DETERMINATION OF SOME FOOD ADDITIVES

USING

POLYION-SENSITIVE MEMBRANE SENSORS

ALİ KAYANSALAN

AUGUST 2005

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by

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I certify that this thesis satisfies all the requirements as a thesis for the degree of Master of Science.

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This is to certify that we have read this thesis and that in our opinion it is fully adequate, in scope and quality, as a thesis for the degree of Master of Sciences.

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ABSTRACT

DETERMINATION of SOME FOOD ADDITIVES

using polyion-sensitive membrane sensors

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This dissertation work is dealing with the determination of xanthan gum, sodium alginate, and carrageenan. These compounds are highly sulfated polysaccharides and are used in food industry as food additive stabilizer, homogenizer, thickening agent or gel-forming agent. The primary focus of the work was to explore the use of polyion sensitive membrane based sensors for the some food additives analysis. It was found that sensors prepared with polymer membranes containing dinonylnaphthalenesulfonate (DNNS) as the ion-exchanger are also capable of detecting polyanionic xanthan gum and sodium alginate used as food additives.

These food thickening agents were determined in the various food products which are sold at the Turkish markets. Ten different food samples were tested. The total polyanion levels in these samples were measured by

potentiometric titration technique using standard protamine as a titrant and polycation-sensitive membrane sensors. The method accuracy is confirmed by recovery experiments of spiked samples. The data obtained are found within the expected ranges of the nominal values for these types of food products.

Keywords: Polymer membran sensors, thickeners, protamine titrant, food analysis.

ÖZET

POLİİYON DUYARLI MEMBRAN SENSÖRLER KULLANILARAK BAZI GIDA KATKI MADDELERİNİN TAYİNLERİ

Kayansalan,Ali

Yüksek Lisans, Kimya Bölümü

Danışman: Doç. Dr. Nedime Dürüst

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Bu tez çalışması, ksantan, sodyum alginat ve karragenan'ın tayini ile ilgilidir. Bu maddeler, yüksekce sülfatlanmış polisakkaritlerdir ve besin endüstrisinde stabilizatör, homojenleştirici, kıvam artırıcı veya jel oluşturma reaktifi olarak kullanılır. Bu çalışmanın asıl hedefi, poliiyon duyarlı membran sensörlerin, bazı besin katkı maddelerinin analizi için kullanımını incelemektir. İyon değiştirici dinonilnaftalinsülfonat (DNNS) içeren polimer membranlar ile hazırlanan sensörler ile besin katkı maddeleri olarak kullanılan polianyonik ksantan gum ve sodyum alginatın da tayin edilebildiği görülmüştür.

Bu tür kıvam artırıcı maddeler, Türk marketlerinde satılan değişik besin maddelerinde tayin edilmiştir. On farklı besin örneği test edilmiştir. Bu örneklerdeki toplam polianyon miktarları, titrant olarak standart protaminin ve polikatyon duyarlı membran sensörlerin kullanıldığı potansiyometrik titrasyon yöntemiyle ölçülmüştür. Yöntemin doğruluğu, geri kazanım deneyleriyle

ortaya konmuştur. Elde edilen veriler, bu tür besin ürünleri için beklenen sayısal değer aralıklarında bulunmuştur.

Anahtar kelimeler: Polimer membran sensörleri, kıvam artırıcılar, protamin titrant, besin analizi.

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TO MY ENDLESS LOVE RAIN,

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CHAPTER 1. Introduction

Chemical sensors are simple devices that can selectively and reversible monitor a chemical species in a complex media without sample pretreatment. They are extremely important tools for monitoring chemical processes that occur in many areas including the environment, industry, food production, agriculture, biology / clinical chemistry, etc. The development of chemical sensors for the direct monitoring of key ions, gases and biochemical molecules in complex biological samples is a rapidly growing avenue of research.

Polymer membrane based ion-selective electrodes (ISEs) were first introduced in the 1960s. They have become very successful tools for measuring biomedically important electrolyte species. Modern clinical analyzers employ such electrodes routinely for detecting calcium, potassium, sodium, and chloride ions[1-3]. The main advantage of such devices is the ability to selectivity and directly detect free ion activities in complex samples. Over the last decade it has been found that thin polymer films (PVC, polyurethanes etc.) doped with lipophilic anion and cation exchangers (e.g., tridodecylmethylammonium ion, dinonylnapthalene sulfonate (DNNS), etc.) exhibit unexpectedly large and reproducible potentiometric responses to low levels of certain biomedically important polyionic species.

Over the past ten years, a wide range of selective chemistries used previously to devise potentiometric polymer membrane type ion-selective electrodes have been adapted successfully to develop new optical sensors (optodes)[4-7]. This ever increasing interest in optodes can be justified, on

the other hand, by the progress in the development of optical hardware, and on the other, by the hope of eliminating certain disadvantages of electrochemical sensors [8].

Multiply charged polyionic species play important roles in numerous fields. For example, many biologically significant macromolecules such as RNA, DNA, highly charged proteins, polysaccharides and other species (e.g., polyphosphate) are all polyions. Several drugs used in modern medicine are also polyions. One of the most prominent and important polyions is heparin. The main function of heparin is to regulate blood coagulation, which occurs through a very complicated biological cascade. Heparin is commercially available. The average molecular weight of the commonly used beef lung and porcine heparin is around 15,000. Protamine is a low molecular weight (ca 4,500) protein rich in basic arginine residues which are positively charged under physiological conditions. Protamine sulfate is used as titrant.

Other polyionic species are of industrial importance. For example, carrageenan, a negatively charged polysaccharide, is used extensively by food, pharmaceutical and cosmetic industries as a thickener, stabilizer, and gelling agent. Figure 1.1 shows structures of some important polyions.

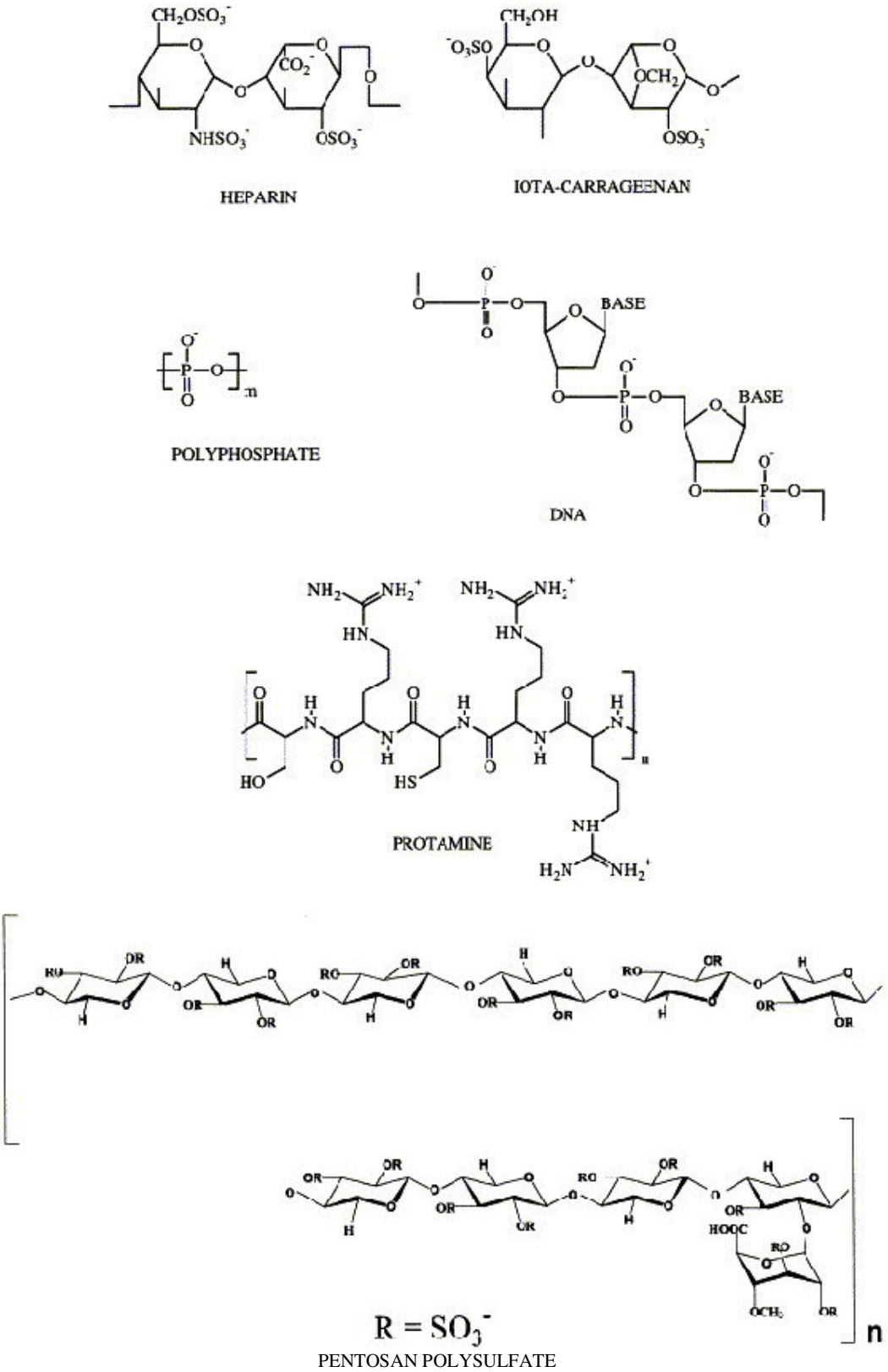

Figure 1.1 The structures of some important polyions.

CHAPTER 2. Membrane Sensors

Miniaturized sensors are commonly used analytical devices in many fields of applications. Polymers have gained tremendous recognition in the field of artificial sensor in the goal of mimicking natural sense organs. Better selectivity and rapid measurements have been achieved by replacing classical sensor materials with polymers involving nano technology and exploiting either the intrinsic or extrinsic functions of polymers. Semiconductors, semiconducting metal oxides, solid electrolytes, ionic membranes, and organic semiconductors have been the classical materials for sensor devices. Both intrinsically conducting polymers and non-conducting polymers are used in sensor devices.Polymers used in sensor devices either participate in sensing mechanisms or immobilize the component responsible for sensing the analyte.

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2.1.Electrochemical Sensors

2.1.1.Ion-Selective Membrane Electrodes

Ion-selective electrodes are electrochemical half-cells, consisting of an ion-selective membrane, an internal filling solution, and an internal reference electrode or of an ion-selective membrane and a solid contact. Ion-selective electrodes (ISE), such as the glass pH electrode, function by using a membrane that reacts selectively with a single ion. Figure 2.1 shows a generic diagram for a potentiometric electrochemical cell equipped with an ion-selective electrode.

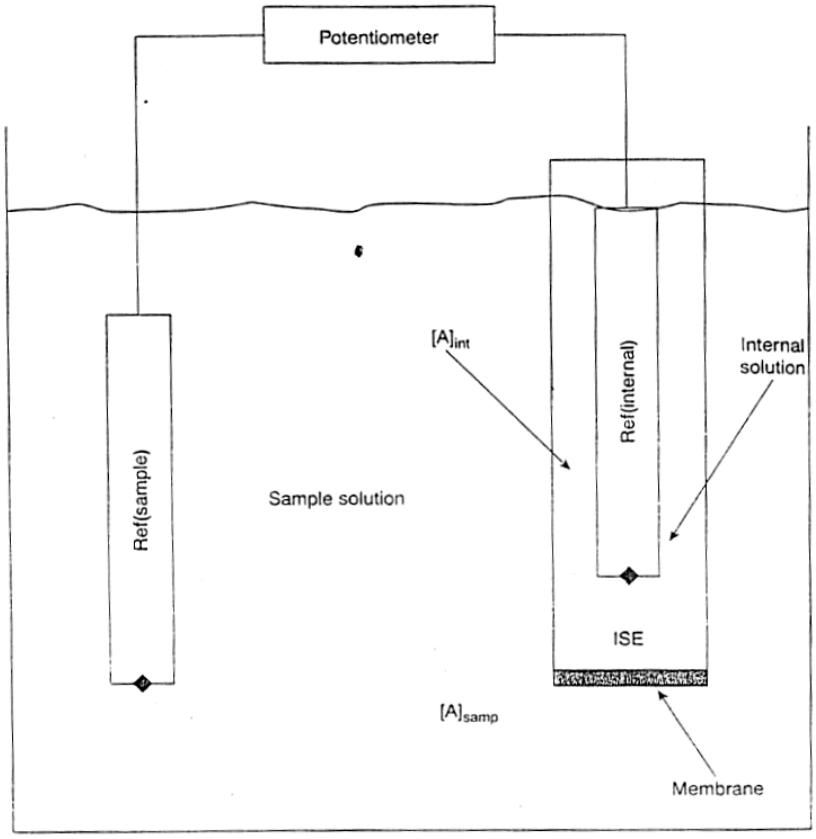

Figure 2.1. Electrochemical cell for potentiometry with an ion-selective membrane electrodes.

The shorthand notation for this cell is

$$Ref_{(sample)} \,||\, [A]_{sample} \mid [A]_{int} \,||\, Ref_{int}$$

Two reference electrodes are used; one positioned within the internal solution, and one in the sample solution. The cell potential, therefore, is

$$E_{cell} = E_{Ref(int)} - E_{Ref(samp)} + E_{mem} + E_{lj}$$

where E_{mem} is the potential across the membrane. Since the liquid junction potential and reference electrode potentials are constant, any change in the cell's potential is attributed to the membrane potential. The membrane potential is given by a Nernst-like equation

$$E_{mem} = E_{asym} - RT / zF \ln [A]_{int} / [A]_{sample}$$

where $[A]_{samp}$ and $[A]_{int}$ are the concentrations of analyte in the sample and the internal solution, recpectively, and z is the analyte's charge. Ideally, E_{mem} should be zero when the concentrations of analyte on both sides of the membrane are equal. The term E_{asym}, which is called an asymmetry potential, accounts for the fact that the membrane potential is usually not zero under these conditions. Substituting this equation into previous equation, and rearranging gives

$$E_{cell} = K + 0.05916 / z \log [A]_{samp}$$

where K is a constant accounting for the potentials of the reference electrodes, any liquid junction potentials, the asymmetry potential, and the concentration of analyte in the internal solution. The last equation is a general equation, and applies to all types of ion-selective electrodes.

Ion-selective membrane electrodes may be classified according to the nature of the basic membrane materials, into the following categories:

Primary ion-selective membrane electrodes;

- glass electrodes
- solid-state (or crystalline) electrodes
- liquid-membrane electrodes
  - ion-exchanger membrane electrodes
  - neutral carrier membrane electrodes

Compound or multiple membrane ion-selective electrodes;

- molecular sensing devices such as gas-sensitive and
- enzyme electrodes

Ion-selective field effect transistors .

2.1.1.1. Liquid Membrane Electrodes

Liquid membrane type ISEs, based on water-immiscible liquid substances impregnated in a polymeric membrane, are widely used for direct potentiometric measurements. The polymeric membrane [commonly made of plasticized poly(vinyl chloride) (PVC)] separates the test solution from the inner compartment, containing a standard solution of the target ion (into which a silver/ silver chloride wire is dipped). The filling solution usually contains a chloride salt of the primary ion, as desired for establishing the potential of the internal silver/ silver chloride wire electrode. The membranes are commonly prepared by dissolving the recognition element, a plasticizer (e.g., o-nitrophenyl ether) that provides the properties of liquid phase, and the PVC in a solvent such as tetrahydrofuran. The extraction properties of the membrane can be further improved by adding ion-pairing agents to the plasticizer. The PVC matrix provides mechanical strength and permits diffusion of analytes to the recognition sites. The hydrophobic nature of the membrane prevents leaching of the sensing element and the plasticizer into the aqueous sample solution.

Ion-exchanger liquid membrane electrodes: One of the most successful liquid-membrane electrode is selective toward calcium. It uses a liquid cation exchanger, consisting of an aliphatic diester of phosphoric acid [$(RO)_2PO_2^-$ with R groups in the C_8-C_{16} range] that possesses high affinity for calcium ions.

$$Ca^{2+} + 2(RO)_2PO_2^- \leftrightarrow [\,(RO)_2PO_2\,]_2Ca$$

Calcium activities as low as 5×10^{-7} M can be measured, with selectivity coefficients $K_{Ca,Mg}$ and $K_{Ca,K}$ of 0.02 and 0.001, respectively.

Liquid anion exchangers, such as lipophilic quaternary ammonium salts or phosphonium salts, have been employed for the preparation of anion-selective sensors. A very successful example is the use of the quaternary ammonium salt tridodecylmethylammonium chloride (TDMAC) for detecting the clinically important drug heparin. The polyionic heparin is favorably extracted into the membrane through ion-pairing interaction with the positively charged nitrogen atoms (Figure 2.2).

Cation-exchange electrodes have also been described. For example, PVC membranes containing dinonylnaphthalenesulfonic acid (DNNS) have been used for the detection of drugs of abuse (e.g., opiate alkaloids).

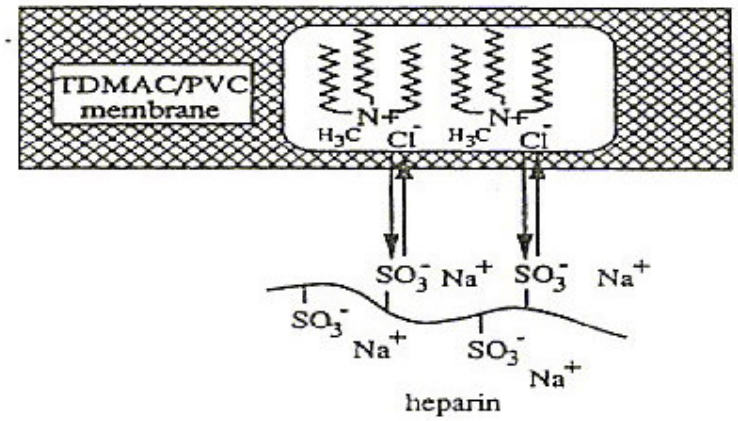

Figure 2.2. The recognition process occuring at the TDMAC / PVC membrane / sample interface used for measurements of polyanion.

Neutral carrier liquid membrane electrodes: A great number of electrically neutral ligands with different structures, called ionophores, were designed for ion-selective potentiometry. The neutral carriers are antibiotic, synthetic acyclic compounds, cyclic polyethers (crown compounds) (Figure 2.3) or more recently calix(4)-arene derivatives. The ionophore is incorporated most commonly into a plasticized poly(vinyl chloride)

membrane, typically consisting of wt% ionophore, 66 wt% nonpolar solvent (plasticizer), and 33 wt% PVC. Lipophilic salt additive are added to the membrane with the aim of decreasing membrane resistance and improving ion selectivity [9-11].

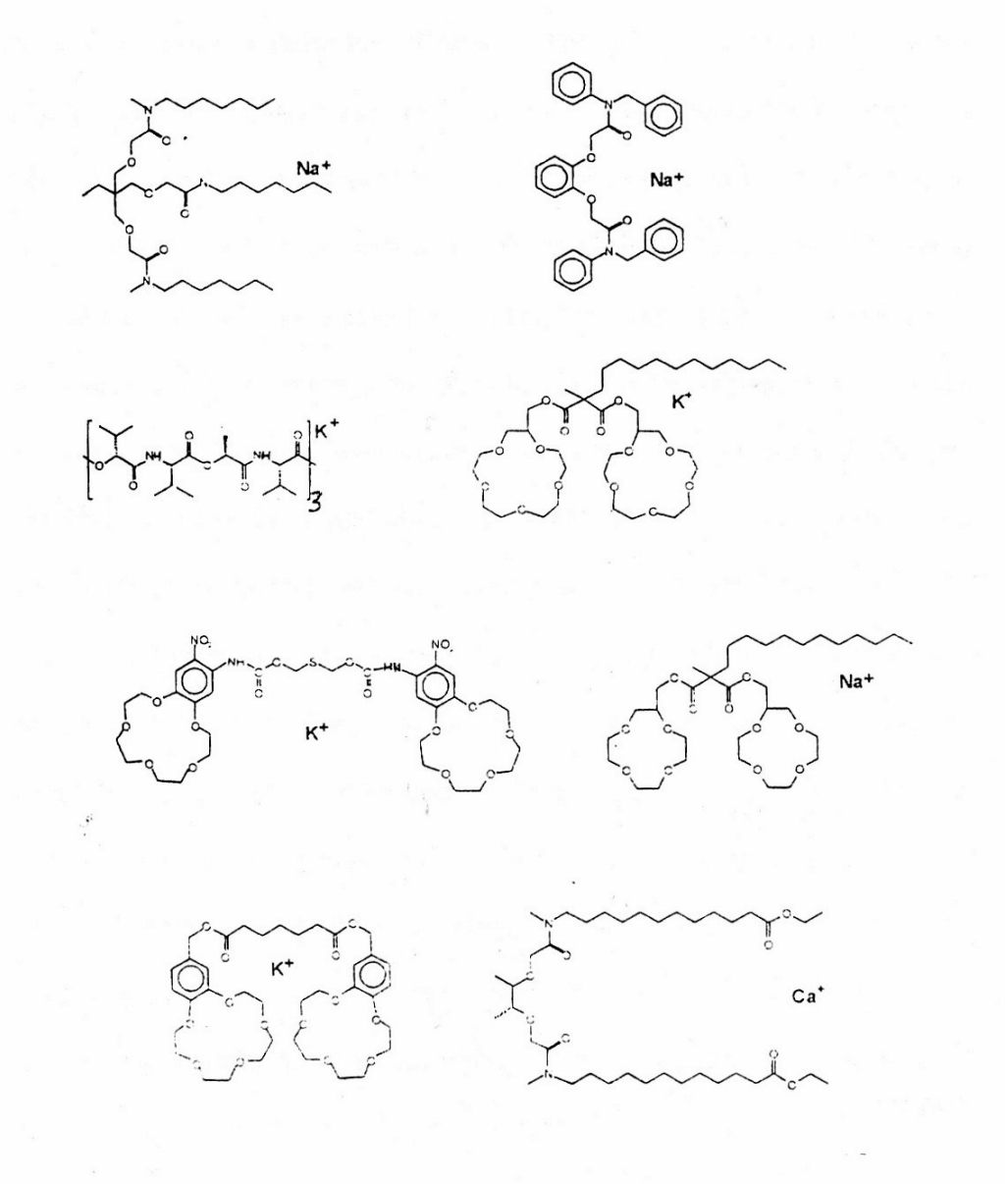

Figure 2.3. Chemical structure of different ionophores for liquid membrane electrodes.

2.1.2. Polyion-Selective Membrane Electrodes

More recently, polymer membrane-based electrodes have also been devised to detect multiply charged clinically important polyions, most notably heparin and protamine, two drugs commonly used during cardiopulmonary bypass surgery [12-15]. These electrodes have been termed polyion-sensitive membrane electrodes (PSEs). Heparin is a complex, highly negatively charged polysaccharide with an average molecular weight of 15,000. Approximately 33 metric tons of heparin, representing 500 million doses, are used world-wide each year. Pharmaceutical industry production figures indicate that clinical use of the drug continues to grow rapidly [16].

The pioneer work to develop electrochemical polyion-sensitive electrodes into a novel analytical tool was initiated by Meyerhoff's group [12], who adopted the ion-selective electrodes diagram to design such a device. After screening with 14 quaternary ammonium salts, 24 plasticizers and 13 polymeric matrices, it was found that properly formulated polymeric membranes containing lipophilic anion exchangers could exhibit an unexpectedly large and reproducible potentiometric response (EMF) to polyanionic heparin molecules [17]. Durust et al. employed a titration scheme to devise an assay to detect pentosan polysulfate (PPS) quantitatively in plasma samples [18]. Polyion-sensitive electrodes are also potentially useful detectors in the design of novel electrochemical enzyme assay . Using PSEs, it is possible to determine the activity of given enzymes that cleave polyionic substrate molecules into smaller fragments of less charge and lower molecular weight. Since the EMF response of PSEs is

dependent on charge and molecular weight, the activity of the enzymes can be directly monitored by observing the reversal in potentiometric response of PSEs to specific polyionic substrates. The first assay based on this novel detection method was designed for trypsin, which cleaves arginine rich peptides, such as protamine, into smaller fragments [19]. A novel electrochemical method to detect protease activities was demonstrated. The assay is based on the use of a macromolecular polycation / polyanion substrate; specifically, a complex of the arginine- rich peptide protamine and pentosan polysulfate (PPS), a highly sulfated polysaccharide. As the protease of interest cleaves the protamine within the complex into smaller fragments, free PPS is generated and detected potentiometrically via a polyanion sensitive membrane electrode. Thus, the rate of free PPS generation is proportional to the activity of the protease in the assay solution. The effect of the substrate concentration is examined, as is the influence of the protamine / PPS stoichiometry on the assay performance [20]. The potentiometric response characteristics of polycation-sensitive membrane electrodes toward two classes of polycationic dendrimers were also examined. Using appropriately formulated polymer membrane electrodes composed of a dinonylnaphthalenesulfonate (DNNS) salt in a plasticized polyurethane matrix, it was shown that poly(amidoamine) (PAMAM) and poly(propylenimine) (PPI) dendrimers are readily detected at submicrogram per milliliter levels via a nonequilibrium response mechanism. The relationship between the total EMF response (at equilibrium) and the specific dendrimer structure was also examined. [21].

The electrochemical cells used for detecting polyions are essentially the same as for simple ions (Figure 2.4). Both ISEs and PSEs consist of a polymer membrane that serves as the sensing element, an internal reference electrode, and an internal electrolyte solution. However, for PSEs, the polymer membrane typically consists of a low weight percentage of ionophore or ion-exchanger (0.5-1.0 wt%), which interacts with the target polyion, and higher weight percentages of polymer (66 wt% for heparin sensor). When operated under equilibrium conditions, the internal electrolyte will contain a fixed concentration of the target polyion. When these electrodes are used in conjuction with an external reference electrode and immersed in a solution, a complete galvanic cell is formed. The overall cell potential (EMF) for polyions also follows the Nernst equation under equilibrium conditions.

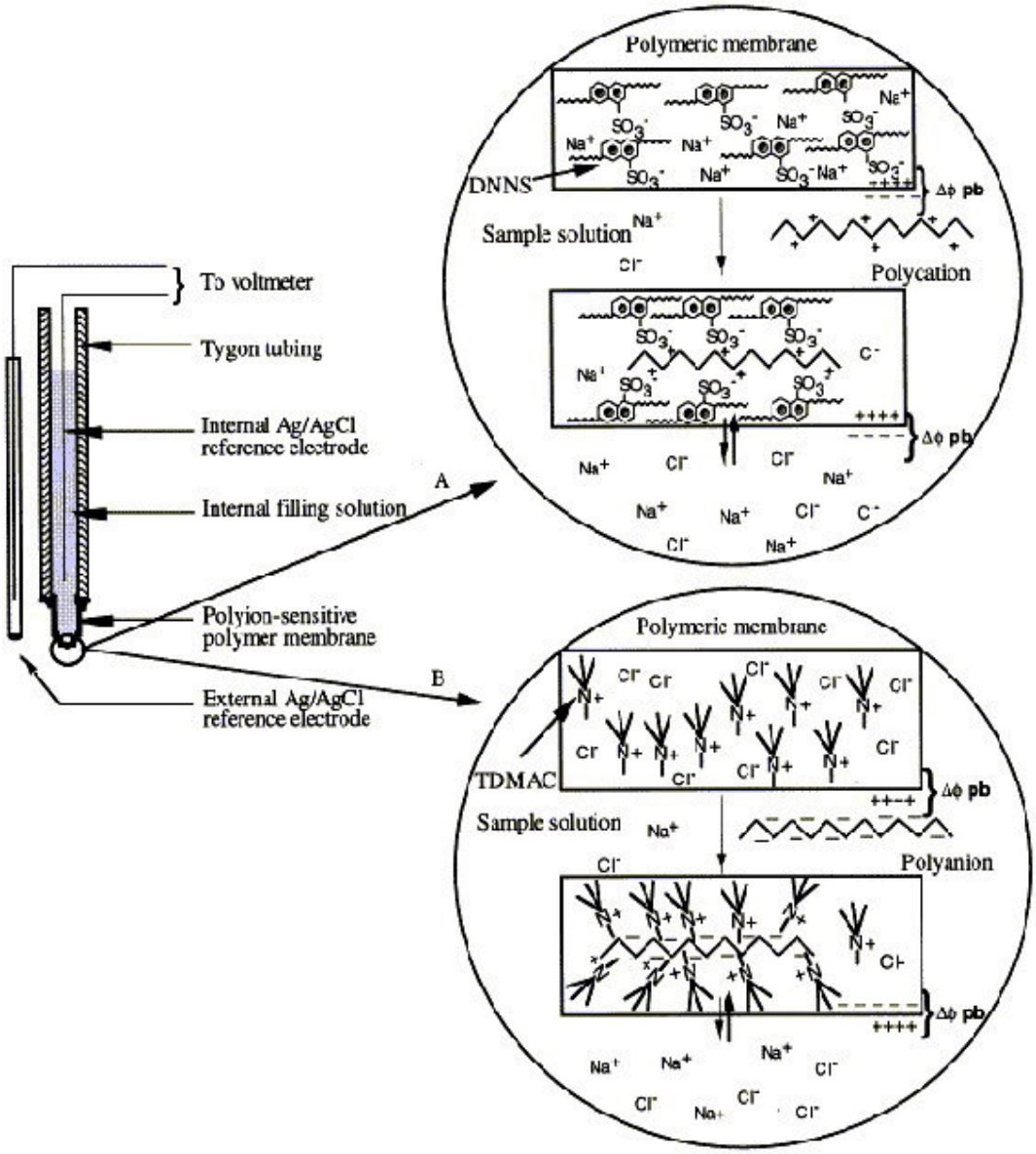

Figure 2.4. Schematic of simple cylindrical PSE configuration, as well as an expanded view of the extraction and ion-pairing reactions of (A) polycations and (B) polyanions into and within the organic membrane phases of such devices.

2.2. Optical Sensors

The chemistry of the ion-selective optode has recently been recognized as a highly selective ion-sensing methodology which involves a highly selective complexation between an ionophore and a primary ion. This simple methodology allowed the construction of several kinds of ion-sensing devices which enabled highly selective ion determination for analytical purposes. Sensing devices are divided into the following four types with different analytical uses: (1) flow-through optode (flow analytical devices), (2) waveguide optode (waveguide devices), (3) sensing plate optode or film optode (sensing plate devices), (4) fiber optode (fiber-optic devices).

The ion-selective optode is one of the optical chemical sensors (so-called optodes or optrodes) which have attracted interest since the 1970s. Generally, the essence of the usefulness of the ion-selective optode is the "ion selectivity" associated with the highly selective complexation of an ionophore (either neutral or charged) with an analyte ion in optical chemical sensing. For the preparation of an ion-selective optode membrane, an ionophore can be incorporated into a hydrophobic membrane such as plasticized poly(vinyl chloride) membrane, together with a lipophilized pH indicator dye, and utilized in contact with a sample solution containing a primary ion. In this case (when a cation is the primary ion, for example), extraction of the primary cation causes a lipophilic pH indicator to be deprotonated to maintain the electroneutrality in the membrane phase. Therefore, most ion-selective optodes measure this proton-releasing process into the sample solution phase upon extraction of the primary cation into the lipophilic membrane phase by a spectrophotometric method [22].

This ever-increasing interest in optodes can be justified on the one hand by the progress in the development of the optical hardware, and on the other by the hope of eliminating certain disadvantages of electrochemical sensors. In general, these optical sensors are prepared by incorporating appropriate lipophilic ionophores and pH indicators into thin (2-5 μm) plasticized poly(vinyl chloride) (PVC) films cast on glass plates or the distal end of optical fibers. In the case of cation sensors, selective extraction of the target cation by neutral ionophore (e.g., valinomycin for K^+) is coupled to a simultaneous deprotonation of a lipophilic pH indicator in the bulk of the organic membrane film, resulting in a change in the film absorbance at a given wavelength [23-24]. The same basic strategy has also been applied for detection of specific anions [25-28]. Unlike the response of polymer membrane electrodes, which is governed by electrochemical equilibria at phase boundaries, the response of ion-selective optical sensors is dependent on a mass/charge balance reaction within the bulk of the polymeric film, and thus, a stable optical signal can be achieved only when the entire film is in chemical (analyte) equilibrium with sample solution [6,29]. Various polymeric membrane based optical sensors have been successfully developed for small mono- and divalent ions, using chromogenic ionophores (chromoionophores) [30-32] or a pH indicator/neutral carrier pair ion-exchange systems [33-36] .

Ion-selective optodes have potential advantages over ISEs, such as ease of fabrication into various thickness and shapes, good sensitivity and reversibility, and no need for a reference electrode. Ion-selective optodes have been utilized as flow-through analytical devices for routine analysis that

requires continuous measurements [37], and they have also been applied to construct miniature/micro fiber-optic devices capable of measuring intracellular ion levels, such as Na^+ and K^+ [38]. More recently, ion-selective optodes have found additional applications in clinical chemistry. Seiler et al. Have developed sodium-selective optodes based on neutral ionophores, which allow the reversible quantification of total sodium concentration in human plasma with accuracy comparable to ISE measurements [39].

Recently, Wang et al. [40,41] demonstrated that certain chromoionophores can be incorporated into similar polymeric matrices to yield thin films that respond optically with high selectivity toward polyions. For example, the polymeric films containing a specific quaternary ammonium cation and a lipophilic pH indicator can efficiently extract rather hydrophilic polyanions, such as heparin. An optical sensing system for the detection of these polyanions has been reported previously using thin films doped with tridodecylmethylammonium chloride (TDMAC) and 3-hydroxy-4-(4-nitrophenylazo)-phenyl octadecanoate (ETH 2412) [40]. Also, a film containing a lipophilic fluorescein ester (2',7'-dichlorofluorescein octadecyl ester (DCFOE)) has been shown to respond with high specificity toward the polycation protamine [41].

Very recently, comprehensive review papers concerning ion-selective electrodes and optodes were compiled in Chemical Reviews[42,43]. These papers not only dealt with the general theoretical aspects of optodes but also introduced many recent research papers concerning optodes and empha sized mainly the response mechanisms and ionophores employed in ion-selective electrodes and optodes.

2.2.1. Principles and Mechanisms of Ion-selective Optodes

To understand how these sensors function, it is important to distinguish between two different types of sensing layers for charged species:

2.2.1.1. Optodes based on surface phenomena

The active components are immobilized near the interface or on the surface of the optical element or porous matrix, so that the active site is located in the sample solution. Very often, the recognition molecule is immobilized, either by a covalent binding [44], or by its physical adsorption on the surface [45], or it is part of a Langmuir-Blodgett film [46]. The response of ph optodes based on conventional pH indicators immobilized on the surface was thoroughly elucidated by Janata [47]. Errors in the determination of the sample's pH are mainly associated with a change in ionic strength of the sample. It influences both the activity coefficient of the charged form of the indicator and the surface potential, which is responsible for the relationship between the measured pH at the surface and the pH of the bulk.

The response of an optode with a negatively charged metal-ion complexing agent, covalently bound to the surface of silica, was studied in detail by Weaver and Harris [44]. In this system, the surface potential was also found to play an important role. Since the ligand itself participates in acid-base reactions, not only the ionic strength, but also the pH of the sample contributes to the optical signal.

2.2.1.2. Bulk optodes

The active components are uniformly entrapped in the bulk of a homogeneous sensing layer. A complete theoretical description of the response of the bulk optodes, which are based on plasticized PVC membranes, has been given earlier by Morf et al. Such optodes are based on a mass transfer of the ions from the sample into the bulk of the sensing layer to get an adequate optical signal. Since the electroneutrality is conserved in the sensing layer, these optodes respond to products (extraction system) or to ratios (ion-exchange system) of the ion activities [48].

CHAPTER 3. Polyions

3.1. Food Thickening Polyions

The agricultural and food sector is in constant need of improved analytical techniques that can be used to control manufacturing processes and the safety and quality of the products. These techniques should be rapid, reliable, specific and cost-effective in their provision of information about physical and chemical characteristics of food. A few biosensors play a prominent role in food processing or quality control. Considerable effort must be made to develop sensors that are inexpensive, reliable, and robust enough to operate under realistic conditions. Reliable and cost-effective analytical methods are increasingly needed in the food industry for the determination ofspecific chemical compounds in foods and food products. The need arises from increased regulatory action and heightened consumer concern about food composition and safety. It has been estimated that the food industry spends, on average, 1.5 to 2% of the value of its total sales on quality control and appraisal. For example, several million penicillin assays for mastitis are performed on cattle milk in US dairy farms every year. Contamination of foods by toxins (e.g. domoic acid in mussels, tetrodotoxin in fish), viruses (e.g. hepatitis A) or microorganisms (e.g. Escherichia coli, Salmonella, Listeria) is a serious concern. The recent bovine spongiform encephalopathy (BSE) outbreak in the UK, as a result of which several million cattle and dairy products were ordered to be destroyed, has not been quickly forgotten. According to the Center for Disease Control and Prevention

(Atlanta, USA), 6.5 million illnesses and 9000 deaths occur every year in the USA because of foodborne bacterial contamination. Food products now require extensive labelling of all their major and minor constituents – artificial flavours and sweeteners, antimicrobial agents and allergens, glucose in sugars and syrups, alcohols in wine, hydrogen peroxide and sulphites as preservatives, antibiotics in milk, cholesterol, glutamate and vitamins, to name just a few [49,50].

Thickening agents and stabilizers have ability to increase a system's viscosity, to form gel or to stabilize emulsion, suspensions or forms. Many pollysacaharides are used as thickening agent and stabilizer for their unique textural, structural and functional characteristics in systems. Some of these are strach, pectin, gum arabic, guar gum, xanthan gum, alginate, carrageenan and agar. These substances are hydrocolloid materials. It means they are hyrophilic and are dispersed in solution as colloid. Good hydrocolloid compounds are able to increase viscosity and they have significant solubility in water.

3.1.1. Carrageenan

Carrageenans are one of the effective hydrocolloid compounds which are naturally occuring family of sulfated linear pollysaccharides [51]. They are extracted form red algea or seaweed [52]. There are several types of carregeenans with different chemical structure and properties.Three of them are most important types, kappa (κ), iota (ι) and lamda (λ) carrageenans [53]. These variations are come from the number/position of sulphate groups of molecules.

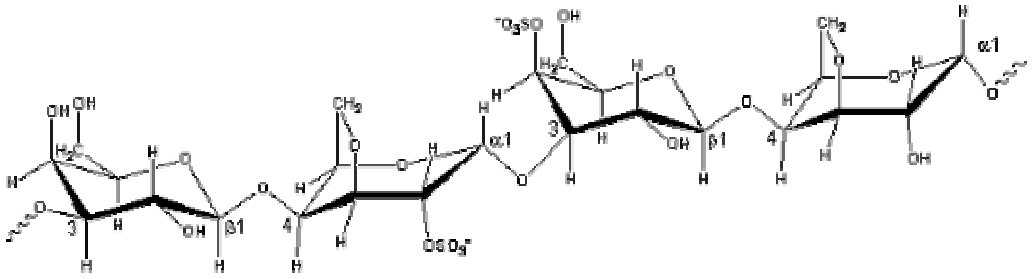

Figure 3.1. Carrageenan consists of alternating 3-linked-β-D-galactopyranose and 4- linked-α-D-galactopyranose units.

Type of carrageenans are changed by which seawed they are extracted, because composition of carragenan in red seawed changed from one species to another.

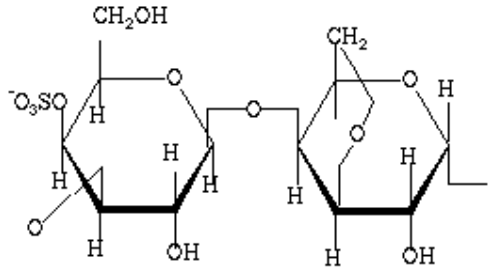

Figure 3.2. κ-carrageenan (kappa-carrageenan) -(1→3)-β-D-galactopyranose-4- sulfate-(1→4)-3,6-anhydro-α-D-galactopyranose-(1→3)-

Kappa carragenan is produced by alkaline extraction from Kappaphycus alvareti [52]. Na^+ salts of of kappa type are soluble in cold water but, K^+ and Ca^{++} salts are insoluble. At high temperature greater than 65 ^oC all salts are soluble in water.

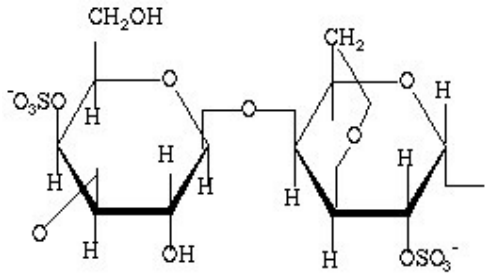

Figure 3.3. *ι-carrageenan (iota-carrageenan)* -(1→3)-β-D-galactopyranose-4-sulfate-(1→4)-3,6-anhydro-α-D-galactopyranose-2-sulfate-(1→3)-

Iota carrageenan is mostly produced by alkaline extraction from the philippins seawed Echeuma denticulatum. Na^+ salts of iota type are soluble in cold water but K^+ and Ca^{++} salts are insoluble in cold water. At high temperature greater than 55 ^oC all salts are soluble.

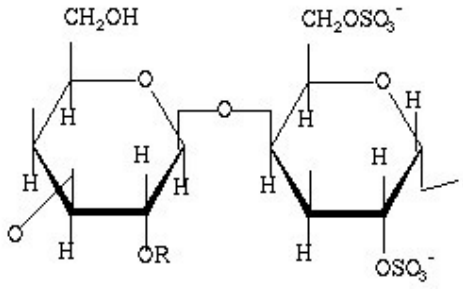

Figure 3.4. λ-*carrageenan* *(lambda-carrageenan)* -(1→3)-β-D-galactopyranose-2- sulfate-(1→4)-α-D-galactopyranose-2,6-disulfate-(1→3)

Lambda carrageenans are produced by Gigartina pistilla or Chondrus crispus by alkaline extraction with **κ**-carrageenan and **ι-**carrageenan**.** Na^+, K^+, and Ca^{++} salts are both soluble in hot and cold water. Solubility of molecule increases as the sulphate contents increases.

The molecular weights of **κ**-carrageenan and **λ** –carrageenans are changed in a range between 200 to 800 kdal.

Carrageenans are widely used as an additive by the food industry for their gelling, thickening and stabilising properties. Kappa and iota carrageenans

can form gels with potassium and calcium salts. Lambda carrageenan cannot form gel but it can form high viscosity solutions. It is especially used in diary products, such as cottage cheese, ice creamand chocolate milk. Very small amounts are enough, 0.01-0.05 percent, to application of products above. Also it is used in salad dressing, meat products, alcholic beverages, souces, dressing, toothpaste and pudding [51-54].

Carrageenan has a molecular weight of 20-30 kDa is called degraded carrageenan or poligeenan and they have been implicated gastrointestinal malignancy in animal models [55]. But undegraded carrageenans, molecular weight of above 100 kDa have not.

3.1.2. Xanthan Gum

The other important hyrocolloid, Xanthan gum, is a high moleculer weight anionic polysaccharide gum produced by the bacterium Xanthomonas campestries, and recovered from the medium by isopropanol precipitation.

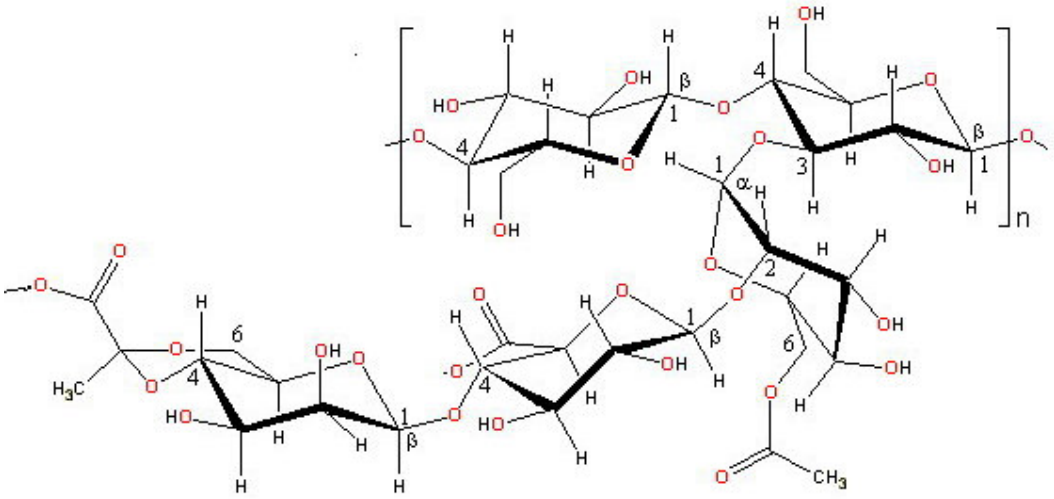

Figure 3.5. Structural unit is a β-(1→4)-D-glucopyranose glucan backbone with side chains of -(3→1)-α-linked D-mannopyranose-(2→1)-β-D-glucuronic acid-(4→1)-β-D -mannopyranose on alternating residues.

Slightly less than half (~40%) of the terminal mannose residues are 4,6-pyruvated and the inner mannose is mostly 6-acetylated (*i.e.* the side chains are mainly β-D-mannopyranosyl-(1→4)- (α-D-glucuronopyranosyl)-(1→2)- β -D-mannopyranoside-6-acetate-(1→3)-.

Xanthan gum is quite soluble in hot and cold water, However its molecular weight is greater than 10^6 dal , and form highly viscous solution [53]. Unlike the other gums xanthan is very stable under a wide range of temperature, 0 to 100 ^oC, and pH, 0 to 12. Except of some water miscible alcohol, xanthan is insoluble in organic solvents. Fennema (1996) lists it as emulsion stabilizer; holds water; enhances freeze-thaw stability; inhibits starch

retrogradation; improves shelf life and serves to bring about stabilization of dispersions, suspensions, and emulsions, thickener.

For its special characteristics above, xanthan is widely used in food industry as a thickenning agent and stabilizer such as in pudding, sauces and dressing, soups, dairy products, and prepared foods.

3.1.3. Sodium Alginate

Alginate is an other important hydrocolloid that is extracted from brown seaweed. They are linear unbranched copolymers containing β-(1→4)-linked D-mannuronic acid (**M**) and α-(1→4)-linked L-guluronic acid (**G**) residues. arranged in different **M** / **G** ratios. These residues are arranged in a blockwise fashion, constructed not only of homopolymer blocks of **MM** or **GG**, but also consist of alternating blocks of **M** and **G.**

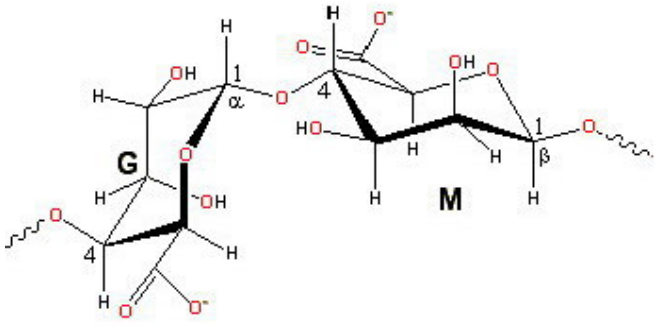

Figure 3.6. G and M units of alginate copolymer

The ammonia and alkali metal salts of alginic acid readily dissolve in cold water at low concentrations to give viscous solutions. Alginate's solubility and water-holding capacity depend on pH (precipitating below about pH 3.5), molecular weight (lower molecular weight calcium alginate chains with less than 500 residues showing increasing water binding with increasing size),

ionic strength (low ionic strength increasing the extended nature of the chains) and the nature of the ions present.

Alginate has been widely used as food and pharmaceutical additives, such as a tablet disintegrant and satabilizing agent for dressings,toppings, sauces, gravies, soups, milk shakes, gelling agent for puddings, mousses, pie and pastry fillings, dessert gels, restructured foods and thickenning agent for frozen foods, pastry fillings, bakery icings, dry mixes, fruit preparations [53].

3.2. Drug Polyions

Heparin and protamine are two important polyionic drugs used widwly in clinical procedures [56-57]. Heparin, a highly negatively charged polysaccharide with an average molecular weight of 15 000 Da, is the anticoagulant of choice for numerous surgical procedures, especially those involving extracorporeal blood circulation (e.g. open heart surgery). Approximately 30 metric tons of heparin, representing 500 million doses, are used annualy world wide [58]. The use of heparin is often associated with severe, potentially fatal bleeding complications, especially in patients undergoing extracorporeal therapies where considerably higher doses and prolonged heparin use are encountered. To prevent such bleeding complications, protamine, the antidote of heparin, is usually administered at the end of the extra corporeal operation to reverse the anticoagulant activity of heparin. Protamine is a low molecular weight (ca. 4500 Da) protein rich in basic arginine residues, and thus is highly positively charged under physiological conditions. The polycationic structure of protamine permits it to bind tightly with the negatively charged heparin through an electrostatic interaction [57].

Pentosan polysulfate (PPS) is a new polyanionic anti-arthritis drug. Pentosan polysulfate is a heparin-like highly sulfated polysaccharide. It is a semisynthetic product. The hemicellulose is isolated from the wood of beech trees (*Fagus sylvatica*) and is then subjected to sulfate esterification using chlorosulfonic acid. PPS consists of repeating units of (1–4)-linked β-D-xylanopyranoses in which α-D-4-methylglucopyranosyluronic acid residues are linked to the 2-position of every tenth xylanopyranose unit. The average molecular weight of PPS is reported to be 5700 . PPS has already been shown to exhibit numerous pharmacological activities. For example, it has been reported that PPS is an effective inhibitor of heparin-binding growth factors released from tumor cells and can actually inhibit the growth of tumors in animals [18].

CHAPTER 4. Experimental

Dinonylnaphthalene sulfonate (DNNS), 2-nitrophenyloctyl ether (NPOE), polyurethane (M48), λ-carrageenan, xanthan gum, sodium alginate, and tetrahydrofuran (THF) were purchased from Fluka. Protamine sulfate (from Herring, grade III) and heparin are obtained from Sigma. All other reagents were analytical grade. Solutions were prepared with deionized water. The buffer solution used in some experiments was 50mMTris–HCl, pH 7.4, containing 120mM NaCl. Food products containing thickening agents were obtained from the markets.

Polycation-sensitive membrane electrodes were prepared as previously described [59-60]. The membrane compositions for polycation -sensitive membrane electrodes were 1 wt./wt.% DNNS, 49.5 wt./wt.% NPOE, 49.5 wt./wt. % M48, respectively. All membranes were cast from a cocktail solution containing a total of 1 g of the components dissolved in 8ml THF. To construct cylindrical electrodes, glass capillaries were inserted into narrow bore Tygon tubing. The casting solution was then dip coated over the glass capillaries (10 times at 25 min intervals) and dried in air to form a cylindrical membrane. After soaking in 15mM NaCl for about 6 h, the glass rods were carefully removed. The tubes were filled with 50mM Tris–HCl, pH7.4, containing 120mM NaCl (for Heparin titrations). The tubes were filled with saline solution (0.12 M NaCl) for titrations of other polyions. A Ag/AgCl wire was inserted into the inner bore of the tube to serve as the internal reference electrode.These cylindrical electrodes were used in all experiments and disposed of after a single use. Potentiometric responses of the disposable cylindrical electrodes were measured versus an Ag/AgCl wire using an Orion

digital pH/mV meter (Model 720A). After mixing for 1 min, the sensors were immersed into the sample solution. The potential change of the membrane electrode was recorded 2 min following each sequential addition of standard protamine solution. All measurements were conducted with constant stirring of the sample solution (magnetic stir-bar) and at room temperature. Calibration curves were prepared by plotting the total potential change from the baseline value (EMF) versus the concentration (or log concentration) of protamine solution. To simulate real food products samples, given amounts of λ-carrageenan were spiked into food product solution, and these samples were titrated with protamine.

4.1. Potentiometric titrations of Xanthan gum, Sodium alginate, Carrageenan, and Heparin

Quantitative potentiometric titrations of polyions were performed by adding small aliquots of a concentrated protamine sulfate solution to a well-stirred saline solution containing a fixed and given amount of λ-carrageenan, xanthan gum, sodium alginate. The polycationic sensor in conjunction with a single junction Ag/AgCl wire reference electrode was immersed in a 5 mL of polyanion solution in 0.12 M NaCl. After potential stabilization, standard protamine solution was added and thepotential reading was recorded after 2 minutes of the addition. The end points were calculated from sigmoidal titration curves. Quantitative potentiometric titrations of heparin were done similarly. For heparin, the background solution was tris-buffer, pH 7.4 . Very good and proportional sigmoidal curves were obtained. Summary of data for potentiometric titration of various levels of different polyanions is shown in Table 1. Figures 4.1 - 4.4 also present typical titration curves of these four polyanions.

Table 4.1. Determination of neutralization stoiciometries of polyanions with protamine by PSE detection.

Polyanions	Mass ratio (Protamine / polyanions)
Heparin	1.53 ± 0.03
λ- Carrageenan	1.05 ± 0.06
Xanthan Gum	0.70 ± 0.03
Sodium Alginate	1.41 ± 0.06

4.2. Determination of Xanthan gum, Sodium alginate and Carrageenan in Food products

Known weights of the food product samples (100-500mg) were dissolved in a 10 mL portion of 0.12 M NaCl solution, and titrated with a standard protamine solution. A polycation-sensitive membrane electrode based on DNNS was used to monitor the titration end point. After potential stabilization, standard protamine solution was added and the potential reading was recorded after 2 minutes of the addition. The end points were calculated from sigmoidal titration curves. Titrations were repeated three times for each food products. Polyanion concentrations were calculated from the end point of the sigmoidal titration curves. Results were given as average of 3 measurements. Thickening additive levels in food products are expressed as % m/m in Table 2. Figures 4.5 - 4.14 also present the titration curves for each food product.

Table 2. Carrageenan, Xanthan Gum, and Sodium Alginate Concentration levels measured in some food samples using polycation-sensitive membrane sensor.

Sample types	Polyanion (% m/m)		
	Carrageenan	Xanthan	Sodium Alginate
Prepared pudding (Ülker)	0.654 ± 0.007	_____	_____
Pudding (Dr Oetker)	0.383 ± 0.010	_____	_____
Salad dressing (Salatfix, Köhne)	0.189 ± 0.009	_____	_____
Salep (Sek)	0.571 ± 0.027	_____	_____
Ice-cream (Algida)	0.606 ± 0.058	_____	_____
Yogurt with Fruit (Danone)	0.233 ± 0.066	_____	_____
Banana aromated milk (Nestle)	0.215 ± 0.054	_____	_____
Pastry Cream (Dr Oetker)	0.293 ± 0.057	_____	_____
Chocolate sauce (Dr Oetker)	_____	5.109 ± 0.058	_____
Dessert with chocolate cream (Dr Oetker)	_____	_____	1.996 ± 0.346

* Average of three measurements.

4.3. The validity of the used potentiometric method

The ability of the titrimetric method to detect carrageenan in samples was assessed, using recovery measurements of spiked real food product solutions. The same weights of each sample of the five test samples were spiked with various concentrations of λ-carrageenan. The titrations of these samples were carried out using protamine titrant and a polycation-sensitive membrane sensor. Curves related to these titrations are shown in figure 4.15.

CHAPTER 5. Results and Discussion

Since the discovery that polymer membrane based ion-selective electrodes can be made to respond toward heparin and protamine [59-62], extensive research has been carried out to apply these novel electrochemical sensors in different applications. The response of these membrane electrodes is attributed to the development of a non-equilibrium steady-state change in the phase boundary potential arising from the selective extraction of polyions into the organic membrane phase. The non-equilibrium EMF responses of polyion electrodes are affected by both the content and the geometry of the polymer membrane. For example, higher polymer and lower plasticizer content will lower the electrode detection limits toward polyions [12], and also a tubular electrode design exhibits larger EMF responses compared with planar electrodes [13] .

The present work is dealing with the determination of xanthan gum, sodium alginate, and carrageenan. These compounds are highly sulfated polysaccharides and are used in food industry as food additive [63] stabilizer, homogenizer, thickening agent or gel-forming agent.

The primary focus of this dissertation work was to explore the use of polyion sensitive membrane based electrodes for the some food additives analysis. It was found that electrodes prepared with polymer membranes containing dinonylnaphthalenesulfonate (DNNS) as the ion-exchanger are capable of detecting polyanionic xanthan gum and sodium alginate used as food additives. It was also determined the various food thickeners in some food products sold at the Turkish markets. The method accuracy is confirmed by recovery experiments of spiked samples.

Food thickening agents were determined in a variety of food products. Ten different food samples were tested: Prepared pudding (Ülker), Pudding (Dr Oetker), Salad dressing (Salatfix, Köhne), Salep (Sek) , Ice-cream (Algida), Yogurt with Fruit (Danone), Banana aromated milk (Nestle), Pastry Cream (Dr Oetker), Chocolate sauce (Dr Oetker), Dessert with chocolate cream (Dr Oetker).

The total polyanion levels in these samples were measured by potentiometric titration technique using standard protamine as a titrant and polycation-sensitive membrane electrodes for the end point detection. The data obtained are found within the expected ranges of the nominal values for these types of food products.Determination of this kind of reagents is so important because their excess doses have harmful gastrointestinal effects [55].

Finally we can say that these polyions are satisfactory determined. Data clearly demonstrate the potential analytical applications of potentiometric polyion sensors for the measurement of this kind of polyions, too. The method has several advantages. The method is simple, accurate and applicable for routine analysis of these food additives.

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APPENDICES

1.Titration Curves

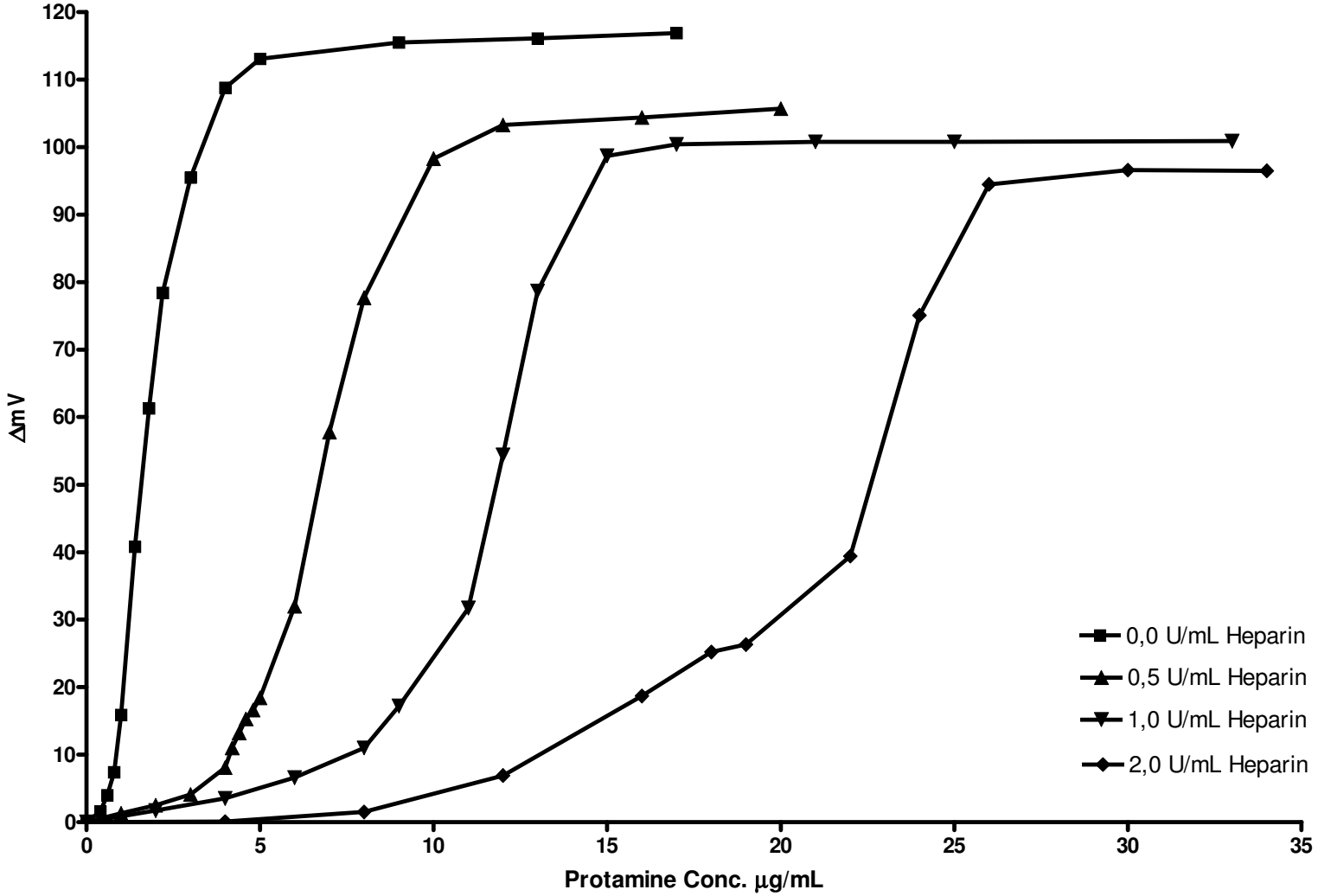

Figure 4.1 Potentiometric Titration of Heparin

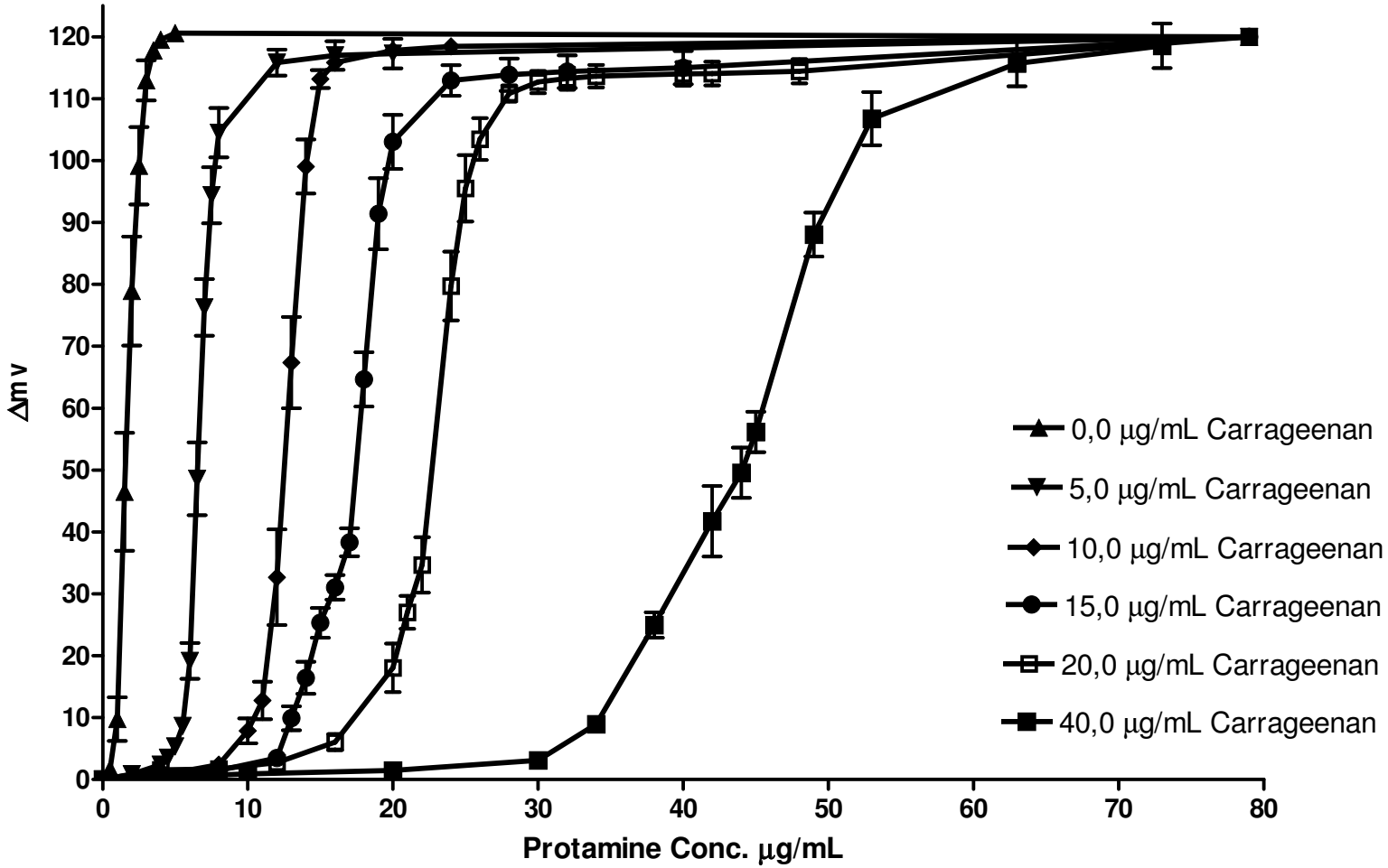

Figure 4.2 Potentiometric titration of Carrageenan

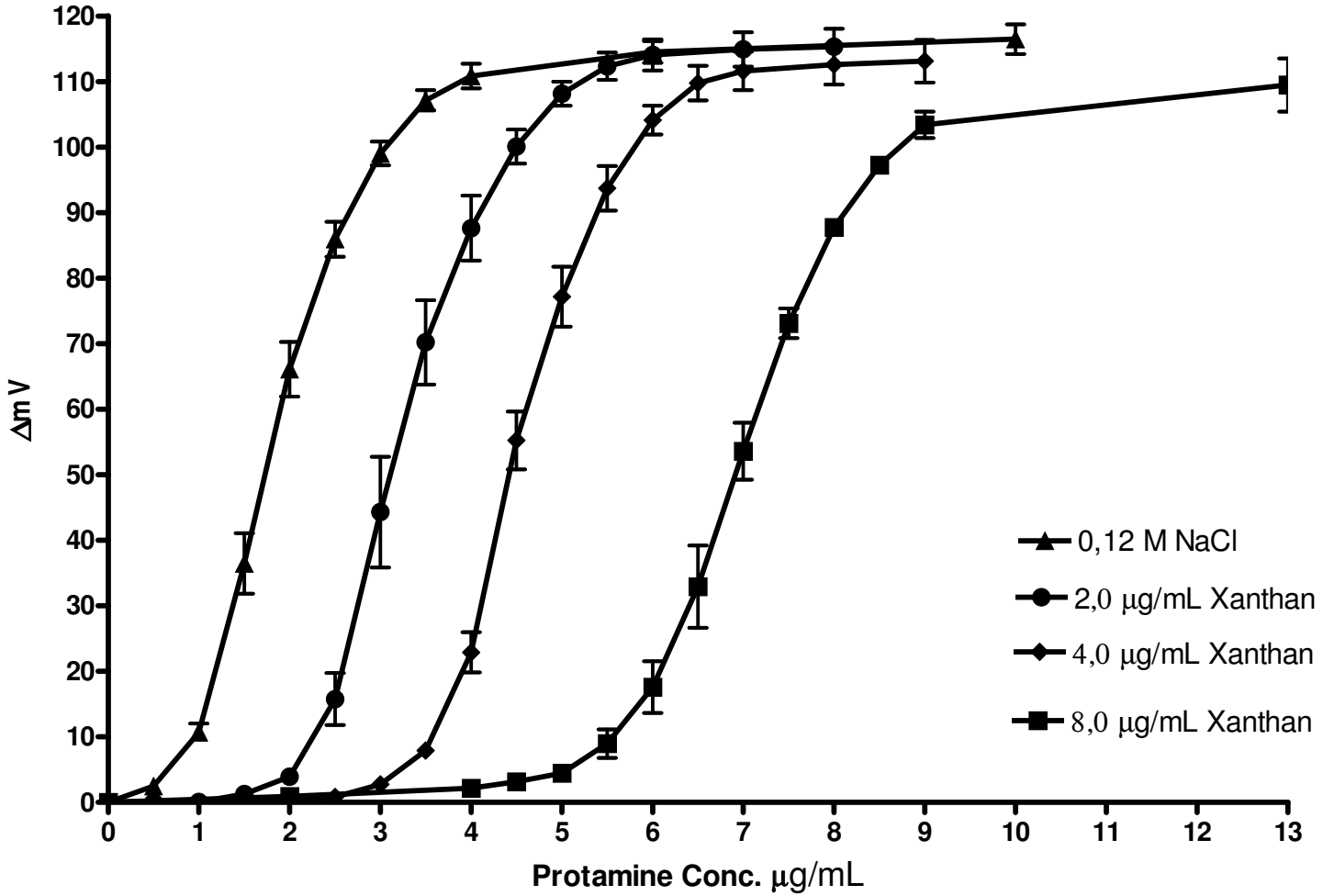

Figure 4.3 Potentiometric titration of Xanthane Gum

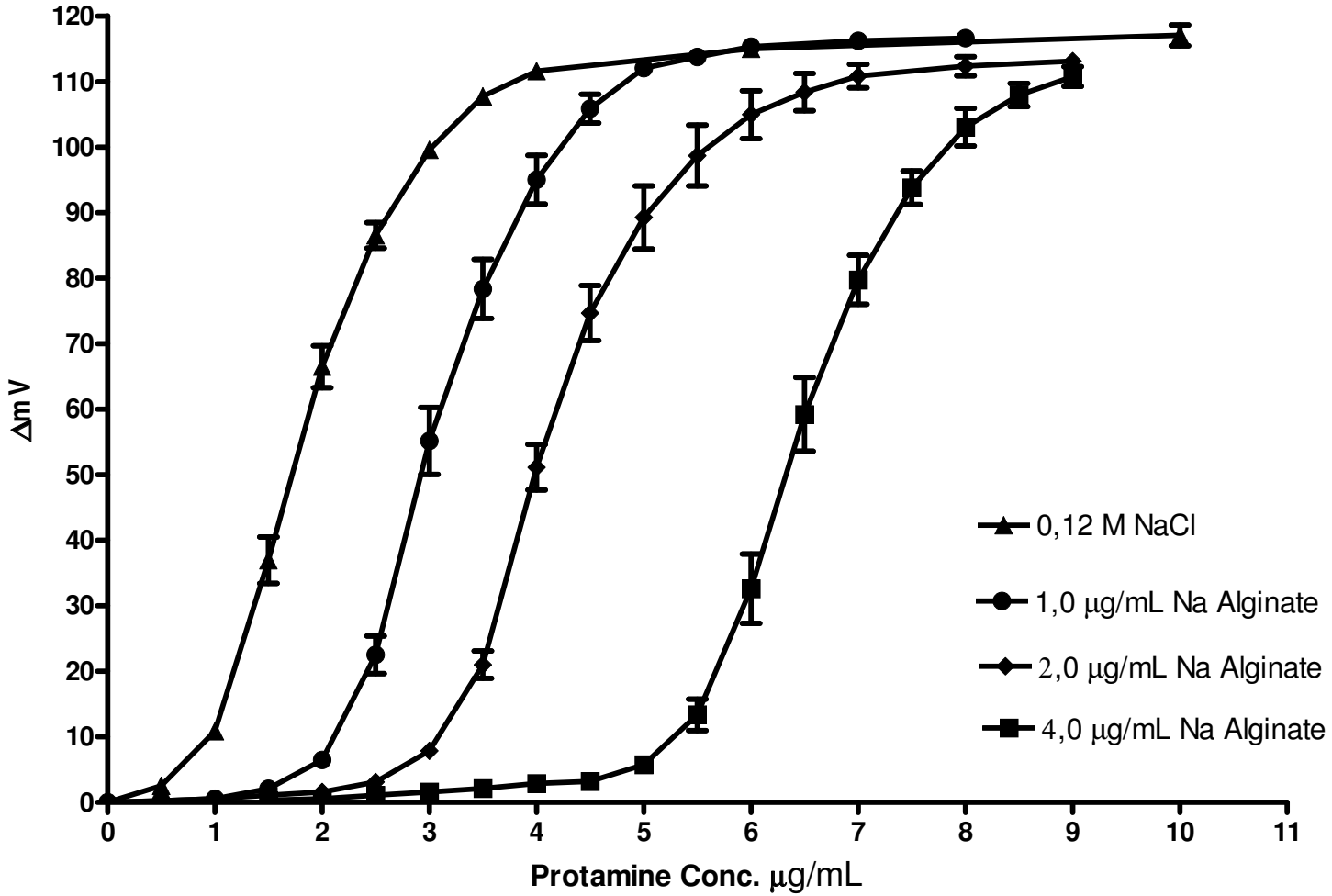

Figure 4.4 Potentiometric titration of Sodium Alginate

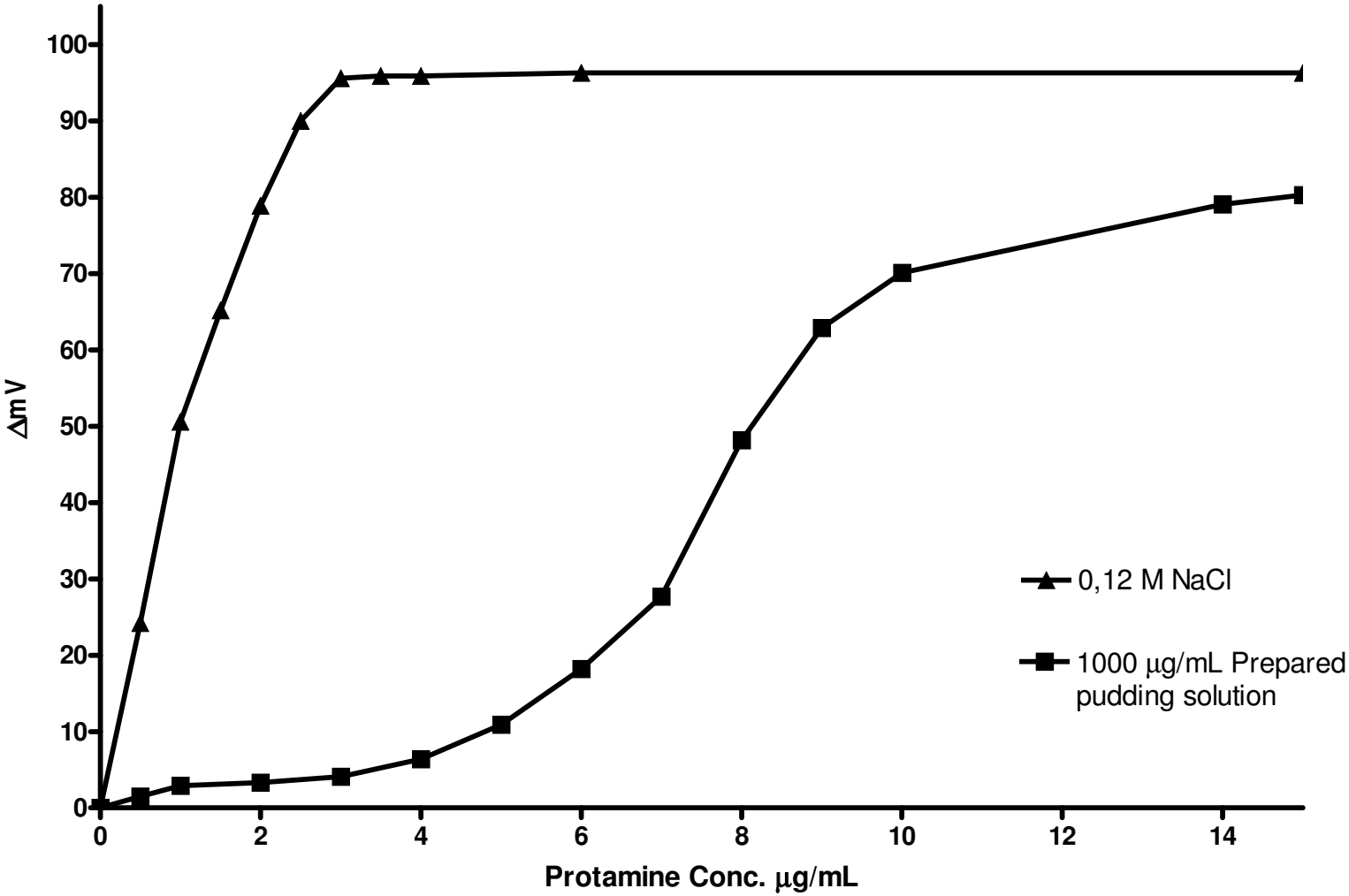

Figure 4.5 Potentiometric titration of Prepared Pudding

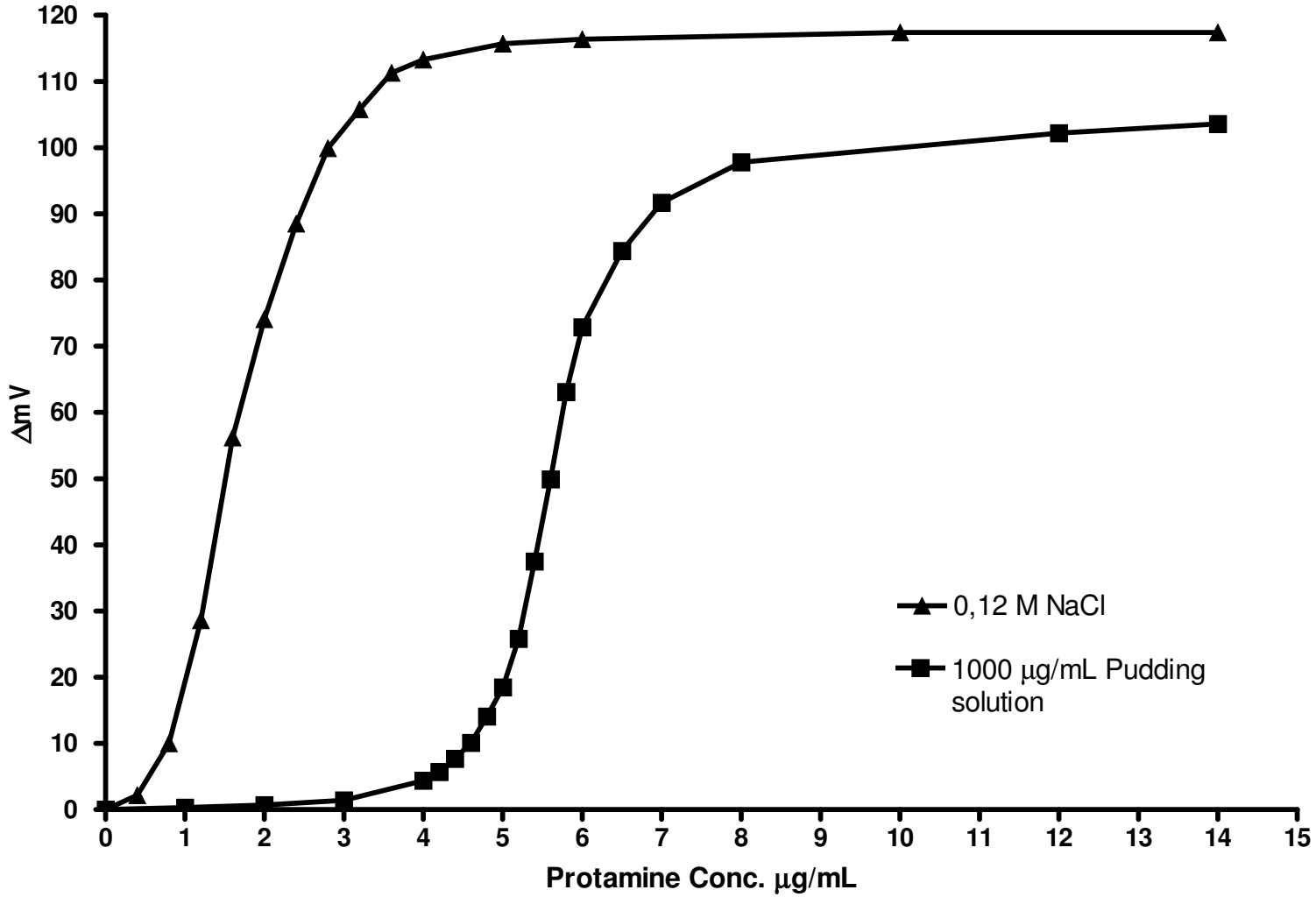

Figure 4.6 Potentiometric Titration of Pudding

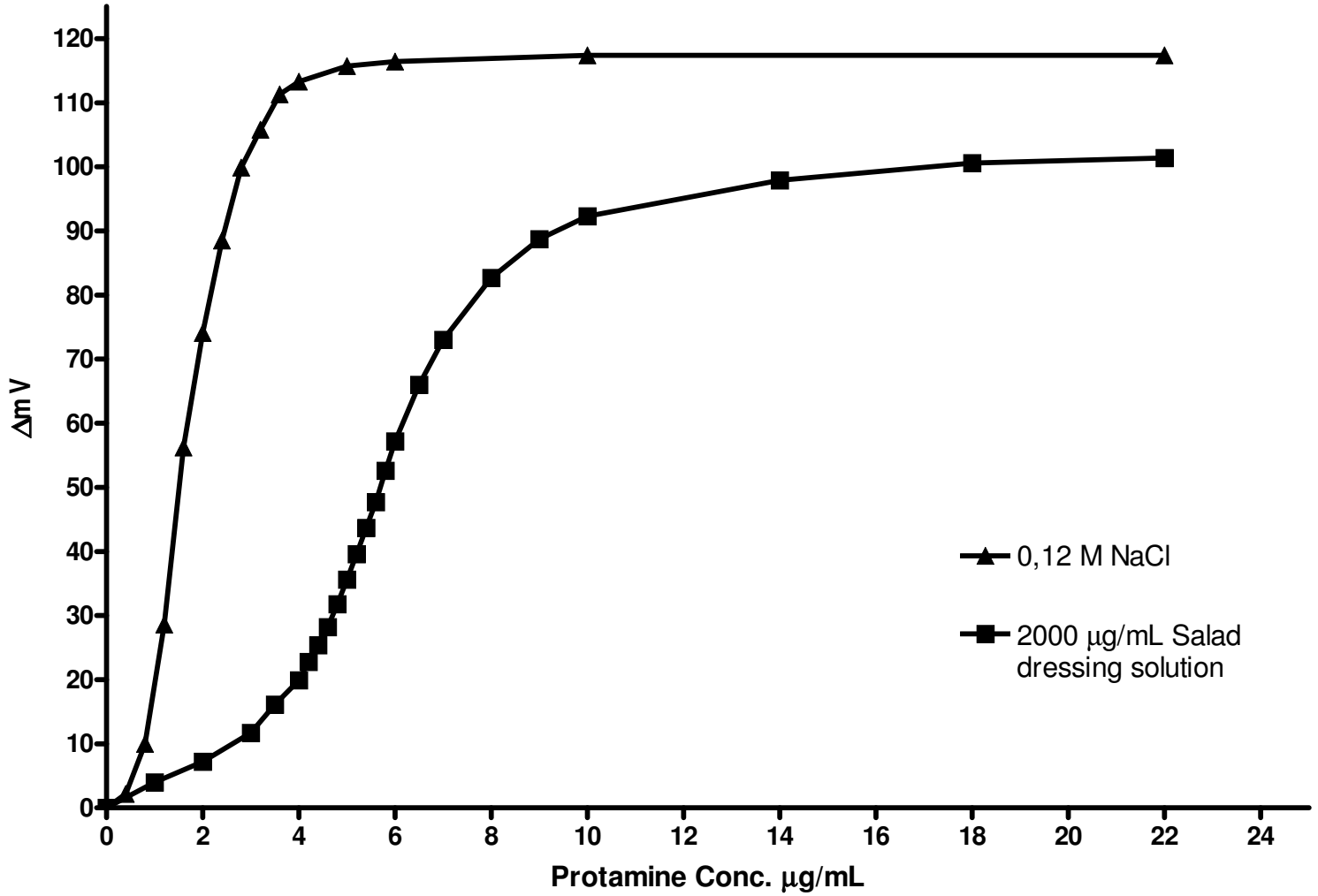

Figure 4.7 Potentiometric titration of Salad dressing

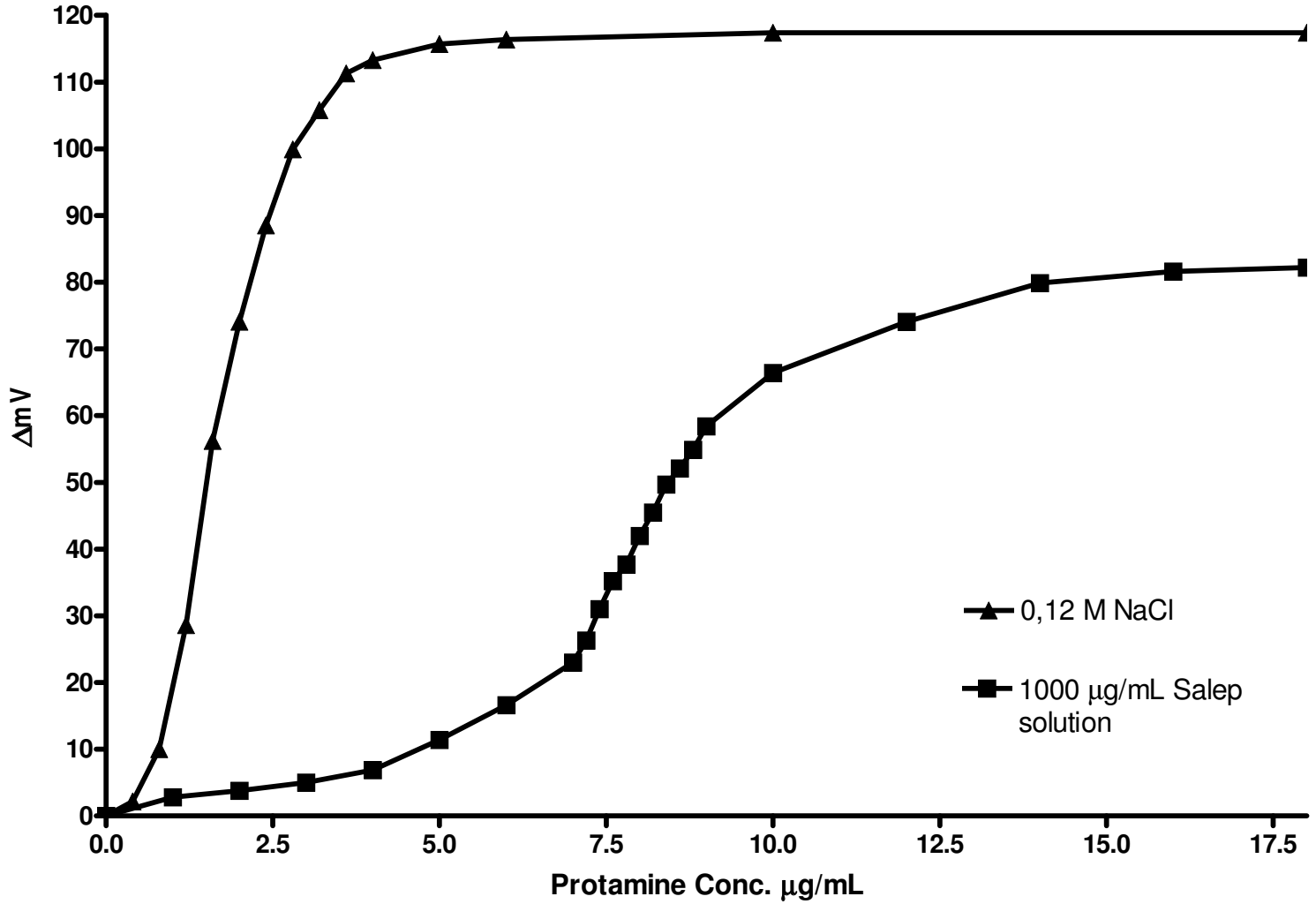

Figure 4.8 Potentiometric titration of Salep

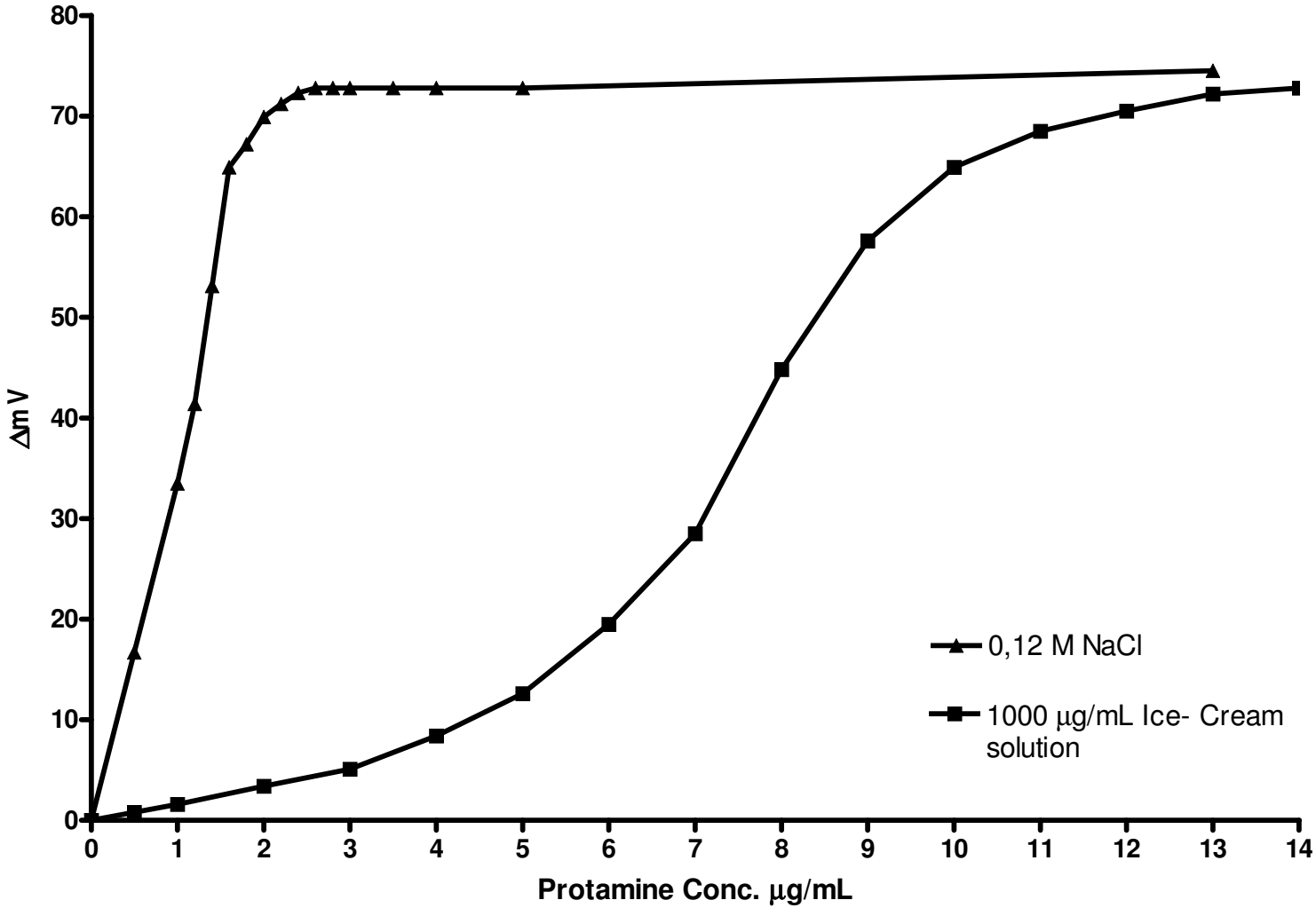

Figure 4.9 Potentiometric Titration of Ice-Cream

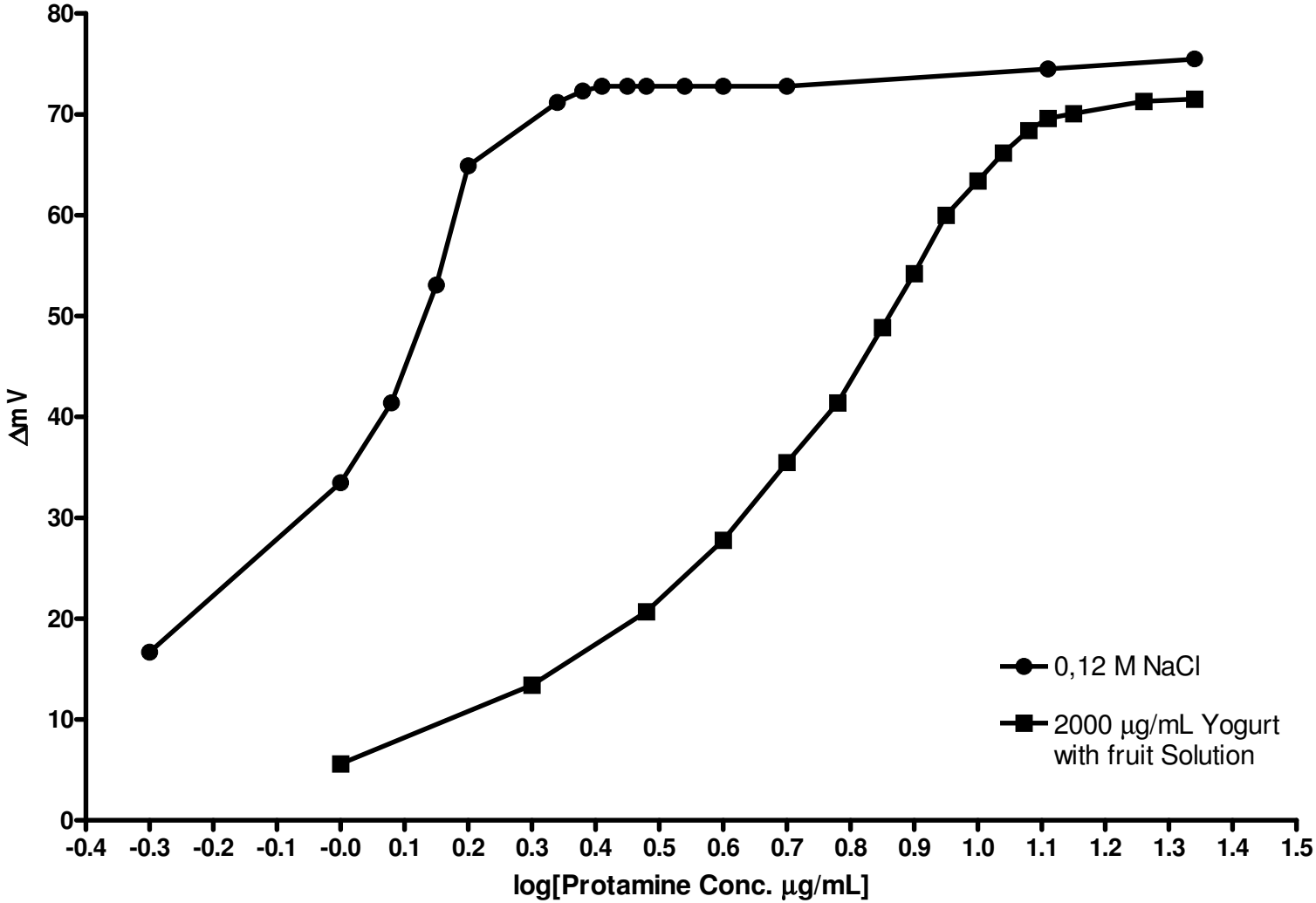

Figure 4.10 Potentiometric Titration of Yogurt with fruit

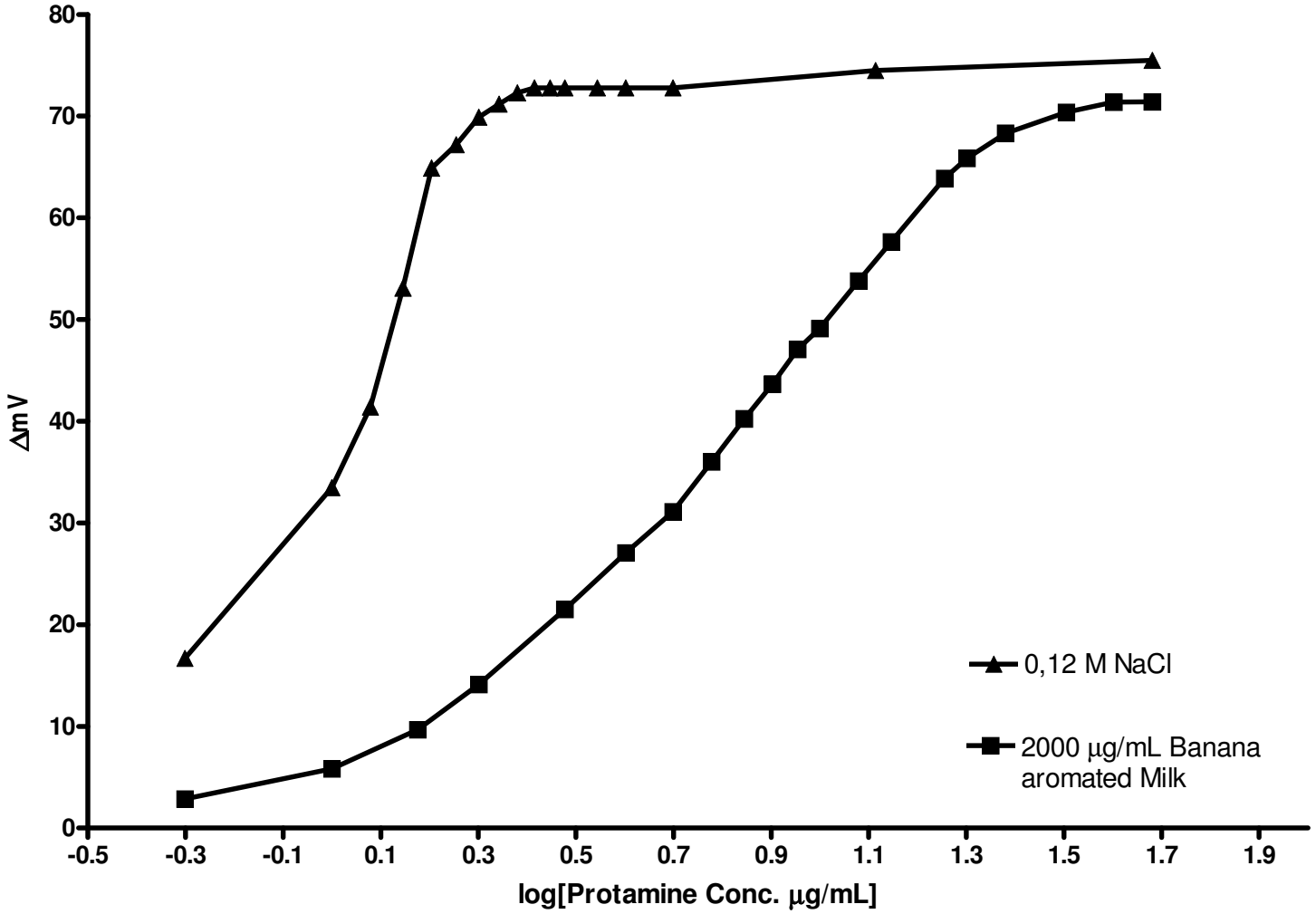

Figure 4.11 Potentiometric Titration graph of Banana aromated Milk

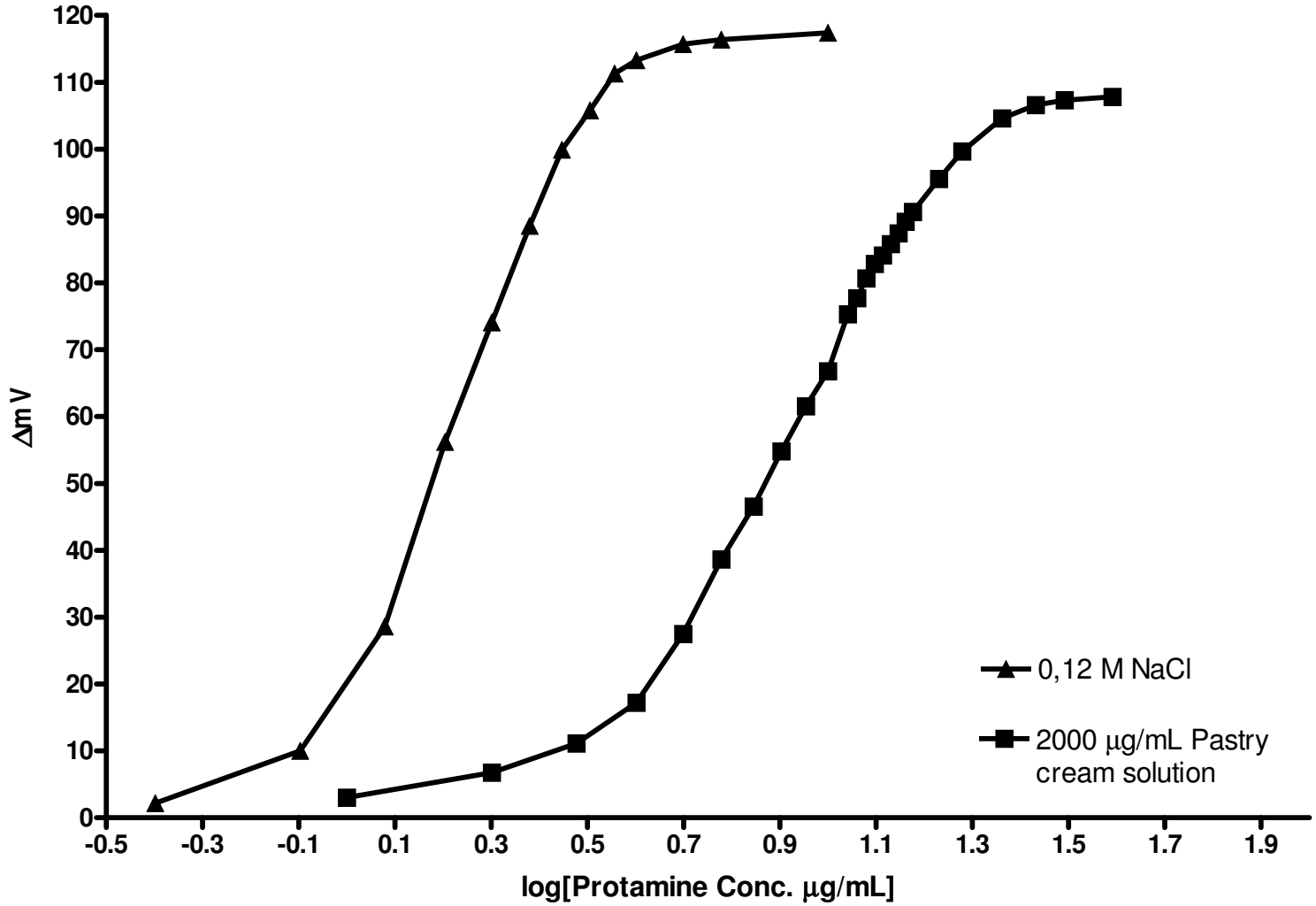

Figure 4.12 Potentiometric Titration of Pastry cream

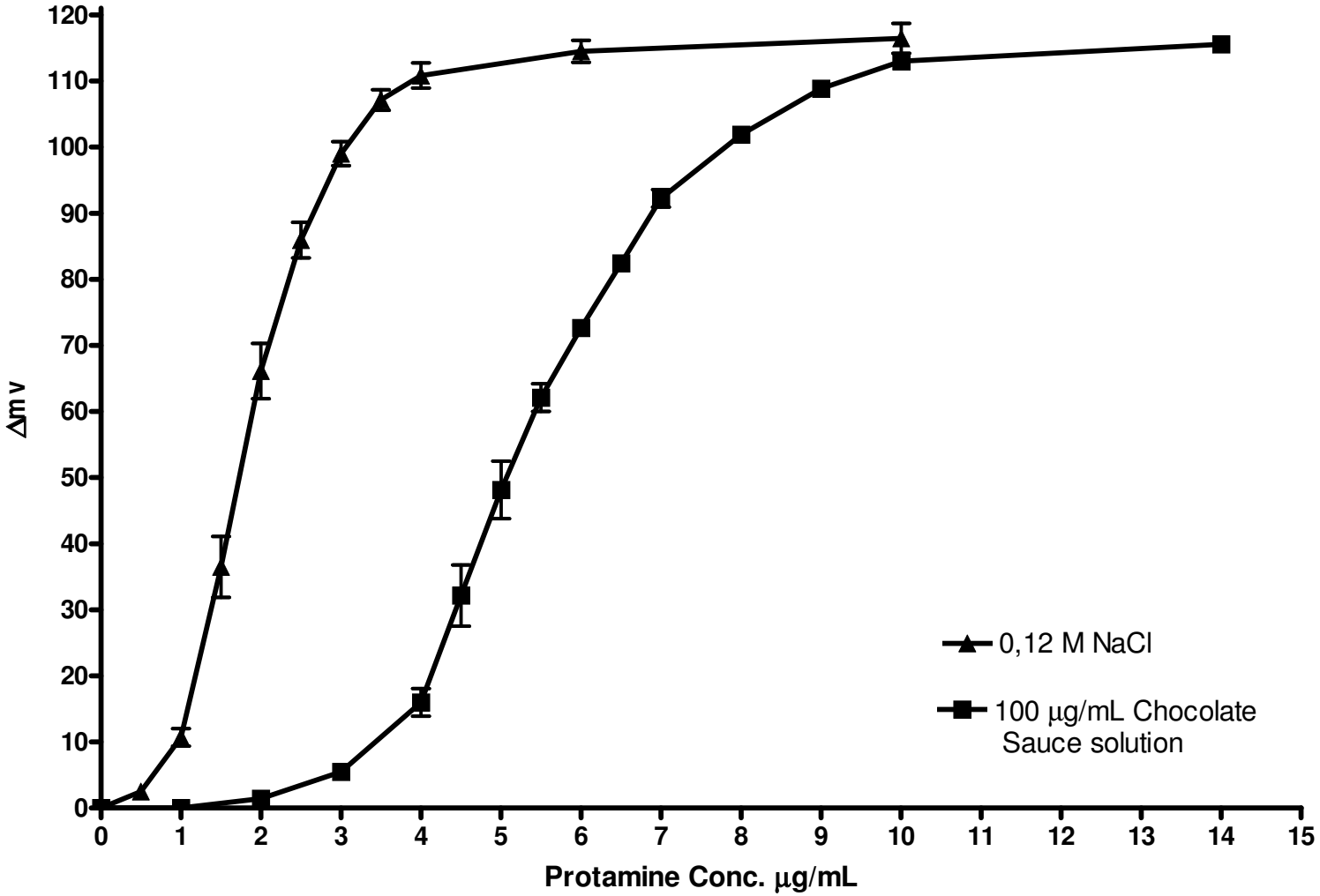

Figure 4.13 Potentiometric titration of Chocolate sauce

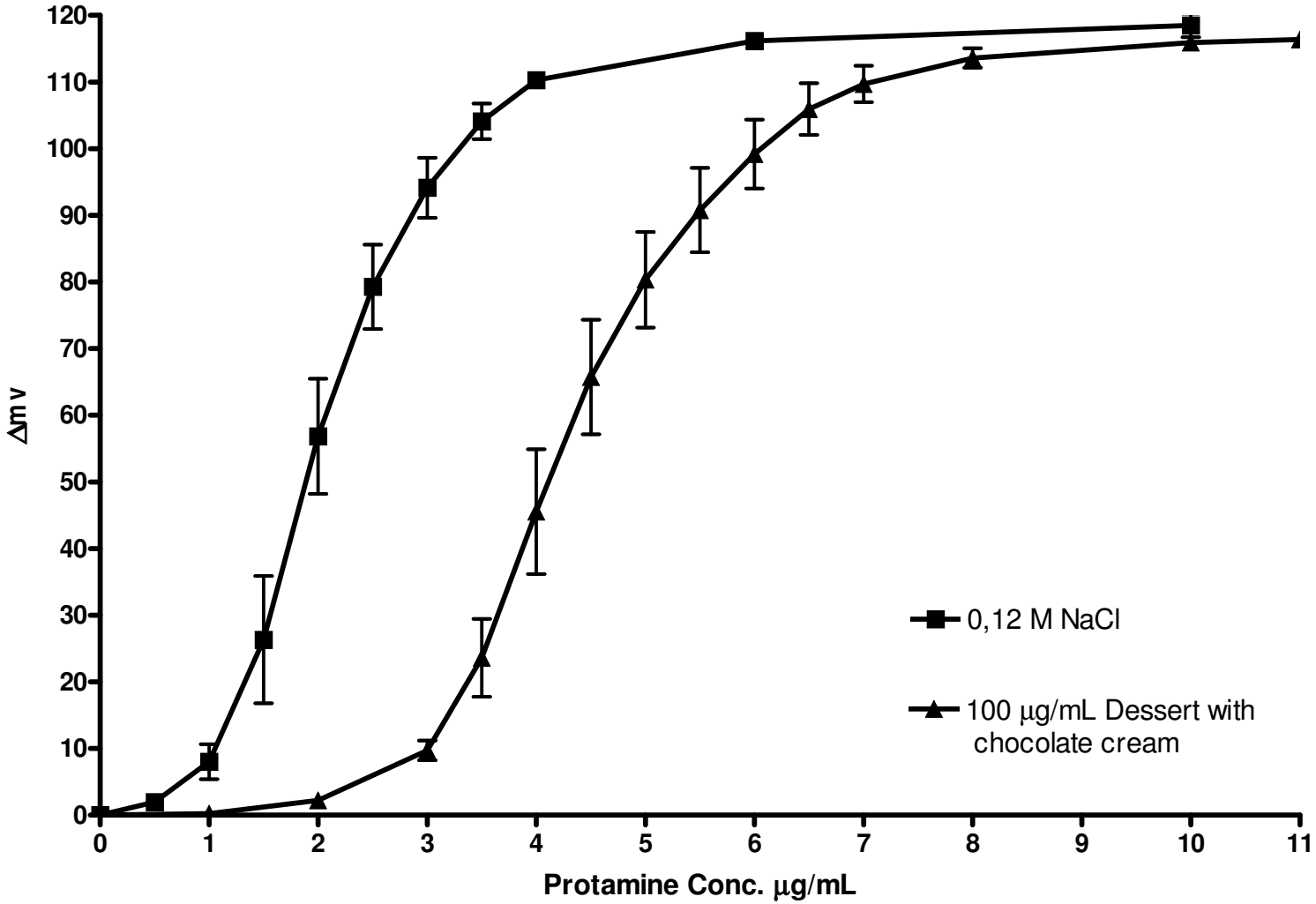

Figure 4.14 Potentiometric titration of Dessert with chocolate cream

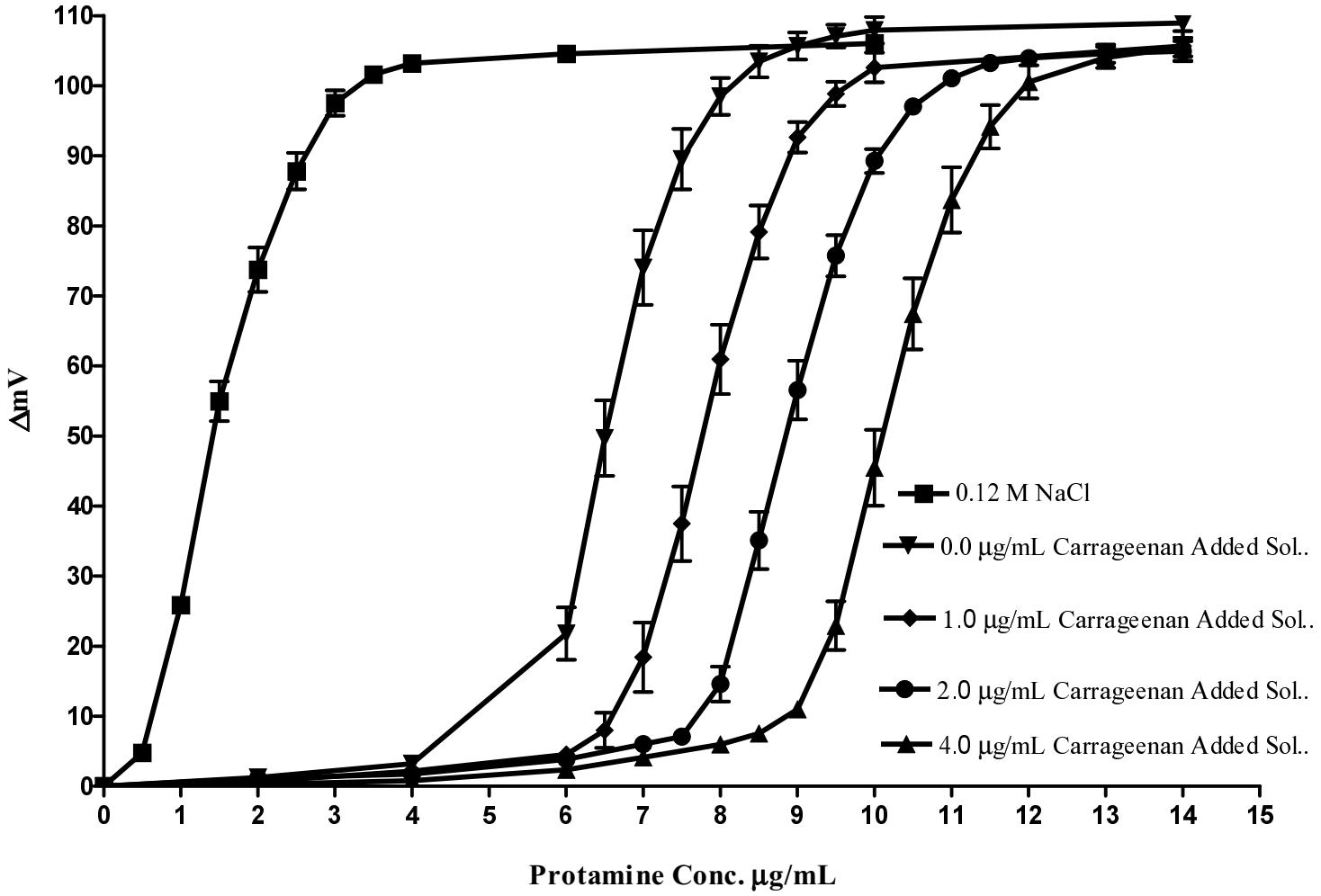

Figure 4.15 Potentiometric titration of Carrageenan added pudding Solutions