Optimization of processing parameters of poly (2-hydroxyethyl methacrylate) for embryo culture chambers

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Optimization of processing parameters of poly (2-hydroxyethyl methacrylate) for embryo culture chambers

by

Monawar Hussain

A Thesis Submitted to the
Graduate Faculty in Partial Fulfillment of the
Requirements for the Degree of
MASTER OF SCIENCE
Department: Industrial Education and Technology
Major: Industrial Education and Technology
(Industrial Technology)

Signatures have been redacted for privacy

Iowa State University
Ames, Iowa
1991

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# TABLE OF CONTENTS

**CHAPTER 1. INTRODUCTION**

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>The Problem of the Study</td>
<td>1</td>
</tr>
<tr>
<td>Purpose of the Study</td>
<td>2</td>
</tr>
<tr>
<td>Need for the Study</td>
<td>3</td>
</tr>
<tr>
<td>Research Questions</td>
<td>4</td>
</tr>
<tr>
<td>Assumption of the Study</td>
<td>5</td>
</tr>
<tr>
<td>Limitations of the Study</td>
<td>5</td>
</tr>
<tr>
<td>Procedure</td>
<td>6</td>
</tr>
<tr>
<td>Definition of Study</td>
<td>6</td>
</tr>
</tbody>
</table>

**CHAPTER 2. REVIEW OF LITERATURE**

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methodology</td>
<td>14</td>
</tr>
<tr>
<td>Preparation of reactants</td>
<td>15</td>
</tr>
<tr>
<td>Procedure</td>
<td>15</td>
</tr>
<tr>
<td>Properties</td>
<td>17</td>
</tr>
<tr>
<td>Measurements</td>
<td>17</td>
</tr>
<tr>
<td>Gas chromatography</td>
<td>17</td>
</tr>
<tr>
<td>Thin layer chromatography</td>
<td>18</td>
</tr>
<tr>
<td>Sulfites and sulfates</td>
<td>19</td>
</tr>
</tbody>
</table>
CHAPTER 3. METHODOLOGY

Research Questions ........................................ 22
The Research Design ...................................... 24
  The variables .............................................. 24
  The research design .................................... 24
Hypotheses of Study ....................................... 25
  Theoretical model ....................................... 25
  Research hypothesis I .................................... 26
  Statistical hypothesis I ................................ 26
  Research hypothesis II .................................. 26
  Statistical hypothesis II ................................. 26
  Research hypothesis III ................................ 27
  Statistical hypothesis III ............................... 27
The Materials .................................................. 27
Preparation of the Mold .................................. 28
The Molding Process ....................................... 28
The Pressurizing ............................................. 30
The Sample Size ............................................ 31
Sample Washing and Control ............................. 31
Measurements ................................................ 35
  Gas chromatography .................................... 35
  Thin layer chromatography ............................. 36
LIST OF TABLES

Table 2.1: Coping with storage and handling of hazards .............. 20
Table 3.1: The research design ................................................. 25
Table 3.2: Relative amount of components used for PHEMA hydrogel chambers. .................................................. 28
Table 4.1: HEMA and EG data for existing technique ................. 41
Table 4.2: HEMA and EG data for group 1: agitation ................. 41
Table 4.3: HEMA and EG data for group 2: no agitation ............. 42
Table 4.4: ANONA for HEMA ...................................................... 42
Table 4.5: ANONA for EG ......................................................... 43
# LIST OF FIGURES

| Figure 3.1: Process diagram for production of PHEMA hydrogel (100 chambers) | 23 |
| Figure 3.2: The insulin syringe mold | 29 |
| Figure 3.3: The pressure device used for pressurized polymerization | 32 |
| Figure 3.4: The PHEMA hydrogel | 33 |
| Figure 3.5: PHEMA hydrogel chamber | 34 |
| Figure 3.6: Important components of all glass model 3920 gas chromatograph | 37 |
| Figure 4.1: Plot of concentration of HEMA vs time for different washing groups | 45 |
| Figure 4.2: Plot of concentration of EG vs time for different washing groups | 46 |
CHAPTER 1. INTRODUCTION

The plastic industry is one of the fastest growing industry today. The use of plastics (i.e., polymers) as biomedical materials has become increasingly important in the last decade. Polymerized 2-hydroxyethyl methacrylate (PHEMA) hydrogel, an insoluble hydrophilic gel, was first synthesized in 1960 by Wichterle & Lim and since then it has been widely used as one of the best materials for a number of biomedical applications. Hydrogels are commonly used without support (e.g., soft contact lenses, drug release systems, embryo culture cells or chambers), with mechanical support (e.g., urethral anastomotic nipples on the artificial urinary bladder), or with chemical bonding (e.g., graft polymerization of hydrogels to suitable polymers such as polyurethanes).

A hydrogel is a substance having polymeric structure that exhibits the ability to swell in water and retain a significant weight fraction (e.g., 20-90%) of water within its structure, but does not dissolve in water. PHEMA hydrogel is an inert, translucent, biocompatible, diffusible, permeable, and nonbiodegradable resin or polymer with a long history of clinical application in medicine. PHEMA hydrogel chamber is a small cylindrical tube (i.e., with a cavity) used to culture embryo, in this case.

The reactants used in the formulation of PHEMA hydrogel are not totally utilized in the polymerization reaction and thus they become contaminants. These
contaminants of the hydrogel chambers are or could be biologically detrimental, in this case for the embryo. Therefore it is of paramount importance that these non utilized (or non polymerized) components are washed out of the PHEMA hydrogel to the level which is not harmful for the embryo. With the current technology, this is a tedious and lengthy procedure, thus insufficient and expensive; particularly because we do not even know if it is necessary to eliminate them, they may not be harmful for the embryo. Total time consumed to make 100 PHEMA chambers with the existing technique is about 116 hours which includes 108 hours to wash the PHEMA hydrogel. Out of these 108 hours, 96 hours are consumed while washing in 75% ethanol and 12 hours for washing in water. This time factor involved at both processing stages is a drawback in the existing practice. Any shortening of the time now spent in washing out non reacted components, that might not be even there, is or would be beneficial and of immediate application to biotechnology.

This study will investigate the processing parameters for the production of PHEMA hydrogel chambers used for the embryo culture and develop a washing procedure for PHEMA hydrogel to find out the actual time required for washing these chambers in 75% ethanol.

**The Problem of the Study**

The problem of this study was five fold:

1. To investigate and establish an optimization among the processing parameters of poly (2-hydroxyethyl methacrylate hydrogel chambers used for embryo culture.
2. To determine the time required to wash unpolymerized HEMA out of PHEMA hydrogel by using 75% ethanol.

3. To determine the time required to wash remaining ethylene glycol (EG) out of the hydrogel using 75% ethanol.

4. To determine the time required to wash remaining Tetraethylene glycol dimethacrylate (TGD) out of the PHEMA hydrogel using 75% ethanol.

5. To determine the time required to wash sulfite and any sulfate formed during polymerization of HEMA.

**Purpose of the Study**

The purpose of this study is to establish an optimization of processing parameters to develop an alternate process for the production of PHEMA hydrogel chambers for embryo culture. This study seeks a better method to wash PHEMA chambers, which will help to reduce the amount of time involved in the existing technique. The information gathered during the investigation of processing parameters will be used to develop a production process for PHEMA chambers used for embryo culture, with less time involved in the washing stage.

The objective of this study are:

1. Efficiency: To minimize the washing time leading to the reduction in total processing time.

2. Reduction in processing time can lead to reduction in processing cost.
With all these considerations, the new process developed would result in reduced costs and increased productivity over the existing processing procedure.

Need for the Study

PHEMA hydrogel is a biomaterial that offers great potential for clinical application. The existing technique utilized to make PHEMA hydrogel chambers is laboratory based and is low in reliability and efficiency. The human errors cause production defects. The chemical reaction completes very fast, which causes the polymerization to complete before even the batch of the reactant components is cast into the chambers and hence the cost of the chambers increases due to wastage of components of the formulation. On the other hand the porosity of the chambers is unknown for many compounds, which is very important for these chambers to be used in embryo culture. Total time consumed to make a batch of 100 chambers with existing technique is 116 hours, which is another cost increasing factor and hence an important drawback. Most of this time is spent on washing stages (108 hours). This factor increase the processing time and hence processing cost which leads to limit their use.

The processing parameters for PHEMA hydrogel chamber are pressure, time, temperature, initial water, crosslinker, initiator, and co-initiator contents. There is a need of research to develop a manufacturing process and establish an optimization of processing parameters of PHEMA hydrogel chambers. This optimization of the processing parameter is needed to develop a manufacturing process with better efficiency and the least processing time.
Research Questions

The questions to be investigated are:

1. What are the processing parameters for the production of PHEMA hydrogel chambers and how can these be optimized?

2. What is the actual time required to wash out into 75% ethanol, the non-polymerized components and unused reactants from PHEMA chambers?

Assumption of the Study

It is assumed that:

1. The chemical composition of the reactants remained constant during the storage, before polymerization.

2. The concentration of the sample taken out from the washing mixture will not change before and during its measurement stage.

3. The amount of the remaining reactants (monomer—unpolymerized HEMA, EG, TGD, and sulfites) present in ethanol after 96 hours of wash for control group (the existing washing technique) establish the maximum amount of non-reacted components which should be washed out of PHEMA hydrogel in order to make them useable. And hence, it defines limits for analysis and the level of these remaining reactants permitted in the hydrogel.

4. Attaining the same or lesser level of the remaining reactants in the experimental groups during the washing procedure as attained in the final wash of the control
group, establish the actual time needed to wash the hydrogel in the ethanol solution.

Limitations of the Study

1. The study is limited to the effects of one processing parameter e.g., time required to wash these chambers in 75% ethanol.

2. Other processing parameters like pressure, temperature, cross-linker, initiator, co-initiator, and monomer-water ratio are held constant and are not considered factors of the experiment.

Procedure

1. Review of literature in order to identify:
   - The parameter of the study.
   - The processing technique for the PHEMA hydrogel chambers.
   - The measures of the experiment.

2. Select a measurement technique to determine levels of the remaining reactants concentration in the wash sample.

3. Replication of the existing laboratory process for the PHEMA chambers for better understanding of the processing technique and the parameters.

4. Identify companies who supply the materials used for the production of PHEMA chambers.
5. Obtain materials from companies.

6. Select a mold for casting of PHEMA chambers.

7. Select a pressure device for pressurized polymerization of PHEMA hydrogel.

8. Determine sample size with the use of pilot study. This sample size will be determined based on the selection of Type I Error $\alpha = 0.05$, Type II Error $\beta = \text{unknown}$. Variance from these Error rates may be necessary if the sample size required exceeds the cost, time and/or resources of the investigator.

9. The following procedure was used to make the PHEMA hydrogel (Pollard, 1987; Pollard & Pineda, 1988):

   - Prepare the reactants which include monomer, crosslinker, initiator, co-initiator, and distilled water into three tubes A, B, and C as described in the literature.
   - Purge the reactants with nitrogen for 15 minutes in order to achieve inert atmosphere.
   - Prepare and lubricate mold with Silicon.
   - Mix the reactants (above three tubes) into a 5 ml glass tube, according to the required composition.
   - Cast the polymerizing mixture into three molds.
   - Insert 5.6 cm long and 0.9 mm OD stainless steel rod coated with teflon tubing, into the center of the polymerizing mixture in each of the mold.
   - Apply pressure of 40 psi for 15 minutes to the polymerizing mixture in order to achieve bubble free surface and to complete the polymerization.
• Take the polymerized hydrogel out of mold.

10. Repeat the above process to get total of 18 hydrogel samples.

11. Using a randomization procedure, divide the above hydrogels in 3 groups having 6 hydrogels in each group.

12. Place each group of the hydrogel in a glass beaker containing 50 ml of 75% ethanol to wash the unpolymerized reactants and un-crosslinked polymer. Subject one of the group to slow agitation.

13. Take specimen (observation) of wash sample according to time schedule given in the research design for the existing technique (control group) and the experimental groups (e.g., agitation and no agitation). And replace the 75% ethanol in the beaker after each observation.

14. Number these specimens as they are taken and seal them.

15. Use the following steps to analyze each sample in all groups for possible content of EG using Gas Chromatograph (Star, 1991; Meyer, 1988):

   • Select a Gas Chromatograph (GC) machine on campus.
   • Prepare 1 μg/ml EG standard.
   • Calibrate the machine to adjust its parameters.
   • Run EG standards to calculate its retention time and to draw a standard curve.
   • Run the samples.
• Calculate the contents of EG in each sample from the standard curve of peak height or area vs concentration.

16. Repeat the above procedure of GC to calculate the contents of HEMA for each sample in each group.

17. Use the following steps to analyze each sample in each group to calculate the content of TGD using Thin Layer Chromatography (Star, 1991):
   • Concentrate the samples from 100 μl to dryness and dissolve the residue in 10 μl methanol.
   • Prepare 1 μg/ml of TGD standard solution.
   • Use reverse-phase plate with indicator and spot it in such a way that the standards are in the middle and samples are on outside.
   • Use reverse-phase solvent (ethanol: water+acetic acid = 65:35+1).
   • Observe the migration of the spot under short range ultra violet light.
   • Use a densitometer to scan the plate using appropriate filter.
   • Construct a standard curve of peak height (density) vs concentration.
   • Calculate the contents of TGD from the standard curve.

18. Use the following steps to calculate the sulfate content by using a spectrophotometer:
   • Prepare the standard solution (100 μg/ml of ammonium sulfate by dissolving 0.1188 gm of ammonium sulfate in 1 liter of 75% ethanol.
   • Prepare samples.
• Add conditioning agent to each of above.

• Add a spatula of barium chloride and stir.

• Pour into 2 cm absorbance cell and record absorbance after 4 minutes.

• Repeat for each standard and sample.

• Construct a standard curve with absorbance on Y-axis and concentration on X-axis.

• Report ppm (=1 μg/g) of sulfate in 75% ethanol.

19. Statistically analyze the data using groups by replication ANOVA.

20. Make conclusions based on findings.

21. Make recommendations relevant to the study.

22. Report the findings.

Definition of Study

ANOVA: Analysis of variance.

APS: Ammonium persulfite.

Attenuator: This is an electrical component made up of a series of resistances that is used to reduce voltage to the recorder by a particular ratio.

Baseline: This is the portion of a detector record resulting from only eluant or carrier gas emerging from GC column.

Biodegradable: Describing a substance that can be decomposed into its natural components by natural influences (e.g. biological action or sunlight).
Chamber: An enclosed space, compartment or hollow cavity. In the present case, chamber is a cylindrical shape (length=1 cm, outside diameter=1 mm, inside diameter=1.7 mm) made of PHEMA hydrogel, as shown in figure 3.5.

Carrier gas: This term is synonymous with mobile or moving phase. This is the phase that transports the sample through a gas chromatographic column.

Chromatogram: This is the plot of detector response vs effluent volume or time.

Chromatography: It is a separation process in which the sample mixture is distributed between two phases in the chromatographic bed (column). The stationary phase is either a solid, porous, surface-active material in small particle form or a solid support covered with a thin film of liquid. The mobile phase is a gas or liquid.

Column: This is a metal, plastic or glass tube packed or internally coated with the column material through which sample components and mobile phase (carrier gas) flow and in which chromatographic separation takes place.

Cross-linking: The linking of two independent polymeric chains by a grouping that spans or links two chains (e.g., EG and TGD).

Diffusivity: The rate of propagation of gaseous or liquid substance due to their molecular movement.

Efficiency: The ratio of useful work to energy supplied.

EG: Ethylene glycol.

Exothermic reaction: A spontaneous reaction in which heat is liberated.

Flame ionization detector, FID: This detector utilizes the increased current at a collector electrode obtained from the burning of a sample component from the column effluent in a hydrogen and air jet flame.

Gas chromatography: This term is used for those chromatographic methods in which moving phase is gas (i.e., nitrogen).

GC: Gas chromatograph.

Heterogeneous: A substance which is not uniform or similar in nature, throughout.
Homogeneous: A substance which is uniform or similar in nature, throughout.

Hydrogel: Water swollen, rigid, 3-dimensional network of cross-linked, hydrophilic macromolecules (e.g., 20 - 95% water).

Hydrophilic: Having an affinity for attracting, adsorbing, or absorbing water.

Homogeneous polymerization: A type of polymerization which involves only single phase of matter.

Inert atmosphere: A sluggish gaseous atmosphere (nobel gases) that is resistant to chemical reaction.

Initiator: The substance or molecule (other than reactant) that initiates a chain reaction as in polymerization: an example is Ammonium persulfate.

Inhibitor: Any substance that retards or reduces the rate of chemical reaction.

Macroporous: A substance having pores size of its membrane between 0.5 to 1.0 micron.

Molarity, M: Measure of the number of gram-molecular weights of a compound present (dissolved) in 1 liter of solution.

Monomer: A basic unit substance, which when an unsaturated bond is broken to form available bonds by catalytic or activator influence, may form basic mer of a polymer.

Parts per million, ppm: 1 μg/g.

Peak: This is the portion of differential chromatogram recording the detector response or elute concentration when a compound emerges from the GC column.

Peak area, a: The area enclosed between peak and peak base.

Peak height, h: This is the distance between peak base and height measured in the direction parallel to detector response axis.

Permeable: The membrane that allows the passage of one type of material particles/molecules and prevent the other one is called as permeable.

PHEMA: Poly (2- hydroxyethyl methacrylate).
Plastics: Finished articles that are made from polymeric materials by molding techniques.

Polymer: A relatively high molecular weight substance composed by repetitively linking together small molecules, called monomeric units, in sufficient number such that the addition or removal of one or several units does not change the properties of the substance.

Polymerization: The process that convert monomers into polymers.

Porosity: The state or property of a substance of being porous.

Reactant: A substance participating in a chemical reaction, at the initiation of the reaction.

Resin: The polymeric material, either pure or with appropriate additives, is called a resin.

RS: Reverse phase.

Si: Silicon.

SMS: Sodium metabisulfite.

Teflon: Trademark for polytetrafluoroethylene. It resists chemical attack and has electrical insulating properties.

TGD: Tetraethylene glycol dimethacrylate.

TLC: Thin layer chromatography.

Translucent: Transmitting light but causing sufficient diffusion to eliminate perception of distinct images.

W: Water.
CHAPTER 2. REVIEW OF LITERATURE

The use of Poly (2-hydroxyethyl methacrylate), PHEMA hydrogel as biomedical materials has become increasingly important due to its use in soft contact lenses, medical implants, surgical dressings, and embryo culture chambers; in the last decade (Hoechner, 1988; Pollard & Pineda, 1988). PHEMA is an insoluble hydrophilic gel. A hydrogel can be defined as a polymeric substance that exhibits the ability to swell in water and retain a significant fraction (e.g., 20-90%) of water within its structure, but does not dissolve in water (Ratner & Hoffman, 1975; Hsiue & coworkers, 1988; Korbelar & coworkers, 1988).

Methodology

The following components were used to construct PHEMA chambers by Lee, 1978 and Pollard, 1987:

- **Monomers:** Low acid, 2-Hydroxyethyl Methacrylate (HEMA) = 10 ml
- **Crosslinkers:** Tetraethylene glycol dimethacrylate (TGD) = 0.1 ml
  Ethylene glycol (EG) = 3.0 ml
- **Distilled water:** = 2.0 ml
- **Initiator:** Ammonium persulfate, 40g/100ml water = 1.0 ml
- **Co-initiator:** Sodium metabisulfite, 15g/100ml water = 1.0 ml
Preparation of reactants

**Tube A:** Mix in 20 ml glass tube: 10 ml HEMA + 0.1 ml TGD + 3.0 ml EG + 2.0 ml distilled water. Molarity of crosslinker in HEMA is 3.72M.

**Tube B:** 1.0 ml of 1.75M ammonium persulfate solution in 5ml glass tube.

**Tube C:** 1.0 ml of 0.76M sodium metabisulfite solution in 5 ml glass tube (Pollard, 1987).

Procedure

1. Purge all of the above reactants with nitrogen.
2. Make polymerization mixture in 3.0 ml polyethylene syringe by mixing 1.5 ml, 0.1 ml, & 0.1 ml from tubes A, B, & C respectively.
3. Cast hydrogel chambers in the 3.0 ml polyethylene syringe by taking 0.5 ml of polymerizing mixture.
4. Apply pressure of about 40 psi for 15 minutes (Pollard, 1987).

In the presence of an initiator, such as ammonium persulfate and sodium metabisulfate, the polymerization of HEMA is an exothermic reaction. At this high temperature the solubility of gases decreases leading to bubble formation. The bubbles trapped in the wall structure of the hydrogel decrease the strength of the polymer. They can provide stagnant pools and become infected or encrusted. The polymerization at pressures above 700 kPa (100 psi) produces completely bubble-free PHEMA hydrogel chambers (Pinchuk & Eckstein, 1981). The reaction kinetics do not change significantly under varying pressures. Maximum exothermic temperature is attained in 4 minutes following the initiator and the reaction completes in about 15 minutes.
The effect of monomer-water ratio on porosity of the polymer is such that, by changing the monomer-water ratio in the starting mixture, it is possible to pass smoothly from the homogeneous course of polymerization to heterogeneous one, when phase separation takes place and the resulting polymer is porous. The increase of water concentration in the starting mixture, cause an increase in the porosity of the resulting polymer from microporous to macroporous (pore size 300–400 μm) spongy gel (Korbelar & coworkers, 1988).

The relative permeabilities of PHEMA membrane decrease significantly on increasing the contents of crosslinker. Over the range of low crosslinker content (0-1.6 mole %), the relatively permeabilities depend on the molecular weights. In other words, smaller the molecular weight, larger the permeability. Over range of low crosslinker contents acetamide (molecular weight = 59.07) shows greater permeability as compared to urea (molecular weight = 60.06). But over the range of higher crosslinker contents, urea which has a larger molecular weight than that of acetamide, tends to have higher permeability (Lee & coworkers, 1978). The diffusion coefficient also decreases by increasing crosslinker content in the hydrogel membrane. Over the range of low crosslinker contents (i.e. 0.4 mole %), the diffusion coefficient of acetamide is 38.65 cm²/sec, whereas urea has diffusion coefficient equal to 15.36 cm²/sec. But on increasing the crosslinker contents (i.e. 6.4 mole %), the diffusion coefficients of acetamide and urea are 6.44 and 6.50 cm²/sec, respectively. This discussion shows that the solute is considered to travel through the pores of the hydrogel memberane, and the pore size is controlled by the crosslinker contents. Whereas, pores are the water containing regions of hydrogel and the become smaller on in-
creasing the crosslinker contents, causing the hydroxyl group density to increase and thus resulting in increased diffusivities. (Lee & coworkers, 1978). A decrease in the diffusion coefficient was observed up to 6 mole % crosslinker. Below 2.5 mole %, a sharp decrease was observed (Wisniewski & coworkers, 1976).

Properties

Polymerized HEMA (PHEMA) is a translucent, permeable, diffusible, and non-biodegradable hydrogel. It can be easily polymerized and cast into any shape (Pollard & Pineda, 1988). PHEMA hydrogel has poor mechanical properties. Tensile strength of wet PHEMA is 2.2 – 2.6 kg/cm², and its percent elongation is 40 – 50. In dried state, PHEMA is brittle and rigid, it has a hydrophilic surface. The contact angle between water drop and PHEMA membrane is 75° and the water content is 40% (Hsiue & coworkers, 1988).

Measurements

Gas chromatography

The chromatographic system enables the analyst to separate and quantitatively evaluate complex sample mixtures in amount of microgram or nanograms depending on type of chromatographic column, detector and/or attenuation used. Gas chromatography has a major role among the methods for the analysis of organic compounds and gases. GC is widely used for the analysis of complex mixture. The basic units of the chromatograph are the chromatographic column and the detector. The column separates the test mixture into its components and the detector records
(carrier gas flow) the concentrations of the separated components. The results of the separation are recorded automatically. Separation in GC is based on different distributions of molecules of the components being separated between the mobile phase and the stationary phase. The quantitative analysis in GC is carried out on the basis of the retention times or retention volumes (Berzkin, V.R. & coworkers, 1977).

Ethylene glycol in urine is determined by direct injection into Porapak Q column on a gas chromatograph (Stahr, 1991). Same procedure can be applied to calculate amount of HEMA or EG in ethanol or water. The ppm (=1 µg/g) EG can be calculated as:

\[
\text{ppm of EG} = \frac{\mu g \text{ of EG from standard curve}}{\text{sample injected}} \times \text{ml of dilution}
\]

The retention time of a component is always constant under identical chromatographic conditions. A peak can be identified by injecting the relevant substance and then comparing the retention times. The area of a peak is proportional to the amount of a compound injected. A calibration graph can be derived from peak areas obtained for various solutions of precisely unknown concentration of an unknown sample and a peak area comparison can then be used to determine the concentration of an unknown sample (Meyer, 1988).

**Thin layer chromatography**

The necessary elements of thin layer chromatography (TLC) are the same in principle as those of gas chromatography, but differ in their purpose. The common means of introducing a sample for TLC is by spotting the sample on the TLC plate. To spot the TLC plate a small disposable capillaries or reusable microliter syringes
are used. Once the chromatography plate is developed, the bands must be visualized. The compound of interest is then detected by the reduction in fluorescence when the plate is irradiated by UV light. Densitometers are used to directly detect TLC bands by densitometry fluorescence or fluorescence quenching, color or reflectance, spectrometry (Stahr, 1991).

**Sulfites and sulfates**

Ammonium persulfate decomposes in the presence of moisture and heat evolving its oxygen and forms a sulfate. Ammonium sulfate is insoluble in alcohol. Sodium metabisulfite is slightly soluble whereas sodium sulfate is insoluble in alcohol (The Merck Index, 1983).

**Safety and Health Information**

While dealing with chemicals, it is important to understand techniques for storage and handling hazards, as given in table 2.1: Coping with storage and handling of hazards\(^1\).

**First aid measures**

- After contact with eyes: Immediately begin flushing with plenty of water, call physician.

- After contact with skin: Wash immediately and thoroughly with soap and cold water, call a physician, remove wetted clothing and launder before re-wearing.

\(^1\)Source: Rohm and Haas company, Philadelphia, PA
Table 2.1: Coping with storage and handling of hazards

<table>
<thead>
<tr>
<th>HAZARD</th>
<th>EFFECT</th>
<th>PREVENTION</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heat</td>
<td>Polymerization</td>
<td>Store below 90° out of sun</td>
</tr>
<tr>
<td>Aging</td>
<td>Polymerization</td>
<td>Store 1 year maximum, replenish air in drums</td>
</tr>
<tr>
<td>Contamination</td>
<td>Polymerization, Hydrolysis</td>
<td>Close containers not in use, keep dry</td>
</tr>
<tr>
<td>Light</td>
<td>Polymerization</td>
<td>Close containers not in use</td>
</tr>
<tr>
<td>Sparks</td>
<td>Fire</td>
<td>Ground containers, vessels, pipe</td>
</tr>
<tr>
<td>Flame</td>
<td>Fire</td>
<td>Do not permit in work, storage area</td>
</tr>
<tr>
<td>Vapor, mist in work place</td>
<td>Irritation of personnel</td>
<td>Provide adequate ventilation, wear respiratory devices</td>
</tr>
</tbody>
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- If overcome by vapors: Move victim to fresh air, administer artificial respiration or oxygen if needed.

- If swallowed and victim is conscious: Dilute by giving water to drink, call physician (Hoechner, 1988).
CHAPTER 3. METHODOLOGY

This study is centered on the optimization of processing parameters of PHEMA hydrogel chambers and time required to wash these chambers in 75% ethanol. The reactants used to polymerize 2-hydroxyethylene methacrylate (HEMA) are not fully utilized during this chemical reaction and thus if present, these reactants (HEMA, TGD, EG, ammonium persulfite, sodium metabisulfite, etc.) can become the contaminants of the PHEMA hydrogel. Until the present time, the amount of these non utilized or non polymerized reactants present in the hydrogel were unknown. These contaminants are or could be harmful for the embryo. Therefore, they must be removed from PHEMA hydrogel. The current procedure used to wash out these remaining reactants from hydrogel into 75% ethanol takes not only 96 hours, we even don’t know if its washing them or not. Therefore it is of significant importance to find out, the washing pattern of these remaining reactants and actual time they may take to wash out into 75% ethanol. Any shortening of time now spent in washing out these remaining components of formulation of PHEMA hydrogel is or could be beneficial and of immediate application in biotechnology.

Data for the time required to wash remaining reactants like HEMA, TGD, EG, ammonium persulfate, sodium metabisulfite, etc. was collected and analyzed. To achieve this aim, many activities were coordinated and performed. This chapter lists
those activities showing the order in which they occurred and explains what was involved in each step.

The figure 3.1 systematically represents a block diagram of different processing steps involved in production of PHEMA hydrogel (100 chambers) and approximate time involved in each step, according to the current procedure. The abbreviations HEMA, EG, TGD, W, APS and SMS represent 2-hydroxyethyl methacrylate, ethylene glycol, tetraethylene glycol dimethacrylate, water, ammonium persulfate and sodium metabisulfite, respectively.

**Research Questions**

The questions to be investigated were:

1. What are the processing parameters for the production of PHEMA hydrogel chambers and how can these be optimized?

2. What is the actual time required to wash out into 75% ethanol, the non polymerized components and unused reactants from PHEMA chambers?

The hypotheses given in the subsequent section are used to test these questions. The hypotheses are tested on results of analysis employing the mixed research design ANOVA model (groups by replications). The research design compares 8 levels of washing time with two agitation levels.
Figure 3.1: Process diagram for production of PHEMA hydrogel (100 chambers)
The Research Design

The variables

The independent variables that were manipulated in the experiment are the time required to wash PHEMA chambers in ethanol and experimental group effects. The effect of those independent variables was determined on the dependent variable which is concentration of the remaining reactants (EG, HEMA, TGD, sodium persulfate and sodium metabisulfite) in 75% ethanol while washing PHEMA hydrogel in it.

The research design

While performing this research, the monomer composition (HEMA, TGD, EG, initiator, co-initiator, and water contents), pressure and temperature were kept constant. The time and experimental group were the only variables which were manipulated. The table 3.1 illustrates the 2 x 8 research design for washing of PHEMA hydrogel in 75% ethanol, for group 1 and group 2. Group 1 was subjected to slow agitation while group 2 was not subjected to agitation during washing PHEMA hydrogel in 75% ethanol. The washing procedure used in the existing technique (Group 3) demonstrated the acceptable amount of non polymerized components in the PHEMA hydrogel. Or in other words Group 3 was used to find the limits of the experiment and was not direct part of experimental groups.

At each time interval, a sample of ethanol was taken from each beaker for both groups and ethanol was changed in each of the beaker. After 96 hours of washing these samples were tested using measurement techniques which are described later. Thus there was one sample or observation for each experimental group (i.e., agitation
and no agitation) with one measurement for each observation. The experiment was replicated three times to confirm the results.

Table 3.1: The research design

<table>
<thead>
<tr>
<th>Concentration of Remaining Reactants</th>
<th>Washing Time (in hrs)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Groups</td>
<td>0.5 1.5 3 6 12 24 48 96</td>
</tr>
</tbody>
</table>

Hypotheses of Study

Following hypotheses are used to analyze remaining reactants present in 75% ethanol, with two agitation levels. These hypotheses are applied to ethylene glycol, HEMA, TGD, sulfite and sulfate, one by one.

Theoretical model

The theoretical model for above research design is given below:

\[ X_{ijk} = \mu + \alpha_j + \beta_k + \pi_i(j) + \alpha \beta_{jk} + \beta \pi_{ki}(j) + e_{i(jk)} \]

where \( \mu \) is the population mean of observations,
\( \alpha_j \) is the effect of treatment group \( j; j=1,2 \)
\( \beta_k \) is the effect of repeated observations \( k; k=1,...,8 \)
\( \pi_i \) is the effect of observation \( i \) within group \( j \),
\( \alpha \beta_{jk} \) is the interaction of group \( j \) with repeated measure \( k \),
\( \beta \pi_{ki}(j) \) is the interaction of observation \( i \) and repeated measure \( k \) in the \( j \)th group,
and \( e_{i(jk)} \) is the error for observation \( i \) in group \( j \) and repeated measure \( k \).
Research hypothesis I

There are significant differences among the mean concentration of remaining reactants present in ethanol at different agitation levels, while washing PHEMA chambers in 75% ethanol.

Statistical hypothesis I

There are differences among the population means of remaining reactants in 75% ethanol, at different agitation levels.

\[ H_T: \mu_1 = \mu_2. \]
\[ H_A: \mu_1 \neq \mu_2. \]

Research hypothesis II

There are significant differences among the mean concentration of remaining reactants present in ethanol at different time intervals, while washing PHEMA chambers in 75% ethanol.

Statistical hypothesis II

There are differences among the population means of remaining reactants in 75% ethanol, at different washing times.

\[ H_T: \mu_{.1} = \mu_{.2} = \mu_{.3} = \ldots = \mu_{.8} \]
\[ H_A: \mu_{.j} \neq \mu_{.k} \text{ for at least one } j, \ k \text{ where } j \neq k \]
Research hypothesis III

There are significant differences among the mean concentration of remaining reactants present in ethanol at different combinations of washing time and agitation levels, while washing PHEMA chambers in 75% ethanol.

Statistical hypothesis III

There are differences among the population means of remaining reactants in 75% ethanol, at different combinations of washing time and agitation levels.

\[ H_T: (\mu_{11} - \mu_1, - \mu_1 + \mu_1, - \mu_2, + \mu_2, - \mu_8, - \mu_8) = \cdots = (\mu_{28} - \mu_2, - \mu_8 + \mu_8) \]
\[ H_A: (\mu_{11} - \mu_1, - \mu_1 + \mu_1, - \mu_2, + \mu_2, - \mu_8, - \mu_8) \neq \cdots \neq (\mu_{28} - \mu_2, - \mu_8 + \mu_8) \]

The Materials

As described in chapter 2, the PHEMA chambers were made from the mixture of low-acid HEMA, as a monomer, tetraethylene glycol dimethacrylate (TGD) and ethylene glycol (EG), as crosslinkers (Polysciences, Inc. Warrington, PA). Ammonium persulfate 40g/100ml distilled water (1.75M) and Sodium metabisulfite, 15g/100ml distilled water (0.79M), were used as initiator and co-initiator respectively. The relative amounts of the components is given in table 3.2.

The reactants were prepared in three tubes according to the procedure described in chapter 2.
Table 3.2: Relative amount of components used for PHEMA hydrogel chambers.

<table>
<thead>
<tr>
<th>Components</th>
<th>Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Monomer: 2-Hydroxyethyl methacrylate</td>
<td>10</td>
</tr>
<tr>
<td>Crosslinkers: Tetraethylene glycol dimethacrylate</td>
<td>0.1</td>
</tr>
<tr>
<td>Ethylene glycol</td>
<td>3.0</td>
</tr>
<tr>
<td>Distilled water:</td>
<td>2.0</td>
</tr>
<tr>
<td>Initiator: Ammonium persulfate solution</td>
<td>1.0</td>
</tr>
<tr>
<td>Co-initiator: Sodium metabisulfite solution</td>
<td>1.0</td>
</tr>
</tbody>
</table>

Preparation of the Mold

A 71-mm-long, 3.5-mm ID, 0.5-ml insulin syringe (No. 8471, single use, plastipak LO-dose U-100; Becton Dickinson and Co, Rutherford, NJ) was used as a mold for casting hydrogel chambers at room temperature. The plunger was withdrawn from the syringe and the rubber gasket at the tip of the plunger was removed, inverted and reinserted into the syringe so that its end with cavity is directing inside. A 5.6-cm-long, 0.9-mm-OD stainless steel rod along with 5.6-cm-long and 1.7-mm-OD, Teflon tubing (Colo-Parmer International, Chicago, IL) on its outer surface was used to form a hollow cavity in the hydrogel chamber. This steel rod was inserted into center of the mold, after the polymerizing mixture was poured into it (Pollard, 1987). The figure 3.2 illustrates a typical mold used to make PHEMA hydrogel chambers, which consists of an insulin syringe.

The Molding Process

The following procedure was used for the molding process by Pollard, 1987 and Pollard & Pineda, 1988):
Figure 3.2: The insulin syringe mold
1. Take 1.5 ml from tube A contents and 0.1 ml from each of tubes B and C contents into a 5 ml glass tube.

2. Use 5 ml syringe to mix the above reactants thoroughly by pumping the mixture in and out of it, twice. Watch out for air bubbles.

3. With the help of above 5 ml syringe, pour the polymerizing mixture into three molds described above, while holding them vertically.

4. Inset 5.6 cm long and 0.9 mm OD stainless steel rod coated with teflon tubing, into the center of the polymerizing gel through needle connector end, in each of three molds. The end of stainless steel rod should rest into the cavity of rubber gasket at the bottom of the mold.

The Pressurizing

A simple hydraulic pressure device with two vertical jaws having neoprene stopper at the ends was utilized. The above three syringe molds holding the polymerizing mixture were inserted into three holes of plastic holder of pressure device. A 4 cm long, 3.5 cm OD stainless steel rod was inserted into the flanged end of the barrel of each of the syringes and then the whole arrangement was placed between neoprene stoppers attached to the jaws of the pressure device. A pressure of 40 psi was applied for 15 minutes. Then the cast hydrogel was taken out of the mold. The pressure device used for this purpose is given in figure 3.3. A total of 18 hydrogels were made and then they were divided into three equal groups. Each of the group was placed in a glass beaker containing 50 ml of 75% ethanol for washing. Cover the glass with aluminum foil. The figure 3.4 illustrate a PHEMA hydrogel made by this process.
and the figure 3.5 illustrates a drawing of the PHEMA hydrogel chamber (Pollard, 1987):

The Sample Size

On the basis of the resources (i.e., time, money, and availability of measurement facilities), review of literature, and the experiment study by the researcher, the sample size was selected as following:

**Group 1, agitation:** Three samples of ethanol wash (with agitation) with 8 observation of each sample.

**Group 2, no agitation:** Three samples of ethanol wash (without agitation) with 8 observations of each sample.

**Group 3, existing technique:** Three samples of ethanol wash with 4 observations.

Total number of observations was 60. The calculations were based on the three observations in each combination of time and agitation grouping. Group 1 and group 2 were the only groups compared, where as the group 3 described the existing technique and was only used to find the limits of the measurements. Type I error was selected equal to 0.05 or in other words the level of the confidence to test the hypothesis was selected 95%. Type II error was unknown because there was no control on this due to fixed sample size.

Sample Washing and Control

Each of the PHEMA hydrogels were subjected to similar washing treatment because samples needed to be identical in order to produce significant differences.
Figure 3.3: The pressure device used for pressurized polymerization
Figure 3.4: The PHEMA hydrogel
Figure 3.5: PHEMA hydrogel chamber

Hydrogel Chamber Body
Silastic Solid Plugs

End View

Approximate Measures
Chamber length = 1 cm
Outside diameter = 3.5 mm
Inside diameter = 1.7 mm
Wall thickness = 0.2 mm
Chambers were kept in 50 ml of 75% ethanol for 96 hours to wash off unpolymerized reactants and other remaining reactants which could be harmful for the embryo. According to time schedule described in the research design, a specimen (e.g., observation) of each of the 75% ethanol wash was taken and then ethanol was changed immediately in the wash beaker. The specimen was sealed in a glass test tube. Each specimen was numbered in the order they are taken out of the sample. The specimens were resorted into groups according to numbers for data collection.

**Measurements**

Appropriate measurement techniques available to the researcher were mass spectroscopy, infrared spectrometry, and high performance liquid chromatography, gas chromatography (GC) and thin layer chromatography (TLC). The best suited technique for the analysis of HEMA and EG was by the use of a gas chromatograph (model 3920, Perkin-Elmer) equipped with Porapak Q column and flame ionization detector (i.e., hydrogen). The carrier gas was nitrogen. TLC was selected for TGD measurement. This decision was based upon the review of literature, test of few specimens using above techniques, and the research design.

**Gas chromatography**

Following procedure was used to analyze the samples for EG using a gas chromatograph equipped with flame ionization detector and Porapak Q column:

1. Prepare ethylene glycol standard solution (1 μg/ml: dilute 12.5 μl ethylene glycol in 10 ml of 75% ethanol.
2. Light the hydrogen flame of the Gas Chromatograph (Perkin-Elmer 3920) and calibrate the GC and recorder parameters such as temperature=230°C, attenuation=4 × 10, chart speed=25 cm/hr and chart sensitivity=0.001 volts.

3. Inject different standards to calculate the retention time and the standard curve of concentration (X-axis) vs peak height (Y-axis).

4. Now run the specimen into the injection unit of the machine and repeat the standard after every 4th sample.

5. From the standard curve or slope factor calculate the concentration of ethylene glycol and use the formula given in chapter 2 to calculate ppm (=1 μg/g) of EG in each sample:

The same procedure was followed for the analysis of HEMA. The only difference was in column temperature 340°C. The peak area was used to draw the standard curve instead of peak height, because of fat peak due to less sensitivity of detector toward HEMA. The figure 3.6 illustrates the important components of all glass model 3920 gas chromatograph.

**Thin layer chromatography**

The following procedure of TLC was used for the analysis of TGD in ethanol samples:

1. Prepare the TGD standard solution (1 μg/ml: dilute 12.5 μl of TGD to 10 ml of 75% ethanol).
Figure 3.6: Important components of all glass model 3920 gas chromatograph
2. Concentrate 100 μl of each of the samples in glass tubes to dryness, using nitrogen flow.

3. Add 5 μl of methanol to redissolve the TGD residue and spot it on a reverse phase TLC plate (with indicator). Rinse the glass tube using another 5 μl of methanol and spot it on the same above spot.

4. Spot 0.5, 1.0, 2.0, 3.0, 4.0, 5.0, 10.0, 20.0 and 30.0 μl of standard solution on the TLC plate such that the standards are in the middle and all concentrated samples are on their sides.

5. Develop the plate in a reverse phase solvent (ethanol:water + acetic acid = 65:35+1), in a glass cell.

6. After the plate is developed, dry it and examine it under UV light. Locate all the spots moved with capillary action of the above solvent.

7. Use densitometer—Fiber Optic Scanner (Knotts, Scientific Instrument Group, NJ) to quantitatively analyze the samples. Calibrate the densitometer and recorder. Use short range UV filter. Use scan rate = 5 and attenuation = 16.

8. Draw a standard curve of TGD.

9. Report the ppm (=1 μg/g) of TGD from the standard curve.

Disposal of Spills and Wastes

1. Contaminated monomers may be unstable. This contamination could cause its polymerization or and hydrolysis. To inhibit these effects, close containers not in use, keep dry and store below 90°F out of sun.
2. Cover spilled material with inert solid absorbent (clay, vermiculite, earth). If necessary dike it with absorbent to contain spill.

3. Shovel soaked absorbent into plastic-lined containers for disposal.

4. Discard all recovered contaminated monomer.

5. Dispose of containers and absorbent in approved landfill or burn in approved incinerator.

6. After cleanup, hose down area with plenty of water. Treat runoff in chemical wastage-disposal facility with activated sludge. In treating runoff, comply with pertinent federal, state and local regulations.
CHAPTER 4. PRESENTATION OF DATA

The results of this study are presented in this chapter. Each of the hypotheses listed in chapter 3 were tested at 95% confidence level for the significant difference between the two groups of agitation and no agitation. The method of analysis chosen was groups by replication (mixed design) Analysis of Variance (ANOVA) since this method would efficiently test the effects of the both independent variables—time and experimental group effect (i.e., agitation and no agitation).

Description of Data and Statistical Analysis

Groups by replication Analysis of Variance (ANOVA) was performed on the results obtained from GC in terms of concentration (ppm=1 μg/g) of EG and HEMA, using S.A.M.P.L.E. package by Miller written in PASCAL on Iowa State University, Ames, IA. Ethylene glycol was not detected in 4th sample for group 1 (agitation), therefore it was extrapolated from the graph of EG for time vs concentration. Due to the same reason, the 3rd value of HEMA for group 1 (agitation) was extrapolated from the curve of HEMA for time vs concentration.

The existing washing technique (change of ethanol after every 24 hours) was used to find out how much of the remaining reactants were washing out during this procedure to make PHEMA hydrogel unharful for the embryo culture. Raw data
calculated from the standard curve of concentration versus peak height or peak area is
given in the appendix C. Table 4.1 gives time versus mean values of parts per million
(ppm) concentration of HEMA and ethylene glycol found in the each of ethanol
wash or in other words mean ppm of HEMA and EG washing out of hydrogel into
the ethanol for the existing technique. The minimum detectable limit for both EG
and HEMA was 100 ppm. Therefore for those samples for which EG or HEMA were
not detected an abbreviation ND will be used.

Table 4.1: HEMA and EG data for existing technique

<table>
<thead>
<tr>
<th>Washing Time (hours)</th>
<th>24</th>
<th>48</th>
<th>72</th>
<th>96</th>
</tr>
</thead>
<tbody>
<tr>
<td>Concentration of HEMA (ppm)</td>
<td>250</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Concentration of EG (ppm)</td>
<td>4583</td>
<td>1177</td>
<td>118</td>
<td>ND</td>
</tr>
</tbody>
</table>

Ammonium sulfate or sodium sulfate were not detectable because of their insol-
ubility in alcohol which caused difficulties in making sulfate standard solution in 75%
ethanol. As far as TGD is concerned, it was not detectable beyond 5 ppm. In other
words if TGD was present in any of the sample, it was below 5 ppm.

The variation of concentration (mean) of HEMA and EG is given in table 4.2
and table 4.3 for agitation and no agitation groups, respectively.

Table 4.2: HEMA and EG data for group 1: agitation

<table>
<thead>
<tr>
<th>Washing Time (hours)</th>
<th>0.5</th>
<th>1.5</th>
<th>3</th>
<th>6</th>
<th>12</th>
<th>24</th>
<th>48</th>
<th>96</th>
</tr>
</thead>
<tbody>
<tr>
<td>Concentration of HEMA (ppm)</td>
<td>288</td>
<td>208</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Concentration of EG (ppm)</td>
<td>3845</td>
<td>1201</td>
<td>240</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

1 μg/g

2 The remaining reactants were assumed to be 100 ppm, because they were unde-
tectable below this limit
Table 4.3: HEMA and EG data for group 2: no agitation

<table>
<thead>
<tr>
<th>Washing Time (hours)</th>
<th>0.5</th>
<th>1.5</th>
<th>3</th>
<th>6</th>
<th>12</th>
<th>24</th>
<th>48</th>
<th>96</th>
</tr>
</thead>
<tbody>
<tr>
<td>Concentration of HEMA (ppm)</td>
<td>166</td>
<td>150</td>
<td>121</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Concentration of EG (ppm)</td>
<td>1548</td>
<td>845</td>
<td>365</td>
<td>150</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

A groups by replication ANOVA (mixed design) was performed on each of the experimental group data. Table 4.4 shows the results of analysis of variance of HEMA, whereas table 4.5 shows the analysis of variance of ethylene glycol. These tables show the variance between the treatment groups, between the time (within the treatment groups) or repeated measures, interactions between treatment groups and time, and error factor.

Table 4.4: ANOVA for HEMA

<table>
<thead>
<tr>
<th>Source of Variance</th>
<th>DF</th>
<th>Sum of Squares</th>
<th>Mean of Squares</th>
<th>F Value</th>
<th>Prob &gt; F</th>
</tr>
</thead>
<tbody>
<tr>
<td>AMONG PHEMA PREPARATION</td>
<td>5</td>
<td>10025</td>
<td>2005</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Between Treatment. Groups</td>
<td>1</td>
<td>8978</td>
<td>8978</td>
<td>34</td>
<td>&lt; 0.0056</td>
</tr>
<tr>
<td>Between Error</td>
<td>4</td>
<td>1047</td>
<td>262</td>
<td></td>
<td></td>
</tr>
<tr>
<td>WITHIN PHEMA PREPARATION</td>
<td>12</td>
<td>73002</td>
<td>6084</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Times</td>
<td>2</td>
<td>50776</td>
<td>25388</td>
<td>634</td>
<td>&lt; 0.00001</td>
</tr>
<tr>
<td>Times × Treatment Groups</td>
<td>2</td>
<td>21905</td>
<td>10953</td>
<td>274</td>
<td>&lt; 0.00001</td>
</tr>
<tr>
<td>Residual</td>
<td>8</td>
<td>320</td>
<td>40</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>17</td>
<td>83027</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Testing of Hypotheses

It was decided from the beginning of this study to accept or reject the hypotheses on the bases of the statistical analysis of remaining reactants present in the 75% ethanol specimens taken during the washing procedure of PHEMA hydrogel in it. Now research hypotheses will be discussed for HEMA and EG.
Table 4.5: ANOVA for EG

<table>
<thead>
<tr>
<th>Source of Variance</th>
<th>DF</th>
<th>Sum of Squares</th>
<th>Mean of Squares</th>
<th>F Value</th>
<th>Prob &gt; F</th>
</tr>
</thead>
<tbody>
<tr>
<td>AMONG PHEMA PREPARATION</td>
<td>5</td>
<td>2209055</td>
<td>441811</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Between Treatment Groups</td>
<td>1</td>
<td>2208873</td>
<td>2208873</td>
<td>48547</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>Between Error</td>
<td>4</td>
<td>182</td>
<td>46</td>
<td></td>
<td></td>
</tr>
<tr>
<td>WITHIN PHEMA PREPARATION</td>
<td>18</td>
<td>30964128</td>
<td>1720229</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Times</td>
<td>3</td>
<td>25025227</td>
<td>8341742</td>
<td>85850</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>Times x Treatment Groups</td>
<td>3</td>
<td>5937735</td>
<td>1979245</td>
<td>20370</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>Residual</td>
<td>12</td>
<td>1166</td>
<td>97</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>23</td>
<td>33173184</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Research hypothesis I

There are significant differences among the mean concentration of remaining reactants present in ethanol at different time intervals, while washing PHEMA chambers in 75% ethanol.

For significant F ratio and reasonable differences of concentration of remaining reactants (HEMA and EG) present in ethanol specimens taken at different time intervals, the test hypothesis is rejected at the 0.05 level and alternate hypothesis is accepted at 95% confidence as true. In other words, with the passage of time ethanol did wash the hydrogel as far as HEMA and EG are concerned. Although concentration of HEMA reached below detection limit after the 3rd wash where as EG took longer time (after 4th wash).

Research hypothesis II

There are significant differences among the mean concentration of remaining reactants present in ethanol at different agitation levels, while washing PHEMA chambers in 75% ethanol.
For significant F ratio and reasonable differences of concentration of remaining reactants (HEMA and EG) present in ethanol specimen for agitation and no agitation groups at different washing levels, the test hypothesis is rejected at the 0.05 level and alternate hypothesis is accepted at 95% confidence as true. By examining these results, introduction of agitation cleaned greater amount of HEMA and EG out of the hydrogel as compared to that of no agitation.

**Research hypothesis III**

There are significant differences among the mean concentration of remaining reactants present in ethanol at different combinations of washing time and agitation levels, while washing PHEMA chambers in 75% ethanol.

For significant F ratio and differences in concentration of remaining reactants at different combinations of washing time and agitation levels, the test hypothesis is rejected at the 0.05 level and alternate hypothesis is accepted at 95% confidence as true. HEMA and EG washed out of hydrogel, more rapidly with the introduction of agitation as compared to no agitation during the course of washing of hydrogel in 75% ethanol. This variation of concentration of HEMA and EG for agitation and no agitation groups is plotted in figure 4.1 and figure 4.2, respectively.

The concentration of TGD in specimens was not detected more than 5 ppm, after even concentrating the specimen 100 times. If there were any traces of TGD present it could have been less than 5 ppm, because this was the minimum possible concentration of TGD which was detectable under the circumstances. The detectable amount of sulfates could not had been washed out because of there insolubility in ethanol.
Figure 4.1: Plot of concentration of HEMA vs time for different washing groups.
Figure 4.2: Plot of concentration of EG vs time for different washing groups
CHAPTER 5. SUMMARY, DISCUSSION AND RECOMMENDATIONS

Summary and Conclusions

The purpose of this study was to establish an optimization of processing parameters to develop an alternate process for the production of PHEMA hydrogel chambers for the embryo culture. This study sought a better method to wash PHEMA hydrogel which could help to reduce the amount of time involved in the existing technique, particularly during the stage of washing PHEMA hydrogel in 75% ethanol. This research was designed to study the rate at which different remaining reactants (HEMA, EG, TGD and sulfates) wash out of the hydrogel into the solvent (75% ethanol). It was assumed that the larger amount of remaining reactants found in the ethanol, the more of those remaining reactants will be washed out of the hydrogel. To find out the level of remaining reactants which can be washed out of the hydrogel and is not harmful to the embryo, the existing technique was investigated first. This also provided the limits of remaining reactants acceptable if the hydrogel had to be made according to the research design using small intervals of washing (changing ethanol at less time as compared to the existing technique) and agitation and no agitation. To determine the possible effect of time and agitation, the groups by replication ANOVA (mixed design) was used to analyze the data.
This section summarizes and concludes the study based on the statistical analysis reported in the previous chapter, other evidence from literature, and other investigations. The research questions will be restated for better understanding of the research problem followed by discussion and conclusion.

Restatement of the problem

The problem of this study was five fold:

1. This study was designed to investigate and establish an optimization among the processing parameters of poly (2-hydroxyethyl methacrylate hydrogel chambers used for embryo culture.

2. This study was also designed to determine the time required to wash unpolymerized HEMA out of PHEMA hydrogel by using 75% ethanol.

3. This study was also designed to determine the time required to wash remaining ethylene glycol (EG) out of the PHEMA hydrogel using 75% ethanol.

4. This study was also designed to determine the time required to wash remaining Tetraethylene glycol dimethacrylate (TGD) out of the PHEMA hydrogel using 75% ethanol.

5. This study was also designed to investigate the time required to wash any sulfite remained or any sulfate formed during polymerization of HEMA.
Conclusions

Time required to wash HEMA

1. The contents of HEMA washed out of the hydrogel and reached below the detection limit after the 2nd washing (48 hours) for the existing technique.

2. The contents of HEMA washed out and reached below the detection limit after 4th washing (11 hours), by decreasing the time intervals between the washes (no agitation group).

3. The contents of HEMA washed out of the hydrogel and reached below the detection limit after 3rd washing (5 hours), by decreasing the time intervals between the washes but with the introduction of agitation. The rate of washing was increased and there was more concentration of HEMA found in the ethanol solution as compared to that of above two procedures.

Time required to wash EG

1. The contents of EG washed out of the hydrogel and reached below the detection limit after 4th washing (96 hours) for the existing technique.

2. The contents EG washed out and reached below the detection limit after 5th washing (23 hours), by decreasing the time intervals between the washes (no agitation group).

3. The contents of EG washed out of the hydrogel and reached below the detection limit after 4th washing (11 hours), by decreasing the time intervals between the washes but with the introduction of agitation. The rate of washing was
increased and there was more concentration of EG found in the 75% ethanol as compared to that of above two procedures.

**Time required to wash TGD**

There was no data available for the washing pattern of TGD. Its relative concentration was less as compared to other reactants. It was not detected in any of the ethanol specimens for any group. Its contents could have been below 5ppm, which was the minimum detectable limit.

**Time required to wash sulfites and sulfates**

The relative amount of ammonium persulfate and sodium metabisulfite in the starting mixture was low as compared to the monomer. The sulfates forming during the polymerization could not have been washed into ethanol solution because of their insolubility in it. Remaining contents of ammonium persulfate and sodium metabisulfite was assumed to be trapped in to the hydrogel during polymerization. Even if traces of sulfate were washed in to 25% water part of 75% ethanol it could not have been considerable in concentration.

**Optimization of processing parameters**

The processing parameters for PMEMA hydrogel chamber are pressure, time, temperature, agitation, initial water, crosslinker, initiator, and co-initiator contents. In this research, only two parameters time of washing in 75% ethanol and agitation were studied. The concentration of the remaining reactants found in ethanol solution was relatively higher when ethanol solution was changed at shorter time intervals
along with the introduction of agitation, as compared to the existing technique. The rate of wash of the remaining reactants was higher with the introduction of agitation.

Discussion

This study was investigated the time needed to wash the PHEMA hydrogel in 75% ethanol as compared to 96 hours spent using the existing technique. The HEMA washed out of hydrogel into the ethanol solution within 5 hours while EG took 11 hours to wash out for the agitation group. Where as TGD was very low (less than 5 ppm) in concentration, if even it was present, it could have been washed out while HEMA and EG were washing into the ethanol solution. As far as sulfates are concerned, the ethanol could not help them to wash out, because of their insolubility in it. This hydrogel had successfully been used for embryo culture and the presence of this much traces of sulfate did not harm the embryo. To summarize this discussion, the findings of this study are that if 75% ethanol had to be used for washing purpose it should be washed out by changing ethanol at 0.5, 1.0, 1.5, 3.0, and 6 hours intervals along with agitating the solvent (75% ethanol). It should take only 11.0 hours to wash out the remaining reactants by this procedure as compared to 96 hours taken by the existing technique.

Recommendation for Further Research

This research generated many interesting questions which could be answered by future researchers. The following recommendations are made in this regard.
1. It is recommended that research should be carried out using smaller intervals of time. Further reduction in the washing time may be obtained.

2. It is recommended that research should be carried out to study the effect of concentration of ethylene glycol on the washing time of hydrogel. Reduction in amount of EG used might help to reduce the washing time.

3. It is recommended that other processing parameters like contents of initiator and co-initiator, pressure, etc. be studied to fully explore their effects on hydrogel.

4. It is recommended that research should be carried out to study the effects of agitation wash on the physical properties of the PHEMA hydrogel.

5. It is recommended that before using the suggested washing procedure to make PHEMA hydrogel chambers, research should be carried out to study its effects on the embryo.

6. It is recommended that research should be carried out to study the washing of hydrogel in the water followed by the washing in ethanol solution. It may help to understand the washing pattern of sulfates into water and may help to further reduce the washing time which can lead to the improvement of the hydrogel and help save the material resources.

7. It is recommended that the aspects of mechanizing the processing of hydrogel should be carried out which may help in further time reduction, accuracy and least material wastage.
BIBLIOGRAPHY


ACKNOWLEDGEMENTS

I wish to take this opportunity to express my sincere appreciation to those individuals who assisted and often motivated me to reach this major goal of my life.

It is with sincere gratitude that I thank my major professor, Dr. Robert Gelina, and the members of my committee: Drs. William G. Miller, Mauricio H. Pineda and William D. Wolansky. A special thanks to Dr. Wolansky for working closely with me to see this through to completion during Dr. Gelina's sabbatical. I would like to take this opportunity to thank Dr. Pineda for letting me use his laboratory and resources.

Grateful appreciation is extended to Dr. Henry M. Stahr who assisted me on the analytical measurement procedures: also to Paula Immerman, Dr. Mike Dooley and all other scientists at ISU Veterinary College for advice and consultation.

I would like to acknowledge and thank Dr. John C. Dugger, the Chair of the Department of Industrial Education and Technology for his support, and to Pat Hahn, secretary, who persistently kept me on task and shared her confidence in my ability to complete my Master of Science program of studies.

Finally, this accomplishment would not be possible without the encouragement of the many friends I have made in the community of Ames, Iowa. Without such a warm, caring environment, it would have been difficult to complete my studies.
A special thanks to my parents, Muhammad Siddique and Ghulam Fatima, rest of family members and friends who supported me during the entire course of my education.
APPENDIX A. A TYPICAL CHROMATOGRAM FOR ETHYLENE GLYCOL

430°C, 4x10^-11, 25 cm/hr, 0.00 volts
APPENDIX B. A TYPICAL CHROMATOGRAM FOR HEMA

240 C, 4x10 Att
25 cm/hr, 0.011 volts
APPENDIX C. RAW DATA

The concentration (μg/g) of ethylene glycol as calculated from standard curve is given below (2nd column shows time intervals in hours):

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<th>1.5</th>
<th>3.0</th>
<th>6.0</th>
<th>12.0</th>
<th>24.0</th>
<th>48.0</th>
<th>96.0</th>
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<td>75</td>
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<td>ND</td>
<td>ND</td>
<td>ND</td>
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<td></td>
<td>3850</td>
<td>1195</td>
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<td>75</td>
<td>ND</td>
<td>ND</td>
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The concentration (μg/g) of HEMA as calculated from standard curve is given below (2nd column shows the time intervals in hours):

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<td>ND</td>
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<td>ND</td>
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APPENDIX D. MEANS AND GROUPS BY REPLICATION ANOVA (MIXED DESIGN) FOR HEMA

Replication Means in Groups

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<th>Mean</th>
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</thead>
<tbody>
<tr>
<td>Group 1</td>
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<td>121.67</td>
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Means for Groups Across Replications:
- Group 1 Mean = 190.56
- Group 2 Mean = 145.89

Means for Replications Across Groups:
- Replication 1 Mean = 227.00
- Replication 2 Mean = 179.33
- Replication 3 Mean =  98.33

ANALYSIS OF VARIANCE RESULTS
Alpha Selected = 0.0500

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<th>MS</th>
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<th>Sig.</th>
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APPENDIX E. MEANS AND GROUPS BY REPLICATION ANOVA
(MIXED DESIGN) FOR EG

Replication Means in Groups

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<tr>
<td>Group 2</td>
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</table>

Means for Groups Across Replications:

- Group 1: Mean = 1334.00
- Group 2: Mean = 727.25

Means for Replications Across Groups:

- Replication 1: Mean = 2696.50
- Replication 2: Mean = 1023.17
- Replication 3: Mean = 302.67
- Replication 4: Mean = 100.17

ANALYSIS OF VARIANCE RESULTS

Alpha Selected = 0.0500

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<th>MS</th>
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<th>Prob &gt; F</th>
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