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PRODUCTION OF SYNTHETIC AMPHOLYTES

FOR

ISOELECTRIC FOCUSING

By

KENNETH A. PEARSON

AND

DAVID W. OATES

NEBRASKA GAME AND PARKS COMMISSION 1979

*Production of Synthetic Ampholytes
for Isoelectric Focusing **

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A B S T R A C T

Synthetic ampholytes can be prepared inexpensively in a small laboratory. With approximately \$200.00 of glassware (less if some components available), vacuum source, heat source and \$5.00 worth of chemicals, one can prepare liters of ampholytes (ph range 3-10). Commercial prices now range from \$75.00 to \$100.00 for 25 ml, so a substantial savings can be realized.

In our search for a technique suitable for differentiating white-tailed and mule deer blood and tissue, isoelectric focusing (IEF) was initially avoided because of the cost of ampholytes. When other techniques failed to differentiate serum proteins we finally turned to IEF. Subsequent success in differentiating white-tailed and mule deer blood and tissue by this technique (Oates et. al. 1979) and the intention to use a similar technique for differentiating species of waterfowl provided sufficient incentive for us to attempt to produce our own ampholytes.

IEF is a relatively new high-resolution technique used for electrophoretic separations of proteins and other amphoteric substances. It is an equilibrium technique in which the isolation of amphoteric macromolecules is based upon their relative isoelectric points in a ph gradient. This phenomenon was observed by Ikeda and Suzuki (1912) following electrolysis of a mixture of amino acids. The ascending sequence taken by those amino acids between the anode and cathode correlated directly to their isoelectric points.

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IEF concepts were further advanced by Krolin (1954, 1955) and Svensson (1961, 1962). Svensson's criteria for useful ampholytes included: low molecular weight and chemical properties that differed from the substance to be separated. Good conductivity, buffering capacity and water solubility at the isoelectric point were also essential. A procedure for synthesizing molecules meeting many of Svensson's criteria of a natural pH gradient was developed by Vesterberg (1969). Vesterberg's process along with refinements are currently employed in production of the expensive commercially available ampholytes. Vinogradov (1973) and Righetti (1975) attempted to duplicate Vesterberg's work to obtain a more economical source of ampholytes. They succeeded in producing a product comparable to commercially available ampholytes in the pH 3-10 range. Modifications of Vesterberg, Vinogradov and Righetti's techniques were employed in our laboratory.

These ampholytes were prepared by coupling carboxylic acid to polyethylene polyamines to produce a series of homologues and isomers of polyamino carboxylic acids with closely spaced pI values. The reaction producing these isomers and homologues proceeded via an anti-Markovnikov addition or more specifically a Michael addition.

Materials

Chemicals:

Ethyleneamine (E-100)

(A mixture of polyethylene polyamines primarily with six or more amine groups). Available by request for research purposes in sample lots from Dow Chemical, Organic Chemicals Department, Midland, Michigan 48640.

Acrylic Acid 99% (Propenoic acid)

Available from Aldrich Chemical Company. Approximate cost: 500 g for \$4.00.

Nitrogen (ideally oxygen free)

Glassware: Costs can vary depending upon available glassware and desired sophistication of the purchased components. Vacuum distillation apparatus and reaction chamber components are available for approximately \$200.00 (see Appendix I).

Equipment:

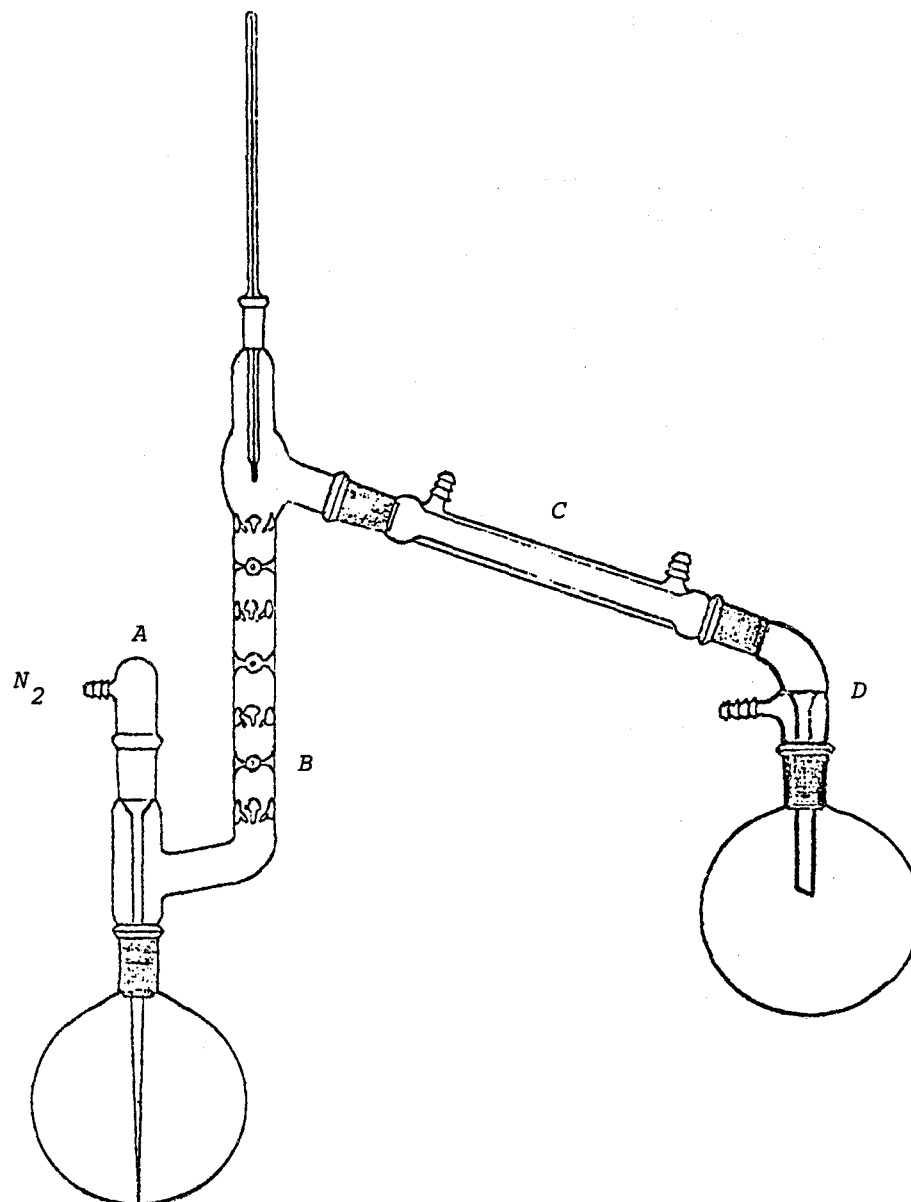
Magnetic stir or mechanical stir
Hot plate or heating mantle
Vacuum source [Pump (should be trapped) or aspirator]

The reactive nature of the reagents necessitated distillation prior to the coupling process. Polyamines can react with CO_2 to form carbamic acid-like or urea-like compounds which can contaminate the final product thus effecting reproducibility. Acrylic acid readily self-polymerizes in the presence of oxygen so commercial preparations contain an inhibitor (hydroquinonemonoethyl ether). This inhibitor should be removed just prior to coupling with the polyamine by vacuum distillation. Distillation of reagents is best accomplished under reduced pressure and a N_2 atmosphere in a system similar to the one reported by Righetti, et al. (1975). Since we used a water aspirator and a hood the traps described by Righetti were not employed. The boiling points for the reagents that could be used to produce ampholytes are listed in appendix II.

The coupling process is also conducted under a N_2 atmosphere to limit competing reactions with CO_2 and O_2 .

Procedure

The distillation apparatus (Fig. 1.) was set up in a hood. Approximately 500 ml. of ethyleneamine was placed in a 1 l. distillation flask and the system was purged with nitrogen. The nitrogen inlet port (A) was



Apparatus for Distillation at Reduced Pressures

Figure 1. A) Capillary for nitrogen purging-replaced with stopper;
B) Vigreux distillation head; C) Condenser; and
D) Vacuum takeoff adapter.

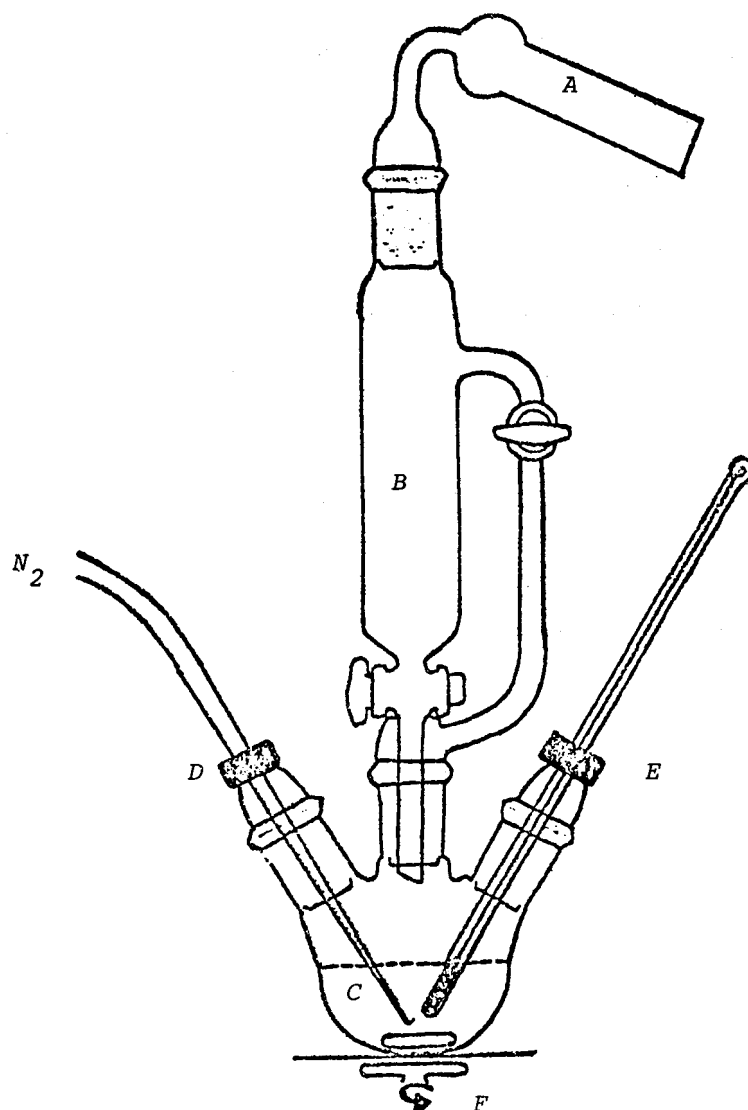
replaced with a glass stopper and a 10-15 mm Hg vacuum was placed on the system with an aspirator. Heat was supplied by a 450 watt heating mantle. The system was finally wrapped in glass wool up to the thermometer to prevent excessive heat loss. Approximately 250 ml of a yellow-brown distillate was then collected.

Maximum temperature was between 260° - 270° C. The remaining tar-like residue was discarded.

This procedure was repeated with the distillate. Small amounts of lower boiling point products were observed at 70° C, 115 - 120° C, 130 - 135° C, 140° C, 147° C, 154° C, 163° C. A fair volume was collected between 200° and 215° C. The fraction collected between 234° and 252° C constituted the major portion of the distillate and it, along with the lower boiling point fractions, was stored in a glass stoppered flask until ready for use.

Acrylic acid readily polymerizes in the presence of oxygen. Due to the highly reactive and self-polymerizing nature of acrylic acid it was vacuum distilled just prior to use to remove the commercial inhibitor.

A ratio of Vesterberg's (1969) formulation (0.09 M acrylic acid to 0.03 M pentaethylenhexamine (PEHA) dissolved in 30 ml water) as used for ampholyte production. Freshly distilled acrylic acid (26 ml) was added dropwise to the PEHA (28 ml PEHA dissolved in 120 ml of distilled water). The system (Fig. 2.) was continuously flushed with nitrogen which, along with the stirring bar, aided in the agitation of the reaction mixture. The acrylic acid was slowly added dropwise over a period of 60 minutes to the recommended N:COOH ration of 2:1 [For calculations the distillate was assumed to have a specific quantity of 1.0 and to be entirely $C_{10}H_{28}N_6$ (PEHA)]. More or less acid will result in an increase in the fractions of ampholytes with pI's either above or below pH 7.0.



Reaction Chamber for Ampholyte Production

Figure 2. A) CaCl_2 trap; B) Acrylic acid in a sidearm burette;
 C) Polyamine solution; D) Capillary for nitrogen purging;
 E) Thermometer for temperature monitoring if Dubnoff shaker
 not available and F) Combination magnetic stir - hot plate.

The mixture was then transferred into glass stoppered Erlenmeyer flask and placed in a Dubnoff shaker (a magnetic stir-hot plate, or water-bath and mechanical stir, may also be suitable) for 16 to 20 hours at 70° C. The ampholytes obtained were diluted to 40% w/v and frozen in amber vials.

These ampholytes when compared to commercial ampholytes (pH 3-10) gave similar results when white-tailed and mule deer tissue esterases were compared to one another. These esterase patterns could easily be distinguished from one another.

By distilling E-100 and utilizing some of the lower boiling fractions in the reaction mixture one can obtain a fairly smooth pH gradient between pH 3-9.5. There may be some areas where there are low ampholyte concentrations; but if necessary, spiking with small amounts of narrow range commercial ampholytes should improve the pH transition in these areas. The ampholyte we produced was suitable for our purposes without spiking, or removal of chromophoric ampholytes.

APPENDIX I

Components for distillation apparatus

and

reaction chamber (all joints 24/40)

Distillation Apparatus (Figure 1)

<u>Quantity</u>	<u>Item</u>	<u>Vendor</u>	<u>#</u>	<u>Approx. Cost (1978)</u>
2	Flasks	Kontes	601000	\$ 4.00-6.00
1	Vigreux distillation head	Kontes	503500	40.00
1	Condenser	Kontes	452000	18.00
		or	447000	
1	Vacuum takeoff adapter	Kontes	205500	13.00
1	Gas inlet adapter	Kontes	179000	10.00
1	Thermometer	Fisher	15-002F	13.00
1	Stopper	Kontes	851000	5.00
		or	850800	

Reaction Chamber (Figure 2)

<u>Quantity</u>	<u>Item</u>	<u>Vendor</u>	<u>#</u>	<u>Approx. Cost (1978)</u>
1	Boiling flask (250 ml--3 neck)	Kontes	606020	\$22.00
1	Separatory funnel (60 ml)	Kontes	634530	47.00
1	Drying tube	Kontes	562500	8.00
1	Inlet tube	Kontes	180200	4.00
2	Adapters	Kontes	1990-0 @	5.50
2	Stoppers	Kontes	851000 @	5.00
		or	850800	
1	Gas inlet adapter	Kontes	179000	10.00

APPENDIX II

Physical constants of chemicals

which can be used for ampholyte production

<u>Compound Formula</u>	<u>Compound Name</u>	<u>M.W.</u>	<u>d(g/ml)</u>	
$C_3^H_4O_2$	Acrylic Acid (Propenoic Acid)	72.06	(a) 1.0511 ²⁰	(b) 141.0 ₇₆₀
			(b) 1.0621 ¹⁶ ₄	(b) 122.0 ₄₀₀
				(b) 103.3 ₂₀₀
				(b) 86.1 ₁₀₀
				(b) 75.0 ₆₀
				(b) 66.2 ₄₀
				(b) 52.0 ₂₀
				(a) 48.5 ₁₅
				(b) 39.0 ₁₀
				(b) 27.3 ₅
$C_2^H_8N_2$	Ethylenediamine	60.10	(a) 0.8995 ²⁰ ₂₀	(a) 116.5 ₇₆₀
	1, 2-Diaminoethane			
$C_4^H_{13}N_3$	Diethylenetriamine	103.17	(a) 0.954 ²⁰ ₂₀	(a) 208.0 ₇₆₀
$C_6^H_{18}N_4$	Triethylenetetramine	146.24	(c) 0.982	(c) 266.7 ₇₆₀
				(a) 157.0 ₂₀
$C_8^H_{23}N_5$	Tetraethylenepentamine	189.31	(c) 0.998	(c) 340 ₇₆₀
$C_{10}^H_{28}N_6$	Pentaethylenehexamine	282.38	(d) 1.004	(e) 384-400 ₇₆₀
				(f) 189-193 ₂
				(f) 180-185 ₀₅

(a) Chemical Rubber Co. Handbook of Chemistry and Physics, 49th ed.

(b) Merck Index. 9th ed.

(c) Aldrich Chemical Co. reagent bottle

(d) Private communication, Dow Chemical Co.

(e) Approximate values derived from use of Figure 3

(f) Vesterberg (1969)

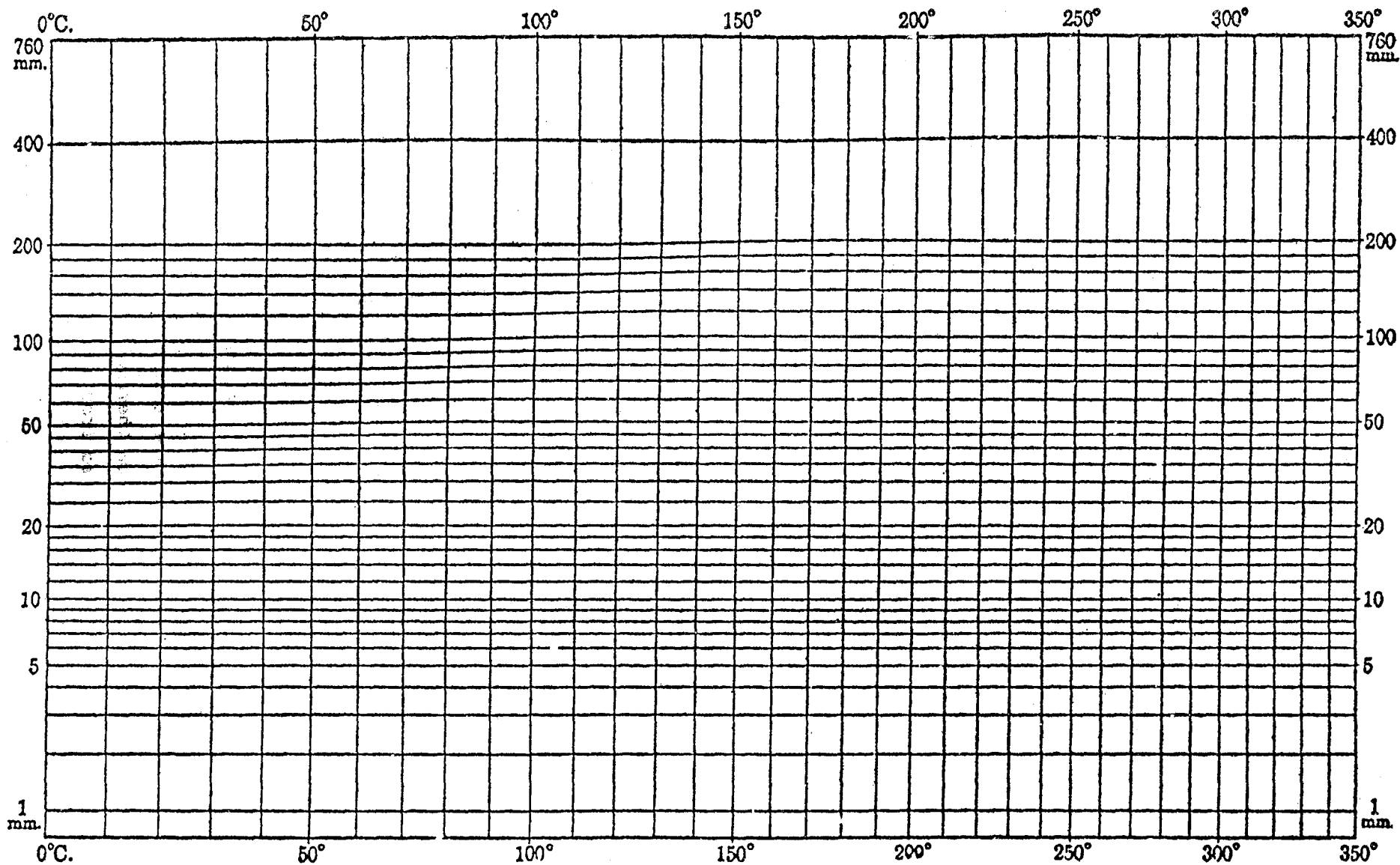


FIG. 3. Chart for Plotting Boiling Points at Reduced Pressures

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