University of Reading

Role of electrophoresis in protein analysis

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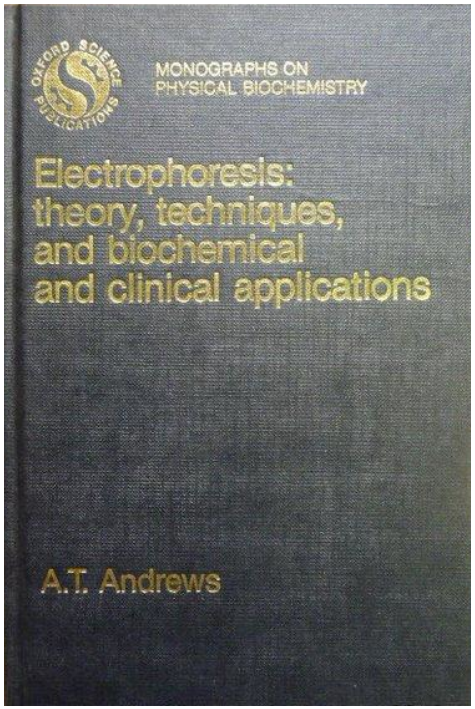
Professor Tony Andrews

- Biochemistry of Milk Products
- Chemical Aspects of Food Enzymes
- Electrophoresis: Theory, Techniques, and Biochemical and Clinical Applications

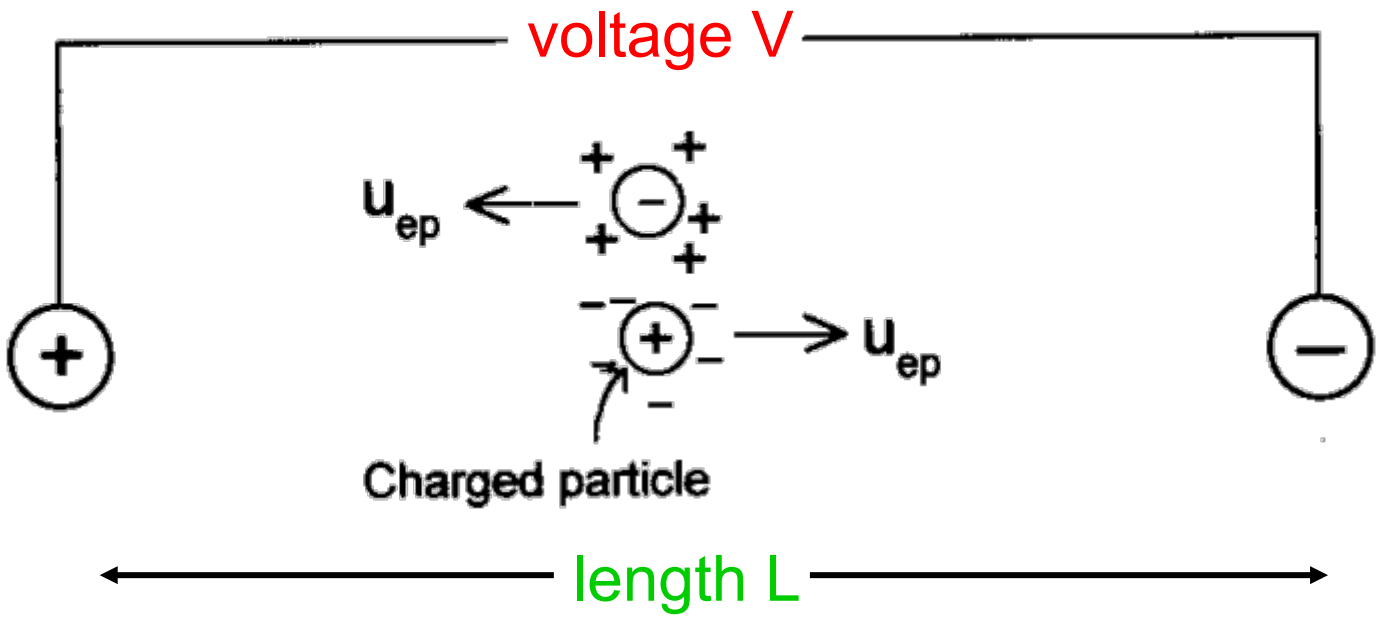


- "Dr. Andrews is to be congratulated on providing under one cover, succinct, clear, and practical descriptions of electrophoresis.” *Nature*
- "A must for biologists, immunologists, microbiologists, and any scientist interested in modern separation science.” *Journal of the American Chemical Society*

Electrophoresis

- What is it?
- How does it work?
- What can we do with it?

Electrophoresis

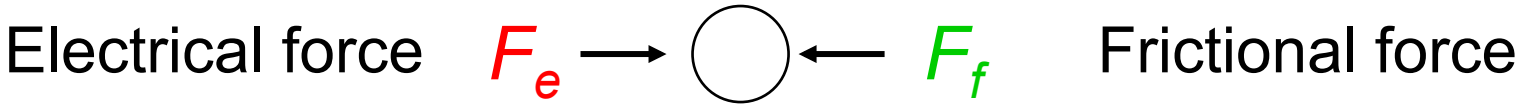


$$u_{ep} = \mu_{ep} \frac{V}{L}$$

V : applied voltage / V

μ_{ep} : electrophoretic mobility / $m^2/(s\ V)$

Electrophoretic Mobility



$$F_e = qE \qquad F_f = 6\pi\eta r u$$

$$u_{ep} = \frac{q}{6\pi\eta r} E \qquad \mu_{ep}$$

In general : $\mu_{ep} \propto$ q / MW

q : charge
r : radius
η : viscosity

pH and Protein Charge

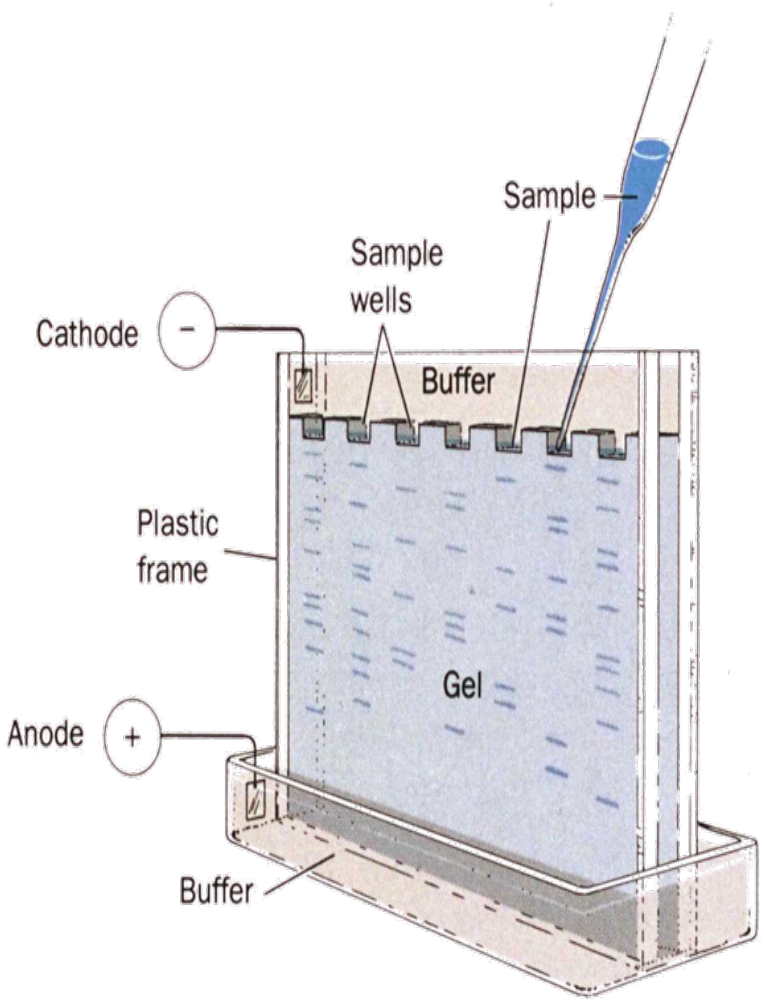
- Charge is determined by acidic and basic groups in residue side chains (*e.g.*, COOH, NH_2)
  - ionisation of these groups is pH dependent
- Isoelectric point of proteins is the pH at which the molecule has no net charge
  - above isoelectric point – net negative charge
  - below isoelectric point – net positive charge
- Migration velocity is proportional to charge, therefore electrophoresis is carried out in a buffered medium

Techniques

- Gel electrophoresis
  - Agarose
  - PAGE
  - SDS-PAGE
  - Isoelectric focusing
  - 2D-electrophoresis
- Capillary electrophoresis
  - Hyphenation to other techniques
- Lab-on-a-Chip

Gel Electrophoresis

- Support is a slab gel
  - gel is cast as a thin slab (1-3 mm thick x 10 x 10 cm)
- Porous and chemically stable gel
  - chromatographic nature of gel may be used to enhance separation (control pore size)



Agarose Gels

- Linear polysaccharide extracted from seaweed
  - average molecular mass about 12,000
  - alternating units of galactose and 3,6-anhydrogalactose
- Very large "pore" size
  - used primarily to separate molecules with molecular mass greater than 200 kDa
  - “true” electrophoresis - molecules are not retarded by adsorption on support
- Applications
  - analysis of PCR products (*e.g.*, in detection of bovine tuberculosis)

Polyacrylamide (PAGE)

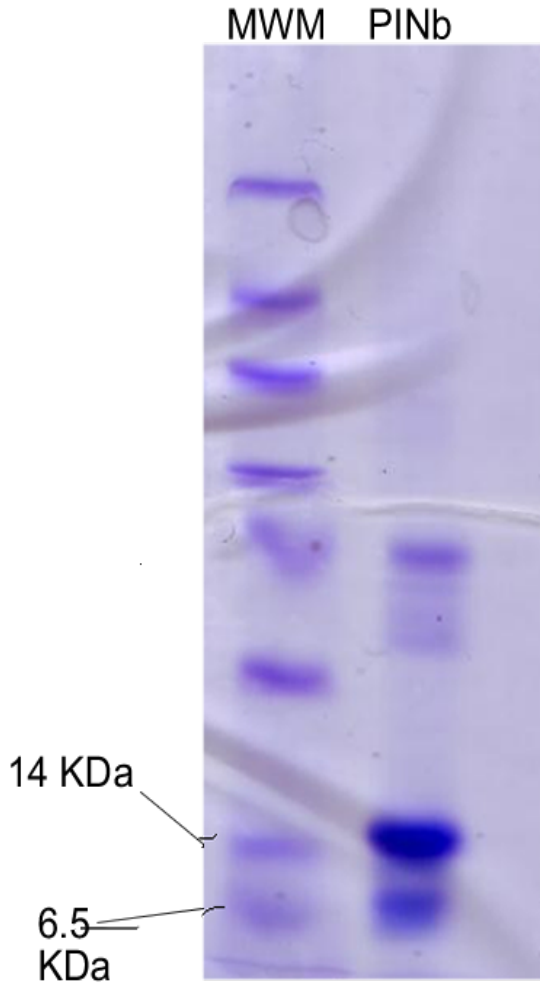
- Prepared by the polymerisation of acrylamide in the presence of N,N´-methylene-bis-acrylamide
- Pore size of gel can be controlled
- Mobility of charged molecules depends on:
  - electrophoretic mobility
  - molecular sieving through pores of support
- Polyacrylamide gels offer greater flexibility and more sharply defined banding than agarose gels

SDS-PAGE

- Sodium dodecyl sulfate is an anionic surfactant (detergent)
- SDS binds to hydrophobic sites in proteins and can improve separation by:
  - reducing aggregation of protein molecules
  - inducing large negative charge on proteins, thus allowing separation by molecular size rather than charge

Detection & Recovery

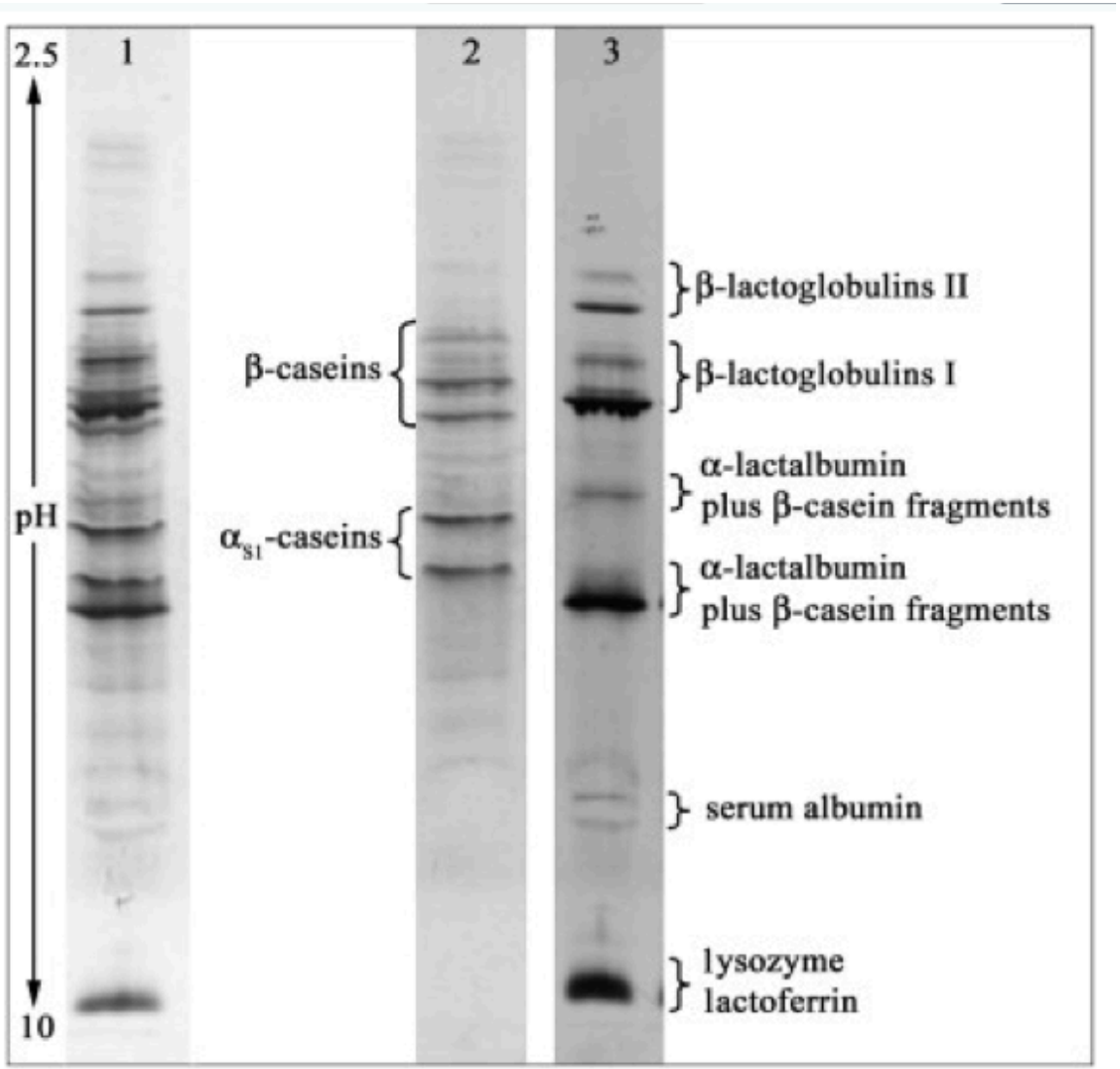
- Staining techniques
  - choice of stain depends on sample components
  - e.g., Coomassie blue binds to proteins
- UV or fluorescence
  - complex with ANS (1-anilinonaphthalen-8-sulfonate), fluorescamine or dansyl chloride
- Blotting techniques
  - mechanically or by electrodilution (current passed perpendicular to gel)



Limitations of SDS-PAGE

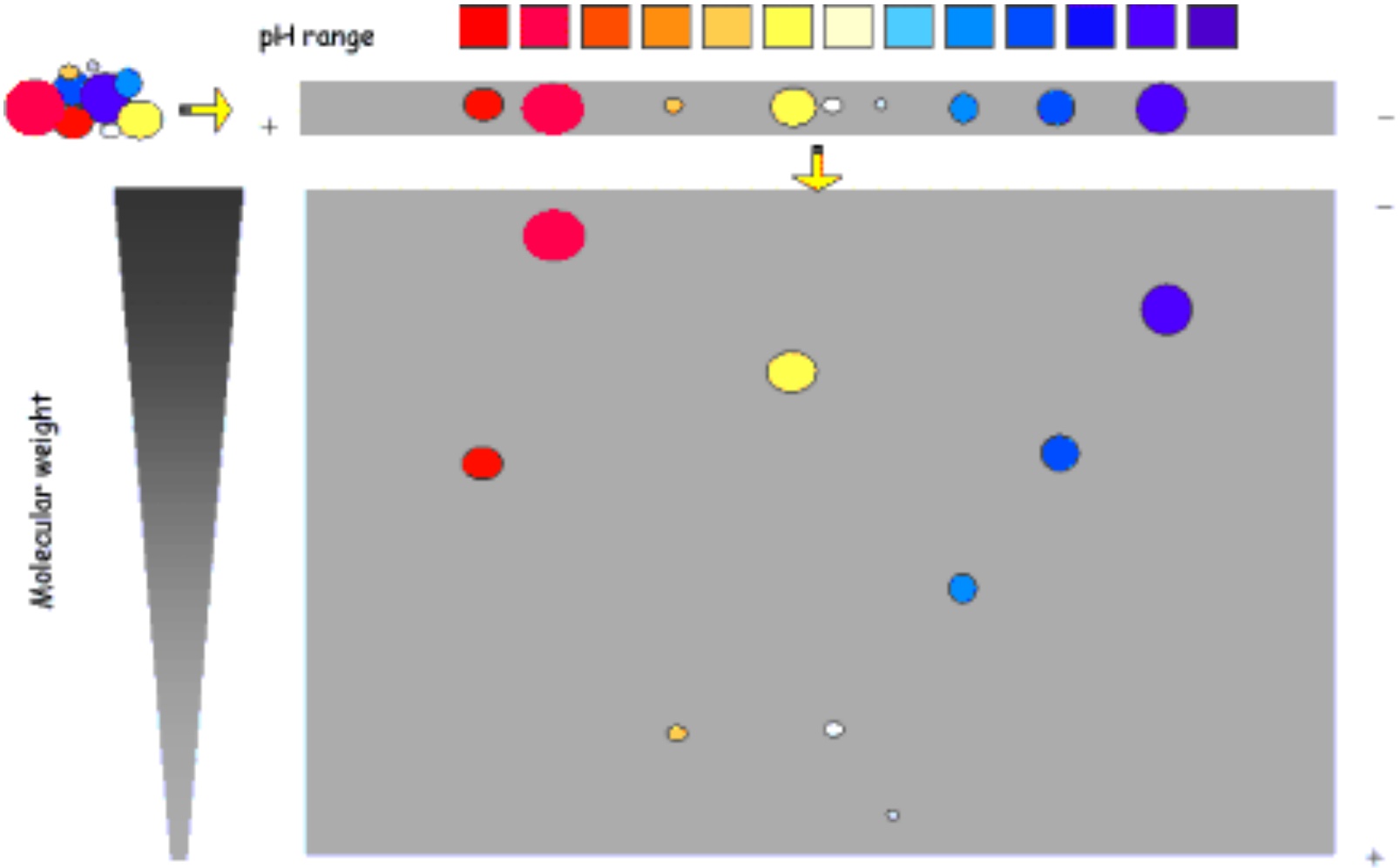
- Failure to separate small peptides
- Inability to resolve proteins with similar molecular masses
- Can be hard to detect less abundant proteins in complex protein mixtures having some very abundant proteins
- Protein is denatured

Isoelectric Focusing



Criscione et al., *Int. Dairy J.* 2009, *19*, 190.

Two-dimensional Protein Electrophoresis (2DE)



Protein Identification

- Use of markers to identify by molecular weight, isoelectric point, etc.
- Peptide mass fingerprinting – trypsin digestion (cleaves at arginine or lysine) followed by MALDI-ToF-MS
- Direct quantification is difficult

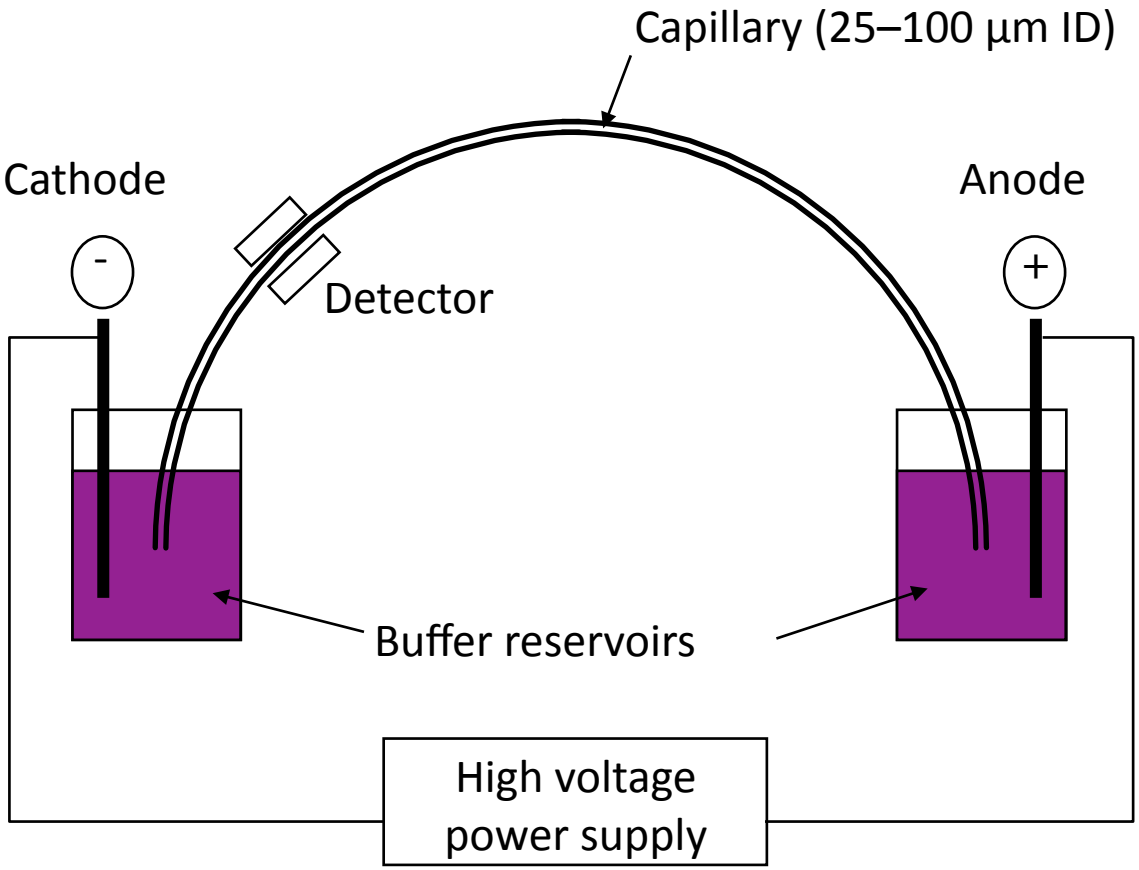
Protein Applications of Gel Electrophoresis

- Detection of milk adulteration (*e.g.*, by analysis of milk tryptic digests)
- Detection of genetic polymorphism
- Proteolysis
- Changes during processing

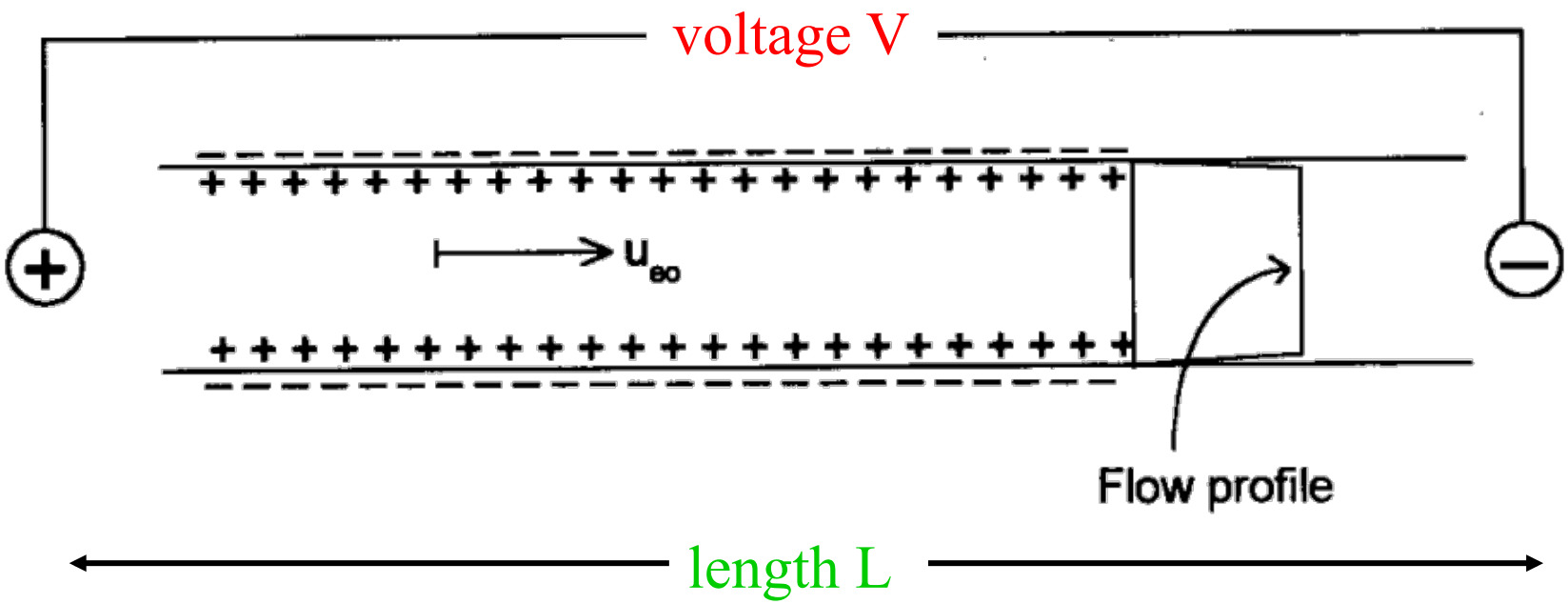
Capillary Electrophoresis

- Electrophoresis performed through narrow bore (25–100 μm ID), buffer-filled capillaries
- Combines advantages of HPLC and electrophoresis in one technique
  - automated
  - can operate electrophoretic AND chromatographic separation mechanisms
- Less labour-intensive than traditional electrophoresis and higher sample throughput
- Easy quantification

Instrumentation



Electro-osmotic Flow (EOF)



$$u_{eo} = \mu_{eo} \frac{V}{L}$$

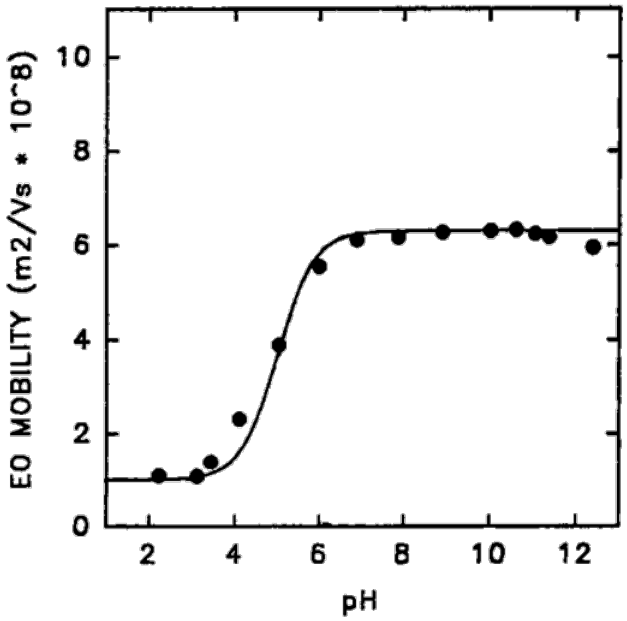
u : velocity / m/s

V : applied voltage / V

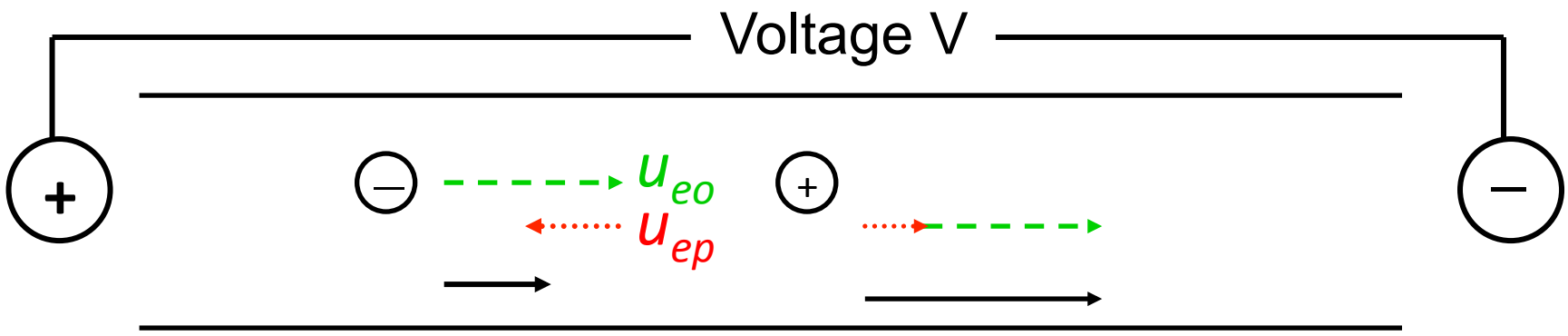
μ_{eo} : electroosmotic mobility / $m^2/(s\ V)$

Electro-osmotic Mobility

- Increases with wall charge
- Fused silica: wall charge from silanol groups
- Dissociation of silanol groups is pH-dependent
- μ_{eo} decreases with pH
- Also: μ_{eo} decreases with solution ionic strength



Separation Principle



$$u = u_{eo} + u_{ep} = \mu_{app} E$$

$$E = \frac{V}{L}$$

$$\mu_{eo} + \mu_{ep} = \mu_{app}$$

Typical values

u_{eo} 1.5 mm/s

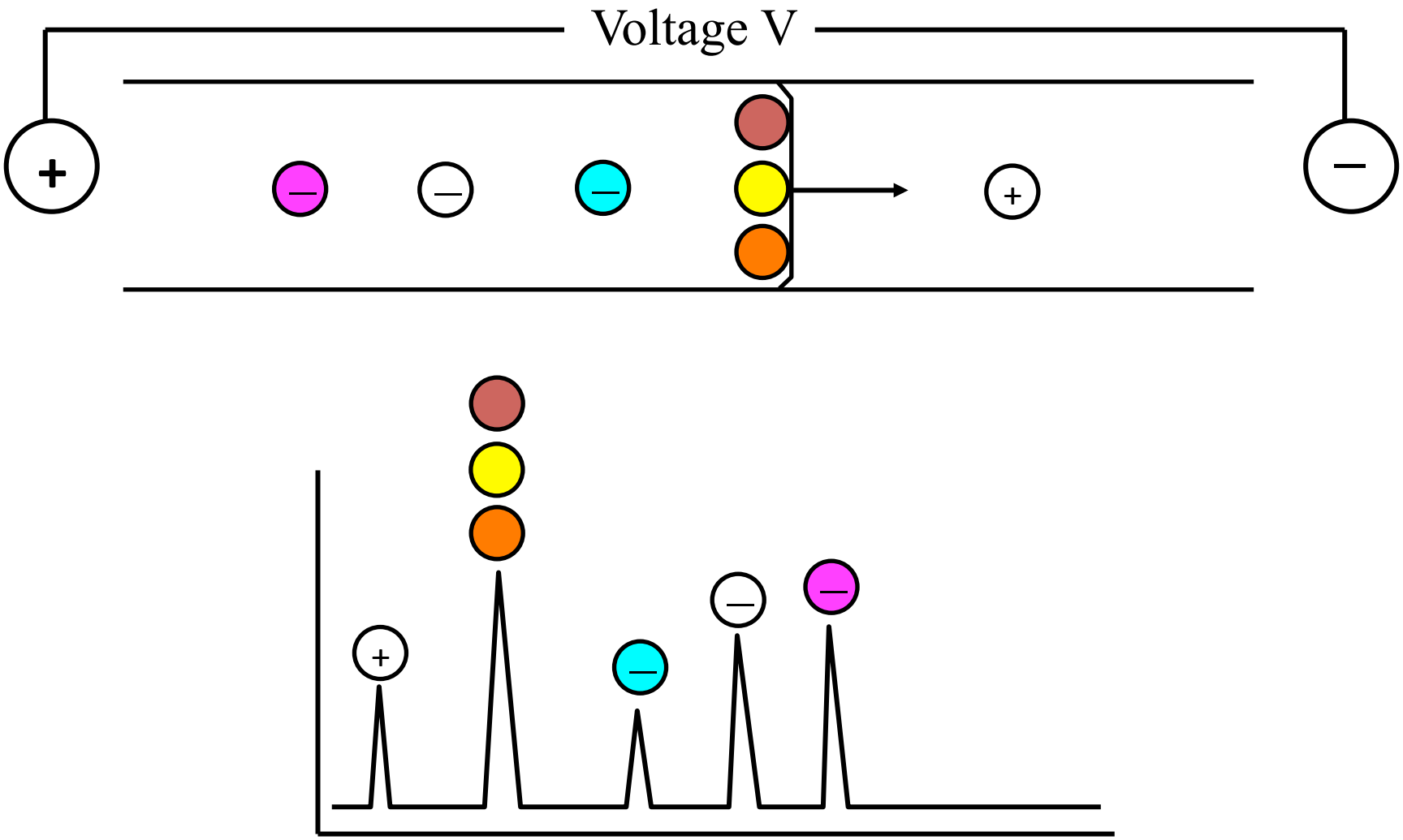
u_{ep} ± 1 mm/s (small ions)

Hence all species (also uncharged) move towards cathode

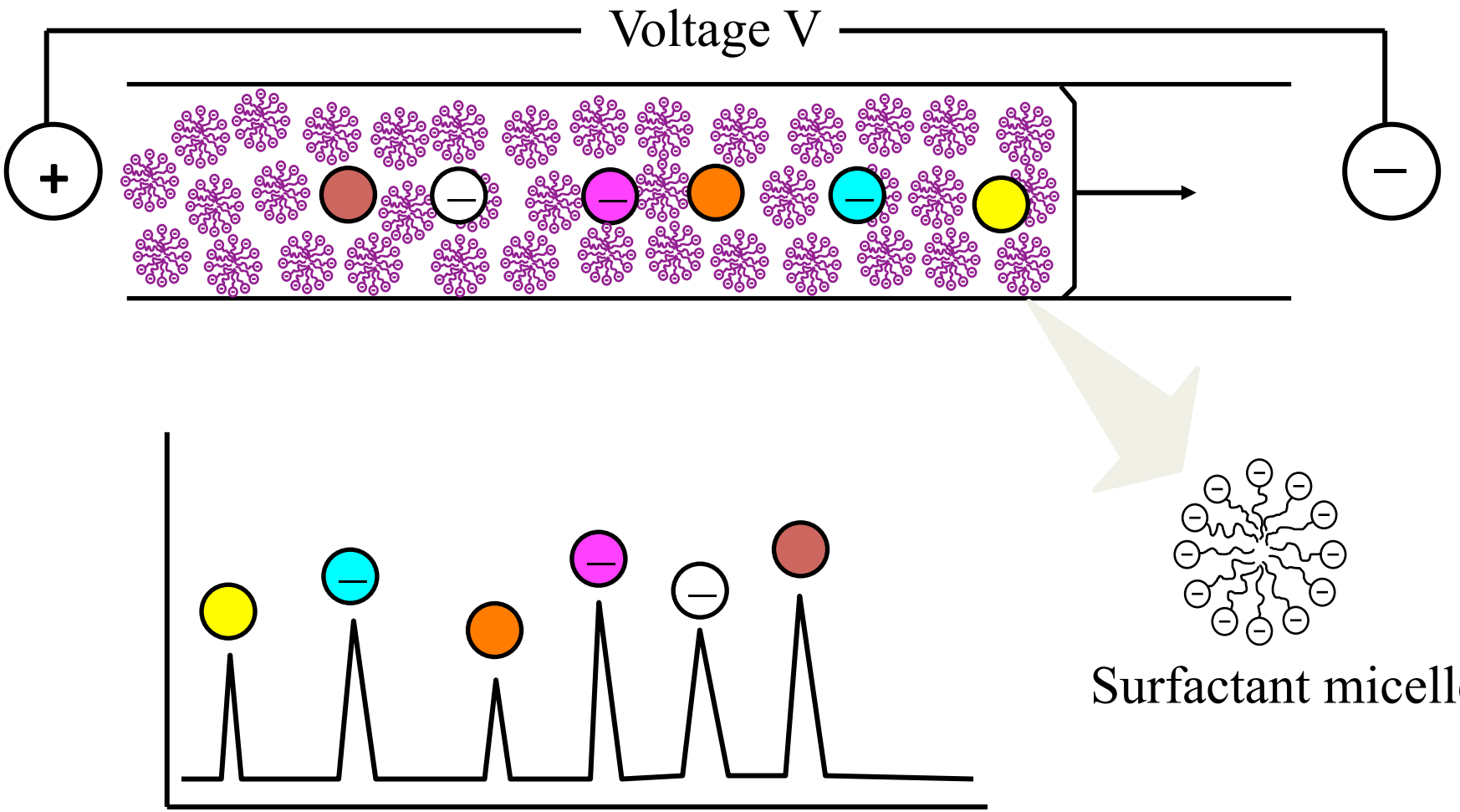
Modes of Separation

- There are several modes of separation in CE
- These modes differ by the buffer system used
- Capillary zone electrophoresis (CZE):
  - simplest and most commonly used mode of separation
  - separates charged sample components
- Micellar electrokinetic chromatography (MEKC):
  - surfactant is added to buffer at conc^{n} above CMC
  - separates charged and neutral components

CZE



MEKC



Performing a Separation

- Method development:
  - mode of separation
  - buffer conditions (type, pH, conc^{n})
  - capillary conditions (length, ID, coating)
  - instrument conditions (voltage, temperature)
  - detection method (UV-vis, fluorescence, MS)
- Commercial instruments are automated
  - buffer and sample vials are placed in a carousel
  - required analyses are programmed into a computer

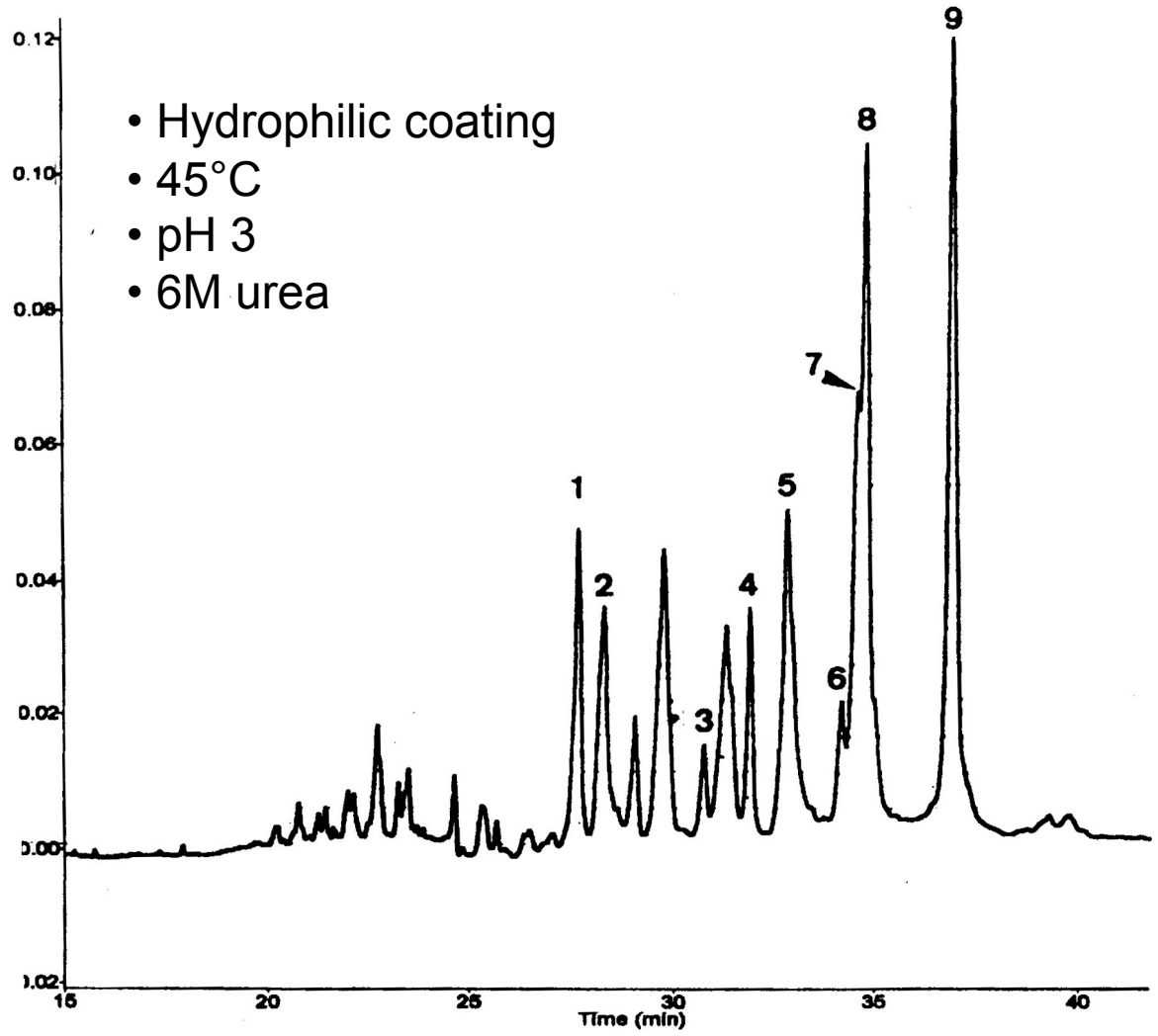
Challenges & Remedies

- Electrostatic adsorption
  - Hydrophilic coatings
  - Extreme pH in uncoated capillaries
  - High ionic strength, non-ionic surfactants
- Protein solubility
  - Urea
- Detection limits
  - Sample preconcentration
  - Alternative detection methods

Milk Proteins

- Adulteration
- Genetic polymorphism
- Changes during processing
  - *e.g.*, heat treatments, storage
- Cheese production
  - Breakdown of proteins through proteolysis
- Quality control

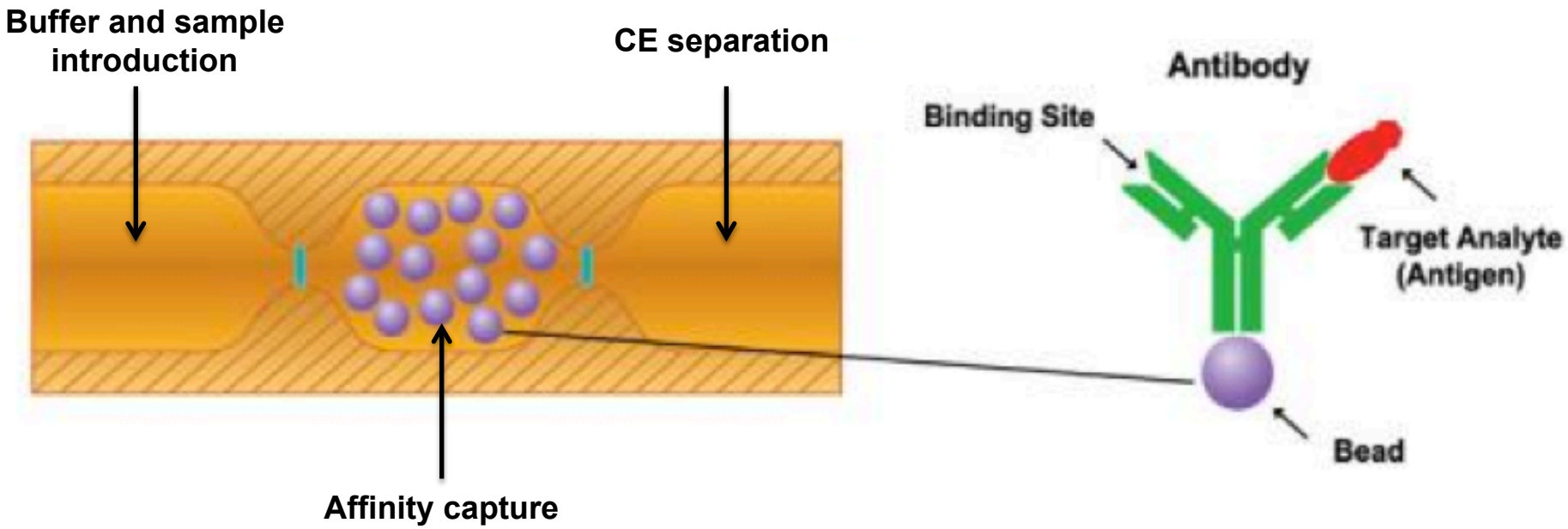
Caseins from Cow, Ewe & Goat Milk



1,3,5,7 – bovine
2,4 – ovine
6 – caprine
8,9 – ovine/caprine

Molina, Martin-Alvarez, Ramos, *Int. Dairy J.* 1999, *9*, 99.

Immunoaffinity CE

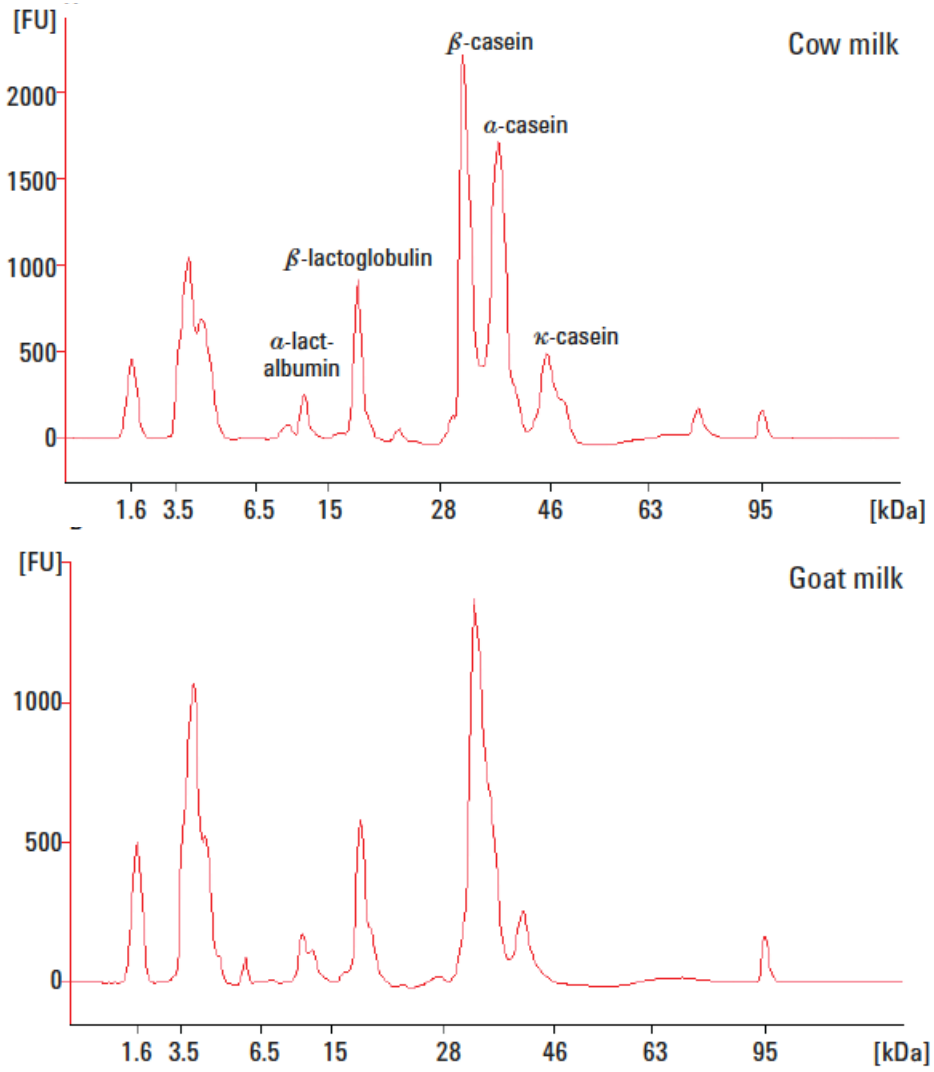


Immunoaffinity CE coupled with MALDI-MS

- Magnetic beads functionalized with appropriate antibodies used for β-lactoglobulin and α-lactalbumin immunocapture inside the capillary
- After elution from the beads, analyte focusing and separation performed followed by MALDI-MS analysis
- Limit of detection in the low nanomolar range

Lab-on-a-Chip

- Based on capillary electrophoresis
- Applicable to wide range of protein separations
- Rapid quantitative analysis



Summary

- Electrophoresis is a powerful technique for protein analysis
  - Wide range of separation mechanisms available for protein characterisation
- A wide range of instrumental approaches are available
  - Advances in lab-on-a-chip making automation and rapid analysis a reality

Thank you for your attention…

Questions?