Journal of Visualized Experiments

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--Manuscript Draft--

Article Type:	Invited Methods Article - JoVE Produced Video
Manuscript Number:	JoVE61101R2
Full Title:	Separation of Bioactive Small Molecules, Peptide from Natural Source and Proteins from Microbe by Preparative Isoelectric Focusing (IEF) Method
Section/Category:	JoVE Immunology and Infection
Keywords:	Candida albicans; small molecules; Gurmarin Peptide; Gymnema sylvestre; Gymnemic acids; Natural Products Purification; Biomolecules separation; isoelectric focusing (IEF); Thin layer chromatography.
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Additional Information:	
Question	**Response**
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TITLE:

Separation of Bioactive Small Molecules, Peptides from Natural Sources and Proteins from Microbes by Preparative Isoelectric Focusing (IEF)

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KEYWORDS:

Candida albicans, small molecules, Gurmarin Peptide, *Gymnema sylvestre*, natural product purification, biomolecules separation, isoelectric focusing (IEF), thin layer chromatography,

SUMMARY:

The objective is to fractionate and isolate bioactive small molecules, peptides from a complex plant extract, and proteins from pathogenic microbes by employing liquid-phase isoelectric focusing (IEF). Further, the separated molecules were identified and their bioactivity confirmed.

ABSTRACT:

Natural products derived from plants and microbes are a rich source of bioactive molecules. Prior to their use, the active molecules from complex extracts must be purified for downstream applications. There are various chromatographic methods available for this purpose yet not all labs can afford high performance methods and isolation from complex biological samples can be difficult. Here we demonstrate that preparative liquid-phase isoelectric focusing (IEF) can separate molecules, including small molecules and peptides from complex plant extracts, based on their isoelectric points (pI). We have used the method for complex biological sample fractionation and characterization. As a proof of concept, we fractionated a *Gymnema sylvestre* plant extract, isolating a family of terpenoid saponin small molecules and a peptide. We also demonstrated effective microbial protein separation using the *Candida albicans* fungus as a model system.

INTRODUCTION:

The purification of biomolecules from complex biological samples is an essential and often difficult step in biological experiments[1]. Isoelectric focusing (IEF) is well-suited for high resolution separation of complex biomolecules where carrier ampholytes travel according to their charge

and establish the pH gradient in an electric field[3]. The first commercial carrier ampholyte for IEF was developed by Olof Vesterberg in 1964 and patented[4,5]. Carrier ampholytes are aliphatic oligo-amino oligo-carboxylic acid molecules of varying length and branching[6]. Subsequently, Vesterberg and others improved the carrier ampholytes for their expanded use in separating biomolecules[6,7].

Methods to separate biomolecules include agarose and polyacrylamide gel electrophoresis, two-dimensional gel electrophoresis (2-DE), isoelectric focusing, capillary electrophoresis, isotachophoresis and other chromatographic techniques (e.g., TLC, FPLC, HPLC)[2]. Liquid-phase IEF performed in an instrument called a "Rotofor" was invented by Milan Bier[8]. He pioneered the concept and design of this instrument and contributed extensively to the theory of electrophoretic migration. His team also developed a mathematical model of electrophoretic separation process for computer simulations[9].

The liquid-phase IEF apparatus is a horizontally rotating cylindrical cell consisting of a nylon core divided into 20 porous compartments and a circulating water cooling ceramic rod. The porous chambers allow molecules to migrate through the aqueous phase between the electrodes and permit collection of purified samples under vacuum in fractions. This purification system can provide up to 1000-fold purification of a specific molecule in <4 hours. A valuable feature of this instrument is that it can be applied as a first step for purification from a complex mixture or as a final step to achieve purity[10]. If the molecule of interest is a protein, another advantage is that its native conformation will be maintained during the separation.

The use of liquid-phase IEF has been reported widely for proteins, enzymes and antibody purification[6,10-14]. Here we describe the use of this approach for separating and purifying small molecules and peptides from the medicinal plant *Gymnema sylvestre*. This protocol will help researchers concentrate and purify active small molecules from a plant extract for downstream applications at low cost. In addition, we also demonstrate that enrichment of proteins from a complex protein extract from *Candida albicans* fungus[15] in this IEF-based system as a second example.

PROTOCOL:

1. Setup and prerunning of standard liquid-phase IEF unit

1.1. Assemble the liquid-phase IEF electrodes (anode-red button and cathode-black button) with their respective exchange membranes according to the instruction manual (see **Table of Materials**). Equilibrate the anion exchange membranes with 0.1 M NaOH and the cation exchange membranes with 0.1 M H_3PO_4 at least for 16 h when new membranes are used.

1.1.1. Store the membranes in electrolytes (0.1 M NaOH or 0.1 M H_3PO_4) between runs and do not allow to dry out.

1.2. Assemble the inner and outer portion of the electrode by aligning three oblong holes in the ion-exchange gaskets. Fill the electrodes with respective electrolytes (~25-30 mL) to prevent their membranes from drying out.

1.2.1. Do not add excess (more than 1/3rd of electrode chamber volume) electrolyte that may build up pressure inside the electrode and cause leakage.

1.3. Cover the sample collection ports with sealing tape that comes with the instrument assembly parts (see **Table of Materials**). The sample collection ports side can be identified by the two vertical metal aligning pins. Alternatively, use standard sealing tape to seal the ports.

1.4. Assemble all parts of the focusing chamber assembly in sequence (anode electrode, nylon membrane core, focusing chamber and cathode electrode) over the ceramic cooling finger.

1.5. Fill the focusing chamber with precooled distilled water (total volume of 60 mL for the standard IEF cell) using a 50 mL syringe.

1.6. Connect the liquid-phase IEF instrument to a circulating cooling water at 4 ^{o}C and prerun the unit at 15 W and 3,000 V for 3-5 min or until the voltage stabilizes.

NOTE: Generally, within one minute, the voltage will reach the maximum set values. Prerunning with distilled water helps to remove the residual ions from the focusing chamber and the nylon membrane core.

1.7. Switch off the power source and remove the water from the cell using the fraction collector. Re-seal the collection ports with sealing tape.

NOTE: Now the instrument is ready for use in step 2.4.

2. Separation and purification of small molecules and peptides from *Gymnema sylvestre* extract

2.1. Measure 0.6 g of plant extract and dissolve in distilled water (60 mL) by mixing in a roller tube for 5 min.

NOTE: Any biological sample that is soluble and free of salt can be used for separation and purification using this IEF instrument. Samples with buffering salt concentration up to 10 mM can be used with slightly decreased resolution. We are interested in the saponin family of triterpenoid compounds, the gymnemic acids, from *G. sylvestre* plant for their unique antifungal properties[16].

2.2. Centrifuge the solubilized plant extract at 10,000 x *g* for 5 min to remove insoluble particles.

2.3. Transfer the supernatant (~60 mL) to an 80 mL centrifuge tube, and mix it with 0.6 mL of ampholyte (pH 3-10) to 1% (v/v).

2.4. Follow steps 1.1 – 1.7 to prepare the liquid-phase IEF unit. The chamber is now ready to load the sample.

2.5. Use a 50 mL syringe with a 1-1/2 inch 19 G blunt end needle (comes with the instrument) and load the prepared sample with ampholyte (60 mL total) in the cell through sample collection ports.

2.6. Remove air bubbles from the sample cell by removing the focusing cell from the stand and tapping the electrode chamber to dislodge the bubbles. The presence of air bubbles will cause voltage and current fluctuations and affect the run.

2.7. Connect the unit to the water coolant (4 °C) and start fractionation with the power supply at a constant 15 W.

2.8. Run the apparatus for 3 h or until the voltage reaches a constant value.

NOTE: As the sample starts focusing, the voltage will start climbing gradually until it reaches a constant value.

2.9. After the run, prepare to collect the fractions in the harvest box (containing 20 plastic tubes, 12 mm x 75 mm, 5-6 mL volume) connected to the vacuum pump by pressing the harvest button **ON** in the IEF unit.

2.10. Align the 20-collection pins with the 20-collection ports of the focusing cell that is sealed with the tape.

2.11. Push the collection pins through the sealing tape and turn the vacuum pump **ON** simultaneously (see **Figure 2B, 2C & 2D**).

NOTE: About 3 mL fractions from each chamber will be collected into the tubes for each time. Fractions can be used for subsequent downstream applications (SDS-PAGE for peptide quantification, TLC and bioassay for small molecules).

3. Separation and purification of proteins from *C. albicans*

3.1. Grow single colony of *C. albicans* in yeast-peptone-dextrose (YPD) broth[16] at 30 °C with shaking (200 rpm) for overnight.

3.2. Collect the yeast cells by centrifugation (10,000 x *g* for 5 min).

3.3. Suspend *C. albicans* yeast cells in ammonium carbonate (1.89 g/L) buffer containing 1% (v/v) beta-mercaptoethanol (β-ME) (1/10th of the culture volume) and rotate in a roller tube for 1 h at 5 °C[15].

3.4. Remove the yeast cells by centrifugation (10,000 x *g* for 5 min) and filter the protein extract through a 0.45 µm filter.

NOTE: Protein samples from *C. albicans* cytosol, membrane or cell wall can be prepared and used for IEF fractionation. Similarly, proteins from bacteria or other biological samples (animal tissue extract) can be used after removing any salt by appropriate methods (*e.g.*, by dialysis or using desalting columns).

3.5. Dialyze the protein extract in a dialysis tubing (3,500 MWCO) against water for 15 h at 4 °C. Estimate the protein concentration by the Bradford dye-binding method using gamma globulin as a standard[17].

3.6. Use 500 mg of total protein in 60 mL of water containing 1% (v/v) ampholyte (pH 5-8) for the fractionation.

NOTE: Since a broad-range ampholyte (pH 3-10) does not enrich certain non-glucan attached cell wall proteins of *C. albicans* well, use a narrow-range (pH 5-8) ampholyte. An ampholyte concentration of up to 2% can be used if the sample protein concentration is more than 2 mg/mL; this minimizes protein aggregation during focusing. Always keep the samples, ampholytes, and water precooled on ice.

3.7. Repeat steps 1.1- 1.7 to prepare the IEF unit and use the protein solution from step 3.6 to load into the IEF cell.

3.8. After 4 h of focusing at a constant 15 W, harvest protein fractions (1-20) as described above (steps 2.9-2.11) and analyze on 12.5% SDS-PAGE after reducing and boiling the protein samples[18].

3.9. Stain the resolved proteins by staining the SDS-PAGE gel with the Coomassie blue dye (0.01%) solution for 2-3 h at room temperature on a rocker. Destain the gel and record the gel image using a gel imager.

NOTE: Coomassie blue dye (0.01%) can be prepared by mixing 0.01 g of Coomassie blue powder in a destaining solution containing 40% methanol and 10% acetic acid.

4. Bioactivity of purified small molecules from plant extract *Gymnema sylvestre*

4.1. Grow *C. albicans* in yeast cells as in step 3.1.

4.2. To prepare cell suspension, dilute overnight culture of *C. albicans* yeast cells (1/1000 dilution) into a fresh RPMI cell culture medium supplemented with 50 mM glucose.

NOTE: *C. albicans* converts from yeast growth to hyphae under hyphal inducing conditions (RPMI at 37 °C). Gymnemic acid small molecules are shown to inhibit the conversion of yeast cells into hyphae under hypha inducing conditions[16]. We aimed to determine if liquid-phase-IEF-separated *G. sylvestre* extract contain these bioactive molecules.

4.3. From the prepared cell suspension, add 90 µL to each well of the 96-well plate.

4.4. From each fraction (1-20 harvested fractions of *G. sylvestre* extract obtained from liquid-phase IEF, **Figure 4**), add 10 µL into the wells with 90 µL of the above cell suspension (step 4.2). Perform the assay in triplicate.

4.5. Add 10 µL of water that contains ampholyte (1%) into separate wells with 90 µL of C. *albicans* yeast cell suspension as a negative control.

NOTE: Ampholytes have bioactivity potential and so it is essential to include an ampholyte control while performing any bioassay[19].

4.6. Incubate the 96-well plate at 37 °C for 12 h and observe the inhibition of *C. albicans* yeast-to-hypha conversion under the microscope[16]. Also, determine the percentage of inhibition of yeast-to-hypha conversion.

REPRESENTATIVE RESULTS:

Separation and purification of small molecules and peptides from *Gymnema sylvestre* plant extract

Using the preparative liquid-phase IEF method, we fractionated medicinal plant extracts and cell surface proteins from a human pathogenic fungus, *C. albicans*. A schematic of these fractionation protocols is shown in **Figure 1**.

From 20 fractions of *G. sylvestre* extract obtained from liquid-phase IEF, the dark-colored molecules (terpenoid saponins) were found to migrate and be enriched at the anode end (pH 2-3) and the light-yellow clear fractions were observed at the cathode end (pH 8-9) (**Figure 2**). Aliquots (20 µL) from each fraction (1-20) were resolved on 15% SDS-PAGE after reducing and boiling the samples. A Coomassie blue-stained gel shows the diffused polypeptide band of about 5 kDa that is enriched in fractions 16-19 (**Figure 3**). It has been reported that the *G. sylvestre* plant contains a 35 amino acid gurmarin basic polypeptide with the predicted molecular weight of 4,209 Da[20]. Bacteria, plants and animals contain peptides; many of them are circular (knottins) and stable with wide range of biological activities such as insecticidal and antimicrobial properties[21,22].

Biological activity of separated gymnemic acids

The *G. sylvestre* plant also contains gymnemic acids (terpenoid saponins) as major constituents[16,23,24]. As expected, these small molecules in fraction 1 and the next few fractions were not detected by SDS-PAGE and Coomassie staining (**Figure 3**) since they are non-proteinaceous. However, these small molecules can be separated by TLC and detected under UV light (**Figure 4A**, lane F1). Fraction 10 did not contain a detectable amount of these small molecules on TLC suggesting most of the organic small molecules were enriched in fractions 1-3. Gymnemic acids (GAs) molecules were shown to inhibit *C. albicans* yeast-to-hypha transition[16,25]. We assayed all 20 fractions collected in this study for their inhibitory activity against *C. albicans* yeast-to-hypha conversion and hyphal growth[16]. The results are shown in **Figure 4B,4C**. The highest activity is observed in fraction 1, which agrees with the TLC results where several spots can be seen. Isomers of gymnemic acids exist and all have similar biological activities[10]. These isomers were separated in fraction 1-3 and show inhibition of *C. albicans* hyphal growth (**Figure 4A,4C,** fraction 1). The degree of hyphal inhibition was gradually decreased as it goes from 1 to 10. Little or no activity was obtained in fractions 10 and above.

Separation of cell surface proteins from pathogenic fungus, *C. albicans*

Results from liquid-phase IEF fractionation of *C. albicans* cell surface proteins are shown in **Figure 5**. These cell surface proteins play important roles in *C. albicans* adhesion and pathogenesis[26]. Several enriched proteins (arrows) in different fractions were observed. This may allow identification of their immunological reactions with Candida infected human serum and/or their identification by mass spectrometry. Similarly, proteins from other cellular fractions (e.g., cytoplasm and cell wall) can be fractionated using this IEF method. The liquid-phase IEF-based purification will allow identification of low abundance proteins from complex biological samples, when coupled with mass spectrometry analysis.

Figure 1: Flow chart showing the experimental workflow. Stepwise liquid-phase IEF fractionation procedures and subsequent downstream assays are depicted. Samples include *Gymnema sylvestre* leaf extract (sample 1) and *Candida albicans* non-glucan attached yeast proteins (sample 2).

Figure 2: Liquid-phase IEF apparatus setup and fractionation of *G. sylvestre* plant extract. (**A**) During the run, (**B**) during fraction collection, (**C**) after fraction collection, and (**D**) liquid-phase IEF apparatus parts, 1) ion exchange membranes, 2) focusing chamber and membrane core, 3) electrode assembly (negative), 4) electrode assembly (positive).

Figure 3: SDS-PAGE separations of the IEF focused plant extract fractions. L- ladder, PC- Positive control (peptide), 0 - input sample, 1-20 separated fractions. SDS-PAGE (15% resolving gel) was stained by Coomassie blue dye to visualize the resolved peptides (~5 kDa) from fractions 1-20. Fractions 1-3 contain small molecules (fraction 1 has darker color indicating enriched compounds) which cannot be stained/detected by Coomassie blue dye.

Figure 4: Analysis of IEF fractionated small molecules by TLC and determination of bioactivity against *C. albicans*. (**A**) Shows TLC analysis of small molecules from fraction #1 and #10. Activated silica gel plate was used to spot ~5 µL of samples and ran with toluene: chloroform: methanol

solvent (5:8:3 ratio) until the solvent front reached the margin. TLC separated compounds were detected under an epifluorescence UV light (310 nm). (**B**) Shows the % inhibition of *C. albicans* yeast-to-hypha conversion by different fractions. (**C**) Demonstrates the cell morphology of *C. albicans* under hypha inducing conditions. Fraction #1 shows maximum (98%) inhibition of yeast-to-hypha conversion. Other fractions and controls show no inhibition of *C. albicans* hyphal growth after 12 hours of incubation at 37 °C.

Figure 5: SDS-PAGE analysis of *C. albicans* cell surface proteins (non-glucan attached). L- ladder, 0 - input sample, 1-18 fractions collected after IEF in a standard liquid-phase IEF cell using a narrow range (pH 5-8) ampholyte. The image shows SDS-PAGE (12.5%) resolved proteins after staining with Coomassie blue dye. Several proteins were enriched in certain fractions (arrows).

DISCUSSION:

Small molecules from natural product sources (e.g., plants) include complex secondary metabolites that are highly diverse in chemical structure. They are believed to be involved in plant defense mechanisms. In addition, polypeptides are also present in plant tissues[22]. These natural product small molecules are rich sources of test molecules for drug discovery and development. However, the difficult and tedious methods required for their isolation and purification limit their use for therapeutic applications. The liquid-phase IEF approach used in this report highlights the ability to separate these small molecules and polypeptides without compromising their bioactivities.

This IEF based method offers several advantages in separating biological molecules including concentration of purified proteins from a complex mixture, maintenance of native conformation during and after their focusing, and collection of samples as individual purified fractions without cross-contamination. When necessary, samples can be re-focused with a narrow pH range to purify protein isoforms. Since a miniature IEF focusing cell (~15 mL) is available, it can be used for smaller volumes of samples as well. The new finding from this report is that organic small molecules and peptides can be separated from a complex plant extract. Though it is difficult to agree that small molecules can be separated from natural product extracts by IEF, it is plausible for those compounds that are amphoteric. The gymnemic acids that were separated from the gurmarin peptide in the *G. sylvestre* extract appear to be amphoteric as they contain a carboxylic acid group or they behave so at least in the presence of the ampholyte used. Since glycosides are bioactive natural molecules similar to gymnemic acids, the IEF method can be used to separate them from complex natural sources. Similarly, peptides from natural products may also be isolated using this liquid-phase IEF approach.

Some of the limitations in this approach are that not all small molecules can be fractionated by the IEF method as they must be water-soluble and weakly amphoteric, at least. The extract used here was prepared by a 50% methanol extraction of dried plant material but is water-soluble. The use of the IEF method for solvent-soluble and amphoteric compounds remains to be seen as some of the organic solvents are incompatible with the liquid-phase IEF instrument components. The tendency of proteins to precipitate at their isoelectric points (pI) in low ionic

strength solutions is well known. However, in a rotating IEF system, protein precipitation is reduced as the focused proteins remain in circulation at their pI point.

If one uses a high concentration of proteins in this IEF separation, precipitation may occur. To minimize protein precipitation and to improve protein focusing, urea can be used up to 3-5 M. Nonionic detergents such as CHAPS, digitonin and low concentration of detergents (0.1-1%) can also be used to reduce protein aggregation during IEF. However, urea and detergents need to be removed before analyzing the proteins for their activity and in some cases, these agents may affect protein functions. A few critical steps to consider during a liquid-phase IEF run include loading the IEF focusing cell without air bubbles, replacing the ion exchange membranes if they were damaged, and replacing the vent buttons after a certain number of repeated uses.

In conclusion, using the liquid-phase IEF method, we have shown the separation of bioactive gymnemic acids and gurmarin polypeptide from the *G. sylvestre* leaf extract. Further, liquid-phase IEF can be useful to enrich selective proteins from the complex crude extracts of pathogenic microbes.

DISCLOSURES:

The authors would like to declare no competing financial interests.

ACKNOWLEDGMENTS:

We are thankful for the funding sources from the Division of Biology and Johnson Cancer Research Center for BRIEF and IRA awards, respectively to GV. We also thank the K-INBRE postdoctoral award to RV. This work was supported in part by the Institutional Development Award (IDeA) from the National Institute of General Medical Sciences of the National Institutes of Health under grant number P20 GM103418. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institute of General Medical Sciences or the National Institutes of Health. We thank the anonymous reviewers for their helpful comments.

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Figure 1

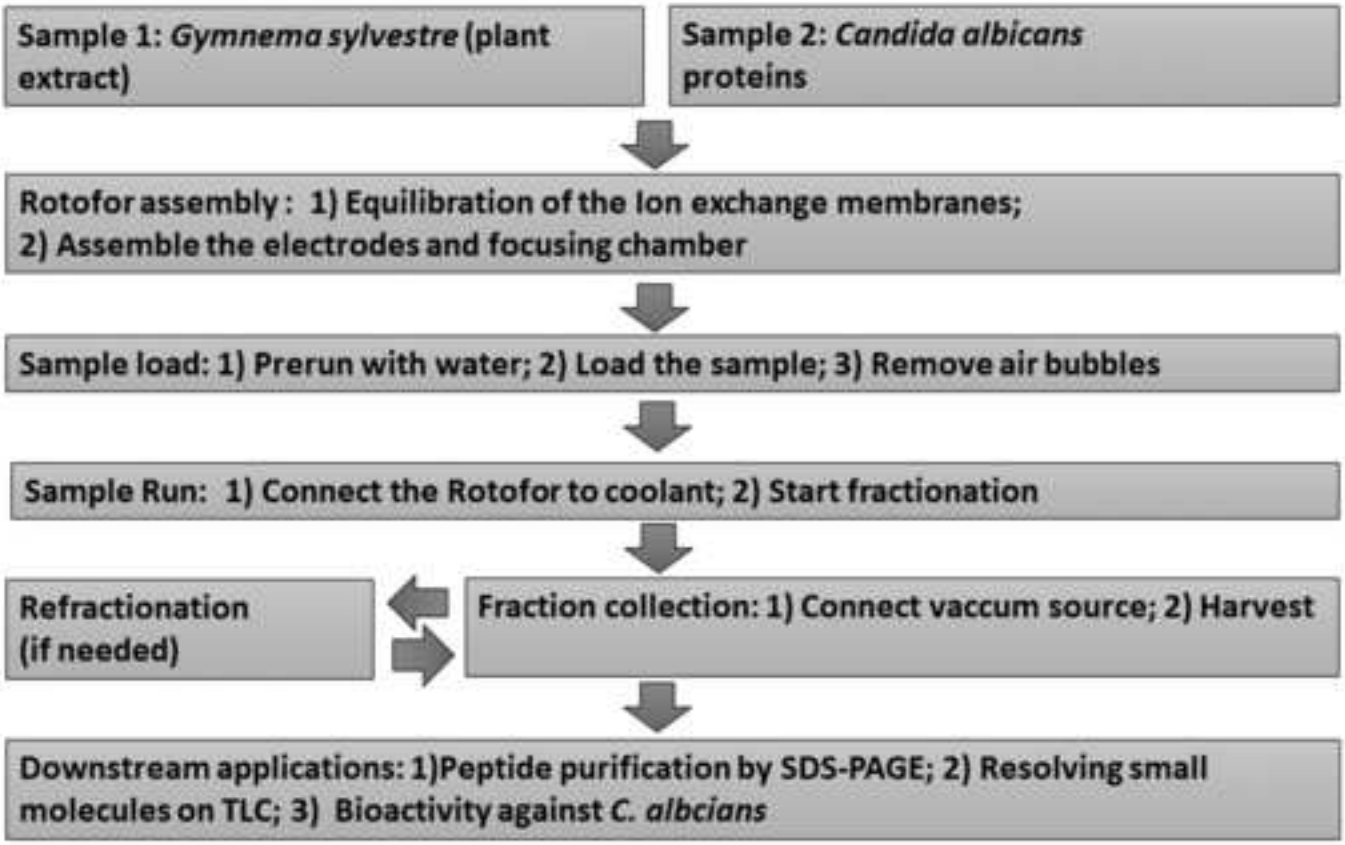

Figure 2

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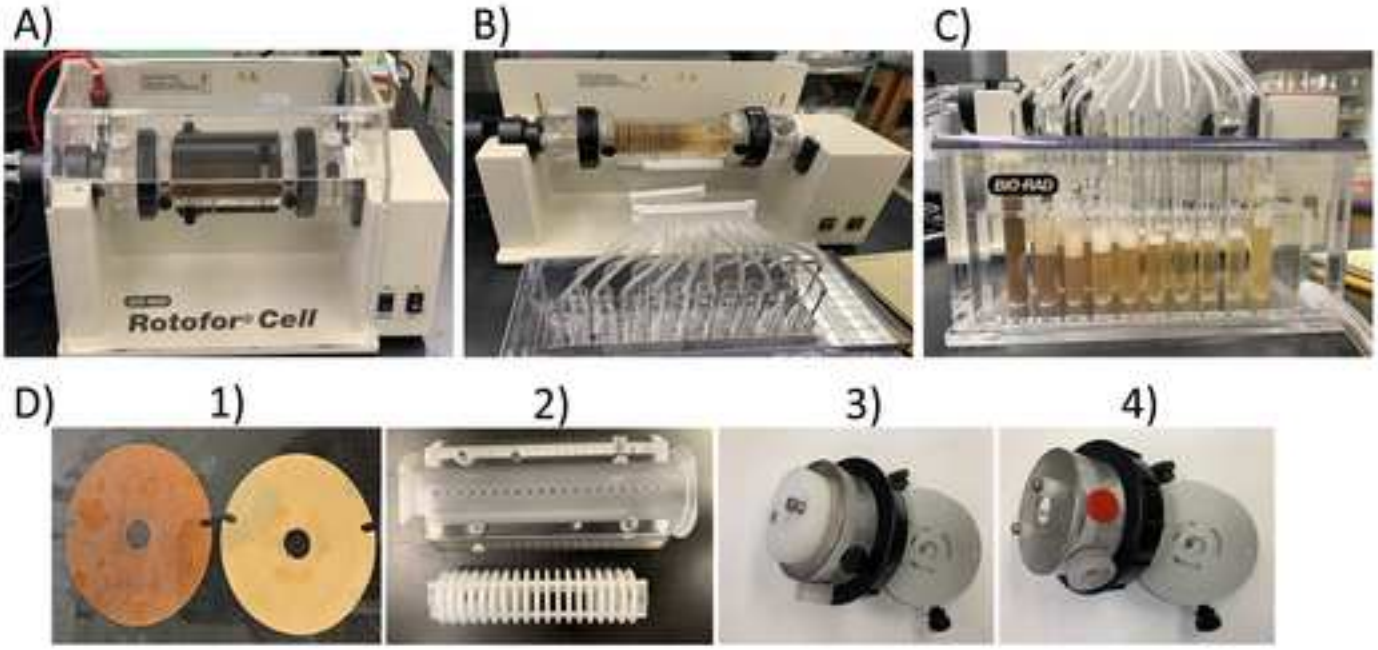

Figure 3

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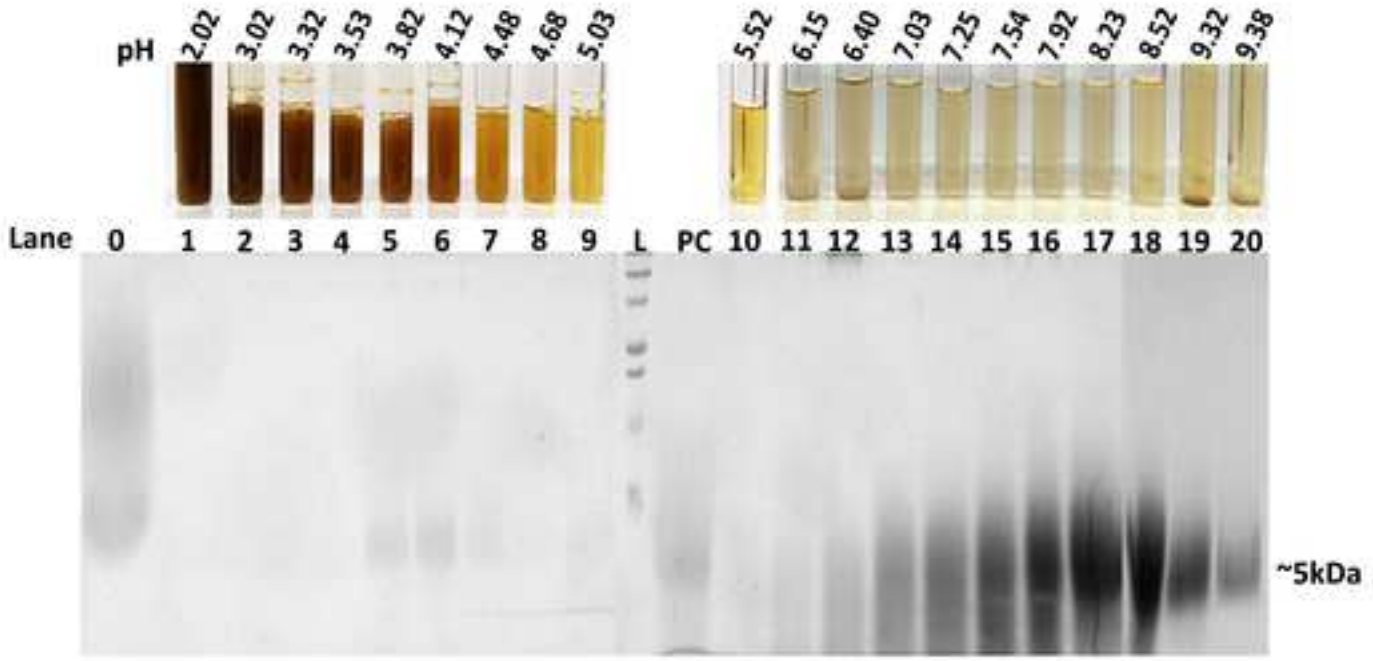

Figure 4

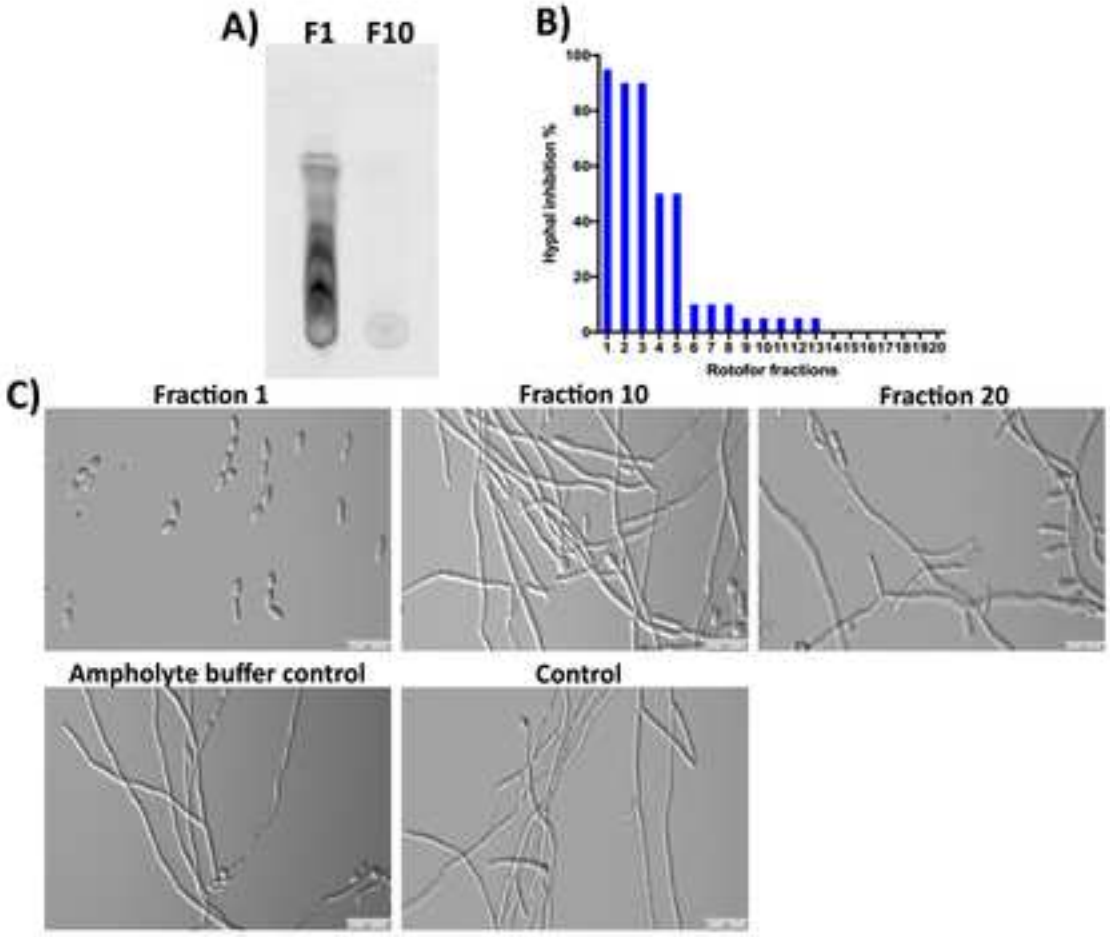

Figure 5

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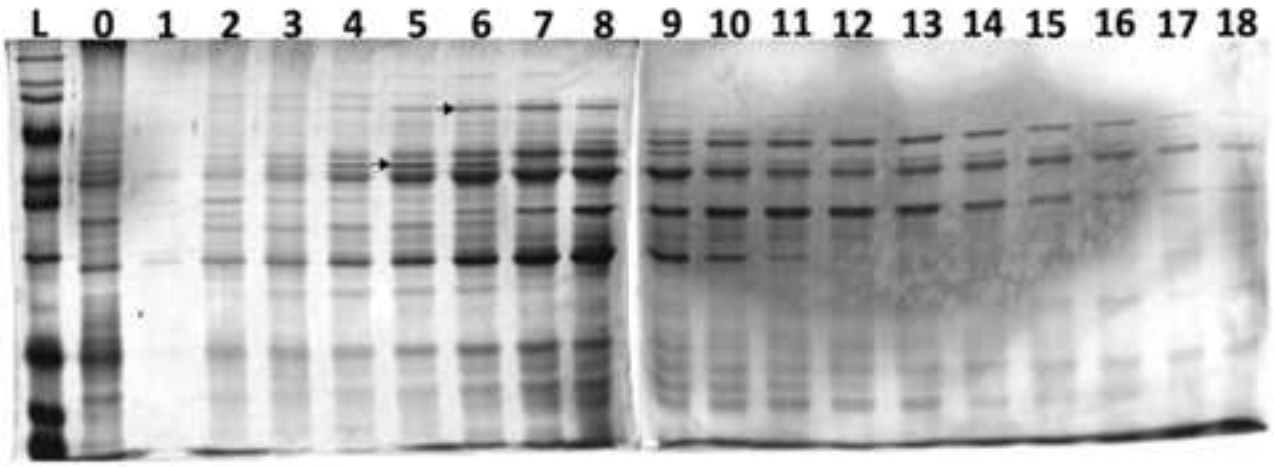

Name of Material/Equipment	Company	Catalog Number	Comments/Description
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2-Mercaptoethanol	Sigma	M3148	
Ammonium carbonate	Sigma-Aldrich	207861-500	
Bio-Lyte 3/10 Ampholyte	Bio-Rad	163-1113	
Bio-Lyte 5/8 Ampholyte	Bio-Rad	163-1192	
Compact low temperature thermostat	Lauda -Brinkmann	RM 6T	Set water cooling to 4 oC and it can be run even at 0 oC a
Coomassie Brilliant Blue R	Sigma-Aldrich	B7920	
Dialysis tubing (3,500 MWCO)	Spectrum Spectra/Por	132112T	
Gymnema plant leaf extract powder (>25% Gymnemic acids)	Suan Farma, NJ USA		
Incubator	Lab companion	SI 300R	
Microscope	Leica	DM 6B	
Mini protean electrophoresis	Bio-Rad		
pH meter	Mettler Toledo	S20	Useful to determine the pH of the Rotofor (liquid-phase I
Rotofor	Bio-Rad	170-2972	http://www.bio-rad.com/webroot/web/pdf/lsr/literatur
RPMI-1640 Medium	HyClone	DH30255.01	
Sealing tape	Bio-Rad	170-2960	Scotch tape may also be used.
Sorvall legend micro 17 centrifuge	Thermo scientific	75002432	
TPP tissue culture plate -96 well flat bottom	TPP	TP92696	

s when it passes through the Rotofor cooling core, the circulating water temperature will be around 5 or more depending on the voltage.

EF) fractions
e/M1702950E.pdf (Rotofor Instruction manual for assembling the unit)

Editorial comments: (01/24/2020)

1. Please employ professional copy-editing services. The language in the manuscript is not publication grade. There are many missing articles and some subject-verb agreement issues. Please avoid colloquial language and superlatives. The writing must be objective and without bias.

Response: We thank the Review Manager/Editor for excellent editorial assistance and helpful suggestions. The entire manuscript is edited and corrected for grammatical errors by a professional service member. These changes are marked with track changes in the revised manuscript (with track changes).

2. JoVE policy states that the video narrative is objective and not biased towards a particular product featured in the video. The goal of this policy is to focus on the science rather than to present a technique as an advertisement for a specific item. To this end, we ask that you please reduce the number of instances of "Rotofor" within your text. The term may be introduced but please use it infrequently and when directly relevant. Otherwise, please refer to the term using generic language.

Response: We have modified several statements throughout the manuscript to reflect the unbiased experimental narrative. We used the term “Rotofor” minimally in the text and used the generic term, ‘liquid-phase IEF’ instead in the revised version. The changes can be seen in “with track change” of the manuscript version.

3. Additional details are needed in the protocol. Please see the comments in the attached manuscript.

Response: More details and additional steps are included in the revised version as recommended (please see the “comments” version of the manuscript.

4. Please do not abbreviate journal titles in the References.

Response: Full names of the Journal titles are included in all the manuscript versions.

Responses to Reviewer’s comments (Earlier version)

Reviewer #1:

Manuscript Summary:
The manuscript by Vediyappan et al. describes an original and new approach they successfully used to separate small molecules based on an Iso-Electrical Focusing. This principle is well

known for its use to separate proteins not based on size but pI. However, but the application in small molecules is novel. Moreover, instead of using more common and well known separation on gels (which very much limits down-stream applications), here, they use an instrument manufactured by Bio-rad, known as a Rotofor. The manuscript and technique used is particularly well suited for JoVE, as these instruments are rather complex and the visualization will greatly benefit (novel) users.

Response: We thank the Reviewer for listing out the major strength of our manuscript. We read the comments carefully and addressed them in the revised manuscript.

Minor Concerns:

Q1. -The abstract mentions "rotofor-based preparative IEF" without explanation. This is the technique used and the authors should explain what it entail in one or two sentences. Like "here we demonstrate that these samples be separated based on their pI using an iso-electric focusing techniques using the instrument Rotofor, which allows to collect fractions" or something like that.
Response: The abstract has been updated and suggested changes were included. (lines, 18-19, 23).

Q2. -in abstract; not sure what "high application potential" means.

Response: The sentence was modified like “The Rotofor based IEF has appealing features such as low cost and simplicity. It can be performed in any laboratory with access to a Rotofor” (lines 29-31).

Q3.-Authors should remove superlatives on p2:
"any biological experiment" purification is important when the goal is to gain a molecular understanding in molecular biology or biochemistry, but there are many scientist doing biological experiment without purifying biomolecules.
"one of the finest"

Response: These types words were removed in the revised manuscript as the reviewer suggested.

Q4- "ampholytes travel to their charge in the established pH gradient"

Response: The sentence is updated.

Q5- P2 "to purify complex biological samples" should be to separate or to purify a compounds out of complex.

Response: The sentence is modified in the revised version (line, 72)

Q6 - P3 sealing tape. Please specify if this is specific tape or some type of tape from stationary store.

Response: We used the sealing tape from Bio-Rad (Cat #1702960). Since commercial name/language can't be used in the manuscript text, this item is referred to Table 1. Alternatively, the standard clear adhesive tape (scotch tape) can also be used for this purpose.

Q7 - After point 1.4 at p 3 may be added as point 1.5, 'Now instrument is ready for use in step 2.4'?

Response: We added a subsection as the reviewer suggested (subsection 1.7, line 112)

Q8 - Authors state any complex biological sample that are soluble and free of salt can be used. First, do authors have some idea what levels of salt are tolerated? most lysates will have some salt so that would be useful information. Second, for gel IEF, samples are dissolved in urea and chaps, while SDS and other charged detergents are detrimental. I assume detergents will be bad here as well?

Response: The rotofor is good to run without salt for obtaining better results. Samples with buffering salt concentration up to 10mM can also be used with slightly decreased resolution.

Q9- Maybe it would be useful for readers if the authors could comment on the difference with gel IEF. As here samples retain their native fold (and maybe remain in quaternary complex for protein complexes?) the location their migration might not correlate with the pI as it would on gels IEF, where polypeptides are denatures by urea?

Response: This point is discussed in the discussion section (lines 272-273).

Q10- Step 2.4 what is the total volume that can be loaded? Also, pre-run is in part described in section 1 and then in part in section 2.4, maybe move all pre-run info and condition to section 1.

Response: The total volume of the standard Rotofor cells is 60 ml and it is mentioned in sub section 1.5.

Q11- Step 2.7 "with the constant power supply" please explain what this means.

Response: This is a power setting that is available in a high voltage power source. This setting provides up to or over 3,000 voltage that is needed for IEF-Rotofor. Under this setting, power (Watts) remains constant.

Q12- Do the isolated samples become "contaminated" with ampholytes? Is this a problem and can they be removed if desired?

Response: The ampholyte may or may not interfere with bioactivity, and it also depends on the type of assay. In our study, we used ampholyte control and it did not affect the assay (step 4.4 & respective note, lines 182-184). If the user finds an ampholyte problem, the ampholyte in the focused protein solution can be removed by dialyzing it against a 1% ammonium sulfate solution. These details are described in the Bio-Rad's manual and we refer the reader to Table 1 for manual access freely.

Q13- Figure 4 please specify what the control is.
Response: Control is *Candida albicans* alone without bioactive compounds (a type of negative control). Since hyphal growth is not inhibited, we see only hyphae.

Q14- Do the authors have any estimate of the complexity of their plant extract and their level of purification? Clearly, they separate the dark compound as seen in solution from the ~5 kDa component, but the SDS-PAGE gel is not ideal to detect all compounds.

Response: Plant extract is complex! The small molecules that separated by Rotofor are isomers (closely related by their chemical nature and mass). This study was aimed to determine if IEF based Rotofor can separate the small molecules from other compound such as peptide (~5kDa) in the plant extract. Indeed, we can. Since the small molecules are non-protein, they can't be detected by CBB staining. However, these small molecules can be detected on TLC, a standard method for those compounds.

Reviewer #2:

Manuscript Summary:
Presents an inexpensive purification scheme from Natural product sources

Major Concerns:
Language and style need work

Response: The manuscript has been revised thoroughly and included all the suggestions recommended by the reviewer.

Minor Concerns:
Attached

Response: We thank this reviewer for helpful comments and edits. We have included all the suggestions in the revised manuscript.

Reviewer #3:

Major Concerns:
In their manuscript Veerapandian et al. show the application of the Rotofor (a very well ingrained instrument in biological separations) to the small-scale separation of bioactive small molecules and for proteins.

There are several flows with this manuscript, which apparently would be used as a basis for a JoVE film. Here is a list:
Response: We thank the Reviewer for listing out several critical points to improve the manuscript. We have addressed most of the questions raised by the reviewer. We sincerely hope the revised manuscript has improved significantly.

Q1 - It is appalling that the authors do not even quote Milan Bier, the inventor of Rotofor as well as of other flowing devices based on isoelectric focusing (IEF). Their ignorance in the field has no excuses;

Response: We regret our oversight. We have included the significant contribution of the inventor and cited appropriate references in the introduction section (lines 60-64).

Q2- They do not seem to know much on the literature of IEF, including a proper description of the properties of the carrier ampholytes, a classical piece of work done by Olof Vesterberg, a pupil of Svensson-Rilbe. It might be helpful for them to read some classical books on these topics, such as P:G: Righetti "Isoelectric Focusing: Theory, Methodology and Applications", Elsevier, Amsterdam, 1983 (aged but still fundamental). They might also take advantage of P.G. Righetti "Immobilized pH Gradients: Theory and Methodology", Elsevier, Amsterdam, 1990, a book that will enlarge their knowledge in the general field of IEF.

Response: Again, we thank the reviewer for insightful comments. We have revised the sentences and included the outstanding contributions of the discoverer of the Ampholyte (lines 52-55).

Q3- Having stated that, it is surprising to me that this topic should be the object of yet another publication and film, since all that they observe and recommend is to be found in the Bio Rad Rotofor system instruction manual. It is a fact that they have to refer the reader to this basic manual. I confess too that I have heard several times lectures by Bio-Rad scientists showing in extenso the set-up of the instrument and its applications, even better detailed than as presented here.

Response: As per the JoVE's guidelines, we have updated the protocols for Rotofor use and its assembly.

Q4- The authors do not seem to appreciate and do not even discuss the limitations and possible drawbacks of this methodology. To start with, focusing of small molecules is almost an impossible dream, doe to the fact that most of them are not even amphoteric. It is a fact that they

collect the small molecules either in the anodic or in the cathodic chambers, where they will NOT be separated, of course, and will probably be oxidized/reduced by their respective environment. Even if some of such molecules were amphoteric, by all means it is not guaranteed that they will be "good carrier ampholytes", able to focus in a very narrow pH region. For that to occur, they would have to have a very small value of pI-pKprox, something very rare in these small molecules. The same applies to small peptides. Most of them do not have a sharp pI value but focus over 3-4 pH units, something that I hope the authors do understand.

Response: We thank the reviewer for his expert inputs/prediction. We agree with the reviewer that small molecules generally are not amphoteric but it may not be true for all. There may be some exceptions as demonstrated in our study. Since the gymnemic acids contain COOH-in their sugar moiety, they may act as amphoteric. We have discussed some of these points in the discussion. Further, we showed separation of a peptide from these small molecules in the plant extract. As the reviewer indicated, the peptide may not focus as a sharp band which we understood. With all due respect, our aim was to separate the peptide and small molecules from the same plant extract. We hope that with further study one may find more interesting aspects with this type of new results.

Q5- The problem is aggravated in the case of focusing proteins. If they want to separate large amounts of proteins, they would have to understand the fact that plenty of proteins, above a certain concentration, will precipitate at their pI, this aggravating matters further. In order to prevent isoelectric precipitation, one would have to dissolve them in 6-8 M urea and surfactants, this naturally destroying their biological activity. The authors do not seem to be aware of such drawbacks and do not bother to discuss them.

Response: We have included these aspects in the discussion section.

Q6- Thus, as presented, this manuscript has not real value and would definitely NOT help any potential user. I repeat, the instruction manual of Bio Rad would be much more informative and helpful!

Response: We regret for this conclusion! We have updated several steps in the revised manuscript so that our protocol will be simple and informative for the users.