Cook Your Own Ampholytes For Use In Isoelectric Focusing And Chromatofocusing

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Abstract: Isoelectric focusing and chromatofocusing are techniques to separate amphoteric compounds based on their isoelectric points. The commonest used compounds to generate a pH gradient in these techniques are ampholytes. The prohibitively expensive cost of carrier ampholytes limits the application of these techniques in less advantaged parts of the world. A simple and inexpensive method is described in this paper to prepare a broad pH range (pH 3 to 11) ampholytes for use in isoelectric focusing or chromatofocusingin any routine laboratory. The method involves crosslinking polyethylene polyamine to create complex cross-linked polyaminesin the first step. Whereas, the second step involves reacting the cross-linked polyamines with an unsaturated carboxylic acid to produce ampholytes. [Kalaria T Natl J Integr Res Med, 2021; 12(3):105-109]

Key Words:ampholytes, isoelectric focusing, chromatofocusing

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Introduction: Proteins including antigens, antibodies and enzymes are amphoteric in nature and can react with both acids and alkalis depending on the pH. This property is used in iso-electric focusing (IEF) [1] and chromatofocusing[2,3] to separate, analyse and purify the proteins. Carrier ampholytes are the commonest compounds used to generate a pH gradient in both these methods. For IEF, immobilised pH gradient is an alternative[4].

Ampholytes are ionisable substances that could form either anions or cations based on the pH and have buffering capacity at their iso-electric point (pI). Ampholytes migrate to their pI to form a pH gradient in IEF and chromatofocusing. As they approach their pI, they gradually become less charged.

If they diffuse from their pI, they gain a net charge and, as a result, return to the pI. Commercial ampholytes mixtures contain heterogeneous mixtures of numerous zwitterionic species with a differing number, and often different types, of acidic and alkaline groups.

Most biological proteins have their isoelectric points in the pH range of 3-10 and therefore ampholytes covering this pH range are useful in separation techniques. Ampholytes covering the broad pH range of 3-10 as well as pH intervals of 2-4, 4-6, 6-8, 8-10 and 9-11 for better resolution and more targeted separation are available from various manufacturers[5].

Generally, a more uniform distribution of ampholytes species in the pH range of interest would improve the separation of molecules of interest. And therefore, the ampholyte production methods that achieve more heterogeneous ampholyte species are believed to be more desirable. Primarily uniform buffering ability and pI distribution; and secondarily molecular size and conductivity decide the suitability of an ampholyte mixture for IEF or chromatofocusing.

Undoubtedly, the separation techniques of IEF and chromatofocusing are useful tools for advanced diagnostics and research laboratories. Also, they are valuable in university laboratories as they aid understanding of principles of these separation techniques. However, the prohibitively expensive cost of commercial ampholytes coupled with relatively short shelf-life limit the availability and applicability of these methods in wider laboratories especially in the less developed parts of the world. A simple and inexpensive in-house ampholyte preparation procedure is described in this manuscript to mitigate these limitations.

Material & Methods: Ampholyte Synthesis: Earlier methods of ampholyte mixture synthesis involved selecting a few polyamine compounds and a few polycarboxylic acid compounds and reacting them together to form polyamine-polycarboxylic acid mixtures [6]. However, the resulting mixtures were not very heterogeneous as the limited number of starting compounds

used limited the number of product species formed. This could be overcome by incorporating a preliminary step to synthesize a mixture of complex polyamines by crosslinking one or more starting polyamines and thereafter in the second step reacting these cross-linked polyamines with one or more carboxyl group compounds[7]. The method described in this paper is adapted and simplified from the previously described ampholyte synthesis method by McWhinney et al [7] to make it suitable for ampholyte synthesis in a resource-limited routine laboratory. This adaptation significantly simplifies materials required, solvents used, and the technique used to create anaerobic incubation condition. An alternate, relatively easy, approach to produce a more heterogeneous ampholyte mixture involves the reaction of a carboxylic acid ester with polyethylene polyamine followed by hydrolysis of the product [8].

Materials Required:

- Amino Compound(S): Compound used in the method described here is Triethylenetetramine (TETA, MW-146.24 gm/mol, density- 0.981 gm/mL) as it was the largest polyamine easily available to the author from a laboratory chemical supplier and it was cheap. TETA is corrosive to tissues and metals, and due care should be taken to avoid any human or metallic surface contact. Using a mixture of more than one starting polyamine and even a mixture of polyamines and amino acids would yield more complex cross-linked polyamines and a more heterogeneous mixture of ampholytesthereafter. The same holds for larger starting polyamines.

- Cross Linker: The compound used here is diethyl malonate (MW-160.17 gm/mol, density-1.05 gm/mL) due to its low cost and easy availability. It is the same cross-linker used in many epoxy adhesives. Diethyle malonate is a colourless liquid with an apple-like odour and naturally occurs in grapes and strawberries. However, it could cause eye irritation on direct contact. Many acid esters are suitable to use as cross-linkers and using a mixture of more than one cross-linkers will produce more heterogeneous ampholytes.

- α-β Unsaturated Carboxylic Acid: Acrylic acid (MW-72.06 gm/mol, density-1.05 gm/mL). Acrylic acid is a severe irritant either on skin contact or on inhalation. It should be handled in a fume hood.
- Methanol
- Deionized water
- Volumetric flasks- 200 or 250 mL and 500 mL
- Glass beakers- 100 mL, 250 mL
- Weighing balance
- Parafilm

The quantities specified and the method described here is to prepare 500 mL of concentrated(42% w/v) ampholyte mixture using a simple procedurewith the least intervention.

Step 1 Preparation of Complex Crosslinked Polyamines: The procedure was started by weighing 73.12 gm (0.5 mol, 71.73 mL) of TETA in a 250 mL glass beaker. An equimolar quantity of diethyl malonate (80.09 gm, 0.5 mol, 84.09 mL) was slowly added to it. As the reactants were thick in consistency, volume measurement was difficult andweighing was preferred. A minor error in weighing should not affect the results. Diethyl malonate spontaneously reacts with air and loses its reactivity and therefore it should be handled as quickly as possible to minimise air exposure. Thereafter, 20-25 ml of methanol was added to make a slightly flowing (thin) mixture.

The mixture was transferred to a 200 or 250 mL glass volumetric flask andadditional methanol was added to make the volume up to the narrow part of the flask with thorough mixing.

Carefully 7 to 8 cm height methanol was overlaid on top of the mixture (figure 1). This was done to cut-off air exposure of the underlying mixture in the flask to create an anaerobic incubation condition. The flask opening was covered with a layer of parafilm with a pin-hole to allow methanol vapours to escape. To avoid pressure built up from methanol vapours,the glass stopper of the flask was not used.

The mixture was incubated at 40°C for 18 hours in a vented oven. The reaction temperature was limited to 40°C as the mixture containeda significant proportion of methanol.

The reaction mixture changed colour from light yellow to bright red within 1 to 2 hours (figure 1B). As complex cross-linked polyamines were produced, the reaction mixture further changed

colour to reddish yellow with a few white/yellow precipitates as well as yellow turbidity (figure 1C).

Figure 1: Changes In The Appearance Of The Reaction Mixture During The Production Of Complex Cross-Linked Polyamines.

(A) At the beginning of the reaction. Note the methanol layer in the neck of the flask to facilitate anaerobic incubation. (B) After one hour- cross linking in progress and the reaction mixture turns red. (C) Cross-linked polyamines- after 20 hours at 40°C. Some yellow-white precipitates at the bottom, an opalescent yellowish lower portion, orange middle portion, reddish top portion and clear methanol above that.

Some methanol was carefully topped up after 8-10 hrs to compensate for evaporation to ensure the mixture underneath is not exposed to air.

Step 2: Preparation Of Ampholytes: On average, there remain three available polyamine groups after cross linking per molecule of TETA added and therefore it was estimated that 0.5 mol TETA would have yielded 1.5 mol polyamine amino groups[7]. The desired molar ratio of available polyamine amino: acrylic acid is 2:1[7]. Therefore, for 1.5 mol of polyamine amino groups, 0.75 mol of acrylic acid was added.

At the end of 20 hours, the flask was removed from the oven and its contents were carefully transferred to a 500 mL glass flask. Approximately 100 mL of deionized water was added to it and mixed. Thereafter, 54.05 gm (0.75 mol) of acrylic acid was weighed in a 100 mL glass beaker, in a fume hood, while avoiding skin contact or vapour inhalation. Again, a small measurement error is unlikely to affect the end products ignificantly. Very slowly, the acrylic acid was added to the 500 mL flask in a fume hood using a 10 mL glass pipette with a rubber teat attached on top. The mixture was mixed while adding the acid slowly, with breaks, for the generated heat to dissipate.

After all the acid was added, extra deionised water was added to make the volume just below the narrow part of the flask and the contents were carefully mixed. Thereafter, some deionised water was overlaid carefully in the top narrow portion of the flask to recreate anaerobic incubation condition for the contents underneath.

The mixture was incubated in vented hot air oven at 70 °C for 16 hours. It was mixed 2 to 3 times during the last six hours of incubation. The flask was not covered in step 2; however, extra water was added every few hours to compensate for evaporation. Yellow coloured ampholytes were obtained after 16 hours.

The produced ampholytes were cooled to room temperature and werealiquotedin 50 mL dark bottles to avoid light exposure. The ampholyte solution formed was 42% w/v. The bottles werefilledto the brim and closed air-tight to minimise carbon dioxide from the air dissolving in the solution.The bottles were stored at 2 to 8 °C. Ampholytes are generally stable for over two years if stored properly. Preservatives, if desired, could be added to the prepared ampholytes before aliquoting.

Results: The ampholytes produced by this method were acidic and might require pH adjustment before they are used for analytical purposes. The yellow-brown colour of the ampholytes does not interfere with their function. However, If desired, the colour can be removed by passing the ampholytes through activated charcoal [7]. The author has not tried it.

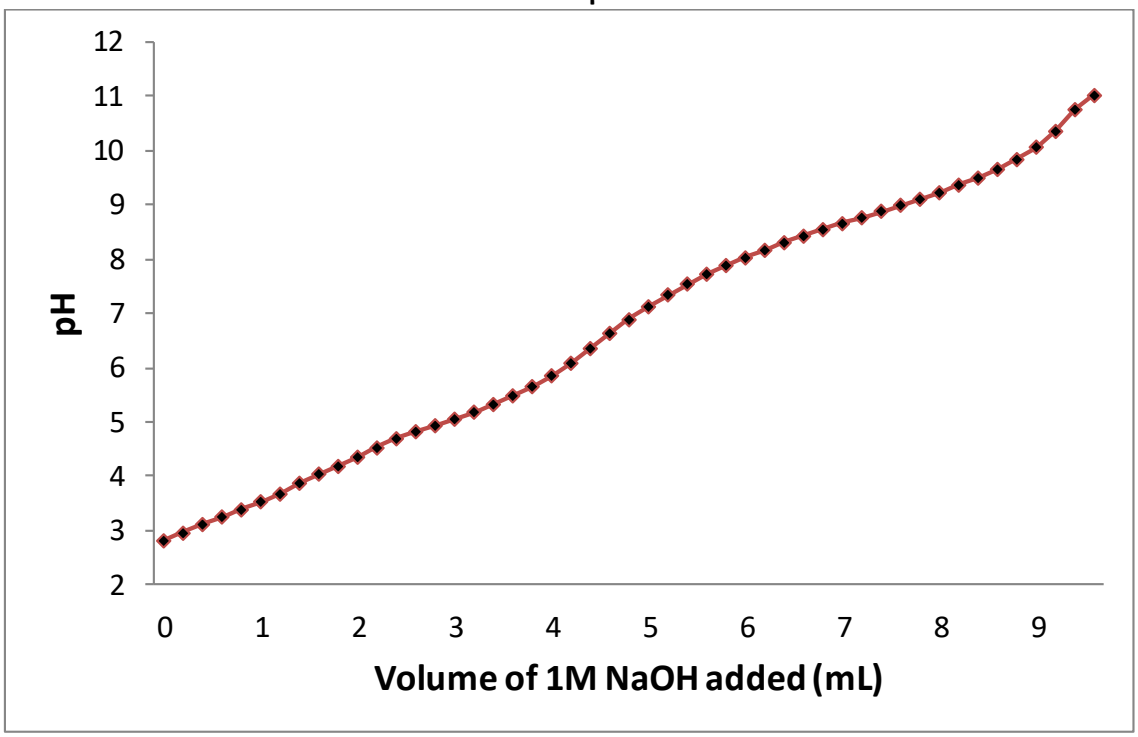
The ampholytes were titrated to access the uniformity of their buffering capacity (figure 2).

Five millilitres of the ampholytes were diluted with approximately 30 millilitres of distilled water and the pH was taken to <3.0 using concentrated hydrochloric acid.

The volume was then made to 50 mL using distilled water. The diluted mixture was titrated with 1M sodium hydroxide at 25°C till pH 11

while continuously stirring and measuring pH (calibrated Systronics pH meter model 362). The volume of NaOH added at each step was 200 µL.

Figure 2: Titration Of 50 mL 1:10 Diluted Ampholytes With NaOH. Each Point On The Graph Represents An Addition Of 200 µL of 1 M NaOH.



As can be seen from figure 2, the ampholytes provided uniform buffering capacity over a pH range of 3 to 11.

Additionally, the ampholytes produced using this method produced good results in both IEF and chromatofocusing of haemoglobins in the author's experiments. It is to be noted that the ampholytes produced with this method would not achieve very good resolution when the target pH range is very narrow (e.g. pI of all the target proteins in pH range 6 to 7). Production of an even more uniform mixture or production of ampholytes with a specific narrow pH range could be achieved by electrolysis or other fractionation techniques to form narrow pH range groups and thereafter mixing the fractions in desired proportions[5,9]. However, this is a complex process requiring expensive electrolysis equipment and is beyond the scope of this manuscript.

In summary, broad pH range ampholytes for use in IEF or chromatofocusing could be produced in-house by this simple and inexpensive method.

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