Another known method digests the polysorbate molecules then characterizes the degradants, typically by liquid chromatography coupled to mass spectrometry, followed by liquid chromatography coupled to charged aerosol detection and liquid chromatography coupled to evaporative light scattering detection. In some embodiments, the characterized degradant is free fatty acids. Therefore, most known methods for measuring polysorbate levels in samples are indirect and require destruction of polysorbate to characterize the resulting degradants or subspecies.

[0008] Moreover, prior methods identified higher than expected polysorbate levels in samples during robustness studies, including overestimations of 30% compared to expected results. Prior attempts to identify gradient programs did not resolve this issue.

[0009] Previous attempts to generate robust, simple, and cost-effective methods for accurately measuring levels of specific polysorbates have been unsuccessful. One previous attempt required solid-phase extraction to remove protein prior to further separation steps for accurate measurements, which is inefficient, time-consuming, and labor-intensive.

[0010] A need exists for an improved method for directly and accurately measuring polysorbate levels in a sample.

[0011] The present disclosure satisfies this need by providing a robust, simple, cost-effective method for directly and accurately measuring polysorbate levels.

SUMMARY

[0012] The disclosure is directed to methods for measuring levels of polysorbate(s) in a sample. The methods of the disclosure directly and accurately measure polysorbate levels in a sample in a robust, simple, and cost-effective manner.

[0013] In some examples, the disclosure is directed to a method for measuring polysorbate levels in a sample. In some examples, the method comprises applying an aliquot of the sample onto a high-performance liquid chromatography system with a mixed-mode column, eluting the sample with a gradient from a first mobile phase to a second mobile phase, and applying evaporative light-scattering detection to the eluant after exiting the column, thereby measuring polysorbate levels. In some examples, the first mobile phase comprises 0-20% of an acid, 1-70% of an organic solvent selected from of an organic solvent selected from acetonitrile, methanol, tetrahydrofuran, isopropyl alcohol, or mixtures thereof, and water. In some examples, the second mobile phase comprises 0-20% of an acid, 80-100% of an

organic solvent selected from acetonitrile, methanol, tetrahydrofuran, isopropyl alcohol, or mixtures thereof, and optionally water.

[0014] In some examples, the acid is a volatile acid.

[0015] In some examples, the volatile acid is formic acid.

[0016] In some examples, the first mobile phase and/or the second mobile phase comprises 0-2% acid.

[0017] In some examples, the organic solvent in the first mobile phase is different from the organic solvent in the second mobile phase.

[0018] In some examples, the first mobile phase comprises 2% formic acid, 20% acetonitrile, and 78% purified water.

[0018a] In an aspect, the disclosure is directed to a method for measuring polysorbate levels in a sample, the method comprising:

applying an aliquot of the sample onto a high-performance liquid chromatography system with a mixed-mode column;

eluting the sample with a gradient from a first mobile phase to a second mobile phase; and

applying evaporative light-scattering detection to the eluant after exiting the column, thereby measuring polysorbate levels;

wherein the first mobile phase comprises: 2% formic acid, 20% acetonitrile, and 78% purified water; and

wherein the second mobile phase comprises: 0-20% of an acid; 80-100% of an organic solvent selected from acetonitrile, methanol, tetrahydrofuran, isopropyl alcohol, or mixtures thereof; and optionally water.

[0019] In some examples, the second mobile phase comprises 2% formic acid and 98% isopropyl alcohol.

[0020] In some examples, the measured polysorbate comprises polysorbate 20, polysorbate 40, polysorbate 60 and/or polysorbate 80.

[0021] In some examples, the measured polysorbate comprises polysorbate 20 and/or polysorbate 80.

[0022] In some examples, the measured polysorbate comprises polysorbate 20.

[0023] In some examples, the measured polysorbate comprises polysorbate 80.

[0024] In some examples, before applying the aliquot to be measured to the column, the method further comprises applying a blank onto a high-performance liquid chromatography system with a mixed-mode column, eluting the blank with a gradient from a first mobile phase to a second mobile phase and applying evaporative light-scattering detection to the blank after it exits the column. In some examples, the first mobile phase comprises 0-20% of an acid, 1-70% of an organic solvent selected from acetonitrile, methanol, tetrahydrofuran, isopropyl alcohol, or mixtures thereof, and water. In some examples, the second mobile phase comprises 0-20% of an acid, 80-100% of an organic solvent selected from acetonitrile, methanol, tetrahydrofuran, isopropyl alcohol, or mixtures thereof, and optionally water.

[0025] In some examples, applying evaporative light-scattering detection to the blank produces a baseline signal.

[0026] In some examples, after the first blank has been applied to the column and before applying the aliquot to be measured to the column, the method further comprises applying a

second blank onto a high performance liquid chromatography system with a mixed-mode column, eluting the blank with a gradient from a first mobile phase to a second mobile phase, and applying evaporative light-scattering detection to the blank after it exits the column, wherein applying evaporative light-scattering detection to the second blank sample produces a stable baseline signal. In some examples, the first mobile phase comprises 0-20% of an acid, of an organic solvent selected from 1-70% acetonitrile, methanol, tetrahydrofuran, isopropyl alcohol, or mixtures thereof, and water. In some examples, the second mobile phase comprises 0-20% of an acid, of an organic solvent selected from 80-100% of an organic solvent selected from acetonitrile, methanol, tetrahydrofuran, isopropyl alcohol, or mixtures thereof, and optionally water.

[0027] In some examples, the first mobile phase and/or the second mobile phase was prepared within 2 weeks of performing the method.

[0028] In some examples, the method further comprises quantifying the measured level of polysorbate in the sample.

[0029] In some examples, quantifying the measured level of polysorbate in the sample comprises comparing the measured amount of polysorbate against a calibration curve.

[0030] In some examples, comparing the measured amount of polysorbate against the calibration curve identifies a quantity of polysorbate in the aliquot.

[0031] In some examples, the calibration curve is generated by separately applying one or more concentration standards comprising known amounts of polysorbate onto a high performance liquid chromatography system with a mixed-mode column, eluting the concentration standards with a gradient from a first mobile phase to a second mobile phase, applying evaporative light-scattering detection to the concentration standards after each exits the column, thereby measuring polysorbate levels, and generating a calibration curve from the measured levels of polysorbate in the concentration standards. In some examples, the first mobile phase comprises 0-20% of an acid, 1-70% of an organic solvent selected from acetonitrile, methanol, tetrahydrofuran, isopropyl alcohol, or mixtures thereof, and water. In some examples, the second mobile phase comprises 0-20% of an acid, 80-100% of an organic solvent selected from acetonitrile, methanol, tetrahydrofuran, isopropyl alcohol, or mixtures thereof, and optionally water.

[0032] In some examples, one or more of the concentration standards produces a larger signal than the aliquot and one or more of the concentration standards produces a smaller signal than the aliquot.

[0033] In some examples, the amount of polysorbate in the one or more concentration standards does not exceed the maximum detectable signal for evaporative light-scattering detection.

[0034] In some examples, the amount of polysorbate in the concentration standard comprising the largest amount of polysorbate has a peak height of about 80% of the maximum detectable signal.

[0035] In some examples, applying evaporative light-scattering detection to a blank produces a signal that is lower than a signal produced by the concentration standard comprising the smallest amount of polysorbate.

[0036] As used herein, a “blank” does not contain polysorbate. In some examples, the blank is water.

[0037] In some examples, the calibration curve is generated using three or more, four or more, five or more, or six or more concentration standards.

[0038] In some examples, the calibration curve is prepared gravimetrically.

[0039] In some examples, the calibration curve has a coefficient of determination (R^2) greater than or equal to 0.995.

[0040] In some examples, the coefficient of determination (R^2) is greater than or equal to 0.998.

[0041] In some examples, the calibration curve is prepared using a quadratic fit through zero.

[0042] In some examples, the percentage relative standard deviation of two or more aliquots of the sample is 10 or less.

[0043] In some examples, the gradient comprises 100% first mobile phase and 0% second mobile phase from 0.0 minutes to 2.0 minutes, and/or 85% first mobile phase and 15% second mobile phase from 2.1 minutes to 5.0 minutes, and/or 30% first mobile phase and 70% second mobile phase from 5.1 minutes to 7.6 minutes, and/or 0% first mobile phase and 100% second mobile phase from 7.7 minutes to 9.0 minutes, and/or 100% first mobile phase and 0% second mobile phase from 9.1 minutes to 10.0 minutes.

[0044] In some examples, the sample is applied onto the mixed-mode column at a temperature of 22-28 ^{o}C.

[0045] In some examples, the eluant is eluted from the column with a flow rate of 0.9-1.1 mL/min.

[0046] In some examples, the evaporative light-scattering detection utilizes an Alltech 3300 detector.

[0047] In some examples wherein the method utilizes an Alltech 3300 detector, the method is performed using a nebulizer temperature of the detector of 68-72 ^{o}C.

[0048] In some examples wherein the method utilizes an Alltech 3300 detector, the method is performed using a gas flow rate of the detector of 2.3-2.7 L/min.

[0049] In some examples, the evaporative light-scattering detection utilizes an Agilent 1260 evaporative light-scattering detector.

[0050] In some examples wherein the method utilizes an Agilent 1260 evaporative light-scattering detector, the method is performed using a nebulizer temperature of the detector of 43-47 ^{o}C.

[0051] In some examples wherein the method utilizes an Agilent 1260 evaporative light-scattering detector, method is performed using a heating tube temperature of the detector of 70-90 ^{o}C.

[0052] In some examples wherein the method utilizes an Agilent 1260 evaporative light-scattering detector, the method is performed using a gas flow of the detector of 1.3-1.7 L/min.

[0053] In some examples, the sample is a drug substance.

[0054] In some examples, the sample is a drug product.

[0055] In some examples, the method comprises: applying a first polysorbate concentration standard onto a high performance liquid chromatography system with a mixed-mode column, eluting the sample with a gradient from a first mobile phase to a second mobile phase, and applying evaporative light-scattering detection to the eluant after it exits the column, thereby measuring polysorbate levels; applying one or more further polysorbate concentration standards comprising different known amounts of polysorbate compared to the first concentration standard onto a high performance liquid chromatography system with a mixed-mode column, eluting the sample with a gradient from a first mobile phase to a second mobile phase, and applying evaporative light-scattering detection to the eluant after it exits the column,

thereby measuring polysorbate levels; generating a calibration curve from the measured polysorbate levels in the polysorbate concentration standards; applying an aliquot of the sample onto a high performance liquid chromatography system with a mixed-mode column, eluting the sample with a gradient from a first mobile phase to a second mobile phase; applying evaporative light-scattering detection to the eluant after it exits the column, thereby measuring polysorbate levels; and quantifying the amount of polysorbate in the aliquot of the sample by comparison against the calibration curve. In some examples, the first mobile phase comprises 2% formic acid, 20% acetonitrile, and 78% purified water and the second mobile phase comprises 2% formic acid, and 98% isopropyl alcohol.

[0056] In some examples, the method is practiced according to any of the features disclosed.

BRIEF DESCRIPTION OF DRAWING(S)

[0057] The drawings are for illustration purposes only and do not provide any limitation on the disclosure.

[0058] Fig. 1 illustrates an exemplary water blank chromatogram.

[0059] Fig. 2 illustrates an exemplary chromatogram of a local reference standard.

[0060] Fig. 3 illustrates an exemplary chromatogram for a concentration standard.

[0061] Fig. 4a and Fig. 4b illustrate exemplary polysorbate 80 chromatograms. Fig. 4a provides an exemplary chromatogram from a drug substance sample. Fig. 4b provides an exemplary chromatogram from a drug product sample.

[0062] Fig. 5a and Fig. 5b illustrate JMP statistical output for S2. Fig. 5a depicts S2 distributions. Fig. 5b depicts variability charts for S2. Fig. 5c depicts response results for S2.

[0063] Fig. 6a, Fig. 6b, and Fig. 6c illustrate JMP statistical output for S3. Fig. 6a depicts S3 distributions. Fig. 6b depicts variability charts for S3. Fig. 6c depicts response results for S3.

[0064] Fig. 7a, Fig. 7b, and Fig. 7c illustrate JMP statistical output for D1. Fig. 7a depicts D1 distributions. Fig. 7b depicts variability charts for D1. Fig. 7c depicts response results for D1.

[0065] Fig. 8 illustrates eluant peaks for multiple samples comprising polysorbate 80 and a Check standard.

CLAIMS

1. A method for measuring polysorbate levels in a sample, the method comprising:

applying an aliquot of the sample onto a high-performance liquid chromatography system with a mixed-mode column;

eluting the sample with a gradient from a first mobile phase to a second mobile phase; and

applying evaporative light-scattering detection to the eluant after exiting the column, thereby measuring polysorbate levels;

wherein the first mobile phase comprises: 2% formic acid, 20% acetonitrile, and 78% purified water; and

wherein the second mobile phase comprises: 0-20% of an acid; 80-100% of an organic solvent selected from acetonitrile, methanol, tetrahydrofuran, isopropyl alcohol, or mixtures thereof; and optionally water.

2. The method according to claim 1, wherein the acid is a volatile acid; optionally wherein the volatile acid is formic acid.

3. The method according to claim 1 or 2, wherein the second mobile phase comprises 0-2% acid; and/or

the organic solvent in the first mobile phase is different from the organic solvent in the second mobile phase; and/or

the second mobile phase comprises 2% formic acid and 98% isopropyl alcohol.

4. The method according to any one of the preceding claims, wherein the measured polysorbate comprises polysorbate 20, polysorbate 40, polysorbate 60, and/or polysorbate 80.

5. The method according to any one of the preceding claims, further comprising, before applying the aliquot to be measured to the column:

applying a blank onto a high-performance liquid chromatography system with a mixed-mode column;

eluting the blank with a gradient from a first mobile phase to a second mobile phase; and

applying evaporative light-scattering detection to the blank after exiting the column.

6. The method according to claim 5, wherein applying evaporative light-scattering detection to the blank produces a baseline signal; and/or

wherein the method further comprises after the first blank has been applied to the column and before applying the aliquot to be measured to the column:

applying a second blank onto a high performance liquid chromatography system with a mixed-mode column;

eluting the blank with a gradient from a first mobile phase to a second mobile phase; and

applying evaporative light-scattering detection to the blank after exiting the column;

wherein applying evaporative light-scattering detection to the second blank produces a stable baseline signal.

7. The method according to any one of the preceding claims, wherein the first mobile phase and/or the second mobile phase was prepared within 2 weeks of performing the method.

8. The method according to any one of the preceding claims, further comprising quantifying the measured level of polysorbate in the sample; optionally wherein

quantifying the measured level of polysorbate in the sample comprises comparing the measured amount of polysorbate against a calibration curve; and/or

comparing the measured amount of polysorbate against the calibration curve identifies a quantity of polysorbate in the aliquot.

9. The method according to claim 8, wherein the calibration curve is generated by:

separately applying one or more concentration standards comprising known amounts of polysorbate onto a high-performance liquid chromatography system with a mixed-mode column;

eluting the concentration standards with a gradient from a first mobile phase to a second mobile phase; and

applying evaporative light-scattering detection to the concentration standards after each exits the column, thereby measuring polysorbate levels; and

generating a calibration curve from the measured levels of polysorbate in the concentration standards.

10. The method according to claim 9, wherein one or more of the concentration standards produces a larger signal than the aliquot and one or more of the concentration standards produces a smaller signal than the aliquot; and/or

wherein the amount of polysorbate in the two or more concentration standards does not exceed the maximum detectable signal for evaporative light-scattering detection; and/or

wherein the amount of polysorbate in the concentration standard comprising the largest amount of polysorbate has a peak height of about 80% of the maximum detectable signal; and/or

wherein applying evaporative light-scattering detection to a blank produces a signal that is lower than a signal produced by the concentration standard comprising the smallest amount of polysorbate; and/or

wherein the calibration curve is generated using three or more, four or more, five or more, or six or more concentration standards.

11. The method according to any one of claims 8-10, wherein the calibration curve is prepared gravimetrically; and/or

wherein the calibration curve has a coefficient of determination (R^2) greater than or equal to 0.995, optionally

wherein the coefficient of determination is greater than or equal to 0.998; and/or

wherein the calibration curve is prepared using a quadratic fit through zero.

12. The method according to any one of the preceding claims, wherein the percentage relative standard deviation of two or more aliquots of the sample is 10 or less; and/or

wherein the gradient comprises:

100% first mobile phase and 0% second mobile phase from 0.0 minutes to 2.0 minutes;

85% first mobile phase and 15% second mobile phase from 2.1 minutes to 5.0 minutes;

30% first mobile phase and 70% second mobile phase from 5.1 minutes to 7.6 minutes;

0% first mobile phase and 100% second mobile phase from 7.7 minutes to 9.0 minutes; and/or

100% first mobile phase and 0% second mobile phase from 9.1 minutes to 10.0 minutes; and/or

wherein the sample is applied onto the mixed-mode column at a temperature of 22-28^{o}C; and/or

wherein the eluant is eluted from the column with a flow rate of 0.9-1.1 mL/min.

13. The method according to any one of the preceding claims, wherein the evaporative light-scattering detection utilizes an Alltech 3300 detector, optionally wherein the method is performed using a nebulizer temperature of the detector of 68-72^{o}C.

14. The method according to claim 13, wherein the method is performed using a gas flow rate of the detector of 2.3-2.7 L/min.

15. The method according to any one of claims 1-12, wherein the evaporative light-scattering detection utilizes an Agilent 1260 evaporative light-scattering detector, optionally wherein the method is performed using a nebulizer temperature of the detector of 43-47°C.

16. The method according to claim 15, wherein method is performed using a heating tube temperature of the detector of 70-90 °C; and/or wherein the method is performed using a gas flow of the detector of 1.3-1.7 L/min.

17. The method according to any one of the preceding claims, wherein the sample is a drug substance or drug product.

18. The method according to any of claims 1, 4-7 or 10-17, the method comprising:

applying a first polysorbate concentration standard onto a high-performance liquid chromatography system with a mixed-mode column, eluting the sample with a gradient from a first mobile phase to a second mobile phase, and applying evaporative light-scattering detection to the eluant after exiting the column, thereby measuring polysorbate levels;

applying one or more further polysorbate concentration standards comprising different known amounts of polysorbate compared to the first concentration standard onto a high-performance liquid chromatography system with a reversed-mixed-mode column, eluting the sample with a gradient from a first mobile phase to a second mobile phase, and applying evaporative light-scattering detection to the eluant after exiting the column, thereby measuring polysorbate levels;

generating a calibration curve from the measured polysorbate levels in the polysorbate concentration standards;

applying an aliquot of the sample onto a high-performance liquid chromatography system with a mixed-mode column, eluting the sample with a gradient from a first mobile phase to a second mobile phase; and applying evaporative light-scattering detection to the eluant after exiting the column, thereby measuring polysorbate levels; and

quantifying the amount of polysorbate in the aliquot of the sample by comparison against the calibration curve;

wherein the first mobile phase comprises 2% formic acid, 20% acetonitrile, and 78% purified water and the second mobile phase comprises 2% formic acid and 98% isopropyl alcohol.