by the carrier gas. The column is maintained in a temperature controlled oven.

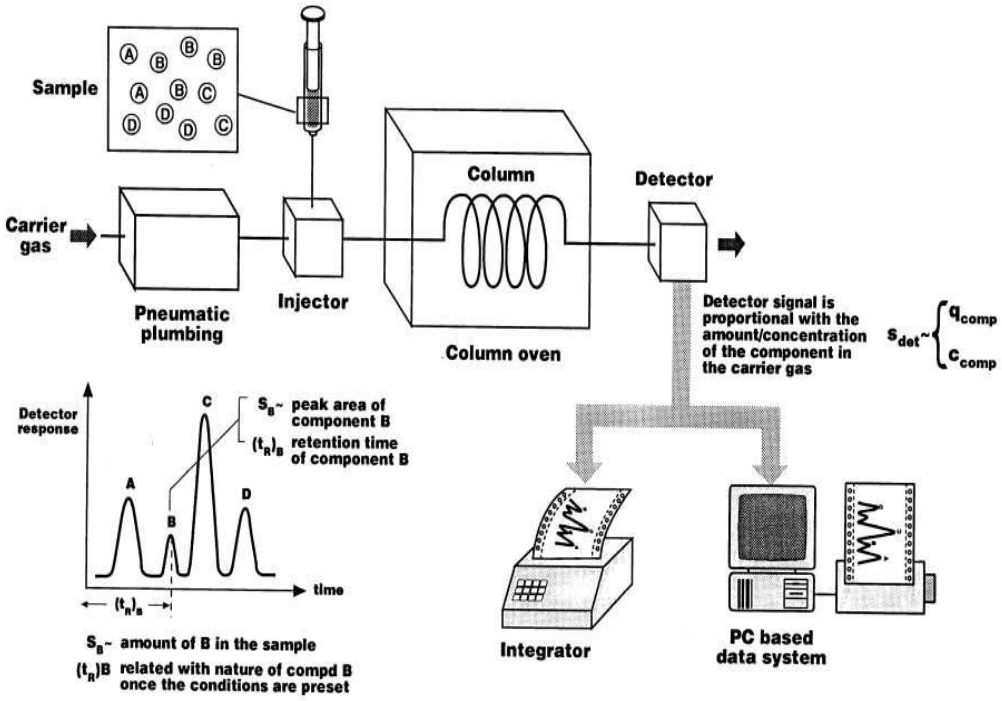

Figure 1.13: Schematic diagram of GC instrumentation

The solutes travel through the column at a rate primarily determined by their physical properties, and the temperature and composition of the column. The various solutes travel through the column at different rates. The fastest moving solute exits (elutes) the column first then is followed by the remaining solutes in corresponding order. As each solute elutes from the column, it enters the heated detector. An electronic signal is generated upon interaction of the solute with the detector. The size of the signal is recorded by a data system and is plotted against elapsed time to produce a chromatogram. The Schematic of GC instrumentation is given in Figure 1.13.

1.7.2 GC stationary phases

1.7.2.1 Polysiloxanes

Polysiloxanes are the most common stationary phases. They are available in great variety and are the most stable, robust and versatile. The most basic polysiloxane is the 100% methyl substituted. When other groups are present, the amount is indicated as the percent of the total number of groups. For example, a 5% diphenyl-95% dimethyl polysiloxane contains 5% phenyl groups and 95% methyl groups. The "di-" prefix indicates that each silicon atom is coupled to two of that particular group. Sometimes this prefix is omitted even though two identical groups are present. If the methyl percentage is not stated, it is understood to be present in the amount necessary to make 100% (e.g., 50% phenyl-methyl polysiloxane contains 50% methyl substitution). Cyanopropylphenyl percent values can be misleading. A 14% cyanopropylphenyl-dimethyl polysiloxane is comprised of 7% cyanopropyl and 7% phenyl (along with 86% methyl). The cyanopropyl and phenyl groups are on the same silicon atom, thus their amounts are summed.

1.7.2.2 Polyethylene glycols

Polyethylene glycols (PEG) are widely used as stationary phases. Stationary phases with "wax" or "FFAP" in their name are some type of polyethylene glycol. Polyethylene glycol stationary phases are not substituted, thus the polymer is 100% of the stated material. They are less stable, less robust and have lower temperature limits than most polysiloxanes. With typical use, they exhibit shorter lifetimes and are more susceptible to damage upon over-heating or exposure to oxygen. The unique separation properties of polyethylene glycol make these liabilities tolerable. Polyethylene glycol stationary phases must be liquids under GC temperature conditions.

1.7.2.3 Gas-solid (PLOT columns)

Gas-solid stationary phases are comprised of a thin layer (usually < 10 µm) of small particles adhered to the surface of the tubing. These are porous layer open tubular (PLOT) columns. The sample compounds undergo a gas-solid adsorption/desorption process with the stationary phase. The particles are porous, thus size exclusion and shape selectivity processes also occur. Various derivatives of styrene, aluminium oxides and molecular sieves are the most common PLOT column stationary phases. PLOT columns are very retentive. They are used to obtain separations that are impossible with conventional stationary phases. Also, many separations, requiring sub-ambient temperatures with polysiloxanes or polyethylene glycols, can be easily accomplished above ambient temperatures with PLOT columns. Hydrocarbon and sulphur gases, noble and permanent gases, and low boiling point solvents are some of the more common compounds separated with PLOT columns. Some PLOT columns may occasionally lose particles of the stationary phase. For this reason, using PLOT columns that may lose particles with detectors negatively affected by particulate matter is not recommended. Mass spectrometers are particularly susceptible to this problem due to the presence of a strong vacuum at the exit of the column.

1.7.2.4 Bonded and cross-linked stationary phases

Cross-linked stationary phases have the individual polymer chains linked via covalent bonds. Bonded stationary phases are covalently bonded to the surface of the tubing. Both techniques impart enhanced thermal and solvent stability to the stationary phase. Also, columns with bonded and cross-linked stationary phases can be solvent rinsed to remove contaminants. Most polysiloxanes and polyethylene glycol stationary

phases are bonded and cross-linked. A few stationary phases are available in a non-bonded version.

1.7.2.5 Chiral stationary phases

The first effective chiral stationary phase utilised derivatised amino acids to provide chiral selectivity.[23,24] The simple dipentylation of the cyclodextrins produces a very dispersive (hydrophobic) environment around the chiral centres of cyclodextrin and, thus, the enantiomer that fits closest to the structure is held more strongly due to dispersive (hydrophobic) forces and, thus, preferentially retained. The selectivity will depend on the size and shape of the analyte as well as the functional groups present.

The substitution of the hydroxyl groups of a cyclodextrin with pure "S" hydroxypropyl groups followed by permethylation yields a polar (hydrophilic) surface that will afford polar interactions with the closest fitting enantiomer and, thus, provide selective retention. All three cyclodextrins can be treated in this way but it has been shown that the β derivative is significantly more selective than either the α or the γ derivatives. These stationary phases can be used isothermally up to 200 ^{o}C and can be programmed up to 220 ^{o}C. The permethylated β cyclodextrin has been shown to have broad chiral selectivity, based largely on polar (hydrophilic) interactions and in some cases inclusion/size selectivity. This material is thermally stable up to temperatures of 230-250 ^{o}C and appears to have the potential to separate over 30% of the GC chiral separations published to date.

1.7.3 GC detectors

After the components of a mixture are separated using gas chromatography, they must be detected as they exit the GC column. Thermal-conductivity (TCD) and flame-ionisation (FID) detectors are the two most common detectors on commercial gas chromatographs. The requirements of a GC detector depend on the separation application. For example, one analysis might require a detector that is selective for chlorine-containing molecules; another analysis might require a detector that is non-destructive so that the analyte can be recovered for further spectroscopic analysis.

1.7.3.1 Flame ionisation detector (FID)

The FID consists of a hydrogen/air flame and a collector plate. The effluent from the GC column passes through the flame, which breaks down organic molecules and produces ions. The ions are collected on a biased electrode and produce an electrical signal. The FID is extremely sensitive with a large dynamic range.

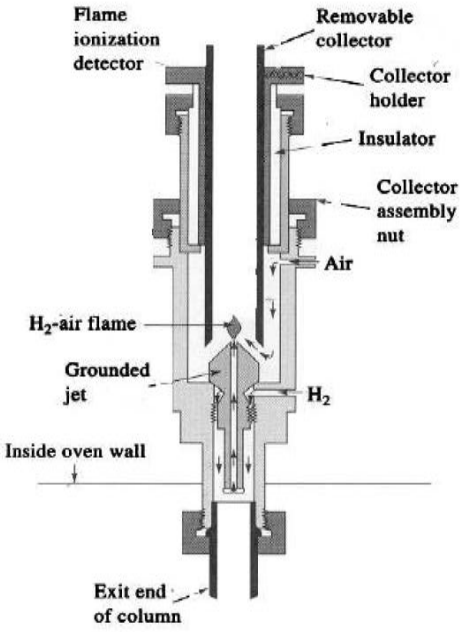

Figure 1.14: Schematic diagram of flame ionisation detector

Its only disadvantage is that it destroys the sample. The schematic of FID is shown in Figure 1.13.

1.7.3.2 Thermal conductivity detector (TCD)

A TCD consists of an electrically-heated wire or thermistor. The temperature of the sensing element depends on the thermal conductivity of the gas flowing around it. Changes in thermal conductivity, such as when organic molecules displace some of the carrier gas, causes a temperature rise in the element which is sensed as a change in resistance. Two pairs of TCDs are used in gas chromatographs. One pair is placed in the column effluent to detect the separated components as they leave the column, and another pair is placed before the injector or in a separate reference column. The resistance of the two sets of pairs are then arranged in a bridge circuit. The bridge circuit allows amplification of resistance changes due to analytes passing over the sample thermo-conductors and does not amplify changes in resistance that both sets of detectors produce due to flow rate fluctuations, etc. The TCD is not as sensitive as other detectors but it is non-specific and non-destructive. The schematic of a bridge circuit for TCD detection is given in Figure 1.15.

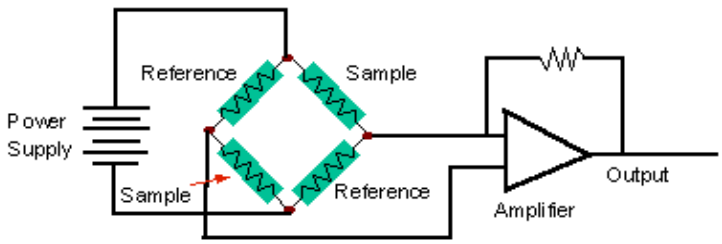

Figure 1.15: Schematic of a bridge circuit for TCD detection

1.7.3.3 Electron capture detector (ECD)

The ECD uses a radioactive β emitter (electrons) to ionise some of the carrier gas and produce a current between a biased pair of electrodes. When organic molecules that have electronegative functional groups, such as halogens, phosphorous, and nitro groups pass the detector, they capture some of the electrons and reduce the current measured between the electrodes. The ECD is as sensitive as the FID but has a limited dynamic range and finds its greatest application in analysis of halogenated compounds.

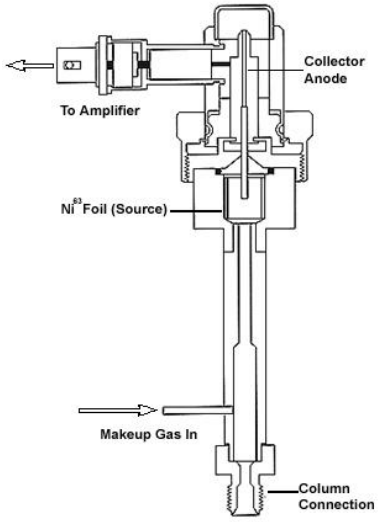

Figure 1.16: Schematic of electron capture detector

1.7.3.4 Nitrogen-phosphorous detector (NPD)

The overall design of a nitrogen-phosphorus detector (NPD) is similar to a flame-ionisation detector (FID). The major difference is that the hydrogen/air flame of the FID is replaced by a heated rubidium silicate bead in the NPD. The effluent from the GC column passes through the hot bead. The hot rubidium salt emits ions when nitrogen and phosphorus-containing compounds pass over it. The ions are collected on a collector

above the heated bead to produce a current, similar to the FID. The Schematic of nitrogen-phosphorous detector is given in Figure 1.17.

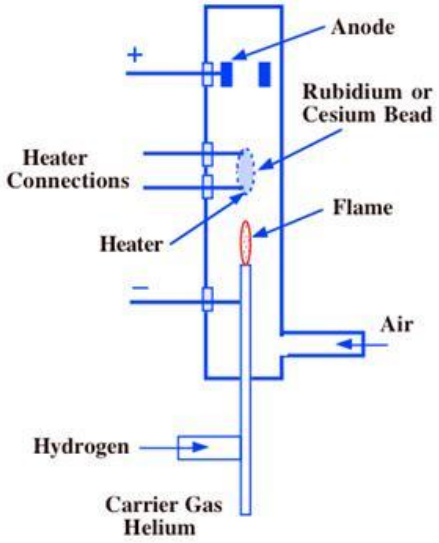

Figure 1.17: Schematic of nitrogen-phosphorous detector

1.7.3.5 Flame photometric detector (FPD)

Flame photometric detector is used to analyse sulphur or phosphorous containing compounds and metals such as tin, boron, arsenic and chromium. An FPD uses a hydrogen/air flame into which the sample is passed.

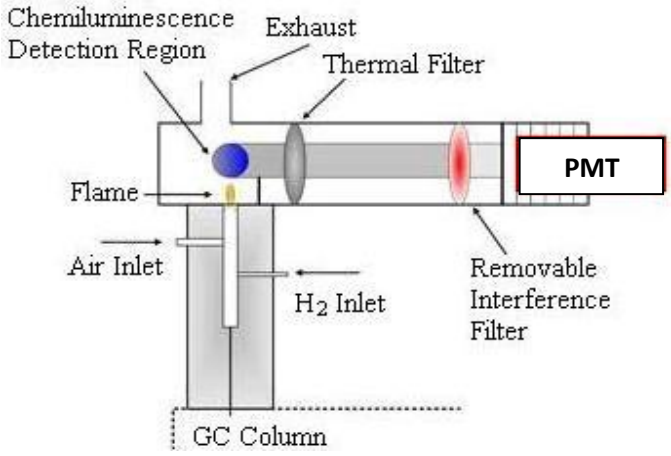

Figure 1.18: Schematic of flame photometric detector

Phosphorous and sulphur containing hydrocarbons generate chemiluminescene at specific wavelengths which when passed into a photo-multiplier give a measurable electrical signal. The schematic of FPD is given in Figure 1.18.

1.8 Method Validation

Method validation can be briefly defined as the process to confirm that the analytical procedure employed for a specific test is suitable for its intended use. The objective of any analytical measurement is to obtain consistent, reliable and accurate data. Validated analytical methods play a major role in achieving this goal. The results from method validation can be used to judge the quality, reliability and consistency of analytical results, which is an integral part of any good analytical practice. Validation of analytical methods is also required by most regulations and quality standards that impact laboratories.

1.8.1 Method validation literature overview

There are various guidelines published in literature explaining terminology and definitions for validation parameters, references of which are given below.

- The Laboratory of the Government Chemist (LGC) developed a guide for internal method validation.[25] It includes a discussion of related laboratory accreditation requirements.
- The United States Food and Drug Administration developed two industry guidelines: one for the validation of analytical methods[26] and one for the validation of bioanalytical methods.[27]
- ICH published two guidelines for method validation. Q2A[28] describes terminology and definitions for eight validation parameters that should be considered for validation.

Q2B[29] includes methodology but allows flexibility through the statement "It is the responsibility of the applicant to choose the validation procedure and the protocol most suitable for their product".

- IUPAC[30] published "Harmonised Guidelines for Single-laboratory validation of methods of analysis".
- EURACHEM[31] published a detailed guide for method validation. This is the most detailed official guide for theory and practice of method validation.
- Huber[32] authored a validation reference book for the analytical laboratory with a chapter on method validation.
- AOAC[33] has published a technical document for the verification of analytical methods for the ISO 17025 accreditation.
- Viswanathan and co-authors[34] developed an overview for validation of bioanalytical methods.

1.8.2 Parameters and tests for method validation

The parameters for method validation have been defined in different working groups of national and international committees. Unfortunately some of the definitions are different between different organisations. An attempt for harmonisation was made for pharmaceutical applications through the International Conference on Harmonisation[28,29] where representatives from the industry and regulatory agencies from USA, Europe and Japan defined parameters, requirements and, to some extent, also methodology for analytical methods validation. The parameters, as defined by the ICH and other organisations and authors, are discussed below in brief.

1.8.2.1 Specificity/Selectivity

Specificity can be defined as "the ability to assess unequivocally the analyte in the presence of other components. Typically this might include impurities, degradants, matrix, etc."USP <1225> refers to the same definition but also comments that other reputable authorities such as IUPAC and AOAC use the term "selectivity" for the same meaning. This reserves the use of "specific" for those procedures that produce a response for a single analyte only. ISO/IEC most likely has the same understanding because it requires a method to be "selective" rather than specific. Analytical techniques that can measure the analyte response in the presence of all potential sample components should be used for specificity validation. In practice, a test mixture is prepared that contains the analyte and all potential sample components. Specificity in liquid chromatography is obtained by choosing optimal columns and setting chromatographic conditions, such as mobile phase composition, column temperature and detector wavelength. Besides chromatographic separation, the sample preparation step can also be optimised for best selectivity.

1.8.2.2 Precision

The precision of an analytical procedure is defined as the closeness of agreement (degree of scatter) between a series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed conditions. Precision may be considered at three levels:

- Repeatability,
- Intermediate precision, and
- Reproducibility.

Repeatability expresses the precision under the same operating conditions over a short interval of time. Repeatability is also termed intra-assay precision. Intermediate precision expresses variations within laboratories, such as different days, different analysts, different equipment, and so forth. Reproducibility expresses the precision between laboratories (collaborative studies usually applied to standardisation of methodology).

1.8.2.3 Accuracy and recovery

The accuracy of an analytical procedure is defined as the closeness of agreement between the conventional true value or an accepted reference value and the value found. Accuracy can also be described as the extent to which test results generated by the method and the true value agree. The true value for accuracy assessment can be obtained in several ways. One alternative is to compare the results of the method with results from an established reference method. This approach assumes that the uncertainty of the reference method is known. Secondly, accuracy can be assessed by analysing a sample with known concentrations (for example, a control sample or certified reference material) and comparing the measured value with the true value as supplied with the material. If certified reference materials or control samples are not available, a blank sample matrix of interest can be spiked with a known concentration by weight or volume. After extraction of the analyte from the matrix and injection into the analytical instrument, its recovery can be determined by comparing the response of the extract with the response of the reference material dissolved in a pure solvent. Because this accuracy assessment measures the effectiveness of sample preparation, care should be taken to mimic the actual sample preparation as closely as possible. If validated correctly, the

recovery factor determined for different concentrations can be used to correct the final results.

1.8.2.4 Linearity and calibration curve

Linearity of an analytical procedure is its ability (within a given range) to obtain test results that are directly proportional to the concentration (amount) of analyte in the sample. Linearity may be demonstrated directly on the test substance (by dilution of a standard stock solution) or by separately weighing synthetic mixtures of the test product components. Linearity is determined by a series of five to six injections of five or more standards whose concentrations span 80–120 percent of the expected concentration range. The response should be directly proportional to the concentrations of the analytes or proportional by means of a well-defined mathematical calculation. A linear regression equation applied to the results should have an intercept not significantly different from zero. If a significant non-zero intercept is obtained, it should be demonstrated that this has no effect on the accuracy of the method. ICH recommends the linearity curve's correlation coefficient, y-intercept, slope of the regression line and residual sum of squares for accuracy reporting. A plot of the data should be included in the report. In addition, an analysis of the deviation of the actual data points from the regression line may also be helpful for evaluating linearity. Some analytical procedures, such as immunoassays, do not demonstrate linearity after any transformation. In this case, the analytical response should be described by an appropriate function of the concentration (amount) of an analyte in a sample. In order to establish linearity, a minimum of five concentrations is recommended. Other approaches should be justified.

1.8.2.5 Range

The range of an analytical procedure is defined as the interval from the upper to the lower concentration (amounts) of analyte in the sample (including these concentrations) for which it has been demonstrated that the analytical procedure has a suitable level of precision, accuracy and linearity. The range of an analytical method is the interval from the upper to the lower levels (including these levels) that have been demonstrated to be determined with precision, accuracy and linearity using the method as written. The range is normally expressed in the same units as the test results (for example percentage, parts per million) obtained by the analytical method.

1.8.2.6 Limit of detection

The detection limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be detected but not necessarily quantified as an exact value. The limit of detection (LOD) is the point at which a measured value is larger than the uncertainty associated with it. It is the lowest concentration of analyte in a sample that can be detected but not necessarily quantified. The limit of detection is frequently confused with the sensitivity of the method. The sensitivity of an analytical method is the capability of the method to discriminate small differences in concentration or mass of the test analyte. In practical terms, sensitivity is the slope of the calibration curve that is obtained by plotting the response against the analyte concentration or mass. In chromatography, the detection limit is the injected amount that results in a peak with a height at least two or three times as high as the baseline noise level.[35]

1.8.2.7 Limit of quantification

The limit of quantification or quantitation (LOQ) of an individual analytical procedure is the lowest amount of analyte in a sample which can be quantitatively determined with suitable precision and accuracy. The quantification limit is a parameter of quantitative assays for low levels of compounds in sample matrices, and is used particularly for the determination of impurities or degradation products. The quantification limit is generally determined by the analysis of samples with known concentrations of analyte and by establishing the minimum level at which the analyte can be quantified with acceptable accuracy and precision. In chromatography, the quantification limit is the injected amount that results in a peak with a height equal to ten times the baseline noise level.[35]

1.8.2.8 Ruggedness

Ruggedness is defined as the degree of reproducibility of results obtained under a variety of conditions, such as different laboratories, analysts, instruments, environmental conditions, operators and materials. Ruggedness is a measure of the reproducibility of test results under normal, expected operational conditions from laboratory to laboratory and from analyst to analyst. Ruggedness is determined by the analysis of aliquots from homogeneous lots in different laboratories.

1.8.3 Selecting validation parameters and limits

For an efficient validation process, it is important to specify the right validation parameters and acceptance criteria. The method's performance parameters and limits should be based on the intended use of the method. It is not always necessary to validate all analytical parameters available for a specific technique. For example, if the method is

to be used for qualitative trace level analysis, there is no need to test and validate the method's limit of quantification, or the linearity over the full dynamic range of the equipment. The more parameters, the more time it will take to validate. It is not always essential to validate every analytical performance parameter, but it is necessary to define the ones that are required.

1.9 Gauge Repeatability and Reproducibility (R&R)

All measurement systems have error. The error may be so small as to be irrelevant or it may be so large that we cannot trust our data. Regardless, they all have error. This means that if we want to choose a gauge (instrument or device) we can trust, we need to understand the extent of this error, and we can do that through Measurement System Analysis (MSA). MSA is a set of techniques that allow us to assess how much error is being introduced by the measurement system. Six Sigma is a quality goal, where sigma is a statistical measure of the variability in a measurement system. ANOVA gauge R&R is an important tool within the Six Sigma methodology.[36-43]

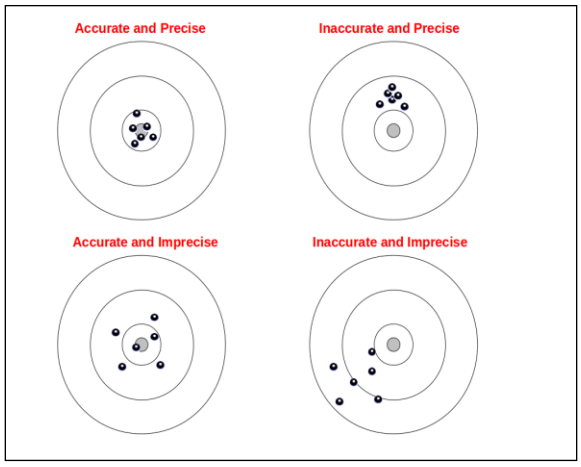

Figure 1.19: Pictorial representation of accuracy and precision

ANOVA gauge (repeatability and reproducibility) R&R is a measurement systems analysis technique which estimates the amount of variability induced in measurements by the measurement system itself, and compares it to the total variability observed in order to determine the viability of the measurement system.

Two fundamental aspects can be defined for a gauge: precision (repeatability and reproducibility) and accuracy. Figure 1.19 gives pictorial representation of accuracy and precision.

Accuracy is a measure of how closely the result of an experiment agrees with the expected result. The difference between the obtained result and the expected one is usually divided by the expected result and reported as a percent relative error as given in equation 1.10.

$$\%Error = \frac{(Obtained\ result - Expected\ result)}{Expected\ result} \text{ x } 100 \qquad 1.10$$

When a sample is analysed several times, the individual results are rarely the same. Instead, the results are randomly scattered. Precision is a measure of this variability. The closer the agreement between individual analyses, the more precise the results.

A Gauge R&R Study is a particular type of designed experiment used to estimate:

- What is the total variation, or spread, in the measurement system? - What is the Gauge R&R?
- How much of the variation is due to differences between the Appraisers?
- How much of the variation is due to the Gauge itself?

- Is there a link between the size of the parts measured, and the measurement each appraiser gets? - Is there an Part / Appraiser interaction?

1.9.1 Main factors affecting a measurement system

The main factors affecting a measurement system are given below. Gauge R&R tries to estimate and/or relate all these components with each other to assess the "goodness" of the measurement system.

- *Measuring instruments:* the gauge or instrument itself and all mounting blocks, supports, fixtures, load cells, etc.
- *Operators or Analysts:* the ability of a person to follow the written or verbal instructions to perform the measurements.
- *Parts (what is being measured)*: some items are easier to be measured than others; this affects the precision of measurement system.
- *Specification*: the measurement is reported against a specification or a reference value.

Here, the number and the type of Parts (Samples) to be used must be chosen so as to cover all the operative process range and the variability within it. The measurement condition is usually chosen as operators' alternation (Operators or Analysts). The number of repeated measurements in different times, after previous points are set; this number usually ranges from 2 to 5. In this study, the measurements order is randomised by varying previous points. This is required to minimise systematic errors.

1.9.2 Analysis of variance (ANOVA) method

This method is more flexible than other methods because it can be used with any number of samples, operators/analysts and repetitions. Also, this method is used to

estimate repeatability and reproducibility of measurement systems. The total variability of a measurement method can be given by equation 1.11

$$\sigma^2\ (Total) = \sigma^2\ (Product) + \sigma^2\ (Gauge) \quad 1.11$$

where, σ^2 *(Product)* is a variability component intrinsic to the product (or process), and σ^2 *(Gauge)* is a variability component due to the measurement error, or Gauge variability. The starting point is the "basic" identity:

$$\sigma^2\ (Total) = \sigma^2\ (Parts) + \sigma^2\ (Gauge) \quad 1.12$$

where,

$$\sigma^2\ (Gauge) = \sigma^2\ (Reproducibilty) + \sigma^2\ (Repeatability) \quad 1.13$$

and then,

$$\sigma^2\ (Total) = \sigma^2\ (Parts) + \ \sigma^2\ (Reproducibilty) + \sigma^2\ (Repeatability) \quad 1.14$$

Here, σ^2*(Reproducibility)* is the variability due to change in experimental conditions, i.e., to operators/analysts change, and to their interactions with the measured parts. The components of Gauge R&R total variation is given in Figure 1.20.

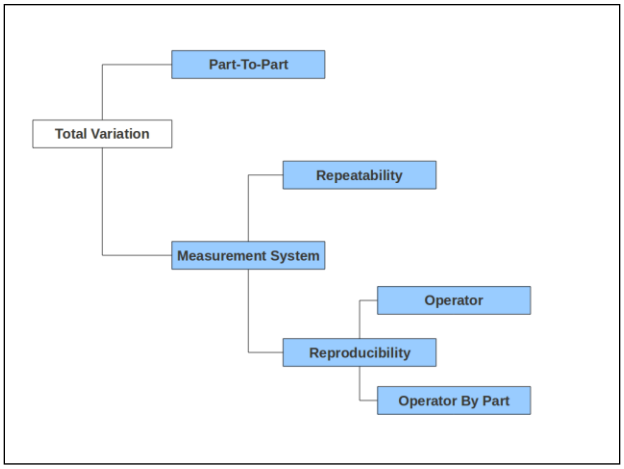

Figure 1.20: Gauge R&R total variation

In a 'two-ways' ANOVA analysis, using the data from R&R study, the factors are Analyst (with "*a*" levels) and Parts (with "*b*" levels); when *n* measurements are drawn for each crossing between Analyst and Parts levels, the ANOVA table obtained is shown in Table 1.6.

Table 1.6: ANOVA table parameters

Source of variability	**Sum of squares (SS)**	**Degrees of freedom (DF)**	**Mean of squares (MS)**	**F statistics**
Analyst	SS_A	(*a*-1)	$MS_A = \frac{SS_A}{a-1}$	$F = \frac{MS_A}{MS_E}$
Part or Sample	SS_B	(*b*-1)	$MS_B = \frac{SS_B}{b-1}$	$F = \frac{MS_B}{MS_E}$
Interaction (Part x Analyst) or (Sample x Analyst)	SS_{AB}	(*a*-1) (*b*-1)	$MS_{AB} = \frac{SS_{AB}}{(a-1)(b-1)}$	$F = \frac{MS_{AB}}{MS_E}$
Error	SS_E	*ab*(n-1)	$MS_E = \frac{SS_E}{ab(n-1)}$	$F = \frac{MS_{AB}}{MS_E}$
Total	SS_T	*a.b.*n-1		

At the beginning, the simplest quantity to estimate is the variance due to repeatability, i.e., the variance due only to the measurement process, with all other factors (analyst and part) held fixed. This quantity may then be estimated with MSE. Then:

$$\sigma^2\ (Repeatability) = MS_E \qquad 1.15$$

Also, it may be shown that "good" estimates for σ^2 *(Parts)*, σ^2 *(Analyst)* and σ^2 *(Analyst* x *Parts)* are given by equations 1.16, 1.17 and 1.18, respectively:

$$\sigma^2\ (Parts) = \frac{MS_B - MS_{AB}}{a.n_{rep}} \qquad 1.16$$

$$\sigma^2\ (Analyst) = \frac{MS_A - MS_{AB}}{b.n_{rep}} \qquad 1.17$$

$$\sigma^2\ (Analyst \text{ x } Parts) = \frac{MS_{AB} - MS_E}{n_{rep}} \quad 1.18$$

where, n_{rep} is the number of replications, within each Part, Analyst. As a consequence, the variance component due to reproducibility is estimated as the sum of estimated variance due to analysts and of estimate variance due to analysts by part interaction as given by equation 1.19:

$$\sigma^2\ (Reproducibilty) = \sigma^2\ (Analyst) + \ \sigma^2\ (Analyst \text{ x } Parts) \quad 1.19$$

The ANOVA method, unlike the Range method, allows estimating the variance component due to Analyst by Part interaction. For example, one may assess if some analysts over-estimate the measurements on small parts and under-estimate large parts, while others do not. In other words, one may assess if different operators measure differently the same parts. If the interaction term is not significant or if σ^2 *(Analyst* x *Parts)* ≤ 0) then, σ^2 *(Reproducibility)* = σ^2 *(Analyst)*.

In this case, the σ^2 *(Analyst)* estimate will become:

$$\sigma^2\ (Analyst) = \frac{MS_A - MS_{E_{pool}}}{b.n_{rep}} \quad 1.20$$

where, $MS_{E\,pool} = MS_E$ is obtained by setting equal to zero the interaction effect. In above example, the variance component due to Analysts is not significant, while the component due to interaction between Analyst and Part is significant. This implies that σ^2 *(Reproducibility)* is greater than 0.

Another parameter to evaluate the measurement system is the number of distinct categories (*NoDC*) which is given by equation 1.21:

$$NoDC = \frac{\sigma\,(Parts)}{\sigma\,(Gage)}.\sqrt{2} \qquad 1.21$$

That index is the "number of non-overlapped confidence intervals" that is needed to cover all the product variability. This index may be thought as the number of groups, within the process, that the measurement system is able to distinguish. When the number of distinct categories is less than 2, the measurement system is unable to measure the process. A number of distinct categories greater or equal to 5 is indicative of an acceptable measurement system.

1.10 Internal Standard Method

Internal standards are widely used in chromatography because the small quantity of sample solution injected into the chromatograph is not very reproducible in some experiments. Internal standards are also desirable when sample loss can occur during sample preparation steps prior to analysis. If a known quantity of standard is added to the unknown prior to any manipulations, the ratio of standard to analyte remains constant because the same fraction of each is lost in any operation.

An internal standard is a known amount of a compound, different from analyte, that is added to the unknown. Signal from analyte is compared with signal from the internal standard to find out how much analyte is present. Internal standards are especially useful for analyses in which the quantity of sample analysed or the instrument response varies slightly from run to run for reasons that are difficult to control. For example, gas or liquid flow rates that vary by a few percent in a chromatography experiment could change the detector response. A calibration curve is only accurate for the one set of conditions under which it is obtained. However, the relative response of the detector to the analyte and standard is usually constant over a wide range of conditions. If

signal from the standard increases by 8.4% because of a change in solvent flow rate, signal from the analyte usually increases by 8.4% also. As long as the concentration of standard is known, the correct concentration of analyte can be derived.

1.11 Present Work

In this thesis, new analytical methodologies based on chromatographic techniques were developed for estimation of monomers which are useful to address certain important issues related to industrial production of poly(lactic) acid and polymerisation processes that include acrylic and styrenic high internal phase emulsions (HIPEs). The measurements done for various monomers in the present work fulfill the specific needs of polymer chemists. While developing the analytical methodologies, the answers or solutions to the following questions were explored:

1. What is the problem to be solved?
2. What type of information is needed to solve the problem?
3. How will the solution to this problem be used?
4. Which analytical method (or technique) will be suitable to solve this problem?
5. What are physico-chemical properties of analytes and matrix under study?
6. What configuration of equipment is needed and what criteria (accuracy, precision, sensitivity, detection limit, the number of samples to be analysed, etc.) were considered in designing the experimental procedure?
7. Were there any potential interferences that had to be eliminated? If so, how were they treated?
8. Is there a plan for validating the experimental method and for assessing method performance?
10. How were the samples collected?
11. Was there a successful conclusion to the problem?

The aims and objectives of the thesis have been presented in Chapter 2.

1.12 Summary

Analytical chemistry is important and useful aspect of chemical research in industry, academia, and government. Analytical chemists have to apply their knowledge of chemistry, instrumentation, computers, and statistics to make measurements to solve problems in chemical industries and various fields of chemistry.

The recently developed analytical methods have the advantage of not only using small amounts of sample, reagents and less time, but also produce accurate results. A number of separation systems can be used to determine organic compounds in complex matrices. The most important techniques are based on chromatographic methods like LC and GC, which use a large variety of stationary phases and detection systems for achieving separation and quantification of analytes under study. To choose the best option from a large number of possibilities one needs to have the clear understanding and requirement of the problem to be solved. The most suitable method can be decided by taking into consideration the physico-chemical properties of the analyte and of the matrix as well as the objectives of the overall method. The chemical structure of the analyte and the physico-chemical properties of the matrix, together with the desired selectivity and sensitivity, determine the most suitable isolation and quantitation system.

Developing an analytical method involves selecting an appropriate method of analysis based on established criteria, such as accuracy, precision, sensitivity, and detection limit; the urgency with which results are needed; the cost of a single analysis; the number of samples to be analysed; and the amount of sample available for analysis. Finding an appropriate balance between these parameters is important.

The objective of any analytical measurement is to obtain consistent, reliable and accurate data. Validated analytical methods play a major role in achieving this goal. The results from method validation can be used to judge the quality, reliability and consistency of analytical results, which is an integral part of any good analytical practice. Validation of analytical methods is also required by most regulations and quality standards that impact laboratories.

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Chapter 2

AIMS AND OBJECTIVES

2.1 Aims and objectives

The aim of this thesis was to develop new analytical methodologies for the estimation of monomers to address certain important issues related to industrial production of poly(lactic acid) and polymerisation processes that include acrylic and styrenic HIPEs.

Chapter 1 outlined the importance of analytical chemistry, analytical perspective - analytical approach to solving problems in various fields of chemistry. It also outlined how chromatographic techniques could be efficiently used to carry out analysis of various analytes in complex matrices. It summarised about the availability of various chromatographic techniques with the options of a wide range of stationary phases and detection systems available today.

As in all scientific research, a rational systematic approach is essential for solving problems related to analytical method development. This requires a sound knowledge of chemical and physical sciences for thorough understanding of analytical instrumentation, accurate judgement of analyte behaviour and an active approach to address possible technical and chemical analytical pitfalls. While developing analytical methodologies for generating meaningful data, analytical chemist has to overcome challenges which demand working with smaller samples, with more complex materials/matrices, with processes occurring on shorter time scales, and with components present at lower concentrations. While addressing specific needs of a particular problem, an analytical chemist has to choose the right analytical technique, configure components of instrument appropriately and optimise various parameters to suit typical analysis requirements. This process is called analytical method development.

Each analysis problem chosen in the present study is unique in terms of requirement and complexity of the analysis which demands choosing an appropriate chromatographic technique and configuration of equipment, developing sample preparation technique and optimising other parameters of analysis accordingly.

Once analytical method is developed, it needs to be validated to demonstrate that the proposed method is suitable for its intended purpose. The present work was also aimed to perform method validation for all the analytical methods developed in this work. Validated analytical data are used for the evaluation of original investigations, medical diagnosis, environmental assessment, purity estimation for important chemicals and other varied purposes. In this context, method validation has received considerable attention in literature and from industrial committees and regulatory agencies.

The objectives of this thesis were to develop analytical methodologies for:

- Estimation of chemical purity of lactic acid monomer by impurity profiling.
- Estimation of optical purity of lactic acid.
- Measurement of 2-ethylhexyl acrylate (EHA), ethylene dimethacrylate (EGDMA) and 2-ethylhexyl methacrylate (EHMA) for estimating monomer conversions in HIPE polymerisation reactions.
- Measurement of trace levels of EHA, EGDMA and EHMA in HIPE process water.
- Measurement of styrene, divinyl benzene (DVB) and EHA for estimating monomer conversions in HIPE polymerisation reactions.

The chapter-wise summary of objectives and the significance of present work are given below.

2.1.1 Estimation of chemical purity of lactic acid monomer

Lactic acid produced by fermentation process contains carboxylic acid impurities which are detrimental to the polymerisation ability of lactic acid resulting in low molar mass PLA, which is not useful for most applications. PLA of high molecular weight is needed to produce devices of high mechanical strength. Evaluation of chemical purity of lactic acid monomer (in terms of carboxylic acid impurities) is therefore important parameter in PLA production.

Chapter 3 deals with the establishment of a suitable analytical method for the impurity profiling of lactic acid monomer. An analytical method based on polar-embedded reverse phase HPLC was developed and various carboxylic acid impurities in lactic acid samples were identified and quantified. The other components present in lactic acid, the PLA precursors, viz. lactoyl lactic acid and the cyclic dimer (3,6-dimethyl-*p*-dioxane-2,5-dione, also called as dilactide) were identified by indirect method (by monitoring changes in peak heights of dilactide and lactoyl lactic acid on hydrolysis of lactic acid sample).

A separate GC method was also developed to measure ethanol and methanol, the byproducts of hydrolysis of lactic acid ester, which may appear as impurities in final product, if not completely removed in reactive distillation step of its downstream purification.

A thorough method validation was performed by investigating system precision, specificity, linearity (calibration studies), limits of detection and quantification, ruggedness of method, 'method precision and accuracy', and 'Gauge R & R' study.

2.1.2 Estimation of optical purity of lactic acid monomer

The small amounts of enantiomeric impurities drastically change properties such as crystallinity or biodegradation rate of the polymer. L (+) lactic acid is required for production of PLA suitable for most applications. Therefore, besides evaluation of chemical purity, estimation of optical purity of lactic acid monomer is also a crucial factor in deciding physical properties of PLA.

Chapter 4 deals with the establishment of an analytical method for estimation of optical purity of lactic acid monomer. The HPLC method based on CLEC (chiral ligand exchange chromatography) was developed for the separation of lactic acid enantiomers. In this work, enantiomeric separation of lactic acid was accomplished by employing stationary phase ligand, which employs L-hydroxy proline as the immobilised chiral selector. The chromatographic parameters such as column temperature, flow rate, detection wavelength and solvent composition were investigated and optimised to achieve separation of lactic acid enantiomers. System precision was investigated by studying repeatability of retention times and peak area. The method was applied to estimate the optical purity of lactic acid synthesised in our laboratory as well as commercial ones.

2.1.3 Estimation of monomers in acrylic HIPEs

Synthesis of PolyHIPE materials using HIPE methodology has gained considerable commercial interest in recent years. Acrylic monomers, viz. EHA, EGDMA

and EHMA are used to produce Functional Absorbent Materials (FAM) using HIPE polymerisation. Studying the rate of monomer conversion (reaction kinetics) is important for process optimisation. This necessitates development of an analytical method, which can estimate time-dependent monomer conversions by analysis of unreacted monomers (EHA, EGDMA and EHMA) in HIPE polymerisation reactions.

Part A of chapter 5 deals with the establishment of an analytical method for measurement of EHA, EGDMA and EHMA in high internal phase emulsions. For this, an analytical method based on gas chromatography (GC), employing flame ionisation detector, was developed and its application was demonstrated by estimating monomer conversions in acrylic HIPE polymerisation under certain selected reaction parameters. The chromatographic system was configured and the protocol for extracting monomers from high internal phase emulsion, sample preparation technique and GC column oven temperature programming were developed and optimised. Method validation was carried out by investigating system precision, specificity, linearity, limit of detection (LOD), limit of quantification (LOQ), 'method precision and accuracy', and Gauge R & R.

Part B of chapter 5 deals with the establishment of sensitive HPLC-UV method, which can estimate EHA, EGDMA and EHMA at low ppm levels in HIPE process water. The HIPE process water samples were generated in laboratory and investigated for contents of residual monomers at trace levels. This method is useful to assess the quality of process water (in terms of residual monomers) before being recycled in industrial process pertaining to production of Functional Absorbent Materials (FAM) using HIPE methodology.

The reverse phase (RP)-HPLC method was employed for achieving chromatographic separations. The chromatographic conditions like mobile phase composition, flow rate and the detection wavelength were optimised and complete baseline separation of analytes was achieved. The method was established by performing method validation, which included investigation of system precision, specificity, linearity, response factors, limit of detection, limit of quantification, and 'method precision and accuracy'.

2.1.4 Estimation of monomers in styrenic HIPEs

Chapter 6 deals with the establishment of an analytical method for estimation of monomer conversions in styrenic HIPE polymerisation. The monomers selected for styrenic HIPE system were styrene, DVB and EHA. The procedure for extraction of monomers from high internal phase emulsion was developed and validated. The sample preparation technique and GC column oven temperature programming were developed and optimised for achieving complete baseline separation of analytes. Method validation was carried out by investigating system precision, specificity, linearity, limit of detection (LOD), limit of quantification (LOQ), 'method precision and accuracy'. A Gauge R & R study was conducted to assess the performance of method. The usefulness of method was demonstrated by estimating monomer conversions for HIPE polymerisation reactions that used thermal and redox initiator systems.

The thermal initiators used in this work included sodium persulphate and cumene hydroperoxide; and redox initiator systems included "sodium persulphate: sodium dithionite", "sodium persulphate : ascorbic acid", "cumene hydroperoxide : ascorbic acid", all used in 1:1 molar proportion.

Chapter 3

ESTIMATION OF CHEMICAL PURITY OF LACTIC ACID MONOMER

3.1 Introduction

3.1.1 Lactic acid and bio-degradable polymers

Lactic acid is a non-volatile odourless organic compound found in many natural products. It has a hydroxyl group adjacent to the carboxyl group, making it an alpha hydroxy acid (2-hydroxypropanoic acid). With the presence of these functional groups, lactic acid can be used as a key starting material in a variety of chemical reactions like esterification, condensation, polymerisation, reduction and substitution and thus, has become as a platform chemical for a wide range of important products with applications in various areas like food industries, leathers, biomedicals,[1-6] pharmaceuticals,[7-8] cosmetics[9] and chemical industries.

Polylactic acid (PLA) is a promising biopolymer and has been extensively discussed in several research articles, reviews and book chapters.[1-6] Until the last 10 to 15 years, the uses of PLA have been mainly limited to medical applications such as implant devices, tissue scaffolds, and internal sutures, because of its high cost, limited availability and low molecular weight. In the last decade, the developments of new polymerisation routes have resulted in the growing economical production of high molecular weight PLA. Traditionally, all plastic materials are manufactured from non-renewable petroleum resources. The two conventional disposal methods for these non-biodegradable plastics are incineration and secured landfill, which subsequently leads to production of dioxins, posing a serious threat to environment.[10] The continued paucity of landfill space and problems associated with incineration has prompted to investigate and manufacture alternative polymers which are biodegradable.[11-16]

PLA is synthesised from lactic acid which can be produced through either chemical synthesis route or microbial (fermentation) method. However, in chemical industry, about 90% of total production of lactic acid is achieved through fermentation process.[17] The fermentation route can form stereo specific acid (depending on the strain being used) by carbohydrate fermentation.

Lactic acid thus is a naturally occurring and renewable monomer and lactic acid based polymers are therefore considered as sustainable materials and promising alternative to petroleum based polymers for certain end applications. The synthesis of lactic acid into high molar mass PLA can follow two different routes of polymerisation as depicted in Figure 3.1.

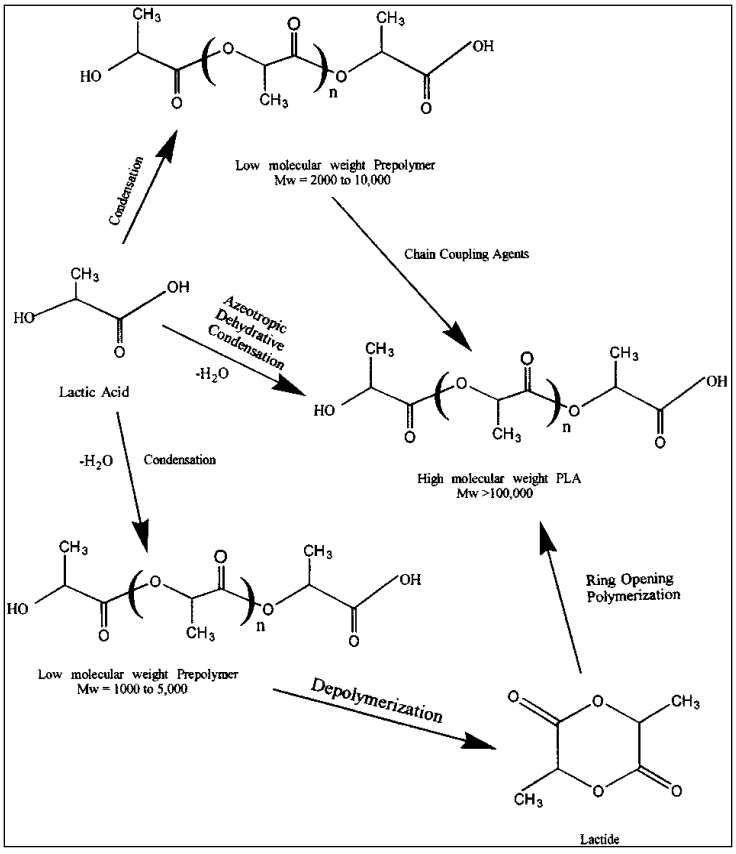


Figure 3.1: Synthesis of high molar mass PLA

The present industrial production of lactic acid is based on carbohydrate fermentation. This is both chemically and economically more feasible and enables the production of optically pure lactic acid. This is one of the most essential requirements for PLA production for certain applications. The lactic acid fermentation broth contains impurities such as carboxylic acids (e.g., oxalic, formic, pyruvic, citric, succinic, fumaric acids, etc.), alcohols (methanol or ethanol), amino acids (e.g., alanine, valine, serine, glutamine, lycine, etc.), protein fragments derived from the cell mass, colour bodies, residual nutrients, metabolic intermediates and fermentation byproducts.[18-20] Most of these impurities are removed by downstream purification. However, the presence of residual impurities, mostly carboxylic acids at ppm levels, is always there. The major technology barrier in cost-effective production of high purity lactic acid is its down-stream separation and purification. It has been reported[21] that the total amount of impurities in lactic acid monomer should preferably be less than 0.05 mol%. The impurities (especially that of carboxylic acids) present in lactic acid can inhibit polymerisation ability of lactic acid resulting in low molecular weight PLA. A recent article[22] reported the effect of tricarboxylic acid on the resulting properties of functionalised lactic acid (LA) polycondensates. The presence of citric acid leads to a significant reduction of molecular weight of the polycondensation products. When PLA is used for orthopaedic and oral surgeries as fixation of augmentation devices, PLA of high molecular weight is needed to produce devices of high mechanical strength.

The identification and removal of residual impurities (especially carboxylic acids) from lactic acid is therefore very crucial because they can drastically affect the

properties of the PLA polymer. Hence, from an industrial point of view, estimating purity of lactic acid in terms of carboxylic acid impurities is important and critical.

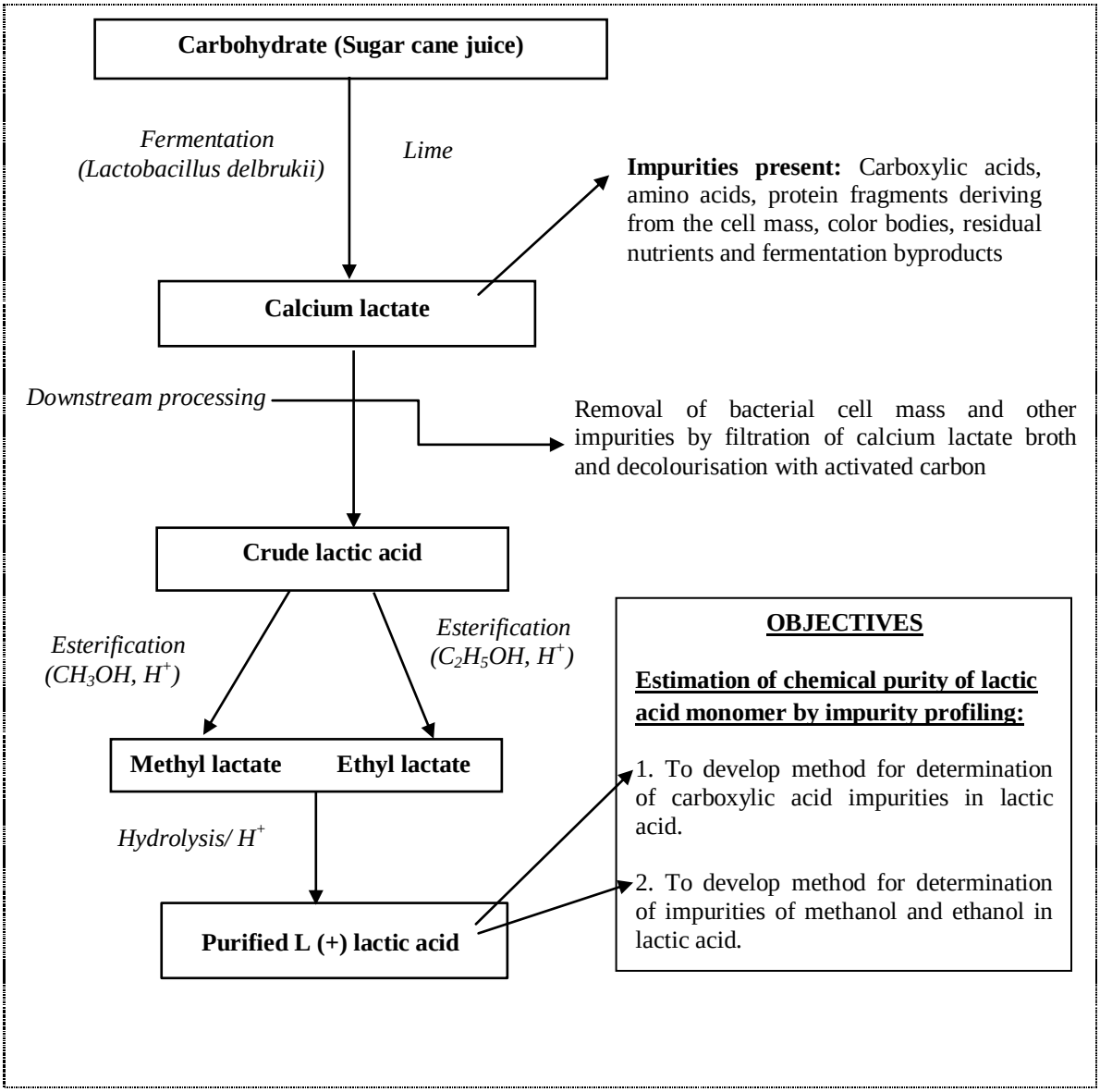


Figure 3.2: Synthesis and purification of LA obtained from fermentation route

Figure 3.2 describes the synthesis and purification of lactic acid obtained by fermentation of sugar cane juice. The objective of the present work was to estimate the purity of lactic acid monomer by impurity profiling. The lactic acid samples were procured from various commercial sources as well as synthesised by fermentation of

sugarcane juice. The percent purity of lactic acid samples was estimated by impurity profiling and their quality was compared. An analytical method based on high performance liquid chromatography was developed to identify and quantify the carboxylic acid impurities present. In addition, a separate GC method was also developed to measure ethanol and methanol, the byproducts of hydrolysis of lactic acid ester, which may appear as impurities in final lactic acid product if not completely removed in reactive distillation step of its downstream purification.

The most commonly found carboxylic acid impurities in lactic acid are oxalic, formic, pyruvic, citric, fumaric, succinic, *cis*-aconitic, *trans*-aconitic, acrylic, propionic, itaconic, butyric, and citraconic acids. A simple isocratic HPLC method, based on polar embedded reverse phase, was developed for this purpose. The method employs purely an aqueous mobile phase. It gave a very stable resolution even in 100% aqueous mobile phase which is difficult to achieve with conventional reverse phases. A detailed validation was performed for the evaluation of parameters such as specificity, system precision, linearity (calibration studies), limit of detection (LOD), limit of quantification (LOQ), accuracy and ruggedness. The performance of method was assessed by conducting Gauge R&R study.

Besides determining carboxylic acid impurities, a GC method was developed to determine methanol and ethanol. PLOT-Q capillary column, compatible with aqueous samples, was chosen. A detailed method validation was conducted prior to analysis of real samples.

3.1.2 Scope of the Study

Purity specifications for food, pharmaceutical, and other grades of lactic acid are available. However, literature concerning 'polymer grade' lactic acid, especially with respect to carboxylic acid impurities, is not available. A thorough literature survey revealed that the fermentation based process for pure (polymer grade) lactic acid has been described,[23] but method of quantitative analysis of carboxylic acids in lactic acid has not yet been established. There are a number of reports on analysis of organic acids in a variety of matrices (e.g., environmental,[24,25] plant root exudates,[26,27] fruit juices,[28,29] red wine,[30] tobacco,[31] honey,[32] Bayer liquors,[33] vinegars,[34] tea,[35] and *Garcinia cambogia*[36]), but a method of quantitative analysis of 'carboxylic acids in lactic acid matrix' is not available. There is but one report which describes the detection method (i.e. only qualitative analysis) of a few carboxylic acids in technical grade lactic acid.[37]

3.2 Determination of Carboxylic acid Impurities in Lactic acid

3.2.1 Experimental

3.2.1.1 Materials

The lactic acid samples were procured from Purac (Lincolnshire, IL, USA), Aldrich (St. Louis, MO, USA), Merck (Mumbai, India) and Lactochem (Mumbai, India). Oxalic acid [ethanedioic acid], formic acid [methanoic acid], acetic acid [ethanoic acid], pyruvic acid [2-oxopropanoic acid], citric acid [2-hydroxypropane-1,2,3-tricarboxylic acid], fumaric acid [trans-butenedioic acid], succinic acid [butanedioic acid], *cis*-aconitic acid [cis-1-propene-1,2,3-tricaroboxylic acid], *trans*-aconitic acid [trans-1-propene-1,2,3-tricaroboxylic acid], acrylic acid [prop-2-enoic acid], malic acid [2-hydroxybutanedioic acid], propionic acid [propanoic acid], itaconic acid [2-methylidenebutanedioic acid],

butyric acid [butanoic acid], and citraconic acid [cis-2-methyl-2-butene-dioic acid] (all having purity ≥ 98%) were purchased from Aldrich Chemicals (St. Louis, MO, USA). Ammonium dihydrogen phosphate and orthophosphoric acid of AR grade were purchased from Merck Ltd. (Mumbai, India). Ultra pure water (Milli-Q System, Millipore, Milford, MA, USA) was used for preparing the mobile phase and conducting hydrolysis experiments.

3.2.1.2 Synthesis of lactic acid samples

Lactic acid was prepared[38,39] using acid-tolerant homolactic (e.g. *Lactobacillus delbrukii*) bacteria, which produce pure L(+) form of lactic acid. During the fermentation, broth was continuously fed with calcium hydroxide (lime) to neutralise and maintain *pH* of the fermentor approximately between 5- 6. Lactic acid formed and accumulated during fermentation decreases the pH of the fermentor. This affects the productivity of microorganisms as they fail to function at lower pH medium. Therefore, the pH of fermentor is continuously maintained by addition of lime to neutralise lactic acid, which thus results into calcium lactate.

The fermented broth comprises of residual sugar compounds, colour and other organic acids. The broth containing calcium lactate was filtered to remove bacterial cell mass and decolourised by treatment with activated carbon. The filtrate was concentrated by evaporation and acidified with sulphuric acid to obtain lactic acid and insoluble calcium sulphate (gypsum). Calcium sulphate was filtered off and lactic acid was converted to methyl lactate, which was distilled off leaving behind high-boiler impurities of methyl esters of other organic acids. Methyl lactate was hydrolysed to obtain pure

lactic acid and the byproduct (methanol) was removed by distillation. A number of samples were prepared and purified which are designated as 'in-house (IH)'.

3.2.1.3 Preparation of lactic acid samples and standards

Lactic acid samples for analysis were prepared by dissolving the required quantity in mobile phase (20 mM of aqueous ammonium dihydrogen phosphate at pH 2.20) to obtain 2.0% (w/v) solution. Separate standard stock solutions (1000 mg L^{-1}) of carboxylic acids (oxalic, formic, pyruvic, citric, fumaric, succinic, *cis*-aconitic, *trans*-aconitic, acrylic, propionic, itaconic acid, and citraconic acids) were prepared by dissolving approximately 100 mg of each acid in 100 mL of mobile phase. The working standards were freshly prepared by appropriate dilution of stock solutions. All solutions were filtered through a 0.45 μm Nylon membrane syringe filter (Whatman, Clifton, NJ, USA) before injecting them into HPLC system.

3.2.1.4 Equipment and parameters of analysis

The analysis was carried out on HPLC system (Waters Corporation, Milford, MO, USA) equipped with a 515 binary HPLC pump, a 717 plus auto sampler and a 996 photodiode array (PDA) detector. The column temperature was maintained at 40 ^{o}C with the help of a column oven controller (HCO-02, PCI Services, Mumbai, India). The mobile phase was degassed by an ultrasonic bath (Transsonic T660/H, Elma Hans Schmidbauer GmbH & Co., Singen, Germany) and an on-line vacuum degasser. A pH meter (Systronics, Mumbai, India), with the combined glass and reference electrodes, was used to adjust the pH of the mobile phase. The diode array detector (DAD) was set to scan 190–300 nm and the chromatogram was extracted from 210 nm. The resolution of the detector was 1.2 nm and the sampling period was 15 ms. Data acquisition and peak

integration were performed with the help of Millenium 32® software (Waters Corporation, Milford, MO, USA).

A polar embedded reverse phase column (250 × 4.6 mm ID, 5 µm particle size, YMC-Pack ODS-AQ, YMC Co. Ltd., Kyoto, Japan) along with a matching guard column (YMC Co. Ltd., Kyoto, Japan) was used. The mobile phase was prepared daily in ultrapure (HPLC grade) water and filtered through 0.2 µm Nylon membrane filter (Whatman, Inc., Sanford, USA) before use. The 13 lactic acid samples analysed are presented in Table 3.1

Table 3.1: Description of lactic acid samples analysed

S. No.	Lactic acid (Source)
1	Commercial source 1
2	Commercial source 2
3	Commercial source 3
4	Commercial source 4
5	IH-1
6	IH-2
7	IH-3
8	IH-4
9	*IH-5, Sample feed
10	**IH-5, Purified
11	IH-6
12	IH-7
13	IH-8

IH: Lactic acid batches synthesised at our laboratory (In-house samples).
*Lactic acid feed sample from reactive distillation purification step.
**Lactic acid purified sample after reactive distillation purification step.

3.2.2 Results and discussion

3.2.2.1 Method development and optimisation

Generally, the methods based on ion chromatography (IC)[35,36,39] and ion exclusion chromatography (ICE)[24,25,27,28,34,40] are applied for the analysis of aliphatic carboxylic acids. However, these methods may not be suitable for the separation of carboxylic acids present in lactic acid matrix, where the analytes and the matrix are of similar nature. It has been reported[25] that between these two methods, the ICE has less separation efficiency which may result in insufficient resolution of analytes having similar chromatographic behaviour. Besides this, small elution volumes and limited separation window are the other limitations of ICE. In addition, the ICE may have lower sensitivity due to relatively large column dimensions.

The conventional reverse phase HPLC was therefore evaluated to separate and analyse various carboxylic acids in lactic acid. This chromatographic separation was difficult due to inadequate retention of carboxylic acids (particularly the more polar and hydrophilic acids) on conventional reverse phase columns (C_8 or C_{18}). When pure aqueous mobile phase buffered at pH 2.2 was used, the retention was substantially increased, but this resulted in hydrophobic collapse of brush like structure of carbon chains (also called as “matting”), and deteriorated both the resolution and repeatability of retention time. There was also the problem of poor peak shapes especially with more polar compounds. A number of trial runs were performed to overcome the hydrophobic collapse by adding 1 to 5% (v/v) methanol as well as acetonitrile to the mobile phase. However, this also deteriorated the resolution of adjacent peaks in all the cases.

Since conventional reverse phase did not work, the possibility of other options was assessed such as normal phase chromatography, reverse phase chromatography with using ion-pairing, hydrophilic interaction chromatography (HILIC) and polar embedded reverse phase chromatography, which are known to give good retention for polar analytes. Although normal phase chromatography offers better separation capabilities, the method was not suitable because the water in lactic acid deactivates the polar stationary phase. The next option of using ion-pairing reverse phase chromatography was avoided because apart from very reproducibility, it shortens column life. HILIC chromatography is another approach in which the polar selectivity in HILIC arises due to (1) partition of polar analyte between adsorbed water (or polar solvent) layer on the stationary phase and mobile phase, and (2) electrostatic interaction between polar analyte and the stationary phase. The analytes herein elute in order of increasing hydrophilicity. HILIC option could be much better than normal phase and ion-pairing reverse phase chromatography. However, with HILIC, it is sometimes difficult to analyse polar and non-polar compounds in the same sample and robustness of method may be an issue. The polar embedded reverse phase was therefore tried so as to circumvent inadequate retention problem encountered with conventional reverse phase column.

The polar embedded reverse phase works in the same way as typical reverse phase. However, besides reverse phase retention mechanism, it also exhibits polar selectivity due to the polar groups (e.g. amide, phenols, carbamate, etc.) embedded in it as shown in Figure 3.3. The embedded reverse phase was found to be suitable because it could retain the relatively more polar and hydrophilic compounds e.g. oxalic, formic, pyruvic acids (due to interaction between polar moiety embedded in the reverse

stationary phase and these analytes) though the predominant retention mechanism remains reverse phase.

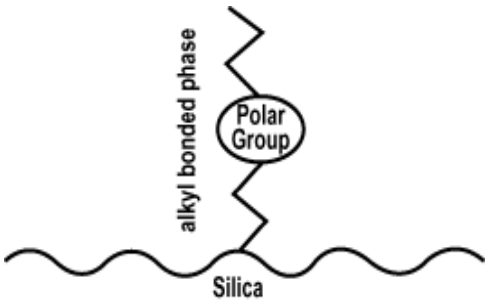
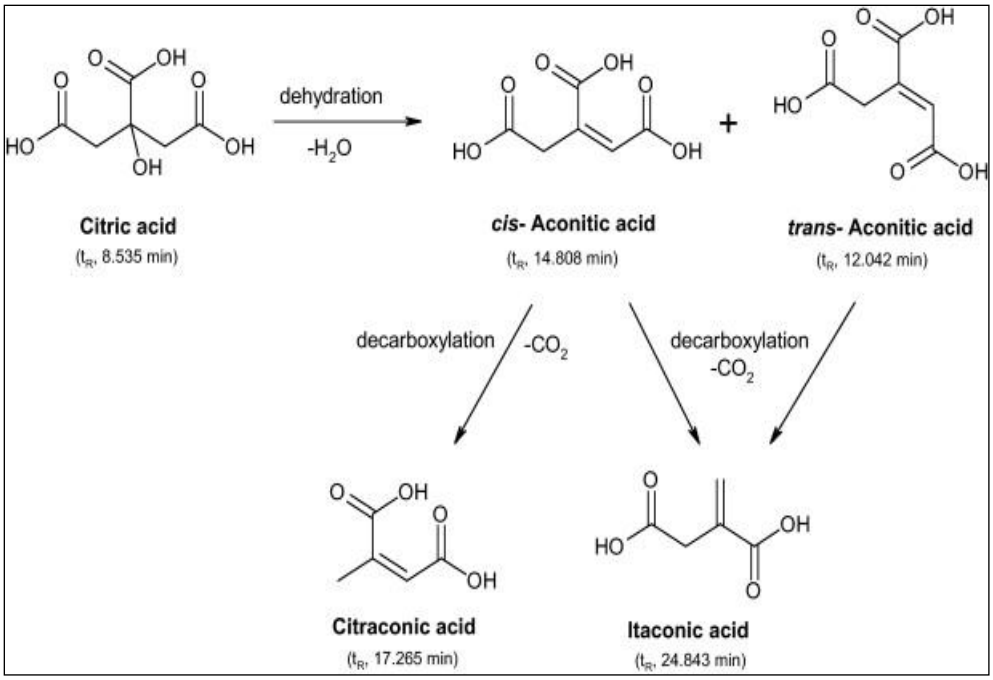


Figure 3.3: 'Polar embedded' reverse phase

Also, the embedded polar moiety prevented the hydrophobic collapse of the hydrocarbon chains even in 100% aqueous mobile phase. For organic acids with lesser hydrophilic character, non-polar interaction plays a predominant role than polar interaction and consequently as the hydrophilic character decreased, the retention time increased. This can be seen in the retention behaviour of citric, aconitic, citraconic and itaconic acids. Citric acid can get converted into citraconic acid and itaconic acid via aconitic acid, as shown in Scheme 3.1. [41]



Scheme 3.1: Retention behaviour of citric, aconitic, citraconic and itaconic acids as a consequence of decreasing hydrophilic character

It may be seen from Scheme 3.1 that the hydrophilic nature of the resulting molecule (as a result of each transformation) gradually decreases as these transformations remove –OH and –COOH groups, which are hydrophilic. Therefore, it is expected that citric acid should elute first followed by aconitic acid (*cis*- and *trans*- forms), and then citraconic or itaconic acids. The observed retention time (t_R) for these compounds matches with the expected retention behaviour.

It was observed that the *cis*- form of aconitic acid is adsorbed more strongly (retained longer) than its *trans*- form. This was attributed to the availability of higher hydrophobic surface area in *cis*- form as the polar moieties are lying on one side. The solvophobic theory[42] predicts that in reverse phase chromatography, the retention of the *cis*-form is greater than that of the *trans*- form, which has a smaller hydrophobic surface area.

A number of experiments were conducted with this column to optimise pH, buffer concentration (in mobile phase) and the flow rate to achieve the required resolution. An adequate separation was achieved when an aqueous mobile phase buffered with 20 mM of ammonium dihydrogen phosphate at pH 2.20 (± 0.05) was used at 1 mL min^{-1} flow rate, isocratically. The column was maintained at constant temperature (40 ^{o}C) to have better repeatability of retention times. The pH of mobile phase was adjusted to suppress the ionisation of organic acids. The column was equilibrated for at least 4 hours prior to sample injection to get a steady baseline, when used for the first time or after a long storage. In this context, the isocratic mode of elution was advantageous as the column continuously runs in the mobile phase of fixed composition. In case of gradient elution, it would have necessitated otherwise a longer (column) equilibration time before every injection.

3.2.2.2 Method validation

After an analytical method is developed, it should be validated to ensure that it is useful for its intended purpose. The present HPLC method was validated by studying various parameters such as system precision (assessment of repeatability of retention time and peak area), specificity, linearity (calibration studies), limit of detection (LOD), limit of quantification (LOQ), accuracy, ruggedness, and Gauge R&R (repeatability and reproducibility).

3.2.2.2.1 System precision

The assessment of system performance is important for an unambiguous identification and quantification of analytes. System performance can be assessed by studying precision. Precision is the measure of how close the data values are to each other

for a number of measurements under identical analytical conditions. Repeatability is the precision of a method under the same operating conditions over a short period of time. One aspect of this is instrumental precision. This is measured by the sequential repetitive injection of the same homogenous sample followed by the averaging of the retention time (t_R) and peak area values and determination of the relative standard deviation of all replicate runs.

After the HPLC system was configured, the preliminary trials with running a test sample were conducted to check the system performance. For this study, 10 nos. of replicate injections of a mixture of two closely eluting analytes (succinic acid, t_R 9.392 min and fumaric acid, t_R 9.929 min) were made into HPLC system (runs spread across morning, afternoon and evening periods of a day) and data on retention time and peak area counts were analysed for checking run to run variation. The variation for peak area counts for both the analytes was found to be within acceptance limit (%RSD $\leq$ 1.0). However, considerable variation (%RSD > 1.0) was noticed for retention time. This observed variation of retention time was treated as significant since for a multi-component separation analysis (wherein the analytes are closely eluting), retention time variation having %RSD > 1.0 cannot be compromised. This may otherwise pose difficulty in identification of certain analytes (especially those having closer retention times).

The variation in retention time can occur due to (i) insufficient column equilibration, (ii) erratic flow rate, (iii) variation in pH of mobile phase, and (iv) variation in column temperature. Taking care of all these parameters, the column oven controller was provided externally as the HPLC system used for this study did not have an in-built

column oven. All the runs were repeated under identical chromatographic conditions. The retention time repeatability was found to improve after controlling column temperature to a constant value (40 ^{o}C). Figures 3.4 - 3.7 depict comparison of repeatability of retention time when runs were performed with and without use of column oven controller, for two typical analytes, succinic and fumaric acids.

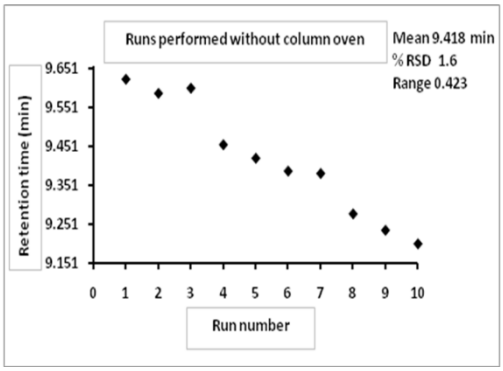


Figure 3.4: Retention time variation in succinic acid analysis (without column oven controller)

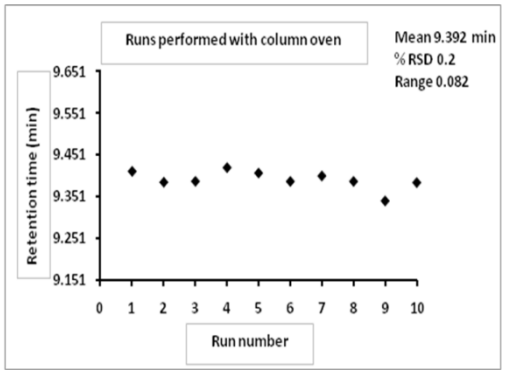


Figure 3.5: Retention time variation in succinic acid analysis (with column oven controller)

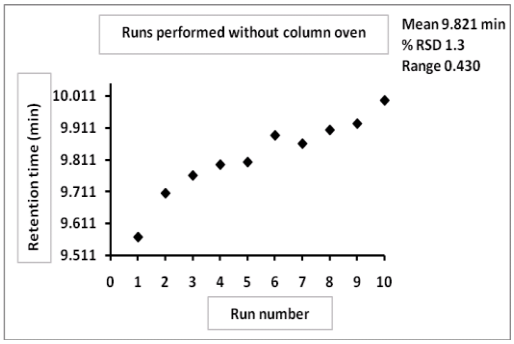


Figure 3.6: Retention time variation in fumaric acid analysis (without column oven controller)

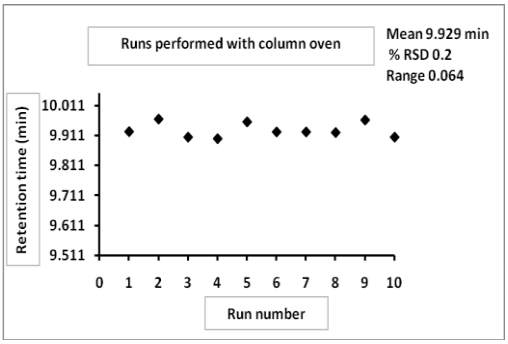


Figure 3.7: Retention time variation in fumaric acid analysis (with column oven controller)

After the problem of retention time variation was rectified, the system performance was evaluated thoroughly by calculating intra- and inter-day precision for all the analyte components under study. A mixture of carboxylic acids (prepared in mobile phase) was injected into HPLC system ten times a day. This procedure was repeated for three consecutive days. The data of retention time and area for corresponding

acid was collected and percentage relative standard deviation (%RSD) was calculated for runs performed on Day 1, Day 2, and Day 3 (intra-day precision, n =10, mean). The data obtained for Day 1, Day 2, and Day 3 were collected together and used for calculating %RSD to assess inter-day precision (n = 30, mean). The %RSD values for retention time and peak areas are given in Tables 3.2 and 3.3, respectively. The low %RSD values obtained for both retention time and peak area, indicated good precision.

Table 3.2: Intra- and inter-day precision for retention time (t_R)

Acid	Intra-day precision (n = 10, Mean)						Inter-day precision (n = 30, Mean)	
	Day 1		Day 2		Day 3		Day 1 + 2 + 3	
	t_R (min)	%RSD	t_R (min)	%RSD	t_R (min)	%RSD	t_R	%RSD
Oxalic acid	3.394	0.56	3.393	0.59	3.388	0.21	3.392	0.44
Formic acid	4.000	0.25	3.994	0.18	3.988	0.20	3.992	0.23
Pyruvic acid	4.539	0.29	4.534	0.09	4.531	0.20	4.535	0.20
Citric acid	8.529	0.21	8.544	0.15	8.531	0.22	8.535	0.35
Succinic acid	9.385	0.05	9.384	0.13	9.386	0.05	9.385	0.08
Fumaric acid	9.935	0.17	9.937	0.16	9.939	0.16	9.937	0.15
trans-Aconitic acid	12.036	0.41	12.050	0.35	12.040	0.32	12.042	0.33
Acrylic acid	12.272	0.07	12.27	0.03	12.271	0.06	12.271	0.05
Propionic acid	13.803	0.04	13.803	0.06	13.807	0.07	13.804	0.05
cis-Aconitic acid	14.805	0.34	14.815	0.34	14.806	0.34	14.808	0.34
Citraconic acid	17.260	0.24	17.263	0.20	17.272	0.19	17.265	0.25
Itaconic acid	24.84	0.17	24.844	0.07	24.845	0.05	24.843	0.10
Butyric acid	41.235	0.48	41.265	0.61	41.258	0.53	41.252	0.49
Dilactide	39.300	0.85	39.172	0.51	34.273	0.37	37.582	0.59

Table 3.3: Intra- and inter-day precision for peak area

Acid	Intra-day precision (n = 10, Mean)						Inter-day precision (n = 30, Mean)	
	Day 1		Day 2		Day 3		Day 1 + 2 + 3	
	Area	%RSD	Area	%RSD	Area	%RSD	Area	%RSD
Oxalic acid	11769	0.07	117554	0.08	117620	0.08	117622	0.10
Formic acid	38098	0.94	38350	0.90	37845	0.82	38098	1.05
Pyruvic acid	10585	0.01	105881	0.41	105824	0.08	105865	0.32
Citric acid	40070	0.15	39988	0.24	41865	0.28	40641	0.30
Succinic acid	25920	1.29	26185	1.08	25000	0.92	25701	1.10
Fumaric acid	15589	0.24	156166	0.20	154420	0.34	155494	0.29
trans-Aconitic acid	48924	1.07	49369	0.98	46519	0.88	48271	1.14
Acrylic acid	62759	0.18	621890	0.22	661598	0.14	637028	0.24
Propionic acid	30478	1.22	30214	1.04	30159	0.99	30284	1.18
cis-Aconitic acid	59464	0.06	594885	0.08	595249	0.10	594925	0.12
Citraconic acid	52574	0.34	55672	0.28	58912	0.22	55720	0.21
Itaconic acid	61185	0.22	610923	0.28	612921	0.34	611901	0.30
Butyric acid	33279	1.14	33547	1.08	32009	0.99	32945	1.20

3.2.2.2.2 Specificity

The method was tested for specificity for various acids present in lactic acid matrix. The chromatograms showed an adequate resolution of all the peaks with an acceptable repeatability of retention time as evident from Table 3.2. In addition, the peak purity test conducted with diode array detector showed that each peak was attributable to only one component. This indicated that the method was specific for analytes under study.

3.2.2.2.3 Linearity (calibration studies)

Three sets of calibration curves (each having eight data points) were generated for each acid by regression method. The relative standard deviation (%RSD) of slope for the three curves was determined, in all the cases. The linearity was studied over a wide range so as to cover the impurity levels that might be present in lactic acid samples. The three sets for a particular acid were combined and linearly regressed to obtain the

working calibration curve. The regression lines were calculated as $y = mx$, where x was the mass of the acid injected, m was the slope, and y was the detector response. The data on linear mass range, %RSD of calibration slope, regression coefficient (R^2) and calibration equation are reported in Table 3.4, and the calibration curves of carboxylic acids are given in Figures 3.8 - 3.19. The relationship between detector response (peak area) and injected mass was linear for all the acids, in the mass ranges studied. The linearity between peak area and analyte concentration was very good, as indicated by value of regression coefficient (R^2) which was equal to or greater than 0.9956 in all the cases.

Table 3.4: Regression (calibration) equations and coefficients (R^2)

Analyte	**Range (ng)**	**Regression equation**	**%RSD of slope**	**R^2 value**
Oxalic acid	11-5320	$y = 459.95x$	0.4	0.9970
Formic acid	11-5400	$y = 27.829x$	2.9	0.9956
Pyruvic acid	2-5016	$y = 648.14x$	3.1	0.9987
Citric acid	20- 4961	$y = 70.805x$	3.7	0.9975
Fumaric acid	3-3000	$y = 7374.2x$	0.2	1.0000
Succinic acid	10-5000	$y = 35.477x$	2.7	0.9998
trans-Aconitic acid	1- 8000	$y = 3180.2x$	2.0	0.9993
Acrylic acid	1- 4970	$y = 2943.6x$	3.3	0.9983
Propionic acid	50-5020	$y = 28.732x$	4.0	0.9983
cis-Aconitic acid	2- 8600	$y = 3179.1x$	2.1	0.9996
Citraconic acid	5-5900	$y = 4043.1x$	1.6	0.9976
Itaconic acid	2-10280	$y = 3028.5x$	1.6	0.9995

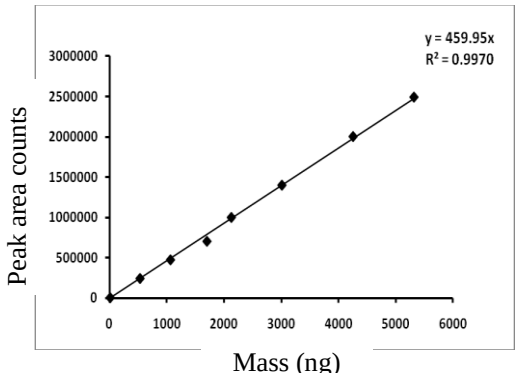


Figure 3.8: Calibration curve for oxalic acid

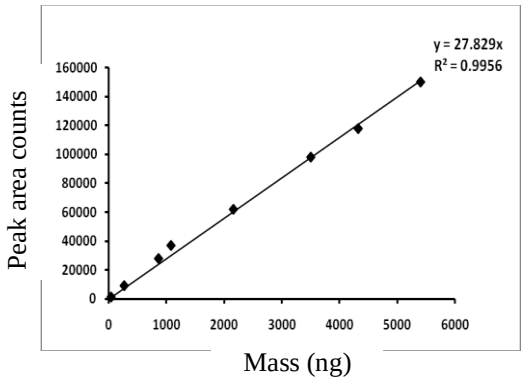


Figure 3.9: Calibration curve for formic acid

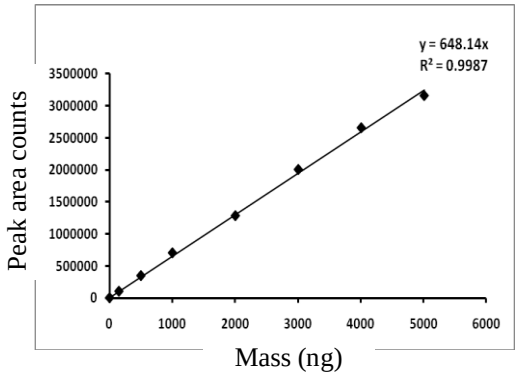


Figure 3.10: Calibration curve for pyruvic acid

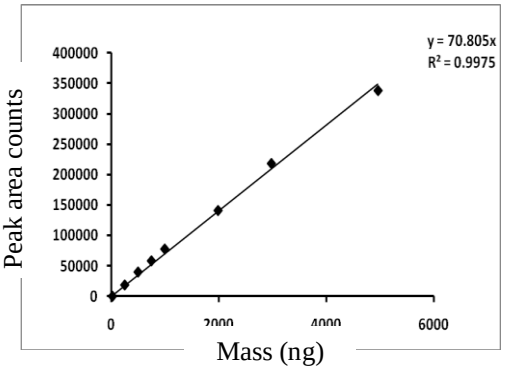


Figure 3.11: Calibration curve for citric acid

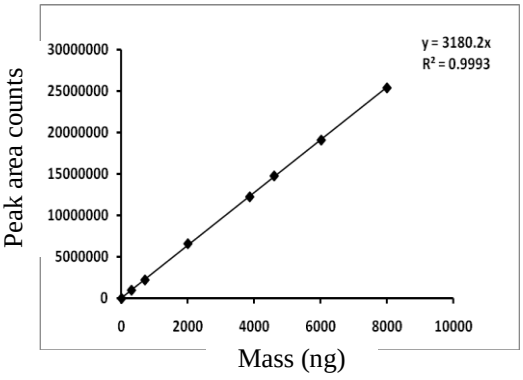


Figure 3.12: Calibration curve for *trans*-aconitic acid

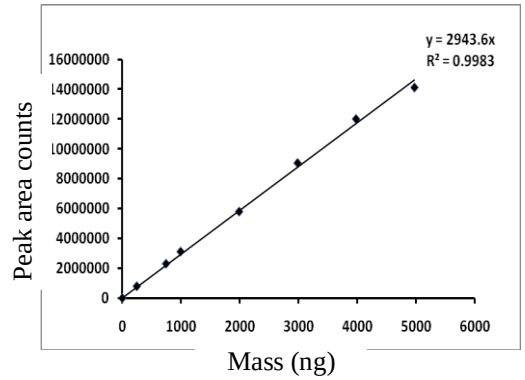


Figure 3.13: Calibration curve for acrylic acid

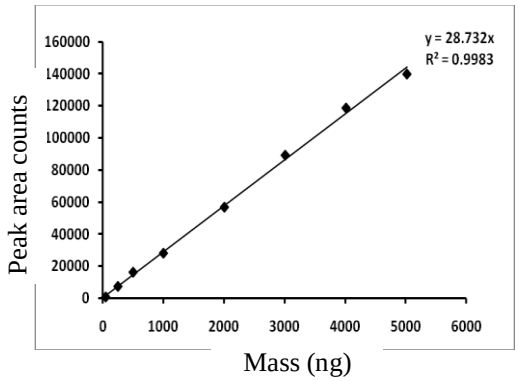


Figure 3.14: Calibration curve for propionic acid

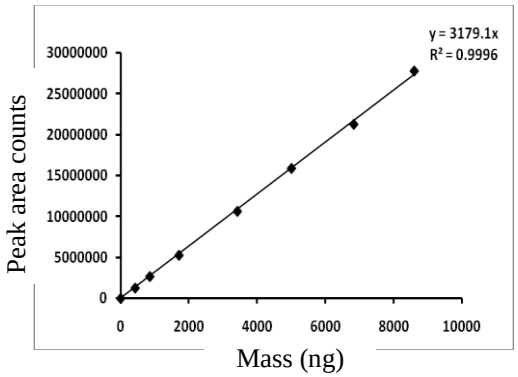


Figure 3.15: Calibration curve for *cis*-aconitic acid

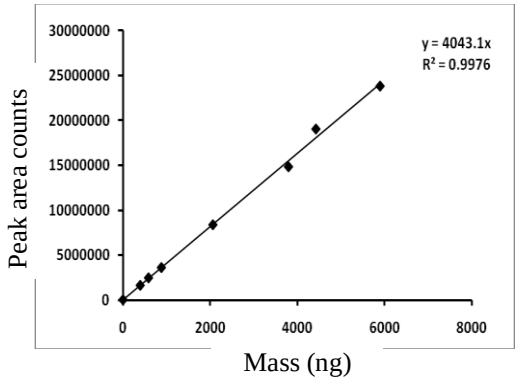


Figure 3.16: Calibration curve for citraconic acid

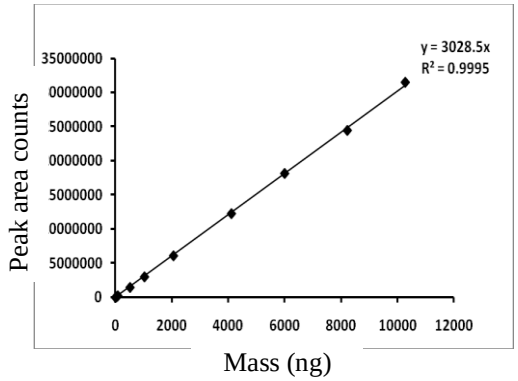


Figure 3.17: Calibration curve for itaconic acid

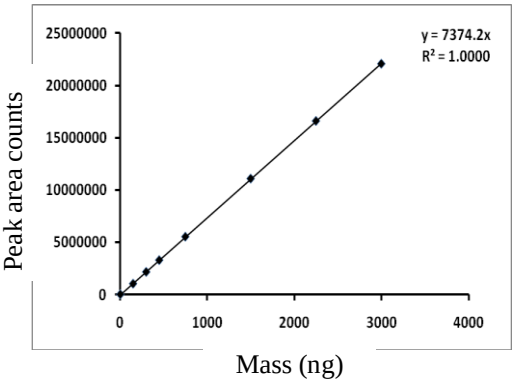


Figure 3.18: Calibration curve for fumaric acid

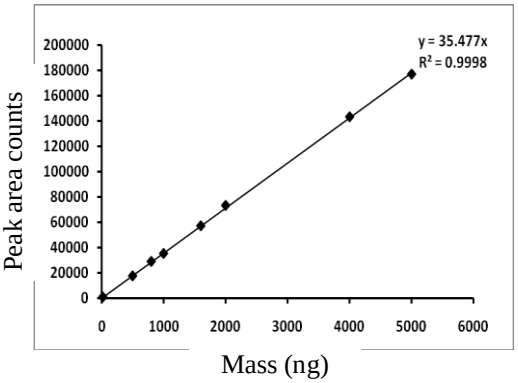


Figure 3.19: Calibration curve for succinic acid

3.2.2.2.4 Limits of detection and quantification

The limit of detection (LOD) and limit of quantitation (LOQ) for analytes were determined on basis of signal-to-noise ratio of 3 and 10, respectively.[43] The LOD and LOQ (data given in Table 3.5) were calculated for average as well as maximum noise, which was measured peak-to-peak. It can be seen that the fumaric acid shows highest sensitivity towards UV-detection, whereas propionic acid shows the least. Citraconic, *trans*-aconitic, acrylic, *cis*-aconitic and pyruvic acids show almost similar sensitivity.

Table 3.5: LOD and LOQ for carboxylic acids

Analyte	**LOD (ng)**		**LOQ (ng)**	
	I[a]	II[a]	I[b]	II[b]
Fumaric acid	0.2	0.3	0.8	1.1
Citraconic acid	0.7	1.0	2.3	3.2
trans-Aconitic acid	0.7	1.0	2.4	3.4
Acrylic acid	0.7	1.1	2.5	3.5
cis-Aconitic acid	0.9	1.2	2.9	4.1
Itaconic acid	1.2	1.7	4.0	5.7
Pyruvic acid	1.7	2.4	5.6	8.0
Oxalic acid	3.6	5.1	12.0	17.0
Citric acid	16.9	24.0	56.3	79.9
Formic acid	42.6	60.5	141.9	201.5
Succinic acid	58.8	83.1	195.1	277.0
Propionic acid	80.0	113.6	266.7	378.7

[a]: w. r. t. Average noise (46 μAU, peak-to-peak)
[b]: w. r. t. Maximum noise (66 μAU, peak-to-peak)

3.2.2.2.5 Method precision and accuracy

The precision and accuracy of the method was tested by running lactic acid sample spiked with a known amount of oxalic, formic, pyruvic, succinic, fumaric, acrylic, propionic and citraconic acids. These acids encompassed low, medium and high sensitivity (Table 3.5, LOD data) and were chosen as representative cases for the recovery experiments. The spiked sample was analysed by three analysts at least in duplicate to determine the average recovery,

which ranged between 98.4 and 104.0%, which was appropriate for present study, as shown in Table 3.6.

Table 3.6: Analytical data on recovery of carboxylic acids from lactic acid

Run No.	Oxalic	Formic	Pyruvic	Succinic	Fumaric	Acrylic	Propionic	Citraconic
1	196.4	172.5	241.5	189.5	2.6	3.1	521.2	23.5
2	190.0	159.1	241.3	122.1	2.6	3.4	577.6	23.7
3	193.9	177.4	241.7	152.7	2.6	3.3	495.6	23.3
4	194.1	164.3	254.0	176.5	2.5	2.6	506.9	23.6
5	194.2	166.7	255.0	201.6	2.7	3.6	557.1	24.7
6	197.1	169.7	257.3	217.3	2.6	3.6	566.6	24.2
7	199.8	181.6	259.6	236.7	2.6	3.4	557.9	25.2
8	202.5	175.5	262.7	264.4	2.7	4.1	584.5	26.1
Found (Mean)	**197.3**	**175.3**	**253.0**	**204.0**	**2.6**	**3.5**	**552.4**	**24.6**
SD	**5.3**	**14.9**	**9.2**	**50.3**	**0.1**	**0.5**	**36.9**	**1.4**
%RSD	**2.7**	**8.5**	**3.6**	**24.7**	**2.5**	**13.5**	**6.7**	**5.5**
Added, ppm	**190.0**	**175.0**	**250.0**	**200.0**	**2.5**	**3.5**	**550.0**	**25.0**
%Recovery	**103.8**	**100.2**	**101.2**	**102.0**	**104.0**	**100.0**	**100.4**	**98.4**

3.2.2.2.6 Ruggedness

The ruggedness of the method was tested by checking the reproducibility of calibration linearity. This was evaluated by running two separate sets of calibration standards on two different columns (of the same type) by two analysts on different days (at least three months apart). The two sets of calibration curves were linear ($R^2 > 0.9950$) having fairly comparable slopes, in most of the cases. The variation observed in slopes might arise due to batch-to-batch variation in columns, and other factors. The summary of linearity ruggedness is given in Table 3.7.

Table 3.7: Summary of linearity ruggedness

Analyte	Calibration I		Calibration II	
	Regression equation	**R^2 value**	**Regression equation**	**R^2 value**
Oxalic acid	$y = 459.95x$	0.9970	$y = 550.96x$	0.9993
Formic acid	$y = 27.829x$	0.9956	$y = 42.837x$	0.9999
Pyruvic acid	$y = 648.14x$	0.9987	$y = 536.78x$	1.0000
Citric acid	$y = 70.805x$	0.9975	$y = 68.187x$	0.9993
Fumaric acid	$y = 7374.2x$	1.0000	$y = 7877.6x$	0.9999
Succinic acid	$y = 35.477x$	0.9998	$y = 32.122x$	0.9985
trans-Aconitic acid	$y = 3180.2x$	0.9993	$y = 3041.8x$	0.9997
Acrylic acid	$y = 2943.6x$	0.9983	$y = 3155.5x$	0.9997
Propionic acid	$y = 28.732x$	0.9983	$y = 34.234x$	0.9992
cis-Aconitic acid	$y = 3179.1x$	0.9996	$y = 3145.9x$	1.0000
Citraconic acid	$y = 4043.1x$	0.9976	$y = 4012.6x$	0.9984
Itaconic acid	$y = 3028.5x$	0.9995	$y = 2984.9x$	0.9999

3.2.2.3 Gauge R & R Study

The method was subjected to Gauge R&R (repeatability and reproducibility) study to determine variability in the measuring system and to identify various sources that contribute to it. The measuring system comprises: (1) measuring device i.e. HPLC instrument and the method of analysis (Repeatability component) and (2) the operators (analysts) who perform the analyses (Reproducibility component). The R&R study indicates how the method performs in the hands of different analysts besides the performance of measuring device, and if there is any interaction between samples and analysts. The %R&R is calculated as follows:

$$\%R\&R = \left(\frac{R\&R}{Total\ variation}\right) \times 100 \qquad 3.1$$

$$R\&R = \sqrt{EV^2 + AV^2} \qquad 3.2$$

$$Total\ variation = \sqrt{EV^2 + AV^2 + SV^2} \quad 3.3$$

where, *EV*, *AV* and *SV* are standard deviation due to repeatability, reproducibility and sample-to-sample variation, respectively. For this purpose, three different samples of lactic acid were analysed in triplicate (one run at a time by one analyst) by three analysts for three acids viz., pyruvic acid (140, 250 and 260 ppm), oxalic acid (75, 100 and 195 ppm) and succinic acid (160, 200 and 700 ppm) in a completely randomised manner on the same HPLC system (measuring device). These acids were chosen as representative cases since they were found in most of the lactic acid samples and have high, medium and low sensitivity (Table 3.5, LOD data). The analytical data were subjected to statistical method of analysis of variance (ANOVA) to delineate the contributions from various sources, and their significance was judged by p-values ($P < 0.05$ indicates significant contribution). The data coverage factor for the R&R study was 5.15 σ (i.e. 99% area under the normal distribution curve). The R&R values up to 10% are considered as very good (which means variation contributed by the measurement process is not more than 10%) and any value between 10-20% is generally acceptable as this indicates good stability of the measuring system.

The results of the R&R study are shown (Figures 3.20 – 3.22) as 'x bar chart by analyst', 'range chart by analysts', 'analyst-sample interaction', 'scatter plots by samples and by analysts', and 'components of variance'.

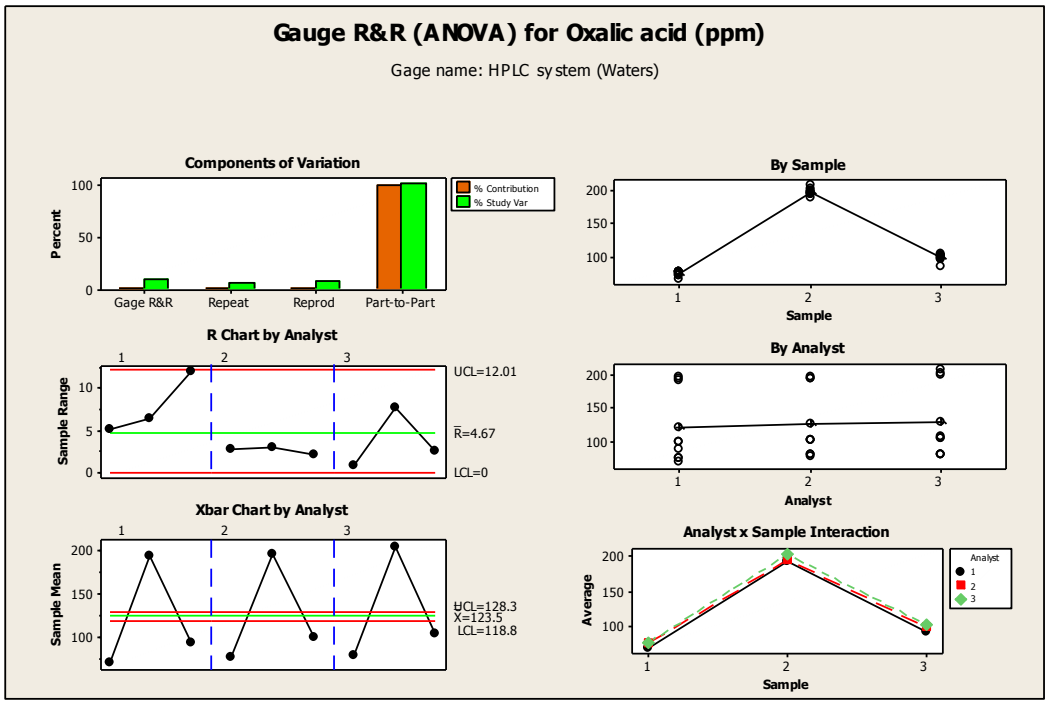


Figure 3.20: ANOVA results for three different oxalic acid analyses by three analysts

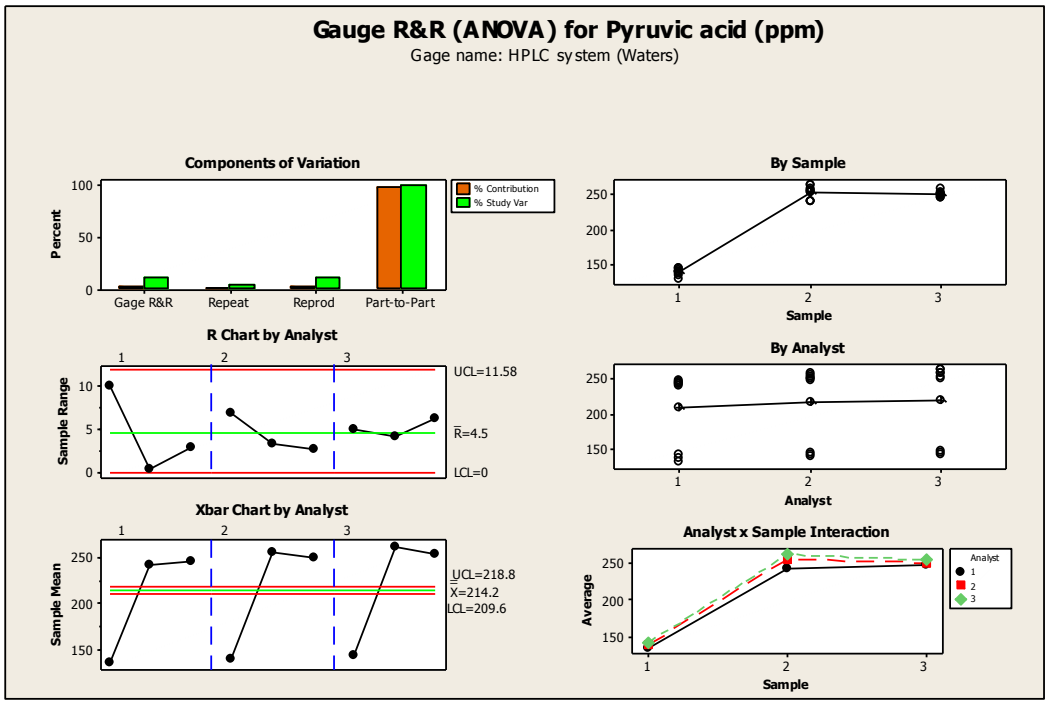


Figure 3.21: ANOVA results for three different pyruvic acid analyses by three analysts

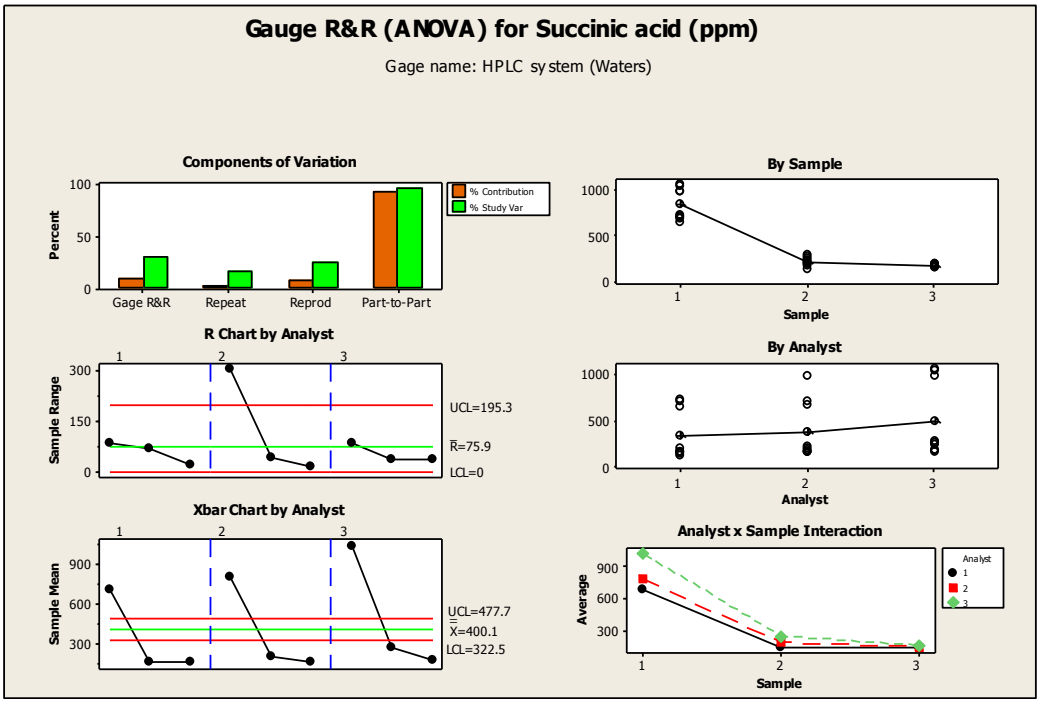


Figure 3.22: ANOVA results for three different succinic acid analyses by three analysts

The R&R values for pyruvic, oxalic and succinic acids were 10.9, 8.3 and 11.9%, respectively. These values are very close to 10% and indicate a very stable measuring system. It may be noted that in all the three cases, the larger contributions to the R&R values originated from the analysts (i.e. Reproducibility component) rather than from the measuring system (i.e. Repeatability component) that included the analytical method under investigation. Further, the sample-analyst interaction (i.e. sample handling by analysts) contributed significantly to the Reproducibility component in the case of pyruvic and succinic acids, and it was insignificant for oxalic acid. The ANOVA data of Gauge R & R study for all the three components (oxalic, pyruvic, succinic acids) is given in Tables 3.8 - 3.13.

Table 3.8: Sources of variation in oxalic acid analysis (by ANOVA method)

Source	DF	SS	MS	F	P
Samples	2	76049.4	38024.7	3522.13	0.00000
Analysts	2	370.8	185.4	17.17	0.01088
Analyst x Sample	4	43.2	10.8	1.15	0.36630
Repeatability	18	169.4	9.4	-	-
Total	26	76632.7	-	-	-

Table 3.9: Repeatability and reproducibility (R&R) data for oxalic acid analysis

Source	Var comp	Std Dev (SD)	Study Var (5.15*SD)	%Contribution	%Study Var
Total R&R	29.2	5.40	27.82	0.69	8.28
Repeatability	9.7	3.11	16.01	0.23	4.77
Reproducibility	19.5	4.42	22.76	0.46	6.78
Analyst	19.5	4.42	22.76	0.46	6.78
Sample-to-Sample	4223.9	64.99	334.71	99.31	99.66
Total variation	4253.1	65.22	335.86	100.00	100.00

Table 3.10: Sources of variation in pyruvic acid analysis (by ANOVA method)

Source	DF	SS	MS	F	P
Samples	2	76068.5	38034.2	853.725	0.00001
Analysts	2	653.7	326.9	7.337	0.04589
Analyst x Sample	4	178.2	44.6	6.282	0.00240
Repeatability	18	127.7	7.1	-	-
Total	26	77028.0	-	-	-

Table 3.11: Repeatability and reproducibility (R&R) data for pyruvic acid analysis

Source	Var comp	Std Dev (SD)	Study Var (5.15*SD)	%Contribution	%Study Var
Total R&R	50.9	7.14	36.76	1.19	10.92
Repeatability	7.1	2.66	13.72	0.17	4.07
Reproducibility	43.9	6.62	34.10	1.03	10.13
Analyst	31.4	5.60	28.84	0.73	8.57
Analyst x Sample	12.5	3.53	18.20	0.29	5.41
Sample-to-Sample	4221.1	64.97	334.59	98.81	99.40
Total variation	4272.0	65.36	336.61	100.0	100.00

Table 3.12: Sources of variation in succinic acid analysis (by ANOVA method)

Source	DF	SS	MS	F	P
Samples	2	1620317	810159	427.405	0.00002
Analysts	2	9698	4849	2.558	0.19253
Analyst x Sample	4	7582	1896	3.721	0.02238
Repeatability	18	9169	509	-	-
Total	26	1646766	-	-	-

Table 3.13: Repeatability and reproducibility (R&R) data for succinic acid analysis

Source	Var comp	Std Dev (SD)	Study Var (5.15*SD)	%Contribution	%Study Var
Total R&R	1300	36.05	185.66	1.43	11.94
Repeatability	509	22.57	116.24	0.56	7.48
Reproducibility	790	28.11	144.77	0.87	9.31
Analyst	328	18.12	93.29	0.36	6.00
Analyst x Sample	462	21.50	110.70	0.51	7.12
Sample-to-Sample	89807	299.68	1543.34	98.57	99.28
Total variation	91107	301.84	1554.47	100.00	100.00

It may also be noted that the three lactic acid samples were distinctly different, as indicated by the ANOVA data ($P < 0.05$).

3.3 Determination of Methanol and Ethanol in Lactic acid

Since methanol and ethanol are not amenable to HPLC-UV method due to absence of chromophores, a separate method, based on GC-FID technique, was developed for their separation and quantification. All the commercial lactic acid samples were analysed on GC to check the presence of both methanol and ethanol since it was not known whether methyl or ethyl esterification was used for their downstream purification. Methyl lactate purification route was used for purification of in-house samples, so these samples were checked for presence of methanol only. Among in-house (laboratory) prepared samples, only four samples (IH-2, IH-4, IH-7 and IH-8) were found to contain

methanol and none of the commercial samples was found to contain either ethanol or methanol.

3.3.1 Experimental

3.3.1.1 Materials

The methanol standard (AR grade, assay 99.7%) was purchased from Merck Ltd. (Mumbai, India). The ethanol standard (AR grade, assay 99.9%) was purchased from Changshu Yangyuan Chemicals (Changshu, China) and AR grade isopropanol (purity 99.8%, used as an internal standard) was purchased from SDFL (Mumbai, India).

3.3.1.2 Preparation of standards and samples

The standard solutions comprising methanol and ethanol were prepared in distilled water, covering concentration range of 514- 6034 ppm (w/v) for methanol, and 502- 6018 ppm (w/v) for ethanol. An equal amount of isopropanol (internal standard) was added into each standard solution such that its concentration in all standard solutions becomes equal to 3000 ppm (w/v). Lactic acid samples for analysis were prepared by dissolving the required quantity in distilled water to obtain 25.0% (w/v) solution.

3.3.1.3 Equipment and parameters of analysis

A gas chromatograph (GC-14B, Shimadzu Corporation, Kyoto, Japan) equipped with an auto sampler (AOC20*i*) and FID was used for the experiments. Hydrogen gas (purity $\geq$ 99.9995) was purchased from Vadilal Chemicals (Baroda, India). The AR grade zero air and UHP grade nitrogen gas were from Inox Ltd. (Mumbai, India). The data acquisition and processing of chromatographic data were carried out on Shimadzu's C-R7A plus Chromatopac Software. The weighing of standards and samples was done on digital balance (Mettler Toledo AG 245, Switzerland). The description of lactic acid

samples analysed is given in Table 3.1. The samples were analysed for ethanol and methanol contents with chromatographic parameters given in Section 3.3.2.1

3.3.2 Results and discussion

3.3.2.1 Method development and optimisation

GC columns like DB-1 or DB-5 (dimethyl polysiloxane based phase) are most commonly used for analysis of volatile organic compounds because they provide better sensitivity and selectivity for analytes. However, aqueous samples can not be injected with these columns, because water present in sample hydrolyses the dimethyl polysiloxane coated on stationary phase, causing damage to the column. Hence, Porapack Q capillary column (Q-PLOT column, 30 m length x 0.32 mm i.d., Supelco, USA) was chosen which is compatible with aqueous samples. De-activated glass wool was placed in GC glass injection liner which acts as a filter for non-volatile component thereby restricting its entry into GC column and thus avoiding possible damage to the column.

Internal standards are widely used in chromatography, especially in gas chromatography, where samples are injected in small volumes (0.5 µL to 1.5 µL). This primarily produces non-reproducible peak area leading to an erratic result. The purpose of using internal standard method was explained in Section 1.10 of Chapter 1. In the present analysis, isopropanol (IPA) was chosen as an internal standard. The GC temperature conditions were optimised to get the baseline separation of methanol, ethanol, and isopropanol and also, separation of other components present in sample matrix to avoid their interference with peaks of interest.

The optimised parameters of GC separation program are given below:

- Carrier gas (nitrogen) flow: 1.88 mL/min
- Make up gas flow: 40 mL/min
- Carrier gas head pressure: 200 kPa
- Hydrogen gas pressure: 60 kPa
- Zero air pressure: 50 kPa
- Column initial temp.: 100 ^{o}C
- Column initial hold time: 0 min
- Column final temp.: 190 ^{o}C
- Column final hold time: 57 min
- Temperature program rate: 30 ^{o}C/ min
- Injector temp.: 200 ^{o}C
- FID temp.: 250 ^{o}C
- Split ratio: 1:50
- Total run time: 60 min
- Injection volume: 1.0 µL

3.3.2.2 Method validation

After method was developed, it was validated to ensure that it was useful for its intended purpose. The validation parameters investigated were system precision, specificity, linearity (calibration studies), limit of detection (LOD), limit of quantification (LOQ) and response factors for ethanol and methanol.

3.3.2.2.1 System precision

System precision was assessed by studying repeatability of retention time and peak area. For this study, 10 replicate injections of a test sample containing mixture of ethanol, methanol and IPA were made on GC system. The data on retention time variation is given in Table 3.14. The retention times for all the components were quite repeatable as evident from the low %RSD values.

Table 3.14: Retention time repeatability

Inj. No.	Retention time (min)		
	Methanol	**Ethanol**	**IPA**
1	0.928	1.184	1.456
2	0.926	1.181	1.458
3	0.928	1.187	1.456
4	0.924	1.186	1.459
5	0.928	1.181	1.458
6	0.927	1.189	1.452
7	0.923	1.187	1.445
8	0.922	1.184	1.457
9	0.928	1.182	1.458
10	0.926	1.181	1.451
Mean	**0.926**	**1.184**	**1.455**
SD	**0.002**	**0.003**	**0.004**
%RSD	**0.2**	**0.3**	**0.3**

The data on peak area variation is given in Table 3.15. Internal standards are useful for analyses wherein the quantity of sample analysed or the instrument response varies slightly from run to run for reasons that are difficult to control. An internal standard is a known amount of a compound, different from analyte that is added to the unknown. Signal from analyte is compared with signal from the internal standard to find out how much analyte is present. When a known amount of internal standard is used, peak area ratio (of a particular component to internal standard) is used for quantification of that particular component which nullifies run- to- run area variation. In this study, the %RSD for peak area ratio was found to be around 1.0, which shows good peak area repeatability. It may be noted that %RSD ($\leq$ 1.0) calculated for peak area ratio (w. r. t. internal standard) for both methanol and ethanol is considerably less than the %RSD (around 3.0) calculated based on their individual area counts. This study demonstrates that the use of internal standard helps reducing error which is likely to occur due to injection variability and other various reasons.

Table 3.15: Peak area repeatability

Inj. No.	Peak area (counts)			Peak area ratio	
	Methanol	**Ethanol**	**IPA**	**Methanol (A_S/A_{IS})***	**Ethanol (A_S/A_{IS})****
1	37865	48000	74618	0.5075	0.6433
2	39856	49623	78612	0.5070	0.6312
3	37895	47985	74521	0.5085	0.6439
4	38562	49978	76523	0.5039	0.6531
5	38101	48978	75412	0.5052	0.6495
6	37545	48578	74586	0.5034	0.6513
7	37215	47801	74618	0.4987	0.6406
8	35612	45124	70121	0.5079	0.6435
9	37986	47986	74618	0.5091	0.6431
10	36985	48101	74568	0.4960	0.6451
Average	**37762**	**48215**	**74820**	**0.5047**	**0.6445**
SD	**1094.976**	**1320.556**	**2109.255**	**0.004**	**0.006**
%RSD	**2.9**	**2.7**	**2.8**	**0.9**	**1.0**

(A_S/A_{IS})*: Peak area of methanol/ Peak area of IPA (IS)
(A_S/A_{IS})**: Peak area of ethanol/ Peak area of IPA (IS)

3.3.2.2.2 Specificity

Specificity of method was tested by spiking lactic acid sample with methanol, ethanol and IPA (internal standard) and run under optimised chromatographic conditions. The method was found to be specific, because it produced the baseline separation for methanol, ethanol and isopropyl alcohol. These peaks were well separated from each other (complete baseline separation) and other interfering components from lactic acid sample matrix. The representative chromatogram showing the chromatographic separation of methanol, ethanol and IPA is given in Figure 3.23.

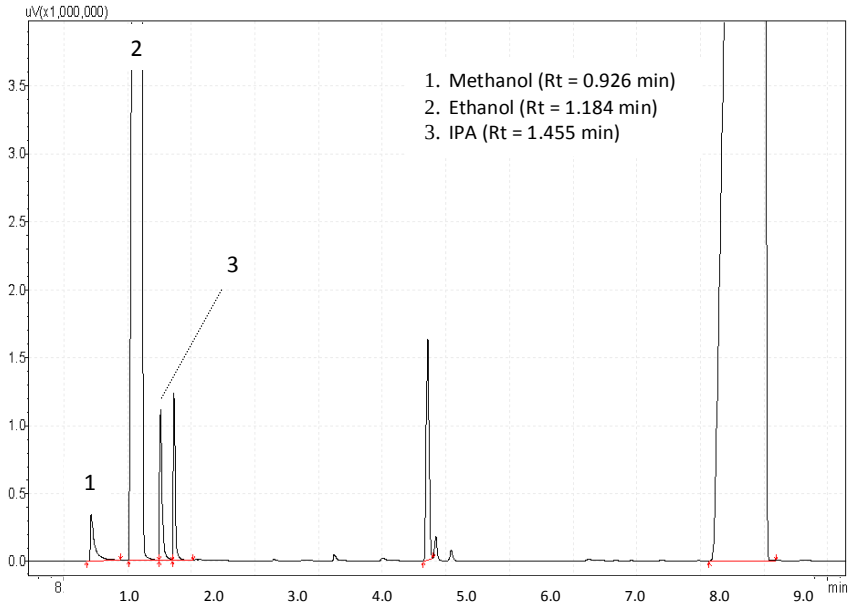


Figure 3.23: Chromatogram showing separation of ethanol, methanol, IPA (IS)

3.3.2.2.3 Linearity (calibration studies)

The detector response was found to be linear in the range of 514 to 6034 ppm (w/v) for methanol, and 502 to 6018 ppm (w/v) for ethanol. The regression coefficient (R^2) values for both methanol and ethanol were equal to or greater than 0.9992, which shows a good fit of regression lines.

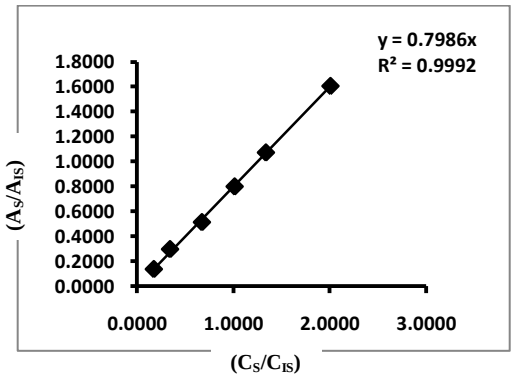


Figure 3.24: Calibration curve for methanol

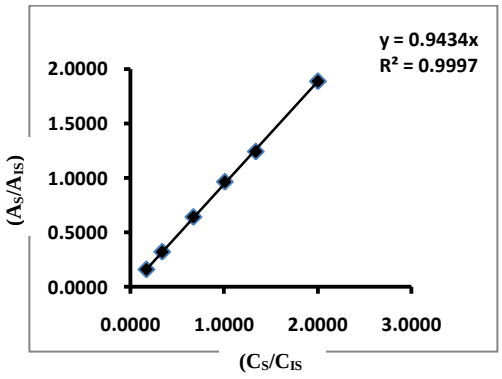


Figure 3.25: Calibration curve for ethanol

The regression equations for methanol and ethanol were obtained from calibration curves by plotting ratio of concentration of analyte to internal standard

(C_S/C_{IS}) against corresponding ratio of peak area of analyte to internal standard (A_S/A_{IS}). The calibration curves of methanol and ethanol are given in Figures 3.24 and 3.25, respectively.

3.3.2.2.4 Response factors

The response factors (R_f) for ethanol and methanol were calculated from calibration data using equation 3.4. Internal standard method, presented in Section 1.10 of Chapter 1, was used for this measurement.

$$R_f = \left(\frac{C_S}{C_{IS}}\right) \times \left(\frac{A_{IS}}{A_S}\right) \qquad 3.4$$

where, C_S is the concentration (ppm, w/v) of analyte standard (methanol or ethanol), C_{IS} is the concentration (ppm, w/v) of internal standard (IPA), A_S is the peak area of analyte standard and A_{IS} is the peak area of internal standard. The calibration data is given in Tables 3.16 and 3.17 and data on response factors is given in Table 3.18.

Table 3.16: Calibration data 1

Run No.	Concentration (ppm, w/v)			Peak area (counts)		
	Methanol	Ethanol	IPA (IS)	Methanol	Ethanol	IPA (IS)
1	514	502	3000	10112	12000	74918
2	1020	1010	3002	22000	24000	74500
3	2014	2012	3002	38318	48000	74800
4	3032	3018	3000	59624.3	72000	74618
5	4012	4016	3004	80214	92978	74816
6	6034	6018	3008	120000	140998	74700

Table 3.17: Calibration data 2

Run No	Methanol (C_S/C_{IS})	Ethanol (C_S/C_{IS})	Methanol (A_S/A_{IS})	Ethanol (A_S/A_{IS})
1	0.1713	0.1673	0.1350	0.1602
2	0.3398	0.3364	0.2953	0.3221
3	0.6709	0.6702	0.5123	0.6417
4	1.0107	1.0060	0.7991	0.9649
5	1.3356	1.3369	1.0722	1.2428
6	2.0060	2.0007	1.6064	1.8875

Methanol (C_S/C_{IS}): concentration of methanol/ concentration of IPA (IS), Methanol (A_S/A_{IS}): peak area of methanol/ peak area of IPA (IS), Ethanol (C_S/C_{IS}): concentration of ethanol/ concentration of IPA (IS), Ethanol (A_S/A_{IS}): peak area of ethanol/ peak area of IPA (IS)

Table 3.18: Response factors (R_f) for methanol and ethanol

Sr. No.	Response factor (R_f)	
	Methanol	Ethanol
1	1.2694	1.0447
2	1.1506	1.0444
3	1.3096	1.0444
4	1.2648	1.0426
5	1.2457	1.0757
6	1.2487	1.0599
Mean	1.2481	1.0520
SD	0.053	0.013
%RSD	4.2	1.3

The %RSD < 5.0 for response factors (calculated from replicate measurements, *n = 6*) shows adequate accuracy for the present method. The average R_f values were used to determine corresponding analyte concentration in lactic acid samples.

3.3.2.2.5 Limits of detection and quantification

(A) Limit of detection (LOD)

The LOD of a method of analysis is the lowest concentration of analyte in a sample that can be detected and confirmed, but not necessarily quantified, under the stated conditions of the test. In the present study, a signal-to-noise ratio of 3:1 was considered, which is generally accepted for reliably establishing this limit.[43]

(B) Limit of quantification (LOQ)

The LOQ of a method of analysis is the lowest concentration of analyte in a sample at which the analyte can be quantified with an acceptable degree of certainty. Here, a signal-to-noise ratio of 10:1 was considered to establish the limit of quantitation.[43]

A blank sample containing internal standard (IPA) with its concentration equal to 3000 ppm (w/v) was run on GC system. The noise was measured randomly at three different places as 9, 13 and 8 (as peak area counts) and the average value was determined as 10, which was used for calculating LOD and LOQ. These limits were determined using corresponding regression equations of ethanol and methanol. The LOD and LOQ values are reported in Table 3.19.

Table 3.19: LOD and LOQ values

Component	LOD (ppm)	LOQ (ppm)
Methanol	38	125
Ethanol	32	106

3.3.2.2.6 Method precision and accuracy

Method precision and accuracy was tested by running lactic acid sample spiked with a known amount of methanol and ethanol each at three (low, medium and high) levels. Three synthetic samples which comprised of a mixture of methanol and ethanol, each having concentrations around 500, 2000, and 5000 ppm, were prepared and the method precision (intra-day) was evaluated by replicate analyses of samples ($n = 5$) on same day and the inter-day precision by comparing the assay on five different days. The %RSD values and relative errors for accuracy are given in Table 3.20. The low values for %RSD and relative errors indicate that the method has good accuracy and precision.

Table 3.20: Method precision and accuracy

Synthetic sample		Spiked in LA sample (ppm)	INTRA-DAY, *n = 5*			INTER-DAY, *n = 5*		
			A ± SD[a]	Precision %RSD[b]	Acc.[c]	A ± SD[a]	Precision %RSD[b]	Acc.[c]
1	Methanol	512	508 ± 15.7	3.1	-0.9	510 ± 13.3	2.6	-0.4
	Ethanol	508	490 ± 12.9	2.6	-3.7	506 ± 12.0	2.0	-3.5
2	Methanol	2024	2016 ± 16.5	0.8	- 0.4	2015 ±	0.8	- 0.8
	Ethanol	2008	2011 ± 14.8	0.7	0.6	2024 ±	1.7	0.8
3	Methanol	5004	5062 ± 42.4	0.8	1.2	5050 ±	0.9	0.9
	Ethanol	5010	5041 ± 32.3	0.6	0.6	5088 ±	2.6	1.5

A: Mean of determined concentration (ppm, w/v), *n = 5*,
[a]**SD**: Standard deviation, [b]**RSD**: Relative standard deviation,
[c]**Acc.**: Accuracy (i.e. %Relative error) = [(Determined conc.− Added conc.) / Added conc.] × 100

3.4 Analysis of Lactic acid Samples

After completing method development and validation, the methods (GC and HPLC) were applied to analyse all commercial and in-house samples for estimating chemical purity of lactic acid by impurity profiling. An HPLC method was employed to determine carboxylic acid impurities and GC method was used to determine impurities of ethanol and methanol. Since the PLA precursors, linear and cyclic dimers of lactic acid, ultimately end up as PLA, they were not considered as impurities and hence, only the identification was done and no effort was made to quantify them. The impurity profile data for individual samples is reported in subsequent sections.

3.4.1 Carboxylic acid impurities in lactic acid

After the HPLC method was established, the lactic acid samples from various sources were analysed for carboxylic acid impurities. The lactic acid samples were prepared in the laboratory and purified as described in Section 3.2.1.2.

Lactic acid, having hydroxyl and carboxyl functional groups, undergoes intramolecular or self-esterification to form linear polyesters, lactoyl lactic acid and

higher poly(lactic acid)s or the cyclic dimer (3,6-dimethyl-*p*-dioxane-2,5-dione), which is also called as dilactide. The "commercial (technical) grade" lactic acid is actually a mixture of free lactic acid, linear dimer (or lactoyl lactic acid), short chain oligomers, a small quantity of dilactide, water, and other carboxylic acid impurities. When this lactic acid is diluted in excess of water, the dimers and oligomers slowly hydrolyse to free lactic acid.

When lactic acid is produced by fermentation of sugarcane juice, impurities, especially of aliphatic carboxylic acids e.g. pyruvic, acetic, malic, fumaric, succinic, propionic and formic acids may be formed. Further, the impurities of aconitic, itaconic, oxalic, citric, malic, formic and acetic acids may also come from sugarcane juice itself.[44]

In the presence of sulphuric acid, following transformations may also take place in lactic acid obtained by fermentation: lactic acid into acrylic acid and also lactic acid into formic acid; malic acid into fumaric acid; citric acid into aconitic acid; oxalic acid into carbon dioxide, carbon monoxide and water. Aconitic acid may further decompose to itaconic acid, which may subsequently get converted to citraconic acid.[45]

If broth or the bacterial culture used is contaminated with *Butyric bacilli* or *Clostridia bacteria*, butyric acid may also be produced during the fermentation. However, acetic, malic and butyric acids were not detected in any of the samples analysed. The individual acids were identified by matching the retention times as well as by spiking with the authentic standards. The concentrations of individual carboxylic acid impurities (ppm, w/w) were calculated using respective regression equations. A particular acid not found in a sample was reported as BDL (i.e. below detection limit). The other components, linear and cyclic dimers of lactic acid, were identified as discussed below.

3.4.2 Identification of linear and cyclic dimers

The free lactic acid and the cyclic dimer (dilactide) were identified by comparison of retention time with standards (Aldrich, St. Louis, MO, USA). The direct identification of linear dimer (lactoyl lactic acid) was not possible since standard lactoyl lactic acid was not available. It was identified indirectly by conducting hydrolysis experiments. The lactic acid sample was subjected to hydrolysis in mobile phase as well as in water. A 2% (w/v) solution of L (+) lactic acid (commercial source 2) was prepared in mobile phase and an aliquot of 25 mL of this was hydrolysed under reflux for 4 hours. After hydrolysis, the reaction volume was remade up to 25 mL with the mobile phase. The same experiment was repeated with water as well. The samples were analysed before and after the hydrolysis. Typical chromatograms of lactic acid sample before and after the hydrolysis are shown in Figures 3.26 and 3.27, respectively. The data on peak area of free lactic acid, linear dimer and cyclic dimer along with their ratios are given in Table 3.21.

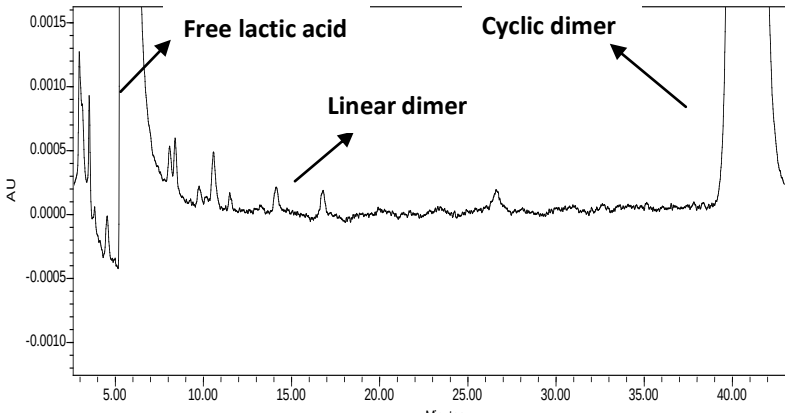


Figure 3.26: Chromatogram of lactic acid sample (commercial source 2) before hydrolysis

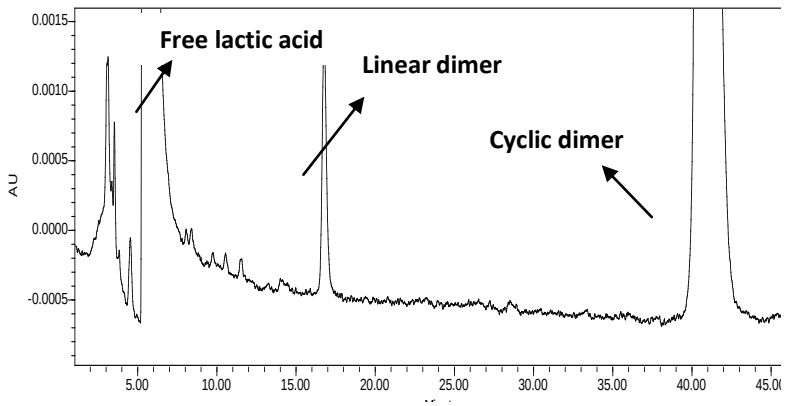
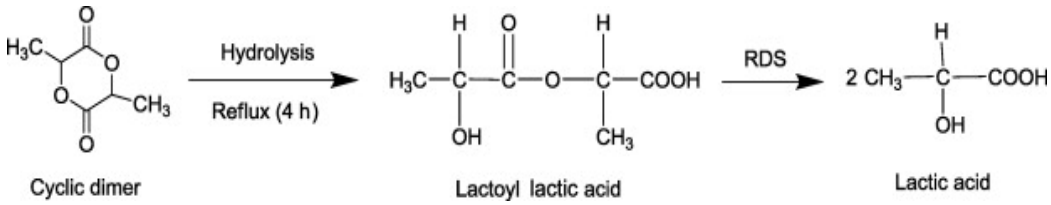


Figure 3.27: Chromatogram of lactic acid sample (commercial source 2) after hydrolysis

It was observed that the peak at 16.5 min enhanced substantially at the expense of cyclic dimer. This can be explained by the following reaction given in Scheme 3.2.



Scheme 3.2: Hydrolysis of cyclic dimer

The enhancement in the peak at 16.5 min is possible only when the rate of conversion of lactoyl lactic acid to free lactic acid is slow (i.e. rate determining step, RDS) as compared to the conversion of cyclic dimer to lactoyl lactic acid. Therefore, the peak at 16.5 min was assigned to lactoyl lactic acid. This explanation is supported by the ratios of cyclic to linear dimer and free lactic acid to cyclic dimer, before and after the hydrolysis, in both water and mobile phase. The identification of linear dimer and the retention behaviour of the two dimers could also be corroborated based on polarity considerations i.e. the linear dimer, which is more polar, elutes before the cyclic dimer. Subsequent to this work, we found that our explanation was also supported by the work of Diaz et al[46] in which they reported the determination of lactoyl-lactic acid in DL-

lactide (cyclic dimer) based on the similar kinetic interpretation of the peak heights ratio obtained by HPLC. Since the linear and cyclic dimers ultimately end up as PLA, they were not considered as impurities and hence, no effort was made to quantify them.

Table 3.21: Hydrolysis of lactic acid (Commercial source 2) sample

Sample	Component	Peak area	Cyc.: linear[e]	LA: Cyc.[f]
LA - HM [a]	Free lactic acid	33617499	38.1	24.1
	Linear dimer	36599.5		
	Cyclic dimer	1394866		
LA - M [b]	Free lactic acid	32425635	796.8	13.7
	Linear dimer	2965.9		
	Cyclic dimer	2363152		
LA - HW [c]	Free lactic acid	32609850	31.1	24.5
	Linear dimer	42703.4		
	Cyclic dimer	1328929		
LA - W [d]	Free lactic acid	32909588	367.0	13.3
	Linear dimer	6766.3		
	Cyclic dimer	2483291		

[a]In mobile phase, after hydrolysis, [b]In mobile phase, before hydrolysis
[c]In plain water, after hydrolysis, [d]In plain water, before hydrolysis.
[e]Ratio of peak area of cyclic dimer to linear dimer, [f]Ratio of peak area of free lactic acid to cyclic dimer.

3.4.3 Quantification of ethanol and methanol

After determining carboxylic acid impurities by HPLC, all the samples were analysed by GC to find out methanol and ethanol contents in them. Each lactic acid sample was prepared by dissolving 12.500 g in distilled water in 50 mL volumetric flask and diluting up to the mark. An amount of about 150.0 mg of internal standard (IPA) was also added to this solution so as to make its concentration equal to 3000 ppm (w/v). The sample was run on GC system under optimised chromatographic conditions. The concentration (ppm, w/v) of analyte in lactic acid sample solution was calculated using equation 3.5:

$$C_S = \left(\frac{A_S}{A_{IS}}\right) \times R_f \times C_S \quad 3.5$$

where, C_S is the concentration (ppm, w/v) of analyte (methanol or ethanol) to be determined, A_S is the peak area of analyte, A_{IS} is the peak area of internal standard (IPA), C_{IS} the concentration (ppm, w/v) of internal standard (IPA) added into sample and R_f is average response factor of corresponding analyte.

Once the analyte concentration in lactic acid sample solution is determined, total amount (mass) of methanol or ethanol in lactic acid sample can be calculated by knowing the total volume of solution. Thus, from quantity of lactic acid sample taken for that particular analysis and the amount of analyte found in it, the concentration of analyte as ppm (w/w) was determined using equation 3.6:

$$Impurity\ (ppm, w/w) = \frac{m}{M} \quad 3.6$$

where, m is the mass of methanol or ethanol (in ng), and M is the total mass (in mg) of lactic acid sample loaded onto the column.

3.4.4 Representative chromatograms of lactic acid samples

Some of the representative chromatograms of lactic acid samples (HPLC analysis for carboxylic acids) are given below in Figures 3.28-3.30.

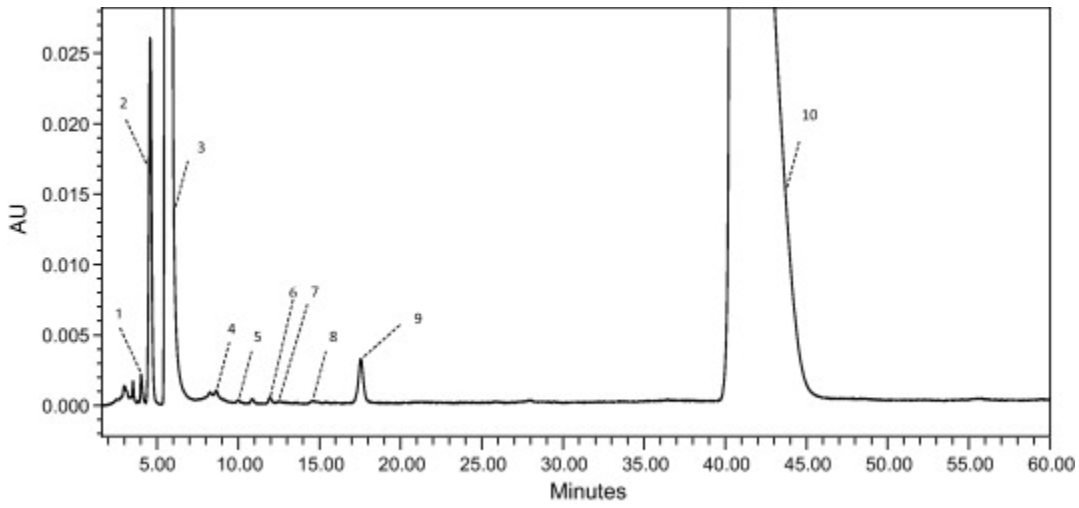


Figure 3.28: Chromatogram of lactic acid sample (commercial source 1): formic acid (1), pyruvic acid (2), lactic acid (3), citric acid (4), succinic acid (5), trans-aconitic acid (6), acrylic acid (7), propionic acid (8), linear dimer (9), cyclic dimer (10)

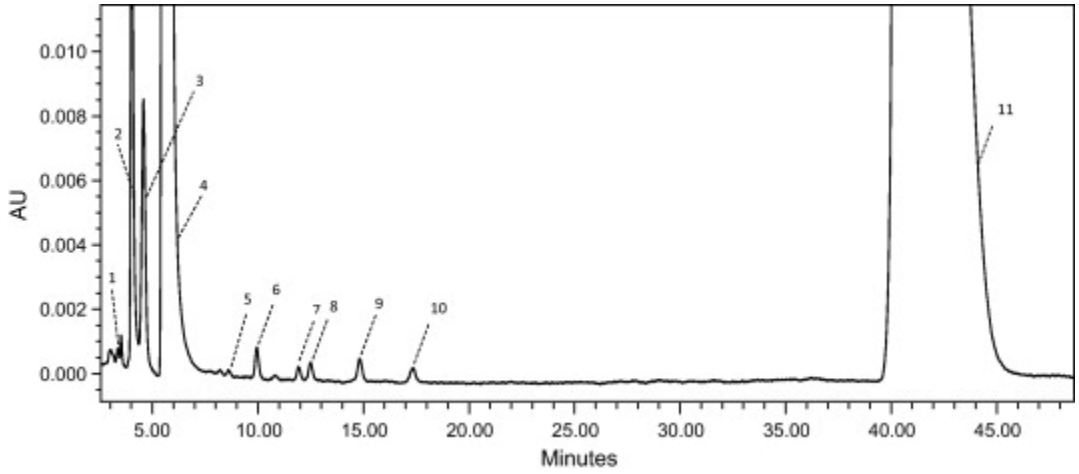


Figure 3.29: Chromatogram of lactic acid sample (commercial source 3): oxalic acid (1), formic acid (2), pyruvic acid (3), lactic acid (4), citric acid (5), succinic acid (6), *trans*-aconitic acid (7), acrylic acid (8), *cis*-aconitic acid (9), linear dimer (10), cyclic dimer (11)

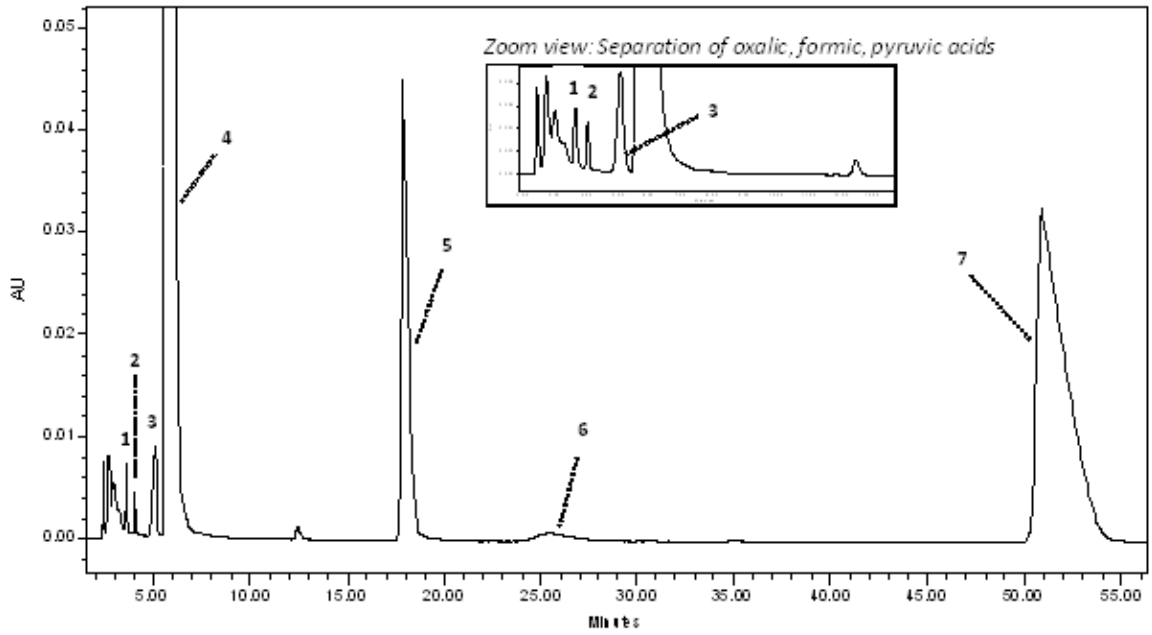


Figure 3.30: Chromatogram of in-house lactic acid sample (IH-8): oxalic acid (1), formic acid (2), pyruvic acid (3), lactic acid (4), linear dimer (5), itaconic acid (6), cyclic dimer (7)

3.4.5 Purity and impurity profile of lactic acid

The impurity profile data of in-house and commercial lactic acid samples are given in Tables 3.22 - 3.24, and Tables 3.25 and 3.26, respectively. All samples were analysed in duplicate (and some in triplicate) results of which were quite repeatable. The results reported below include average values of replicate analyses. The results of duplicate analyses of in-house samples (IH-1, 2, 3 and 4 reported in Tables 3.22 and 3.23) and triplicate analyses of lactic acid sample from Commercial source- 1 (reported in Table 3.25) are cited below to display the repeatability of measurements. The amount (mass) of identified acid was determined by using calibration slope of respective regression equation. A few unknown impurities in traces (other than 13 carboxylic acids under present study) seen in LA samples were quantified using the calibration slope of fumaric acid. The linear and cyclic dimers were not considered as impurities.

Table 3.22: Impurity profiles of in-house lactic acid samples, duplicate analyses

Impurities	**Concentration, ppm (w/w)**								
	IH-1			**IH-2**			**IH-3**		
	I	II	Mean	I	II	Mean	I	II	Mean
Oxalic acid	124.6	128.4	126.5	188.8	190.4	189.6	224.5	230.2	227.35
Formic acid	488.4	492.2	490.3	328.6	344.5	336.6	240.5	215.8	228.15
Pyruvic acid	120.8	116.8	118.8	88.6	92.2	90.4	156.3	172.5	164.4
Citric acid	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL
Fumaric acid	BDL	BDL	BDL	46.2	45.9	46.1	88.5	88.2	88.35
Succinic acid	600.8	628.5	614.65	140.5	154.1	147.3	214.3	220.1	217.2
Aconitic acid	160.4	158.3	159.35	24.7	24.9	24.8	301.6	306.5	304.05
Acrylic acid	202.4	205.4	203.9	40.2	46.2	43.2	240.2	248.7	244.45
Propionic acid	BDL	BDL	BDL	560.2	578.5	569.4	560.2	578.5	569.35
Citraconic acid	10.6	10.9	10.8	BDL	BDL	BDL	*Det.	*Det.	*Det.
Itaconic acid	20.2	21.8	21.0	BDL	BDL	BDL	201.6	218.9	210.3
Butyric acid	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL
Methanol	BDL	BDL	BDL	*Det.	*Det.	*Det.	BDL	BDL	BDL
Unknowns	320.4	318.8	319.6	24.8	25.6	25.2	24.8	25.6	25.2
Total impurities	2048.6	2081.1	2064.9	1442.6	1502.3	1472.5	2252.5	2305.0	2278.8
%Lactic acid	**99.7951**	**99.791**	**99.7935**	**99.8557**	**99.8498**	**99.8528**	**99.7748**	**99.7695**	**99.7721**

*Det.: Peak detected (i.e. analyte signal was observed between LOD and LOQ)

Table 3.23: Impurity profile of in-house lactic acid sample, IH-Batch 4, duplicate analyses

Impurities	**IH-4, Concentration, ppm (w/w)**		
	1	**II**	**Mean**
Oxalic acid	4051.6	4078.5	4065.1
Formic acid	BDL	BDL	BDL
Pyruvic acid	2039.8	2046.6	2043.2
Citric acid	47.0	42.2	44.6
Fumaric acid	0.3	0.3	0.3
Succinic acid	91.1	86.3	88.7
Aconitic acid	0.9	0.9	0.9
Acrylic acid	1.3	1.2	1.3
Propionic acid	159.2	155.6	157.4
Citraconic acid	*Det.	*Det.	*Det.
Itaconic acid	*Det.	*Det.	*Det.
Butyric acid	BDL	BDL	BDL
Methanol	*Det.	*Det.	*Det.
Unknowns	746.3	741.3	743.8
Total impurities	7137.5	7152.9	7145.3
%Lactic acid (w/w)	**99.2863**	**99.2847**	**99.2855**

*Det.: Peak detected (i.e. analyte signal observed between LOD and LOQ)

Table 3.24: Impurity profiles of in-house lactic acid samples

Impurities	**Concentration, ppm (w/w)**				
	IH-5 *(Sample Feed)*	**IH-5** *(Purified)*	**IH-6**	**IH-7**	**IH-8**
Oxalic acid	40.6	343.1	633.5	66.1	31.0
Formic acid	761.8	1442.9	470.7	509.4	452.1
Pyruvic acid	1386.9	788.4	713.9	205.5	217.8
Citric acid	BDL	BDL	BDL	BDL	BDL
Fumaric acid	BDL	BDL	1.5	BDL	BDL
Succinic acid	985.4	203.8	BDL	BDL	BDL
Aconitic acid	280.5	18.2	7.0	BDL	BDL
Acrylic acid	45.6	5.7	BDL	BDL	BDL
Propionic acid	102.9	BDL	BDL	BDL	BDL
Citraconic acid	BDL	BDL	BDL	BDL	BDL
Itaconic acid	BDL	BDL	17.8	30.4	17.1
Butyric acid	BDL	BDL	BDL	BDL	BDL
Methanol	BDL	BDL	5687	1268.9	BDL
Unknowns	312.0	392.0	67.2	23.2	1284.0
Total impurities	3915.7	3194.1	7598.6	2103.5	2002.0
%Lactic acid (w/w)	**99.6084**	**99.6806**	**99.2401**	**99.7897**	**99.7998**

Table 3.25: Impurity profile of Commercial source- 1 lactic acid sample, triplicate analyses

Impurities	**Concentration, ppm (w/w)**			
	I	**II**	**III**	**Mean**
Oxalic acid	BDL	BDL	BDL	BDL
Formic acid	477.8	449.5	455.6	461.0
Pyruvic acid	720.3	718.3	724.5	721.0
Citric acid	77.4	78.3	79.1	78.3
Fumaric acid	BDL	BDL	BDL	BDL
Succinic acid	108.6	105.4	96.5	103.5
Aconitic acid	2.1	2.4	2.1	2.2
Acrylic acid	1.3	1.2	1.7	1.4
Propionic acid	152.5	149.6	122.4	141.5
Citraconic acid	BDL	BDL	BDL	BDL
Itaconic acid	BDL	BDL	BDL	BDL
Butyric acid	BDL	BDL	BDL	BDL
Methanol	BDL	BDL	BDL	BDL
Ethanol	BDL	BDL	BDL	BDL
Unknowns	5.4	5.9	5.8	5.7
Total impurities	1529.5	1500.6	1513.6	1515.0
%Lactic acid (w/w)	**99.8471**	**99.8499**	**99.8486**	**99.8485**

Table 3.26: Impurity profiles of lactic acid samples (Commercial sources 2, 3 and 4)

Impurities	**Concentration, ppm (w/w)**		
	Source 2	**Source 3**	**Source 4**
Oxalic acid	6.3	6.3	10.8
Formic acid	52.3	4335.8	1382.6
Pyruvic acid	8.5	207.5	1739.4
Citric acid	29.5	29.5	BDL
Fumaric acid	BDL	BDL	154.8
Succinic acid	102.1	471.6	1865.2
Aconitic acid	BDL	6.2	BDL
Acrylic acid	BDL	3.2	44.6
Propionic acid	BDL	BDL	125.6
Citraconic acid	BDL	BDL	BDL
Itaconic acid	BDL	BDL	52.0
Butyric acid	BDL	BDL	BDL
Methanol	BDL	BDL	BDL
Ethanol	BDL	BDL	BDL
Unknowns	3.7	2.1	230.4
Total impurities	202.0	5062.2	5605.4
%Lactic acid (w/w)	**99.9798**	**99.4938**	**99.4395**

It was interesting to note that the lactic acid purified by reactive distillation ("IH-5, Purified") showed substantially higher concentration of oxalic acid and formic acid when compared to the feed ("IH-5, Sample feed"). The increase in formic acid concentration could be due to the conversion of lactic acid to formic acid when the former is heated under acidic condition. The increase in oxalic acid concentration can be attributed to the decomposition of aconitic acid to oxalic acid on heating.[47] The aconitic acid present in the feed ("IH-5, Sample feed", Table 3.24), was found to be reduced after purification ("IH-5, Purified", Table 3.24).

The purity of lactic acid (based on impurities of carboxylic acids and methanol) was calculated using following equation:

$$Impurity\ (ppm, w/w)\ = \frac{m}{M} \qquad 3.7$$

where, m is the mass of impurity (in ng), and M is the total mass (in mg) of lactic acid

sample loaded onto the column.

$$\%Purity\ (w/w) = \left\{100 - \sum \frac{Impurities\ (ppm)}{10000}\right\} \quad 3.8$$

where, $\sum$ *impurities (ppm)* is the sum of all the carboxylic acid impurities determined by HPLC and methanol impurity determined by GC method.

The purity results of lactic acid samples (in terms of carboxylic acids and methanol, ppm) are tabulated in Table 3.27.

Table 3.27: Percent purity results of lactic acid samples

S. No.	Lactic acid (Source)	%Purity (w/w)
1	IH-6	99.2401
2	IH-4	99.2855
3	Commercial source 4	99.4395
4	Commercial source 3	99.4938
5	IH-5, Sample feed	99.6084
6	IH-5, Purified	99.6806
7	IH-3	99.7721
8	IH-7	99.7897
9	IH-1	99.7935
10	IH-8	99.7998
11	Commercial source 1	99.8485
12	IH-2	99.8528
13	Commercial source 2	99.9798

The purity of lactic acid samples was found in the following order: Commercial Source 2 > IH-2 > Commercial Source 1 > IH-8 > IH-1 > IH-7 > IH-3 > IH-5, *Purified* > IH-5, *Sample feed* > Commercial Source 3 > Commercial Source 4 > IH-4 > IH-6.

3.5 Summary

Purity of lactic acid (in terms of carboxylic acid impurities) is important in PLA production. The analytical methods developed for impurity profiling of lactic acid give useful information (both qualitative and quantitative) on impurities of carboxylic acids, methanol and ethanol. In this work, a number of carboxylic acid impurities present in lactic acid samples were determined and their quality was compared. The HPLC method presented in this work allows the simultaneous determination of commonly observed carboxylic acids in pure lactic acid, besides the separation of PLA precursors, linear and cyclic dimers of lactic acid. The method employed polar embedded reverse phase for analysis wherein polar-embedded groups provided enhanced stability against phase collapse, which occurred when 100% aqueous phase was used with conventional reverse phase. The method validation including Gauge R&R study proved that the method generates accurate and repeatable results.

Methanol may appear as an impurity (a byproduct of hydrolysis of methyl ester of lactic acid) if not completely removed in downstream purification step. GC-FID method was developed to detect ethanol and methanol. No methanol or ethanol was detected in any of the commercial samples. Methanol was observed in a few in-house samples at trace level. GC-FID method presented in this work can be used as an in-process Quality Control (Q. C.) check to confirm absence of alcohol (ethanol or methanol) used in esterification step of reactive distillation step of downstream purification of lactic acid.

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Chapter 4

ESTIMATION OF OPTICAL PURITY OF LACTIC ACID MONOMER

4.1 Introduction

In 1780, the Swedish chemist Carl Wilhelm Scheele isolated lactic acid for the first time from sour milk. Its structure was later established by Johannes Wislicenus in 1873. Lactic acid exists in two optically active isomeric forms, as shown in Figures 4.1 and 4.2.

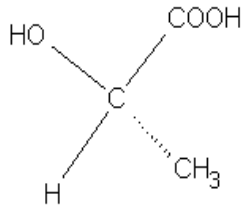


Figure 4.1: L (+) Lactic acid

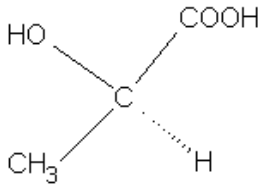
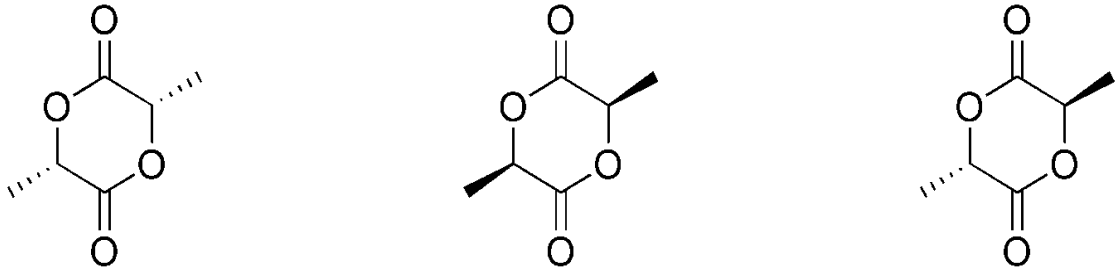


Figure 4.2: D (-) Lactic acid

As discussed earlier in Chapter 3, the chemical purity of lactic acid monomer in terms of carboxylic acid impurities is important as these impurities can retard polymerisation of lactic acid, restricting the growth of the molar mass during poly(lactic acid) (PLA) formation. Another important factor for PLA synthesis is the optical purity of lactic acid.

Lactide, the cyclic dimer of lactic acid, is used to prepare high molar mass PLA, using the ring-opening polymerisation (ROP) route, and is therefore an important intermediate in the industrial production of PLA.[1-12] Because of the chiral nature of lactic acid, lactide exists in three different forms as L-lactide, D-lactide, and *meso*-lactide, as shown in Figure 4.3. A 50:50 mixture of L- and D-lactide is referred to as racemic lactide.

The optical purity of lactide significantly affects the physical properties of PLA produced by ROP. PLA is a thermoplastic polymer that is typically formed into the desired shape by melt processing above its melting temperature (Tm).



L-lactide D-lactide *meso*-lactide

Figure 4.3: Three optical isomers of lactide

The melting point and the glass transition temperature (Tg) of PLA are strongly dependent on molar mass and optical purity of the polymer, among other factors.[13] In order to prepare PLA with desirable physical properties, metal alkoxide catalysts are used for ring-opening polymerisation (ROP) of lactide. When dilactide is prepared from racemic lactic acid, the three isomers that result are D-lactide, L-lactide and meso-lactide. The meso isomer can be removed, but D and L-lactide are enantiomers that comprise the racemic form, rac-lactide. When rac-lactide is polymerised with simple catalysts, an amorphous polymer results from an essentially random incorporation of D- and L- lactide units in the growing chain. The racemic polymer is not crystalline, has very low Tg and hence, not suitable for most applications. At present, commercial processes utilise L-lactide produced from L-lactic acid.

Enantiomeric impurities present in lactide might come from lactic acid. Therefore, besides chemical purity, the optical purity of lactic acid is also crucial as traces of enantiomeric impurities drastically change crystallinity or biodegradation rate of the polymer;[14] for example, it is L (+) lactic acid that can be polymerised to a highly-crystalline PLA suitable for commercial uses such as fibres and films.[15]

The various methods used to estimate enantiomeric purity of chiral compounds are given below.

4.1.1 Optical rotation

This classical method involves measurement of specific optical rotation. The optical purity can be determined by using following equation:

$$Optical\ purity = \frac{[\propto]}{[\propto_0]} \text{ x } 100 \qquad 4.1$$

where, [α] is the specific optical rotation of the mixture and [α_0] is the specific optical rotation of the pure enantiomer. The enantiomeric excess (%ee) can be calculated using equation 4.2 given below:

$$Enantiomeric\ excess\ (\%ee) = \frac{[R_{isomer} - S_{isomer}]}{[R_{isomer} + S_{isomer}]} \text{ x } 100 \qquad 4.2$$

where, R and S are the relative proportions (ratio) of the two enantiomers. In practice, this may often lead to some confusion, since the optical rotation is dependent upon various conditions of measurements such as solvents, temperature, purity, etc. and ambiguity may exist if the compound is new or not well documented in literature.

4.1.2 Nuclear magnetic resonance (NMR)

NMR spectroscopy techniques can determine the absolute configuration of stereoisomers such as *cis* or *trans* alkenes, *R* or *S* enantiomers, and *R*,*R* or *R*,*S* diastereomers.[16] In a mixture of enantiomers, these methods can help quantify optical purity by integrating the area under NMR peak corresponding to each stereoisomer. Accuracy of integration can be improved by inserting a chiral derivatising agent with a nucleus other than hydrogen or carbon, then reading the heteronuclear NMR spectrum: for example fluorine-19 NMR or phosphorus-31 NMR. Mosher's acid contains a -CF_3

group, so if the adduct has no other fluorine atoms, the fluorine-19 NMR of a racemic mixture shows just two peaks, one for each stereoisomer. As with NMR spectroscopy in general, good resolution requires a high signal-to-noise ratio, clear separation between peaks for each stereoisomer and narrow line width for each peak. Chiral lanthanide shift reagents cause a clear separation of chemical shift, but they must be used in low concentrations to avoid line broadening.

4.1.3 Gas-liquid chromatography (GLC)

GLC or gas chromatography (GC) technique, employing chiral stationary phase, is an accurate and reliable method for enantiomeric purity determination. Chiral separations using GC are mainly performed on chiral stationary phases (CSPs) capable of hydrogen bonding, coordination and inclusion, such as amino-acid derivatives, terpene-derived metal-coordination compounds and modified cyclodextrins, respectively. In Chirasil-type stationary phases, the chiral selector is anchored to a polysiloxane backbone, thus improving GC performance. Although GC is a powerful tool, it can only be used for compounds that can be readily vapourised without decomposition.

4.1.4 High performance liquid chromatography (HPLC)

HPLC offers direct separation and analysis of enantiomers. In general, it is more versatile than chiral GC method, because it can be used to separate enantiomers of a wide variety of non-volatile compounds. Current chiral HPLC methods are either direct, which utilise chiral stationary phases (CSPs) and chiral additives in the mobile phase, or indirect, which involves derivatisation of sample.

Several other methods based on techniques such as fluorescence, UV-Visible, calorimetry, and TLC have been used[17-28] for accomplishing chiral separations. Most of

these procedures require chiral hosts created through extensive synthetic efforts. Besides this, many require that the analyte be derivatised before the analysis can be preformed.

All the methods described above are useful for the determination of enantiomeric purity depending on suitability and accuracy of chiral separation; however, HPLC and GC are more powerful tools which serve as efficient and robust analysis methods with appreciable sensitivity. The choice of GC and/or HPLC is a must where precise determination of enantiomeric compositions is important and critical.

HPLC can be used to separate enantiomers either indirectly with chiral derivatisation reagents or directly with chiral stationary phases or chiral mobile phase additives. Each of these techniques has advantages and disadvantages. Indirect separation is based on the use of chiral derivatisation reagents to form diastereomeric derivatives which differ in their chemical and physical behaviour and therefore, can be separated on achiral stationary phases. This approach circumvents the need for expensive columns with chiral stationary phases and is more flexible; however, derivatisation represents an additional step which can involve undesirable side reactions, formation of decomposition products and racemisation. Furthermore, the chiral derivatisation reagent has to be of high enantiomeric purity and the presence of derivatisable groups in the analyte is a prerequisite. A direct approach, using columns with chiral stationary phases, is more convenient and also applicable for separations on preparative scale, but requires a collection of expensive columns to solve a variety of problems. The chiral mobile phase approach represents a simple and flexible alternative, which is, however, not always applicable. Since the mobile phase containing the chiral selector cannot be reused, this technique cannot be applied with expensive reagents.

In the present work, the HPLC method based on chiral ligand-exchange chromatography (CLEC) was developed to determine the optical purity of lactic acid monomer. The method was applied to analyse a rich variety of lactic acid samples.

4.2 Experimental

4.2.1 Materials

Lactic acid samples were procured from Purac (Lincolnshire, IL, USA), Aldrich (St. Louis, MO, USA), Merck (Mumbai, India), Pratishtha Chemicals (Mumbai, India), Lactochem (Mumbai, India), SDFL (Mumbai, India) and Godavari Sugar Mills (Sameerwadi, India). D, L-lactide sourced from Aldrich (St. Louis, MO, USA) was used as a standard for matching the retention times of enantiomers of lactic acid. Copper (II) sulphate pentahydrate of AR grade was purchased from Merck (Mumbai, India). Ultra pure water, obtained from Milli-Q System, Millipore (Milford, MA, USA), was used for preparing mobile phase. Lactic acid samples were also synthesised in the laboratory and designated as "in-house (IH)" samples. Synthesis and purification of in-house samples (IH-1, IH-2, IH-3, IH-4, IH-5: *LA feed for reactive distillation,* IH-5: *LA purified after reactive distillation,* IH-6, IH-7 and IH-8) was described in Section 3.2.1.2.

4.2.2 Preparation of 50:50 mixture of L (+) and D (-) lactic acid standard

The standard solution of 50:50 mixture of L (+) and D (-) lactic acid was prepared by hydrolysis of DL-lactide (racemic). For this, 25 mg of DL-lactide was dissolved in aqueous mobile phase (0.5 mM of copper sulphate pentahydrate) taken in a 50 mL reaction flask. The reaction flask was attached with an air condenser and the reaction mixture was refluxed for 3 to 4 hours. After hydrolysis, the reaction mixture was cooled to room temperature. The condenser was rinsed with mobile phase and the

washings were collected in a reaction flask. The contents of the flask were transferred to a 25 mL volumetric flask (Class "A" type) and diluted up to the mark with mobile phase. This produced a standard test solution of hydrolysed DL-lactide with concentration equal to 1000 ppm (w/v). This solution, in principle, contained 500 ppm (w/v) each of L (+) and D (-) and lactic acid enantiomers. It was confirmed that the racemisation of lactide did not occur during hydrolysis; and as a result, the D- and L-lactate units in lactide were quantitatively converted into D- and L-lactic acids. This standard test solution was used to identify and quantify L (+) and D (-) enantiomers. It was filtered through 0.2 µm Nylon membrane filter disc prior to using it for analysis.

4.2.3 Preparation of L (+) lactic acid standard

An amount of 25 mg of L (+) lactic acid (Aldrich) taken in a 25 mL volumetric flask was dissolved in mobile phase and diluted up to the mark. This produced the standard test solution of 100% L (+) lactic acid with concentration equal to 1000 ppm (w/v). This solution was filtered through 0.2 µm Nylon membrane filter disc prior to using it for analysis.

4.2.4 Preparation of lactic acid samples

Lactic acid samples were prepared by dissolving the requisite amount in requisite volume of mobile phase to obtain 1000 ppm (w/v) solution. All samples were filtered through 0.2 µm Nylon membrane filter and transferred to autosampler vials for automatic injection.

4.2.5 Equipment and parameters of analysis

The optical purity analysis was carried out on HPLC system (Waters Corporation, Milford, MO, USA) equipped with a 515 binary HPLC pump, a 717 plus

auto sampler and a 996 photodiode array (PDA) detector. The column temperature was maintained at 60 ^{o}C with the help of a column oven controller (HCO-02, PCI Services, Mumbai, India). The mobile phase was degassed by an ultrasonic bath (Transsonic T660/H, Elma Hans Schmidbauer GmbH & Co., Singen, Germany) and an on-line vacuum degasser. The photodiode array (PDA) detector was set to scan 200 - 300 nm wavelength regions at 1.0 nm resolution and the chromatograms were extracted from 240 nm. The sampling period was kept as 15 ms. Data acquisition and peak integration were performed with the help of Millenium 32® software from Waters Corporation, USA.

Nucleosil Chiral–1, EC 250/4, column (250 x 4.0 mm I.D., 5 µm particle size, Machery–Nagel, Germany) was employed for achieving chiral separations. A fresh mobile phase was prepared daily in ultrapure (HPLC grade) water and filtered through 0.2 µm Nylon membrane filter (Whatman, Inc., Sanford, USA) before using it for chromatographic elution. The samples were injected with a sample volume of 20.0 µL. The 17 lactic acid samples analysed are presented in Table 4.1

Table 4.1: Description of lactic acid samples analysed

S. No.	Source (Lactic acid)
(A) Commercial samples	
1	*Source-1
2	Source-2
3	Source-3
4	Source-4
5	Source-5
6	Source-6
7	Source-7
8	Source-8
(B) In-house samples	
9	IH-1
10	IH-2
11	IH-3
12	IH-4
13	IH-5 (feed)**
14	IH-5 (Purified)***
15	IH-6
16	IH-7
17	IH-8

***** Hydrolysed DL-lactide sample (Aldrich)
IH: Lactic acid batches synthesised at our laboratory (in-house samples)
** Lactic acid feed sample from reactive distillation purification step
*** Lactic acid purified sample after reactive distillation purification step

4.3 Results and Discussion

4.3.1 Method development and optimisation

In the present work, HPLC technique based on chiral ligand exchange chromatography (CLEC) was developed to accomplish the enantiomeric separation of lactic acid. CLEC technique has been proven to be very useful to separate enantiomers of barbiturates, α-amino acids, dansyl amino acids, hydroxy acids, peptides, amino alcohols, alkaloids, β-blockers and other adrenergic drugs.[29-31]

Basic principle of ligand exchange[32] is the involvement of a complexing metal ion into interaction between the analyte enantiomers to be resolved and the chiral selector, through the formation of diastereomeric ternary complex as "selector/metal-

ion/analyte". It is essential that the complexes be kinetically labile, i.e. they must form and dissociate at a high rate; otherwise the chromatographic column efficiency would be compromised. Complexes of Cu (II), Zn (II), Ni (II), and a few other ions meet this condition while coordinating amino, carboxy, hydroxy, amido, thio, and a few other electron donating functional groups. Herewith, the lone pairs of electrons from hetero atoms (N, O, S) of functional groups, belonging to the analyte and selector, occupy definite positions in the coordination sphere of central metal ion, to result in the formation of ternary complex. During chromatography process, the coordinated ligands are reversibly replaced by other ligands, such as molecules of water, ammonia, or other components of the eluent. Quick exchange of ligands in the metal ion coordination sphere dictates the name of the technique as 'ligand-exchange chromatography' (LEC).

4.3.1.1 Selection of stationary phase

Enantiomers, as is well known, have identical physico-chemical properties until placed in an environment which itself is chiral. Therefore, they must have equal retention on interaction with any achiral stationary phase and cannot be separated in achiral chromatographic systems. To create a chiral environment, one has to add an additional chiral component to the chromatographic system, the so-called chiral selector. The chiral selector may be present in the mobile phase (chiral mobile-phase mode, CMP) or in the stationary phase (chiral stationary-phase mode, CSP). However, in general, the chiral selector is present in both phases and chiral recognition occurs simultaneously in the mobile and stationary phases.

CLEC stationary phases are prepared by grafting or physically adsorbing an optically pure moiety onto a achiral chromatographic matrix. Such grafted ligand

exchange phases are used for enantiomeric separations that include immobilised derivatives of L-hydroxyproline or L-proline. An example of physi-sorbed CLEC stationary phase includes the use of histidine-based chiral selector (N^{τ}-*n*-decyl-L-histidine, LNDH) onto a C_{10} reversed phase matrix, which is used for separating most α-amino acid racemates.[33]

In the present work, Nucleosil Chiral–1 column (250 x 4.0 mm I.D., 5µm particle size, Machery–Nagel, Germany) was selected. This offered an appropriate stationary phase ligand for enantiomeric separation of lactic acid. The chiral column used here employs L-hydroxy proline as the immobilised chiral selector for separation of a racemic mixture of lactic acid, as shown in Figure 4.4.

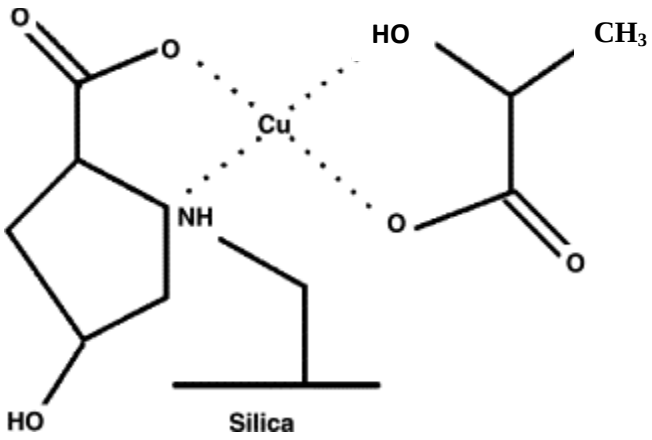


Figure 4.4: Structure of the immobilised L-hydroxyproline ligand bound to the stationary phase of the Nucleosil Chiral-1 column

The stationary phase is composed of silica (particle size 5 µm, pore size 120 Å as base material with chiral selector as *L*-hydroxyproline-Cu^{2+} complexes.

4.3.1.2 Optimisation of chromatographic parameters

Proper selection of the ligand and column operating conditions (*i.e.*, temperature, flow rate, pH, solvent composition, etc.) can have a significant impact on resolution of enantiomers. Cu (II) was selected from the complexes of Cu (II), Zn (II), Ni (II), and Co (II), as it works better. Along with selection of Nucleosil Chiral-1 column, copper ions were included in the mobile phase to ensure that there was no loss of copper. The lactic acid enantiomers were separated by forming diastereomeric copper complexes. Here, water stabilises the complex by coordinating in an axial position. Steric factors then determine which of the two complexes is most stable; one of the water molecules is usually sterically hindered from coordinating with copper. In order to achieve adequate chiral separation, the concentration of copper (II) sulphate pentahydrate in mobile phase was optimised to 0.5 mM. The column temperature was optimised to 60 °C, which gave the best resolution at the given conditions of the chromatographic system. Temperature has a strong effect on retention and selectivity. It was observed that when column temperature was raised from room temperature to 60 ^{o}C, sharp peaks were eluted resulting in shorter retention times and hence, better resolution. However, resolution was lowered when temperature was raised further. In general, an increase in temperature decreases the viscosity of the mobile phase, increasing the diffusion coefficient of the analyte in the mobile phase and liquid within the pore of the stationary phase, and thereby improving the separation performance through what is collectively termed the *kinetic effect*. Raising the column temperature from ambient condition to 60 °C enhances pore diffusion and film mass transfer of the analyte by only a small amount. Instead, changes in separation performance with temperature correlate more closely with changes in

chemical equilibria. The pH of mobile phase is one of the parameters that drives resolution; however, it showed adequate resolution of peaks when mobile phase was used without doing any pH adjustment. The flow rate of mobile phase was optimised to 0.8 mL/min with isocratic elution and the detection wavelength was set at 240 nm. The column was equilibrated with mobile phase for at least 2 hours to get a steady baseline before running the test standards and samples on it.

4.3.2 Method validation

Method validation was accomplished by evaluating system precision (assessment of repeatability of retention time and peak area) for which the replicate injections (*n = 10*) of a test standard of 50:50 mixture of L (+) and D (-) lactic acid were made into HPLC system under optimised chromatographic conditions. The data on retention time and peak area were collected and standard deviation (SD) and %RSD were calculated.

Table 4.2: Repeatability of retention times

Run no.	Retention time (min)	
	L(+) isomer	D (-) isomer
1	5.960	6.982
2	5.994	6.856
3	6.005	6.882
4	5.986	6.788
5	5.950	6.789
6	5.949	6.964
7	6.146	6.991
8	5.956	6.888
9	6.012	6.985
10	6.011	6.924
Mean	5.997	6.905
SD	0.061	0.082
%RSD	1.0	1.2

The %RSD ≤ 1.2 (Table 4.2) for retention time and %RSD ≤ 2.5 (Table 4.3) for peak area indicated good system precision leading to an unambiguous identification and accurate determination of enantiomers.

Table 4.3: Repeatability of peak area

Run no.	Peak area (Counts)	
	L(+) isomer	**D (-) isomer**
1	1607489	1610422
2	1692456	1689564
3	1756478	1749856
4	1724567	1719562
5	1709880	1714587
6	1749812	1749018
7	1753331	1748397
8	1729452	1736102
9	1699864	1700104
10	1711428	1724562
Mean	1713476	1714217
SD	43479	39805
%RSD	2.5	2.3

It is well known that optical isomers or enantiomers differ in their three-dimensional arrangements of atoms or groups, but have the same sequence of atoms and bonds (i.e. same chemical formula). Accordingly, both the enantiomers of lactic acid show identical response to UV detector. Percent optical purity of lactic acid, in present analysis, therefore, can simply be calculated by area normalisation method using following equation:

$$\%\ Optical\ purity = \left[\frac{A_x}{(A_x + A_y)}\right] \text{x } 100 \qquad 4.3$$

where, A_x is the peak area counts of L(+) lactic acid and A_y is the peak area counts of D(-) lactic acid.

Area normalisation method fulfills the quantitation purpose with the advantage of similar detector responses of enantiomers. Evaluation of repeatability of retention time and peak area thus establishes the method precision and essentially validates the method.

4.3.3 Optical purity of lactic acid

All the lactic acid samples were run on HPLC system under optimised chromatographic conditions and their percent purity values were determined using equation 4.3. The purity results of samples are given in Table 4.4, and the representative chromatograms are given in Figures 4.5 – 4.7.

Table 4.4: Optical purity results

S. No.	**Source (Lactic acid)**	L (+) enantiomer (%)
(A) Commercial samples		
1	Source-1*	50.0
2	Source-2	100.0
3	Source-3	100.0
4	Source-4	100.0
5	Source-5	100.0
6	Source-6	100.0
7	Source-7	100.0
8	Source-8	99.9
(B) In-house samples		
9	IH-1	100.0
10	IH-2	100.0
11	IH-3	100.0
12	IH-4	100.0
13	IH-5 (feed)**	100.0
14	IH-5 (Purified)***	100.0
15	IH-6	100.0
16	IH-7	100.0
17	IH-8	100.0

***** Hydrolysed DL-lactide sample (Aldrich)
IH: Lactic acid batches synthesised at our laboratory (in-house samples)
** Lactic acid feed sample from reactive distillation purification step
*** Lactic acid purified sample after reactive distillation purification step

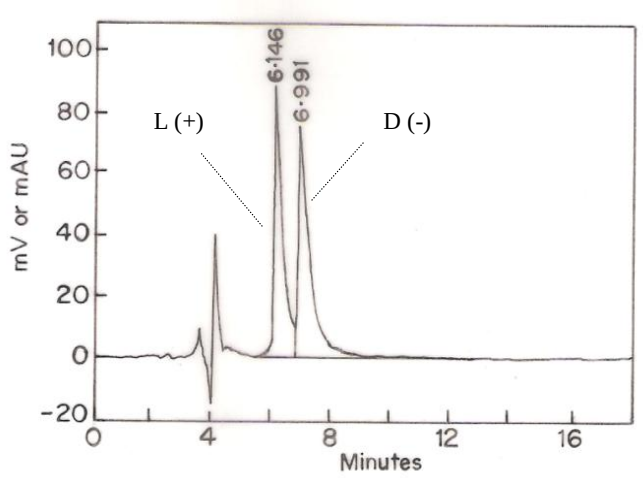


Figure 4.5: Chromatogram of 50:50 mixture of L (+) and D (-) lactic acid

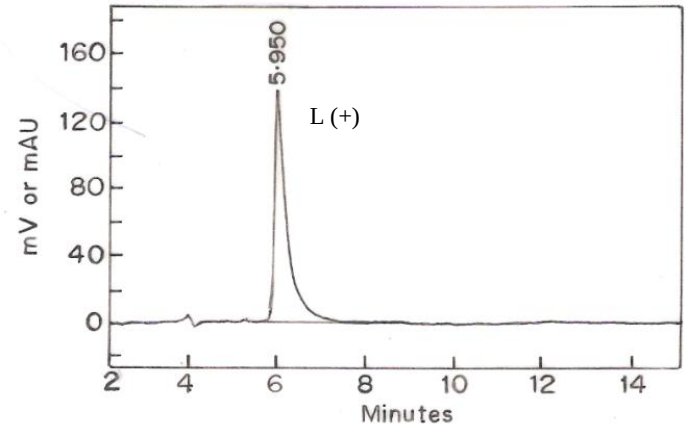


Figure 4.6: Chromatogram of L (+) lactic acid (Aldrich standard)

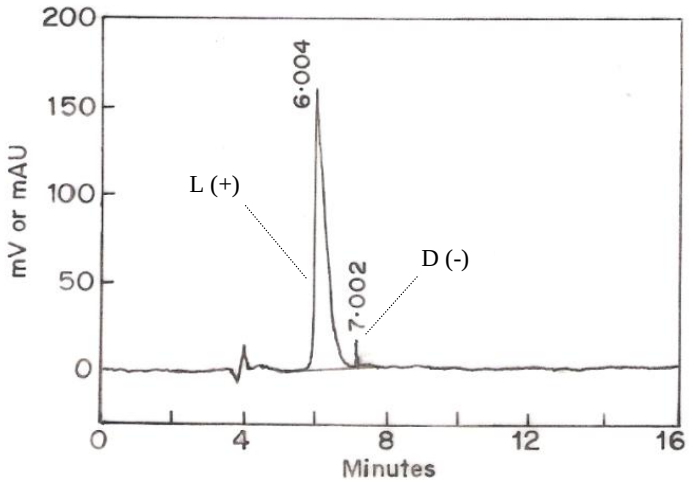


Figure 4.7: Chromatogram of lactic acid (Source-8 sample)

4.4 Summary

Optical purity of lactic acid is a crucial factor in deciding physical properties of PLA. The small amounts of enantiomeric impurities drastically change properties such as crystallinity or biodegradation rate of the polymer. L(+) lactic acid is required for production of PLA which is suitable for most of the applications. Therefore, besides evaluation of chemical purity, estimation of optical purity of lactic acid is also equally important. Analytical method based on chiral-ligand exchange chromatography (CLEC) was developed which determines optical purity of lactic acid. Method was validated by investigating system precision (assessment of repeatability of retention times and peak area). The method is most reliable and serves as an inexpensive chiral HPLC technique due to the simplicity of the procedure and extremely high enantioselectivity of complexation. Method is fast (total run time < 9 min) and produces accurate results. The method was applied to estimate the optical purity of lactic acid samples synthesised in our laboratory, as well as those obtained from other sources. All samples were found to be pure (i.e. containing 100% L (+) enantiomer) except Source-8 sample, which was 99.9% pure (i.e. containing 0.1% impurity of D (-) isomer).

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Chapter 5

ESTIMATION OF MONOMERS IN ACRYLIC HIPEs

5.1 Part A: Estimation of EHA, EGDMA and EHMA in HIPEs

5.1.1 Introduction

5.1.1.1 High internal phase emulsion

High internal-phase emulsion (HIPE) is defined as an emulsion in which the dispersed phase occupies more than 74% of the volume, the maximum packing fraction of monodispersed spheres. The continuous phase, which generally constitutes less than 26% of the final volume, can comprise of monomers and crosslinking comonomers. Lissant[1-3] was one of the first to describe the structure and properties of the non-Newtonian, thixotropic, Bingham fluids that were termed HIPEs (or high internal phase ratio emulsions, HIPREs). These emulsions have also been referred to as concentrated emulsions,[4-6] gel emulsions,[7-9] and hydrocarbon gels.[10]

HIPEs are formed by mixing two immiscible liquids in the presence of an emulsifier, usually a surfactant. One of the liquids is almost always an aqueous solution and the other is usually hydrophobic. The major phase is usually added slowly, under constant agitation, to a solution consisting of the minor phase and the surfactant. HIPEs can also be formed by applying a centrifugal force to an emulsion.[11-12]

5.1.1.2 PolyHIPE

The HIPE can be cured by thermal, UV, and redox methods or by addition of a catalyst to produce poly(HIPE) or foam.[13] PolyHIPE is a microporous material produced by the polymerisation of monomers in the continuous phase of HIPE. Polymerisation of continuous monomer phase of HIPE around emulsion droplets is followed by the removal of dispersed phase, and a product of highly porous low density polymeric material is obtained. During polymerisation process, the emulsion acts as a template for resulting

polymeric foam and interconnecting windows form in the contact points of emulsion droplets entrapped in the HIPE structure. This emulsion templating technique can form foams that have porosities in the range of 75–99%, pore sizes of 5–100 μm and open or closed type cellular nature depending on the emulsion composition.[14] The formation of HIPE to produce PolyHIPE is illustrated in Figure 5.1. A typical SEM micrograph of PolyHIPE foam is shown in Figure 5.2.

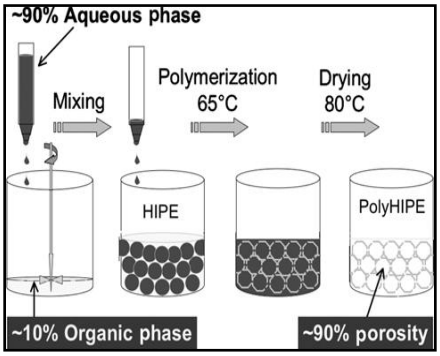

Figure 5.1: Schematic illustration of PolyHIPE formation

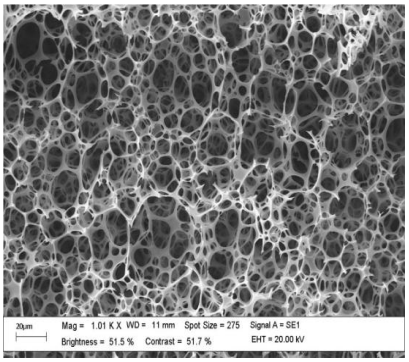

Figure 5.2: A typical SEM micrograph of PolyHIPE foam

Because of their unique cellular architecture, these materials have been used as membrane filters,[15] ion exchange resins,[16] supports for cells,[17-18] and catalytic systems in industry.[19] They are also ideal candidates for chromatographic applications.

The extensive research and development on PolyHIPE has been described in several reviews.[20-22] Making PolyHIPE using HIPE methodology[20-22] has gained considerable commercial interest in recent years. One of the important commercial applications of PolyHIPE foams is manufacturing of functional absorbent materials (FAM). These highly porous foams have the ability of acquiring, distributing, and rapidly desorbing fluids. It is for these attributes they find applications in disposable diapers, adult incontinence pads and briefs, and sanitary napkins.[23] Functional absorbent polymers

are obtained from lightly crosslinked polyacrylates. The selection of monomers for producing such materials are (1) a monofunctional monomer whose atactic amorphous polymer has a Tg of about 25 ºC, or lower,[24] (2) a second monofunctional co-monomer (e.g., styrene, ethyl styrene, methyl methacrylate, etc.) to improve the material toughness, and (3) a polyfunctional crosslinking agent (e.g. divinylbenzenes, divinylxylenes; or acrylate and methacrylate crosslinking agents derived from diols, triols and tetraols, etc.).[23]

In the present work, acrylic HIPE system comprising of monomers viz. 2-ethylhexyl acrylate (EHA), ethylene dimethacrylate (EGDMA) and 2-ethylhexyl methacrylate (EHMA) were chosen which fit the above mentioned criteria. The present work was aimed to develop an analytical methodology to estimate monomer conversions for this acrylic HIPE system.

With increasing competition in the chemical industry, the manufacturers are compelled to find ways to reduce the cost of production without compromising the quality of product. In this context, study of rate of monomer conversions (reaction kinetics) with varying reaction parameters is important for optimising the process parameters, since it has a significant impact on plant operability and economics. In order to study the polymerisation reaction kinetics for the present acrylic HIPE system, it was necessitated to develop an analytical method which can estimate time-dependent monomer conversions by analysis of unreacted monomers (EHA, EGDMA and EHMA).

In order to generate data on monomer conversions, individual HIPEs (containing EHA, EGDMA and EHMA) were polymerised for incremental curing times, with a particular set of reaction parameters, and the monomer conversions at a particular

curing time were calculated by determining unreacted monomers from HIPE reaction mixture cured for that particular curing time.

Determination of EHA, EGDMA and EHMA in HIPE was a difficult problem, because for quantitative measurement of unreacted monomers, breaking of intrinsically extremely stable HIPE (demulsification) was a prerequisite. After demulsification, monomers are released from HIPE matrix, which then have to be efficiently extracted in a suitable solvent and further subjected to gas chromatographic analysis. The method for selective extraction of monomers from HIPE was therefore developed wherein cyclohexane was identified as extraction solvent, which showed excellent selectivity for hydrophobic monomers under present study. The monomer extraction efficiency was assessed by determining recovery (97.3-104.9%), which was appropriate for the present work.

EHA, EGDMA and EHMA are volatile compounds which make them amenable to gas chromatographic (GC) analysis; secondly, due to their long chain alkane backbone, they show appreciable sensitivity towards flame ionisation detector (FID). The selection of analytical method based on GC-FID was therefore the right choice for the present problem. The chromatographic parameters were optimised to achieve complete baseline separation of analytes and avoid interference of components from sample matrix.

Method validation was carried out by studying various parameters such as system precision (assessment of repeatability of retention time and peak area), specificity, linearity (calibration studies), limit of detection (LOD), limit of quantification (LOQ) and 'method precision and accuracy'. The Gauge R & R study was conducted which indicated that the measurement system was stable and acceptable. All these studies

established the efficiency of method and ensured that the method was valid for its intended purpose.

After an analytical method was developed and validated thoroughly, its usefulness was demonstrated by estimating time-dependent monomer conversions in HIPE polymerisation reactions under various reaction parameters, as an example of method application.

5.1.1.3 Scope of the study

There is no analytical method reported in literature for the simultaneous extraction of EHA, EGDMA and EHMA from water-in-oil (W/O) HIPE matrix followed by their gas chromatographic measurement in presence of each other. Therefore, the work was undertaken to develop a simple and reliable analytical method, which can accurately measure EHA, EGDMA and EHMA as unreacted monomers in HIPE polymerisation reactions.

HIPE free radical polymerisation lies between suspension and emulsion methodologies. The kinetics of the HIPE polymerisation has remained relatively unexplored. The analytical methodology explained in this work is useful to derive the rates of HIPE polymerisation which can be further useful to create a phenomenological model for analysing the kinetics of polymerisation. The optimisation algorithms can be utilised to calculate optimal operating conditions for a particular polymerisation process which will eventually help improve polymer plant operability and economics.

5.1.2 Experimental

5.1.2.1 Materials

The analytical standards of monomers viz. 2-ethylhexyl acrylate (EHA), ethylene dimethacrylate (EGDMA), 2-ethylhexyl methacrylate (EHMA) and an internal standard, butyl methacrylate (BMA), had purity equal to 98% and were procured from Aldrich Chemicals (St. Louis, MO, USA). Cyclohexane of AR grade (purity 99%), used as an extraction solvent, was from Rankem Chemicals (Mumbai, India). Hydrogen gas (purity > 99.99%) was from Inox Ltd. (Mumbai, India). Nitrogen and dry air were supplied from Nitrogen-Air generator (Domnik Hunter, North Carolina 28269, USA). Anhydrous magnesium sulphate of analytical grade was from Loba Chemie (Mumbai, India). It was activated by heating at 600 ºC for 6 hours in an oven and kept in desiccators before use. The surfactants, di(hydrogenated tallow alkyl) dimethyl ammonium chloride (Arquad 2HT-75) was from Aldrich Chemicals (St. Louis, MO, USA) and sorbitan monooleate (Span 80) was purchased from Loba Chemie (Mumbai, India). The thermal polymerisation initiator, sodium persulphate (NaPS) of analytical grade was from Loba Chemie (Mumbai, India). Fused calcium chloride (assay ≥ 98%), used as an electrolyte, was from Merck (Mumbai, India). The inhibitors, monomethyl ether hydroquinone (MEHQ) and phenothiazine (purity ≥ 98%) were from Aldrich Chemicals (St. Louis, MO, USA).

5.1.2.2 Preparation of monomer standards

The internal standard (IS) solution with concentration equal to 2.5 mg mL^{-1} (i.e. 2500 ppm, w/v) was prepared by dissolving 5000.0 mg of butyl methacrylate in 2 L of cyclohexane. This IS solution was stored in the refrigerator at 4 ºC when not in use and

was found to remain stable for more than two weeks. For calibration studies, 10 nos. of analytical standards each containing a mixture of EHA, EGDMA, and EHMA were prepared by dissolving requisite amounts of monomers in 25 mL of IS solution taken in a Class "A" volumetric flask. The concentration range for individual monomers was chosen by taking into consideration a batch size of 0.925 g (comprising 370.0 mg of EHA, 175.0 mg of EGDMA, and 380.0 mg of EHMA) and a total volume of extraction solvent (equal to 100 mL). The concentrations of calibration standards were prepared within a given range such that they will cover the levels of monomers in concordance with their conversion from zero (i.e. no conversion at curing start time) to a maximum of 98%.

5.1.2.3 Preparation of HIPE reaction samples

(A) Preparation of HIPE and curing

HIPE was prepared with a volume to weight ratio of aqueous to oil phase of 27:1. A stock solution of organic phase was prepared in bulk such that 1 mL of it contained 0.370 g (2.0078 mM) of EHA, 0.175 g (8.8829 mM) of EGDMA, 0.380 g (1.9162 mM) of EHMA and a mixture of surfactants: 0.065 g (7.03% of oil phase) of Span-80 and 0.008 g (0.86% of oil phase) of Arquad 2HT-75. The individual HIPE samples were prepared by dispensing 1 mL of organic phase from stock solution and transferring in a glass reactor flask stirred with Ruston turbine stirrer. An aqueous phase containing 4% calcium chloride was added drop wise to this mixture under continuous stirring at 1400 rpm. The resultant emulsion formed by this method is called as water-in-oil (W/O) high internal phase emulsion (HIPE). An accurately measured 1 mL of aqueous sodium persulphate (1.35%, w/v), taken in a double walled dropping funnel, was then

added to HIPE, thereby making the concentration of sodium persulphate in total aqueous phase equal to 0.05%, w/v. This concentration of initiator was considered as normal or standard initiator concentration and designated as "X". The temperature of the circulating water in the outer jacket of the dropping funnel was maintained at which HIPE was to be cured. The time at which the addition of initiator completed was noted as curing start time. The reactor flask was then immediately kept in a water bath for curing at 85 ºC.

The same procedure was followed for experiments wherein HIPEs were to be cured in presence of inhibitors (MEHQ or phenothiazine) for which requisite amount of inhibitor was mixed with the oil phase. The amount of inhibitor equal to 1 mol% of initiator was designated as "Y". Thus, multiple experiments were conducted in which HIPEs were cured at varying initiator concentrations from 1X to 4X, and varying inhibitor concentrations from 0.5Y to 4Y at a constant curing temperature (85 ºC). The individual HIPEs were cured for incremental curing times so as to calculate the time-dependent monomer conversions.

(B) HIPE reaction quenching and monomer extraction

After curing a HIPE for a known duration, the reaction was immediately quenched in liquid nitrogen for 5 min. Further, in order to avoid post-polymerisation, MEHQ (twice the moles of initiator used) dissolved in 1 mL of acetone, was added into HIPE reaction mixture. The curing time was measured with a stop watch. A 100 mL of cyclohexane (containing IS) was then added to HIPE reaction mass for extracting monomers. The reaction mixture was shaken manually for a few minutes and kept overnight for extraction of unreacted monomers. After extraction, the reactor flask was sonicated for 5-10 min to release the traces of monomers that were probably trapped in

porous polymer matrix. Further, approximately 10 mL of an aliquot from upper organic layer was removed and placed in a sampling bottle to which anhydrous magnesium sulphate (1-2 g) was added to capture the traces of water, if present. After magnesium sulphate settled down, approximately 1.5 mL of this solution was transferred to a GC autosampler vial for injecting 1.0 μL of sample solution into GC system for measurement of unreacted monomers.

5.1.2.4 Equipment and parameters of analysis

All the chromatographic analyses were performed on Shimadzu GC-2010 Chromatograph (Shimadzu Corporation, Kyoto, Japan), equipped with a flame ionisation detector. The GC separation was carried out on a DB-1 capillary column (J & W Scientific, Folsom, CA, USA) with a dimension of 100 m (L) × 0.25 mm (i.d.) and a film thickness of 0.5 μm. The temperature program was optimised for baseline separation of EHA, EGDMA and EHMA including internal standard, BMA, used in analysis. The operating conditions were set as follows:

Nitrogen was used as carrier gas with flow rate of 1.59 mL/min. Hydrogen and zero air flow were maintained at 40 and 400 mL/min, respectively. The temperature program was set to start from 150 ºC and increased to 210 ºC, with a ramp rate of 5 ºC/min, and held there for 3 min, thus making total chromatographic run time equal to 10 min. The injector port (PTV1) and detector (FID) temperature were kept at 275 and 300 ºC, respectively. The samples (1.0 μL) were injected using HTA autosampler with the split injection mode having split ratio of 25:1. De-activated glass wool was used in glass injection liner to retain non-volatile components in a sample if present.

5.1.3 Results and Discussion

5.1.3.1 Method development and optimisation

The aim of the present work was to develop a simple, reliable, and sensitive analytical method for accurate measurement of EHA, EGDMA, and EHMA to estimate their conversions in HIPE polymerisation reactions. The selection and configuration of equipment, experimental conditions for sample preparation (solvent extraction of monomers) and parameters for GC column oven temperature programming were optimised to achieve required chromatographic separation described below.

5.1.3.1.1 Selection of analytical technique

FID is extremely sensitive, has a large dynamic range, and detects all carbonaceous organic compounds. The response of the detector is generally directly proportional to the carbon content of the analyte.[25]

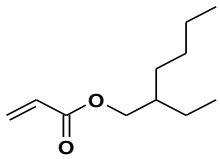

Figure 5.3: Chemical structure of 2-ethylhexyl acrylate (EHA)

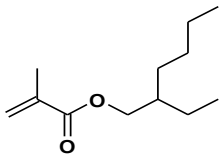

Figure 5.4: Chemical structure of 2-ethylhexyl methacrylate (EHMA)

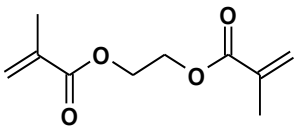

Figure 5.5: Chemical structure of ethylene dimethacrylate (EGDMA)

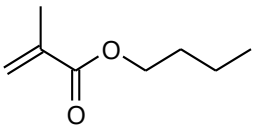

Figure 5.6: Chemical structure of butyl methacrylate (BMA)

Since the monomers in the present study (including internal standard, BMA) have long chain alkane backbone (ample of carbons), they show appreciable

sensitivity towards flame ionisation detector. Secondly, they are volatile enough to be analysed by gas chromatography. The chemical structures of monomers and internal standard (BMA) are given in Figures 5.3 - 5.6. The selection of gas chromatographic technique with FID was therefore appropriate for present analysis.

5.1.3.1.2 Optimisation of chromatographic parameters

In chromatography, the retention of analyte on stationary phase is dependent on many factors such as nature of analyte and stationary phase, various types of interactions taking place between analyte and stationary phase, analyte and mobile phase, and mobile phase and stationary phase, etc. In GC analysis, in general, the predominant parameter responsible for chromatographic separation is the difference in boiling points of analytes to be separated. However, 'analyte-stationary phase' interaction may play significant and pre-dominant role in chromatographic elution process.

In order to achieve adequate resolution, preliminary trials were taken by running a synthetic sample comprised of acetone, MEHQ, cyclohexane, EHA, EGDMA, EHMA and BMA. The GC temperature program conditions along with carrier gas flow rate and injector split ratio were set and optimised by comparing the boiling points and polarity of analytes for achieving adequate chromatographic separation. The boiling point data is given in Table 5.1 and the optimised parameters for chromatographic separation are given in Section 5.1.2.4.

As mentioned earlier, in GC analysis, the compound with low boiling point (b. p.) elutes before the one with high b. p. most of the times, which is the case for EHA (b. p. 216 °C, R_t 11.084 min) and EGDMA (b. p. 240 °C, R_t 12.082 min). However, it may be noted that EGDMA (b. p. 240 °C, R_t 12.082 min) eluted before EHMA (b. p. 218 °C,

R_t 12.251 min) though the former has a higher b. p. than the latter. This may be due to comparatively stronger interaction of EHMA towards dimethylpolysiloxane stationary phase (DB-1 column) than EGDMA (which is relatively more polar than EHMA and hence, shows less interaction). However, it is interesting to note that between EHA and EGDMA, the extent of retention is predominantly due to the difference in their boiling points although EHA has an almost similar chemical structure to that of EHMA except having one methyl group less. (Figures 5.3 and 5.4)

Table 5.1: Boiling point data

Sr. No.	Compound	Boiling point (°C)
1	BMA	160
2	EHA	216
3	EGDMA	240
4	EHMA	218

5.1.3.1.3 Internal standard method

In quantitative analysis, one of the methods is to use internal standard. The usefulness of internal standard method has been explained in Section 1.10 of Chapter 1. In the present study, the internal standard method was used wherein the internal standard was added into reaction mass during extraction step (i.e. added as internal standard solution prepared in cyclohexane).

The internal standard reduces error that can occur due to injection variability, often observed while making small volumes of injections into the column. The internal standard is also desirable when error due to sample loss is likely to occur in multi-step sample preparation. When a known amount of internal standard is used in analysis, area ratio (of a particular analyte to internal standard) is used for quantification instead of absolute peak area of analyte. The cumulative error that may incur in measurement due to

sample handling is therefore nullified, since the ratio of internal standard to analyte always remains constant as the same fraction of each is lost in any operation or step of the analysis method.

5.1.3.1.4 Sample preparation

Sample preparation is one of the important steps in any analysis. In the present study, demulsification of intrinsically extremely stable HIPE was a prime requisite for quantitative measurement of unreacted monomers. After demulsification, the monomers released from HIPE matrix need to be isolated from aqueous HIPE reaction sample by selective extraction using a water-immiscible solvent (as aqueous samples cannot be injected on DB-1 column used for the present work). PLOT-Q capillary column, which is compatible with aqueous samples, could have been used to overcome this problem, if monomers were extracted in a water soluble solvent like methanol. However, the water-soluble initiator and electrolyte (sodium persulphate and calcium chloride), used in HIPE reaction, do not elute and get accumulated in GC column because of their non-volatility. Due to this, the column becomes coated with such contaminants, which interfere with the proper partitioning of solutes between stationary phase and mobile phase. This may result into poor peak resolution and affect the accuracy of measurement.

The method for selective extraction of unreacted monomers from HIPE matrix was therefore developed, wherein cyclohexane was chosen as extraction solvent, which showed excellent selectivity for hydrophobic monomers under present study.

5.1.3.1.5 Solvent for extraction

After screening a number of organic compounds, cyclohexane was chosen as a suitable solvent for extraction of monomers. The advantage of selecting cyclohexane as

extraction solvent was that (i) the monomers EHA, EGDMA and EHMA, being hydrophobic in nature, show appreciable miscibility in it, (ii) it acts as a non-solvent to crosslinked polymer product formed in HIPE reaction mixture, thus avoiding interference of polymer product, and (iii) it does not dissolve initiator (sodium persulphate) and electrolyte (calcium chloride) used in HIPE.

When sample contains dissolved polymer, it may degrade into monomers or oligomers in a heated chamber of GC injector and may therefore cause serious interference in measurement. This problem is completely eliminated here, because cyclohexane selectively extracts only the monomers. The samples, therefore, can directly be injected into GC system without fear of column damage.

5.1.3.1.6 Optimisation of extraction method

Among various solvents considered for extraction purpose, cyclohexane showed a high distribution coefficient and good selectivity for analytes. The monomer extraction protocol was evolved by investigating the extraction efficiency, by determining the recovery of monomers at their low, medium, and high concentration levels. Experiments were conducted by making five synthetic samples as HIPEs prepared with known concentrations of EHA, EGDMA, and EHMA for all the three levels of monomer concentrations. The HIPE as a "sample matrix" was prepared by using initiator (0.05% sodium persulphate), electrolyte (4% calcium chloride), and an inhibitor (MEHQ) equal to 1 mol% of initiator. Further, 100 mL of cyclohexane (containing internal standard, BMA, 2500 ppm, w/v) was added into each synthetic sample for extraction of monomers. The monomer extraction was followed as per the procedure described in Section 5.1.2.3 (B), and the samples were injected into GC system for investigating recovery of

monomers after their extraction. The percent recovery was found to be in the range of 97.3 - 104.9, which was appropriate for the present work. A number of experiments were conducted and the volume of extraction solvent was optimised to 100 mL. The analytical data on percent recovery of monomers from HIPE matrix for their low, medium and high levels is given in Table 5.2. This proved that the monomers could be extracted in a single-step exhaustive extraction. A single-step extraction is advantageous over multi-step extraction, since multi-step extraction may introduce considerable error in measurement due to multiple steps involved in sample handling.

Table 5.2: Analytical data on recovery of monomers from HIPE matrix

Sample	**EHA, ppm (w/v)**			**EGDMA, ppm (w/v)**			**EHMA, ppm (w/v)**		
	Added	Found	*%Rec.	Added	Found	*%Rec.	Added	Found	*%Rec.
Low level									
1	70	72	102.9	38	39	102.6	75	77	102.7
2	110	107	97.3	80	78	97.5	124	130	104.8
3	204	214	104.9	110	114	103.6	240	234	97.5
4	508	510	100.4	300	312	104.0	480	498	103.8
5	640	630	98.4	412	414	100.5	656	672	102.4
Medium level									
1	800	802	100.3	520	510	98.1	824	855	103.8
2	1200	1196	99.7	715	735	102.8	1280	1265	98.8
3	1500	1490	99.3	956	946	99.0	1489	1562	104.9
4	1800	1821	101.2	1200	1250	104.2	1938	2012	103.8
5	2220	2184	98.4	1324	1356	102.4	2150	2198	102.2
High level									
1	2500	2487	99.5	1546	1586	102.6	2634	2700	102.5
2	2965	3012	101.6	1758	1745	99.3	3020	2984	98.8
3	3402	3416	100.4	1956	1986	101.5	3390	3456	101.9
4	3750	3812	101.7	2116	2210	104.4	3652	3597	98.5
5	4200	4284	102.0	2200	2190	99.5	4018	4156	103.4

*%Rec: %Recovery of monomers from HIPE matrix

5.1.3.2 Method validation

After an analytical method is developed, it should be validated to ensure that it is useful for its intended purpose. The present method was validated by studying various parameters such as system precision, specificity, linearity (calibration studies), limit of detection (LOD), limit of quantification (LOQ), and 'method precision and accuracy'. The performance of measurement method was evaluated by conducting Gauge R&R study.

5.1.3.2.1 System precision

System precision was checked by assessing repeatability of retention time and peak area by making 10 replicate injections of a test sample comprising a mixture of EHA, EGDMA, EHMA and BMA (internal standard). The retention times of all the components were quite repeatable as evident from the low values of standard deviation and %RSD, given in Table 5.3.

Table 5.3: Retention time repeatability

Inj. No.	Retention time (min)			
	EHA	**EGDMA**	**EHMA**	**BMA (IS)**
1	11.074	12.071	12.252	8.531
2	11.078	12.078	12.266	8.556
3	11.082	12.082	12.251	8.598
4	11.098	12.073	12.278	8.542
5	11.078	12.086	12.286	8.534
6	11.084	12.102	12.268	8.576
7	11.075	12.074	12.278	8.534
8	11.084	12.078	12.269	8.567
9	11.087	12.087	12.256	8.592
10	11.084	12.108	12.286	8.563
Mean	11.082	12.084	12.269	8.564
SD	0.007	0.012	0.013	0.024
%RSD	0.06	0.10	0.11	0.28

The data on peak area variation is given in Table 5.4. Internal standards are useful for analyses wherein the quantity of sample analysed or the instrument response varies slightly from run to run for reasons that are difficult to control. Signal from analyte is compared with signal from the internal standard to find out how much analyte is present. When a known amount of internal standard is used, peak area ratio (of a particular component to internal standard) is used for quantification of that particular component which minimises run- to- run area variation.

It can be seen that there was a greater variation (%RSD > 3.0) for individual area counts (Table 5.4). However, the %RSD for peak area ratio, for all the three monomers, was found to be approximately 1.0 (Table 5.4), which shows good peak area repeatability and hence, leads to an accurate result. This study demonstrated that the use of internal standard in present analysis helped minimise error which may occur due to above mentioned factors.

Table 5.4: Peak area repeatability

Inj. No.	Peak area (counts)				Peak area ratio		
	EHA	**EGDMA**	**EHMA**	**BMA (IS)**	***EGDMA**	***EHA**	***EHMA**
1	40728	13454	43124	10059	4.05	1.34	4.29
2	42869	13909	45392	10597	4.05	1.31	4.28
3	40760	13450	43159	10045	4.06	1.34	4.30
4	41477	14009	43918	10315	4.02	1.36	4.26
5	42981	14729	45896	10918	3.94	1.35	4.20
6	40383	13616	42760	10054	4.02	1.35	4.25
7	40028	13399	42384	10059	3.98	1.33	4.21
8	38304	12648	40559	9452	4.05	1.34	4.29
9	40858	13450	43262	10061	4.06	1.34	4.30
10	39781	13483	43145	10052	3.96	1.34	4.29
Mean	40817	13615	43360	10161	4.02	1.34	4.27
SD	1396	534	1498	388	0.05	0.01	0.04
%RSD	3.4	3.9	3.5	3.8	1.1	0.9	0.8

***EHA**: Peak area of EHA/Peak area of BMA (IS), ***EGDMA**: Peak area of EGDMA/ Peak area of BMA (IS), ***EHMA**: Peak area of EHMA/ Peak area of BMA (IS)

5.1.3.2.2 Specificity

Method specificity for given monomers was achieved due to both solvent extraction (sample clean-up) step and optimised GC column oven temperature programming. The specificity of this method has been demonstrated by the representative chromatogram (Figure 5.7) of the test sample comprising a mixture of EHA, EGDMA and EHMA, which were extracted from HIPE matrix. All the peaks of analytes along with the peak of internal standard were well resolved with base line separation and there was no interference of components from HIPE sample matrix. The interference of initiator (sodium persulphate), electrolyte (calcium chloride), and polymer product formed in HIPE reaction was eliminated, since they did not come in organic (cyclohexane) layer during extraction. Further, the surfactants (Span-80 and Arquad 2HT-75), used in very small amounts in HIPE reactions, due to their non-volatility, got retained over the glass wool placed in an injector liner and thus, did not go to GC column thereby avoiding their interference (*the glass wool was replaced periodically after every 10 injections to avoid charring of non-volatile components retained over glass wool inside the GC injector liner)*. The monomers had mono methyl ether hydroquinone (MEHQ), a stabiliser, at ppm levels (10 ppm for EHA, 100 ppm for EGDMA, and 50 ppm for EHMA). The MEHQ from monomers and the one (dissolved in acetone) added externally for reaction quenching goes along with monomers during extraction. However, the chromatographic method separated both MEHQ and acetone peaks very well and avoided their interference with the peaks of interest. The interference of one more inhibitor, phenothiazine (b. p. 371 ºC), used in HIPE reactions, was eliminated as it was retained over the glass wool placed in the GC injector liner and thus, did not go to GC

column. Traces of impurities that might arise from solvents were also separated and thus, their interference with the peaks of interest was avoided. This proved that the method was specific to the monomers, viz. EHA, EGDMA and EHMA.

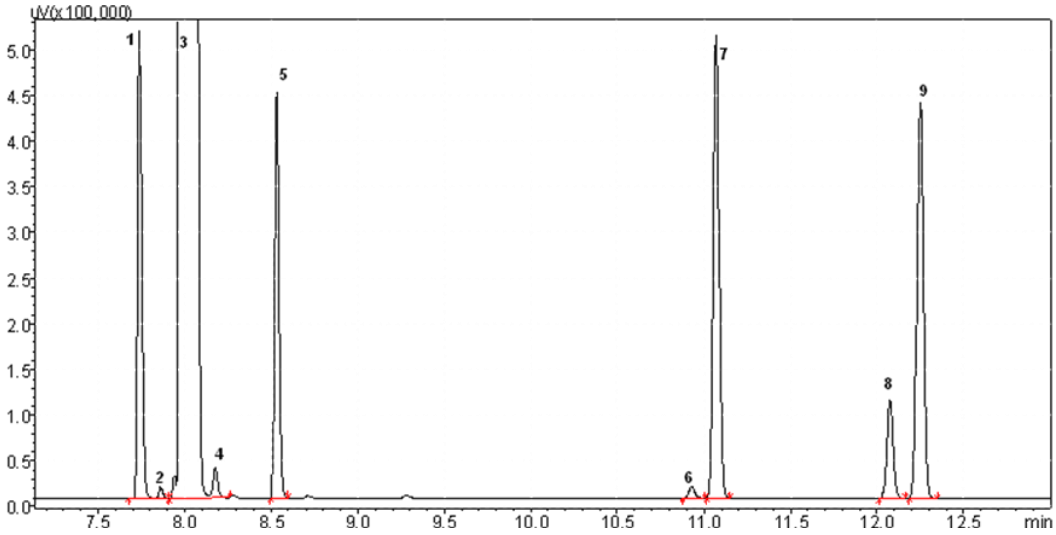

Figure 5.7: The representative chromatogram showing separation of EHA, EGDMA, and EHMA: (1) acetone (Rt 7.740 min); (2) impurity[1] (Rt 7.861 min); (3) cyclohexane (Rt 7.979 min); (4) impurity[2] (Rt 8.177 min); (5) BMA (Rt 8.531 min); (6) MEHQ (Rt 10.928 min); (7) EHA (Rt 11.071 min); (8) EGDMA (Rt 12.074 min); and (9) EHMA (Rt 12.249 min)

5.1.3.2.3 Linearity (calibration studies)

The linearity was assessed by running analytical standards on GC system under optimised chromatographic conditions. For this study, 10 nos. of calibration standards in the given range (Table 5.5) were prepared as per procedure described in Section 5.1.2.2, and injected into the chromatographic system. The calibration curve for a particular monomer was derived by plotting peak area ratio (ratio of peak area of analyte to internal standard) against concentration ratio (ratio of concentration of analyte to internal standard). The regression equation was obtained as $y = mx$, where y was the peak area ratio of analyte to internal standard, m was the slope, and x was the ratio of concentration ($\mu g\ mL^{-1}$) of analyte to internal standard. The linearity analysis showed a good fit of

regression lines, as indicated by the regression coefficient values ($R^2 \geq 0.9990$). The monomer concentration range, regression equations and values of R^2 are reported in Table 5.5, and the calibration curves for EHA, EGDMA and EHMA are given in Figures 5.8, 5.9 and 5.10, respectively.

Table 5.5: Linearity studies of monomers

Monomer	Range (µg mL^{-1})	Regression equation	R^2 value
EHA	40-4000	$y = 1.6414x$	0.9990
EGDMA	20-2000	$y = 1.1852x$	0.9994
EHMA	50-4000	$y = 1.8051x$	0.9997

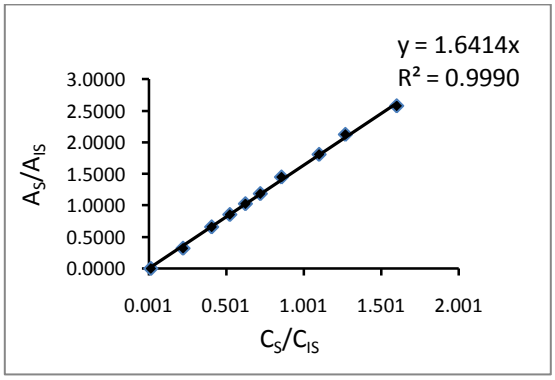

Figure 5.8: Calibration curve for EHA (A_S/A_{IS}: Peak area of EHA/Peak area of IS, C_S/C_{IS}: Conc. of EHA/ Conc. of IS)

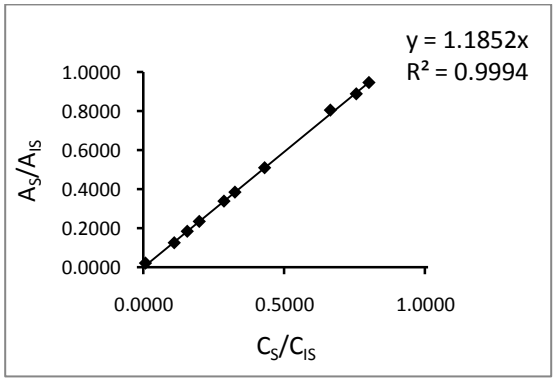

Figure 5.9: Calibration curve for EGDMA (A_S/A_{IS}: Peak area of EGDMA/Peak area of IS, C_S/C_{IS}: Conc. of EGDMA/ Conc. of IS)

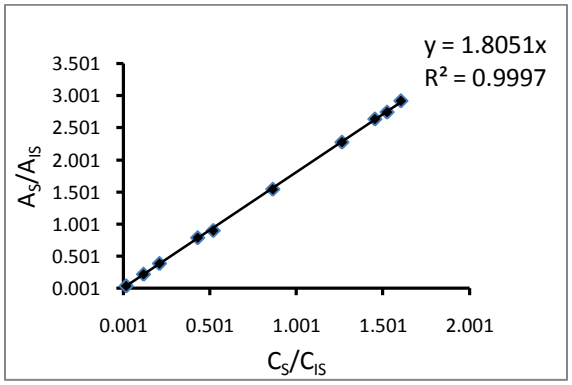

Figure 5.10: Calibration curve for EHMA (A_S/A_{IS}: Peak area of EHMA/Peak area of IS, C_S/C_{IS}: Conc. of EHMA/ Conc. of IS)

5.1.3.2.4 Response factors

The analytes exhibit different responses to a particular detection system. Hence, it is necessary to calculate response factor of an analyte. The response factors (R_f) for EHA, EGDMA and EHMA were calculated from calibration data using the following equation:

$$R_f = \left(\frac{C_S}{A_S}\right) \text{ x } \left(\frac{A_{IS}}{C_{IS}}\right) \quad 5.1$$

where, C_S is the concentration (ppm, w/v) of monomer standard, C_{IS} is the concentration (ppm, w/v) of internal standard (BMA), A_S is the peak area of monomer standard and A_{IS} is the peak area of internal standard. The calibration and statistical data for replicate measurements ($n = 10$) of response factors are given in Tables 5.6 and 5.7, respectively. The %RSD ($n = 10$) ≤ 1.8 shows good precision for measurement of response factors.

The mean R_f values given in Table 5.7 were used to determine corresponding monomer concentration (ppm, w/v) in a particular HIPE reaction sample.

Table 5.6: Calibration data

S. No.	EHA			EGDMA			EHMA		
	C_S/C_{IS}	A_{IS}/A_S	R_f	C_S/C_{IS}	A_{IS}/A_S	R_f	C_S/C_{IS}	A_{IS}/A_S	R_f
1	0.0160	38.0228	0.6084	0.0080	106.5500	0.8524	0.0200	27.4725	0.5495
2	0.2235	2.7580	0.6164	0.1102	7.9114	0.8718	0.1200	4.5720	0.5486
3	0.4079	1.5177	0.6191	0.1564	5.4043	0.8452	0.2119	2.5890	0.5486
4	0.5250	1.1701	0.6143	0.1990	4.2474	0.8452	0.4326	1.2682	0.5486
5	0.6254	0.9748	0.6097	0.2867	2.9482	0.8452	0.5225	1.1109	0.5805
6	0.7214	0.8435	0.6085	0.3256	2.5959	0.8452	0.8664	0.6474	0.5609
7	0.8578	0.6902	0.5920	0.4310	1.9611	0.8452	1.2661	0.4388	0.5556
8	1.1000	0.5533	0.6087	0.6644	1.2430	0.8259	1.4562	0.3788	0.5516
9	1.2695	0.4709	0.5978	0.7564	1.1264	0.8520	1.5264	0.3640	0.5556
10	1.6000	0.3882	0.6210	0.8014	1.0575	0.8475	1.6063	0.3421	0.5495

Table 5.7: Response factors data

Monomer	**EHA *(n = 10)***	**EGDMA *(n = 10)***	**EHMA *(n = 10)***
Mean	0.6096	0.8476	0.5549
SD	0.009	0.011	0.010
%RSD	1.5	1.3	1.8

5.1.3.2.5 Limits of detection and quantification

The limits of detection and quantification (LOD and LOQ) of a method (given in Table 5.8) were determined by signal-to-noise ratio method.[26] The LOD (three times signal-to-noise) and LOQ (10 times signal-to-noise) for individual monomers were estimated from the corresponding slope values of regression equations (Table 5.5).

Table 5.8: LOD and LOQ data

Monomer	**LOD (μg mL^{-1})**	**LOQ (μg mL^{-1})**
EHA	0.9	3.1
EGDMA	1.3	4.2
EHMA	0.8	2.8

It may be noted that the minimum concentration values of EHA, EGDMA, and EHMA (i.e. concentration of unconverted monomers at 98% conversion) are much higher than the LOD and LOQ values. Hence, it can be concluded that this method was sensitive enough for the measurement of given monomers for a HIPE reaction having a batch size of 0.925 g.

The response of FID is generally directly proportional to the carbon content of the analyte.[25] The monomers, EGDMA, EHA and EHMA have 10, 11 and 12 carbon atoms, respectively. Accordingly, they show low, medium and high sensitivity towards FID, respectively. This aspect has been reflected in the values of LOD/LOQ (Table 5.8), response factors (R_f) (Table 5.7) and slopes of regression equations (Table 5.5) calculated for EHA, EGDMA and EHMA.

5.1.3.2.6 Method precision and accuracy

The method precision (intra-day repeatability) and intermediate precision (inter-day repeatability) were tested by multiple analyses of mixtures of monomers prepared with HIPE matrix. Three quality control (Q.C.) solutions considering levels of unreacted monomers at low, medium and high conversions were prepared. The concentrations of monomers in individual mixtures were, (i) EHA (370, 1850 and 3330 μg mL^{-1}), (ii) EGDMA (175, 875 and 1575 μg mL^{-1}) and (iii) EHMA (380, 1900 and 3420 μg mL^{-1}) for low, medium and high levels, respectively. The initiator (sodium persulphate), and MEHQ (dissolved in acetone) were added into each HIPE sample. The monomers in individual samples were extracted in cyclohexane (containing IS) and subjected to GC runs for assay analysis.

Table 5.9: Method precision and accuracy

Q.C. Solution	Added in HIPE (μg mL^{-1})	INTRA-DAY, *n = 5*			INTER-DAY, *n = 5*		
		A ± SD[a]	Precision %RSD[b]	Accuracy[c]	A ± SD[a]	Precision %RSD[b]	Accuracy[c]
1							
EHA	370	369 ± 11.7	3.16	- 0.27	359 ± 10.4	2.90	-2.97
EGDMA	175	173 ± 6.3	3.67	- 1.14	171 ± 7.2	4.20	-2.28
EHMA	380	377 ± 9.7	2.57	- 0.79	370 ± 9.0	2.42	-2.63
2							
EHA	1850	1904 ± 37.7	1.98	2.92	1894 ± 42.4	2.24	2.38
EGDMA	875	865 ± 17.6	2.03	- 1.14	859 ± 11.4	1.33	- 1.83
EHMA	1900	1910 ± 44.0	2.30	0.52	1934 ± 46.5	2.40	1.79
3							
EHA	3330	3318 ± 31.0	0.93	- 0.36	3274 ± 73.7	2.25	- 1.68
EGDMA	1575	1601 ± 21.8	1.36	1.65	1599 ± 14.0	0.88	1.52
EHMA	3420	3458 ± 77.3	2.24	1.11	3383 ± 79.8	2.36	- 1.08

A: Mean of determined concentration (μg mL^{-1}), *n = 5*
[a]**SD**: Standard deviation
[b]**RSD**: Relative standard deviation,
[c]**Accuracy**: (%Relative error) = [(Determined conc.− Added conc.) / Added conc.] × 100

The method precision (intra-day) was evaluated by replicate analyses of samples (n = 5) on same day and the inter-day precision was studied by comparing the assay on five different days. The %RSD values and relative errors for accuracy are given in Table 5.9. This analysis showed good precision and accuracy of the method and proved it to be suitable for its intended use.

5.1.3.3 Gauge R & R study

Any analytical technique or measurement method can have many sources of error. If the total variability in a particular case is higher than desired, then the significant sources of error must be identified and controlled. Analysis of variance (ANOVA) is a statistical technique for estimating the importance of one or more factors suspected of contributing significantly to the total uncertainty in a given situation. Gauge R & R is a means of assessing the repeatability and reproducibility of the measurement system.

The present analytical method was subjected to Gauge R&R (Repeatability and Reproducibility) study to determine variability in the measuring system and to identify various sources that contribute to it. The measuring system consisted of (1) measuring device (i.e. GC instrument) and the method of analysis (Repeatability component) and (2) the operators or analysts who perform the analyses (Reproducibility component). The R&R study indicates how the given method performs in the hands of different analysts besides the performance of measuring device, and if there is any interaction between samples and analysts.

For this experiment, five different HIPE samples (containing each of 2400 ppm of internal standard) were analysed three times (one run at a time by one analyst) by three analysts for three monomer components viz., EHA (532, 2010, 4040, 5970 and 8000

ppm), EGDMA (288, 990, 2000, 2980 and 3970 ppm) and EHMA (512, 2060, 4080, 7200 and 8210 ppm) in a completely randomised manner on the same GC system (i.e. measuring device). The analytical data were subjected to statistical method of analysis of variance (ANOVA) to delineate the contributions from various sources, and their significance was judged by *P*-values ($P < 0.05$ indicates significant contribution). The data coverage factor for the R&R study was 5.15 σ (i.e. 99% area under the normal distribution curve).

The ANOVA data of Gauge R & R study for all the three components (EHA, EGDMA and EHMA) are presented in Tables 5.10 - 5.15, and the results of the R&R study are shown in Figures 5.11 - 5.13 as 'x bar chart by analyst', 'range (R) chart by analysts', 'analyst-sample interaction', 'scatter plots by samples and by analysts', and 'components of variation'.

The R&R values (Tables 5.11, 5.13 and 5.15) for EHA, EGDMA and EHMA were 1.39, 1.68 and 1.73%, respectively. The R&R values less than 10% are considered to be good and indicate that the measurement system is acceptable.[27] The R&R values obtained for all the three monomer components were much lower than 10% which proved that the analysis method was very stable. It may be noted that in all the three cases, the major contribution to the R&R values (Tables 5.11, 5.13 and 5.15) originated from the gauge (GC equipment) itself (i.e. repeatability component) rather than from the analysts (i.e. Reproducibility component). Further, the sample-analyst interaction was insignificant, i.e. sample handling by analysts did not contribute significantly to the Reproducibility component for all the three cases, as evident from *P*-values ($P > 0.05$, Tables 5.10, 5.12 and 5.14). Further, it can be seen from Figures 5.11 - 5.13 ('R chart by

Analyst') that all the three analysts measured almost all samples consistently except measurement of Sample no. 4 by Analyst 2 for which the sample range value falls outside the upper control limit (UCL = 149.1, Figure 5.11).

Another parameter to evaluate the measurement system is the number of distinct categories (described in Section 1.9.2 of Chapter 1). This index is the "number of non overlapped confidence intervals" that is needed to cover all the product variability. When the number of distinct categories is less than 2, the measurement system is unable to measure the process. A number of distinct categories greater or equal to 5 means an acceptable measurement system.[28] The number of distinct categories for EHA, EGDMA and EHMA analyses was found to be 101, 84 and 81, respectively, which showed that the measurement system was quite acceptable.

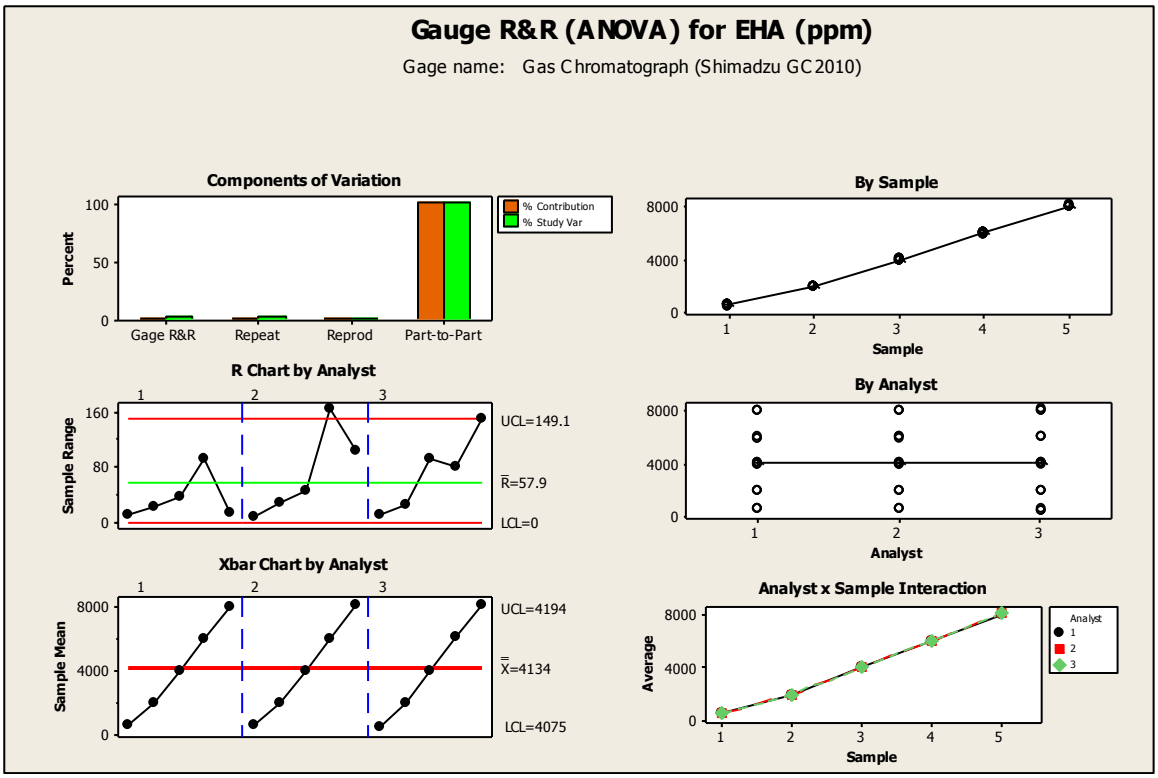

Figure 5.11: ANOVA results for five different EHA analyses by three analysts

Table 5.10: Sources of variation in EHA analysis (by ANOVA method)

Source	DF	SS	MS	F	P
Sample	4	329502486	82375622	45593.1	0.000
Analyst	2	9686	4843	2.7	0.129
Sample x Analyst	8	14454	1807	1.2	0.327
Repeatability	30	44833	1494	-	-
Total	44	329571459	-	-	-

Table 5.11: Repeatability and reproducibility (R&R) data for EHA analysis

Source	Var Comp	%Contribution	Std Dev (SD)	Study Var (5.15*SD)	%Study Var
Total Gauge R&R	1779	0.019	42.18	217.2	1.39
Repeatability	1560	0.017	39.50	203.4	1.31
Reproducibility	219	0.002	14.79	76.2	0.49
Analyst	219	0.002	14.79	76.2	0.49
Sample-To-Sample	9152673	99.981	3025.34	15580.5	99.99
Total Variation	9154453	100.000	3025.63	15582.0	100.00

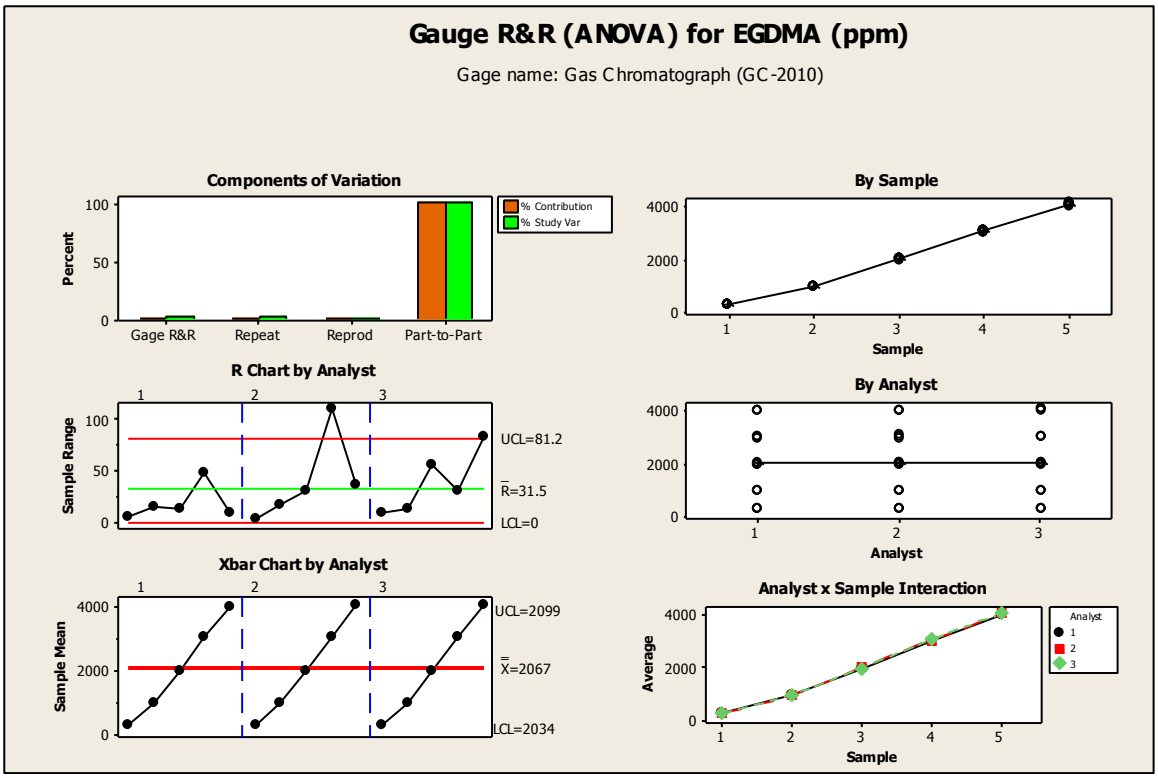

Figure 5.12: ANOVA results for five different EGDMA analyses by three analysts

Table 5.12: Sources of variation in EGDMA analysis (by ANOVA method)

Source	DF	SS	MS	F	P
Sample	4	82340932	20585233	32980.9	0.000
Analyst	2	4747	2373	3.8	0.069
Sample x Analyst	8	4993	624	1.3	0.297
Repeatability	30	14767	492	-	-
Total	44	82365438	-	-	-

Table 5.13: Repeatability and reproducibility (R&R) data for EGDMA analysis

Source	Var Comp	%Contribution	Std Dev (SD)	Study Var (5.15*SD)	%Study Var
Total Gauge R&R	644	0.028	25.37	130.56	1.68
Repeatability	520	0.023	22.80	117.44	1.51
Reproducibility	124	0.005	11.12	57.24	0.73
Analyst	124	0.005	11.12	57.24	0.73
Sample-To-Sample	2287190	99.972	1512.35	7788.58	99.99
Total Variation	2287834	100.000	1512.56	7789.68	100.00

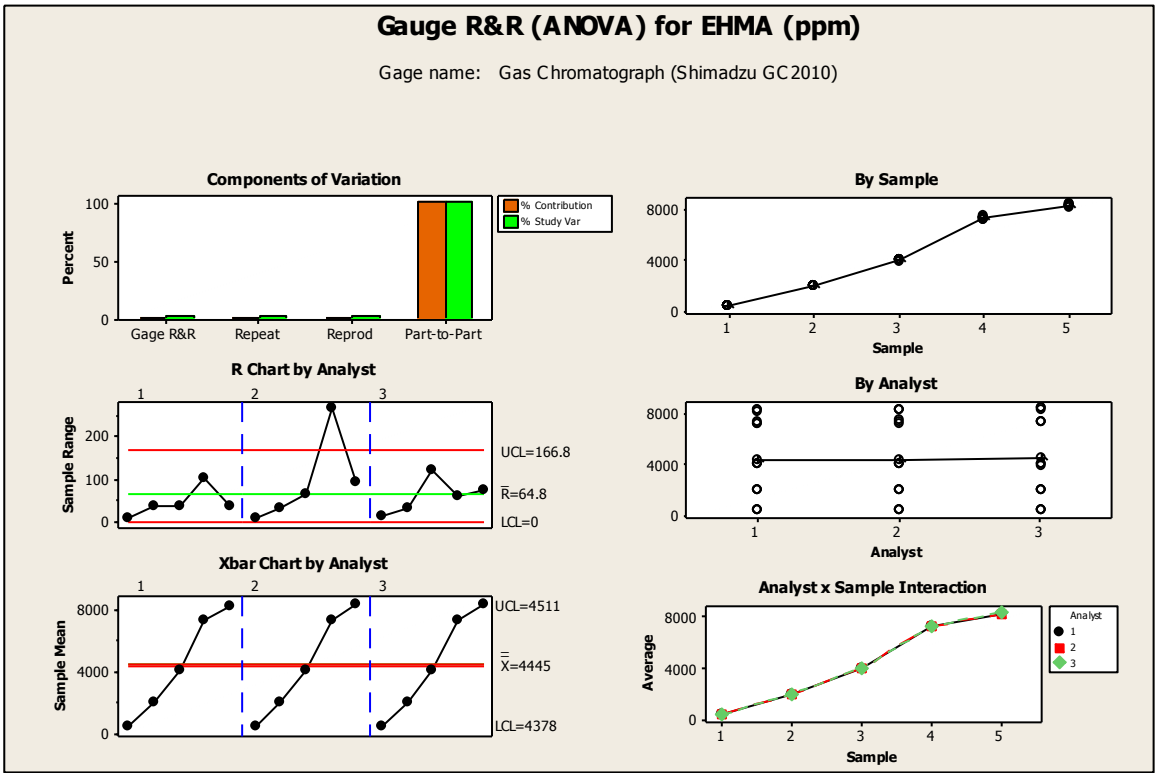

Figure 5.13: ANOVA results for five different EHMA analyses by three analysts

Table 5.14: Sources of variation in EHMA analysis (by ANOVA method)

Source	DF	SS	MS	F	P
Sample	4	404377277	101094319	23335.8	0.000
Analyst	2	23513	11757	2.7	0.126
Sample x Analyst	8	34657	4332	2.0	0.077
Repeatability	30	64088	2136	-	-
Total	44	404499535	-	-	-

Table 5.15: Repeatability and reproducibility (R&R) data for EHMA analysis

Source	Var Comp	%Contribution	Std Dev (SD)	Study Var (5.15*SD)	%Study Var
Total Gauge R&R	3363	0.030	57.99	298.7	1.73
Repeatability	2136	0.019	46.22	238.0	1.38
Reproducibility	1227	0.011	35.03	180.4	1.04
Analyst	495	0.004	22.25	114.6	0.66
Sample x Analyst	732	0.007	27.05	139.3	0.81
Sample-To-Sample	11232221	99.970	3351.45	17260.0	99.99
Total Variation	11235584	100.000	3351.95	17262.6	100.00

5.1.3.4 HIPE reaction quenching

An instant reaction quenching is necessary for inhibiting HIPE polymerisation reaction in order to obtain accurate curing time to relate it to the corresponding monomer conversion. Initially preliminary experiments were conducted with ice-cold water for reaction quenching which however caused a large data scatter in plots of time-dependent monomer conversions. There are various sources that can contribute to errors in measurements. To check if reaction quenching with ice-cold water is not as effective as required and whether this factor is the major source of error causing data scatter, the same experiments were repeated wherein reaction quenching was done using liquid nitrogen. The amounts of unconverted monomers were determined and the data on %monomer conversions were calculated as tabulated in Table 5.16. The monomer conversion data indicated that there was post-polymerisation in case of reaction quenched with ice-cold water. The %conversion values (Table 5.16) and time-dependent %EHA conversion profiles (Figure 5.14) indicated that the reaction quenching by ice-cold water was not as instant as required. Hence, further all experiments were performed using liquid nitrogen reaction quenching, wherein data scatter problem was resolved.

For the sake of simplicity, only %EHA conversion was plotted against curing time as shown in Figure 5.14. A similar trend was observed for EGDMA and EHMA as well.

Table 5.16: Comparison of ice-cold water and liquid nitrogen reaction quenching

Curing time (Min)	%Conversion (w/w)					
	EHA		EGDMA		EHMA	
	IW	LN	IW	LN	IW	LN
2	2.1	1.6	6.8	3.4	4.6	2.1
4	5.2	3.1	26.4	12.6	12.5	7.4
6	9.1	4.1	35.0	22.3	17.6	9.6
8	26.8	10.6	50.4	27.8	42.1	22.6
10	38.6	18.6	70.6	62.8	60.8	32.6
12	52.2	27.5	85.0	71.7	72.7	47.7

IW: Percent conversion data when reaction quenched with ice-cold water.
LN: Percent conversion data when reaction quenched with liquid nitrogen.
Polymerisation conditions: i) Initiator (sodium persulphate): 0.2 wt%, ii) Reactor: Glass (150 mL), iii) Stirrer speed: 1400 rpm, iv) Curing temp.: 65 ^{0}C, v) Surfactants: mixture of Span-80 (0.065 g, i.e. 7.03% of oil phase) and of Arquad 2HT-75 (0.008 g, i.e. 0.86% of oil phase)

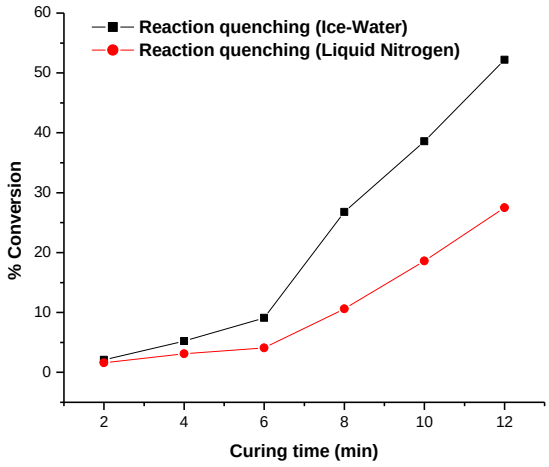

Figure 5.14: The plot of %conversion of EHA against curing time for comparing ice-cold water and liquid nitrogen reaction quenching

5.1.3.5 Estimation of monomer conversions

5.1.3.5.1 HIPE polymerisation reactions

All HIPE polymerisation reactions were conducted with a batch size of 0.925 g, which contained 0.370 g (2.0078 mM) of EHA, 0.175 g of EGDMA (8.8829 mM), and 0.380 g (1.9162 mM) of EHMA. The organic phase was prepared in bulk (stock solution)

to have an uniform composition of its ingredients and the required quantity (0.925 ± 0.005 g) for a particular batch was dispensed from stock solution for conducting a HIPE reaction. The HIPE samples were prepared as described in Section 5.1.2.3. The concentration of surfactants was kept constant in all HIPE reactions. In all 14 experiments were conducted wherein individual batches of HIPEs were polymerised for different curing times and data on time-dependent monomer conversions (weight %) was obtained by measuring unreacted monomers. The details of HIPE reaction parameters are presented in Table 5.17.

Table 5.17: Parameters for HIPE polymerisation reactions

Expt. No.	Initiator concentration	Curing temp. (°C)	Inhibitor Concentration
1	1X	85	No inhibitor used
2	2X	85	No inhibitor used
3	3X	85	No inhibitor used
4	4X	85	No inhibitor used
Inhibitor used: Monomethyl ether hydroquinone (MEHQ)			
5	4X	85	1Y
6	4X	85	2Y
7	4X	85	3Y
8	4X	85	4Y
Inhibitor used: Phenothiazine (PTZ)			
9	2X	85	0.5Y
10	2X	85	1Y
11	2X	85	2Y
12	4X	85	0.5Y
13	4X	85	1Y
14	4X	85	2Y

X: Standard initiator concentration (0.05% w/v).
Y: Standard inhibitor concentration (concentration equal to 1 mol% of initiator).

5.1.3.5.2 Percent monomer conversion

All samples were injected into GC system with the injection volume of 1.0 µL and the chromatographic data of peak area counts of monomers and an internal standard (BMA) were collected to calculate the amounts of unreacted monomers, as given below.

The concentration of a monomer in a particular HIPE reaction sample was calculated using following equation:

$$C_S = \left[\frac{A_S}{A_{IS}}\right] \text{x } R_f \text{ x } C_{IS} \qquad 5.2$$

where, C_S is the concentration (ppm, w/v) of analyte (EHA, EGDMA or EHMA) to be determined, A_S is the peak area of analyte, A_{IS} is the peak area of internal standard (BMA), C_{IS} the concentration (ppm, w/v) of internal standard added into sample and R_f is the mean value of response factor of corresponding analyte.

From the concentration (ppm, w/v) value, the absolute amount of unreacted monomer in total reaction mass (i.e. total "g" in 100 mL of extraction solvent) was calculated and further, the %monomer conversion was calculated using equation 5.3:

$$\%Conversion\ (w/w) = \frac{(A - B)}{A} \text{x } 100 \qquad 5.3$$

where, "A" is the initial amount (g) of monomer taken for a HIPE reaction and "B" is the amount (g) of unreacted monomer determined in a reaction mass.

5.1.3.5.3 Time-dependent monomer conversion profiles

The time-dependent monomer conversion profiles were generated by plotting percent conversion against curing time as shown in Figures 5.15 - 5.28. The percent conversion data for EHA, EGDMA, and EHMA for all the experiments (Expt. Nos. 1 - 14) are reported in Table 5.18 - 5.31.

Table 5.18: Percent conversion (Expt. No. 1)

S. No.	Time (min)	%Conversion (w/w)		
		EHA	EGDMA	EHMA
1	0.0	0	0	0
2	2.0	1.2	8.8	4.5
3	2.5	6.5	16.7	11.8
4	3.5	9.8	29.7	18.1
5	4.0	17.9	47.1	30.2
6	4.5	25.1	56.4	38.7
7	5.0	36.9	67.5	51.0
8	5.5	42.2	76.7	60.4
9	6.0	50.1	82.0	67.2
10	6.5	63.3	89.1	78.3
11	7.0	67.5	90.4	82.0
12	7.5	70.7	91.0	83.7
13	8.0	74.4	91.9	86.4
14	9.0	77.4	92.8	87.7
15	10.0	82.6	95.3	91.1
16	12.0	90.1	98.1	95.2
17	14.0	89.5	95.0	93.2
18	16.0	91.8	97.1	94.1

Initiator conc. 1X, curing temp. 85 ºC, No inhibitor used

Table 5.19: Percent conversion (Expt. No. 2)

S. No.	Time (min)	%Conversion (w/w)		
		EHA	EGDMA	EHMA
1	0.0	0.0	0.0	0.0
2	1.0	2.7	6.3	3.9
3	1.5	17.8	33.4	22.2
4	2.0	19.7	41.5	26.8
5	2.5	29.5	51.8	37.8
6	3.0	42.2	68.9	52.7
7	3.5	53.0	77.6	65.4
8	4.0	58.7	84.9	73.6
9	4.5	63.5	85.7	76.3
10	5.0	65.3	88.6	79.6
11	6.0	73.3	90.1	83.0
12	7.0	80.4	93.4	87.9
13	8.0	84.5	93.9	90.0
14	9.0	84.5	92.4	88.8
15	10.0	90.4	96.0	93.4
16	12.0	86.7	92.6	88.9
17	14.0	89.9	95.1	91.8
18	16.0	93.5	96.4	94.4

Initiator conc. 2X, curing temp. 85 ºC, No inhibitor used

Table 5.20: Percent conversion (Expt. No. 3)

S. No.	Time (min)	%Conversion (w/w)		
		EHA	EGDMA	EHMA
1	0.0	0.0	0.0	0.0
2	1.0	4.5	21.2	9.0
3	2.0	2.7	13.0	6.0
4	2.5	57.8	85.7	70.4
5	3.0	65.6	91.6	80.8
6	3.5	79.6	94.8	89.5
7	4.0	80.4	94.7	90.7
8	4.5	88.4	96.0	94.1
9	5.0	87.5	96.2	93.5
10	5.5	91.3	97.2	94.6
11	6.0	91.5	96.6	94.8
12	6.5	93.5	97.2	95.6
13	7.0	93.3	97.1	95.6
14	9.0	94.2	96.8	96.0
15	10.0	95.0	97.6	96.1
16	12.0	95.8	98.0	96.4
17	14.0	95.9	97.7	96.4
18	16.0	96.7	97.2	97.1

Initiator conc. 3X, curing temp. 85 ºC, No inhibitor used

Table 5.21: Percent conversion (Expt. No. 4)

S. No.	Time (min)	%Conversion (w/w)		
		EHA	EGDMA	EHMA
1	0.0	0.0	0.0	0.0
2	1.0	10.6	22.9	14.7
3	1.5	11.6	28.7	17.1
4	2.0	52.1	79.7	63.3
5	2.5	60.0	88.7	74.1
6	3.0	66.8	91.1	80.2
7	3.5	75.7	93.9	86.9
8	4.0	70.7	91.1	83.9
9	4.5	81.9	95.2	91.3
10	5.0	84.3	94.5	91.3
11	5.5	83.6	94.9	91.4
12	6.0	80.0	88.4	84.2
13	6.5	93.1	97.1	95.5
14	7.5	85.7	91.9	89.0
15	8.0	94.5	97.9	96.0
16	8.5	93.8	97.7	95.5
17	9.0	94.7	97.8	95.7
18	9.5	94.2	97.8	95.6

Initiator conc. 4X, curing temp. 85 ºC, No inhibitor used

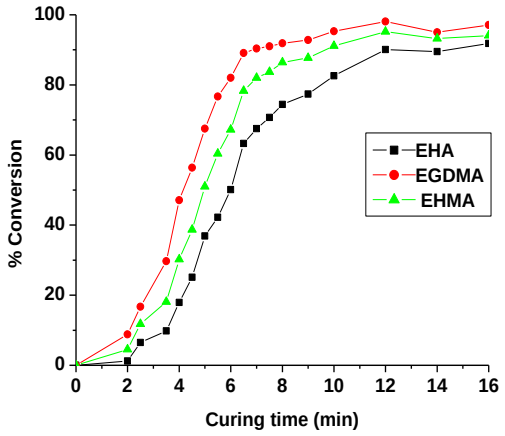

Figure 5.15: Monomer conversion profile (Expt. No. 1: initiator conc. 1X, curing temp. 85 ºC, no inhibitor used)

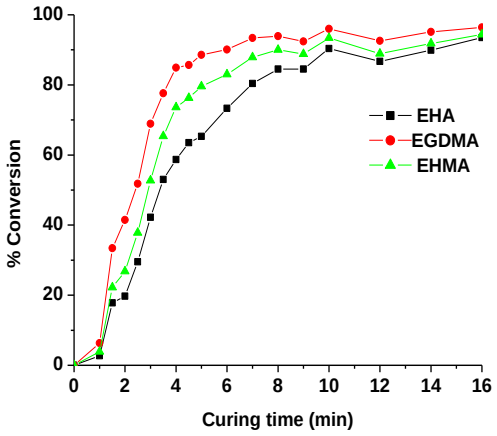

Figure 5.16: Monomer conversion profile (Expt. No. 2: initiator conc. 2X, curing temp. 85 ºC, no inhibitor used)

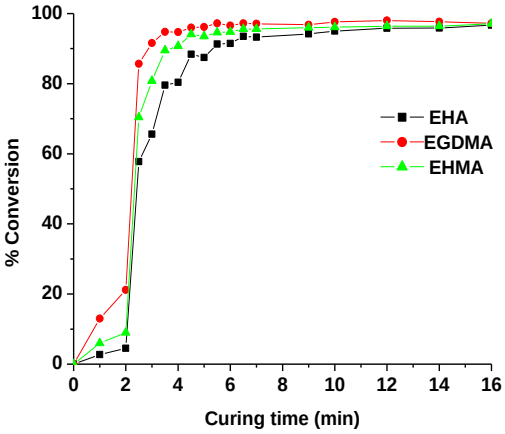

Figure 5.17: Monomer conversion profile (Expt. No. 3: initiator conc. 3X, curing temp. 85 ºC, no inhibitor used)

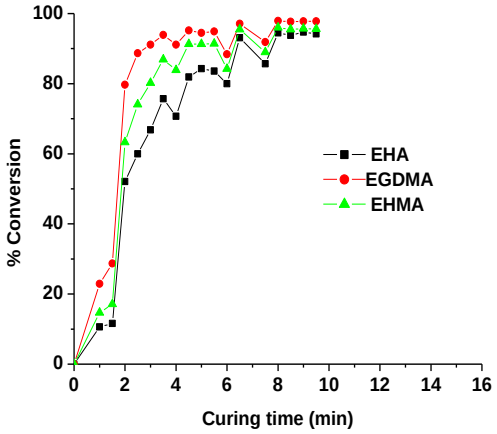

Figure 5.18: Monomer conversion profile (Expt. No. 4: initiator conc. 4X, curing temp. 85 ºC, no inhibitor used)

The time-dependent monomer conversion profiles for Expt. nos. 1, 2, 3 and 4 were generated using %conversion data given in Tables 5.18, 5.19, 5.20 and 5.21, respectively. The time-dependent monomer conversion profiles are given in Figures 5.15 - 5.18. It can be seen that as initiator concentration increased from 1X to 4X, the rate of monomer conversion increased.

Table 5.22: Percent conversion (Expt. No. 5)

S. No.	Time (min)	%Conversion (w/w)		
		EHA	EGDMA	EHMA
1	0	0.0	0.0	0.0
2	1.5	8.7	23.6	13.2
3	2.5	21.7	51.0	32.4
4	3	41.1	72.0	53.9
5	3.5	48.8	82.9	65.6
6	4	58.6	88.2	75.6
7	4.5	70.1	91.8	83.5
8	5	77.8	93.6	88.7
9	5.5	80.4	95.6	90.2
10	6.5	85.8	95.4	92.2
11	7.5	89.2	95.6	92.9
12	8	90.5	96.6	94.4
13	8.5	92.3	97.3	95.5
14	9	87.2	94.3	90.3
15	10	91.9	97.0	94.1
16	12	93.2	96.8	94.2
17	14	94.3	98.0	95.2
18	16	94.5	96.1	94.3

Initiator conc. 4X, curing temp. 85 ºC, MEHQ conc. 1Y

Table 5.23: Percent conversion (Expt. No. 6)

S. No.	Time (min)	%Conversion (w/w)		
		EHA	EGDMA	EHMA
1	0	0.0	0.0	0.0
2	1.5	6.4	22.6	11.0
3	2	6.6	27.0	12.2
4	2.5	14.3	39.9	22.8
5	3	28.2	61.3	40.0
6	3.5	52.1	80.4	63.5
7	4	61.7	88.1	74.0
8	5	74.5	92.7	84.3
9	5.5	85.0	97.0	91.8
10	6.5	87.5	96.5	92.4
11	7.5	89.6	97.4	94.1
12	8.5	93.5	100.0	96.1
13	9	90.0	96.5	92.9
14	10	88.8	96.3	92.5
15	11	92.9	98.0	95.0
16	12	93.9	97.8	95.1
17	14	94.6	97.4	95.4
18	16	95.9	100.0	96.4

Initiator conc. 4X, curing temp. 85 ºC, MEHQ conc. 2Y

Table 5.24: Percent conversion (Expt. No. 7)

S. No.	Time (min)	%Conversion (w/w)		
		EHA	EGDMA	EHMA
1	0	0.0	0.0	0.0
2	1.5	9.9	17.3	13.0
3	2.5	14.1	28.7	19.4
4	3	18.8	40.1	26.1
5	4	31.6	62.4	43.6
6	4.5	39.1	73.9	55.1
7	6	53.1	79.3	66.4
8	6.5	73.2	90.2	83.1
9	7	69.7	87.7	79.6
10	7.5	75.9	91.8	85.7
11	8.5	85.4	94.7	90.9
12	9	86.5	95.1	91.3
13	9.5	87.2	95.8	92.4
14	10	87.7	95.2	91.7
15	11	90.8	96.7	93.8
16	12	92.4	97.0	94.6
17	14	93.1	97.8	94.4
18	16	94.8	97.1	95.2

Initiator conc. 4X, curing temp. 85 ºC, MEHQ conc. 3Y

Table 5.25: Percent conversion (Expt. No. 8)

S. No.	Time (min)	%Conversion (w/w)		
		EHA	EGDMA	EHMA
1	0	0.0	0.0	0.0
2	2	6.9	21.2	10.2
3	3	10.4	33.1	18.1
4	3.5	21.1	49.1	30.0
5	4.5	42.4	69.6	54.1
6	5	54.3	81.2	67.3
7	5.5	69.1	88.4	79.2
8	7	83.8	93.8	90.2
9	7.5	85.9	94.4	91.4
10	8.5	90.0	95.6	93.5
11	9	87.4	95.2	92.0
12	9.5	90.7	96.2	93.8
13	10	91.8	96.8	94.8
14	10.5	93.5	96.9	95.6
15	11	92.3	96.3	94.6
16	14	94.4	96.9	95.8
17	15	93.8	95.9	94.9
18	16	95.0	97.3	95.6

Initiator conc. 4X, curing temp. 85 ºC, MEHQ conc. 4Y

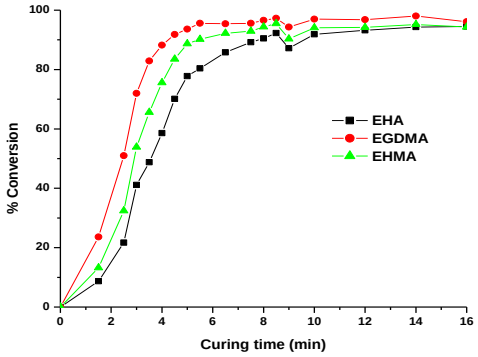

Figure 5.19: Monomer conversion profile (Expt. No. 5: initiator conc. 4X, curing temp. 85 ºC, MEHQ conc. 1Y)

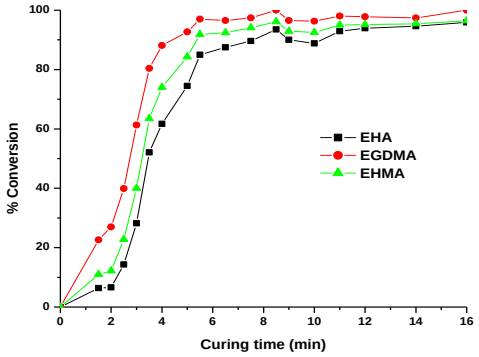

Figure 5.20: Monomer conversion profile (Expt. No. 6: initiator conc. 4X, curing temp. 85 ºC, MEHQ conc. 2Y)

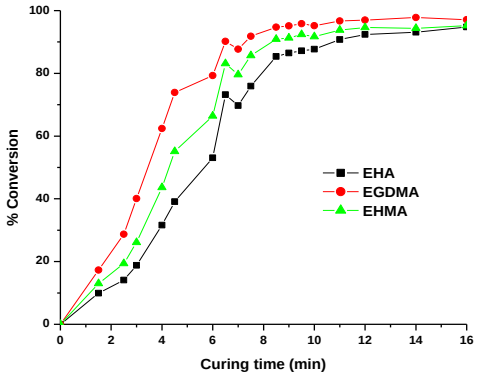

Figure 5.21: Monomer conversion profile (Expt. No. 7: initiator conc. 4X, curing temp. 85 ºC, MEHQ conc. 3Y)

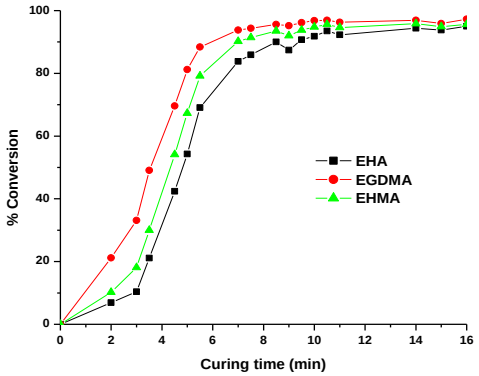

Figure 5.22: Monomer conversion profile (Expt. No. 8: initiator conc. 4X, curing temp. 85 ºC, MEHQ conc. 4Y)

The time-dependent monomer conversion profiles (Figures 5.19 - 5.22) for Expt. nos. 5, 6, 7 and 8 were generated using percent conversion data given in Tables 5.22, 5.23, 5.24 and 5.25, respectively.

These experiments show the effect of inhibitor (MEHQ) concentration on monomer conversions. It can be seen from the conversion profiles that as MEHQ concentration was increased from 1Y to 4Y, the rate of polymerisation was decreased accordingly.

Table 5.26: Percent conversion (Expt. No. 9)

S. No.	Time (min)	%Conversion (w/w)		
		EHA	EGDMA	EHMA
1	0	0.0	0.0	0.0
2	1	3.0	8.7	5.5
3	1.5	7.3	20.5	11.4
4	2	12.1	34.1	19.6
5	2.5	32.2	64.7	45.3
6	3	48.3	80.5	64.5
7	3.5	63.4	86.7	76.7
8	4	79.9	93.4	89.5
9	4.5	83.5	93.4	90.3
10	5	83.6	94.8	91.3
11	6	88.3	94.7	92.9
12	7.5	91.0	95.8	93.9
13	8	92.4	95.8	94.6
14	9	93.3	96.1	95.2
15	10	95.3	100.0	96.3
16	12	96.7	100.0	97.4
17	14	95.4	100.0	96.2
18	16	97.1	100.0	97.4

Initiator conc. 2X, curing temp. 85 ºC, PTZ conc. 0.5Y

Table 5.27: Percent conversion (Expt. No. 10)

S. No.	Time (min)	%Conversion (w/w)		
		EHA	EGDMA	EHMA
1	0	0.0	0.0	0.0
2	1	4.8	4.2	5.4
3	2	13.1	27.3	18.2
4	2.5	24.9	45.1	31.9
5	3	35.9	68.0	49.5
6	3.5	45.5	76.4	60.4
7	4	65.6	87.8	78.5
8	4.5	71.3	89.8	83.4
9	5	83.1	94.8	90.6
10	6	85.0	94.3	91.7
11	6.5	90.0	96.6	94.6
12	7.5	92.7	97.3	95.7
13	8	94.0	96.9	96.0
14	9	95.5	100.0	96.7
15	10	94.8	100.0	96.2
16	12	96.2	100.0	96.7
17	14	98.1	100.0	98.2
18	16	97.3	100.0	97.3

Initiator conc. 2X, curing temp. 85 ºC, PTZ conc. 1Y

Table 5.28: Percent conversion (Expt. No. 11)

S. No.	Time (min)	%Conversion (w/w)		
		EHA	EGDMA	EHMA
1	0	0.0	0.0	0.0
2	2	2.3	13.9	4.9
3	3	21.6	39.9	28.1
4	3.5	25.4	50.8	38.4
5	4	30.8	63.7	44.9
6	4.5	39.0	70.4	53.0
7	5	48.9	78.9	63.2
8	5.5	69.0	88.1	80.0
9	6	79.2	94.0	88.0
10	7.5	83.3	94.0	89.7
11	8	90.6	97.0	94.4
12	8.5	89.1	96.5	93.6
13	9.5	91.8	97.5	94.2
14	10	92.0	97.6	94.6
15	10.5	93.8	97.2	95.2
16	12	92.4	95.9	93.9
17	14	92.8	95.2	94.3
18	16	93.5	96.9	94.4

Initiator conc. 2X, curing temp. 85 ºC, PTZ conc. 2Y

Table 5.29: Percent conversion (Expt. No. 12)

S. No.	Time (min)	%Conversion (w/w)		
		EHA	EGDMA	EHMA
1	0	0.0	0.0	0.0
2	1	2.4	4.5	3.0
3	1.5	5.6	16.1	8.9
4	2	10.0	26.2	15.5
5	2.5	19.4	44.0	28.2
6	3	28.3	58.2	40.4
7	3.5	38.1	74.1	54.8
8	4	55.8	84.2	71.6
9	4.5	73.2	91.9	86.0
10	5	74.1	90.9	85.6
11	5.5	81.4	94.2	90.8
12	6	83.2	94.0	91.3
13	7	89.3	95.2	93.7
14	9	92.0	96.4	94.8
15	10	93.1	96.3	95.0
16	12	93.6	95.9	94.9
17	14	94.6	96.6	95.7
18	16	96.0	96.9	96.5

Initiator conc. 4X, curing temp. 85 ºC, PTZ conc. 0.5Y

Table 5.30: Percent conversion (Expt. No. 13)

S. No.	Time (min)	%Conversion (w/w)		
		EHA	EGDMA	EHMA
1	0	0.0	0.0	0.0
2	1	3.0	8.7	5.5
3	1.5	7.3	20.5	11.4
4	2	12.1	34.1	19.6
5	2.5	32.2	64.7	45.3
6	3	48.3	80.5	64.5
7	3.5	63.4	86.7	76.7
8	4	79.9	93.4	89.5
9	4.5	83.5	93.4	90.3
10	5	83.6	94.8	91.3
11	6	88.3	94.7	92.9
12	7.5	91.0	95.8	93.9
13	8	92.4	95.8	94.6
14	9	93.3	96.1	95.2
15	10	95.3	100.0	96.3
16	12	96.7	100.0	97.4
17	14	95.4	100.0	96.2
18	16	97.1	100.0	97.4

Initiator conc. 4X, curing temp. 85 ºC, PTZ conc. 1Y

Table 5.31: Percent conversion (Expt. No. 14)

S. No.	Time (min)	%Conversion (w/w)		
		EHA	EGDMA	EHMA
1	0	0.0	0.0	0.0
2	1	3.0	8.7	5.5
3	1.5	7.3	20.5	11.4
4	2	12.1	34.1	19.6
5	2.5	32.2	64.7	45.3
6	3	48.3	80.5	64.5
7	3.5	63.4	86.7	76.7
8	4	79.9	93.4	89.5
9	4.5	83.5	93.4	90.3
10	5	83.6	94.8	91.3
11	6	88.3	94.7	92.9
12	7.5	91.0	95.8	93.9
13	8	92.4	95.8	94.6
14	9	93.3	96.1	95.2
15	10	95.3	100.0	96.3
16	12	96.7	100.0	97.4
17	14	95.4	100.0	96.2
18	16	97.1	100.0	97.4

Initiator conc. 4X, curing temp. 85 ºC, PTZ conc. 2Y

The percent conversion data for Expt. nos. 9, 10 and 11 are given in Tables 5.26, 5.27 and 5.28, respectively; and the corresponding time-dependent conversion profiles are given in Figures 5.23, 5.24 and 5.25. The percent conversion data for Expt. nos. 12, 13, 14 are given in Tables 5.29, 5.30 and 5.31, respectively; and the corresponding time-dependent conversion profiles are given in Figures 5.26 - 5.28. These figures show monomer conversion profiles in the experiments wherein the concentration of phenothiazine was varied from 0.5Y to 2Y both at initiator concentration of 2X and 4X, keeping curing temperature constant at 85 ºC. The reaction details of these experiments (Expt. nos. 9 - 14) can be referred from Table 5.17.

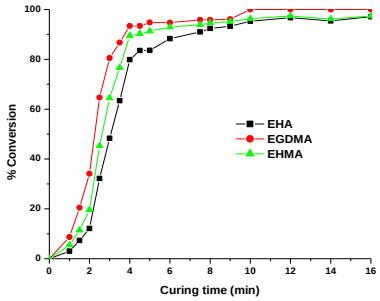

Figure 5.23: Monomer conversion profile (Expt. No. 9: initiator conc. 2X, curing temp. 85 ºC, phenothiazine conc. 0.5Y)

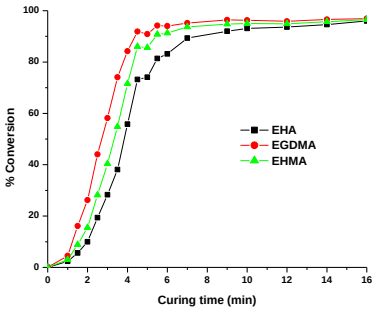

Figure 5.26: Monomer conversion profile (Expt. No. 12: initiator conc. 4X, curing temp. 85 ºC, phenothiazine conc. 0.5Y)

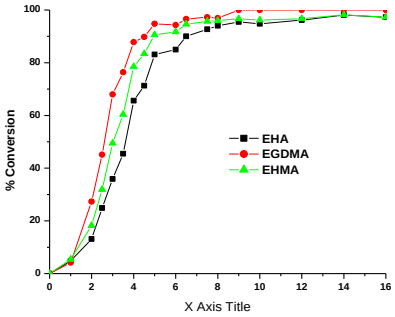

Figure 5.24: Monomer conversion profile (Expt. No. 10: initiator conc. 2X, curing temp. 85 ºC, phenothiazine conc. 1Y)

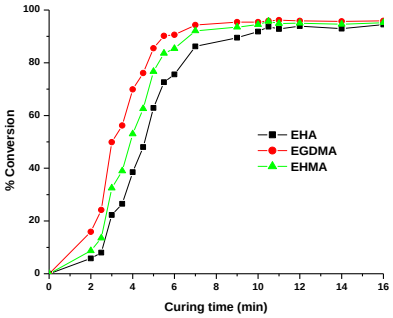

Figure 5.27: Monomer conversion profile (Expt. No. 13: initiator conc. 4X, curing temp. 85 ºC, phenothiazine conc. 1Y)

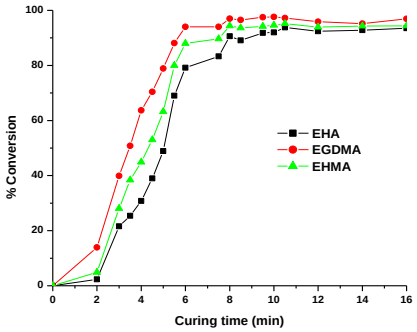

Figure 5.25: Monomer conversion profile (Expt. No. 11: initiator conc. 2X, curing temp. 85 ºC, phenothiazine conc. 2Y)

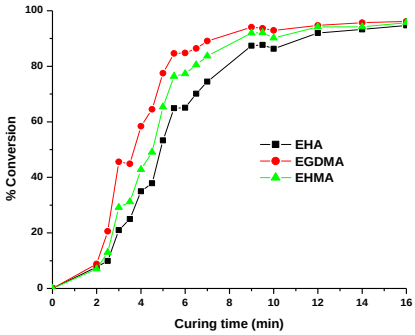

Figure 5.28: Monomer conversion profile (Expt. No. 14: initiator conc. 4X, curing temp. 85 ºC, phenothiazine conc. 2Y)

5.1.4 Summary

In this work, a sensitive and accurate analytical method was developed which was useful for estimating time-dependent monomer conversions by analysis of unreacted monomers (EHA, EGDMA and EHMA) in HIPE polymerisation reactions. The method for extraction of monomers from high internal phase emulsion was developed and validated. The solvent extraction method described here also acts as a sample clean-up, an important step of sample preparation, which isolates potential interferences from HIPE sample matrix. Method validation was carried out thoroughly by investigating system precision, method specificity, linearity (calibration studies), limit of detection (LOD), limit of quantification (LOQ), and 'method precision and accuracy'. This study established the efficiency of method and ensured that the method was valid for its intended purpose. Gauge R & R study proved that the measurement system was very stable and performed very well producing overall less variation and hence, it was quite acceptable. The usefulness of method was successfully demonstrated by estimating monomer conversions in HIPE polymerisation reactions with selected reaction parameters, as an example.

5.2 Part B: Estimation of EHA, EGDMA, and EHMA in HIPE process water

5.2.1 Introduction

Demand of water is continuously increasing due to rapid population growth and industrialisation. This may lead to water scarcity in the near future. Process industries consume huge amount of water and discharge polluted water into the environment. It has been estimated that industrial water use comprises of about 25% of all worldwide water withdrawals.[29] In an industrial setting, water is used as a coolant, for processing, washing, and/or transporting products or materials. Massive increase in water usage and changes in manufacturing methods and products may impact the amount of water withdrawn in the future.[30] Increase in wastewater disposal costs as well as more stringent environmental laws enforces process industries to cut down freshwater consumption and reuse wastewater after proper purification. Polluted water needs proper treatment before recycling and reuse. For this purpose, wastewater passes through different purification processes like coagulation, adsorption and membrane filtration.[31-39]

Reclaimed water use and industrial water recycling provide three major benefits to the industry. (1) a supplemental and reliable water source to augment or replace existing fresh water supplies; (2) reduction in the net amount of water consumed; and (3) reduction in wastewater generation and associated costs of wastewater treatment.

In polymer industries, functional absorbent materials (FAM) are made through the polymerisation of the continuous (oil) phase of a water-in-oil emulsion. Water is used extensively in maintaining the water to oil ratios in the preparation of aqueous phase and initiators. Under the present scenario, where the oil prices are on the rise and the government regulations are becoming more and more stringent, there is a need to recover

this water and recycle or reuse in the FAM making process either as it is or by way of giving pretreatments. This would eventually reflect not only in reducing the operating cost but also the load on the effluent. In this context, it is important to know the levels of residual monomers in the industrial process water before its recycle.

In the present study, an analytical method based on reverse-phase high performance liquid chromatography (RP-HPLC) was developed to measure the trace levels of EHA, EGDMA and EHMA in process water samples emanating from HIPE process used for synthesis of functional absorbent materials (FAM).

The RP-HPLC method developed in this work employs reverse-phase C_8 column for chromatographic separation with flow rate of 1 mL/min in an isocratic mode of elution having mobile phase composition of 80:20 (acetonitrile:water) and detection carried out at 220 nm. Method validation was carried out by evaluation of system precision, method specificity, linearity (calibration studies), response factors, 'limits of detection and quantification', and 'method precision and accuracy'. The usefulness of method was demonstrated by analysing the HIPE process water samples generated in lab.

5.2.2 Experimental

5.2.2.1 Materials

Methyl methacrylate (MMA), used as an internal standard, had purity > 98% and was from Aldrich (St. Louis, MO, USA). HPLC grade acetonitrile (purity > 99%) was from Merck Ltd. (Mumbai, India). The ultra pure (HPLC grade) water used for preparing mobile phase was obtained from Millipore's Milli-Q System (Milford, MA, USA).

5.2.2.2 Preparation of monomer standards

The stock solution of internal standard with concentration equal to 3.0 ppm (w/v) was prepared by dissolving requisite amount of methyl methacrylate (MMA) in requisite volume of mobile phase taken in a class "A" volumetric flask.

The stock solution of monomer standards was prepared by taking requisite amounts of EHA, EGDMA and EHMA and dissolved in an internal standard solution so that the concentration of MMA (internal standard) in each monomer standard remains the same (i.e. 3.00 ppm). The working standards of monomers in the required range (EHA: 0.50 - 8.00 ppm, EGDMA: 0.02 - 2.00 ppm, and EHMA: 0.20 - 6.00 ppm) were prepared by serial dilution of standard stock solution using internal standard solution. These standard solutions were used to study the linearity of monomers in their specified ranges.

5.2.2.3 Preparation of HIPE process water samples

In all 10 HIPE process water samples were prepared. The individual HIPEs (prepared as per procedure given in Section 5.1.2.3, A) were polymerised at 65 ^{o}C by keeping the glass reactor flaks in a constant temperature water bath for a period of 10 hours. After this, the polymer product formed in each flask was squeezed dry to ensure that no residual monomers were trapped in the porous polymeric matrix. The dried polymer product was discarded and thus, HIPE process water samples (each containing 3.0 ppm of internal standard, MMA, added externally) were ready for measurement of traces of residual monomers in them.

5.2.2.4 Equipment and parameters of analysis

All the analyses were carried out on a HPLC system (Thermo Separation Products, USA) equipped with a P4000 Quaternary HPLC pump, an AS100 auto sampler

and a Spectra FOCUS UV detector. The column temperature was kept at ambient conditions. The mobile phase was degassed by an ultrasonic bath (Transsonic T660/H, Elma Hans Schmidbauer GmbH & Co., Singen, Germany) and with helium purging device (TSP) attached with P4000 pump. The chromatograms were extracted at 220 nm and the sampling period was kept as 20 ms. Data acquisition and peak integration were performed with the help of PC1000 system software.

A reverse phase C_8 column (250 × 4.6 mm ID, 5 μm particle size, Zorbax, Hewlett Packard, USA) was used for executing chromatographic separations. The mobile phase was prepared daily in ultrapure (HPLC grade) water and filtered through 0.2 μm Nylon membrane filter (Whatman) before use. The HIPE process water samples (prepared as per procedure given in Section 5.2.2.3) were injected into HPLC system with chromatographic parameters as discussed above. In all 10 samples (sample codes presented in Table 5.32) were analysed.

Table 5.32: Sample codes for HIPE process water samples

Sample No.	Sample code
1	HPWS-1
2	HPWS-2
3	HPWS-3
4	HPWS-4
5	HPWS-5
6	HPWS-6
7	HPWS-7
8	HPWS-8
9	HPWS-9
10	HPWS-10

HPWS: HIPE process water sample

5.2.3 Results and discussion

5.2.3.1 Method development and optimisation

5.2.3.1.1 Chromatographic system suitability

The system suitability test represents an integral part of the method and is used to ensure the adequate performance of the chosen chromatographic system. Factors that can affect chromatographic behaviour include mobile phase composition, temperature, flow rate and column length, and stationary phase characteristics such as porosity, particle size and type, specific surface area, and, in the case of reverse-phase supports, the type of chemical modification, carbon loading, and end-capping. Efficiency, capacity factor, and symmetry factor are the parameters that are normally used in assessing the column performance. These parameters have been described in Chapter 1.

5.2.3.1.2 Column performance test

Prior to its use, the RP column ZORBAX SB-C8 (Hewlett Packard, USA) was tested for its performance using toluene as a test sample. The chromatographic run was performed as per conditions given in Table 5.33. The column performance was assessed by evaluating retention time, capacity factor, symmetry factor, number of theoretical plates (N) and HETP (mm). The significance and the equations for calculation of these parameters have been presented in Chapter 1 (Sections 1.5.2.3, 1.5.2.4, 1.5.2.5 and 1.5.2.7). The obtained values of these parameters are tabulated in Table 5.34, along with the certified values of column test report provided by column manufacturer.

Table 5.33: **Chromatographic conditions for testing column performance**

S. No.	Parameter	Values/conditions
1	Mobile Phase	80 : 20 (Methanol : Water)
2	Detection wavelength	254 nm
3	Column flow rate	1 mL/min
4	Column temperature	Ambient
5	Test sample (Toluene) concentration	800 μg/mL

Table 5.34: The values of column testing parameters

Column test (Values of parameters)	Ret. time (t_R, min)	Capacity factor (K')	Symmetry factor (A_s)	Number of theoretical plates (N)	*HETP* (mm)
Certified by manufacturer	4.567	1.492	1.1673	19874	0.01260
Practically calculated in our lab	4.303	1.349	1.1126	12779	0.00783

It may be noted that the practically calculated values for retention time, capacity factor and symmetry factor matched reasonably well with the certified values. However, the values of *N* and *HETP* were found to be less than the certified values. The diminished number of theoretical plates observed for the HPLC column might be due to its longer use; however, an efficient separation could be worked out on this column for the present work.

5.2.3.1.3 Optimisation of chromatographic parameters

In order to select a suitable chromatographic mode, the physico-chemical properties of analytes should be studied. Since EHA, EGDMA, EHMA are hydrophobic in nature due to their long alkyl chain backbone (Figures 5.3 – 5.5), they show more retention on reverse stationary phase column. Since these monomers are non-polar with Mw < 2000, the reverse phase chromatography was chosen where a C_8 column was

employed, as a suitable mode. The experiments were conducted to optimise the composition of mobile phase to achieve baseline separation of the three monomers (EHA, EGDMA and EHMA) and the internal standard (MMA) along with the separation of other interfering components from sample matrix. To achieve complete resolution of chromatographic peaks, mobile phase with varying compositions of 'methanol: water' and 'acetonitrile: water' were investigated. The best separation was achieved with mobile phase composition of "acetonitrile: water (80: 20, v/v)" with a flow rate of 1 mL/ min at ambient temperature. To optimise the detection wavelength, UV detector was set to scan in the range of 200 to 300 nm. The detection at 200 nm showed the highest peak response but produced a large noise, because mobile phase used in this analysis absorbed UV light at (and below) 200 nm (due to acetonitrile present in mobile phase whose UV cutoff value is 190 nm). The signal to noise ratio at 220 nm was found to be acceptable for the present trace analysis and hence, this was chosen as the detection wavelength.

All the FAM process water samples were analysed by performing isocratic elution under the given optimised chromatographic conditions.

5.2.3.1.4 Internal standard method

Since the level of monomers to be measured in the present problem is in traces (less than 10 ppm), the internal standard method was preferred to enhance and ensure the accuracy of measurement though in HPLC (which uses higher injection volumes, typically 40-60 µL), internal standard method is generally not desirable.

A suitable internal standard to be used in HPLC analysis must fulfill the following conditions:

1. It should mix easily in the sample mixture.

2. It should not react with any of the components in the sample or the mobile phase.
3. It should elute close to the components of interest.
4. It should preferably have similar structure or functional groups as that of analyte components so that it will give comparative absorption at the wavelength used for those particular analyte components.

Methyl methacrylate (MMA) was chosen as an internal standard in this method, since it meets all the criteria of an ideal internal standard for this work, as mentioned earlier. The purpose of internal standard and the quantification of analyte using internal standard method have already been described in section 5.1.3.1.3.

5.2.3.2 Method validation

5.2.3.2.1 System precision

System precision was checked by assessment of repeatability of retention time and peak area by making 10 replicate injections of a test sample comprising a mixture of EHA, EGDMA, EHMA and MMA (internal standard). The data on retention time variation is given in Table 5.35. This study showed that the retention times were quite repeatable, as evident from the low values of standard deviation and %RSD (> 0.2) obtained for all the components under study.

The data on peak area variation is given in Table 5.36. It may be noted that the variation in peak area of individual components was found to be greater (%RSD $\geq$ 5.6); however, when the ratio of peak area of analyte to the internal standard (IS) was calculated, the variation was reduced considerably (%RSD $\leq$ 2.5), which was adequate for the present analysis. This experiment demonstrated that the internal standard method

preferred in this analysis was useful and helped enhancing accuracy of measurement which was important for present trace analysis.

Table 5.35: Retention time repeatability

Inj. No.	Retention time (min)			
	MMA (IS)	**EGDMA**	**EHA**	**EHMA**
1	3.249	3.619	6.094	7.291
2	3.247	3.617	6.093	7.275
3	3.244	3.612	6.078	7.267
4	3.244	3.611	6.072	7.260
5	3.242	3.609	6.070	7.256
6	3.241	3.615	6.072	7.254
7	3.242	3.614	6.069	7.234
8	3.243	3.617	6.072	7.262
9	3.241	3.615	6.075	7.278
10	3.242	3.618	6.084	7.286
Mean	3.244	3.615	6.078	7.266
SD	0.003	0.003	0.009	0.017
%RSD	0.1	0.1	0.1	0.2

Table 5.36: Peak area repeatability

Inj. No.	Peak area (counts)				Peak area ratio		
	MMA (IS)	**EGDMA**	**EHA**	**EHMA**	***EGDMA**	***EHA**	***EHMA**
1	135425	102528	20703	20814	0.7571	0.1529	0.1537
2	133504	101275	20428	20564	0.7586	0.1530	0.1540
3	155738	121694	22760	23838	0.7814	0.1461	0.1531
4	149066	116843	21872	22916	0.7838	0.1467	0.1537
5	143520	108645	20992	22103	0.7570	0.1463	0.1540
6	133814	100897	18474	20578	0.7540	0.1455	0.1538
7	147860	111744	21566	22925	0.7557	0.1459	0.1550
8	136578	104396	21208	21702	0.7644	0.1553	0.1589
9	137865	106365	19164	21705	0.7715	0.1463	0.1574
10	149982	112598	21865	23678	0.7507	0.1458	0.1579
Mean	142335	109255	20903	22035	0.7634	0.1484	0.1552
SD	7936	6925	1293	1233	0.0116	0.0038	0.0021
%RSD	5.6	6.3	6.2	5.6	1.5	2.5	1.4

***EGDMA**: Peak area of EGDMA/ Peak area of MMA (IS)
***EHA**: Peak area of EHA/Peak area of MMA (IS)
***EHMA**: Peak area of EHMA/ Peak area of MMA (IS

5.2.3.2.2 Specificity

The specificity of the method was tested for EHA, EGDMA, EHMA and internal standard (MMA) used in analysis. It showed good resolution of all the peaks with an acceptable repeatability of retention times as evident from data given in Table 5.35. In addition, the peak purity test conducted with diode array detector showed that no peak was attributable to more than one component. It was also ensured that there was no interference of any other components from sample matrix. The representative chromatogram showing separation of EHA, EGDMA and EHMA, and internal standard (MMA) is given in Figure 5.29.

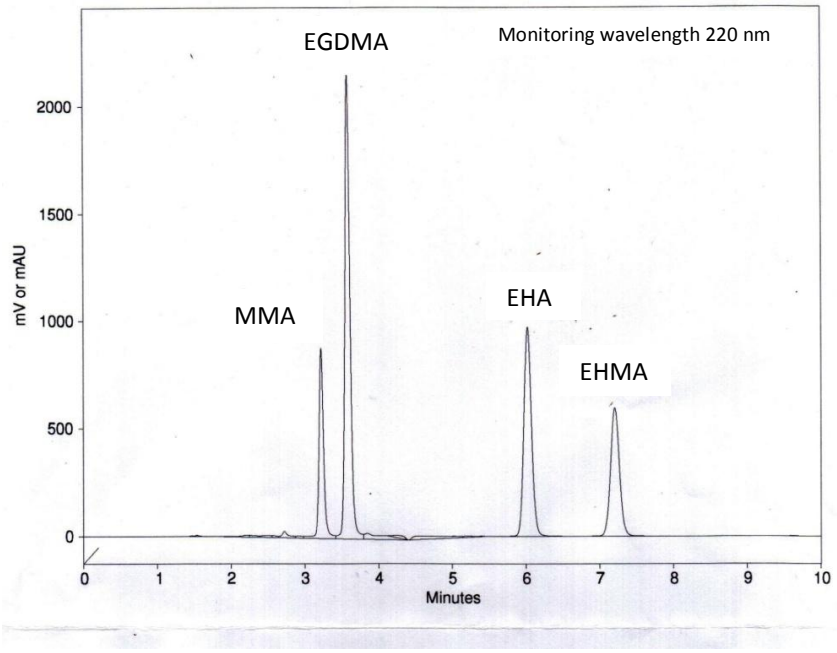

Figure 5.29: Chromatogram showing separation of EHA, EGDMA, EHMA and MMA (IS)

5.2.3.2.3 Linearity (calibration studies)

The linearity was assessed by running analytical standards under optimised chromatographic conditions. The calibration standards (each having mixture of EHA, EGDMA, EHMA and an internal standard) in the given range (Table 5.37) were prepared

as described in section 5.2.2.2 and injected into chromatographic system. The calibration curve for a particular monomer was derived by plotting peak area ratio (ratio of peak area of analyte to internal standard) against concentration ratio (ratio of concentration of analyte to internal standard). The regression equation was obtained as $y = mx$, where y was the peak area ratio of analyte to internal standard (A_S/A_{IS}), m was the slope, and x was the ratio of concentration (ppm, w/v) of analyte to internal standard (C_S/C_{IS}). The values of $R^2 \geq 0.9914$ are quite acceptable for the calibration of monomers in the range of low ppm levels. The range of monomer concentrations, regression equations and values of R^2 are given in Table 5.37. The calibration curves for EHA, EGDMA and EHMA are given in Figures 5.30, 5.31 and 5.32, respectively.

Table 5.37: Linearity data of monomers

Monomer	Range (ppm, w/v)	Regression equation	R^2 value
EHA	0.50 - 8.00	$y = 0.1378x$	0.9965
EGDMA	0.02 - 2.00	$y = 1.1906x$	0.9969
EHMA	0.20 - 6.00	$y = 0.5761x$	0.9914

IS (MMA) conc. in each standard solution was equal to 3.0 ppm (w/v)

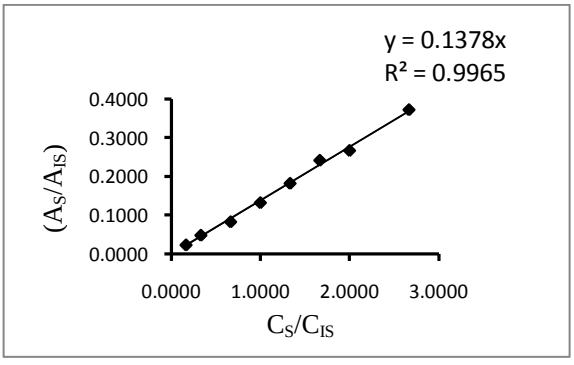

Figure 5.30: Calibration curve for EHA (A_S/A_{IS}: Peak area of EHA/Peak area of IS, C_S/C_{IS}: Conc. of EHA/Conc. of IS)

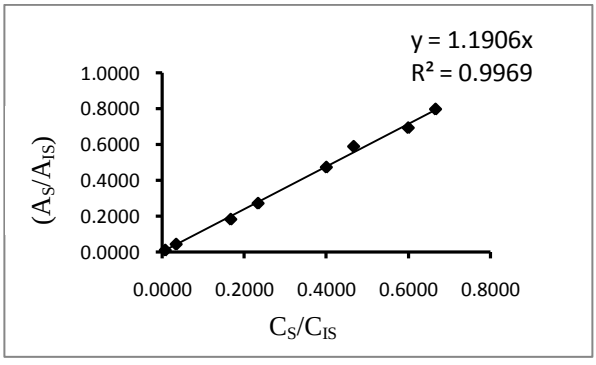

Figure 5.31: Calibration curve for EGDMA (A_S/A_{IS}: Peak area of EGDMA/Peak area of IS, C_S/C_{IS}: Conc. of EGDMA/Conc. of IS)

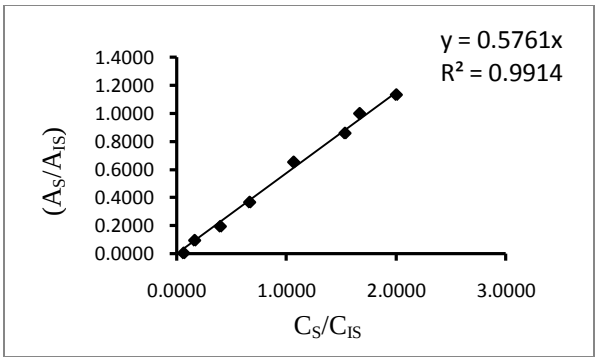

Figure 5.32: Calibration curve for EHMA (A_S/A_{IS}: Peak area of EHMA/Peak area of IS, C_S/C_{IS}: Conc. of EHMA/ Conc. of IS)

5.2.3.2.4 Response factors

The response factors (R_f) for EHA, EGDMA and EHMA were calculated from calibration data using the following equation:

$$R_f = \left(\frac{C_S}{A_S}\right) \text{ x } \left(\frac{A_{IS}}{C_{IS}}\right) \qquad 5.4$$

where, C_S is the concentration (ppm, w/v) of monomer standard, C_{IS} is the concentration (ppm, w/v) of internal standard (MMA), A_S is the peak area of monomer standard and A_{IS} is the peak area of internal standard.

Table 5.38: Response factors data

S. No.	EHA			EGDMA			EHMA		
	C_S/C_{IS}	A_{IS}/A_S	R_f	C_S/C_{IS}	A_{IS}/A_S	R_f	C_S/C_{IS}	A_{IS}/A_S	R_f
1	0.1667	42.6794	7.1132	0.0067	125.0845	0.8339	0.0667	26.8470	1.7898
2	0.3333	20.6596	6.8865	0.0333	24.1623	0.8054	0.1667	10.6413	1.7736
3	0.6667	12.0516	8.0344	0.1667	5.5265	0.9211	0.4000	5.1667	2.0667
4	1.0000	7.5618	7.5618	0.2333	3.6963	0.8625	0.6667	2.7356	1.8238
5	1.3333	5.4913	7.3217	0.4000	2.1148	0.8459	1.0667	1.6462	1.7559
6	1.6667	4.1415	6.9025	0.4667	1.6974	0.7921	1.5333	1.0559	1.6191
7	2.0000	3.7503	7.5006	0.6000	1.4421	0.8653	1.6667	1.0854	1.8090
8	2.6667	2.6902	7.1738	0.6667	1.2530	0.8353	2.0000	0.8417	1.6835
		Mean, R_f	7.3118		**Mean, R_f**	0.8452		**Mean, R_f**	1.7902
		SD	0.383		**SD**	0.0398		**SD**	0.131
		%RSD	5.2		**%RSD**	4.7		**%RSD**	7.3

The calibration and statistical data for replicate measurements (*n = 8*) of response factors are given in Table 5.38. The values of %RSD (*n = 8*) for EHA, EGDMA and EHMA were found to be 5.2, 4.7 and 7.3, respectively, which are acceptable for the calibration range having higher concentration limit less than 10.0 ppm. The mean R_f values given in Table 5.38 were used to determine corresponding monomer concentration (ppm, w/v) in a process water sample.

5.2.3.2.5 Limits of detection and quantification

The limits of detection and quantification (LOD and LOQ) were determined by signal-to-noise ratio method.[26] The LOD (three times signal-to-noise) and LOQ (10 times signal-to-noise) for individual monomers were calculated from the corresponding slope values of regression equations (Table 5.40) that were based on peak heights. The regression equations based on peak height (regressed in the same range as for linearity studies) were used for calculating LOD and LOQ, since peak height is a better parameter in trace analysis.

Table 5.39: LOD and LOQ data

Monomer	LOD (ppm, w/v)	LOQ (ppm, w/v)
EHA	0.02	0.40
EGDMA	0.009	0.030
EHMA	0.04	0.12

Table 5.40: Regression equations based on peak heights

Monomer	Regression equation	R^2 value
EHA	$y = 0.0816x$	0.9990
EGDMA	$y = 1.0796x$	0.9998
EHMA	$y = 0.2646x$	0.9989

$y = H_S/H_{IS}$ (i.e. Peak height of monomer std./Peak height of IS)
$x = C_S/C_{IS}$ (i.e. Conc. of monomer std. /Conc. of IS)

The regression equations were derived (given in Table 5.40) by plotting the peak height ratio (ratio of peak height of analyte to internal standard) against concentration ratio (ratio of conc. of analyte to internal standard). The data on LOD and LOQ are presented in Table 5.39. It can be seen from LOD and LOQ that the present method is very sensitive and can detect, for example, levels of EGDMA as low as 0.009 ppm (i.e. 9 ppb).

Analyte molecule having unsaturated bonds or free nonbonding electrons that can absorb radiation of relatively low energy are called chromophores. The number of chromophores or degree of conjugation in a particular analyte determines its response to UV detector. Among EHA, EGDMA and EHMA (structures in Figures 5.3 - 5.5), EGDMA has the highest degree of conjugation and hence, shows highest response to UV detector. EHA and EHMA have almost similar structures except EHMA having one more methyl group than EHA. Between these two, EHMA shows more UV response than EHA, because the extent of conjugation in EHMA is more than EHA due to the hyperconjugation effect exhibited by methyl group attached to carbon atom adjacent to carbonyl group. This explanation corroborates with the values of response factors (Table 5.38), LOD/LOQ (Table 5.39) and slopes of regression equations (Tables 5.37 and 5.40) obtained for EHA, EGDMA and EHMA.

5.2.3.2.6 Method precision and accuracy

The method precision (intra-day repeatability) and intermediate precision (inter-day repeatability) were tested by multiple analyses of synthetic samples containing mixtures of EHA, EGDMA and EHMA at low, medium and high levels. The samples were prepared by taking known amounts of monomers dissolved in mobile phase, which contained internal standard, MMA (3.0 ppm, w/v). All individual samples were subjected to GC runs for assay analysis. The method precision (intra-day) was evaluated by replicate analyses of samples (n = 5) on the same day and inter-day precision on five different days. The %RSD values and relative errors for accuracy (Table 5.41) show that the method precision and accuracy is good for analysis of monomers at trace levels.

Table 5.41: Method precision and accuracy

Synthetic sample		Conc. of monomer in synthetic sample (ppm)	INTRA-DAY, *n = 5*			INTER-DAY, *n = 5*		
			A ± SD[a]	**Precision %RSD[b]**	**Acc.[c]**	**A ± SD[a]**	**Precision %RSD[b]**	**Acc.[c]**
1	EHA	2.02	1.90 ± 0.14	7.1	- 5.7	1.91 ± 0.15	7.8	- 5.3
	EGDMA	0.50	0.51 ± 0.03	5.2	1.2	0.51 ± 0.02	4.3	2.4
	EHMA	0.62	0.62 ± 0.03	4.5	- 0.3	0.61 ± 0.03	4.5	- 0.5
2	EHA	4.94	5.02 ± 0.18	3.5	1.7	5.04 ± 0.14	0.8	- 0.8
	EGDMA	1.26	1.22 ± 0.03	2.6	- 3.2	1.21 ± 0.03	2.2	- 3.7
	EHMA	1.95	2.07 ± 0.10	4.6	6.1	1.99 ± 0.11	5.6	2.0
3	EHA	7.02	6.98 ± 0.09	1.3	- 0.5	6.99 ± 0.12	1.7	- 0.6
	EGDMA	2.00	1.96 ± 0.11	5.7	- 2.1	1.98 ± 0.12	6.2	- 1.2
	EHMA	5.52	5.54 ± 0.04	0.7	0.3	5.58 ± 0.12	2.2	1.1

A: Mean of determined concentration (ppm, w/v), *n = 5*, [a]**SD**: Standard deviation, [b]**RSD**: Relative standard deviation, [c]**Acc.**: Accuracy (i.e. %Relative error) = [(Determined conc. - Added conc.) / Added conc.] × 100

5.2.4 Analysis of HIPE process water samples

The HIPE process water samples (prepared as per procedure described in Section 5.2.2.3) were injected into HPLC system and chromatographic data was collected. From this data, the amounts of monomers were calculated using following equation:

$$C_S = \left[\frac{A_S}{A_{IS}}\right] \text{ x } R_f \text{ x } C_{IS} \qquad 5.5$$

where, C_S is the concentration (ppm, w/v) of analyte (EHA, EGDMA or EHMA) to be determined, A_S is the peak area of analyte, A_{IS} is the peak area of internal standard (MMA), C_{IS} the concentration (ppm, w/v) of internal standard (MMA) added into sample and R_f is the mean value of response factor of corresponding analyte. The results of analysis of process water samples are tabulated in Table 5.42.

Table 5.42: Results of HIPE process water samples

Sample No.	Sample code	Concentration (ppm, w/v)		
		EHA	EGDMA	EHMA
1	HPWS-1	0.80	0.02	0.21
2	HPWS-2	2.01	0.43	5.74
3	HPWS-3	1.91	0.41	3.84
4	HPWS-4	3.81	0.77	2.48
5	HPWS-5	1.24	0.52	2.24
6	HPWS-6	1.76	0.48	1.89
7	HPWS-7	2.54	0.62	2.02
8	HPWS-8	3.12	0.50	2.76
9	HPWS-9	1.48	0.68	1.12
10	HPWS-10	1.64	0.43	1.08

HPWS: HIPE process water sample

5.2.5 Summary

A new HPLC-UV method was developed and validated for estimation of EHA, EGDMA and EHMA at low ppm levels in HIPE process water. This method is fast, accurate and very sensitive. The HIPE process water samples were generated in our lab and analysed for the contents of monomers (EHA, EGDMA and EHMA) present at trace levels.

The present method is useful to assess the quality of industrial process water before being recycled in FAM making process which is essential not only for reducing the operating cost and also the load on the effluent.

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Chapter 6

ESTIMATION OF MONOMERS IN STYRENIC HIPEs

6.1 Introduction

Chapter 5 described the development of analytical method and its application for estimation of monomer conversions in acrylic HIPEs. This chapter deals with the analytical method development for estimation of monomer conversions in styrenic HIPEs.

Most poly(HIPE) polymers are prepared based on a styrene (St)/divinylbenzene (DVB) monomer mixture. Porous St-DVB beads are converted to or used as adsorbents,[1] support for catalysts,[2,3] anion exchangers,[4,5] cation-exchangers,[6,7]etc. Cation-exchangers are among the most important commercial products derived from St-DVB for use in chromatographic separations[8] and as acid catalysts[9]. The cation-exchangers are obtained by sulphonation, i.e., by the introduction of sulphonic acid ($–SO_3H$) groups with exchangeable protons onto benzene rings of monomer units in St-DVB.[10,11]

The PolyHIPE polymers derived from St-DVB have overall similar mechanical properties to gas-blown PS foams, except that the smaller size and increased spherical symmetry of the cells result in higher compressive strengths.[12] They are, however, rather hard and brittle, due to the relatively high glass transition temperature (Tg) of polystyrene (100 °C). The mechanical properties of PolyHIPE polymers might be improved by introducing an elastomeric comonomer to reduce the overall Tg and thus lend some flexibility to the material. This comonomer must also be sufficiently hydrophobic to form stable HIPEs, should copolymerise readily with styrene and DVB and preferentially should be available commercially at low cost. Both 2-ethylhexyl acrylate (EHA) and 2-ethylhexyl methacrylate (EHMA) fulfill these requirements. Tg values of the

homopolymers are 223 and 263 K, respectively.[13] Acrylates giving low Tg polymers are often used as comonomers to plasticise harder materials.[14]

St-DVB-EHA HIPE systems are important for certain applications, e.g. PolyHIPE membranes prepared from such HIPEs are very promising for the fabrication of sensors with integrated separation in which the ion and electron transport can be effectively controlled by functional, porous PolyHIPE membranes that are impregnated with conducting particles, electron mediators and enzymes.[15] In order to study the polymerisation reaction kinetics for this system, it was necessary to develop a method which can estimate monomer conversions by analysis of unreacted monomers.

In the present study, an analytical method based on GC-FID technique was established for measurement of styrene, DVB and EHA. The protocol for extraction of monomers for acrylic HIPE system (described in chapter 5) works very well for the present styrenic HIPE system as well. Cyclohexane showed excellent selectivity for styrene, DVB and EHA due to their more non-polar nature. The monomer extraction efficiency was assessed by determining recovery (96.7 - 104.9%), which was appropriate for the present work.

The commercial divinylbenzene, used in this work, is a mixture of 80% DVB and 20% ethylvinylbenzene (EVB). Both DVB and EVB exist as their meta- and para-isomers. DVB is manufactured by the thermal dehydrogenation of isomeric diethylbenzenes. Under synthesis conditions, o-divinylbenzene converts to naphthalene and thus is not a component of the usual mixtures of DVB.[16] In the present work, DVB was reported by summing together the detector responses of all its constituents, viz. m-DVB, p-DVB, m-EVB and p-EVB.

Method validation was carried out thoroughly by studying various parameters such as system precision, specificity, linearity (calibration studies), limit of detection (LOD), limit of quantification (LOQ) and 'method precision and accuracy'. Gauge R & R study was conducted to assess the performance of method.

After the method was developed and validated, its usefulness was demonstrated by estimating time-dependent monomer conversions in HIPE polymerisation reactions that used thermal and redox initiator systems. The thermal initiators used in this work included sodium persulphate and cumene hydroperoxide; and redox initiator systems included 'sodium persulphate: sodium dithionite', 'sodium persulphate: ascorbic acid', 'cumene hydroperoxide: ascorbic acid', all used in 1:1 molar proportion.

6.2 Experimental

6.2.1 Materials

Synthesis grade styrene was purchased from Encore Chemicals (Mumbai, India). Divinylbenzene (purity 80%), butyl acrylate (BA, purity ≥ 99%), cumene hydroperoxide (assay 88%) and monomethyl ether hydroquinone (purity 98%) were from Aldrich (St. Louis, MO, USA). Sodium dithionite (assay 85%) and ascorbic acid (assay 99%) were from Loba Chemie (Mumbai, India). The source of other chemicals and gases are presented in Section 5.1.2.1.

6.2.2 Preparation of monomer standards

The internal standard (IS) solution with concentration equal to 2.0 mg mL^{-1} (i.e. 2000 ppm, w/v) was prepared by dissolving 4000.0 mg of butyl acrylate in 2 L of cyclohexane. This IS solution was stored in the refrigerator at 4 ºC when not in use and was found to remain stable for more than two weeks. For calibration studies, 8 nos. of

analytical standards each containing a mixture of styrene, DVB and EHA were prepared by dissolving requisite amounts of monomers in 25 mL of IS solution taken in a Class "A" volumetric flask. The concentration range for individual monomers was chosen by taking into consideration a batch size of 0.917 g (comprising of 225.0 mg of styrene, 168.0 mg of DVB and 524.0 mg of EHA) and a total volume of extraction solvent, i.e. 100 mL. The concentrations of calibration standards were prepared within a given range such that it will cover the levels of monomers in concordance with their conversion from zero (i.e. no conversion at curing start time) to a maximum of 98%.

6.2.3 Preparation of HIPE reaction samples

HIPE was prepared with a volume to weight ratio of aqueous to oil phase of 25:1. A stock solution of organic phase was prepared in bulk such that 1 mL of it contained 225.0 mg (2.1603 mM) of styrene, 168.0 mg (1.2904 mM) of DVB, 524.0 mg (2.8434 mM) of EHA and a mixture of surfactants: 0.0596 g (6.5% of oil phase) of Span-80 and 0.0073 g (0.79% of oil phase) of Arquad 2HT-75.

The individual HIPE samples were prepared by dispensing 1 mL of organic phase from stock solution and transferring in a glass reactor flask with Ruston turbine stirrer placed in it. An aqueous phase containing 4% calcium chloride was added drop wise to this mixture under continuous stirring at 1400 rpm to form water-in-oil (W/O) high internal phase emulsion (HIPE).

In order to generate time-dependent monomer conversions, the HIPEs were cured individually, with incremental curing times, using thermal and redox initiators (added as 1 mL aqueous solution of requisite amounts to HIPE). The details of initiator/redox systems are presented in Table 6.1. The time at which the addition of

initiator(s) completed was noted as curing start time. The reactor flask was then immediately kept in a water bath for curing at 65 ^{o}C.

Table 6.1: Details of thermal and redox initiator systems

1	Inorganic initiator	*Sodium persulphate
2	Organic initiator	*Cumene hydroperoxide
3	Redox system (inorganic: inorganic)	*Sodium persulphate: Sodium dithionite (1:1, molar composition)
4	Redox system (organic: organic)	*Cumene hydroperoxide: Ascorbic acid (1:1, molar composition)
5	Redox system (inorganic: organic)	*Sodium persulphate: Ascorbic acid (1:1, molar composition)

* 0.05 wt% of water phase

HIPE reaction quenching and monomer extraction were accomplished by same procedure as described for acrylic HIPE system in Chapter 5 (Section 5.1.2.3, B). After extraction, the reactor flask was sonicated for 5-10 min to release traces of monomers that were probably trapped in porous polymer matrix. Approximately 10 mL of an aliquot from upper organic layer was removed to which anhydrous magnesium sulphate (1-2 g) was added to capture the traces of water. After magnesium sulphate settled down, the sample was transferred to a GC autosampler vial for injecting 1.0 μL of sample solution into GC system for measurement of unreacted monomers.

6.2.4 Equipment and parameters of analysis

All the chromatographic analyses were performed on Shimadzu GC-2010 Chromatograph (Shimadzu Corporation, Kyoto, Japan), equipped with a flame ionisation detector. The GC separation was carried out on DB-1 capillary column (J & W Scientific, Folsom, CA, USA) having dimension of 100 m (L) × 0.25 mm (i.d.) and a film thickness of 0.5 μm. The temperature program was optimised for the baseline separation of styrene,

DVB (including meta- and para- isomers of DVB and EVB), EHA and an internal standard (BA) used in this analysis. The operating conditions were set as follows:

Nitrogen was used as carrier gas with flow rate of 1.78 mL/min. Hydrogen and zero air flow were maintained at 40 and 400 mL/min, respectively. The column temperature program was set to start from 180 ºC and increased to 210 ºC, with a ramp rate of 5 ºC/min, and held there for 4 min, thus making total chromatographic run time equal to 10 min). The injector port (PTV1) and detector (FID) temperature were kept at 235 and 280 ºC, respectively. The samples (1.0 μL) were injected using HTA autosampler which had split injection mode with split ratio of 20:1. De-activated glass wool was used in glass injection liner to retain non-volatile components in sample, if present.

6.3 Results and Discussion

6.3.1 Method development and optimisation

The primary objective of the present work was to develop a simple, reliable, and sensitive analytical method for the accurate measurement of styrene, DVB and EHA to estimate their conversions in HIPE polymerisation reactions. The selection and configuration of equipment, experimental conditions for sample preparation (solvent extraction of monomers) and parameters for GC column oven temperature programming were optimised for achieving baseline separation of peaks of interest.

6.3.1.1 Selection of chromatographic technique

The monomers, styrene, DVB (which includes m-DVB, p-DVB, m-EVB, and p-EVB) and EHA are hydrophobic in nature and therefore, show greater retention on reverse-phase (HPLC) column. Secondly, they have a higher degree of conjugation

(Figures 6.1 - 6.7) and hence show greater sensitivity towards UV detector. Hence, RP-HPLC technique with UV detector was suitable for present work. However, these monomers can be analysed by GC as well, because they are thermally stable and volatile, and show better sensitivity to FID due to their relatively larger carbon content. If both GC and HPLC options are feasible for a particular analysis, then GC should be the first choice, because the separation power of GC is about 100 times higher than HPLC. Also, GC technique is relatively easy to handle and economical since it does not employ costly organic solvents which are required for HPLC.

The chemical structures of analytes (including an internal standard, BA) to be separated are given in Figures 6.1 - 6.7.

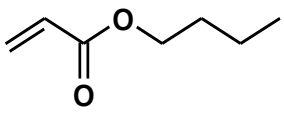

Figure 6.1: Chemical structure of butyl acrylate

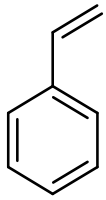

Figure 6.2: Chemical structure of styrene

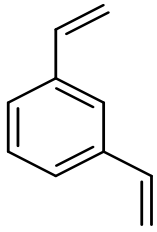

Figure 6.3: Chemical structure of m-divinylbenzene

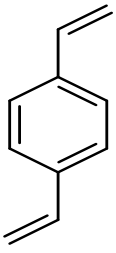

Figure 6.4: Chemical structure of p-divinylbenzene

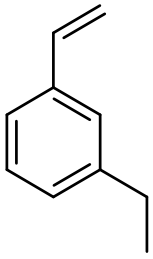

Figure 6.5: Chemical structure of m-ethylvinylbenzene

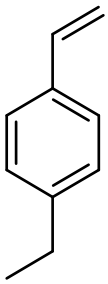

Figure 6.6: Chemical structure of p-ethylvinylbenzene

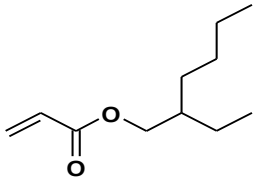

Figure 6.7: Chemical structure of 2-ethylhexyl acrylate (EHA)

6.3.1.2 Optimisation of chromatographic conditions

In order to achieve complete baseline separation of analytes, chromatographic conditions such as carrier gas flow rate, injector temperature, FID temperature, injector split ratio and column oven temperature programming were optimised. These parameters were set by comparing the boiling points of components to be separated. The boiling point data is given in Table 6.2 and the optimised parameters for chromatographic separation are presented in Section 6.2.4.

Table 6.2: Boiling point data

Sr. No.	Compound	Boiling point (°C)
1	Cyclohexane	81
2	Styrene	145
3	BA	147
4	m-EVB	185
5	p-EVB	192
6	DVB (80%)	195
7	EHA	216

The chromatogram (Figure 6.8) shows that the chromatographic elution follows the increasing order of boiling point of components i.e. the component having lowest boiling point eluted first and the one with highest boiling point eluted last, with the exception of BA, which eluted before styrene in spite of having higher boiling point (Table 6.2) than it. This is because styrene, being non-polar, interacts more strongly with

100% dimethylpolysiloxane stationary phase (of DB-1 capillary column), which is non-polar in character.

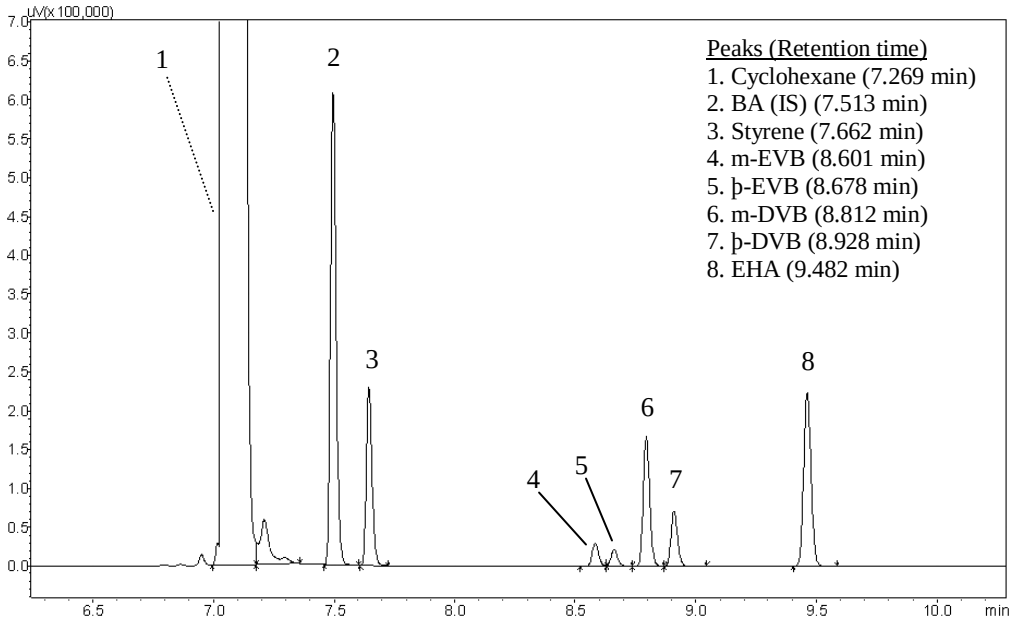

Figure 6.8: Chromatogram showing separation of components under study

The difference of boiling point between styrene (147 °C) and BA (145 °C) is not significant; hence, their elution order is predominantly determined by "analyte-stationary phase" interaction rather than due to difference in boiling points (or vapour pressure). It was seen that the meta- isomer retains more than para- isomer on DB-1 (dimethyl polysiloxane) capillary column for both DVB and EVB. This method separated all these isomers very well (Figure 6.8) with complete baseline separation along with separation of other components. Since EVB standard was not available, it was identified by GC-MS technique.

6.3.1.3 GC-MS analysis of divinylbenzene

Preliminary trials were taken by injecting a standard divinylbenzene solution (prepared in cyclohexane) into GC-MS system and the parameters were set to achieve

chromatographic separation of all isomers followed by measurement of corresponding masses (m/z values). The details of equipment and parameters of GC-MS analysis are given in Table 6.3. TIC chromatogram (Figure 6.9) shows the separation of meta- and para- isomers of DVB and EVB, and Figures 6.10, 6.11, 6.12 and 6.13 show MS spectra of m-EVB, p-EVB, m-DVB and p-DVB, respectively.

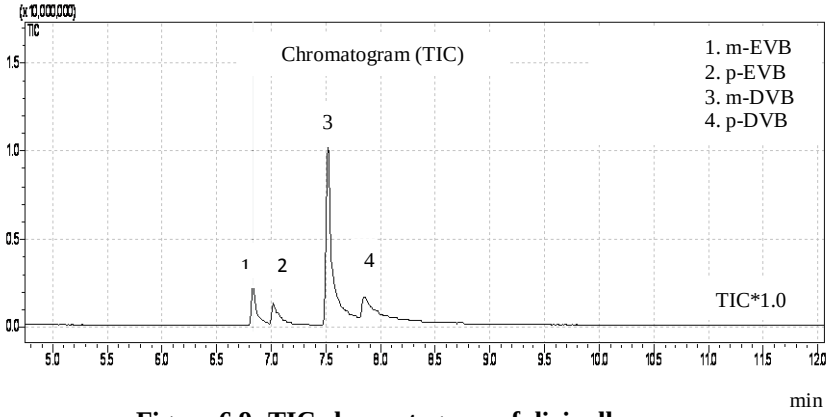

Figure 6.9: TIC chromatogram of divinylbenzene

Table 6.3: Details of GC-MS analysis

Equipment	Shimazdu: GC-17A/GCMS
Capillary column	DB-1ms (30 m x 0.25 mm, 0.25µm film thickness)
GC-MS parameters:	
Column initial temp.	80 ºC
Initial hold time	1.0 min
Column final temp.	80 ºC
Final hold time	5.0 min
Ramp rate	20 ºC/min
Injector temp.	280 ºC
Column flow	1.0 mL/min
Split ratio	20
Interface temp.	280 ºC
Detector voltage	1.5V (interval 0.5 s)

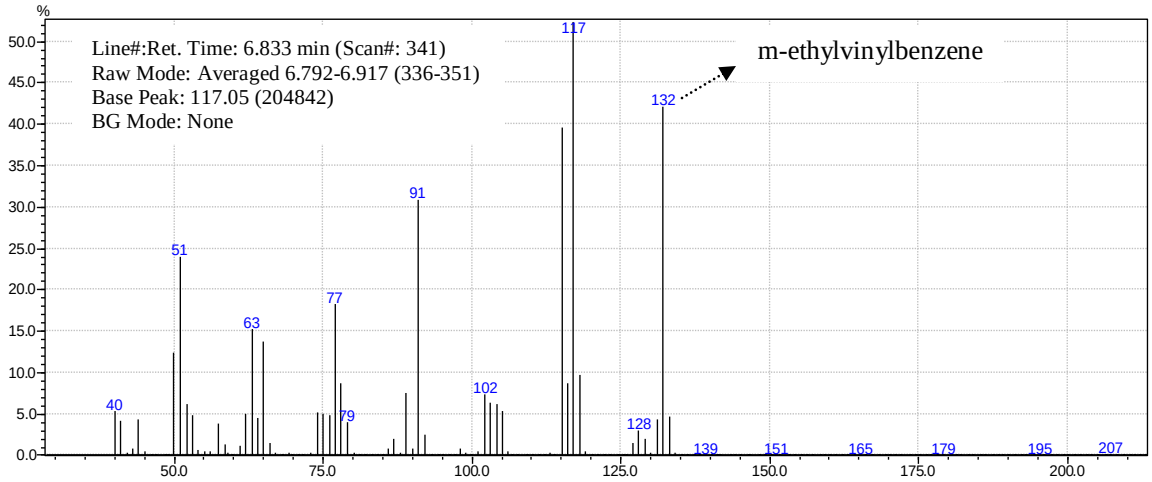

Figure 6.10: Mass spectrum of m-ethylvinylbenzene

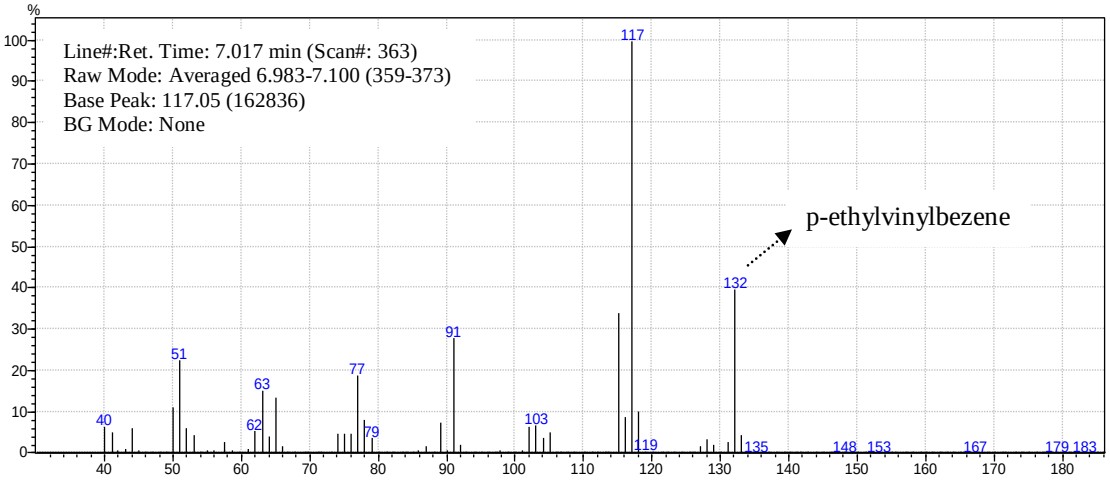

Figure 6.11: Mass spectrum of p-ethylvinylbenzene

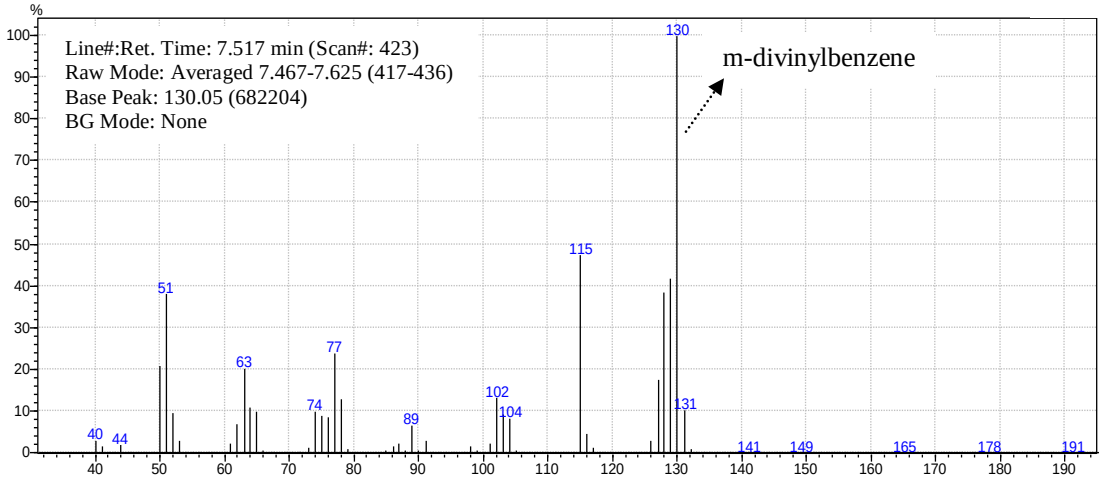

Figure 6.12: Mass spectrum of m-divinylbenzene

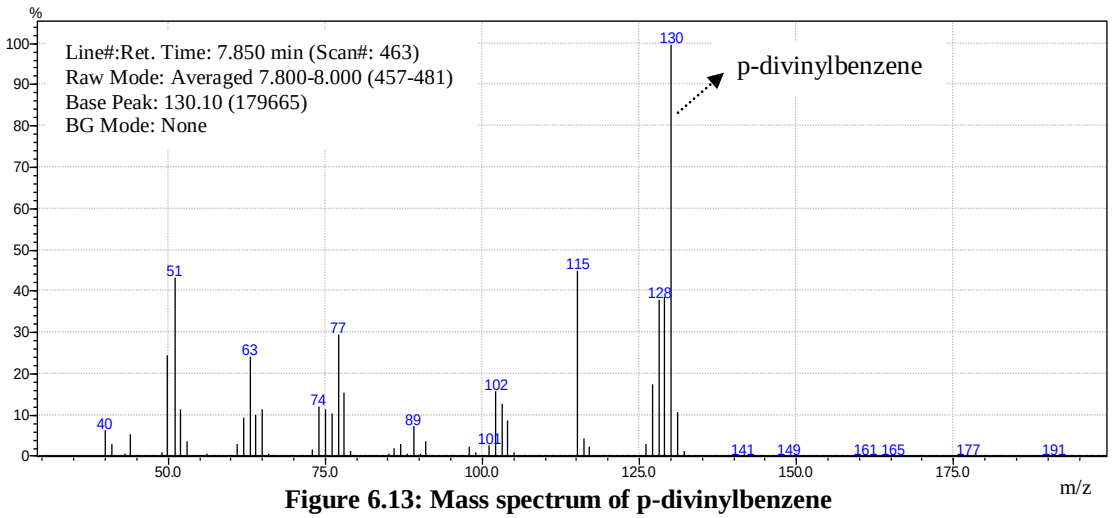

Figure 6.13: Mass spectrum of p-divinylbenzene

The peaks eluted at 6.833 min and 7.017 min (Figure 6.9) belong to m-EVB and p-EVB, respectively, which show m/z values equal to 132 (Figures 6.10 and 6.11). The peaks eluted at 7.517 min and 7.850 min (Figure 6.9) belong to m-DVB and p-DVB, respectively, which show m/z values equal to 130. The mass spectra of all the isomers showed major fragments of m/z as 40, 51, 63, and 77, which is a characteristic degradation pattern of aromatic compounds.[17]

6.3.1.4 Optimisation of extraction method

Similar to monomers used in acrylic HIPE system, cyclohexane showed a high distribution coefficient and good selectivity for styrene, DVB and EHA, as well. The protocol for monomer extraction was evolved by investigating the extraction efficiency, by determining the recovery of monomers at their low, medium, and high levels. The experiments were conducted by making five synthetic samples of HIPEs prepared with known concentrations of styrene, DVB and EHA for all the three levels of monomer concentrations. HIPE as a "sample matrix" was prepared by adding sodium persulphate, sodium dithionite, cumene hydroperoxide, ascorbic acid and calcium chloride. In the next

step, 100 mL of cyclohexane (containing internal standard, 2000 ppm) was added into each synthetic sample for extracting monomers. The sample preparation (solvent extraction) was followed as per procedure described in Section 6.2.3.2, and the samples were injected into GC system for investigating recovery of monomers after their extraction. The %recovery was found to be in the range 96.7-104.9. The volume of extraction solvent was optimised to 100 mL. The analytical data on %recovery of monomers from HIPE matrix for their low, medium and high levels is given in Table 6.4.

Similar to acrylic HIPE system, the advantages of selecting cyclohexane, for present styrenic HIPE system, were that (i) the monomers styrene, DVB and EHA show appreciable miscibility in it, (ii) it acts as a non-solvent to crosslinked polymer product formed in HIPE reaction mixture, thus avoiding interference of polymer product, and (iii) it does not dissolve initiators and electrolyte (calcium chloride) used in HIPE. Therefore, the samples (free from polymer particles and other interfering components) can directly be injected into GC system without fear of column damage.

Table 6.4: Analytical data on recovery of monomers from HIPE matrix

Sample	Styrene, ppm (w/v)			DVB, ppm (w/v)			EHA, ppm (w/v)		
	Added	Found	*%Rec.	Added	Found	*%Rec.	Added	Found	*%Rec.
Low level									
1	50	49	98.0	40	41	102.5	122	128	104.9
2	120	116	96.7	118	115	97.5	250	254	101.6
3	210	214	101.9	205	210	102.4	456	450	98.7
4	250	254	101.6	234	236	100.9	612	602	98.4
5	300	308	102.7	252	248	98.4	878	892	101.6
Medium level									
1	500	492	98.4	310	314	101.3	824	855	103.8
2	700	714	102.0	520	524	100.8	1280	1265	98.8
3	920	945	102.7	730	740	101.4	1489	1562	104.9
4	1056	1058	100.2	804	842	104.7	1938	2012	103.8
5	1200	1196	99.7	1020	1028	100.8	2150	2198	102.2
High level									
1	1412	1418	100.4	1080	1092	101.1	2634	2700	102.5
2	2965	2988	100.8	1214	1196	98.5	3020	2984	98.8
3	3402	3418	100.5	1480	1476	99.7	3390	3456	101.9
4	3750	3698	98.6	1615	1628	100.8	3652	3597	98.5
5	2586	2556	98.8	1800	1802	100.1	4018	4156	103.4

*%Rec: %Recovery of monomers from HIPE matrix

6.3.2 Method validation

The present method was validated by studying various parameters such as system precision, specificity, linearity (calibration studies), limit of detection (LOD), limit of quantification (LOQ), and 'method precision and accuracy'. The performance of measuring system was evaluated by conducting Gauge R&R study.

6.3.2.1 System precision

System precision was checked by assessing repeatability of retention time and peak area by making 10 replicate injections of a test sample comprising of a mixture of styrene, DVB and EHA. The retention times of all the components were quite repeatable as evident from the low values of standard deviation and %RSD, given in Table 6.5.

Table 6.5: Retention time repeatability

Inj. No.	Retention time (min)						
	BA (IS)	Styrene	m-EVB	p-EVB	m-DVB	p-DVB	EHA
1	7.513	7.662	8.601	8.678	8.812	8.928	9.482
2	7.500	7.689	8.585	8.663	8.814	8.926	9.484
3	7.502	7.668	8.612	8.658	8.810	8.924	9.481
4	7.510	7.650	8.602	8.688	8.814	8.928	9.486
5	7.498	7.668	8.603	8.672	8.812	8.923	9.484
6	7.499	7.687	8.604	8.669	8.811	8.922	9.488
7	7.496	7.678	8.608	8.674	8.815	8.919	9.487
8	7.513	7.667	8.598	8.678	8.812	8.921	9.482
9	7.523	7.672	8.604	8.676	8.811	8.922	9.475
10	7.516	7.665	8.601	8.675	8.810	8.924	9.486
Mean	7.507	7.671	8.602	8.673	8.812	8.924	9.484
SD	0.009	0.011	0.007	0.008	0.002	0.003	0.004
%RSD	0.12	0.14	0.08	0.09	0.02	0.03	0.04

Table 6.6: Peak area repeatability

Inj. No.	Peak area (counts)				Peak area ratio		
	Styrene	**DVB**	**EHA**	**BA (IS)**	*** Styrene**	*** DVB**	***EHA**
1	1764460	1338822	2748515	736920	2.3944	1.8168	3.7297
2	1748560	1329196.6	2695689	727450	2.4037	1.8272	3.7057
3	1672089.	1297882	2657907	714568	2.3400	1.8163	3.7196
4	1644267	1244051	2538147	684598	2.4018	1.8172	3.7075
5	1626035	1233958	2530361	678745	2.3957	1.8180	3.7280
6	1699097	1272430	2659256	707456	2.4017	1.7986	3.7589
7	1749276	1324818	2718168	724578	2.4142	1.8284	3.7514
8	1624291	1235216	2515894	678541	2.3938	1.8204	3.7078
9	1684115	1265567	2617359	698745	2.4102	1.8112	3.7458
10	1642601	1238052.9	2546936	685674	2.3956	1.8056	3.7145
Mean	1685479	1278000	2622823	703727	2.3951	1.8160	3.7269
SD	53196	41649	85439	21617	0.0205	0.0091	0.0194
%RSD	3.2	3.3	3.3	3.1	0.9	0.5	0.5

***Styrene**: Peak area of styrene/Peak area of IS, ***DVB**: peak area of DVB/ Peak area of IS, ***EHA**: Peak area of EHA/ Peak area of IS

The data on peak area variation is given in Table 6.6. Internal standard method was used in this work. It can be seen that there was a greater variation (%RSD $\geq$ 3.3) for individual peak area counts (Table 6.6). However, the %RSD for peak area ratio for all

the three monomers was found to be ≥ 0.9 (Table 6.6), which showed good peak area repeatability leading to accurate results.

6.3.2.2 Specificity

Method specificity for given monomers was achieved due to both solvent extraction (sample clean-up) step and optimised GC column oven temperature programming. The specificity of this method has been demonstrated by the representative chromatogram (Figure 6.7) of the test sample comprising of a mixture of components under present study.

All the peaks of analytes along with the peak of internal standard were well resolved with base line separation and there was no interference of components from HIPE sample matrix. The interference of initiators (sodium persulphate, sodium dithionite, cumene hydroperoxide and ascorbic acid), electrolyte (calcium chloride), and polymer product formed in HIPE reaction was eliminated, since they did not come in organic (cyclohexane) layer during extraction. Further, the surfactants (Span-80 and Arquad 2HT-75), used in very small amounts, in HIPE reactions, due to their non-volatility, got retained over the glass wool placed in an injector liner and thus, did not go to GC column thereby avoiding their interference. Traces of impurities that might arise from solvents were separated to avoid their interference with the peaks of interest. This proved that the method was specific to the monomers, viz. styrene, DVB and EHA.

6.3.2.3 Linearity (calibration studies)

The linearity was assessed by running analytical standards on GC system under optimised chromatographic conditions. For this study, 8 nos. of calibration standards in the given range (Table 6.7) were prepared as per the procedure described in Section 6.2.2,

and injected into chromatographic system. The calibration curve for a particular monomer was derived by plotting peak area ratio (ratio of peak area of analyte to internal standard) against concentration ratio (ratio of concentration of analyte to internal standard). The regression equation was obtained as $y = mx$, where y was the peak area ratio of analyte to internal standard, m was the slope, and x was the ratio of concentration (µg mL^{-1}) of analyte to internal standard. The linearity analysis showed a good fit of regression lines, as indicated by the regression coefficient values ($R^2 \geq 0.9974$). The monomer concentration range, regression equations and values of R^2 are reported in Table 6.7, and the calibration curves for styrene, DVB and EHA are given in Figures 6.14, 6.15 and 6.16, respectively.

Table 6.7: Linearity studies of monomers

Monomer	Range (µg mL^{-1})	Regression equation	R^2 value
Styrene	45-2250	$y = 1.7476x$	0.9974
DVB	34-1680	$y = 1.8529x$	0.9998
EHA	105-5240	$y = 1.4682x$	0.9994

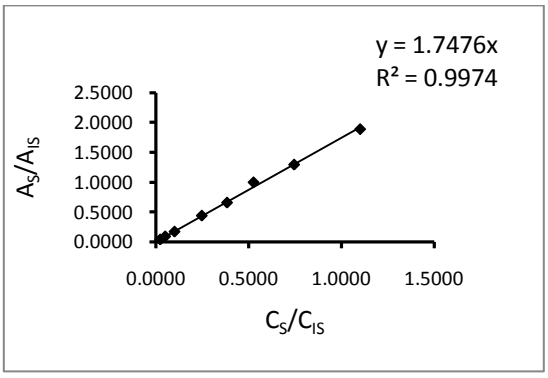

Figure 6.14: Calibration curve for styrene (A_S/A_{IS}: Peak area of styrene/Peak area of IS, C_S/C_{IS}: Conc. of styrene/ Conc. of IS)

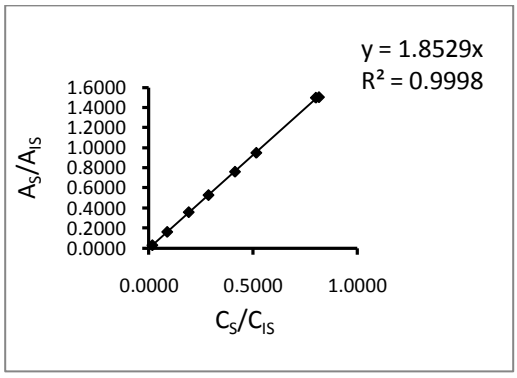

Figure 6.15: Calibration curve for DVB (AS/AIS: Peak area of DVB/Peak area of IS, C_S/C_{IS}: Conc. of DVB/ Conc. of IS)

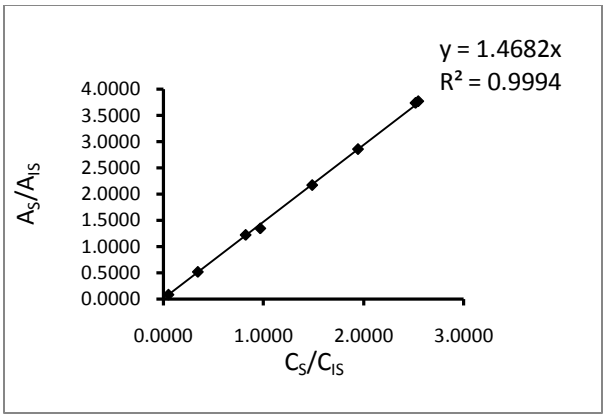

Figure 6.16: Calibration curve for EHA
(A_S/A_{IS}: Peak area of styrene/Peak area of IS, C_S/C_{IS}: Conc. of styrene/ Conc. of IS)

6.3.2.4 Response factors

The response factors (R_f) for styrene, DVB and EHA were calculated from calibration data (Table 6.8) using equation 5.1 (presented in Chapter 5). The calibration and statistical data for replicate measurements ($n = 8$) of response factors are given in Tables 6.8. The %RSD ($n = 8$) ≤ 3.8 showed good precision for the measurement of response factors. The mean R_f values (Table 6.8) were used to determine corresponding monomer concentration (ppm, w/v) in a particular HIPE reaction sample.

Table 6.8: Calibration data

S. No.	Styrene			DVB			EHA		
	C_S/C_{IS}	A_{IS}/A_S	R_f	C_S/C_{IS}	A_{IS}/A_S	R_f	C_S/C_{IS}	A_{IS}/A_S	R_f
1	0.0223	26.1286	0.5821	0.0168	32.1492	0.5411	0.0520	13.0348	0.6776
2	0.0490	11.0870	0.5435	0.0882	6.1169	0.5397	0.8235	0.8226	0.6774
3	0.0993	5.8644	0.5821	0.1916	2.7767	0.5319	2.5211	0.2681	0.6759
4	0.2463	2.2809	0.5618	0.4138	1.3114	0.5427	0.3458	1.9576	0.6770
5	0.3827	1.5218	0.5823	0.2864	1.8873	0.5405	0.9699	0.7466	0.7242
6	0.5253	1.0019	0.5263	0.8171	0.6644	0.5429	2.5486	0.2658	0.6774
7	0.7444	0.7720	0.5747	0.5161	1.0514	0.5427	1.4888	0.4620	0.6878
8	1.0905	0.5338	0.5821	0.8020	0.6668	0.5348	1.9462	0.3509	0.6829
		Mean R_f	0.5669		**Mean R_f**	0.5395		**Mean R_f**	0.6850
		SD	0.0214		**SD**	0.0041		**SD**	0.0163
		%RSD	3.8		**%RSD**	0.8		**%RSD**	2.4

6.3.2.5 Limits of detection and quantification

The limits of detection and quantification (LOD and LOQ) of a method (Table 6.9) were determined by signal-to-noise ratio method.[18] The LOD (three times signal-to-noise) and LOQ (10 times signal-to-noise) for individual monomers were determined from the corresponding slope values of regression equations (Table 6.7).

Table 6.9: LOD and LOQ data

Monomer	LOD (µg mL^{-1})	LOQ (µg mL^{-1})
Styrene	0.15	0.50
DVB	0.14	0.47
EHA	0.18	0.59

It may be noted that the minimum concentration values of styrene, DVB and EHA (i.e. concentration of unconverted monomers at 98% conversion) are much higher than the LOD and LOQ values. This established that the present method was sensitive enough for the measurement of given monomers for a HIPE reaction having batch size of 0.917 g.

The response of FID is generally directly proportional to the carbon content of the analyte.[18] The monomers, styrene, DVB and EHA have 8, 10 and 11 carbon atoms, respectively. Accordingly, FID response should be in the order of EHA > DVB > styrene. However, the calculated values of slope of regressions equations (Table 6.7), response factors (Table 6.8) and LOD/LOQ, (Table 6.9) indicated that the FID response was in the order of DVB > styrene > EHA. As expected, between DVB and styrene, DVB (10 carbon atoms) showed more detector response than styrene (8 carbon atoms). However, EHA had the lowest detector response in spite of having highest carbon content (11 carbon atoms). It has been reported[20] that though, in general, FID response is proportional to the carbon number of the hydrocarbons, it is always less for a substituted hydrocarbon

than that of the parent hydrocarbon. Karasek[20] et al. further reported that the substituted hydrocarbon having heteroatom (like oxygen or nitrogen) shows considerably less FID response of compound compared with that of the parent hydrocarbon. For EHA, the presence of two oxygen (hetero) atoms in its hydrocarbon chain lowers its FID response considerably resulting in lowest values of regression slope, LOD/LOQ and response factor (which are a measure of sensitivity).

6.3.2.6 Method precision and accuracy

The method precision (intra-day repeatability) and intermediate precision (inter-day repeatability) were tested by multiple analyses of mixtures of monomers prepared with HIPE matrix. Three quality control (Q.C.) solutions considering levels of unreacted monomers at low, medium and high conversions were prepared. The concentrations of monomers in individual mixtures were, (i) Styrene (55, 1000, 2200 μg mL^{-1}), (ii) DVB (50, 800, 1600 μg mL^{-1}) and (iii) EHA (110, 2600, 5000 μg mL^{-1}) for low, medium and high levels, respectively. The initiator (sodium persulphate), and MEHQ (dissolved in acetone) were added into each mixture. Monomers in individual mixtures were extracted in cyclohexane with internal standard (BA) added to it and subjected to GC runs for assay analysis. Method precision (intra-day) was evaluated by replicate analyses of samples (n = 5) on same day and the inter-day precision was studied by comparing the assay on five different days. The data on method precision and accuracy (%RSD values and relative errors) are given in Table 6.10. The analysis showed good accuracy and precision of the method and thus proved that the method was suitable.

Table 6.10: Method precision and accuracy

Synthetic sample		Conc. of monomer in synthetic sample (ppm)	INTRA-DAY, *n = 5*			INTER-DAY, *n = 5*		
			A ± SD[a]	**Precision %RSD[b]**	**Acc.[c]**	**A ± SD[a]**	**Precision %RSD[b]**	**Acc.[c]**
1	Styrene	55	53 ± 1.5	2.8	- 4.0	53 ± 1.3	2.4	- 4.4
	DVB	50	51 ± 2.4	4.7	1.6	51 ± 1.7	3.4	1.8
	EHA	110	106 ± 3.2	3.0	- 3.8	106 ± 2.9	2.7	- 3.4
2	Styrene	1000	1010 ± 8.6	0.9	1.0	1019 ± 13.6	1.3	1.9
	DVB	800	818 ± 19.3	2.4	2.2	813 ± 32.3	4.0	1.7
	EHA	2600	2524 ± 52.4	2.1	- 2.9	2538 ± 49.7	2.0	- 2.4
3	Styrene	2200	2236 ± 28.5	1.3	1.7	2243 ± 30.4	1.4	1.9
	DVB	1600	1635 ± 41.7	2.6	2.2	1643 ± 40.1	2.4	2.7
	EHA	5000	4912 ± 166.6	3.4	- 1.8	4966 ± 182.4	3.7	- 1.1

A: Mean of determined concentration (ppm, w/v), *n = 5*
[a]**SD**: Standard deviation
[b]**RSD**: Relative standard deviation,
[c]**Acc.**: Accuracy (i.e. %Relative error) = [(Determined conc.− Added conc.) / Added conc.] × 100

6.3.3 Gauge R & R study

The present analytical method was subjected to Gauge R&R (Repeatability and Reproducibility) study to determine variability in the measuring system and to identify various sources that contribute to it. The measuring system was comprised of (1) measuring device (i.e. GC instrument) and the method of analysis (Repeatability component) and (2) the operators or analysts who performed the analyses (Reproducibility component). The R&R study indicates how the given method performs in the hands of different analysts besides the performance of measuring device, and if there is any interaction between samples and analysts.

For this experiment, five different HIPE samples (containing each of 2400 ppm of internal standard) were analysed three times (one run at a time by one analyst) by three analysts for three monomer components, viz. styrene (840, 1500, 2260, 2880, 3200 ppm),

DVB (640, 980, 1770, 2040 and 2420 ppm) and EHA (2020, 3080, 3980, 5000 and 6990 ppm) in a completely randomised manner on the same GC system (i.e. measuring device). The analytical data were subjected to statistical method of analysis of variance (ANOVA) to delineate the contributions from various sources, and their significance was judged by *P*-values ($P < 0.05$ indicates significant contribution). The data coverage factor for the R&R study was 5.15 σ (i.e. 99% area under the normal distribution curve).

The ANOVA data of Gauge R & R study for styrene, DVB and EHA are summarised in Tables 6.11 - 6.16, and the results of the R&R study are shown in Figures 6.17 - 6.19 as 'x bar chart by analyst', 'range (R) chart by analysts', 'analyst-sample interaction', 'scatter plots by samples and by analysts', and 'components of variation'.

R&R values (Tables 6.12, 6.14 and 6.16) for styrene, DVB and EHA were 7.51, 2.00 and 3.98%, respectively. The R&R values less than 10% are considered to be good and indicate that the measurement system is acceptable.[21] The R&R values obtained for all the three monomer components are below 10%, which proved that the analysis method is stable. The values of %Study Var for DVB (Repeatability: 1.98 & Reproducibility: 0.33, Table 6.14) and EHA (Repeatability: 3.90 & Reproducibility: 0.83, Table 6.16) indicated that the major contribution to R&R values originated from the Gauge (GC equipment) itself (i.e. Repeatability component) rather than from the analysts (i.e. Reproducibility component), for analyses of both styrene and DVB. Whereas, for styrene analysis, gauge (GC equipment, i.e. Repeatability component, %Study Var = 5.61, Table 6.12) and analysts (i.e. Reproducibility component, %Study Var = 4.99, Table 6.12) contributed almost equally to the total R&R values. In analyses of DVB and EHA, the sample-analyst interaction was insignificant (i.e. sample handling by analysts

did not contribute significantly to the Reproducibility component), as evident from the *P*-values ($P > 0.05$, Tables 6.13 and 6.15). However, in case of styrene analyses, the sample-analyst interaction was significant ($P < 0.05$, Table 6.11).

It can be concluded from Figures 6.17- 6.19 ('R chart by Analyst') that all the three analysts measured almost all samples consistently except measurement of Sample no. 1 by Analyst 1 for which the sample range value falls outside the upper control limit (UCL = 287.6).

The number of distinct categories is the "number of non overlapped confidence intervals" that is needed to cover all the product variability. When the number of distinct categories is less than 2, the measurement system is unable to measure the process. A number of distinct categories greater or equal to 5 means an acceptable measurement system.[22] The numbers of distinct categories for styrene, DVB and EHA analyses in present study were found to be 18, 70 and 35, respectively, which showed that the measurement system was quite acceptable. The Gauge R & R study conducted in this work proved that the present measurement system was stable, rugged and performed well producing overall less variation and hence, quite acceptable.

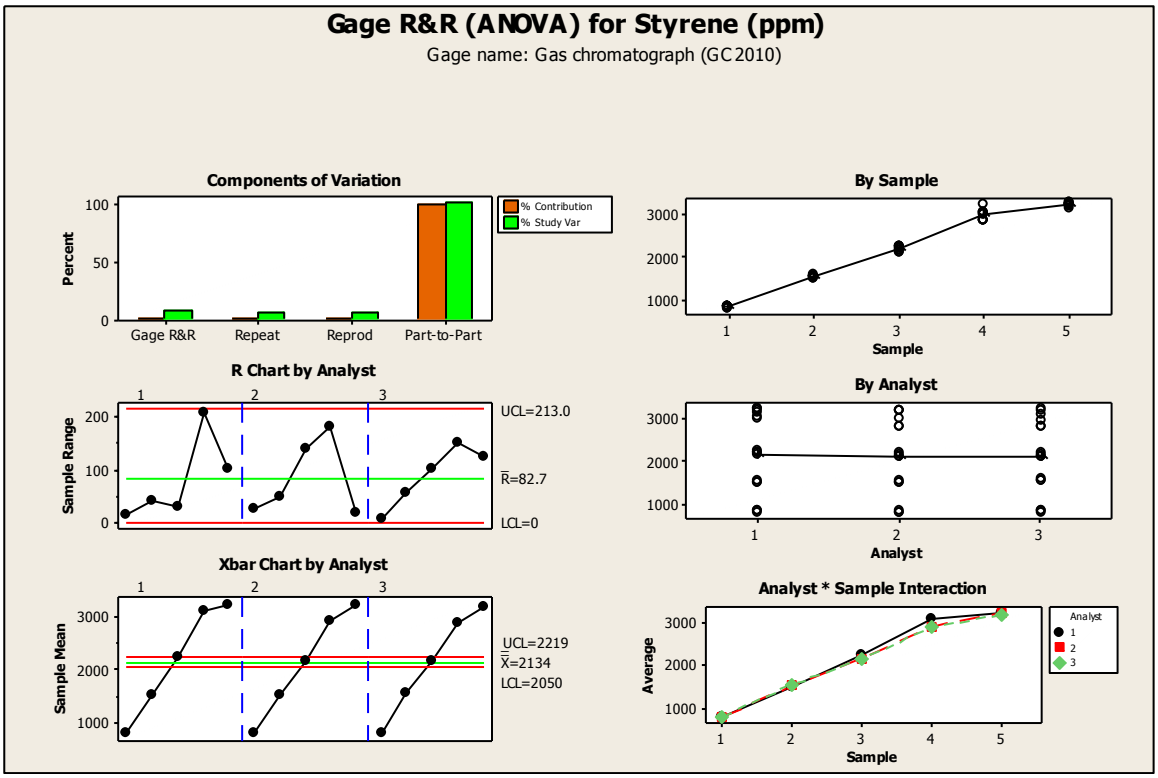

Figure 6.17: ANOVA results for five different styrene analyses by three analysts

Table 6.11: Sources of variation in styrene analysis (by ANOVA method)

Source	DF	SS	MS	F	P
Sample	4	35978430	8994608	1016.16	0.000
Analyst	2	35730	17865	2.02	0.195
Analyst x Sample	8	70813	8852	2.80	0.019
Repeatability	30	94921	3164	-	-
Total	44	36179894	-	-	-

Table 6.12: Repeatability and reproducibility (R&R) data for styrene analysis

Source	Var Comp	%Contribution (of Var Comp)	Std Dev (SD)	Study Var (5.15*SD)	%Study Var
Total Gage R&R	5661	0.56	75.24	387.48	7.51
Repeatability	3164	0.32	56.25	289.69	5.61
Reproducibility	2497	0.25	49.97	257.33	4.99
Analyst	601	0.06	24.51	126.24	2.45
Analyst x Sample	1896	0.19	43.54	224.24	4.35
Sample-To-Sample	998417	99.44	999.21	5145.92	99.72
Total Variation	1004078	100.00	1002.04	5160.49	100.00

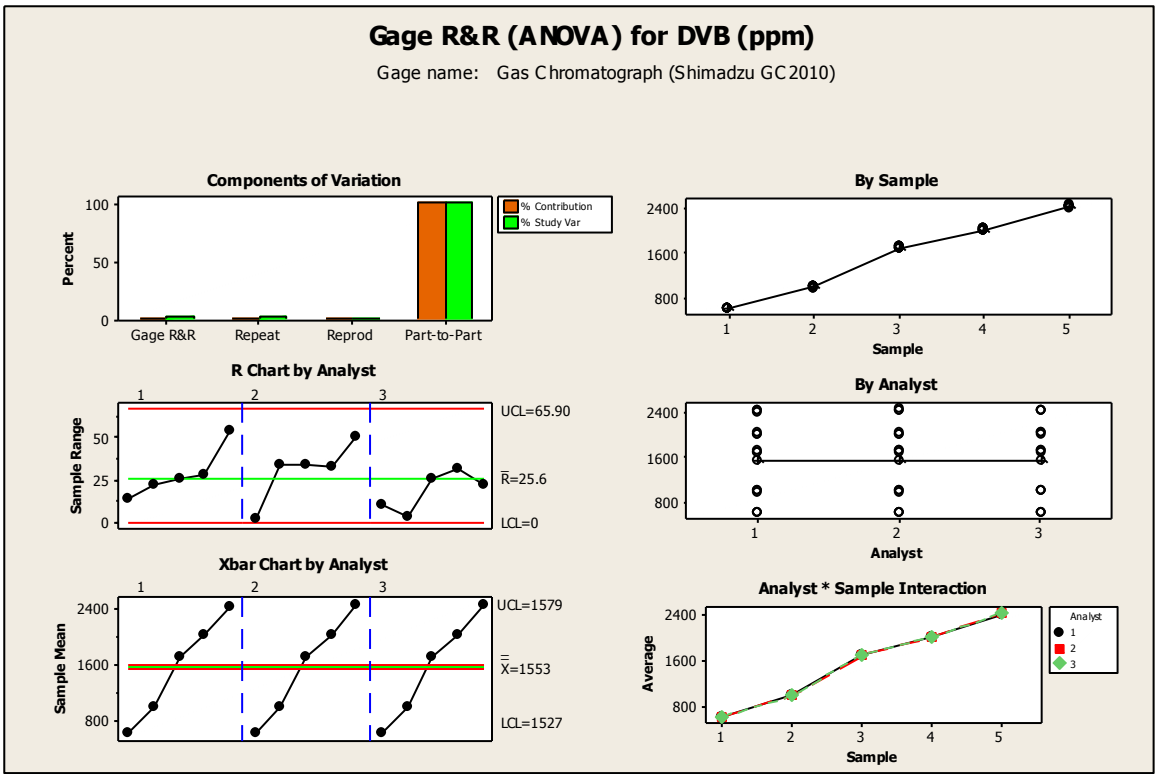

Figure 6.18: ANOVA results for five different DVB analyses by three analysts

Table 6.13: Sources of variation in DVB analysis (by ANOVA method)

Source	DF	SS	MS	F	P
Sample	4	19871397	4967849	33065.1	0.000
Analyst	2	615	307	2.0	0.192
Analyst x Sample	8	1202	150	0.6	0.734
Repeatability	30	6988	233	-	-
Total	44	19880202	-	-	-

Table 6.14: Repeatability and reproducibility (R&R) data for DVB analysis

Source	Var Comp	%Contribution	Std Dev (SD)	Study Var (5.15*SD)	%Study Var
Total Gage R&R	222	0.040	14.888	76.67	2.00
Repeatability	216	0.039	14.681	75.61	1.98
Reproducibility	6	0.001	2.474	12.74	0.33
Analyst	6	0.001	2.474	12.74	0.33
Sample-To-Sample	551959	99.960	742.940	3826.14	99.98
Total Variation	552181	100.000	743.089	3826.91	100.00

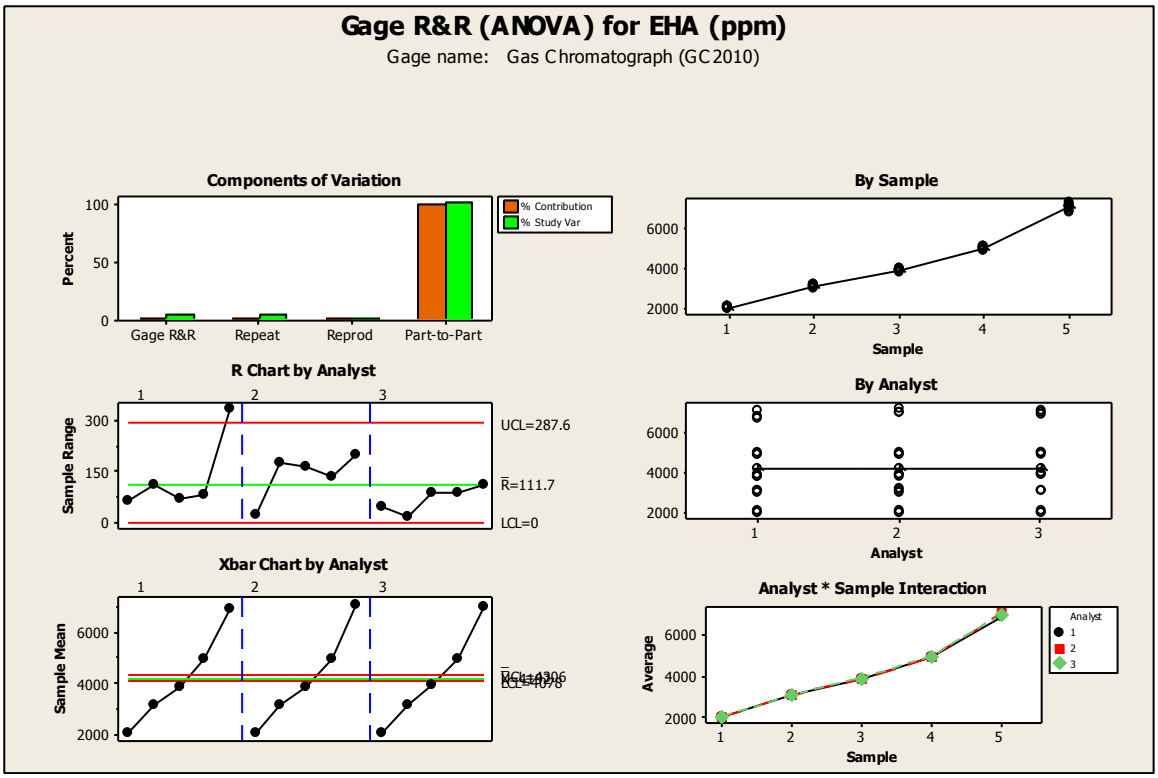

Figure 6.19: ANOVA results for five different EHA analyses by three analysts

Table 6.15: Sources of variation in EHA analysis (by ANOVA method)

Source	DF	SS	MS	F	P
Sample	4	127581966	31895492	5151.43	0.000
Analyst	2	17574	8787	1.42	0.297
Analyst x Sample	8	49533	6192	1.19	0.336
Repeatability	30	155605	5187	-	-
Total	44	127804679	-	-	-

Table 6.16: Repeatability and reproducibility (R&R) data for EHA analysis

Source	Var Comp	%Contribution (of Var Comp)	Std Dev (SD)	Study Var (5.15*SD)	%Study Var
Total Gage R&R	5624	0.16	75.00	386.23	3.98
Repeatability	5398	0.15	73.47	378.39	3.90
Reproducibility	226	0.01	15.03	77.41	0.80
Analyst	226	0.01	15.03	77.41	0.80
Sample-To-Sample	3543344	99.84	1882.38	9694.24	99.92
Total Variation	3548968	100.00	1883.87	9701.93	100.00

6.3.4 Estimation of monomer conversions

6.3.4.1 HIPE polymerisation reactions

All HIPE polymerisation reactions were conducted with a batch size of 0.917 g, which contained 225.0 mg (2.1603 mM) of styrene, 168.0 mg (1.2904 mM) of DVB, 524.0 mg (2.8434 mM) of EHA. The organic phase was prepared in bulk (stock solution) for an uniform composition of its ingredients and the required quantity (0.917 ± 0.005 g) for a particular batch was dispensed from stock solution for conducting a HIPE reaction. The concentration of surfactants was kept constant in all the HIPE reactions conducted for this study. HIPE samples were prepared as described in Section 6.2.3.1. In all 5 experiments were conducted in which individual batches of HIPEs were polymerised, using thermal and redox initiator systems (details given in Table 6.17), for incremental curing times, and data on time-dependent monomer conversions (weight %) was obtained by analysis of unreacted monomers.

Table 6.17: Parameters of HIPE polymerisation reactions

Expt. No.	Thermal initiator/Redox initiator systems	Conc. or Composition
1	Sodium persulphate	0.05 wt% of water
2	Cumene hydroperoxide	0.05 wt% of water
3	*Sodium persulphate: Sodium dithionite	(1:1) molar composition
4	*Sodium persulphate: Ascorbic acid	(1:1) molar composition
5	*Cumene hydroperoxide: Ascorbic acid	(1:1) molar composition

* 0.05 wt% of water phase
Water: Oil = 25:1, Span 80: 0.0596 g (6.5% of oil phase), Arquad 2HT-75: 0.0073 g (0.79% of oil phase), stirring speed = 1400 rpm, curing temperature = 65 ^{o}C.

6.3.4.2 Percent monomer conversion

All the samples were injected into GC system with the injection volume of 1.0 µL and the chromatographic data of peak area counts of monomers and an internal standard (BA) were collected. The concentration of a particular monomer in a particular

HIPE reaction sample was calculated using equation 5.2 (presented in Chapter 5). From the concentration (ppm, w/v) value, the absolute amount of unreacted monomer in total reaction mass (i.e. total "g" in 100 mL of extraction solvent) was calculated and further, the percent monomer conversion was calculated by using equation 5.3.

6.3.4.3 Time-dependent monomer conversion profiles

The time-dependent monomer conversion profiles were generated by plotting percent conversion against curing time as shown in Figures 6.20 - 6.24. The percent conversion data for styrene, DVB and EHA for all the experiments (Expt. Nos. 1-5) are reported in Table 6.18 - 6.22.

Table 6.18: Percent conversion (Expt. No. 1)

S. No.	Time (min)	%Conversion (w/w)		
		EHA	Styrene	DVB
1	0	0	0	0
2	15	18.3	17.2	13.8
3	30	9.5	15.4	12.7
4	45	18.7	25.3	24.5
5	60	18.8	31.3	29.5
6	75	30.7	39.6	43.2
7	90	46.9	57.9	64.5
8	105	60.5	73.2	77.3
9	120	68.2	82.2	79.9

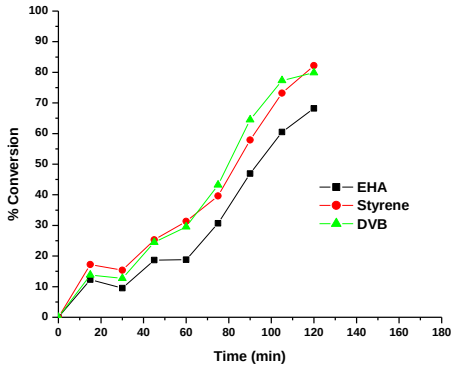

Figure 6.20: Monomer conversion profile (Expt. No. 1, sodium persulphate: 0.05 wt% of water)

Table 6.19: Percent conversion (Expt. No. 2)

S. No.	Time (min)	%Conversion (w/w)		
		EHA	Styrene	DVB
1	0	0.0	0.0	0.0
2	30	8.0	12.8	9.9
3	60	7.6	17.3	15.5
4	90	17.3	25.8	30.7
5	120	26.2	37.6	49.2
6	150	64.5	71.3	81.9

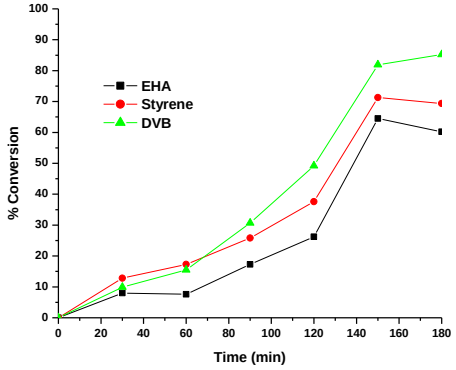

Figure 6.21: Monomer conversion profile (Expt. No. 2, cumene hydroperoxide: 0.05 wt% of water)

Table 6.20: Percent conversion (Expt. No. 3)

S. No.	Time (min)	%Conversion (w/w)		
		EHA	Styrene	DVB
1	0	0.0	0.0	0.0
2	15	19.3	23.2	36.6
3	30	74.3	75.1	81.9
4	45	81.3	86.2	89.7
5	75	84.4	90.2	92.7
6	90	84.7	88.9	91.6

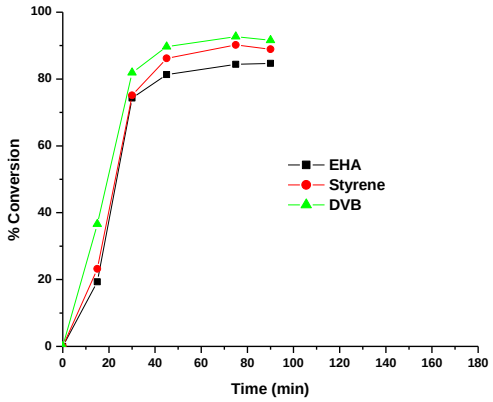

Figure 6.22: Monomer conversion profile (Expt. No. 3, sodium persulphate: cumene hydroperoxide, 1:1 molar composition)

Table 6.21: Percent conversion (Expt. No. 4)

S. No.	Time (min)	%Conversion (w/w)		
		EHA	Styrene	DVB
1	0	0	0	0
2	15	5.3	6.0	11.2
3	30	43.4	56.5	64.0
4	45	56.1	65.6	69.4
5	60	53.6	64.3	72.8
6	90	68.7	77.8	82.3
7	105	70.9	76.4	85.2
8	120	75.1	87.2	88.8

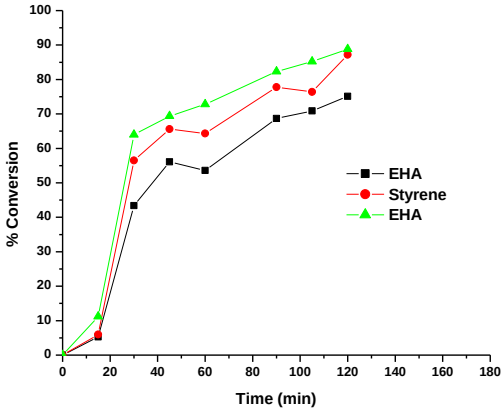

Figure 6.23: Monomer conversion profile (Expt. No. 4, sodium persulphate: ascorbic acid, 1:1 molar composition)

Table 6.22: Percent conversion (Expt. No. 5)

S. No.	Time (min)	%Conversion (w/w)		
		EHA	Styrene	DVB
1	0	0.0	0.0	0.0
2	30	47.9	58.6	70.5
3	60	92.0	95.4	95.8
4	90	96.7	97.6	97.8
5	120	94.5	97.9	98.0
6	150	96.6	98.0	98.0

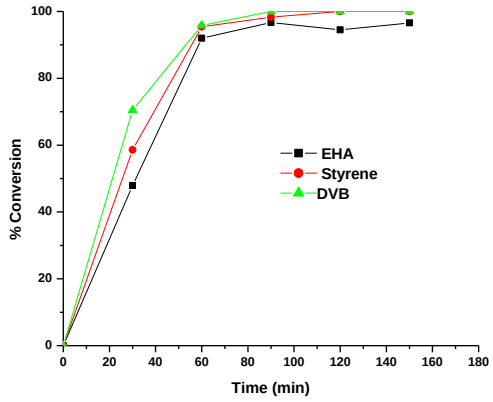

Figure 6.24: Monomer conversion profile (Expt. No. 5, cumene hydroperoxide: ascorbic acid, 1:1 molar composition)

6.4 Summary

In this work, a sensitive and accurate analytical method was developed which was useful for estimating time-dependent monomer conversions by analysis of unreacted monomers (styrene, DVB and EHA) in HIPE polymerisation reactions. The method was based on simple GC-FID technique. The method for extraction of monomers from high internal phase emulsion was developed and validated. Method validation was carried out by investigating system precision (assessment of repeatability of retention time and peak area), method specificity, linearity (calibration studies), limit of detection (LOD), limit of quantification (LOQ), and 'method precision and accuracy'. Gauge R & R study was conducted which proved that the measurement system was stable and performed very well producing overall less variation and hence, quite acceptable.

The usefulness of method was successfully demonstrated by estimating monomer conversions in HIPE polymerisation reactions conducted using certain thermal and redox initiator systems, as an example of method application.

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Chapter 7

SUMMARY AND CONCLUSIONS

7.1 Summary and Conclusions

In this thesis, new analytical methodologies based on chromatographic techniques were developed for estimation of monomers which are useful to address the important issues related to industrial production of poly(lactic) acid and polymerisation processes that include acrylic and styrenic HIPEs. The summary and conclusions of the present work are presented below.

- **Poly(lactic) acid**

(A) Estimation of chemical purity of lactic acid by impurity profiling

The analytical method developed for impurity profiling of lactic acid (discussed in Chapter 3) gives useful information (both qualitative and quantitative) on carboxylic acid impurities present in lactic acid which is a critical factor in PLA production.

- In this work, a number of carboxylic acid impurities present in in-house and commercial lactic acid samples were determined and their quality was compared. Pyruvic, oxalic, formic, succinic, itaconic, aconitic and acrylic acids were present in most of the samples; citric, propionic and fumaric acids were found in a few samples and citraconic acid was detected in only one sample. Acetic, malic and butyric acids were not found in any of the samples.
- The analytical method presented in this work allows the simultaneous determination of commonly observed carboxylic acids in pure lactic acid, besides the separation and identification of PLA precursors, linear and cyclic dimers of lactic acid.
- The method employed polar embedded reverse phase for analysis wherein polar-embedded groups provided enhanced stability against phase collapse, which occurred when 100% aqueous phase was used with conventional reverse phase.

Apart from good selectivity, a major benefit of using polar-embedded stationary phase was that good peak shape and peak symmetry for the (polar carboxylic acid) analytes was obtained.

- This method is novel and unique because there was no method for simultaneous separation and quantification of polar carboxylic acids in lactic acid matrix.
- The analytical validation data including Gauge R&R study demonstrated that the method was precise, stable, accurate and rugged. Duplicate, and in some cases triplicate, analyses demonstrated that the method generates consistent results.
- It is essential that the analysis protocols are simple, readily repeated at different labs and should be easily translated into useful information. The method presented here is simple since it does not require any complicated sample preparation and it is based on isocratic mode of elution. The method uses purely an aqueous phase avoiding use of HPLC grade solvents for chromatographic elution, and hence it is economical. Thus, it can be used routinely for purity evaluation of lactic acid, in terms of carboxylic acid impurities.
- Methanol or ethanol may appear as an impurity (a byproduct of hydrolysis of methyl ester of lactic acid) if not completely removed in the downstream processing step of lactic acid purification. GC-FID method was developed to determine ethanol and methanol. Methanol and ethanol were not present in any of the commercial lactic acid samples. Methanol was found in a few in-house samples at a trace level. GC-FID method presented in this work can be used as an in-process Quality Control check to confirm absence of alcohol (ethanol or methanol) used in esterification step of reactive distillation step of downstream purification of lactic acid. If an impurity of methanol or ethanol is detected, it can then be removed by distillation.

(B) Estimation of optical purity of lactic acid

The chiral HPLC method developed for separation and quantification of lactic acid enantiomers (discussed in Chapter 4) determines optical purity of lactic acid which is a crucial factor in deciding physical properties of PLA. Small amounts of enantiomeric impurities drastically change properties such as crystallinity or biodegradation rate of the polymer. L (+) lactic acid is required for production of PLA which is suitable for most of the applications. Therefore, besides investigating chemical purity, evaluation of optical purity of lactic acid is also equally important. To summarise this work:

- The HPLC method based on chiral ligand-exchange chromatography (CLEC) technique was developed for separation of lactic acid enantiomers. Enantiomeric separation was accomplished by employing stationary phase ligand, which employed L-hydroxy proline as the "immobilised chiral selector".
- The chromatographic parameters such as column temperature, flow rate, detection wavelength and solvent composition were investigated and optimised to achieve separation of lactic acid enantiomers. System precision was investigated by studying repeatability of retention times and peak area, which was appropriate.
- Due to simplicity of the procedure and extremely high enantioselectivity of complexation, CLEC proved to be most reliable and an inexpensive chiral HPLC technique for resolving and analysing enantiomeric composition of lactic acid.
- The method was applied to determine optical purity of commercial and in-house lactic acid samples. All samples were found to be pure (i.e. containing 100% L (+) enantiomer) except Source-8 sample, which was 99.9% pure (i.e. containing 0.1% impurity of D (-) isomer)
- The present method is fast (total run time < 9 min) and produces accurate results.

- **Acrylic HIPEs**

(A) Estimation of monomers in acrylic HIPE polymerisation

The analytical method developed for estimating monomer conversions in acrylic HIPEs (discussed in Chapter 5) can be used to generate kinetics data, which is useful to create a phenomenological model to predict the rate parameters of HIPE polymerisation. This would eventually be useful to optimise the HIPE polymerisation process.

- In order to generate monomer conversions data, individual HIPEs (containing EHA, EGDMA and EHMA) were polymerised for incremental curing times, with a particular set of reaction parameters, and the monomer conversions were calculated by determining unreacted monomers in HIPE reaction mixtures. This analysis was difficult, because it required demulsification of intrinsically stable HIPE so as to release unreacted monomers from HIPE matrix which then have to be efficiently extracted in a suitable solvent and further subjected to gas chromatographic analysis.
- Cyclohexane (water-immiscible solvent) was selected for extraction which, being non-polar, showed good selectivity for EHA, EGDMA and EHMA. It also eliminated potential interference of water soluble salts (sodium persulphate and calcium chloride), which are harmful to DB-1 GC capillary column, and may affect accuracy of measurement. The method for extraction of monomers from high internal phase emulsion was developed and validated by assessing recovery of analytes from HIPE matrix. The solvent extraction method herein acts as a sample clean-up, an important step of sample preparation, which isolates potential interferences from sample matrix, thereby helping make the method more selective.

- The extraction method serves as a single step exhaustive extraction, and therefore, it is efficient and helps determine the unreacted monomers accurately.
- The sample preparation method and the chromatographic conditions (GC oven temperature programming) were optimised to accomplish baseline separation of monomers and avoid interference of components originating from HIPE sample matrix.
- Method validation was carried out by investigating system precision, method specificity, linearity, limit of detection (LOD), limit of quantification (LOQ), and 'method precision and accuracy'. These parameters established the efficiency of method and ensured that the method was valid for its intended use.
- Gauge R & R study proved that the measurement system was stable and rugged, and performed very well producing overall less variation.
- The usefulness of method was demonstrated by estimating monomer conversions in HIPE polymerisation reactions with certain selective reaction parameters, as an example of method application.
- The method is fast (total run time < 15 min) and hence, cost-effective. It serves as an efficient and simple analytical tool to estimate the time-dependent monomer conversions for a given acrylic HIPE system.
- This method can also be used for other applications such as studying the reactivity ratios of co-polymerisation of HIPE systems for the given monomers, such as EHA-EGDMA, EHMA-EGDMA, etc.

(B) Estimation of monomers in HIPE process water

A fast and sensitive HPLC-UV method was developed (discussed in Chapter 5, Part B) for estimation of EHA, EGDMA and EHMA present at low ppm levels in HIPE process water. The application of method was demonstrated by analysing HIPE

process water samples generated in our laboratory. This method is useful to assess the quality of process water (in terms of residual monomers) before recycling it in FAM making process which is important not only for reducing the industrial operating cost, but also the load on the effluent. To summarise this work:

- Reverse phase chromatographic (RP-HPLC) method was developed for achieving separation of EHA, EGDMA and EHMA present in HIPE process water samples.
- Chromatographic conditions like mobile phase composition, flow rate and the detection wavelength were optimised and complete baseline separation was achieved for analytes.
- Method validation was performed by evaluating parameters such as system precision, specificity, linearity, response factors, limits of detection and quantification and 'method precision and accuracy'. These parameters established the efficiency of method and ensured that the method was valid for its intended use.
- The method is fast (total run time < 8 min) and very sensitive (can measure < 0.5 ppm of monomer levels). Thus, it serves as an efficient and economical analytical tool for analysis of trace levels of EHA, EGDMA and EHMA in HIPE process water (effluent) samples.

- **Styrenic HIPEs**

The analytical method for estimating monomer conversions in styrenic HIPE system (discussed in Chapter 6) was developed. The monomer conversions were estimated by measuring unreacted monomers (styrene, DVB and EHA) in HIPE polymerisation reactions. The protocol for monomer extraction for acrylic HIPE system worked very well for styrenic HIPE system as well. The method was based on simple GC-FID technique. The conclusions of this work are summarised below:

- Cyclohexane (water-immiscible solvent) was selected for extraction which, being non-polar, showed good selectivity for styrene, DVB and EHA. It also eliminated potential interference of water soluble (non-volatile) initiators, used in HIPE polymerisation reactions, which are harmful to GC column. The method for extraction of monomers from high internal phase emulsion was validated by assessing recovery of analytes from HIPE matrix. The solvent extraction method herein acts as a sample clean-up, an important step of sample preparation, which isolates potential interferences from HIPE sample matrix, thereby helping to make method more selective.
- The extraction method serves as a single step exhaustive extraction, and therefore, it is efficient and helps determine the unreacted monomers accurately.
- The sample preparation method and the chromatographic conditions (GC oven temperature programming) were optimised to accomplish the baseline separation of analytes and avoid interference of other components that originate from HIPE sample matrix.
- Method validation was carried out thoroughly by investigating system precision, method specificity, linearity (calibration studies), limit of detection (LOD), limit of quantification (LOQ), and 'method precision and accuracy'. This study established the efficiency of method and ensured that the method was valid for its intended use.
- Gauge R & R study proved that the measurement system was very stable, rugged and performed very well producing overall less variation and hence, it is quite acceptable.

- The usefulness of method was demonstrated by estimating monomer conversions in HIPE polymerisation reactions that were conducted using thermal and redox initiator systems.
- The method is fast (total run time < 10 min) and hence, cost-effective.
- This method can also be used for other applications such as studying the reactivity ratios of co-polymerisation of HIPE systems for the given monomers, such as EHA-DVB, Styrene-DVB, etc.