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.