CLAIMS

What is claimed is:

1. A method of identifying an ampholyte composition for capillary isoelectric focusing (CIEF) or imaged capillary isoelectric focusing (iCIEF) of a protein sample, the method comprising:
    a. identifying by mass to charge ratio (m/z) at least one marker in at least one test ampholyte composition and in a reference ampholyte composition using liquid chromatography-mass spectrometry (LC-MS), wherein the at least one marker comprises a covariance that is non-0 when covariance of Accurate Mass/Retention Time (AMRT) and/or collision cross section (CCS) measurements for a plurality of components from the at least one test and reference ampholyte compositions are plotted using an S-plot; and
    b. determining the degree of similarity or difference of the at least one marker between the at least one test ampholyte composition and the reference ampholyte composition, comprising:

        (i) determining if the at least one marker has a negative covariance and the level of the at least one marker is different between the at least one test ampholyte composition and the reference ampholyte composition, or

        (ii) determining if the at least one marker has a positive covariance and the level of the at least one marker is similar between the at least one test ampholyte composition and the reference ampholyte composition;

        thereby identifying the ampholyte composition for CIEF or iCIEF of the protein sample.

2. The method of claim 1, wherein step (a) comprises
    i. determining the AMRT and/or collision cross section measurements for the plurality of components of the at least one test ampholyte composition and the reference ampholyte composition;
    ii. plotting covariance of the AMRT and/or collision cross section measurements for the plurality of components from the at least one test and reference ampholyte compositions using an S-plot; and

iii. selecting at least one component that is different between the at least one test ampholyte composition and the reference ampholyte composition, wherein the difference comprises a covariance that is non-0 in the S-plot;
thereby identifying the at least one marker.

3. The method of claim 2, wherein the at least one component comprises a covariance in the S-plot that is less than the covariance of at least 20%, at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, or at least 80% of the plurality of components in the S-plot.

4. The method of claim 2 or 3, wherein the level of the at least one marker in the at least one test ampholyte composition and the reference ampholyte composition is different.

5. The method of claim 4, wherein the difference in the level of the at least one marker comprises a difference in level of at least 10%, at least 20%, at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, at least 80%, or at least 90% relative to a normalized level of the at least one marker.

6. The method of claim 1, wherein step (a) comprises
   i. determining the AMRT and/or collision cross section measurements for the plurality of components of the at least one test ampholyte composition and the reference ampholyte composition;
   ii. plotting covariance of the AMRT and/or collision cross section measurements for the plurality of components from the at least one test and reference ampholyte compositions using an S-plot; and
   iii. selecting at least one component that is similar between the at least one test ampholyte composition and the reference ampholyte composition, wherein the similarity comprises a covariance that is non-0 in the S-plot;
   thereby identifying the at least one marker.

7. The method of claim 6, wherein the at least one component comprises a covariance in the S-plot that is greater than the covariance of at least 40%, at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 95%, or at least 99% of the plurality of components in the S-plot.

8. The method of claim 6 or 7, wherein the level of the at least one marker in the at least one test ampholyte composition and the reference ampholyte composition is similar.

9. The method of claim 8, wherein the similarity in the level of the at least one marker comprises a level that is at least 20%, at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 95% or at least 99% similar relative to a normalized level of the at least one marker.

10. The method of any one of claims 1-9, wherein the level of the at least one marker is characterized by relative intensity of the *m/z* in a mass spectrum.

11. The method of claim 10, wherein step (b) comprises:
    i. determining a LC-MS mass spectrum of the at least one marker in the at least one test ampholyte composition and the reference ampholyte composition,
    ii. determining the relative intensity of a base peak for the at least one marker in the mass spectrums of the at least one test ampholyte composition and the reference ampholyte composition,
    iii. normalizing the relative intensity of the base peak of the at least one marker to a maximum relative intensity of the base peak measured from the at least one test ampholyte composition or the reference ampholyte composition, and
    iv. comparing the normalized relative intensities of the base peak of the at least one marker in the at least one test and reference ampholyte compositions.

12. The method of any one of claims 1-11, wherein the at least one marker comprises 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15 or 16 markers with different *m/z*.

13. The method of any one of claims 1-11, wherein the at least one marker comprises 16 markers with different *m/z*.

14. The method of claim 13, wherein the 16 markers comprise markers with an *m/z* of 280, an *m/z* of 319, an *m/z* of 329, an *m/z* of 347, an *m/z* of 373, an *m/z* of 375, an *m/z* of 376, an *m/z* of 431, an *m/z* of 504, an *m/z* of 506, an *m/z* of 508, an *m/z* of 520, an *m/z* of 534, an *m/z* of 562, an *m/z* of 906 and an *m/z* of 980.

15. The method of any one of claims 1-14, wherein the mass spectrometry comprises ion mobility Quadrupole Time-of-flight Mass Spectrometry (IMS-Q-ToF-MS).

16. The method of any one of claims 1-15, wherein the liquid chromatography comprises high-performance liquid chromatography (HPLC).

17. The method of claim 16, wherein the HPLC comprises a C4 silica column.

18. The method of any one of claims 1-17, further comprising validating the at least one test ampholyte composition by generating iCIEF electropherograms of reference proteins using the reference ampholyte composition and the at least one test ampholyte composition, thereby generating at least one test electropherogram and a reference electropherogram.

19. The method of claim 18, wherein the test ampholyte composition is validated if the at least one test and the reference electropherograms are similar.

20. The method of claim 19, wherein the similarity of the at least one test and reference electropherograms is determined by number, size, or isoelectric point (pI) of peaks,

or a combination thereof.

21. The method of claim 19 or 20, wherein the similarity of the at least one test and reference electropherograms comprises having an identical number of peaks.

22. The method of any one of claims 19-21, wherein the similarity of the at least one test and reference electropherograms comprises a difference in an area under the peak of a region of the reference and at least one test electropherograms with a p-value of less than 0.05.

23. The method of claim 22, wherein the region comprises a major reference peak, a region that is more acidic than the major reference peak, or a region that is more basic than the major reference peak.

24. The method of any one of claims 1-24, wherein the iCIEF is used to characterize a protein drug product or drug substance.