THE ELECTROPHORETIC ANALYSIS OF COMMON FOOD HYDROCOLLOIDS

A Thesis

Presented in Partial Fulfillment of the Requirements

for the degree Master of Science in the

Graduate School of the Ohio State University

by

Shu-Tai Wang, B. S.

* * * * *

The Ohio State University

1993

Master's Examination

Committee :

P. M. T. Hansen

M. E. Mangino

G. W. Chism

Approved by

Adviser

Food Science and Nutrition

Graduate program

To My Parents and Family

ACKNOWLEDGMENTS

I would like to express my sincere appreciation to Dr. Poul Hansen for his valuable guidance throughout my graduate studies, for the participation of his sport events, and for all the efforts in preparing this thesis, which bring me to the interesting field of research and a very fun way of life. Thanks go to Dr. G. W. Chism for providing me with a part time job during the summer quarter last year and for his asistance and advice during my graduate study. Thanks also go to Dr. M. Mangino for his guidance and to all faculty members of the Department of Food Science and Technology for their advise and hospitality during my graduate life.

Thanks go to my friends around me for all the time we have been together which made my graduate study in the U. S. A. a very special and happy memory. Special love and appreciation is expressed to my parents for their love, encouragement and support

VITA

December 29, 1968	Born - Taipei, Taiwan
1991	B.S., Department of Agriculture Chemistry, National Taiwan University, Taipei, Taiwan
Summer, 1992	Laboratory assistant, Color and Flavor Laboratory, Worthington Foods Inc. Worthington, OH
1993-Present	Vitamin Laboratory, Nestle USA Quality Assurance Laboratory, Dublin, Ohio

Field of study: Food Science and Nutrition

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

Hydrocolloids are applied widely as stabilizers in food products to increase thickness or form aqueous gels. Most of the hydrocolloids are polysaccharides of large molecular weight and characterized by repeating sugar units. The commercial manufacturers of stabilizers supply under brand name selected mixtures for specific purpose. The reason for using mixtures rather than single stabilizers is that each type has its own merits as well as detriments. To optimize the useful properties and to compensate for possible disadvantages, compounding of the ingredients is necessary. Since each component provides specific function in the mixture, the determination of the composition of mixed stabilizers is helpful to extend the application of such blends.

There is no convenient and accurate approach to determine the composition of stabilizer mixtures. Some methods are limited to the analysis of specific components or to the determination of a structural entity within the framework of hydrocolloids. Therefore, a developed analytical protocol for evaluating the hydrocolloid composition of a stabilizer mixture alone or in food products could be very important.

This study has applied electrophoresis to determine the hydrocolloid components in a stabilizer mixture. Hydrocolloids such as carrageenan, carboxymethyl cellulose, alginate, locust bean gum, furcellaran, pectin and xanthan gum are characterized by different structures which exhibit

differences in electrophoretic charge. Since the electrophoretic mobility of colloidal substances depends on the charge-to-weight ratio of the molecules, hydrocolloids differing in this characteristic may be expected to be separated by electrophoresis.

Because of the large molecular size and aggregating shape, hydrocolloids can not migrate into the standard electrophoretic gel used for protein electrophoresis. Therefore, in this study, cellulose acetate membranes were adopted as the support for electrophoresis. The specific aim has been to adapt presently available instrumentation for cellulose acetate electrophoresis for the electrophoretic analysis of common food hydrocolloids and to develop a sensitive staining method to detect the very small amount of hydrocolloids on the strip.

The significance of this study is related to providing a fast and accurate analytic method for identification of stabilizer mixtures. This approach may then be applied to determine the identity of stabilizers in food formulations.

CHAPTER I
LITERATURE REVIEW

A. Hydrocolloids

1. Structure of hydrocolloids

Food hydrocolloids are typically hydrophilic high-molecular-weight molecules, with useful functional colloidal properties. In an appropriate solvent or swelling agent, these hydrocolloids depending on concentration may produce gels, highly viscous suspensions or colloidal dispersion. The functionality of hydrophilic colloids is usually achieved at exceptionally low concentration measured in parts per million. They are used commonly as food stabilizers, and they can be sorted into four groups according to their sources: (a) seaweed extracts, (b) tree exudates and extracts, (c) seed gums, (d) biosynthetic gums, and (e) cellulose derivatives (Klose and Glicksmann. 1968). Major structural components of the various stabilizers are shown in Table 1 (Chang,1968).

A major distinction relates to whether or not the hydrocolloid is linear or branched. This is an important consideration regarding solubility and viscosity. Within these two broad classification groups, a differentiation may be made based upon the presence or absence of electrical charge and the strength of the charge. Some polysaccharides are anionic, possessing acidic functions such as carboxyl groups, sulphate groups, or phosphate

Table 1. Structural components of important stabilizers

Stabilizer	Functional group	Sugar units
Seaweed extracts		
Agar	Ester sulphate	D-Galactose β–(1-4), 3,6 Anhydro-L-Galactose-(1-3),+ Sulphate acid ester group
Algin	Carboxyl	D-Mannuronic acid β–(1-4), L-Guluronic β–(1-4)
Carrageenan	Ester sulphate	D-Galactose, 3-6-Anhydro-D-Galactose, + Sulphate acid ester group
Furcelleran	Ester sulphate	D-Galactose, 3,6-Anhydro-D-Galactose, + D-Galactose-4-sulphate
Tree exudates and extracts		
Gum arabic	Carboxyl	L-Arabinose, D-Galactose, L-Rhamnose, D-Glucuronic acid
Pectin	Carboxyl	D-Galactose, L-Arabinose, (1-4) α–D-galacturonan

Table 1. (Continued)

Stabilizer	Functional group	Sugar units
Seed gums		
Guar gum	Nonionic	D-Mannose β–(1–4), D-Galactose-(1-6) Branches
Locust bean gum	Nonionic	D-Mannose β–(1-4), D-Galactose (1-6) Branches
Biosynthetic gums		
Xanthan	Carboxyl	Repeating pentasaccharide units consisting of two D-glucopyranosyl units, two D-mannopyranosyl units, and one D-glucopyranosyluronic acid units
Gellan		A linear sequence of tetra-saccharide repeating units containing D-glucuronopyra-nosyl, D-glucopyranosyl L-rhamnopyranosyl units and acryl groups.
Cellulose derivatives		
Carboxymethylcellulose	Carboxyl	D-Glucose β–(1-4)

Source: Chang. 1968

groups. Other polysaccharides are neutral with no electrical charge. Hydrocolloids with these various structures possess different functionality when used in food systems.

2. Functional properties of hydrocolloids

The functional properties of hydrocolloids are described by Glicksman (1982) and are shown in table 2. The author explained functionalities of hydrocolloids in the following way:

> VISCOSITY
> "...Viscosity, also defined as "internal friction" or "resistance to flow" s the key factor in distinguishing between the different types of fluid system and is an important factor in the organoleptic acceptability of many food products..."
>
> GELATION
> "....A gel is a mixture, one component of which is a fluid homogeneous down to substantially colloidal dimensions, capable of resisting a finite shearing force...."
>
> DIETARY FIBER
> "....Dietary fiber embraces the algal polysaccharides, such as alginates and carrageenans; gums, such as guar and locust bean; mucilages, such as isphagula and all other polysaccharides which are not digested...."
>
> EMULSION STABILIZATION
> "....An emulsion, by definition, is a two-phase system consisting of two immiscible liquids, one being dispersed as finite globules within the other.Hydrocolloids are not true emulsifiers and do not act by means of the hydrophilic-hydrophilic bridging mechanism of classical emulsifiers. Hydrocolloids function as emulsion stabilizers in several other ways, but primarily by thickening and increasing the viscosity of the aqueous phase so that it inhibits or minimizes the tendency of the dispersed oil globules to migrate and coalesce...."
>
> CRYSTALLIZATION INHIBITION
> "....Crystallization is the process of orientation of atoms, molecules, or other building blocks into rigid, repeating arrangement called a crystal. Hydrocolloids affect crystallization in three ways: compatibility, competition, and combination...."

Table 2. Functional properties of hydrocolloids

Function	Example
Adhesive	Glazes, icing, frostings
Binding agent	Pet foods
Bodying agent	Dietetic beverages
Crystallization inhibitor	Ice cream, sugar syrups, frozen foods
Clarifying agent (fining)	Beer, wine
Cloud agent	Fruit drinks, beverages
Coating agent	Confectionery, fabricated onion rings
Dietary fiber	Cereals, bread
Emulsifier	Salad dressing
Encapsulating agent	Powdered flavors
Film former	Sausage casing, protective coatings
Flocculating agent	Wine
Foam stabilizer	Whipped toppings, beer
Gelling agent	Puddings, desserts, confectionery
Molding	Gum drops, jelly candies
Protective colloid	Flavor emulsions
Stabilizer	Salad dressings, ice cream
Suspending agent	Chocolate milk
Swelling agent	Processed meat products
Syneresis inhibitor	Cheese, frozen foods
Thickening agent	Jams, pie fillings, sauces
Whipping agent	Toppings, marshmallows

Source: Glicksman (1982)

Since individual hydrocolloids exhibit such different properties, it has become industrial practice to formulate stabilizer mixtures or blends for application rather than using them as single entities. Typical hydrocolloids used in the food industry include: algin, carrageenan, furcellaran, gum arabic, guar gum, locust bean gum, pectin, xanthan, dextrans and various cellulose derivatives.

The application of hydrocolloids in small amounts can improve the texture of different food products and can influence the physical property of a food system a great deal. For example, a blend of primary stabilizers (0.3-0.4%) in association with carrageenan as a secondary stabilizer at a concentration of 0.01-0.05% provides creaminess and prevents syneresis and crystal formation in ice cream under freeze-thaw conditions (Nickerson, 1962). Besides their stability function, hydrocolloids may influence some of the food processing parameters. For instance, in research on utilizing carrageenan as a processing aid to enhance cheese yield, Manning et al.(1985) pointed out that cottage cheese yield increased 5-10% with carrageenan compared to controls. There is little doubt that as the properties of hydrocolloids become better understood, their application will become increasing important. The development of analytical methods for determination of food hydrocolloids must be emphasized at the same time.

B. Methods for analysis of hydrocolloids

1. Structure characterization according to FOOD CHEMICALS CODEX

FOOD CHEMICALS CODEX (1981) has assigned standard methods for determining the basic structure, acid-insoluble matter, ash, galactomannans, heavy metals, protein and starch amount in specified hydrocolloids, including alginate, carrageenan, guar gum, gum arabic, sodium carboxymethylcellulose, locust bean gum and xanthan gum. These are fundamental analyses for initial understanding of the characteristics of individual hydrocolloids. The definition and standard methods for determining these hydrocolloids have been described in FOOD CHEMICALS CODEX. For instance, the description for carrageenan are cited below:

> " Carrageenan is obtained by extraction with water or aqueous alkali from certain members of the class Rhodophyceae (red seaweeds). It is a hydrocolloid consisting mainly of the potassium, sodium, magnesium, calcium, and ammonium sulfate esters of galactose and 3,6-anhydrogalactose copolymers. These hexoses are alternately linked α–1,3 and β–1,4 in the polymer. The relative proportion of cations existing in carrageenan may be changed during processing to the extent that one may become predominant.
>
> The prevalent copolymers in the hydrocolloid are designated *kappa*–,*iota*–, and *lambda*–carrageenan. *Kappa*-carrageenan is mostly the alternating polymer of D-galactose-4-sulfate and 3,6-anhydro-D-galactose; iota-carrageenan is similar, except that the 3,6-anhydrogalactose is sulfate at carbon 2.....
>
> The ester sulfate content of carrageenan ranges from 18% to 40%..."

The identification method for carrageenan as stated in FOOD CHEMICALS CODEX is based upon the differential solubility in the presence of potassium ions, and on infra red spectroscopy as cited below:

> A. Add 4 g of sample to 200ml of water, and heat the mixture in a water bath at 80^{0}C, with constant stirring, until dissolved. Replace any water lost by evaporation, and allow the solution to cool to room temperature. It become viscous and may form a gel.
> B. To 50 ml of the solution or gel obtained in A. add 200mg of potassium chloride, then reheat, mix well, and cool. A short-textured ("brittle") gel indicates a carrageenan of a predominatly *kappa*-- type; a compliant ("elastic") gel indicates a predominatly *iota*- type. If the

solution does not gel, the carrageenan is of a predominantly *lambda* type.

C. To 5 ml of the solution obtained in A. add 1 drop of a 1 in 100 solution of methylene blue. A fibrous precipitate forms.

D. Obtain infrared absorption spectra on the gelling and nongelling fractions of the sample by the following procedure: Disperse 2 gm of the sample in 200 ml of 2.5% potassium chloride solution, and stir for 1 hour. Let stand overnight, stir again for 1 h, and transfer into a centrifuge tube. Centrifuge for 15 min at approximately 1000 g's.

Remove the clear supernatant, resuspend the residue in 200 ml of 2.5% potassium chloride solution, and centrifuge again. Coagulate the combined supernatant by adding 2 volumes of 85% ethanol or isopropanol. (Retain the sediment) Recover the coagulum, and wash it with 250 ml of the alcohol. Press the excess liquid from the coagulum, and dry it at 60^{0}C for 2 h. The product obtained is the nongelling fraction (*lambda*- carrageenan).

Disperse the sediment (retained above) in 250 ml of cold water, heat at 90^{0}C for 10 min, and cool to 60^{0}C. Coagulate the mixture, and then recover, wash, and dry the coagulum as describe above. The product obtained is the nongelling fraction (*kappa*- and *iota*-carrageenan).

Prepare 0.2% aqueous solution of each fraction, cast film 0.0005 cm thick (when dry) on a suitable nonsticking surface such as Teflon, and obtain the infrared spectrum of each film. (Alternatively, the spectra may be obtained on potassium bromide pellets if care is taken to avoid moisture.)

Carrageenan has strong, broad adsorption bands, typical of all polysaccharides, in the 1000 to 1100 cm-1 region. Absorption maxima are 1065 and 1020 cm-1 for gelling and nongelling types, respectively. Other characteristic absorption bands and their intensities relative to the absorbance at 1050 cm-1 are as follows:

Wave Number (cm-1)	Molecular Assignment	Absorbance Relative to 1050 cm-1		
		Kappa	Iota	Lambda
1220-1260	ester sulfate	0.7-1.2	1.2-1.6	1.4-2.0
928-933	3,6-anhydrogalactose	0.3-0.6	0.2-0.4	0-0.2
840-850	galactose-4-sulfate	0.3-0.5	0.2-0.4	-
825-830	galactose-2-sulfate	-	-	0.2-0.4
810-820	galactose-6-sulfate	-	-	0.1-0.3
800-805	3,6-anhydrogalactose-2-sulfate	0-0.2	0.2-0.4	-

Shelembe (1992) identified and explained the structure of kappa-, iota- and lambda- carrageenan by using these hydrocolloid films for infrared

analysis and results also fitted in the IR absorbance table of FOOD CHEMICALS CODEX.

The sulfate content of different carrageenans is variable, within the range 18%-40% on a dry weight basis. FOOD CHEMICALS CODEX includes a standard method for measuring the amount of sulfate in carrageenan. This method is based upon a gravimetric determination of hydrolyzed ester sulfate as barium sulfate.

> "Transfer about 500 mg, previously dried at 105°C for 12h and accurately weighed, into a 100 ml Kjeldahl flask. Add 10 ml of nitric acid and heat gentlely for 30 min, adding more of the acid, if necessary, to prevent evaporation to dryness, and to yield a volume of about 3 ml at the end of the heating. Cool the mixture to room temperature and decompose the excess nitric acid by the addition of formaldehyde TS, dropwise, heating, if necessary, until no brown fumes continue to be evolved. Continue the heating until the volume of the reaction mixture is reduced to about 5 ml, and then cool. Transfer the residue quantitatively with the aid of water into a 400-ml beaker, dilute it to about 100 ml, and filter, if necessary, to produce a clear solution. Dilute the solution to about 200 ml, and add 1 ml of hydrochloric acid. Heat to boiling and add, dropwise, with constant stirring, an excess (about 6 ml) of hot barium chloride TS. Heat the mixture for 1 h on a steam bath, collect the precipitate of barium sulfate on a filter, wash it until free from chloride, dry, ignite, and weigh. The weight of the barium sulfate so obtained, multiplied by 0.4116, gives the equivalent of sulfate (SO_4).

2. Structure characterization by GC and HPLC

Modern procedures for the characterization of hydrocolloid chemical structures involve the use of GC (Gas Chromatography) and GC-MS (Gas-Chromatography-Mass Spectrometry) analysis of the monosaccharide mixtures produced by hydrolysis (Bradbury and Halliday, 1985). Hansen and Shelembe (1992) studied on Gas liquid chromatography analysis of carrageenan and HPLC analysis of carrageenan. First, the polymers need

to be hydrolyzed into monosaccharides followed by monosaccharide derivatization after the hydrolysis. The majority of GC-based procedures for the analysis of monosaccharide mixtures involve either trimethylsilylation or acetylation to give volatile derivatives ready for GC analysis.

Most of the common linkages in polysaccharides can be well hydrolyzed by trifluoroacetic acid (typically, 2N, 120°C, 1.5h), which may subsequently be removed by evaporation (Albersheim et al, 1967). However, for various hydrocolloids, additional procedures may be necessary for the complete hydrolysis of polysaccharide chains containing less common components. Adaption of suitable hydrolysis conditions has become a crucial part for sample preparation for GC analysis, because the conditions necessary for quantitative conversion to monosaccharides vary according to the structure and monosaccharide composition of the polysaccharide. Analyses of the hydrolyzate mixtures obtained from gums which contain acid-sensitive moieties, such as the sulfate and anhydro groups in carrageenans, must be interpreted with care. For polymers of this type, the positions of substitution are usually labeled, e.g. by methylation, before being subjected to acid hydrolysis (Bradbury and Halliday, 1985).

Other techniques available for the separation of carbohydrates which does not involve derivazation is HPLC (High Performance Liquid Chromatography). Voragen et al. (1982) applied HPLC analysis to determine the uronide composition of alginate samples. The major advantage of HPLC in sugar analysis can be attributed to its ease of use and simple data interpretation. Voragen et al. (1983) also investigated the

analysis of pectins as anhydrogalacturonic acid (AGA) content by HPLC of the degradation products obtained by enzymic degradation.

The HPLC analysis of hydrocolloids had been done by Shelembe (1992). He used HPLC on a size exclusion chromatography column maintained at 50°C and tried to separated samples of carrageenan and furcellaran. The peak represented the molecular weight distribution of the polysaccharide. However, the results indicated that the different types of carrageenan had almost the same retention times. It was found the HPLC analysis would be ideal for studying the molecular weight distribution of polysaccharides and it would be difficult to identify the different types of carrageenans by this technique (Shelembe, 1992).

3. Methods for analyzing specific food hydrocolloids

Methods are available which can detect specific hydrocolloids present in blends with other food ingredients or in food products. If the hydrocolloid is present in a food system with other components, additional isolation and purification steps before analysis is generally required. The analysis of hydrocolloids in food products, such as infant formula, is a very complex problem and generally requires elaborate isolation procedure. The food stabilizer carboxymethylcellulose (CMC) may be recovered from a tryptic digest of milk by solvent precipitation of the fraction soluble in 12.5% trichloroacetic acid (Hansen and Chang, 1968). The mean recovery from skim milk, whole milk, and ice cream mix containing 0.05 to 0.4% CMC was around 92.7%.

To isolate CMC from milk, the pH of a ten gram of sample was adjusted to 8.5 with four drops of 1N sodium hydroxide, one ml of trypsin solution is added, and the sample is incubated at 40°C for three hours. The hydrolyzed protein was then precipitated by adding 12.5% trichloroacetic acid, and the CMC was recovered may be collected by centrifugation after the addition of ethanol (2:1) and ethyl acetate (1:1).

Identification has been performed by various techniques including microscopy, I.R. spectroscopy, precipitation and color reactions with more or less specific reagents. Anderson and Bowtle (1974) and Allen et al.(1982) stated that thiobarbituric acid was be an ideal reagent for the colorimetric assay of κ–carrageenan in complex polysaccharide mixtures of normal stabilizers routinely used in the food industry. This assay procedure could be applied in the selective assay of galacturonic acid (pectin) in the presence of mannuronic and guluronic acid (alginate).

It has been postulated (Feather and Harris, 1973) that for polysaccharides with 3,6 anhydrogalactose and certain other hexose units an acid catalyzed degradation reaction occurs leading to production of 5-(hydromethyl)-2-furaldehyde. This compound absorbs visible light at 438 nm) after reaction with 2-thiobarbituric acid. In the case of hexuronic acids however the important reactions are decarboxylation and dehydration reactions giving a mixture of products among which are 2-furaldehyde, reductic acid (2,3-dihydroxy-2-cyclopenten-1-one) and traces of 5-formyl-2-furic acid (Feather and Harris, 1973). These are capable of forming a colored complex with 2-thiobarbituric acid. It appeared from this study that

the rate of carboxylation/dehydration for D-galacturonic acid from a pectin source is substantially greater than for either L-guluronic or D-mannuronic acid from alginate source.

Wedlock et al. (1983) applied this technique to the colorimetric assay of pectin. In this study, the researchers exploited the difference in rates of producing aldehydic derivatives upon acid hydrolysis to estimate pectin in the presence of alginate.

Their (1983) used an analytical method based on glass capillary gas-liquid chromatography for identification and quantitative determination of several thickeners and gums of natural origin at the milligram level in a great variety of food stuffs. They analyzed the percentage of monosaccharide units found in some commercial stabilizers such as pectin, guar gum, gum arabic, carrageenan, tragacanth, carob gum, agar, xanthan and algin. The procedure has already proved to be suitable for routine food control in government laboratories for food inspection.

Knutsen and Grasdalen (1992) pointed out that carrageenan could be analyzed by enzymic degradation, gel filtration and ^{1}H NMR spectroscopy. Matsuhiro and Urzua (1992) studied heterogeneity of carrageenan from *Chondrus crispus* which were analyzed by hydrolysis, KCl fractionation and ^{1}H NMR. The method could detect various components in the *Chondrus crispus*.

In the paper " Analysis of hydrocolloids in foods", Oates and Sim pointed out another approach for analyzing hydrocolloids, which is cited below:

"...Immunoassay can be used for the analysis of polysaccharides in foods. These assays requires specific antibodies raised against a polysaccharide. A good antibody will combine rapidly, virtually irreversibly and with little cross reaction. Ideally the situation will arise where, for example, an antibody against κ– carrageenan will have virtually no affinity for ι– or λ– carrageenan and certainly no affinity for other polysaccharides. Typically a known amount of antigen (polysaccharide) and the polysaccharide extracted from the food system are mixed together and reacted with an antibody. Recently more practical methods have been developed by Rittenburg et al. (1983) involving the use of enzymes coupled to the antigen. This was called an "Enzyme Linked Immuno Sorbant Assay" (ELISA), the advantage of such a system was that it could conveniently be purchased in the form of a ready made kite.

Most analytical methods which have been proposed up to now are intended for the quantitative determination of a definite stabilizer. They can only be applied if the identity of the stabilizer in question is taken for granted. Identification of an unknown polysaccharide which may be present as an ingredient of a food commodity is much more difficult..."

C. Electrophoresis

Electrophoresis is the technique used originally to fractionate and purify mixtures of proteins and other charged biopolymers. The charges on the protein molecules arise from dissociation of carboxyl and amino groups, which is a function mainly of pH.

Protein molecules in solution are characterized by a net average charge. At the isoelectric point this charge is zero, but becomes positive at pH values below the isoelectric point and negative at pH values above. This causes the molecule to move in an applied electric field. The force is given by E, the electrical field ($V\ m^{-1}$) times z, the net number of charges on the molecule. This force is opposed by viscous forces in the media, which are proportional to the viscosity n, particle radius r, and the velocity; Thus for a steady state, the equation of Scopes (1988) is cited below:

$$Ez = 6\pi nrv$$

The specific mobility $u=v/E$ is given by

$$u = z/(6\pi nr)$$

Based on this formula, the mobility of molecules is dependent on the charge-size ratio. Free boundary electrophoresis, operated as an an analytical system, rarely produces more than about eight discernable components even from most complex mixtures because of difussion, overlapping of components, and some protein-protein interactions. Interactions between different proteins at low ionic strength may be of many types; electrostatic forces are the most important for many albumin-type, i.e., highly soluble proteins. The electrostatic interactions can be minimized be increasing the ionic strength.

Electrophoresis can be done in solution as free boundary electrophoresis, or by zonal electrophoresis in support systems including paper, cellulose films, or in gels of starch or acrylamide. There is a abundance of variable factors which affect the quality of zonal electrophoresis of proteins. In addition to the differences in support matrices of strips, there are buffer systems which may vary in composition and ionic strength. The buffer chosen depends somewhat on the nature of the proteins, but generally it is slightly alkaline in the range 8-9, where most proteins are negatively charged and therefore move toward the anode. For instance, the general purpose electrophoresis buffer (pH 8.8) containing 32.1% w/w TRIS [2-Amino-2-(hydroxymethyl)-1-,3-propanediol], 13.7% w/w Barbital and 54.2%w/w Sodium Barbital can provide high resolution for most proteins. In the research of bovine serum proteins, Senda (1992) used lithium iodate (pH8.6, ionic strength 0.06) as the buffer for analyzing these proteins on cellulose acetate strips by electrophoresis.

Different staining reagents to visualize separated bands are available. For proteins, coomassie blue, ponseau-S and amido black provide suitable staining intensity. For anionic polysaccharides, toluidine

blue and alcian blue are frequently used. On the other hand, another approach is to stain the strips by some special reaction between the component in the bands and selected reagents, such as by application of the horseradish peroxidase-benzidine staining procedure (Hansen et al. 1989). This staining method utilized the ionic interaction between anionic polysaccharides and a positively charged protein (horseradish peroxidase) to form a complex that can be sensitively and quantitatively detected by staining for peroxidase activity with benzidine.

Generally speaking, the proper choice of strips, buffer, and stain should depend on the characteristics of the molecules to be analyzed.

1. Gel electrophoresis

Gels are the most common supports for conducting electrophoresis of proteins. They are commonly based on gelatinized starch, agarose or polyacrylamide. The polyacrylamide gel is formed by chemical crosslinking. Polyacrylamide gel electrophoresis (PAGE) is a versatile and useful technique available for the food chemist. Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis has become popular for determination of the molecular weight of proteins and polypeptides. SDS will bind to the polymers and make it evenly charged. Molecular weight determination by SDS-polyacrylamide gel electrophoresis was first introduced in 1967 by Shapiro et al.. Subsquently, Weber and Osborn (1969) and Dunker and Rueckert (1969) extended the use of SDS electrophoresis for determination of the molecular weights of proteins.

The electrophoretic mobility is proportional to the molecular weight of a polypeptide or protein only when the charge/mass ratios and the shapes of all the SDS-polypeptide complexs are the same. Therefore, valid molecular weight standard are required for molecular weight determination.

2. Cellulose acetate membrane electrophoresis

Cellulose acetate strips is another media which may serve as support for electrophoresis of proteins. The material may be manufactured as thin strips without additional support, but in this case, the films are fragile, greater structural stability for cellulose acetate is achieved by attachment to myla plastic which does not interfere with the electrophoresis. Cellulose acetate has been used in a wide variety of applications, including electrophoresis of serum proteins and hemoglobins. The thin strips provide a flexible, easy handling electrophoresis medium, and it is very convenient to keep dry cellulose acetate strips without any additional preservation procedure.

Because of the different construction characteristics between gel and cellulose acetate strips, cellulose acetate strips have larger pore size which make them particularly suited for electrophoretic determination of polysaccharides.

D. Electrophoresis of DNA

Electrophoresis through agarose or polyacrylamide gel is the standard method to separate, identify, and purify DNA fragments. Polyacrylamide gels are most effective for separating small fragments of

DNA (5-500 bp). Their resolving power is extremely high, and fragments of DNA that differ in size by as little as 1 bp can be separated from one other. Polyamide gels are run in a vertical configuration in a constant electric field. DNAs from 200 bp to approximately 50 kb in length can be separated on agarose gels of various concentrations. Agarose gels are usually run in a horizontal configuration in an electric field of constant strength and direction. Larger DNAs, up to 10,000 kb in length, can be separated by pulsed-field gel electrophoresis, in which the direction of the electric flux is changed periodically.

The location of DNA within the gel can be determined directly by staining with low concentrations of fluorescent interchelating dye ethidium bromide; bands containing as little as 1-10 ng of DNA can be detected by direct examination of the gel in ultraviolet light (Sharp et al., 1973).

E. Application of cellulose acetate electrophoresis to food hydrocolloids

Electrophoresis can be applied to separation of other molecules besides proteins, such as for example Hidalgo and Hansen (1968) applied free boundary electrophoresis for characterizing locust bean gum, gur gum, arabic gum, carboxymethylcellulose, κ- and λ-carrageenan, alginate and non-dialyzable fraction of corn syrup solids. Carrageenans were determined by zonal electrophoresis on cellulose acetate strips. (Hansen and Chang, 1968). According to Hansen and Chang (1968), zonal electrophoretic separation of several hydrocolloids was possible using

cellulose acetate as a supporting matrix. Neutral compounds will not migrate in an electrical field. However, neutral polysaccharides are capable of complexing borate ions in such a way that the colloid acquires a charge sufficient for electrophoresis but not for staining. In this case, it was possible to visualize the migrating bands by application of the PAS staining technique (Hansen and Chang, 1968). For anionic polysaccharides, Toluidine blue and Alcian blue are frequently used. Another approach is to stain the strips by some special reaction between the component in the bands and selected reagents, such as by application of the horseradish peroxidase-benzidine staining procedure (Dickmann, et al, 1989). This staining method utilizes the ionic interaction between anionic polysaccharides and a positively charged protein (horseradish peroxidase) to form a complex that can be sensitively and quantitatively detected by staining for peroxidase activity with benzidine.

For the analysis of a mixture of different hydrocolloids, boric acid buffer (pH10, ionic strength 0.13) was prepared for neutral polysaccharides and malonate buffer (pH2.9, 0.075M) was prepared for anionic polysaccharides. If an unknown mixture is to be determined, these two buffer systems may be be used to identify both neutral and anionic polysaccharides.

Padmoyo and Miserez (1967) reported that a number of gelling and thickening agents could be separated by electrophoresis on cellulose acetate strips in borate buffer, pH10. After conducting electrophoresis, the hydrocolloids in the strips were visualized by staining by four staining

methods including: amido black, Periodic Acid Schiff reagent (PAS), tannin-PAS reagent and toluidine blue. They indicated that amido black was effective only for gelatin, which is a protein.

Hansen et al. (1974) applied electrophoresis in malonate buffer to determine following hydrocolloids: lambda- and kappa-carrageenan, sodium alginate, sodium carboxymethylcellulose, pectin, gum arabic, guar gum and locust bean gum. They successfully separated a mixture of lambda- and kappa-carrageenan, alginate and carboxymethylcellulose into four sharp bands which could be easily recognized. This provided a very convenient approach to determine the components in a unknown food hydrocolloid mixture. However, the PhoroSlide Electrophoresis system (Millipore Corp., Bedford, Mass.) is no longer available.

In England, Marrs et al. (1985) applied cellulose acetate strip electrophoretic and gel permeation methods for characterising carrageenans. They investigated several buffer systems and molarities which are listed below:

Sodium acetate (0.01M)
Sodium acetate (0.01M)/isopropyl alcohol (10% to 70%)
Magnesium acetate (0.005M, 0.01M, 0.02M and 0.05M)
Zinc acetate (0.005M, 0.01M, 0.02M and 0.05M)
Lithium acetate (0.05M)
Lithium acetate (0.05M)/EDTA (0.05M)
Lithium chloride (0.05M)/EDTA (0.01M)
Barium acetate (0.02M, 0.03M, 0.04M and 0.05M)

Based on the use of the above buffers, Marrs selected for electrophoresis of polysaccharides from red seaweed a 0.02M barium acetate buffer at pH5.8. The electrophoresis was conducted at 100 volts for 60 minutes at 30°C followed by staining in Alcian Blue 8GX solution (1%

Alcian Blue 8GX in 1:1, ethanol:0.02M sodium acetate) for 15 min. Unfortunately, they did not indicate in the paper which kind of cellulose acetate strips was used.

CHAPTER II
SCOPE OF INVESTIGATION

The overall goal for this study has been to explore the possibility of using zonal electrophoresis for analysis of food hydrocolloid mixtures. The specific objectives have been:

1. Selecting an appropriate buffer for electrophoresis of hydrocolloid dispersions on cellulose acetate strips.
2. Determining the proper voltage/time relationship for electrophoresis of hydrocolloids.
3. Selecting a suitable staining method to visualize the bands of hydrocolloid on cellulose acetate strips.

CHAPTER III
EXPERIMENTAL METHODS

A. Materials

1. Hydrocolloid Samples

Various types of hydrocolloid samples including CMC, κ–, λ–, and ι–carrageenan, xanthan, alginate, furcellaran, pectin and locust bean gum were obtained from Hercules Powder Company, Marine Colloids, a division of FMC corporation U.S.A., Miles Lab Inc., Kimitsu Company, Japan, Continental Colloids, and Germantown Manufacture Company. All of the samples and their sources are listed in Table 3.

These samples were commercially produced as food grade stabilizers except that CMC were produced for experimental use only. They were dissolved in water by heating and stirring and made into dispersions at different concentrations. These dispersions were applied for electrophoretic examination individually or in blends for exploring the proper conditions for conducting electrophoresis.

Table 3. Sample sources

Sample	Source
CMC (4M6F),Lot #50535	Hercules Poeder Company (Wilmington, Delaware)
CMC (9M31F),Lot#55951	Hercules Poeder Company (Wilmington, Delaware)
CMC (12M8P),Lot#61815	Hercules Poeder Company (Wilmington, Delaware)
κ-carrageenan,Lot#140900	Marine Colloids (Philadelphia,PA)
λ-carrageenan,Lot#321700	Marine Colloids (Philadelphia,PA)
ι-carrageenan,Lot#490400	Marine Colloids (Philadelphia,PA)
Xanthan Gum,N9570	Miles Lab. Inc. (Elkhart ,IN)
Alginate,Lot#31104	Kimitsu Chem. Industry Co. (Tokyo, Japan)
Furcellaran	Continental Colloids Inc.(West Chicago,IL)
Locust Bean Gum	Germantown Mfg. Co.
Pectin Lot#636912	Continental Colloids Inc.(West Chicago,IL)

2. Reagents:

The sources of the reagents used in this study are listed in Table 4.

Table 4. Reagent sources

Reagent	Manufacturer
Malonic acid ($HOOCCH_2COOH$)	Fisher Scientific Co.
Sodium hydroxide (NaOH)	Ohio State University Lab Store
Calcium hydroxide (Ca(OH))	Fisher Scientific Co.
Ethanol (Ethyl alcohol USP) with 200 proof Aaper	Aaper Alcohol and Chemical Co.
Boric acid (H_3BO_3), 99.9%	J. T. Baker Chemical Co.
Trichloroacetic acid (CCl_3COOH),	Packed by Jenneile Enterpries Co.
Disodium dehydrogen ethylenediaminetra-acetate dihydrate (EDTA)	G. Frederick Smith Chemical Co.
Disodium phosphate	
Citric acid ($CH_3C_6H_5O_7.H_2O$)	Mallincrodt Inc.

Table 4. (continued)

Reagent	Manufacturer
Sulfosalicylic acid ($HOC_6H_3(COOH)SO_3H.2H_2O$)	Fisher Scientific Co.
Urea ($(NH_2)_2CO$	Mallincrodt Inc.
Methanol	
Lithium iodate ($LiIO_3$)	Ventron Alfa Products.
Lithium chloride (LiCl), 99.9%	Mallincrodt Inc.
Horseradish peroxidase	SIGMA Chemical Co.
Benzidine base	Hartman-Leddon Co.
Tris	SIGMA Co.
Acetic acid (Glacial),100%	Mallincrodt Inc.
Imidazole	Nitritional Biochemicals Corporation
Toluidine blue	Ohio State University Lab Store
Alcian blue	Ohio State University Lab Store
Amido black	Ohio State University Lab Store
Ponceau-S	Ohio State University Lab Store
Hydrogen peroxide (H_2O_2),3%	Fisher Scientific Co.

B. Apparatus:

1. Fisher Accumet pH meter model 630 equipped with a Corning 476531 general purpose combination electrode (Fisher Scientific Company).
2. Cellulose acetate strips (Super Sepraphore, product #51040) were obtained from Gelman Sciences Inc.
3. Electrophoresis chamber (Semi-Micro II Chamber, product# 51214) was purchased from Gelman Sciences Inc.
4. Sample applicator was bought from Gelman Sciences Inc.

C. General instrumental technique for zonal electrophoresis

Electrophoresis was conducted with the Gelman Sciences Electrophoresis system at approximately 25°C. The chamber was filled with the chosen buffer and approximately 0.25μl of sample was applied to the strip by the applicator. Electrophoresis was conducted according to the operation procedure provided by Gelman Scienecs Inc. :

1. Place 100 mL buffer in a staining tray.
2. Remove Super Sepraphore Strips from package using forceps.
3. Wet a Super Sepraphore strip by inserting it slowing into the buffer, allowing it to wet completely as it enters. Buffer slowly advances up the membrane as it is edged into the buffer solution. Plunging the membrane into the buffer hinders complete wetting.
4. Submerge strip in buffer and soak at least 10 minutes.
5. Fill chamber with required amount of buffer by measuring and pouring half the amount into each side of the chamber.
6. Load sample applicator with samples. (Either the Sepratek-8 (51118) or the Sepratek-4 (51119) may be used in the Sepratek or Semi-Micro Chambers.)
7. Remove strip from soaking tray, lay flat on an absorbent pad (62093)

and blot with a second absorbent pad.

8. Place strip in electrophoresis chamber.
9. Place drops of samples in sample wells and apply sample by applicator.
10. Attach electrodes and turn on power supply and adjust voltage..
11. Electrophorese for time indicated.
12. Treat the strip by proper staining reagent.
13. Rinse the strip.

D. Electrophoretic analysis of HMR carrageenan

1. Sample preparation

0.4% HMR carrageenan stock dispersion was prepared by sprinkling 0.4 gm of Gelcarin HMR carrageenan powder in 100 ml water at 60°C. The stock dispersion was stored at 0°C to 5°C for no longer than 2 weeks. The following dispersions, 0.5%, 0.4%, 0.3%, 0.2%, 0.1%, 0.05% and 0.025% were made by diluting the 0.5% dilution.

2. Buffer systems for electrophoresis

a. Malonate buffers:

(1) Na-malonate buffer was prepared according to Chang (1968):

> Malonate buffer (pH 2.9, 0.075M) was prepared by disolving 7.8g. malonic acid in 800 ml. distilled water. The pH was adjusted to 2.9 with 1N sodium hydroxide and the volume was adjusted to 1 liter.

(2) Ca-malonate buffer (0.05M) with 15% EtOH was prepared by dissolving 5.2 g malonic acid in 500 ml distilled water, and 150 ml of absolute ethanol was added into the 0.05M malonate solution. The pH was adjusted to 2.9 with 1N $Ca(OH)_2$, and the volume was adjusted to 1000 ml.

(3) Malonate solution (0.075M, pH1.98) was prepared by dissolving 7.8g malonic acid in 1000 ml distilled water.

(4) Malonate solution with 15% EtOH was prepared by adding 150 ml absolute ethanol into 850 ml 0.075M malonate solution.

(5) Malonate buffer with 30% EtOH was prepared by adding 300 ml absolute ethanol in 700 ml 0.075M malonate solution.

b. Boric buffers:

(1) Boric acid buffer (0.1M) with 15% EtOH: 0.1M boric acid solution was prepared by dissolving 6.2 grams boric acid in 1 liter of distilled water, and 150 ml absolute ethanol was added into 0.1M boric acid solution to make 0.1M boric acid solution with 15% EtOH.

(2) Modified TCA/boric acid buffer with 15% EtOH: 0.1M boric acid and 0.075M Trichloroacetic acid solution was prepared by dissolving 6.2 grams of boric acid and 6.15 grams of trichloroacetic acid in 1 liter distilled water. The modified solution was made by adding 150 ml of absolute ethanol into 850 ml of 0.1M boric acid and 0.075M trichloroacetic acid solution

3. Electrophoresis at different temperatures

Electrophoresis of 25°C was operated at room temperature. The run at 40°C was conducted by incubating the assembled cells in a temperature controlled oven (Precision Scientific Co. Model 4) during electrophoresis.

4. Electrophoresis at different voltage/time

Electrophoretic analysis of HMR carrageenan at various concentrations was conducted by using the modified TCA/boric acid buffer under the following conditions:

a. 100 volt/30 min

b. 200 volt/15 min

c. 300 volt/10 min

E. Electrophoretic analysis for common food hydrocolloids

1. Preparation of food hydrocolloid dispersions

Food hydrocolloid dispersions (0.4%) were made by dissolving 0.4 gm of hydrocolloid powder (kappa-, lambda- and iota-carrageenan, HMR carrageenan, alginate, CMC, furcellaran, pectin, locust bean gum and xanthan) into 100ml distilled water at 70°C with stirring for 1 hour. The samples were then cooled and refrigerated until use.

2. Conditions for electrophoresis

a. Electrophoresis in modified TCA/boric acid buffer

Hydrocolloid dispersions (0.4%) including 1) a mixture of kappa- and lambda-carrageenan, alginate and CMC 2) alginate 3) lambda-carrageenan 4) kappa-carrageenan 5) CMC were analyzed by electrophoresis by using the modified TCA/boric acid buffer at 100 V/20 min (25°C). For all the single samples the concentration was 0.4% and the mixture was prepared by

blending equal portions of these mixtures creating a sample solution of 0.4% total hydrocolloid but with only 0.1% of each of the constituents.

b. Electrophoresis in lithium iodate solutions

The same hydrocolloid dispersions as used before were analyzed by electrophoresis by using 0.075M Lithium iodate solution (pH11.75) and 0.15M lithium iodate solution as buffers at 100 V for 60 minutes (25°C).

c. Electrophoresis in lithium chloride solutions

Two lithium chloride solutions were prepared as buffers for electrophoresis: 1) The original 0.1M lithium chloride solution at pH 5.38 2) 0.1M lithium chloride solution at adjusted pH 2.0 by adding 1N HCl. The same hycolloid dispersions as used before were analyzed by electrophoresis by using 0.1M lithium chloride at pH 5.38 and 0.1M lithium chloride at pH 2.0.

3. Staining

a. Toluidine blue

After conducting electrophoresis for 15 min at 200 volts, the strip was placed in 0.2% aqueous toluidine blue for 15 minutes. Then the strip was rinsed with distilled water until the bands were clearly visible against a pale blue background (Padmoyo and Miserez, 1967).

b. Horseradish peroxidase-benzidine staining (Dickmann et al.,1989):

> The strips were placed in a Petri dish and covered with 15 ml of horseradish peroxidase solution (25 mg in 50 ml of distilled deionized water containing 0.5 ml of acetic acid) and incubated at room temperature in the dark for 45 min. The strips were then rinsed in a running water bath for 20 min. The rinsed strips were then placed into a dish containing 15 ml of a solution of benzidine hydrochloride (0.05% in 0.05M Tris, 0.01M imidazole, pH7.6) which had three drops of 3% hydrogen peroxide added immediately before addition of the strips. Strips were stained for 1-2 min, rinsed under running water for 2 min and dried in air.

In this reaction, benzidine is a potent carcinogen which is hazard to health. Though considerable effort was spent to select a less toxic substitute for benzidine, tetramethylbenzidine, pyrogallol, dimethylamine and p-hydroxybenzoic acid had been used and provided unsatisfactory results (Dickmann et al., 1989). It is necessary to use benzidine carefully and avoid the possible pollution of the environment.

c. Modified Horseradish peroxidase-benzidine staining

After electrophoresis for 60 min at 100 volts, the cellulose acetate strip was stained following the procedure by Dickmann et al. (1990) with the following exceptions:

1) 3 ml of 3% hydrogen peroxide was added instead of only three drops.

2) In the procedure of destaining background color, gentle rinsing with distilled water three times was used instead of rinsing under running water.

CHAPTER IV
EXPERIMENTAL RESULTS

A. Electrophoresis of HMR carrageenan

Because the equipment that Chang (1968) used is no longer available, the electrophoretic equipment from Gelman Science Inc., which can resolve proteins sharply (Appendix Fig 17), was adapted for zonal electrophoresis of mixtures of hydrocolloids. In order to optimize the analysis, it was necessary to select the most suitable conditions including choice of buffer, temperature and voltage/time relationship. Various conditions were applied for this particular equipment using a dispersion of HMR carrageenan as the test sample. This material was chosen because it is an alkaline extract of Chondorus Crispus and contains approximately equal amounts of λ- and κ-carrageenan, for which comparative patterns are available in our laboratory (Appendix Fig. 16).

1. Selection of Buffer Systems

a. Ca-malonate buffer

HMR carrageenan dispersions were prepared in the concentration range from 0.025% to 0.5% The buffer used was 0.05M Ca-malonate buffer with 15% ethanol, pH 2.9 (Chang, 1968). The electrophoretic pattern in figure 1 shows the presence of a calcium aggregated stationary zone

Figure 1. Electrophoretic patterns of HMR carrageenan.in Ca-malonate buffer

The concentration of HMR carrageenan from right to left:

0.1%, 0.2%, 0.3%, 0.4%, 0.5%, 0.05%, 0.025%

Voltage: 200 V

Time: 15 min

Stain: Toluidine blue

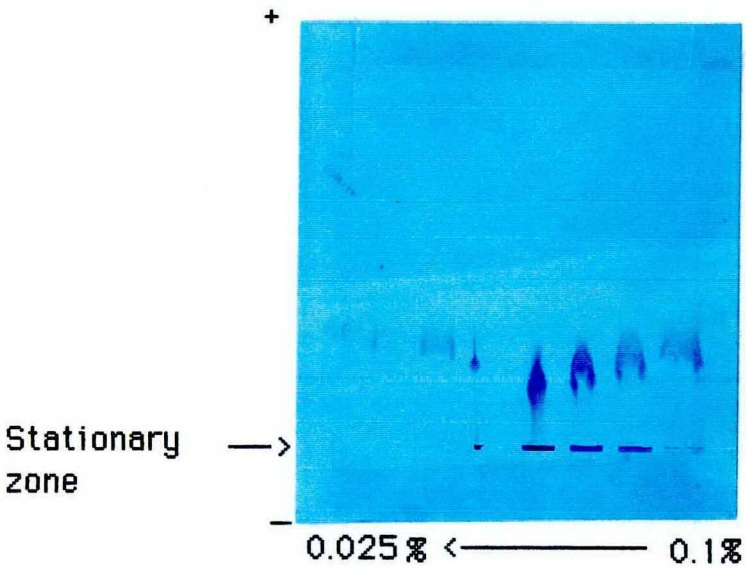


Figure 1

located at the point of application. In addition, there were migrating bands moving toward the positive electrode. These bands, however, were streaky and not uniformly separated. Furthermore, the migrating distances were dependent on the concentration, which is undesirable.

It is reported that Ca^{2+} is capable of causing the formation of calcium aggregated material which stays in the stationary zone. However, some material is still able to migrate electrophoretically but the distance moved appears to be a function of the colloid concentration. Thus, at low concentration a relatively larger portion is free to migrate. This is clearly seen by the results in figure 1. According to Hansen and Renoll (1974) , the formation of a stationary band is evidently the result of the aggregating influence of calcium ions and does not occur when Na- or Li- malonate buffer was used. Therefore, Na-malonate buffer was used in the next experiment to eliminate the calcium aggregated stationary zone, and make the migration less concentration dependent.

b. Na-malonate buffer

0.075M Na-malonate buffer with 15% ethanol, pH 2.9 was used for electrophoresis in this experiment. The concentration of HMR carrageenan dispersions ranged from 0.025% to 0.5%. The result of the electrophoresis shown in figure 2 indicated that there was no aggregated stationary zone except at the highest concentrations, 0.4% and 0.5%. The migrating distances were less concentration dependent. These were the improvement

Figure 2. Electrophoresis pattern of HMR carrageenan in Na-malonate buffer

HMR carrageenan concentration from right to left:

0.025%, 0.05%, 0.1%, 0.2%, 0.3%, 0.4%, 0.5%

Voltage: 200 V

Time: 15 minutes

Stain: Toluidine blue

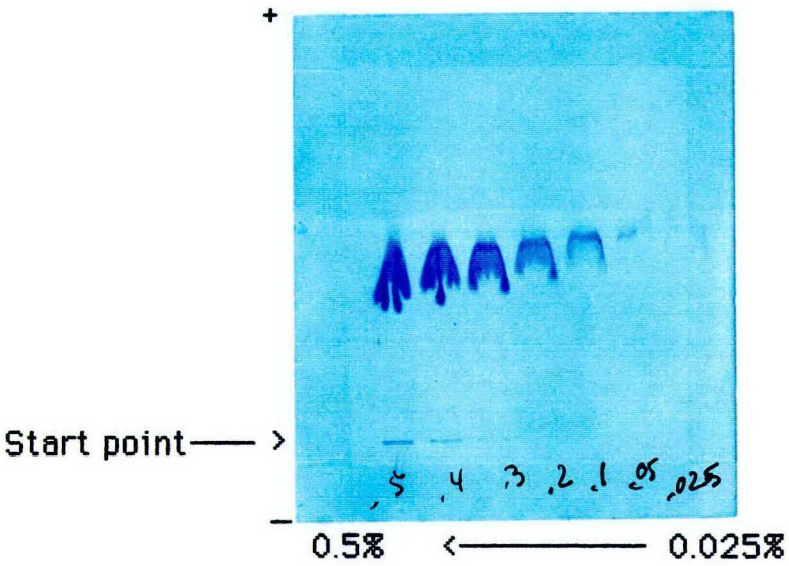


Figure 2

by using the Na-malonate buffer instead of Ca-malonate buffer. However, the resolution was still very poor. In order to examine the effect of eliminating metal ions and the influence of adding ethanol to the buffers, the buffer used in the next experiment was based on 0.075M malonic acid alone or with different percentage of ethanol added.

c. Malonate buffer

The solutions for electrophoresis used in this experiment were 0.075M malonic acid, pH 1.98 with or without 15% or 30% ethanol added. The concentration of HMR carrageenan dispersions applied to the cellulose acetate strips was the same as in the former experiment. The results in figure 3 show that (1) no aggregated stationary zones were formed in this system with or without 15% ethanol, 2) HMR carrageenan showed the most uniform migrating distances in malonic acid with 15% ethanol, (3) ethanol had an effect of decreasing the migration of the bands, (4) the bands of HMR carrageenan from the highest concentration levels became very streaky in the presence of 30% ethanol.

The overall results suggest that the addition of 15% ethanol is sufficient to make the migration of bands less concentration dependent. The functionality of ethanol in the buffer was eliminating the aggregation between hydrocolloids. Because ethanol was not a very good solvent for hydrocolloids, this imcomplete dissolving effect could probanly reduce the interaction between hydrocolloids when they were completely dissolved. However, the unbuffered malonic acid did not provide for better resolution.

Figure 3 Electrophoresis patterns of HMR carrageenan in malonate buffer systems

The concentration of HMR carrageenan from right to left:

0.025%, 0.05%, 0.1%, 0.2%, 0.3%, 0.4%, 0.5%

a. Malonate buffer

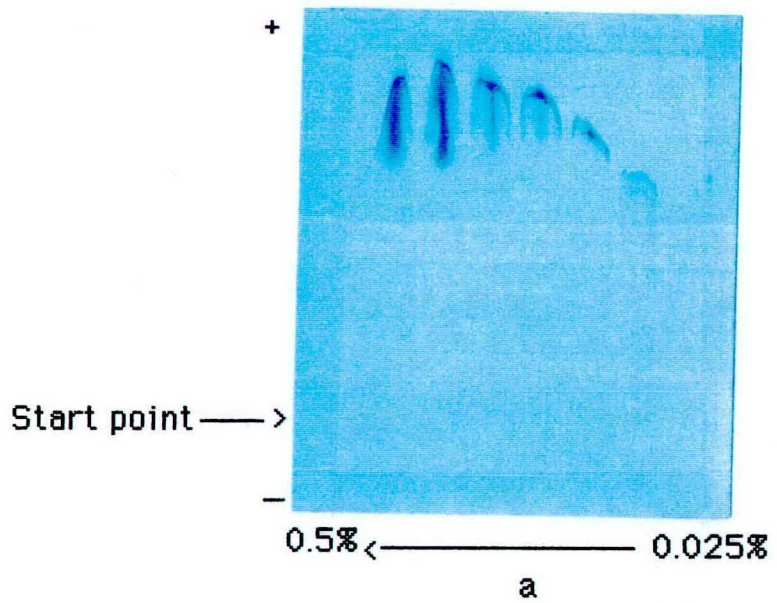
b. Malonate buffer with 15% EtOH

c. Malonate buffer with 30% EtOH

Voltage: 200 V

Time: 15 minutes

Stain: Toluidine blue



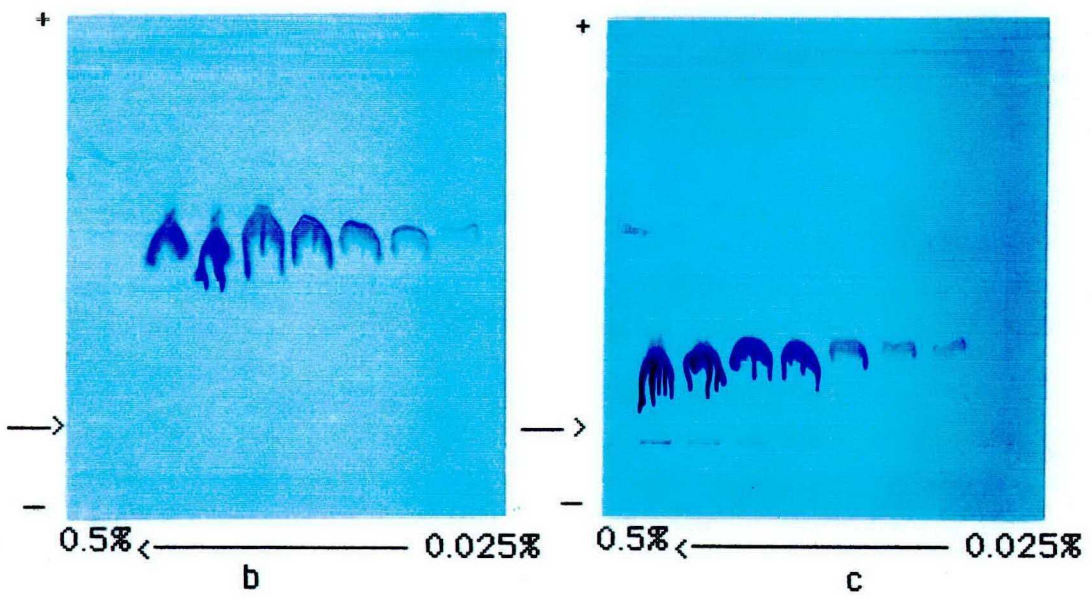


Figure 3

To explore other suitable buffers to resolve HMR carrageenan by electrophoresis, boric acid buffer (Padmoyo and Miserez, 1967) was used in the next experiment.

d. Boric acid solution

The underlying reason for using boric acid is related to the fact that hydrocolloids with cis-hydroxy groups form complexes with boric acid (Schachat and Raymond, 1956). The reaction causes the neutral hydrocolloids, such as locust bean gum and guar gum, to carry negative charges which promote electrophoretic mobility (Padmoyo and Miserez, 1967 and Chang et al., 1974).

In this experiment 0.1M boric acid, pH 5.38 with 15% ethanol was used with HMR carrageenan. The results in Fig 4 a show a diffuse zone migrating with a uniform mobility at all concentrations. There was no aggregated stationary zone at the starting point indicating that this buffer system is a good solvent. At the highest concentration the bands were broader than at low concentration indicating that the support strip had become overloaded. The patterns in Fig 4b were obtained by a applying constant sample concentration (0.2%) in all eight slots. The results show that the migration was uniform and reproducible. The slight curvature evident for the front was probably caused by a tendency for the edges to dry before the center portion. Dehydration causes a build up of electrolytes and an increase in conductivity of the solvent, with a resulting decrease in mobility of the colloid.

Figure 4 Electrophoretic patterns of HMR carrageenan in 0.1M boric acid with 15% ethanol

a. The concentration of HMR carrageenan from right to left: 0.025%, 0.05%, 0.1%, 0.2%, 0.3%, 0.4%, 0.5%

b. The concentration of HMR carrageenan is 0.2%

Voltage: 200 V

Time: 15 minutes

Stain: Toluidine blue

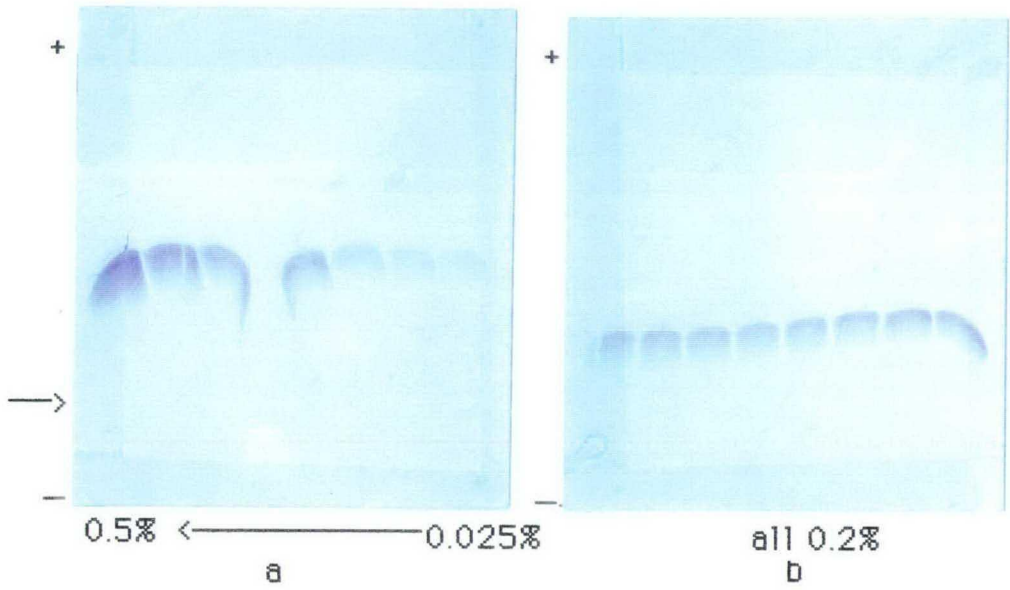


Figure 4

The use of boric acid in combination with ethanol still did not produce the expected separation of HMR carrageenan into two bands. Therefore, in the next experiment a modification of this buffer system was attempted.

e. Modified TCA/boric acid solution

In Chang's study (1968) of separating four hydrocolloids including CMC, alginate, κ- and λ-carrageenan by electrophoresis, he pointed out that the separation was improved at pH 2.9 of malonate buffer over that at pH 10 of the borate buffer by Padmoyo and Miserez (1967). He explained that the reason for improved separation at the lower pH may be found in the lower degree of dissociation of the weakly acidic carboxylic groups in contrast to the completely dissociated sulphate groups. This provides for better separation of carboxylated species from sulfated species. Therefore, the use of a low pH value is an important factor to improve the resolution of hydrocolloids on cellulose acetate strips. It was necessary to adjust 0.1M boric acid solution (pH 5.38) to a lower pH value. The reagent used to lower the pH was trichloroacetic acid (TCA). The reason for choosing this reagent was that TCA is a good solvent for polysaccharides. Hansen and Chang (1968) used 12.5% TCA to precipitate hydrolyzed protein from milk containing CMC and proteins. The recovery of the CMC from the milk was in the range from 89.3% to 96.1%, showing that TCA might be a suitable reagent to reduce the pH of the buffer solution without destroying the hydrocolloids.

The pH of the modified buffer containing 0.1M boric acid, 0.075M TCA with 15% ethanol was pH 1.29. The result of electrophoresis in figure 5 showed two separate migrating bands for HMR carrageenan. This improved resolution suggests that the modified boric acid buffer was suitable for HMR carrageenan. The two bands represent λ- and κ-carrageenan.

Boric acid can react with hydrocolloids, and the difference in reactivity of various hydrocolloids reflects dissimilar configurations leading to unlike charges after combining with boric acid (Boeseken, 1949). The two separate bands are evidently caused by the two components in HMR carrageenan, λ- and κ-carrageenan, carrying different charges after reacting with boric acid.

Though the separation of bands was successful, the resolution was not yet considered to be sufficiently sharp for analytical purposes. Voltage, time and temperature of conducting electrophoresis were considered to be other important factors affecting the electrophoretic resolution. These various conditions were used in the following experiments to explore appropriate conditions for improved resolution.

2. Selection of Electrophoretic Conditions

a. Electrophoresis at different temperatures

Studies on the effect of temperature on zonal electrophoresis of hydrocolloids by Hansen and Renoll (1973) have shown improved resolution of Seakem 2 carrageenan with the Millipore system using 40°C rather than room temperature (25°C). The following experiment was

Figure 5 Electrophoretic pattern of HMR carrageenan in modified TCA/boric acid buffer

The concentration of HMR carrageenan from right to left:

0.025%, 0.05%, 0.1%, 0.2%, 0.3%, 0.4%, 0.5%

Buffer: 0.1M Boric acid, 0.075M TCA w/ 15% EtOH

Voltage: 200 V

Time: 15 minutes

Stain: Toluidine blue

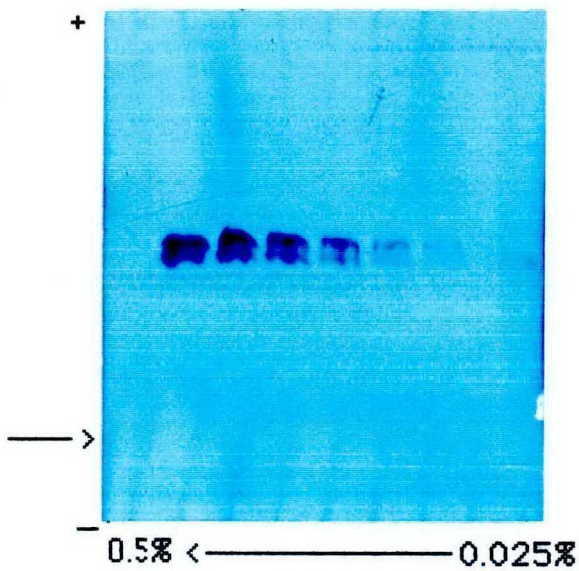


Figure 5

performed by conducting electrophoresis at these two temperatures, 40°C and 25°C using the Gelman system. The run at 40°C was conducted by incubating the assembled cells in a temperature controlled oven during electrophoresis. The buffer system was the modified TCA/boric acid buffer.

The electrophoretic patterns in figure 6 show two migrating bands for HMR carrageenan. However, the migration of the bands was not stable but showed streaming toward the center portion. This might be the effect of heating, which vaporized the moisture from the edges of the cellulose acetate strip and caused HMR carrageenan to migrate toward the wetter center portion. Furthermore, the HMR carrageenan at the highest concentration was poorly retained on the cellulose acetate so that some portions of the bands were missed.

Apparently, no improved resolution of HMR carrageenan could be achieved at 40°C, and this approach was abandoned. The reason for the failure may be related to the fact that the Millipore system provided a sealed environment which prevented evaporation. The Gelman apparatus does not provide for a sealed environment and the poor results may reflect the loss of moisture from the strips.

b. Electrophoresis at different voltage/time

The voltage gradient provides the force to pull molecules along from the negative electrode toward the positive electrode. The following experiment was designed to determine the optimum relationship between voltage and time for resolving HMR carrageenan by electrophoresis.

Figure 6 Electrophoretic pattern of HMR carrageenan at 40°C

The concentration of HMR carrageenan from right to left:

0.025%, 0.05%, 0.1%, 0.2%, 0.3%, 0.4%, 0.5%

Buffer: 0.1M boric acid, 0.075M TCA with 15% EtOH

Voltage: 200 V

Time: 15 minutes

Stain: Toluidine blue

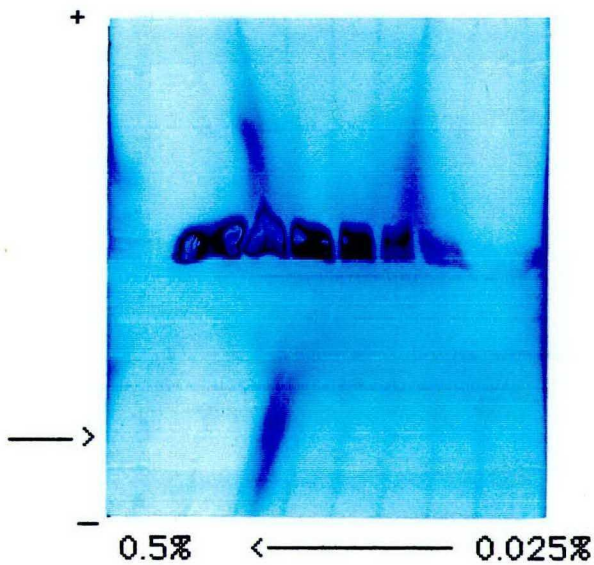


Figure 6

Various conditions including 100 V/30 min, 200 V/15 min and 300 V/10 min for conducting electrophoresis were used in this experiment The results in figure 7 show that the best resolution was achieved at 200 V/ 15 min. Using lower voltage for a longer time resulted in less sharp resolution.

Electrophoresis at 300 V for only 10 minutes resulted in complete failure because the plastic support melted in the center indicating that the Ohmic heating was excessive.

B. Electrophoretic analysis of food hydrocolloid mixtures

1. Selection of Buffer system

a. Modified Boric acid buffer

This experiment was performed to ensure if the modified TCA/boric acid buffer which was suitable for HMR carrageenan was also proper for other hydrocolloids. Hydrocolloid dispersions (0.4%) were prepared including 1) a mixture of kappa- and lambda-carrageenan, alginate and CMC, 2) alginate, 3) lambda-carrageenan, 4) kappa-carrageenan and 5) CMC and analyzed by electrophoresis in the modified TCA/boric acid buffer.

The results in figure 8 show the electrophoretic pattern for these hydrocolloids. For the mixture of four hydrocolloids, there were only two separate bands instead of four, emphasizing the point that the buffer system might not be suitable for a complex mixture of food hydrocolloids. Therefore, other buffer systems for resolving these hydrocolloids needed to be explored. According to the manual for the Gelman electrophoresis system,

Figure 7 Electrophoretic patterns of HMR carrageenan at various conducting conditions:

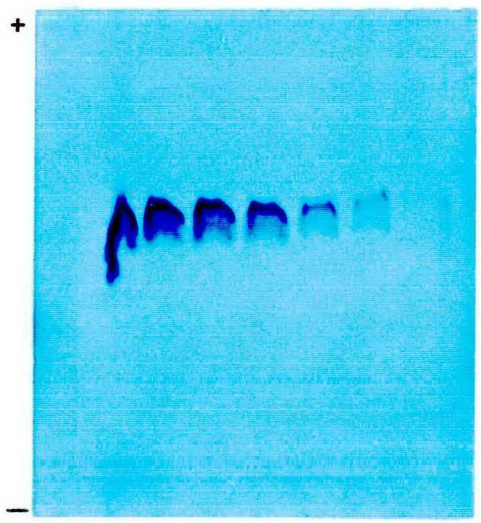
a. 100 volt, 30 minutes

b. 200 volt, 15 minutes

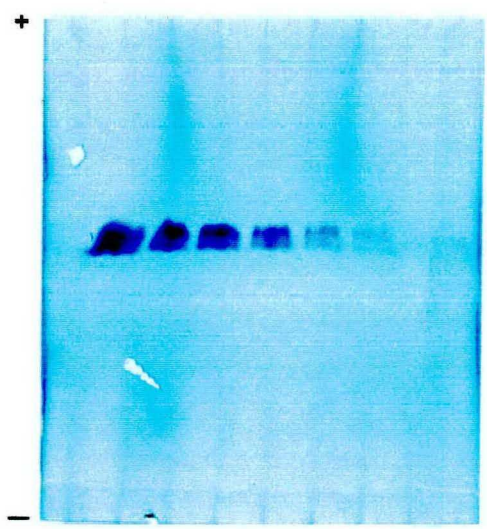
c. 300 volt, 10 minutes

Buffer: Modified TCA/boric acid buffer

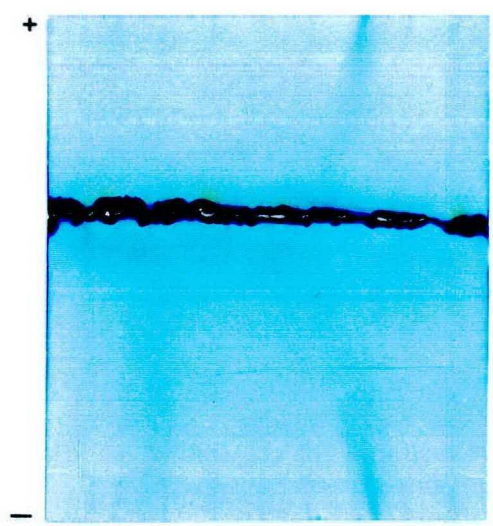
Stain: Toluidine blue



a



b



c

Figure 7

Figure 8. Electrophoretic pattern of hydrocolloids in modified TCA/boric acid solution

Hydrocolloids:

1. Mixture of CMC, alginate, κ-and λ-carrageenan
2. Alginate
3. λ-carrageenan
4. κ-carrageenan
5. CMC
6. Mixture of CMC 4H, CMC 9M and CMC 12H
7. CMC 4H
8. CMC 9M

Buffer: Modified TCA/boric acid buffer

Voltage: 100 V

Time: 20 minutes

Stain: Toluidine blue

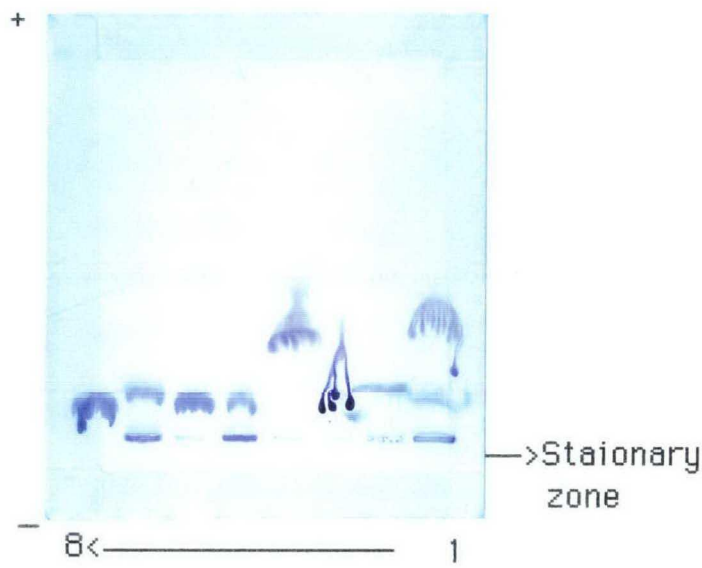


Figure 8

these particular cellulose acetate strips were designed for usage in determining serum proteins. Therefore, a similar buffer used for electrophoretic analysis of bovine serum proteins on cellulose acetate membrane was used with the hydrocolloid samples.

b. Lithium iodate buffer

Lithium iodate solution (Senda, 1992) was used for electrophoresis of bovine serum proteins on cellulose acetate strips. The following experiment was undertaken by using 0.075M and 0.15M lithium iodate solution, pH 11.4, for the same food hydrocolloids as used before. In this experiment, a lower voltage and longer time (100 V/60 min) were used for electrophoresis. The electrophoretic results in figure 9 may be interpreted as follows:

1. This buffer system was capable of separating the mixtures into recognizable bands. However, for most of the samples containing only a single hydrocolloid species, the resolution was poor. The reason for the failure in this case is evidently overloading of the strips with sample material. For all of the single samples the concentration was 0.4% and the mixture was prepared by blending equal portions of these mixtures creating a sample solution of 0.4% total hydrocolloid but with only 0.1% of each of the constituents. Therefore, during electrophoresis the resolved zones in the mixed sample correspond to 0.1%.

Figure 9 Electrophoretic patterns of hydrocolloids in lithium iodate buffer of different concentration

Buffer:

a. 0.075M $LiIO_3$

b. 0.15M $LiIO_3$

Hydrocolloids:

1, 7. and 8. Mixture of alginate, CMC, κ–and λ–carrageenan

2. κ–and λ–carrageenan

3. κ–carrageenan

4. λ–carrageenan

5. Alginate

6. CMC

Voltage/time: 100 V/60 min

Stain: Toluidine blue

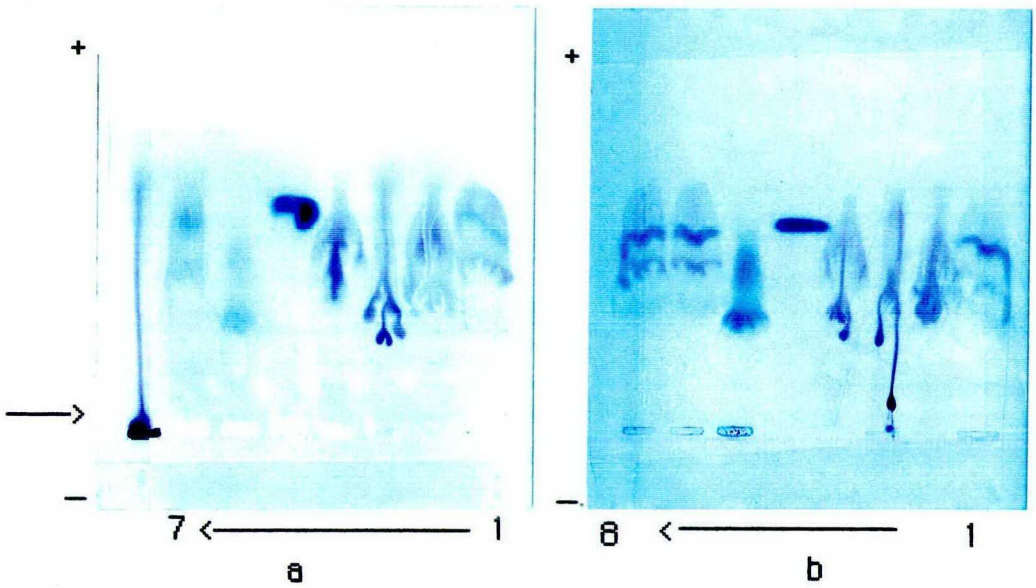


Figure 9

2. Lithium iodate solution produced better resolution for a mixture of the selected hydrocolloids than any other buffers used in the previous experiments.

3. Comparing the resolution effect of figure 9 a and b, it is obvious that the electrophoretic pattern in b (0.15M lithium iodate) has sharper separation of bands than the pattern in a. Considering the buffer for electrophoresis, the determining factor for electrical conductivity is the concentration of the buffer salts (Osterman, 1984). The lithium iodate solution in pattern b contained twice the concentration of the buffer in pattern a and the separation was better in b than in a.

It was concluded that the chosen buffer was able to provide basic resolution of these four hydrocolloids, though the bands were not yet sharply resolved. To explore possible improvement, the following experimant was conducted replacing lithium iodate solution with lithium chloride solution at pH 2.0. This choice was made since it has been shown that the use of a lower pH was advantageous for better resolution (Chang, 1968). Lithium chloride can be adjusted to pH 2 by hydrogen chloride without introducing any cation or anion other than H^+, Li^+ or Cl^-.

c. Lithium chloride buffer

In order to improve the resolution of the buffer, electrophoresis was performed in 0.1 M lithium chloride at two different pH values- pH 7.25 and pH 2. The original pH value of 0.1M lithium chloride was pH 7.25. By the addition of 1N HCl this solution was adjusted to pH 2.0.

The electrophoresis was conducted at 100 V for 60 minutes. According the results in figure 10, the pattern at pH 2 resulted in sharper resolution of the separate bands. Moreover, the bands in pattern b were less streaky than the bands in pattern a. In pattern b, alginate and CMC migrated with lower mobilities than λ- and κ-carrageenans corresponding to the differences in ionization for these colloids. It was easy to identify alginate and CMC at the lower portion of the pattern. The front part of the migrating zones was characterized by a leading zone which was streaky and a second zone which was sharp. Absolute identification was not possible because the bands of pure carrageenans in pattern b were too streaky to recognize.

These results confirm that sample loading is a critical factor for electrophoresis of hydrocolloids on cellulose acetate. Thickness of this membrane may be at issue because the Gelman strips are thinner than the Phoroslide strips originally provided by Millipore. The next experiment was conducted to determine if streakyness was related to sample concentration.

2. Concentration of hydrocolloids

a. Comparison of hydrocolloids at 0.2% and 0.4%

To explore the effect of differences in concentration of hydrocolloids, 0.4% and 0.2% κ- and λ-carrageenan dispersion were applied for electrophoresis at 100 V/60 min using 0.1M LiCl solution (pH2.0). Mixtures were prepared by mixing equal volumes of the individual samples, therefore the actual concentration of κ– or λ–carrageenan became 0.2% in

Figure 10. Electrophoresis of 0.5% hydrocolloids in LiCl buffers at different pH values

a. 0.1M LiCl (pH 7.25)

b. 0.1M LiCl (pH 2)

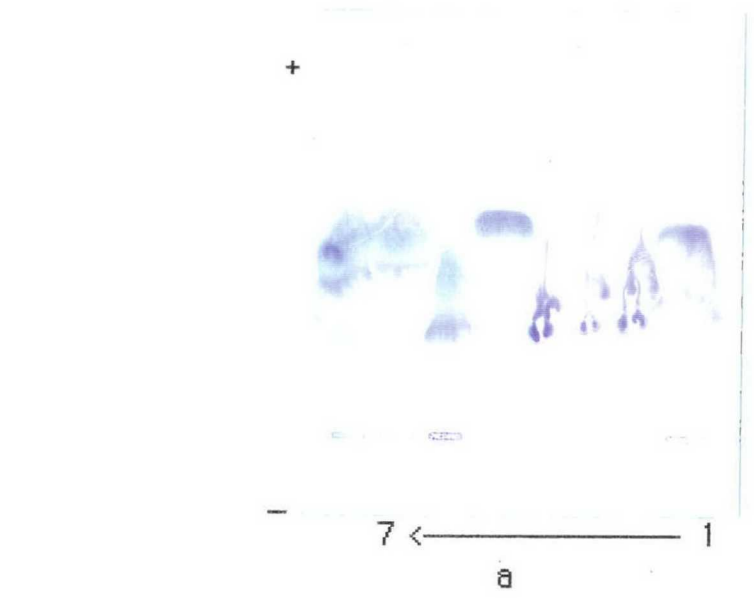
Hydrocolloids:

1. Mixture of κ–and λ–carrageenan, Alginate and CMC.
2. κ–and λ–carrageenan
3. κ–carrageenan
4. λ–carrageenan
5. Alginate
6. CMC
7. Mixture of κ–and λ–carrageenan, Alginate and CMC.
8. Mixture of κ–and λ–carrageenan, Alginate and CMC.

Voltage: 100 V

Time: 60 minutes

Stain: Toluidine blue



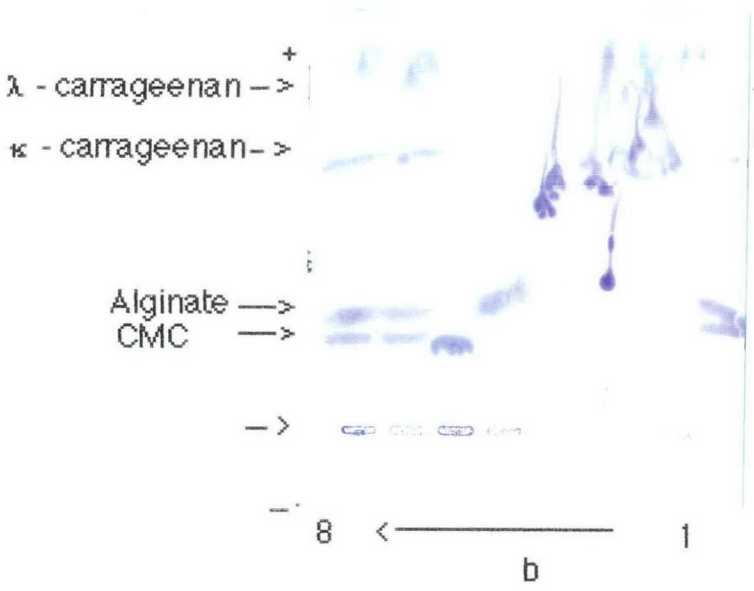


Figure 10

the 0.4% mixed dispersion of these two. In the same manner, the concentration of the individual carrageenans would be 0.1% in the 0.2% mixed disperson. The results in figure 11 indicated clearly that the 0.2% dispersion produced better separation than the 0.4% dispersion which showed an inability to be retained or absorbed by the cellulose acetate matrix. At the high concentration, all of the zones showed a tendency to "bleed" resulting in poor separation so that they could not be distinguished. On the other hand, although the bands in pattern b were not sharp, it was possible to discern two different migrating bands in the mixture. Further inspection of pattern b, showed different shapes for the zones corresponding to kappa- and lambda-carrageenan. Lambda-carrageenan formed a flame-like migrating zone while kappa-carrageenan tended to form a sharper line-like band.

The results suggested that the lower concentration of hydrocolloids produced a sharper separation effect. Therefore, the following experiment was performed to explore even lower concentrations.

b. Hydrocolloids at 0.025%

The results in figure 12 show the electrophoretic pattern of a 0.025% carrageenan dispersion after electrophoresis in LiCl buffer (pH2.0) at 100 V/60 min. Although the stained zones are weak it is possible to make an identification of kappa- and lambda–carrageenan. Tracks 1 and 2 which contained the mixture of kappa- and lambda-carrageenan were satisfactorily resolved. By comparison to the band positions for kappa- and lambda-

Figure 11. Electrophoresis of κ– and λ–carrageenan in 0.4% and 0.2%

a. 0.4%

b. 0.2%

Hydrocolloids:

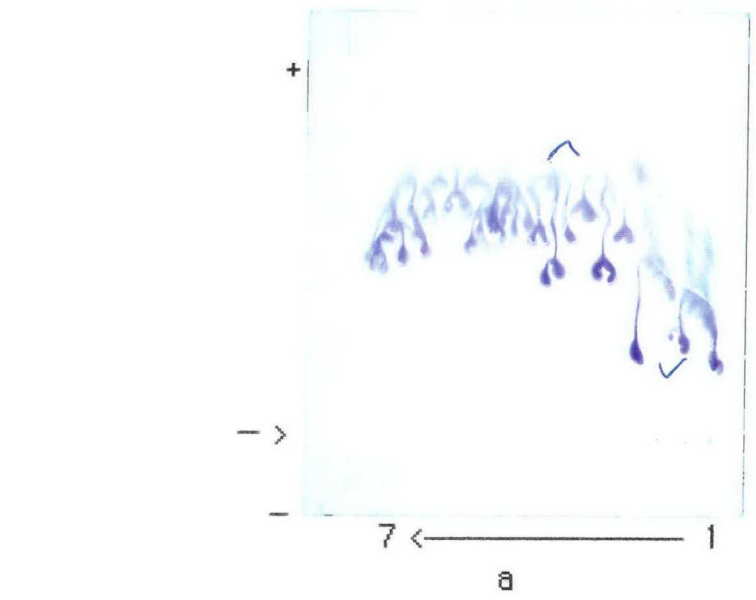
1. κ-carrageenan
2. κ-carrageenan
3. λ-carrageenan
4. λ-carrageenan
5. Mixture of κ– and λ–carrageenan
6. Mixture of κ– and λ–carrageenan
7. Mixture of κ– and λ–carrageenan
8. Mixture of κ– and λ–carrageenan

Buffer: 0.1 M LiCl (pH 2)

Voltage: 100 V

Time: 60 minutes

Stain: Toluidine blue



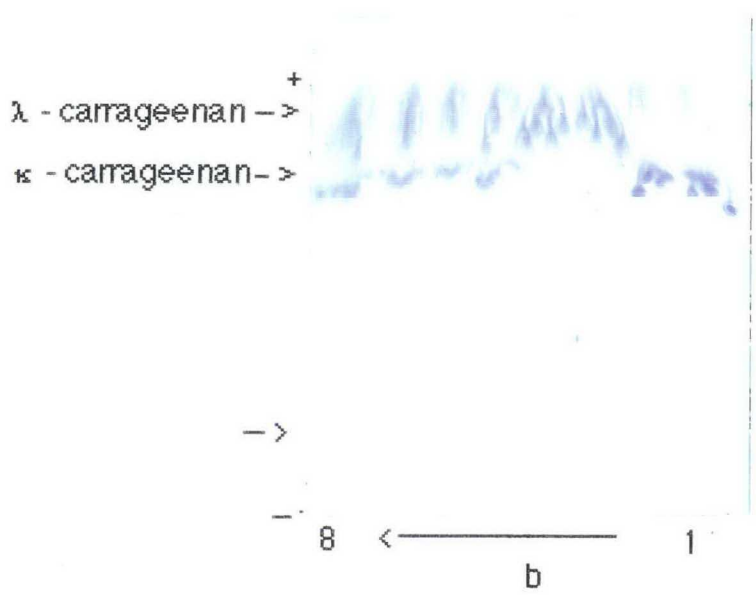


Figure 11

Figure 12. Electrophoresis of 0.025% hydrocolloid dispersions

Hydrocolloids:

1. Mixture of κ- and λ-carrageenan
2. Mixture of κ- and λ-carrageenan
3. κ-carrageenan
4. λ- carrageenan

Buffer: 0.1M LiCl (pH 2)

Voltage: 100 V

Time: 60 minutes

Stain: Toluidine blue

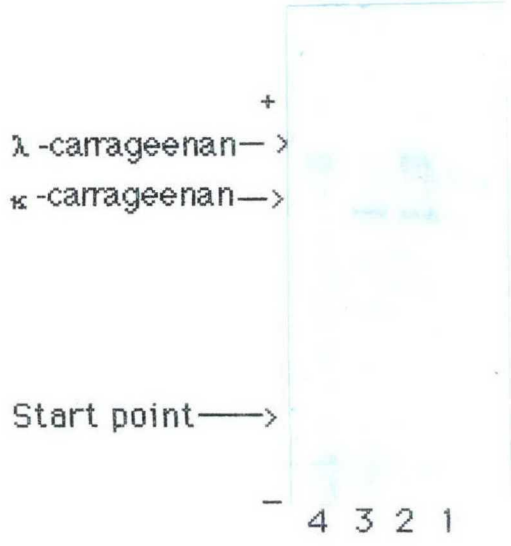


Figure 12

carrageenan at tracks 3 and 4, it is possible to recognize that the upper band of the mixture is lambda carrageenan and the lower band is kappa-carrageenan. It was concluded that the use of low sample concentration in conjunction with the selected buffer and electrophoretic conditions provided for adequate electrophoretic resolution using the Gelman electrophoresis system. The overall result suggested that 0.025% of a kappa- and lambda-carrageenan mixture represented a minimum concentration to be satisfactorily identified by current staining procedures.

Further experiments were conducted to explore the possibility of enhancing the staining intensity of the zones.

3. Staining methods

a. Toluidine blue

Toluidine blue is often used to detect anionic polysaccharides. Hsu et al. (1972) used Toluidine blue to detect glycosaminoglycans. Rothschild and Oliveira (1981) used Toluidine blue to measure the amount of sulfate group in polysaccharides. They used the absorption spectra between 400-700 nm to detect the color compound formed between Toluidine blue and sulfate group of polysaccharides.

For better understanding of the staining effect of Toluidine blue on various hydrocolloids the following experiment was designed. Cellulose acetate membranes were soaked in buffer at pH 6 and then spotted with hydrocolloid dispersions using the sample applicator. No electrophoresis was conducted; instead the strips were directly stained by Toluidine blue.

The different intensity of staining color is shown in figure 13 Locust bean gum could not be stained by Toluidine blue. Locust bean gum is a neutral polysaccharide and is not expected to react with the cationic dye, Toluidine blue. For the remaining, there is a noticeable difference in staining characteristics between the group represented by kappa-, iota carrageenan, furcellaran and xanthan and the group represented by lambda-carrageenan, CMC, alginate and pectin. For the first group the stain appears to have caused a gel-like band which is weaker in the second group. It is possible that these differences represent a tendency for the dye to cause differential precipitation or gelation.

Based on the former experimental result, the lowest concentration of carrageenan which could be readily visualized by Toluidine blue was 0.025%. The next attempt was to determine if less than 0.025% of hydrocolloids can be detected with better resolution using another staining method.

b. Horseradish peroxidase-benzidine staining

Food hydrocolloids on polyamide strips could be detected by using horseradish peroxidase-benzidine staining according to Dickmann et al. (1989). They pointed out that the staining technique was effective for anionic gums, which included algin, carrageenan, carboxymethylcellulose, gum arabic, pectin, propyleneglycol alginate and xanthan, but was ineffective with the neutral gums including guar gum, hydroxypropylmethylcellulose and locust bean gum.

Figure 13. Staining effect of Toluidine blue on various hydrocolloids at pH 6

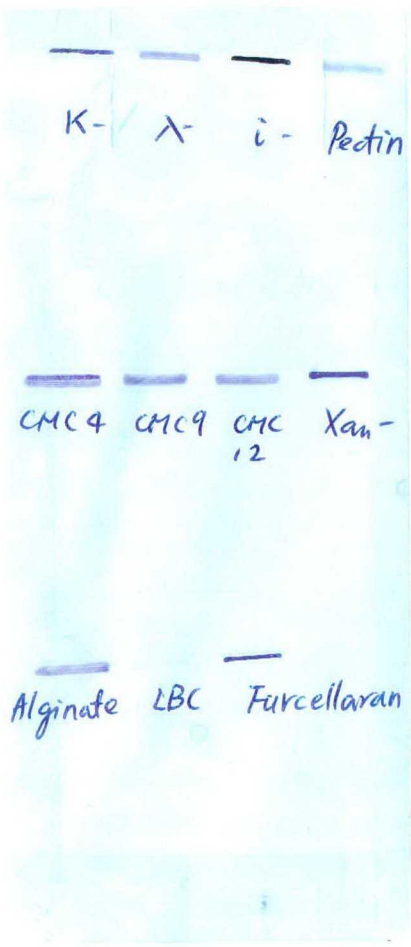


Figure 13

This staining method utilized the ionic interaction between anionic polysaccharides and a positively charged protein (horseradish peroxidase) to form a complex that could be sensitively and quantitatively detected by the enzymatic reactions of peroxidase.

Cellulose acetate strips were also examined for staining by this method, but were less effective because of high background staining (Dickmann et al, 1989). However, this staining method is very promising for detecting very small amounts of hydrocolloid, since the horseradish-benzidine staining method applied to hydrocolloids on polyamide strips could sensitively detect 0.008% carrageenan (Dickmann, 1989).

In this experiment, the Gelman cellulose acetate strips were used for electrophoresis instead of polyamide strips. Polyamide would not be a suitable support system for electrophoresis because of its strong absorbtion characteristics. Hydrocolloid dispersions (0.05%)were prepared as in the former experiment. The mixed dispersion of alginate, CMC, κ- and λ-carrageenan was prepared by blending equal portions of the individual dispersions so that the individual hydrocolloid concentration in the mixture became 0.0125%. The electrophoresis was conducted by using 0.1M lithium chloride solution as the buffer at 100 V for 60 minutes. Visualization of the bands followed the procedure described in the Method section except for 0.1M Lithium chloride (pH2) was used as the buffer for electrophoresis and soaking.

Figure 14. Electrophoresis of hydrocolloid dispersions

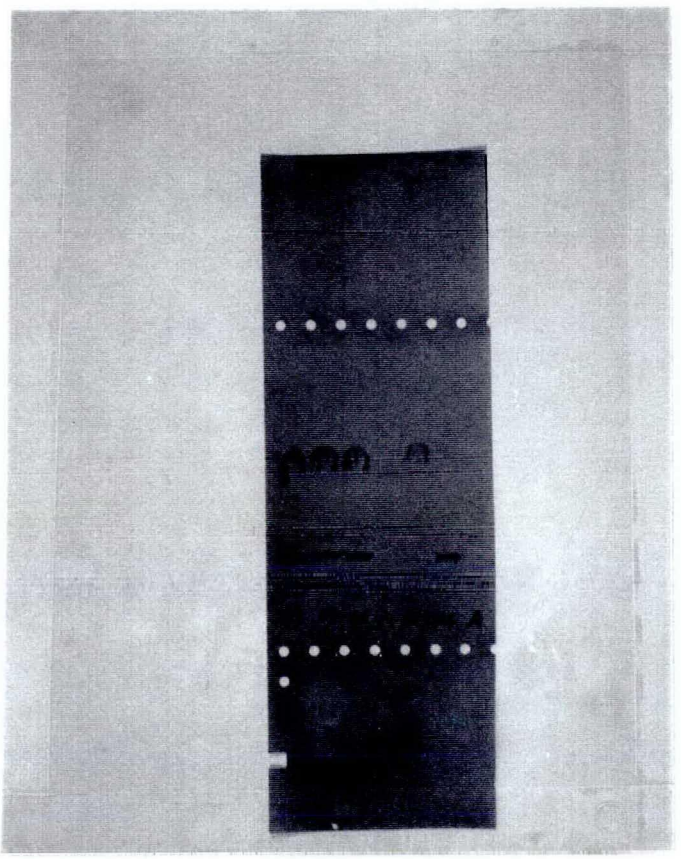
1. Alginate (0.05%)
2. CMC (0.05%)
3. κ-carrageenan (0.05%)
4. λ-carrageenan (0.05%)
5. Mixture of Alginate, CMC, κ–and λ–carrageenan
6. Mixture of Alginate, CMC, κ–and λ–carrageenan
7. Mixture of Alginate, CMC, κ–and λ–carrageenan
8. Mixture of Alginate, CMC, κ–and λ–carrageenan

Voltage: 100 V

Time: 60 minutes

Stain: Horseradish peroxidase-benzidine

[ERRATUM: λ– and κ–carrageenan should be reversed]



8 ——————— 1

Figure 14

The picture in figure 14 was photographically recorded when the strip was wet, because the color intensity would became weak after drying. The patterns in figure 14 indicated that the bands could be visualized by this method, regardless of the strong background stain. There were three migrating bands in this electrophoresis pattern. Alginate and CMC were readily identified. Kappa- and lambda-carrageenan could be identified in the different migrating positions when the pure dispersions were applied. Some aggregating effect seemed to occur when they were electrophoresed in a mixture because only one diffuse band was apparent in the migrating zone for kappa- and lambda-carrageenan. However, additional experiments using lower concentrations, showed that the high background staining precluded the visualization of the bands. Generally speaking, 0.05% was the preferred concentration for staining by the Horseradish peroxidase-benzidine staining approach.

In the following experiment a comparison was made of the Horseradish peroxidase-banzidine method with the standard Toluidine blue method.

c. Comparison of Toluidine blue stain and Horseradish peroxidase-benzidine stain

The following experiment was performed by applying 0.05% furcellaran and xanthan gum to two strips for electrophoresis in 0.1M lithium chloride solution (pH2) at 100 V for 60 minutes. After electrophoresis, these

two strips were separately stained by 0.2% Toluidine blue and Horseradish peroxidase-benzidine staining method.

The electrophoretic patterns in figure 15 indicated no evidence for Horseradish peroxidase-benzidine method being superior to Toluidine blue. In fact, Toluidine blue produced the more intensive staining effect. It was concluded that Toluidine blue would be the better staining reagent.

Figure 15. Horseradish peroxidase-benzidine stain and Toluidine blue

a. Horseradish peroxidase-benzidine stain

b. Toluidine blue

Hydrocolloids:

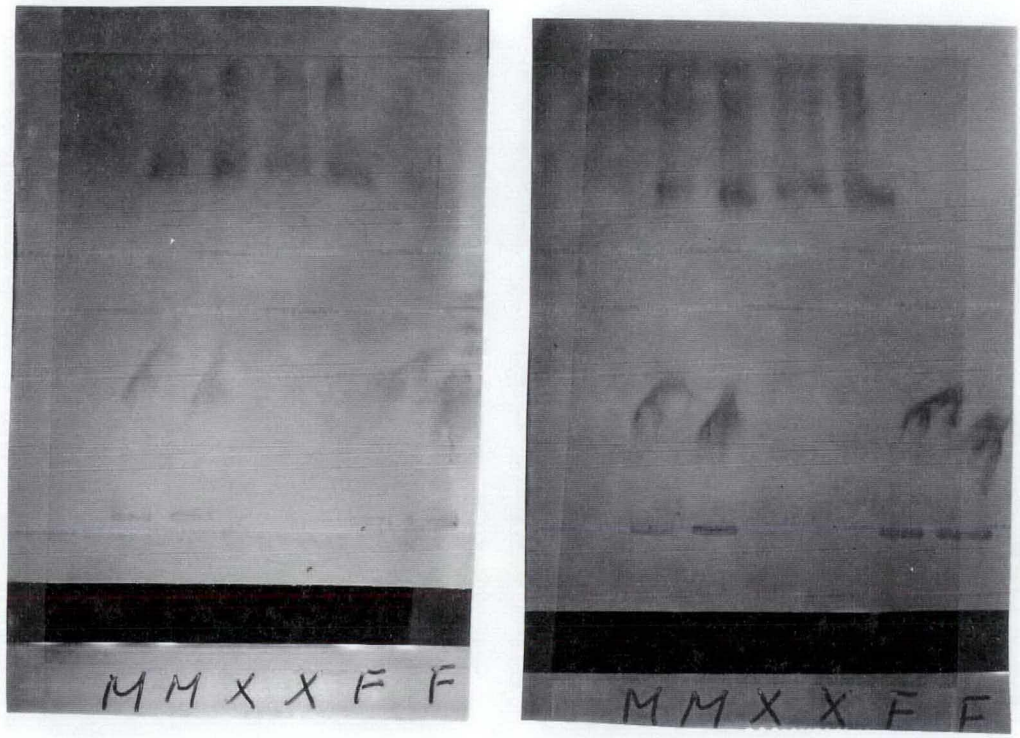
F: Furcellaran

X: Xanthan

Buffer: 0.1M LiCl (pH 2)

Voltage: 100 V

Time: 60 minutes



a b

Figure 15

CHAPTER V
DISCUSSION

This study has been concerned with the development of an electrophoretic procedure for analysis of food hydrocolloids. All of the experiments performed in this study were basically for the purpose of adapting the Gelman electrophoresis system for electrophoresis as a replacement for the Millipore electrophoresis system, which is no longer commercially available. The main difference between these two systems are (1) The electrophoresis chamber of the Millipore system was a sealed cell while the Gelman chamber is a larger unsealed chamber which can not prevent evaporation (2) The cellulose acetate strips of the MIllipore system was thicker than the ones designed for the Gelman system and possibly of a different degree of substitution. Therefore, proper electrophoretic resolution could not be achieved by applying the electrophoretic conditions used by Chang and Hansen (1968) for the Millipore system without modification.

An appropriate method for the of Gelman electrophoresis system has been developed in this study, which is using 0.1M LiCl (pH 2) buffer to conduct electrophoresis at 100 V for 60 minutes on cellulose acetate strips. The bands of various hydrocolloids were visualized by staining in 0.2% Toluidine blue for 15 minutes. The results have shown that the application of these electrophoretic conditions provide satisfactory resolution on the type of cellulose acetate strips provided with Gelman system. The analyzed

hydrocolloids in this study were alginate, CMC, pectin, furcellaran, xanthan, lambda- and kappa-carrageenan.

The first task in the developmental work was to select a proper buffer for electrophoretic resolution of hydrocolloids on the Gelman cellulose acetate strips. It was found that the relative mobilities of hydrocolloids were very similar in buffer systems of alkaline pH values and for this reason it was not possible to identify the species in a typical borate buffer at pH 10.

Results showed that the individual anionic polysaccharides possessed sufficiciently different mobilities at low pH value for satisfactory separation and identification. It was shown that 0.1M LiCl at pH 2 provided sharper resolution of bands than the same buffer at pH 7.25. The underlying reason is that the pKa values for various hydrocolloids are different. For the hydrocolloids containing carboxylic groups, such as alginate, pectin and CMC, the pKa values are around pH 3.8-4.5. In contrast, the hydrocolloids containing sulfate groups would be expected to exhibit pKa values around 1-2 corresponding to the dissociation constant for sulfric acid. When these hydrocolloids find themselves in an electrolyte system which is buffered between pH2 and pH 4 the degree of dissociation will be different for the various species and for this reason the bands become better resolved than when all species are completely dissociated such as is the case in an alkaline environment. Therefore, a low pH buffer between pH 2 and pH 4 would be the most suitable buffer to produce the best separation of common food hydrocolloids. Electrophoresis at pH 7 or above causes very little separation because there is too little difference in the charge/mass ratio.

Another consideration is the dependence of current and electric field strength on the nature and concentration of the running buffer for gel electrophoresis as well as for electrophoresis on cellulose acetate strips. Osterman (1984) pointed out the importance of the choice of running buffer in the following manner:

> " We shall now discuss the dependence of current and electric field strength on the nature and concentration of the running buffer. It should be noted that pH alone does not influence gel conductivity. For example, at pH 4, i. e. at a concentration of protons equal to 10^{-4}M, their contribution to conductivity is insignificant in comparison with that of the buffer ions which are at least 100 times more concentrated."
> "Conductivity is determined, to a great extent, by the nature of component ions. Thus, for example, sodium and potassium phosphate buffers posses high conductivity, compared with that of Tris.HCl buffer, due to the presence of K^+ and Na^+ ions."
> "Thus, the conductivity of the running buffer is determined by three factors: by concentration, which must be sufficiently high to maintain pH in the protein zones, by the degree of dissociation of the components at a given pH, and by the nature ofthe component ions."

The same considerations apply to the choice of buffer for electrophoresis of common food hydrocolloids on cellulose acetate strips. The concentration of buffer salts and the electrophoretic mobility of colloids are inversely related. At high buffer salt concentration the proportion of the current carried by the colloids is less than at low buffer salt concentrations. Therefore, the electrophoretic mobility of colloids is retarded by increasing the buffer salt concentrations. The final choice of an appropriate buffer for electrophoresis of a mixture of food hydrocolloids was 0.1M LiCl at pH 2.

Difficulties were encountered in separating kappa- and lambda-carrageenan, partly because their pKa values are very close. The results of this study revealed that they can be electrophoretically resolved better at low concentration (0.025%) than at higher concentrations. The reason is related

to the fact that the thin cellulose acetate strips have a limited loading capacity. If that capacity is exceeded the hydrocolloids are no longer retained in the support matrix and show evidence of "bleeding" which makes the two very close bands difficult to distinguish from one another. It is now becoming very clear that the difference between Millipore and the Gelman membrane are related to thickness of the cellulose acetate and to the capacity for binding the hydrocolloids.

The need for using relatively small quantities of samples for electrophoretic separation of hydrocolloids prompted a search for a sensitive staining procedure. The possibility of using the Horseradish peroxidase-benzidine staining method by Dickman et al. (1989) was explored but was eventually abandoned. Instead, Toluidine blue was selected as the better staining reagent. The stained cellulose acetate strips showed high background color by using the Horseradish peroxidase benzidine method which is possibly due to the cellulose acetate itself reacting by binding the enzyme. Toluidine blue at 0.2% concentration can provide satisfactory staining results especially if the bands on the dry strips are inspected under strong background light illumination. Staining by Toluidine blue requires only 15 minutes and is, therefore, more convinient than the Horseradish peroxidase-benzidine method which requires a long incubation time.

Hansen and Chang (1974) reported improved resolution of carrageenans at 40°C over room temperature (25°C). They attributed the improved results to a disaggregation of the hydrocolloids at this high temperature. It was not possible to demonstrate a similar effect in the present study. The likely reasons for this are (1) The chamber of the Gelman

system is unsealed, therefore some dehydration of the cellulose acetate membrane occurs during electrophoresis especially at high temperature or high voltage. (2) Evaporation will make the concentration of the buffer inconsistant.

Various conditions of voltage/ time were applied for electrophoresis of common food hydrocolloids. The results revealed that lower voltage and longer time is more appropriate for resolving a complex mixture of hydrocolloids. The use of lower voltage reduces the ohmic heating effects and the longer time provides for more more apparent separation between the bands, which can be more reasily identified.

The overall results suggested that a buffer of 0.1M LiCl provided satisfactory resolution for common food hydrocolloids at 100 V for 60 minutes, and, that the bands on the cellulose acetate strips could be satisfactorily visualized by staining in 0.2% Toluidine blue and by using intense back lighting of the slide.

Studies were conducted using borate buffer. The use of borate buffers has special significance in electrophoresis of food hydrocolloids and other polysaccharides because interactions between borate and hydroxyl groups of sugars confer electrical charge to neutral polysaccharides. This reaction has been studied in relationship to gelation of locust bean gum and guar gum. According to Schachat and Raymond (1956):

> Compounds which contain cis-hydroxy groups form complexes with boric acid and borates. When the compounds are of high molecular weight, the complex may form as a three-dimensional network (Deuel and Neukom, 1949). Many common hydrophilic gums such as locust bean gum (Deuel and Neukon, 1954), guar (Whistler, 1948), poly (vinyl alcohol), algins, pectins, gum arabic, starch, hemicellulose (consisting of sugars which contain cis-1,2-diols-rhamnose, mannose, galactose), and carboxylate ethers (Moe, 1950) (the

reaction product of galactomannans or glucomannans with glycerol monochlorohydrin), form gels with boric acid and borate (Figure 1). Borax gels form most readily in weakly alkaline solutions and are usually cleaved by acids or by strong alkali. The bonds forming the gels are in a state of dynamic equilibrium, which explains the rapid reformation of the gel when it has been mechanically broken. No syneresis occurs during this procedure.

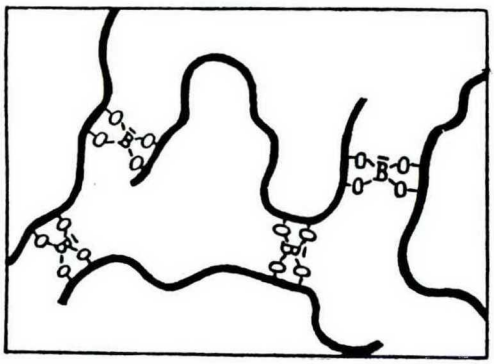


Figure 1. Cross linking of chain molecules of locust bean gum by borax (6)

The system has been used as an analytical tool (Boeseken, 1949), because the complex formed showes greater conductivity than the sum of the conductivities of the boric acid and the diol compound. Favorably situated diols have a pronounced effect in increasing the conductivity. cis-1,2-Diols fixed in position by molecular structure-e.g., o-dihydroxyphenols and cis-dicyclic polyols-are "favorable" structures. The reaction is depicted in figure 2.

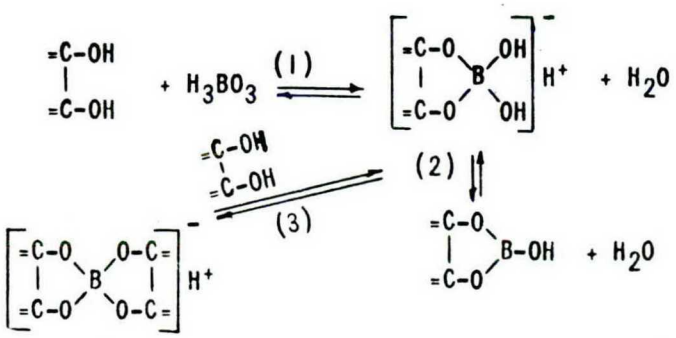


Figure 2. Mechanism of complex formation of diols by borates

1. Goes much to the right
2. Depends upon position of hydroxyls. The final product, a spirane, is a bis-diol boric acid, a fairly strong acid (4)

While the use of borate buffers is important for the detection of neutral polysaccharides, there is little to suggest that borate will interact with carrageenans, CMC or xanthan because they do not contain the required hydroxyl groups in the required protein.

It may be very worthwhile to combine the application of the boric acid reaction and the use of 0.1M LiCl buffer for the purpose of analysis and identification of the hydrocolloid species in a mixture. Reaction with boric acid will confer charges to neutral polysaccharides, including locust bean gum and guar gum as well as sugars with cis-dihydroxyl groups. These charges are sufficient for electrophoretic migration. Further investigation will determine if it is possible to preheat a mixture of hydrocolloids with borax at alkaline pH and subsequently separate the species by electrophoresis in an acidic buffer. Such a sample treatment may prove to be superior to the approaches developed so far.

CHAPTER VI
SUMMARY AND CONCLUSION

This study was undertaken to develope an appropriate protocol for electrophoretic analysis of food hydrocolloids on cellulose acetate membranes using the Gelman electrophoresis system. Because the Gelman cellulose acetate membranes are very thin and the loading capacity is low, it was necessary to stimulate samples with low hydrocolloid concentrations so that the individual components would be in the range of 0.05-0.1% concentration. Experiments were designed to optimize conditions for electrophoresis with respect to the following variables, type of buffers, conductivity of buffer, voltage and time relationships as well as the choice of a staining method. A buffer solution of 0.1M LiCl (pH 2) was found satisfactory for electrophoresis at 100 V for 60 minutes on Super Sepraphore cellulose acetate strips. The bands of various hydrocolloids were visualized by staining in 0.2% Toluidine blue for 15 minutes. Results suggested that it may be very worthwhile to combine the application of the boric acid reaction at alkaline pH value and the use of 0.1M LiCl buffer for the purpose of analysis and identification of the hydrocolloid species in a mixture.

REFERENCES

Allen, J. C., Gardner, R., Wedlock, D. J. and Phillips, G. O. 1982. The use of 2-thiobarbituric acid in the assay of κ-carrageenan in polysaccharide mixtures. In Phillips, G. O., Wedlock, D. J. and Williams, P. A., ed. Gums and stabilizers foe the food industry 6. p.387. Pergamon Press. New York.

Boeseken, J. 1949. The use of boric acid for the determination of the configuration of carbohydrates. Advance in Carbohydrate Chem. 49: 189

Bradbury, A. G. W. and Halliday, D. J. 1983. Structural characterization of polysaccharides using gas chromatography. In Phillips, G. O., Wedlock, D. J. and Williams, P. A., ed. Gums and stabilizers foe the food industry 2. p.45. Pergamon press. New York.

Chang, J. C. 1968. Analysisof food stabilizers in milk products. M.S. Thesis. The Ohio State University

Chang, J. C., Renoll, M. W. and Hansen, P. M. T. 1974. Zonal electrophoresis of food stabilizers in malonate buffer J. Food Sci., 39:97.

Committee on codex specifications. 1981. Food chemicals codex 3rd. pp.7, 13, 74, 141, 174, 280, 347. National Academy Press. Washington, D. C.

Deuel, H., Neukom, H. 1954. "Natural Plant Hydrocolloids" ACS Monograph. p. 54. Reihold, New York.

Dickmann, R. S., Chism, G. W., Renoll, M. W. and Hansen, P. M. T. 1989. Detection of food gums on polyamide strips using horseradish peroxidase-benzidine staining and laser-bean densitometry. Food Hydrocolloids 3:33

Feather, M. S. and Harris, J. F. 1973. Adv. Carb. Chem. Biochem. p. 28, 161

Hansen, P. M. T. and Chang, J. C. 1968. Quantitative recovery of carboxymethylcellulose from milk. Agriculture and Food Chemistry 16:77

Hsu, D., Hoffman, P. and Mashburn, T. A., Jr. 1972. Anal. Biochem., 46:156

Hansen, P. M. T. and Renoll, M. W. 1974. Heat-induced changes in the zone electrophoretic patterns of carrageenan stabilizers. J. Food Sci., 39:103.

Hansen, P. M. T. 1966. Distribution of carrageenan stabilizers in milk (abstract) J. Dairy Sci., 49:698.

Hansen, P. M. T. 1982. Hydrocolloid-protein interactions: relationship to stabilization of fluid milk products. A review. Prog. Fd. Nutr. Sci., 6:127

Hidalgo, J. E. and Hansen, P. M. T. 1968. Interactions between food stabilizers and Beta-Lactoglobulin. J. Dairy Sci., 51:945.

Klose, R. E., and Glicksman, M. 1968. Gums. In Furia, T. E., ed. Handbook of Food Additives. p. 315. The Chemical Rubber Co., Cleveland.

Knutaen, S. H. and Grasdalen, H. 1992. Analysis of carrageenan by enzymic degradation, gel filtration and ^{1}H NMR spectroscopy. Carbohydrate polymers 19:199

Lange, N. A. ed. 1956. Handbook of Chemistry. p. 952. Handbook Publishers, Inc. Sandusky, OH.

Manning Jr, D., Witt, H. and Ames, J. 1985. Increasing cheese yields with carrageenan. In Phillips, G. O., Wedlock, D. J. and Williams, P. A., ed. Gums and stabilizers foe the food industry 3. p.379. Pergamon Press. New York.

Moe, O. A. (to General Mills). 1950. Ibid.2, 496, 670

Nickerson, T. A. 1962. Lactose crystallization in ice cream IV. factors responsible for reduced incidence of sandiness. J. Dairy Sci. 45:354.

Padmoyo, M. and Miserez, A. 1967. Identification of gelling and thicking agents permitted in switzerland by electrophoresis and staining on cellulose acetate strips (in German). Mitt. Geb. Lebensmittelunters. Hyg., 58:31.

Rothschild, Z. and Oliveira, M. 1981. Quantative measurements of sulfated polysaccharides by complex formation with Toluidine blue. *Rev. Fac. Farm. Odont. Ribeirao Preto, 18 (2) : 81-86.*

Schachat, R. and Raymond, L. Z. 1956. Some aspects of hydrocolloid gelation. Physical functions of hydrocolloids. p.11. American Chemical Society, Washington, D.C.

Scopes, R. K. 1988. Protein purification 2nd ed. p.199. Springer-Verlag, New York

See, Y. P. and Jackowski, G. 1990. Estimating molecular weights of polypeptides by SDS gel electrophoresis. In Creighton, T. E. ed. Protein structure. p.1

Senda, H. 1992. Cellulose acetate membrane electrophoretic patterns of bovine serum proteins (in Japanese). Nippon Juishikai Zasshi 45(3), p. 159.

Shelembe, J. S. 1992. Analysis and identification of carrageenan types in carrageenan stabilizer mixtures. The Ohio State University

Thier, H. P. 1983. Identification and quantitation of natural polysaccharides in foodstuffs. In Phillips, G. O., Wedlock, D. J. and Williams, P. A., ed. Gums and stabilizers foe the food industry 2. p.13. Pergamon press. New York.

Voragen, A. G., Schols, H. A. and Pilnik, W. 1982. H.P.L.C. analysis of anionic gums. In Phillips, G. O., Wedlock, D. J. and Williams, P. A., ed. Gums and stabilizers foe the food industry 6. p.379. Pergamon Press. New York.

Voragen, A. G. J., Schols, H. A., Clement, A. J. J. and Pilinik, W. 1983. Enzymic analysis of pectins. In Phillips, G. O., Wedlock, D. J. and Williams, P. A., ed. Gums and stabilizers foe the food industry 2. p.517. Pergamon press. New York.

Wedlock, D. J., Phillips, G. O. and Bachmann, M. 1983. A new colourimetric assay for pectins. In Phillips, G. O., Wedlock, D. J. and Williams, P. A., ed. Gums and stabilizers foe the food industry 2. p. 529. Pergamon Press. New York.

Whistler, R. L. 1948. Chem. Inds. 62: 60

APPENDIX

(Figure 16 and 17, and Table 5)

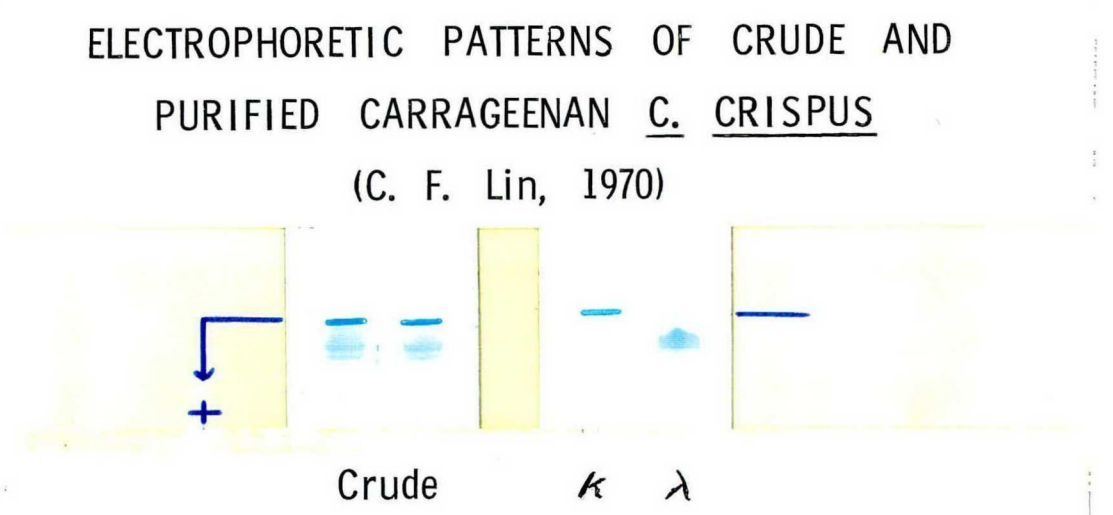


Figure 16. Millipore, Phoroslide cellulose acetate electrophoresis of carrageenans
(from Laboratory records, C. F. Lin/P. M. T. Hansen, 1970)

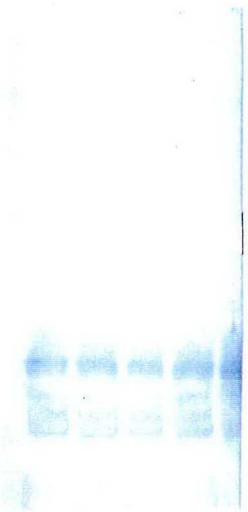


Figure 17. Electrophoretic pattern of skim milk proteins

Buffer: General Purpose Electrophoresis Buffer, pH 8.8

(Gelman Sciences Inc.) with 3.3M urea

Voltage: 200 V

Time: 15 minutes

Stain: Amido black

Table 5. The resolution of different buffers applied to electrophoresis of HMR carrageenan

Buffer	Resolution
0.075M Malonic acid 15% EtOH (pH 1.98)	no separate bands
0.075M Malonic acid 30% EtOH	no separate bands
0.075M Malonic acid 15% DMSO	no separate bands
0.1M Phenol 15% EtOH	no separate bands
10% Lactic acid	no separate bands
10% Trifluoroacetic acid (pH 3.0)	no separate bands
10% Trifluoroacetic acid 15% EtOH	no separate bands
0.1M Boric acid 15% EtOH	no separate bands
0.1M Boric acid 0.075M Trifluoroacetic acid 0.01M EDTA (pH 1.34) 15% EtOH	no separate bands
0.05M Boric acid 0.0375M Trifluoroacetic acid 0.005M EDTA 15% EtOH	no separate bands

Table 5. (Continued)

Buffer	Resolution
0.1M Boric acid 0.075M Trichloroacetic acid 0.01M EDTA (pH 1.29) 15% EtOH	two separate bands
0.1M Boric acid 0.075M Trichloroacetic acid 15% EtOH	two separate bands
0.075M Trichloroacetic acid 0.1M Boric acid 25% Glycerine	no separate bands
0.075M Trichloroacetic acid 0.1M Boric acid 15% EtOH 10% Glycerine (pH 1.24)	no separate bands