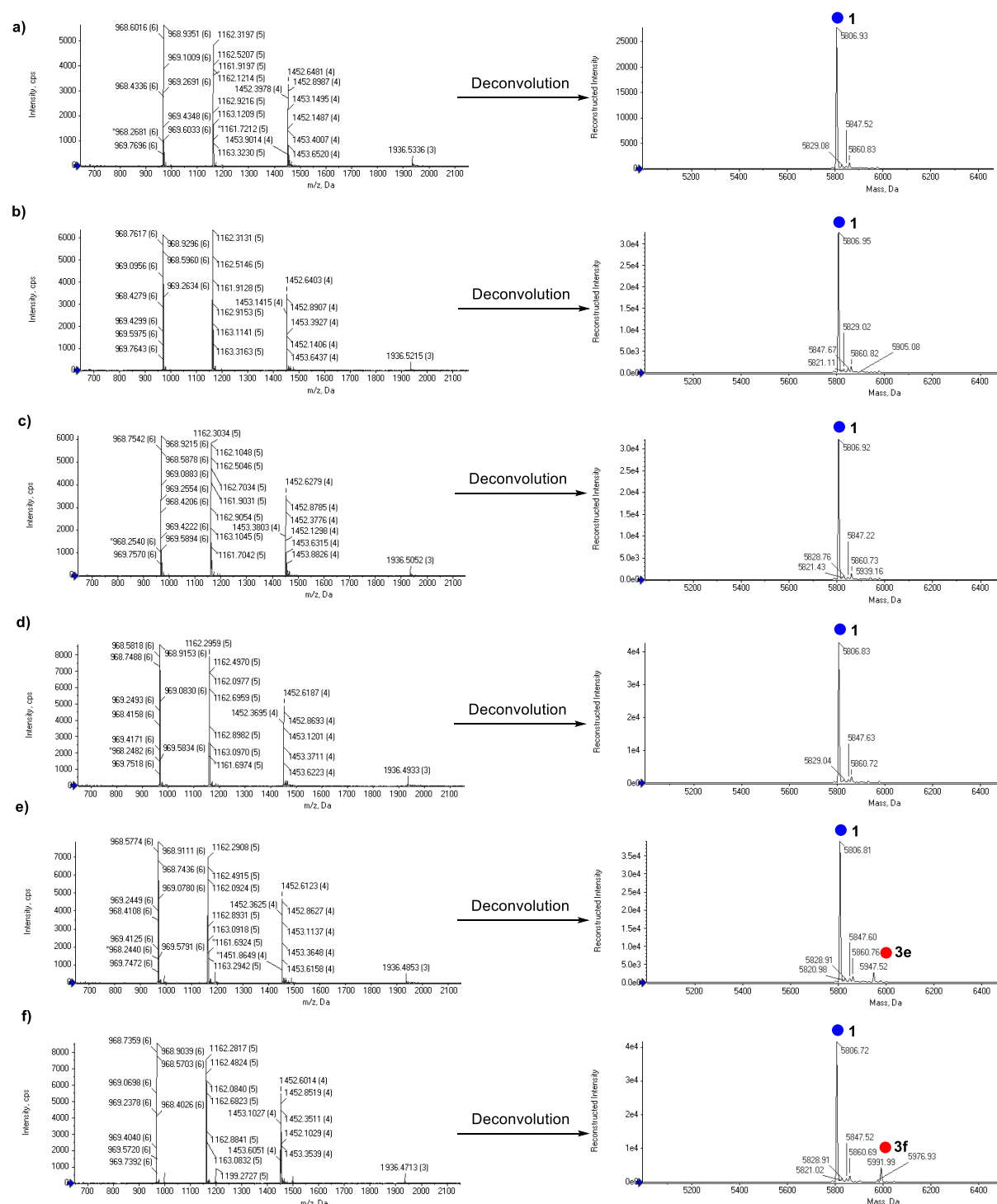


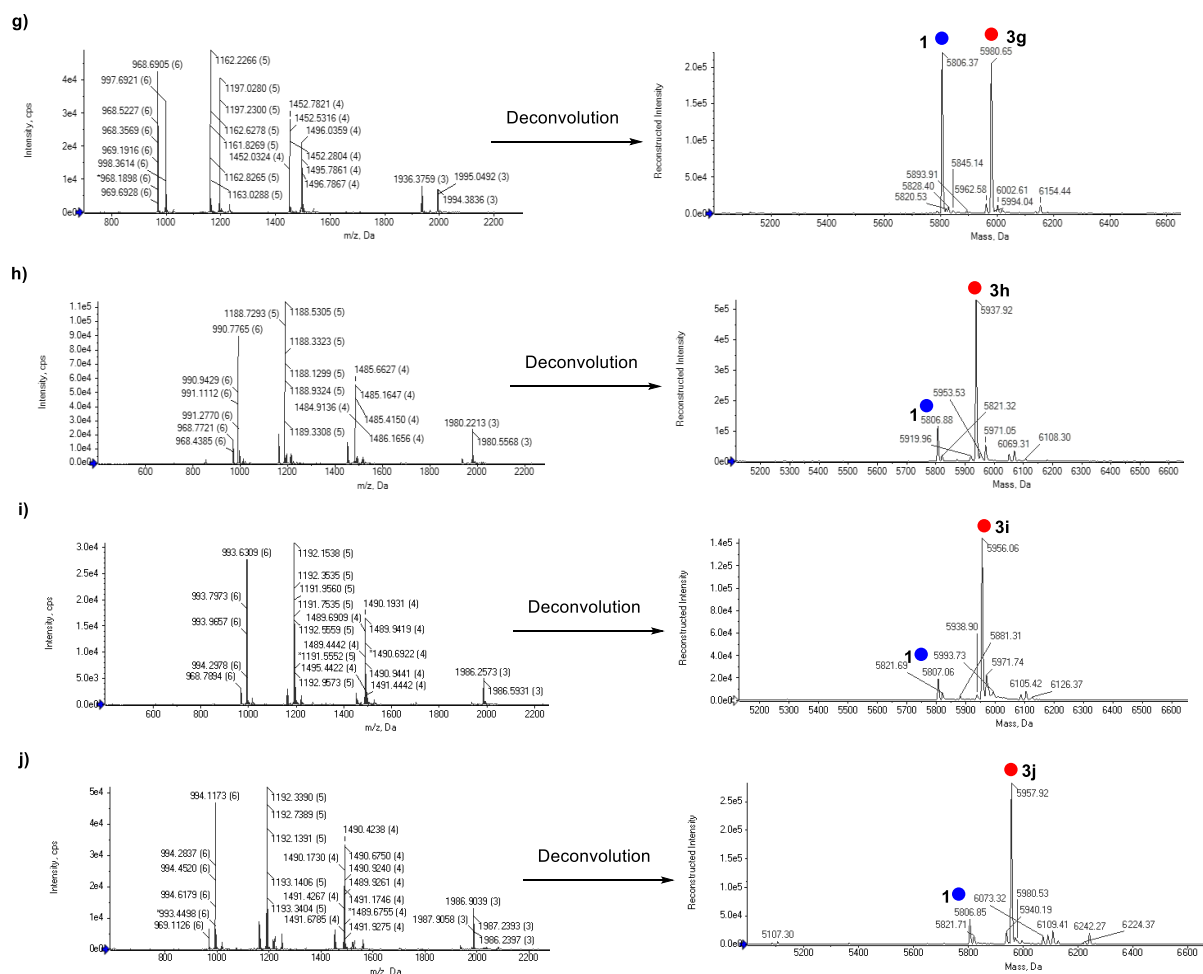
Additional Supplementary Information: MS data of replicates 2 and 3

1. N-Gly bioconjugation

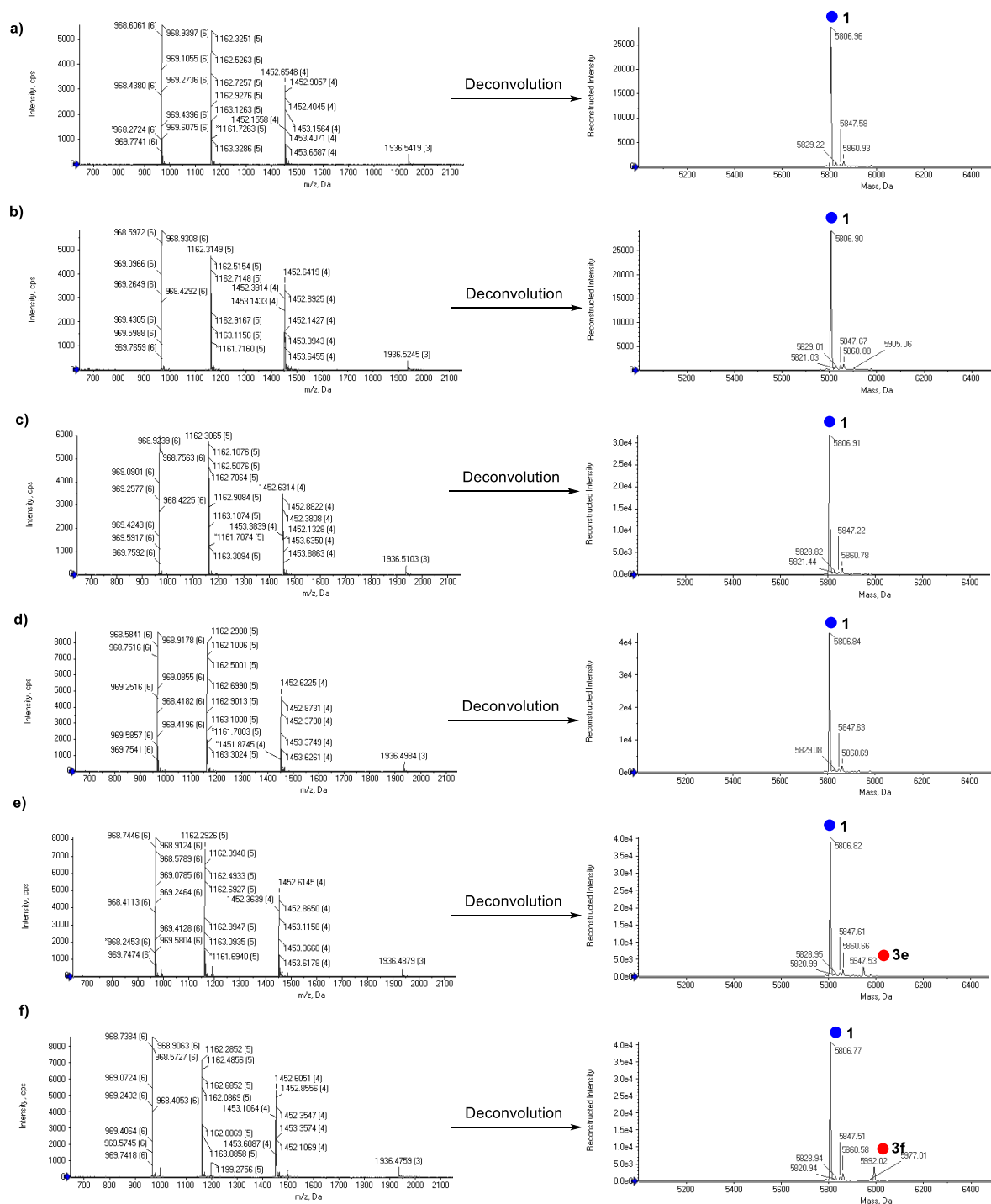
1.1 Screening of aromatic aldehydes 2a-2j for N-terminus Gly modification of insulin



Replicate MS data Fig. 1. Replicate 2: Charged and deconvoluted MS spectra for insulin (1) after reaction with (a) 2a, (b) 2b, (c) 2c, (d) 2d, (e) 2e, and (f) 2f for N-Gly bioconjugation.

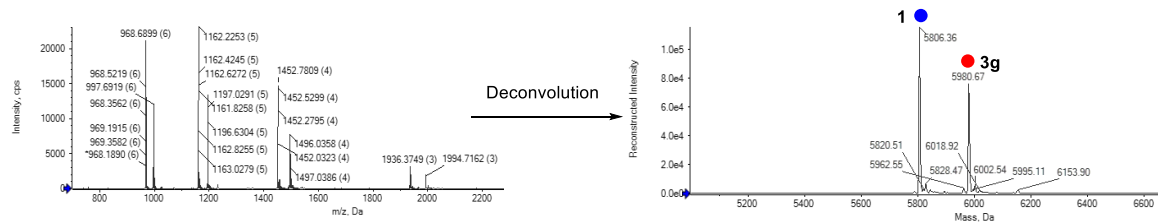


Replicate MS data Fig. 2. Replicate 2: Charged and deconvoluted MS spectra for insulin (1) after reaction with (a) **2g, (b) **2h**, (c) **2i**, and (d) **2j** for N-Gly bioconjugation.**

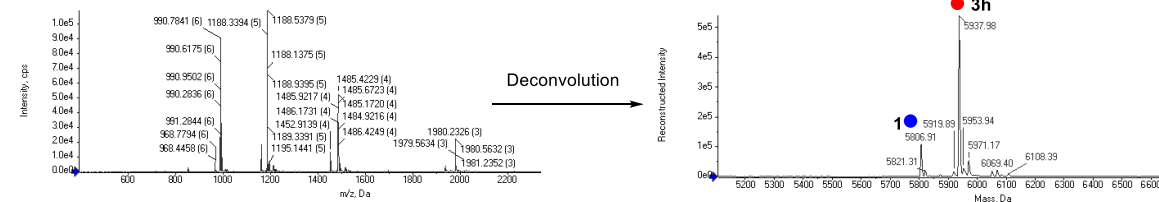


Replicate MS data Fig. 3. Replicate 3: Charged and deconvoluted MS spectra for insulin (1) after reaction with (a) **2a, (b) **2b**, (c) **2c**, (d) **2d**, (e) **2e**, and (f) **2f** for N-Gly bioconjugation.**

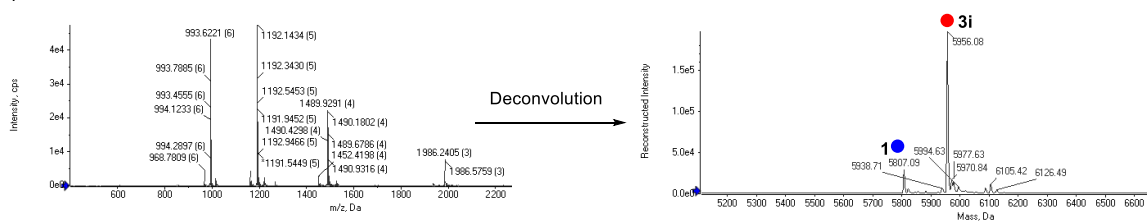
g)



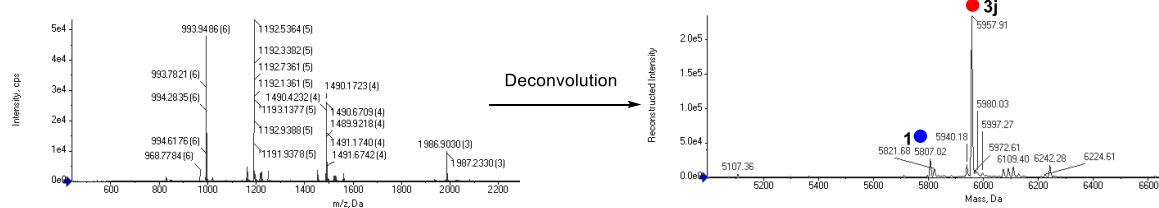
h)



i)

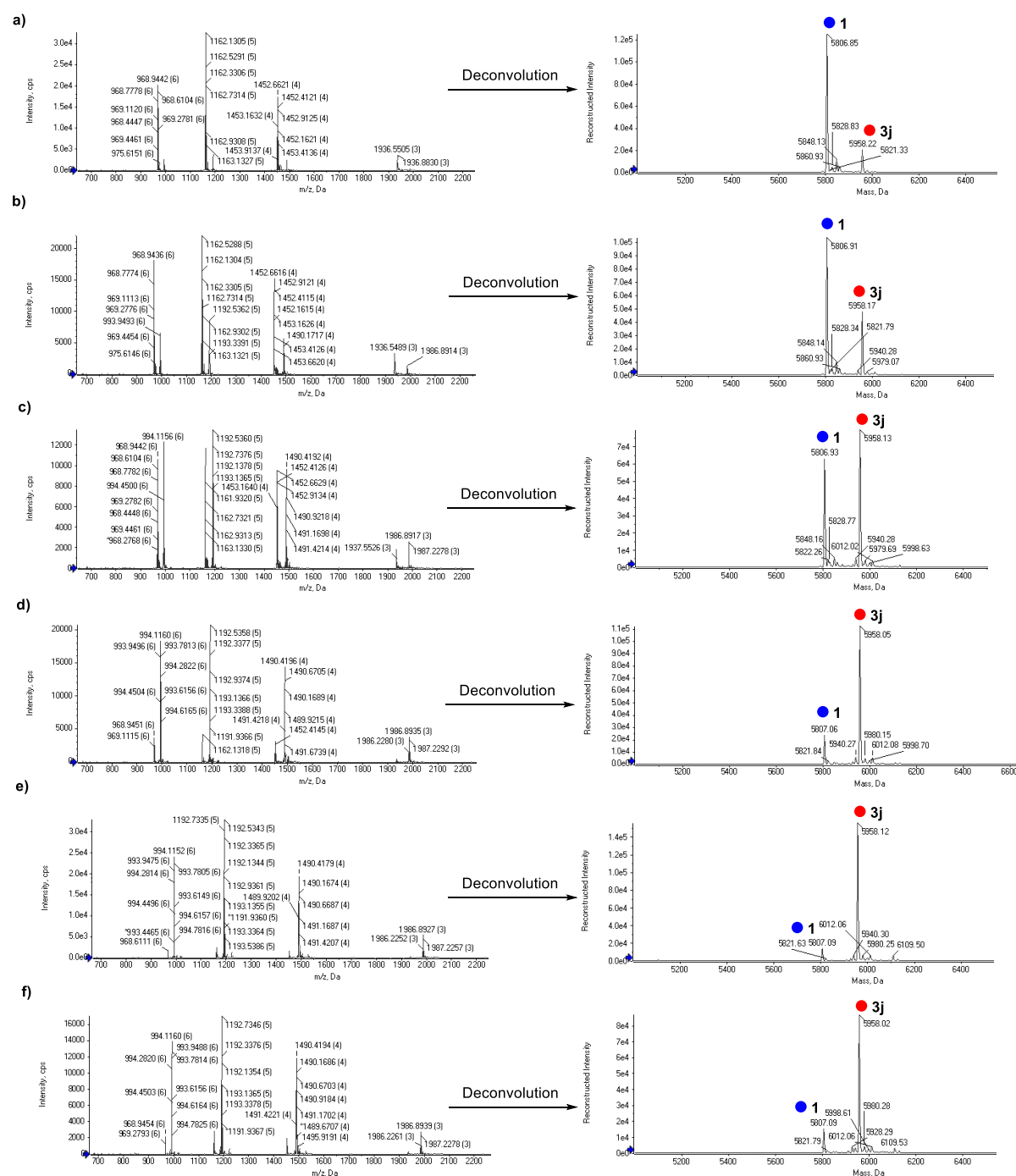


j)

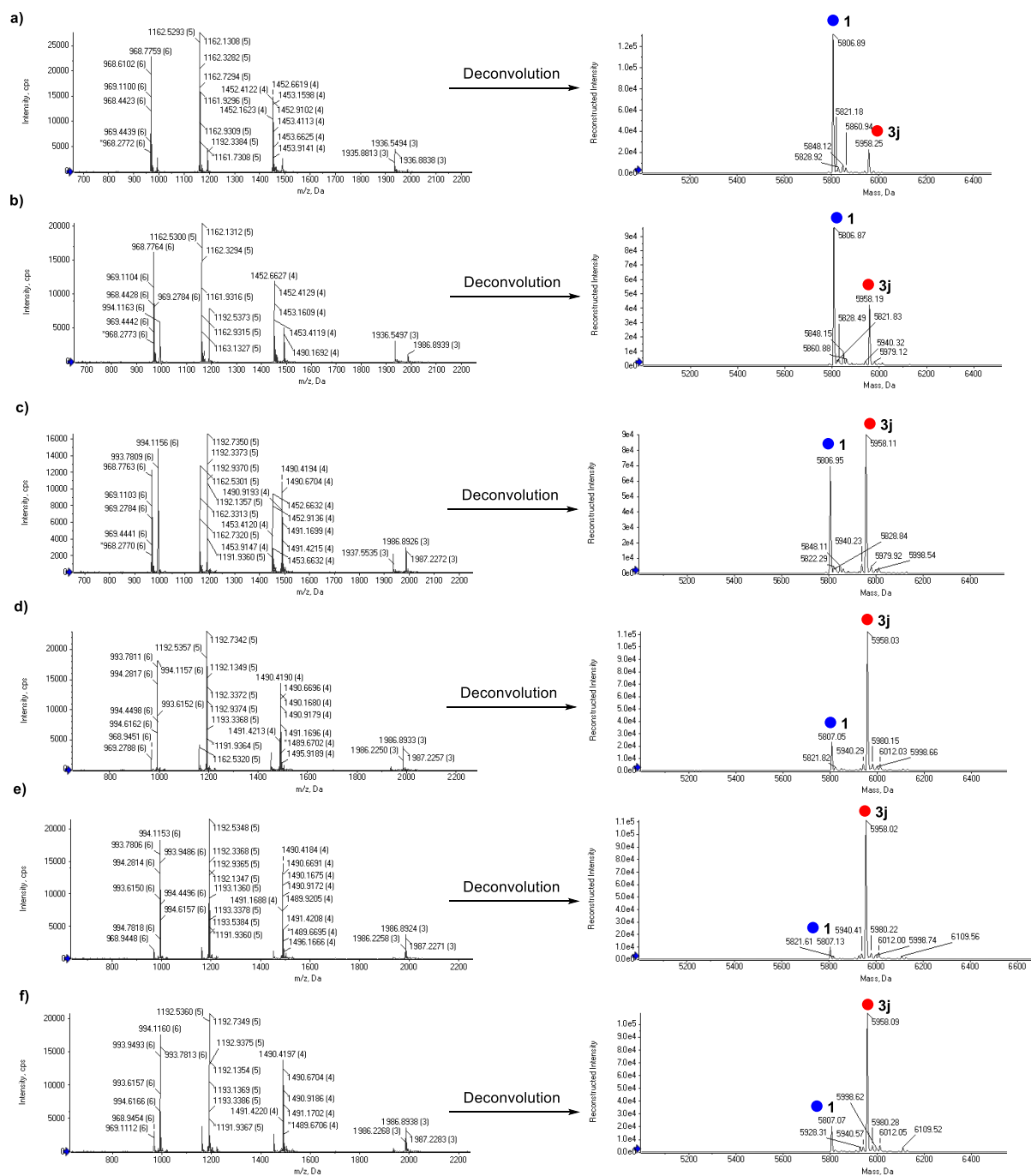


Replicate MS data Fig. 4. Replicate 3: Charged and deconvoluted MS spectra for insulin (1) after reaction with (a) 2g, (b) 2h, (c) 2i, and (d) 2j for N-Gly bioconjugation.

1.2 Effect of concentration of 2j on N-Gly bioconjugation of insulin

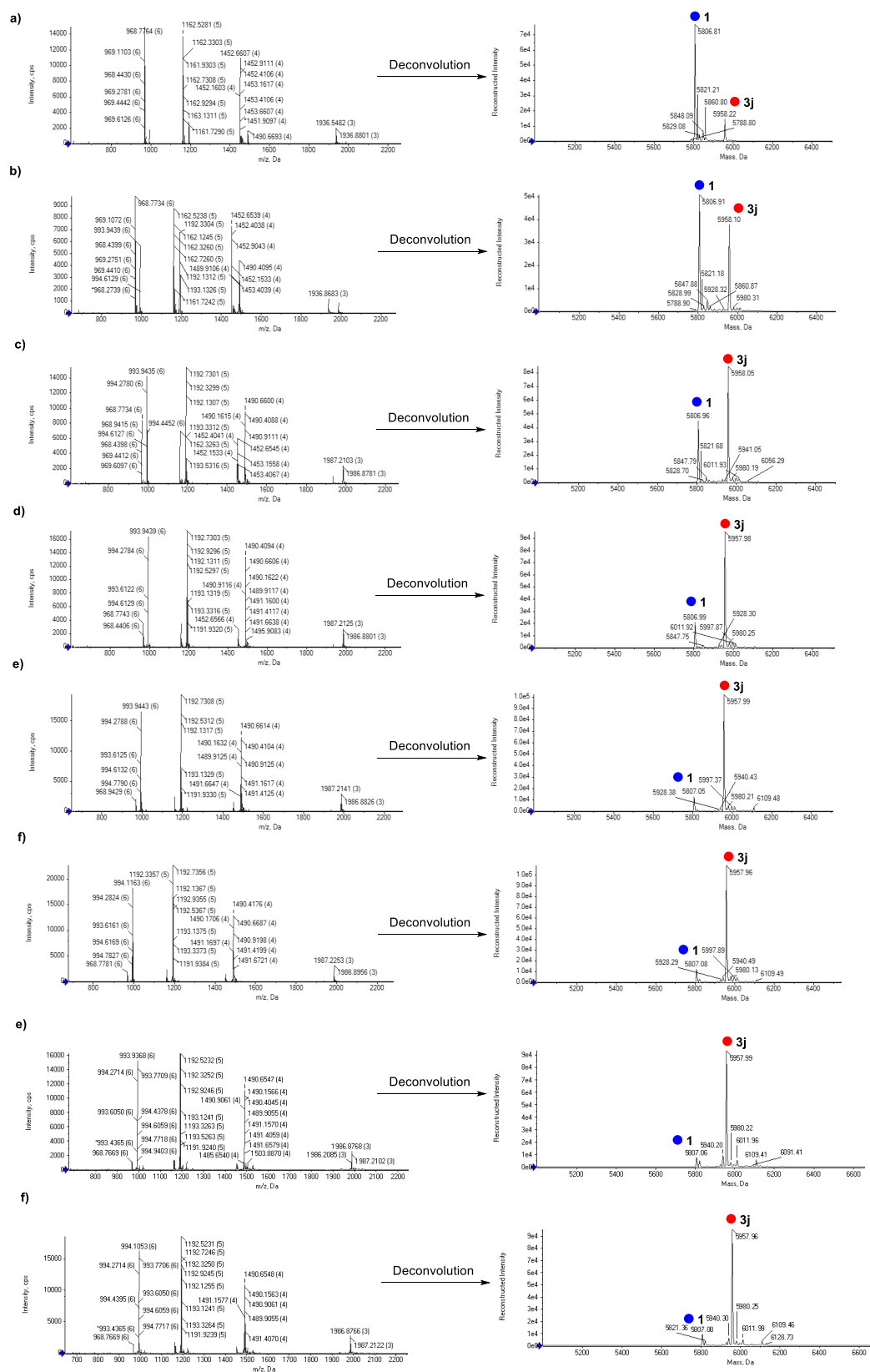


Replicate MS data Fig. 5. Replicate 2: Charged and deconvoluted MS spectra for insulin (**1**) after reaction at different concentrations of **2j**; (a) 0.5 mM, (b) 1 mM, (c) 2 mM, (d) 5 mM, (e) 10 mM, and (f) 20 mM.

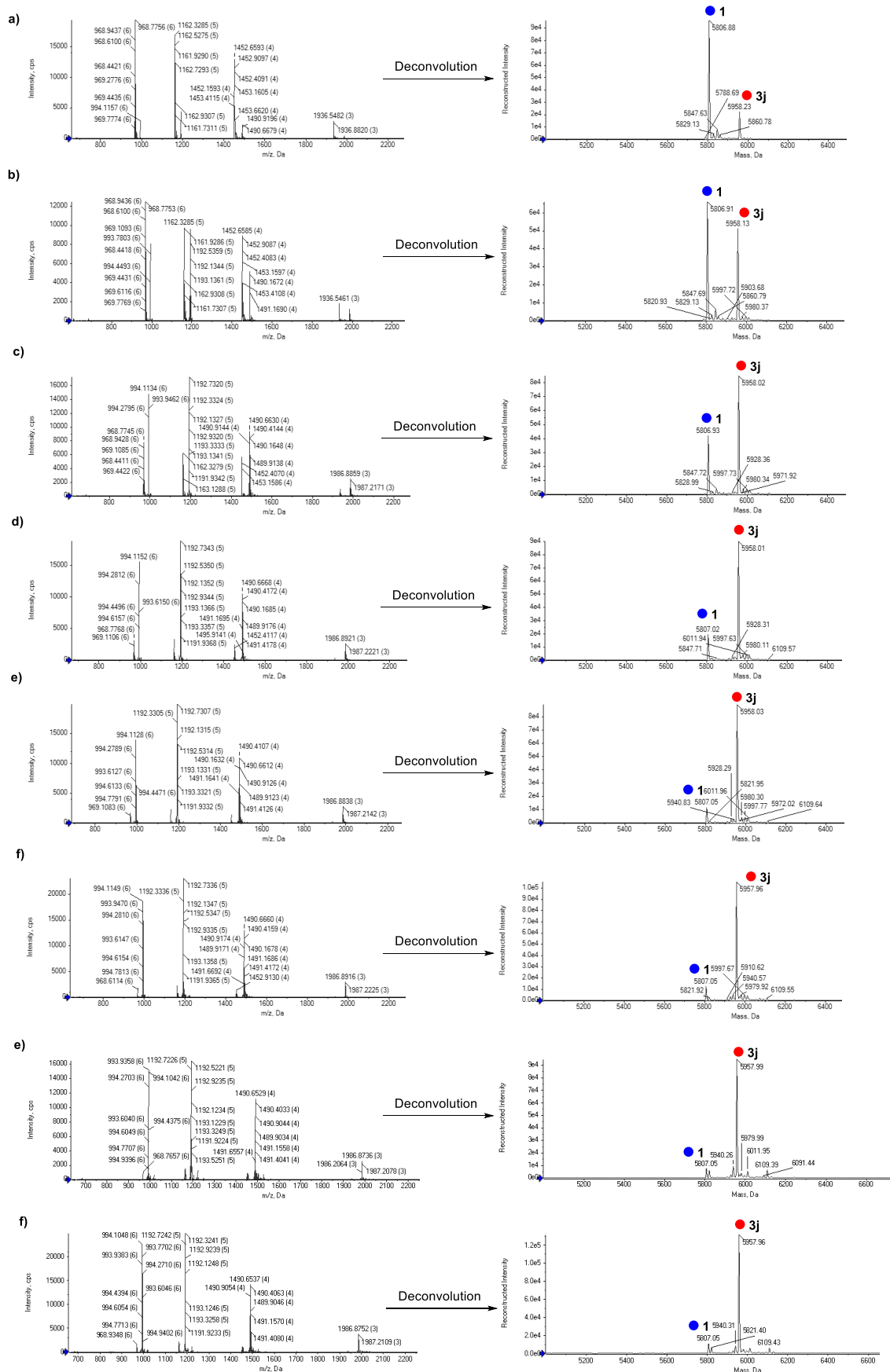


Replicate MS data Fig. 6. Replicate 3: Charged and deconvoluted MS spectra for insulin (1**) after reaction at different concentrations of **2j**; (a) 0.5 mM, (b) 1 mM, (c) 2 mM, (d) 5 mM, (e) 10 mM, and (f) 20 mM.**

1. 3 Progress of N-Gly bioconjugation of insulin as a function of time

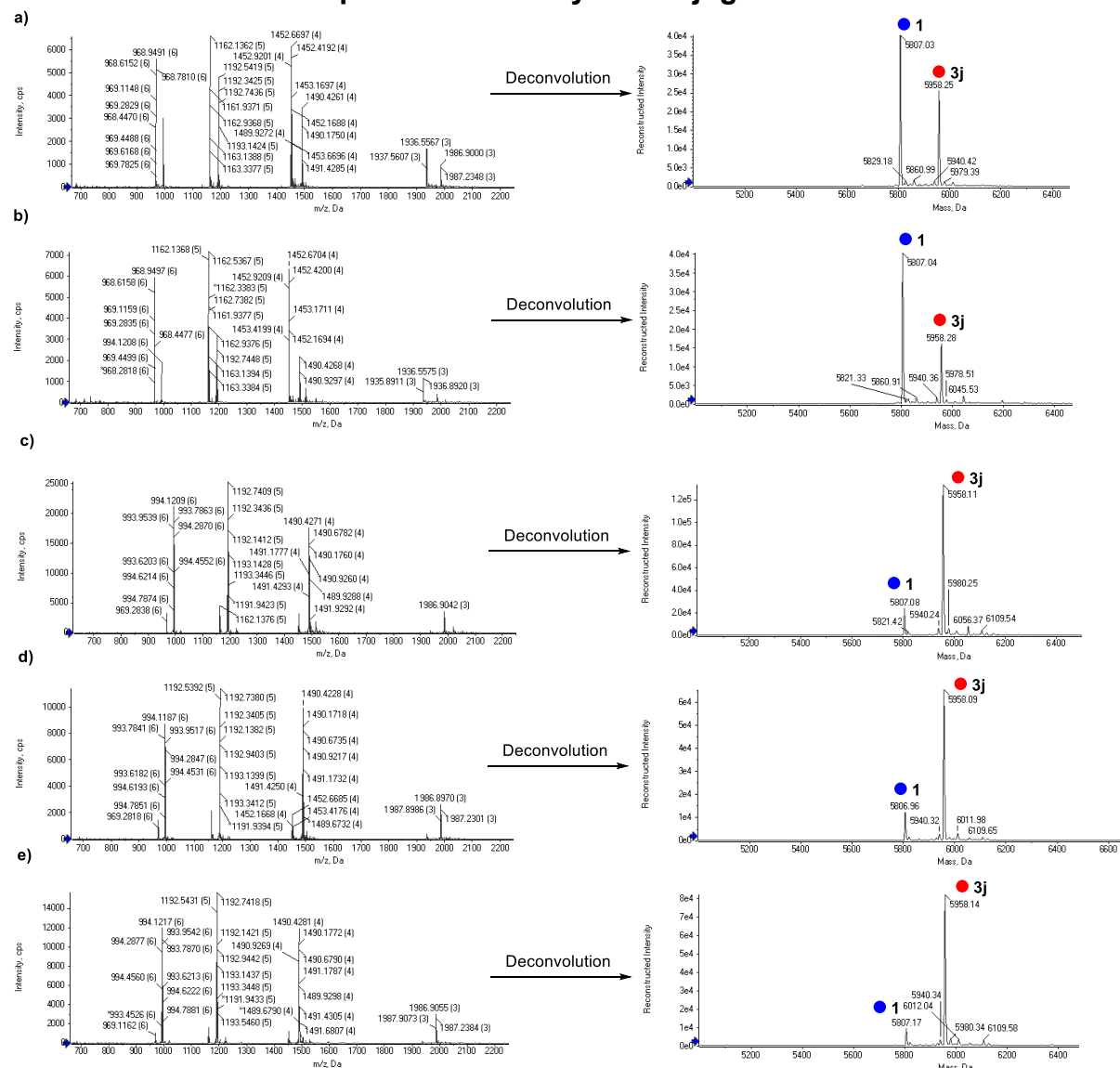


Replicate MS data Fig. 7. Replicate 2: Charged and deconvoluted MS spectra for insulin (1) after reaction with 2j at different time intervals; (a) 2 min, (b) 10 min, (c) 20 min, (d) 40 min, (e) 60 min, (f) 120 min, (g) 240 min, and (h) 360 min.

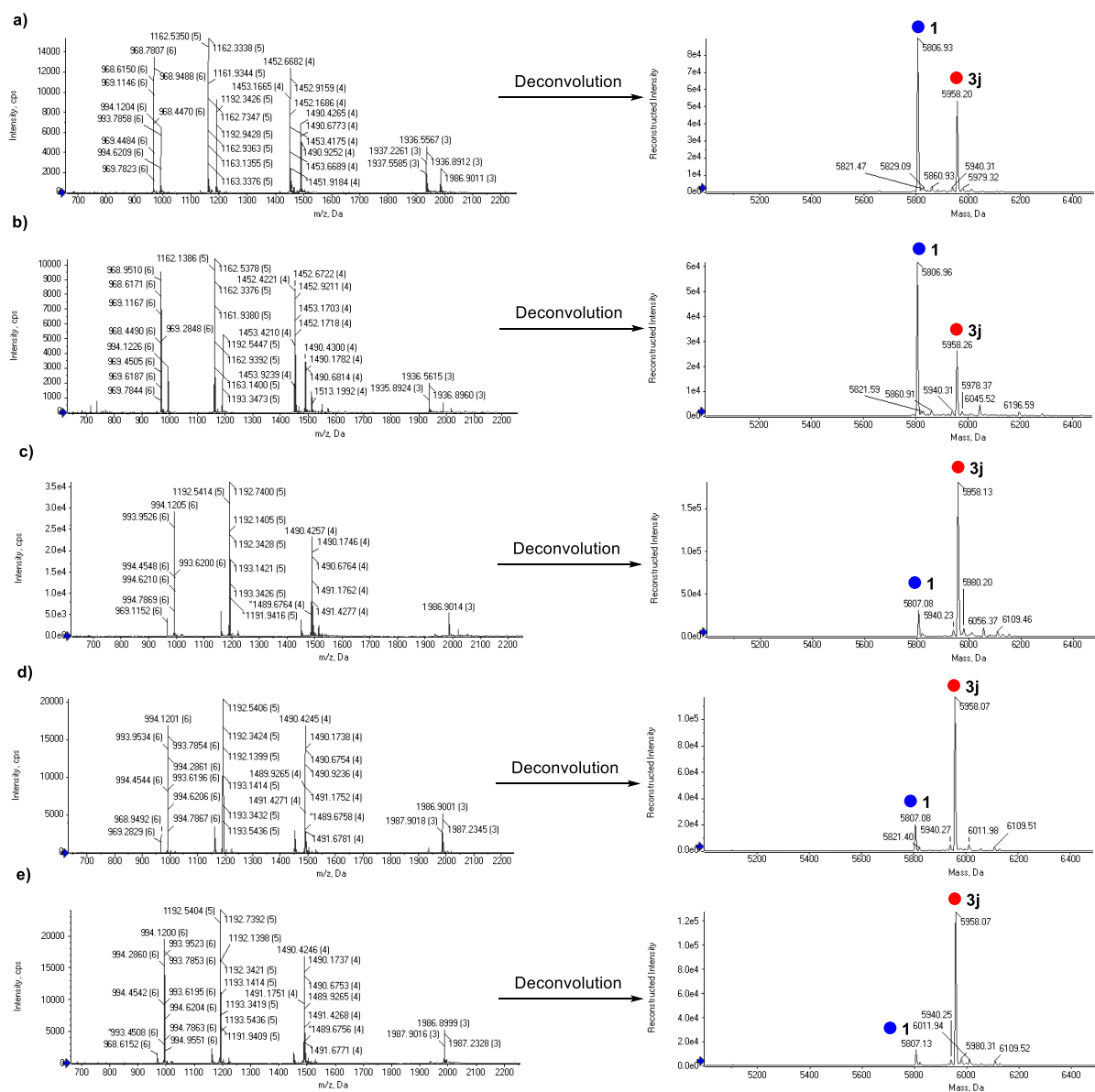


Replicate MS data Fig. 8. Replicate 3: Charged and deconvoluted MS spectra for insulin (1) after reaction with 2j at different time intervals; (a) 2 min, (b) 10 min, (c) 20 min, (d) 40 min, (e) 60 min, (f) 120 min, (g) 240 min, and (h) 360 min.

1.4 Effect of buffer composition on N-Gly bioconjugation of insulin

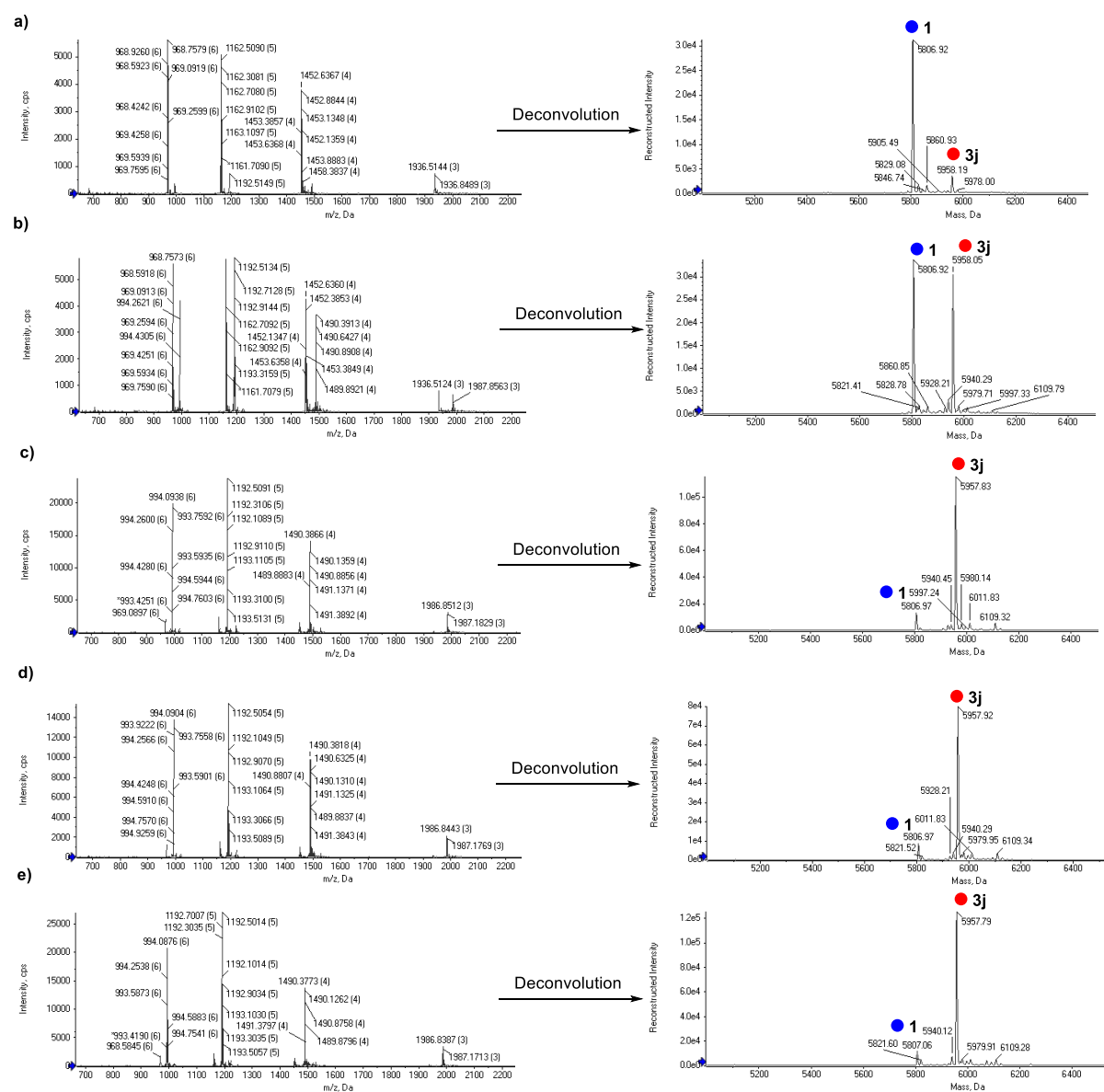


Replicate MS data Fig. 9. Replicate 2: Charged and deconvoluted MS spectra for insulin (1) after reaction with 2j using: (a) Tris buffer, (b) HEPES buffer, (c) PB buffer, (d) NH_4HCO_3 buffer, and (e) NaHCO_3 buffer.

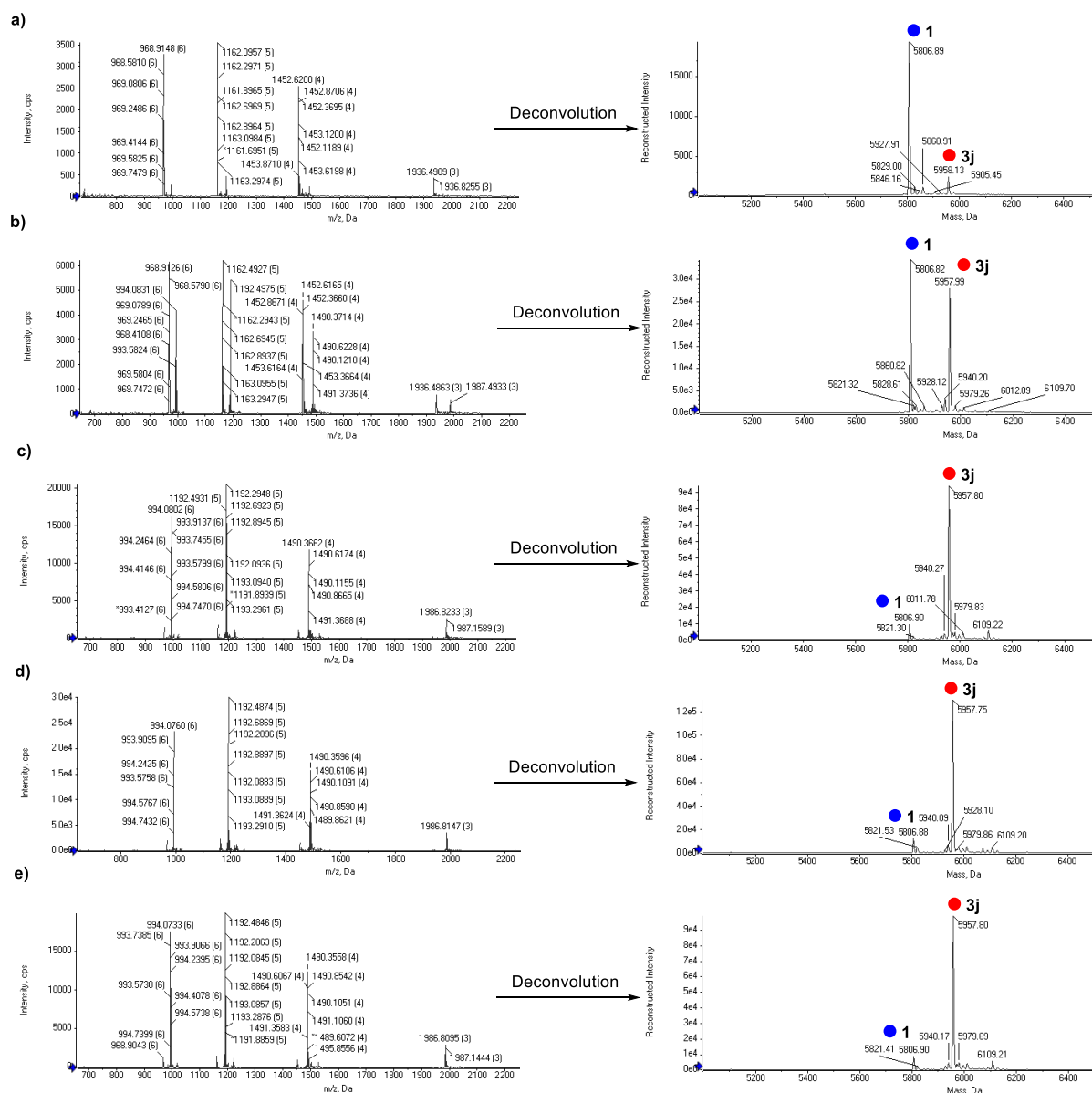


Replicate MS data Fig. 10. Replicate 3: Charged and deconvoluted MS spectra for insulin (1) after reaction with 2j using: (a) Tris buffer, (b) HEPES buffer, (c) PB buffer, (d) NH₄HCO₃ buffer, and (e) NaHCO₃ buffer.

1.5 Effect of pH on N-Gly bioconjugation of insulin

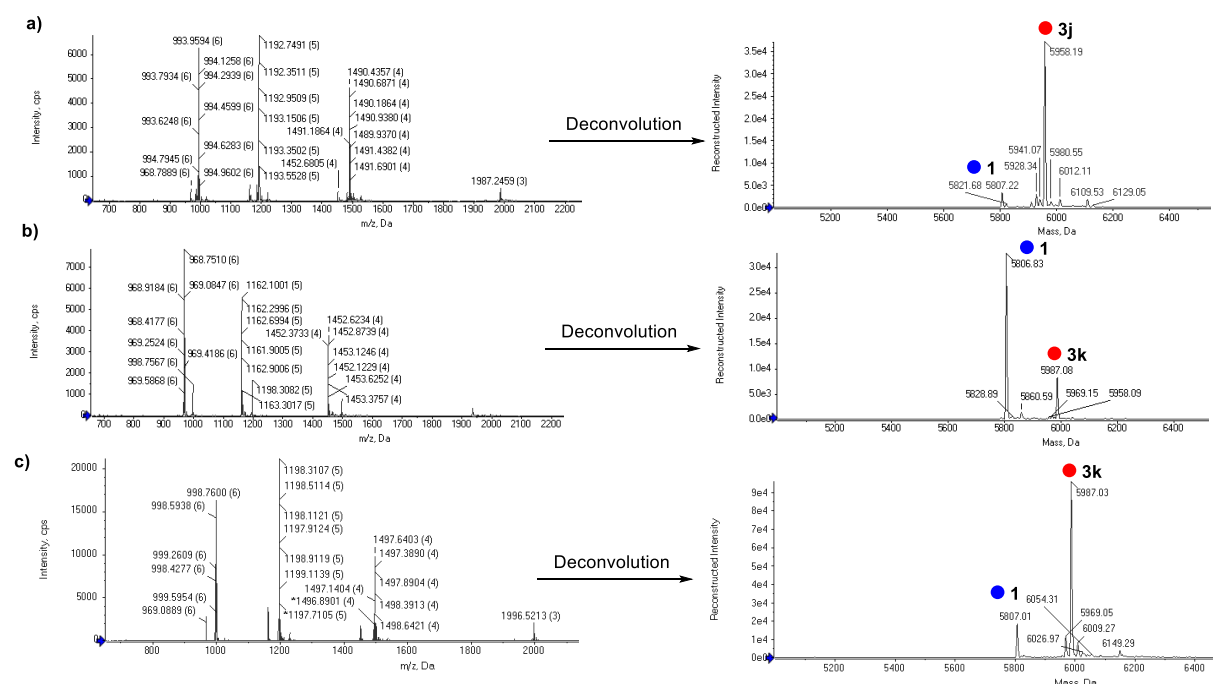


Replicate MS data Fig. 11. Replicate 2: Charged and deconvoluted MS spectra of insulin (**1**) after reaction with **2j** at: (a) pH 5.0, (b) pH 6.0, (c) pH 7.0, (d) pH 7.8, and (e) pH 9.0.

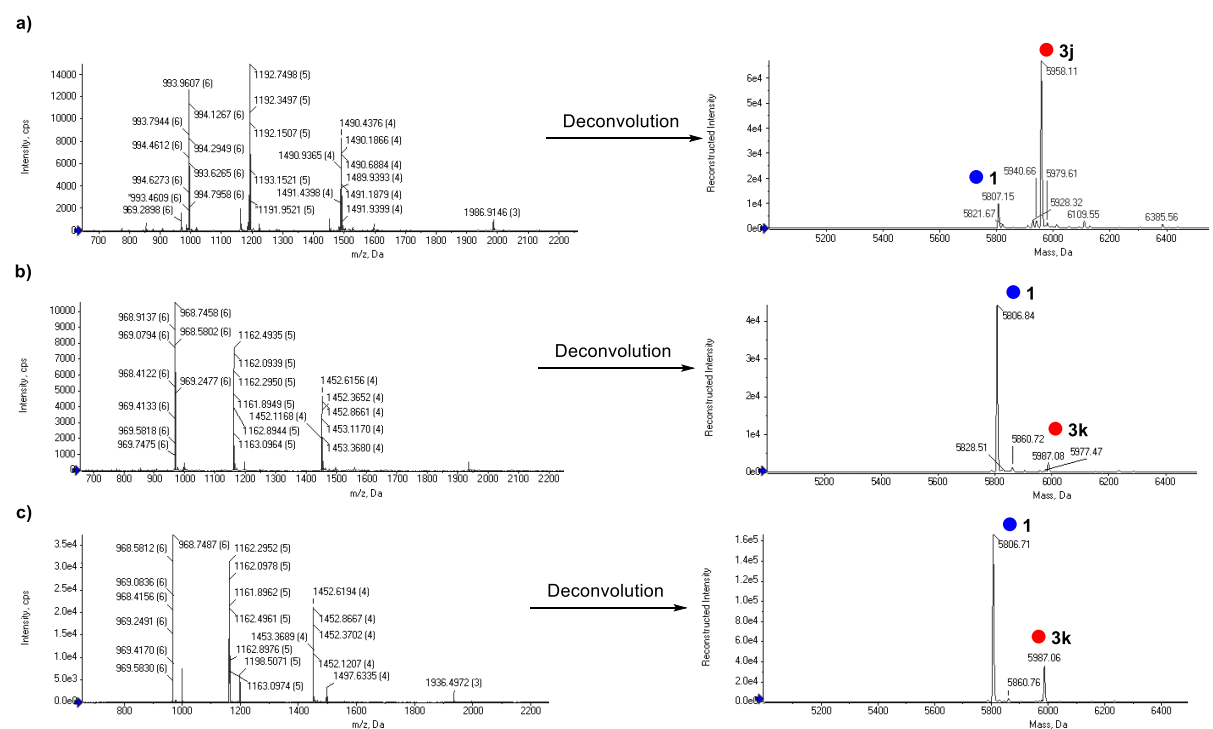


Replicate MS data Fig. 12. Replicate 3: Charged and deconvoluted MS spectra of insulin (**1**) after reaction with **2j** at: (a) pH 5.0, (b) pH 6.0, (c) pH 7.0, (d) pH 7.8, and (e) pH 9.0.

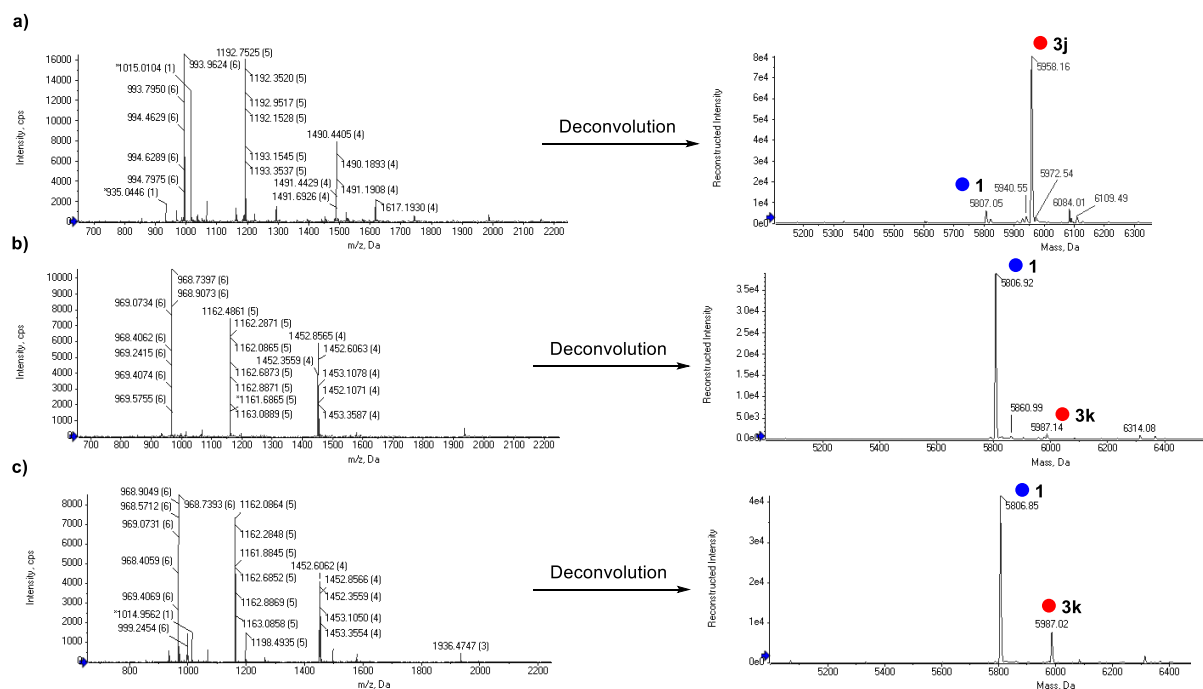
1.6 Control experiments: N-Gly bioconjugation of insulin with 2j versus 2k in the presence of biological additives



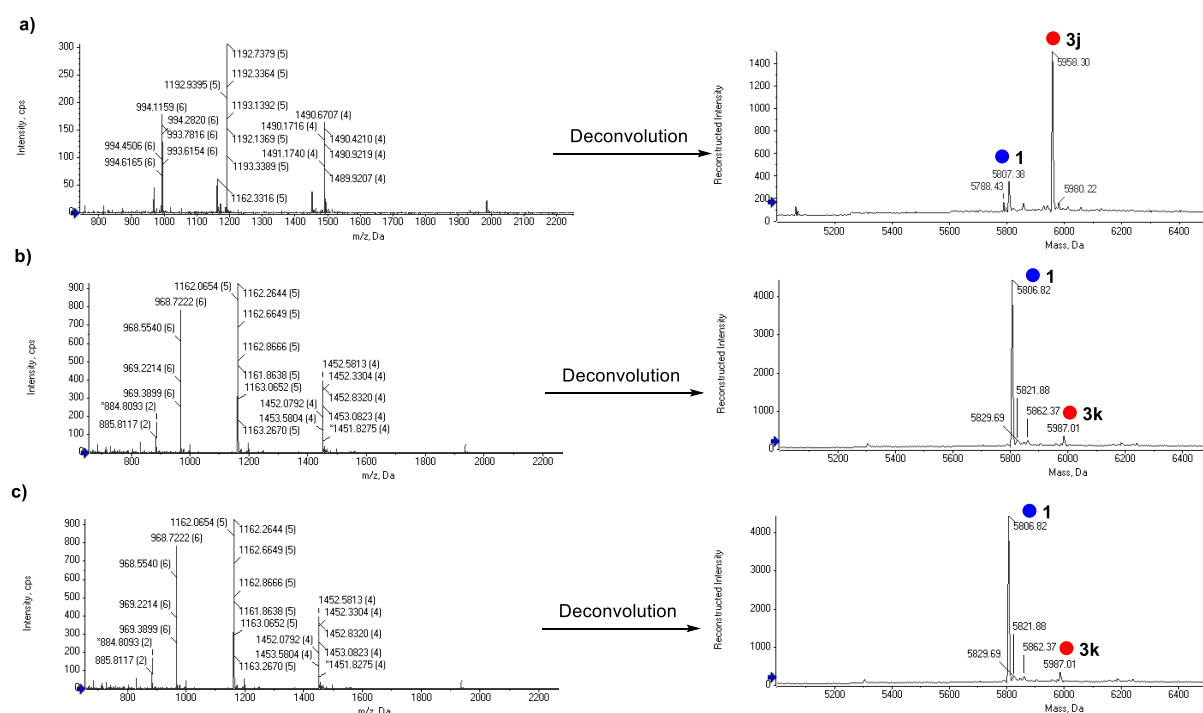
Replicate MS data Fig. 13. Replicate 2: Charged and deconvoluted MS spectra for insulin (1) without any additive after reaction; (a) with 2j for 2 h, (b) with 2k for 2 h, and (c) with 2k for 24 h.



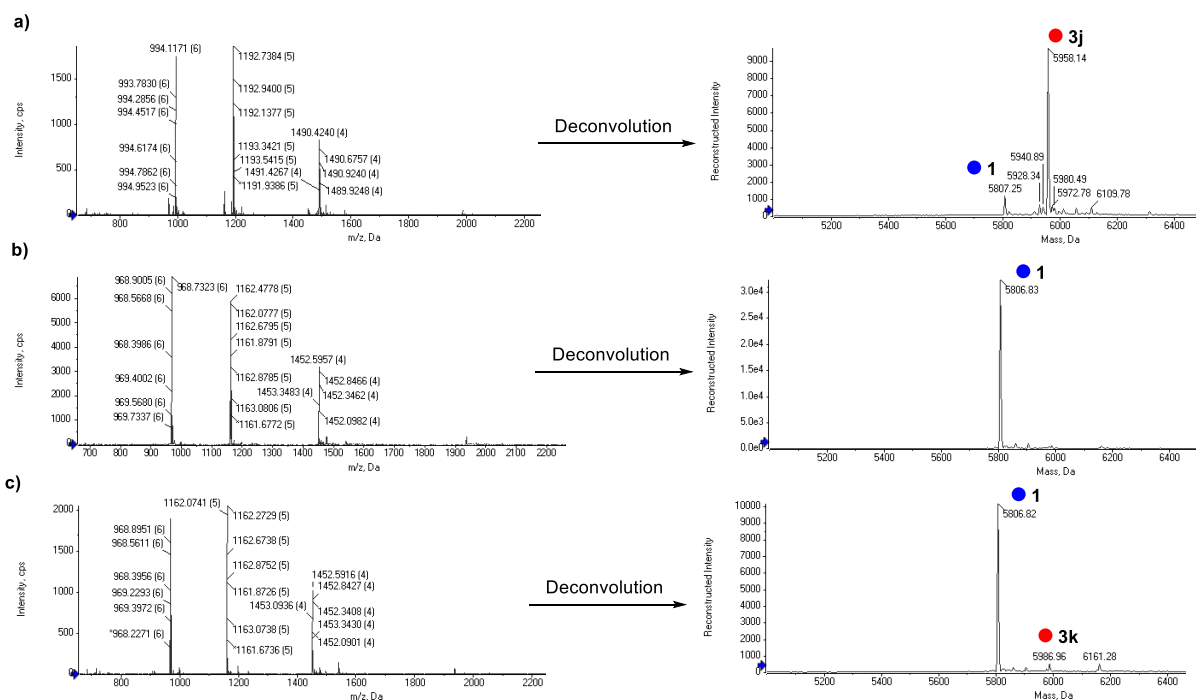
Replicate MS data Fig. 14. Replicate 2: Charged and deconvoluted MS spectra for insulin (1) in the presence of ADP (5 mM) as an additive after reaction; (a) with 2j for 2 h, (b) with 2k for 2 h, and (c) with 2k for 24 h



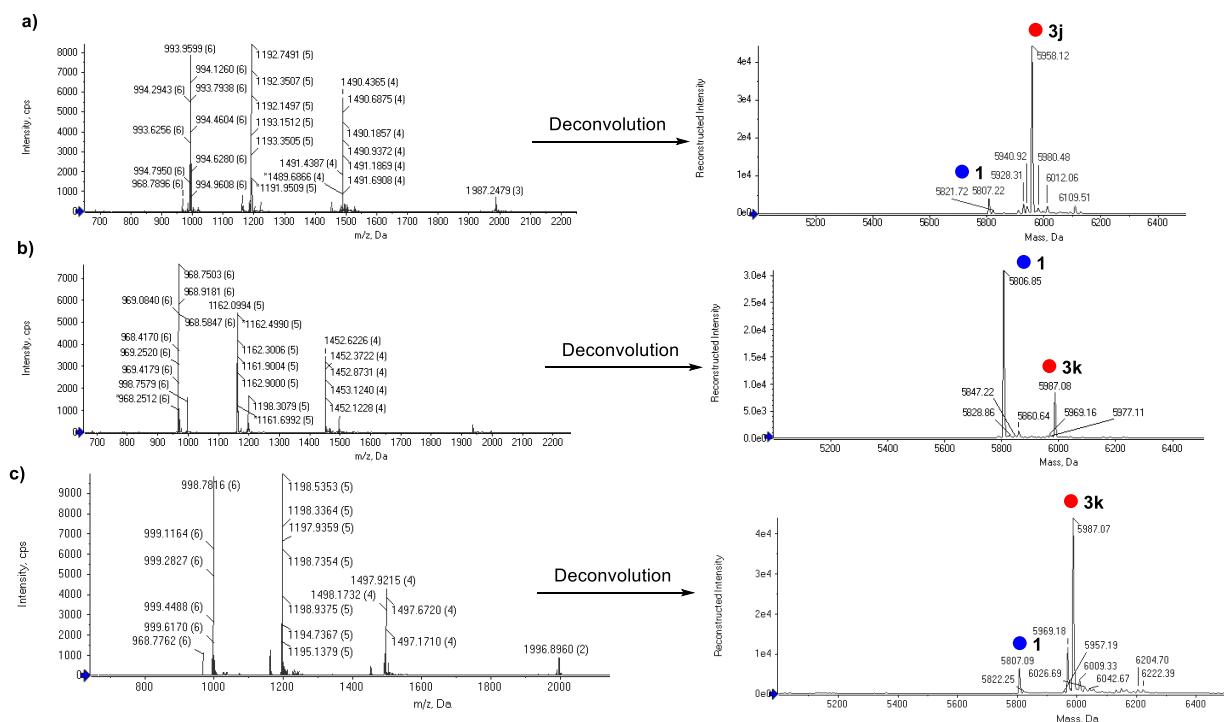
Replicate MS data Fig. 15. Replicate 2: Charged and deconvoluted MS spectra for insulin (**1**) in the presence of ATP (5 mM) as an additive after reaction; (a) with **2j** for 2 h, (b) with **2k** for 2 h, and (c) with **2k** for 24 h.



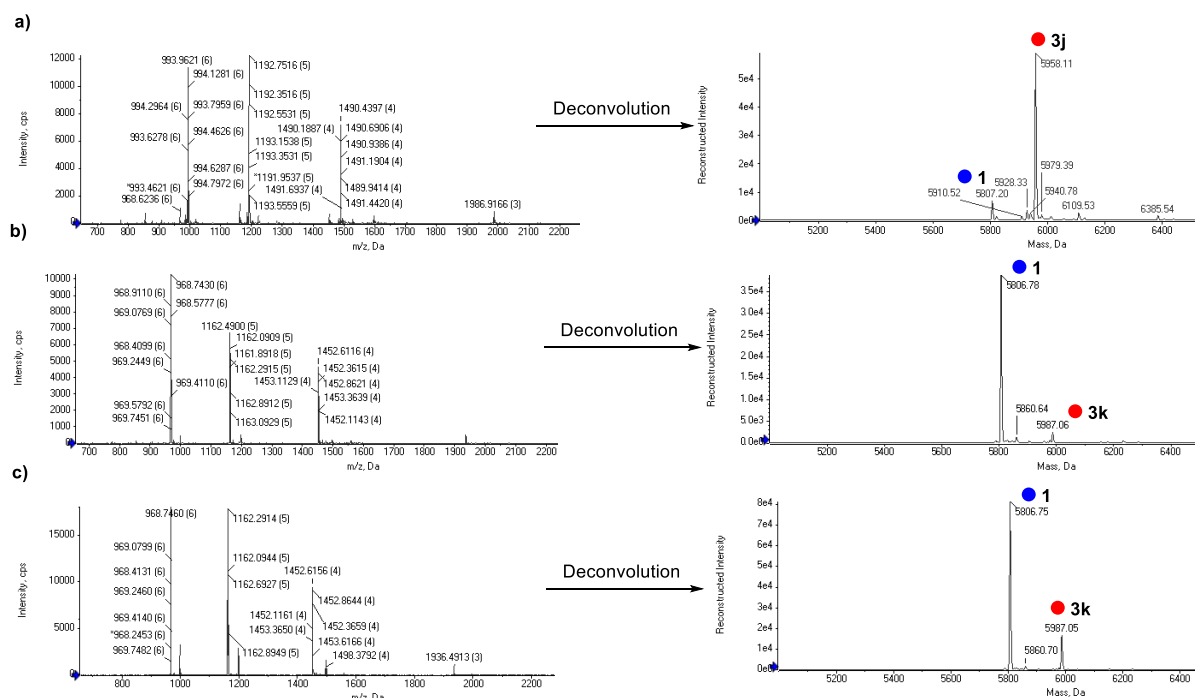
Replicate MS data Fig. 16. Replicate 2: Charged and deconvoluted MS spectra for insulin (**1**) in the presence of 10% human serum as an additive after reaction; (a) with **2j** for 2 h, (b) with **2k** for 2 h, and (c) with **2k** for 24 h.



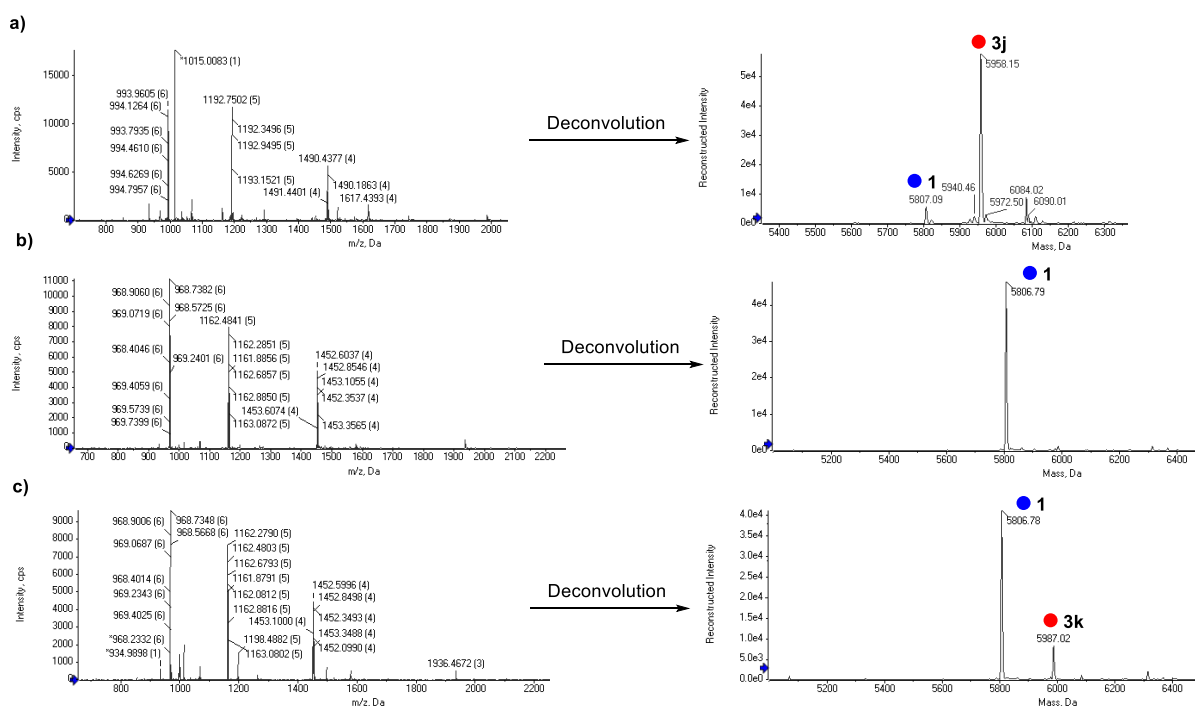
Replicate MS data Fig. 17. Replicate 2: Charged and deconvoluted MS spectra for insulin (1) in the presence of DMEM media as an additive after reaction; (a) with **2j** for 2 h, (b) with **2k** for 2 h, and (c) with **2k** for 24 h.



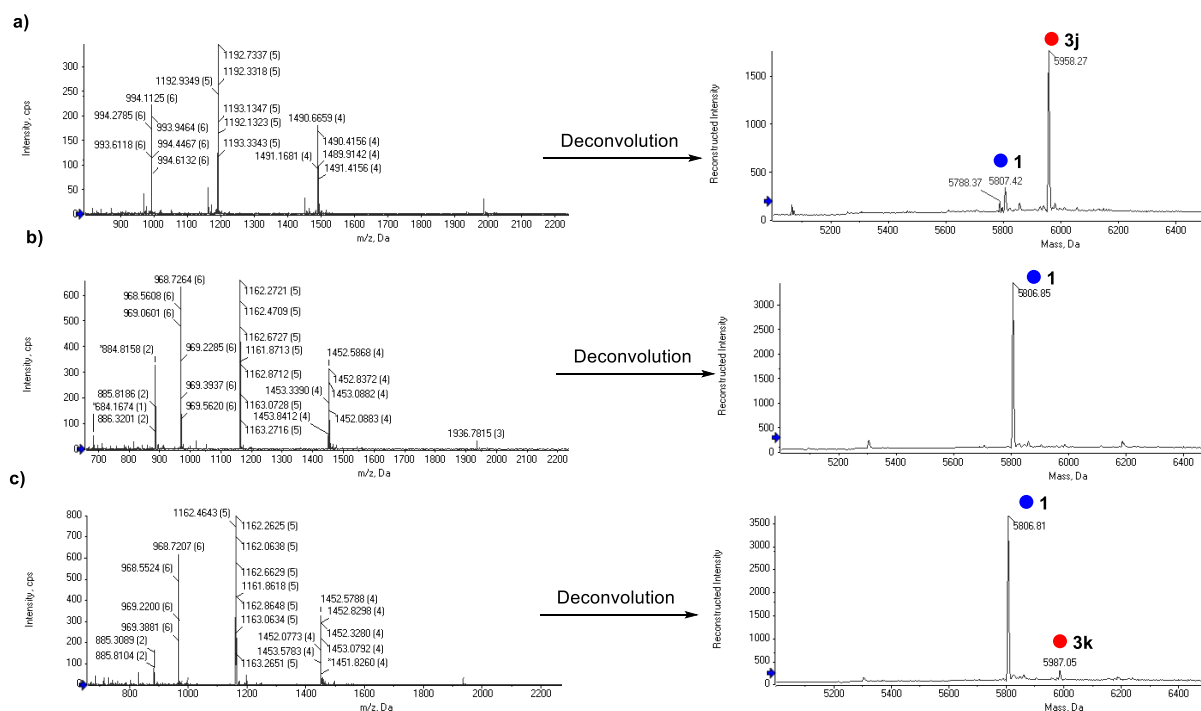
Replicate MS data Fig. 18. Replicate 3: Charged and deconvoluted MS spectra for insulin (1) without any additive after reaction; (a) with **2j** for 2 h, (b) with **2k** for 2 h, and (c) with **2k** for 24 h.



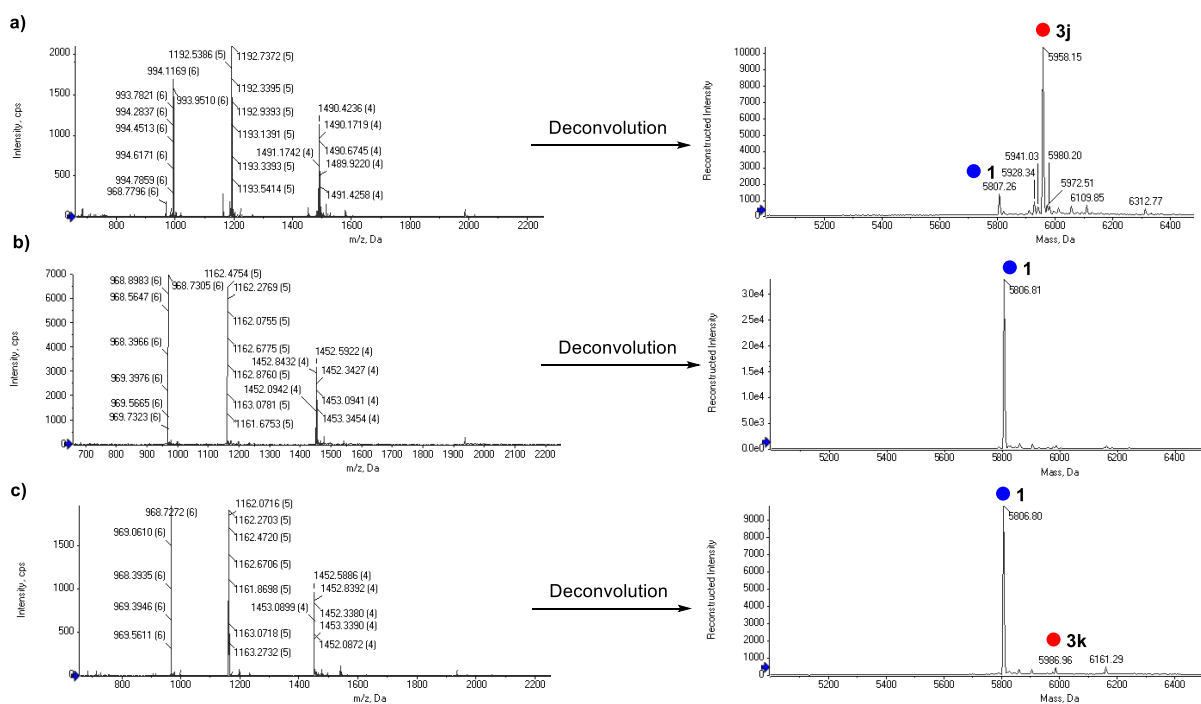
Replicate MS data Fig. 19. Replicate 3: Charged and deconvoluted MS spectra for insulin (**1**) in the presence of ADP (5 mM) as an additive after reaction; (a) with **2j** for 2 h, (b) with **2k** for 2 h, and (c) with **2k** for 24 h



Replicate MS data Fig. 20. Replicate 3: Charged and deconvoluted MS spectra for insulin (**1**) in the presence of ATP (5 mM) as an additive after reaction; (a) with **2j** for 2 h, (b) with **2k** for 2 h, and (c) with **2k** for 24 h.

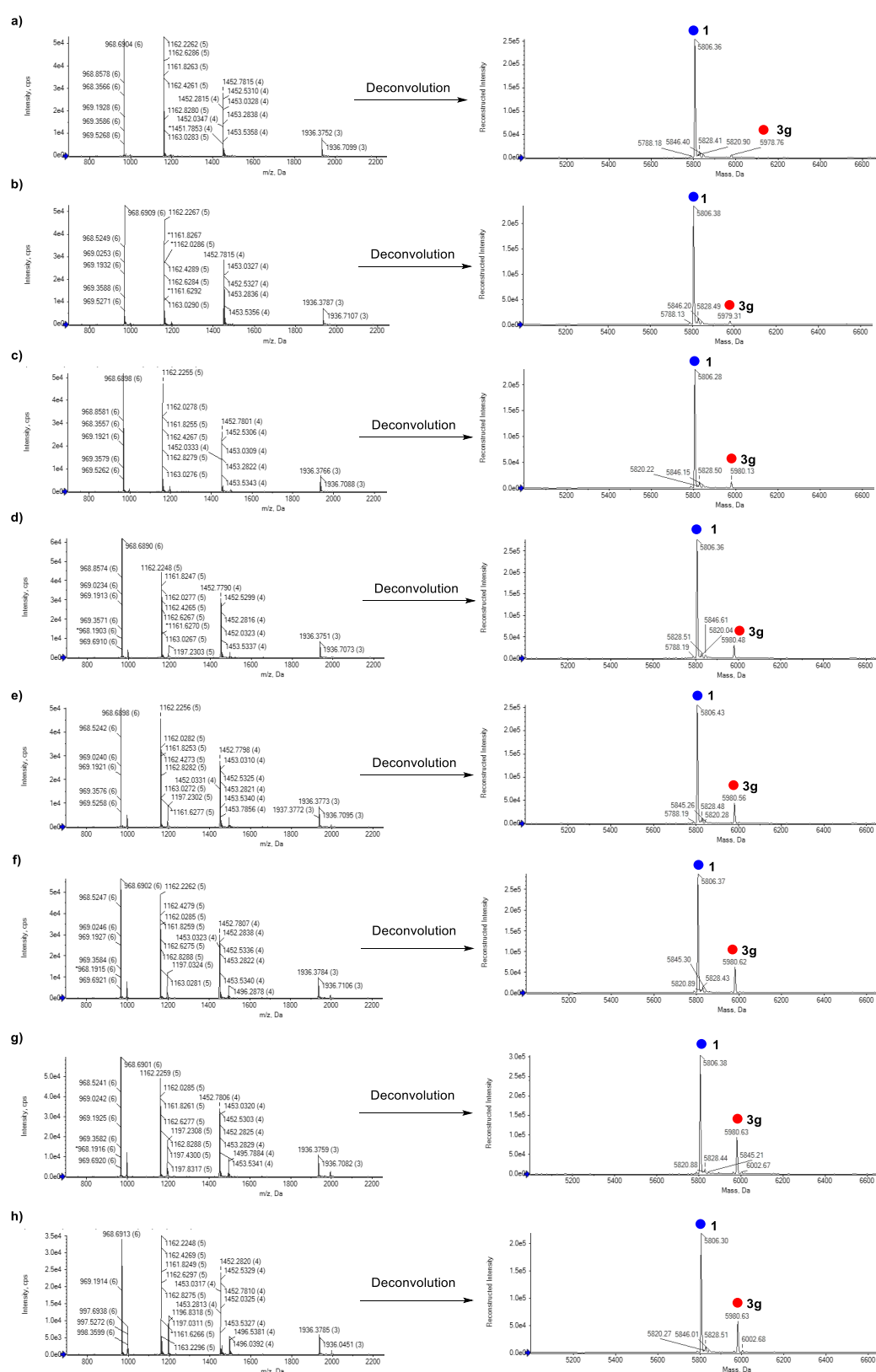


Replicate MS data Fig. 21. Replicate 3: Charged and deconvoluted MS spectra for insulin (1) in the presence of 10% human serum as an additive after reaction; (a) with **2j** for 2 h, (b) with **2k** for 2 h, and c) with **2k** for 24 h.

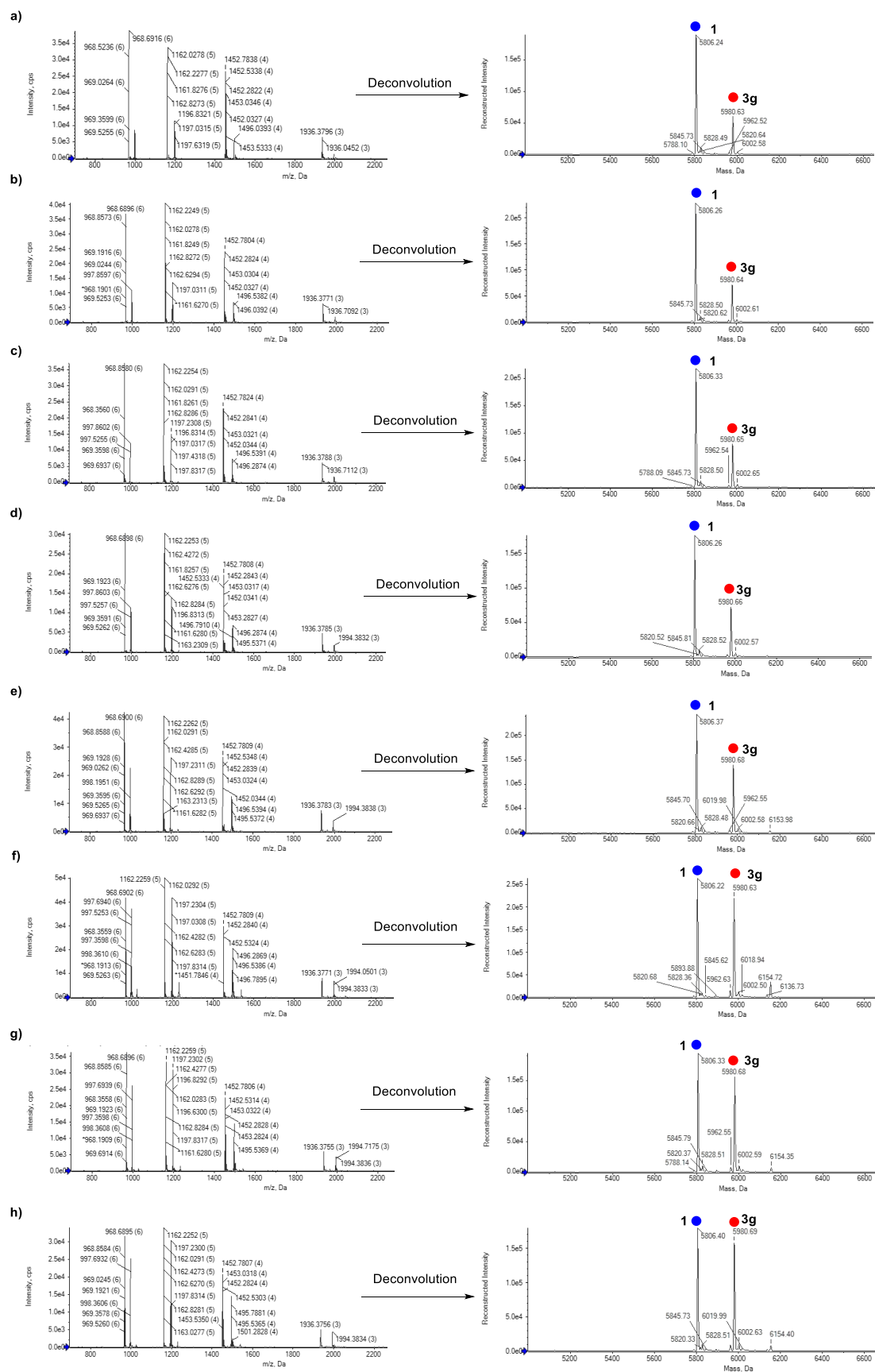


Replicate MS data Fig. 22. Replicate 3: Charged and deconvoluted MS spectra for insulin (1) in the presence of DMEM media as an additive after reaction; (a) with **2j** for 2 h, (b) with **2k** for 2 h, and (c) with **2k** for 24 h.

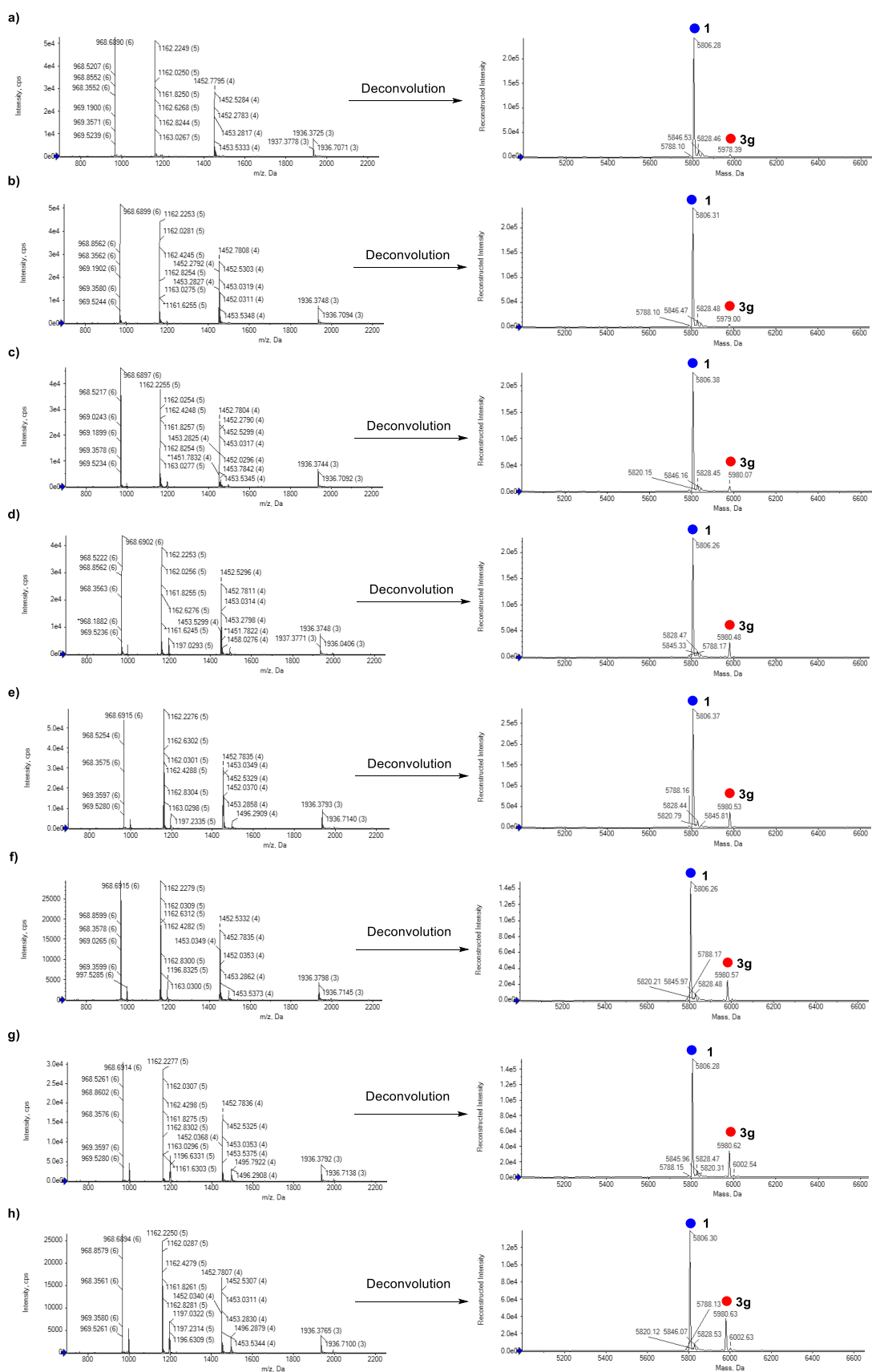
1.7 Kinetics experiments: N-Gly bioconjugation of insulin with 2g



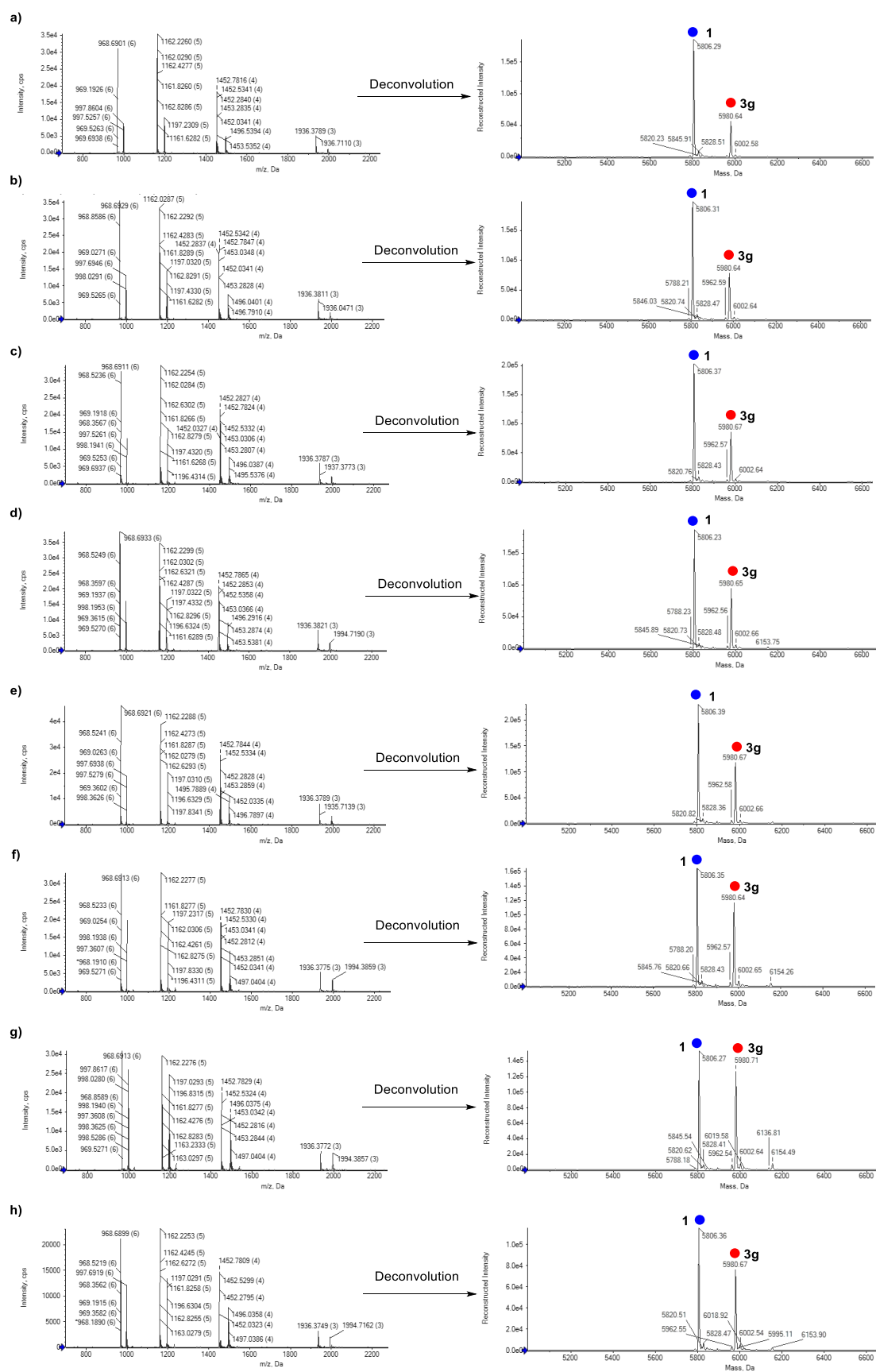
Replicate MS data Fig. 23. Replicate 2: Charged and deconvoluted MS spectra for insulin (1) after reaction with 2g for kinetics at different time intervals (2-35 min). (a) 2 min, (b) 5 min, (c) 10 min, (d) 15 min, (e) 20 min, (f) 25 min, (g) 30 min, and (h) 35 min.



Replicate MS data Fig. 24. Replicate 2: Charged and deconvoluted MS spectra for insulin (**1**) after reaction with **2g** for kinetics at different time intervals (40-120 min). (a) 40 min, (b) 45 min, (c) 50 min, (d) 55 min, (e) 60 min, (f) 75 min, (g) 90 min, and (h) 120 min.

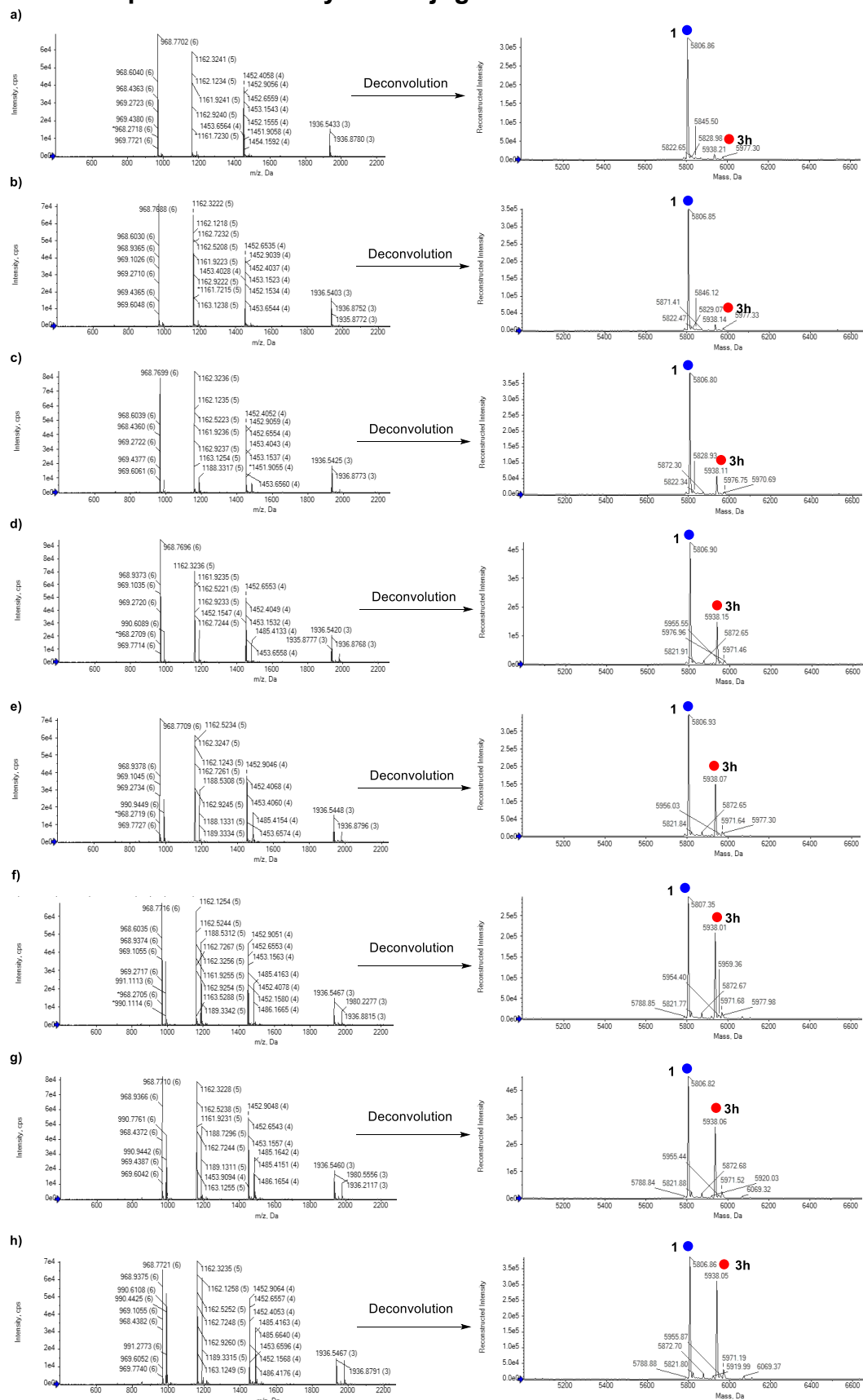


Replicate MS data Fig. 25. Replicate 3: Charged and deconvoluted MS spectra for insulin (**1**) after reaction with **2g** for kinetics at different time intervals (2-35 min). (a) 2 min, (b) 5 min, (c) 10 min, (d) 15 min, (e) 20 min, (f) 25 min, (g) 30 min, and (h) 35 min.

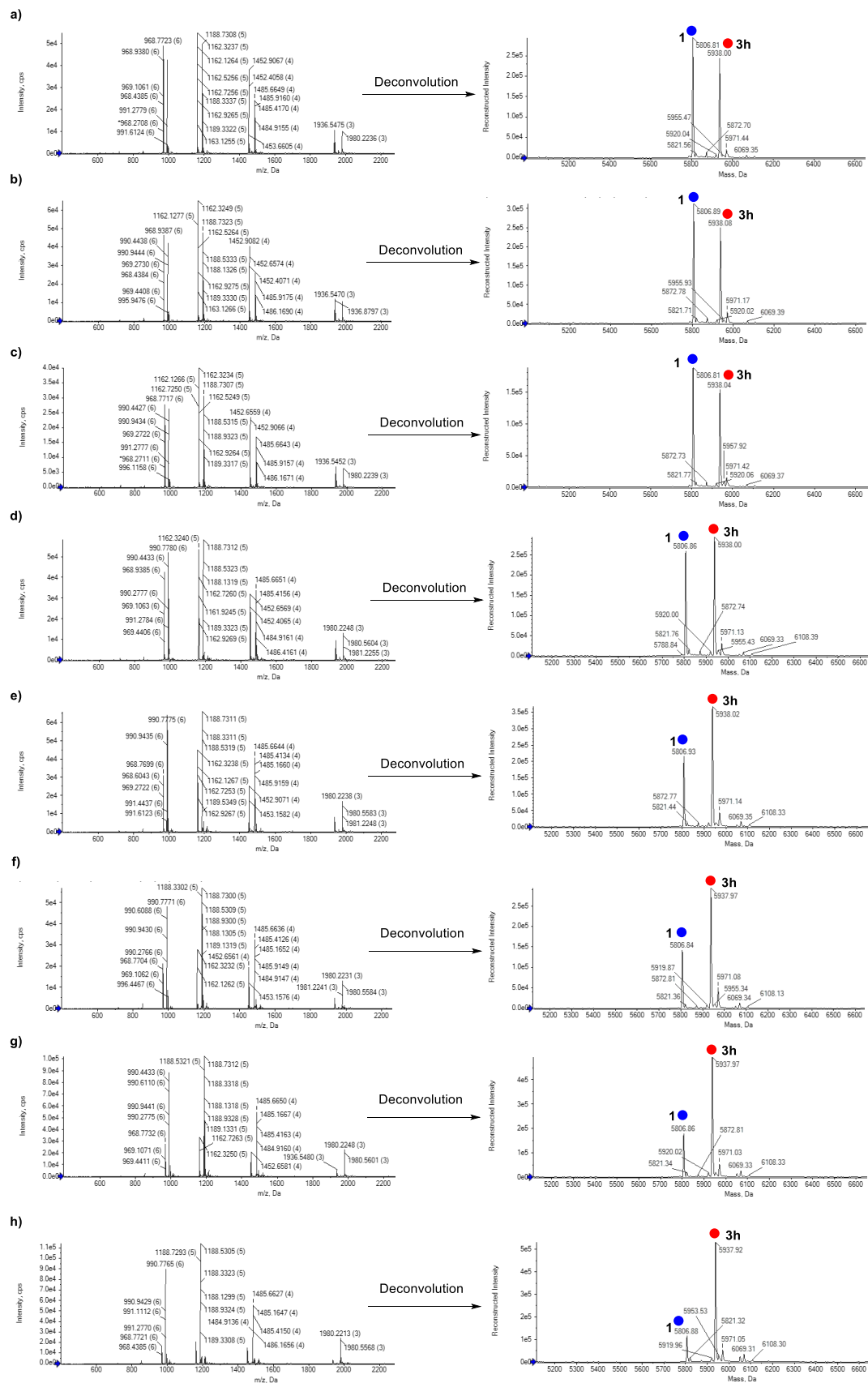


Replicate MS data Fig. 26. Replicate 3: Charged and deconvoluted MS spectra for insulin (**1**) after reaction with **2g** for kinetics at different time intervals (40-120 min). (a) 40 min, (b) 45 min, (c) 50 min, (d) 55 min, (e) 60 min, (f) 75 min, (g) 90 min, and (h) 120 min.

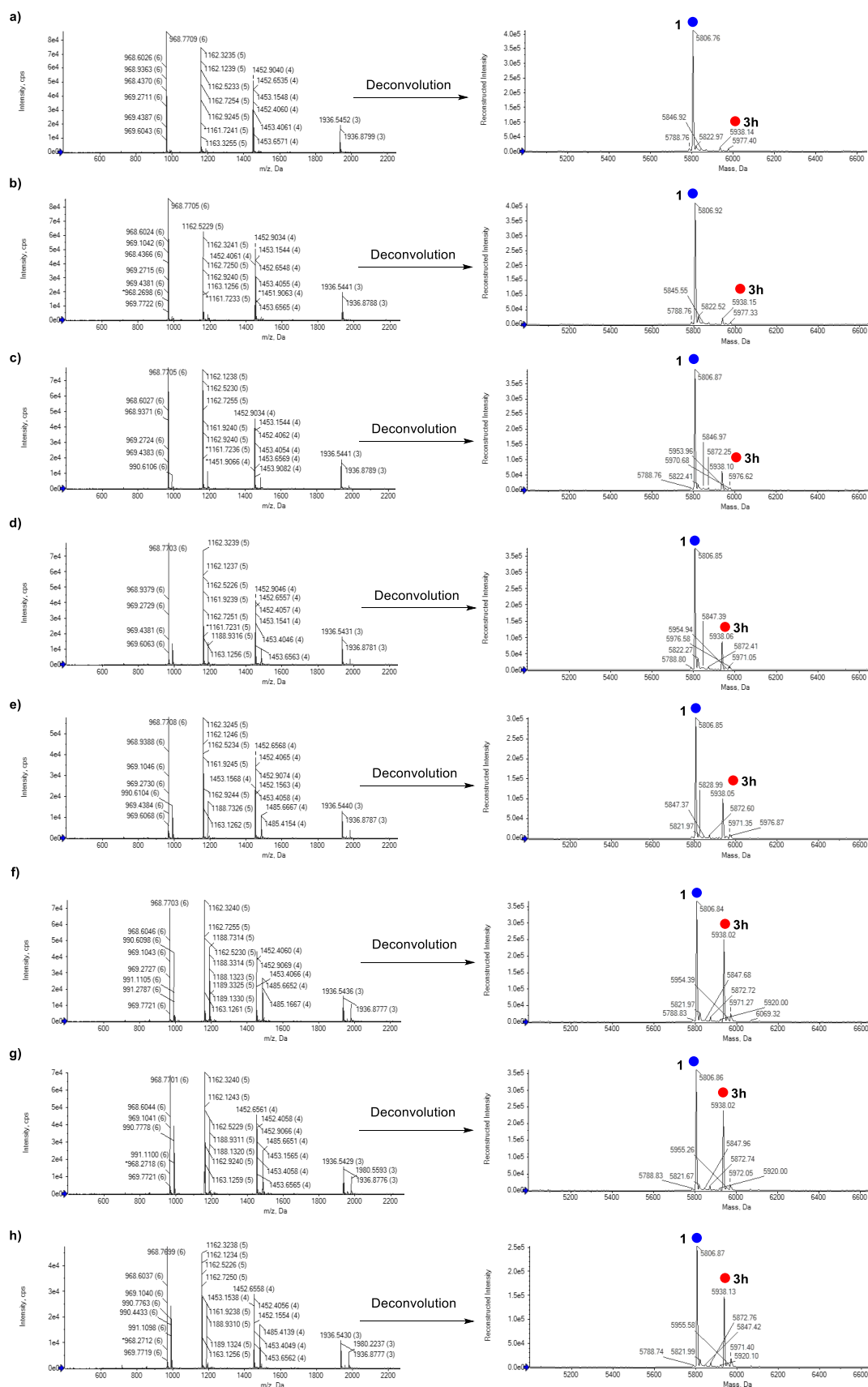
1.8 Kinetics experiments: N-Gly bioconjugation of insulin with 2h



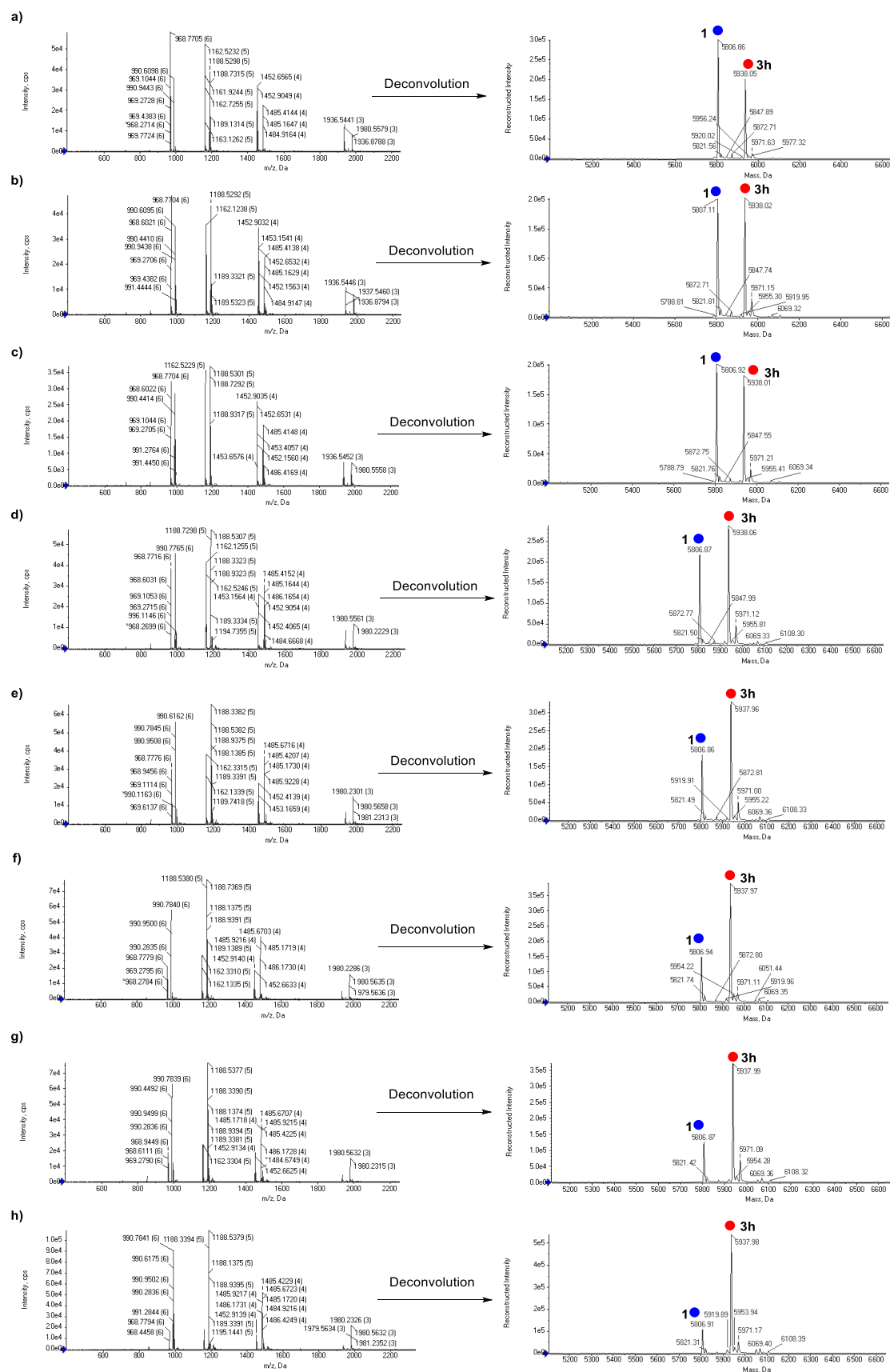
Replicate MS data Fig. 27. Replicate 2: Charged and deconvoluted MS spectra for insulin (1) after reaction with 2h for kinetics at different time intervals (2-35 min). (a) 2 min, (b) 5 min, (c) 10 min, (d) 15 min, (e) 20 min, (f) 25 min, (g) 30 min, and (h) 35 min.



Replicate MS data Fig. 28. Replicate 2: Charged and deconvoluted MS spectra for insulin (1) after reaction with 2h for kinetics at different time intervals (40-120 min). (a) 40 min, (b) 45 min, (c) 50 min, (d) 55 min, (e) 60 min, (f) 75 min, (g) 90 min, and (h) 120 min.

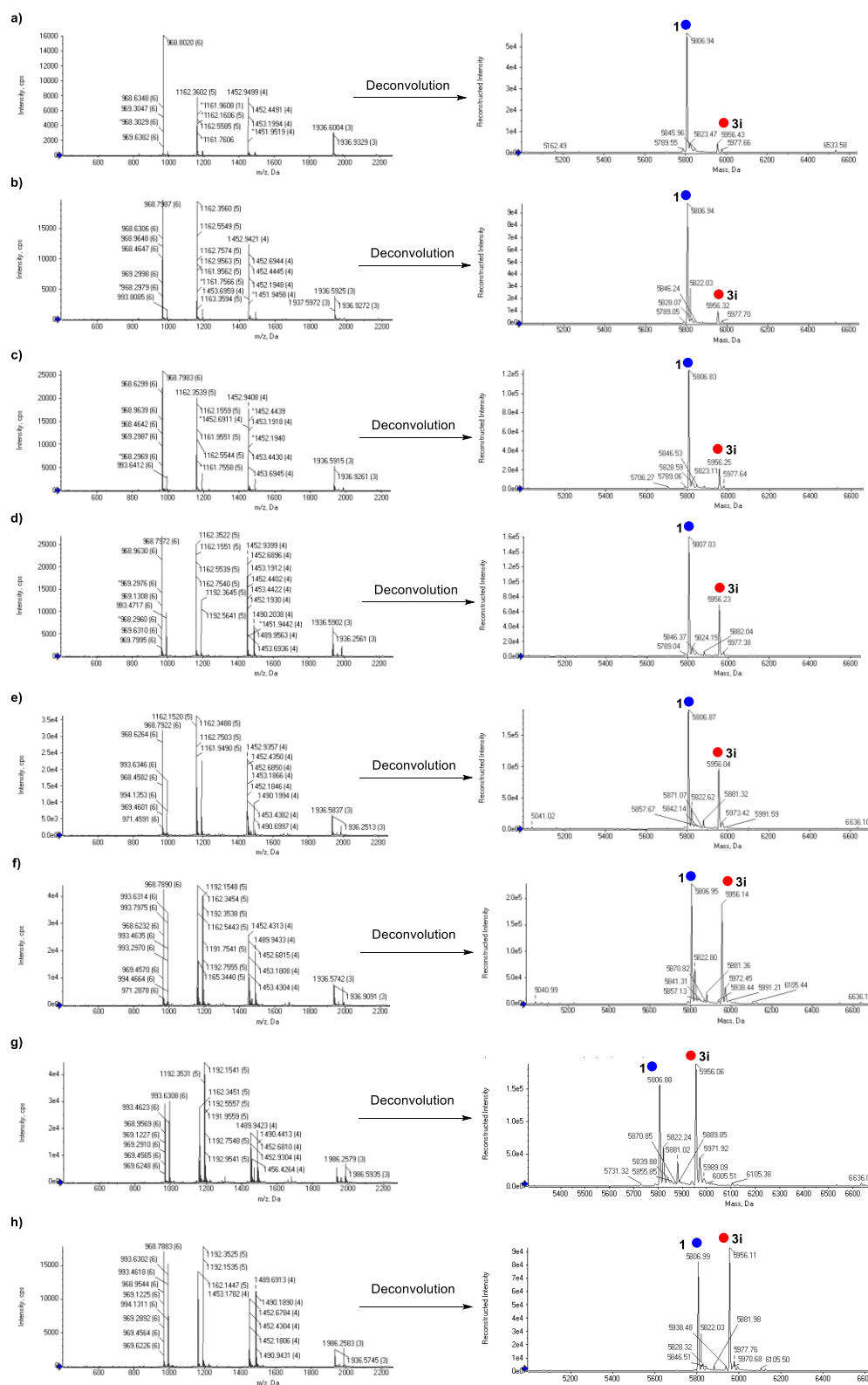


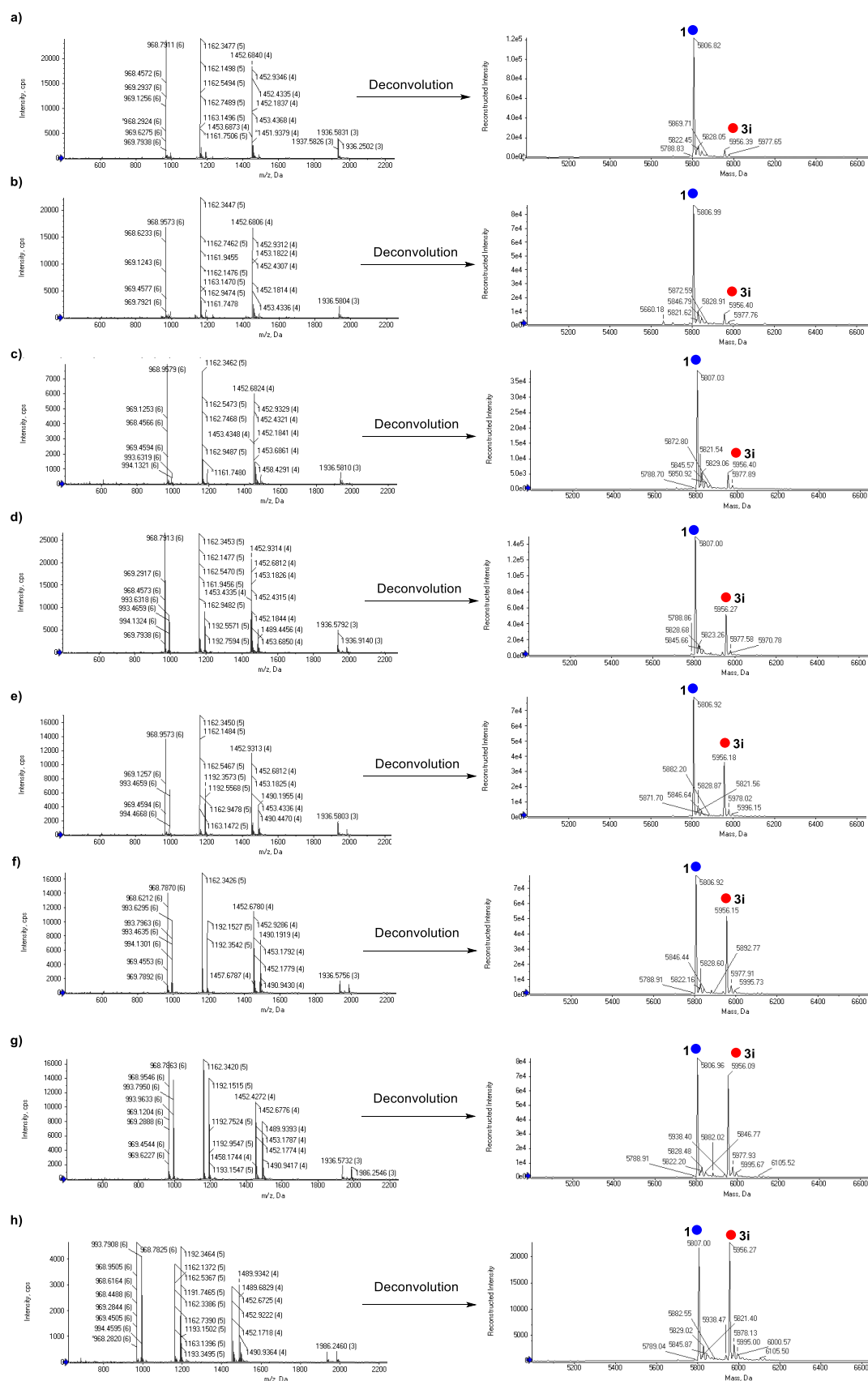
Replicate MS data Fig. 29. Replicate 3: Charged and deconvoluted MS spectra for insulin (**1**) after reaction with **2h** for kinetics at different time intervals (2-35 min). (a) 2 min, (b) 5 min, (c) 10 min, (d) 15 min, (e) 20 min, (f) 25 min, (g) 30 min, and (h) 35 min.



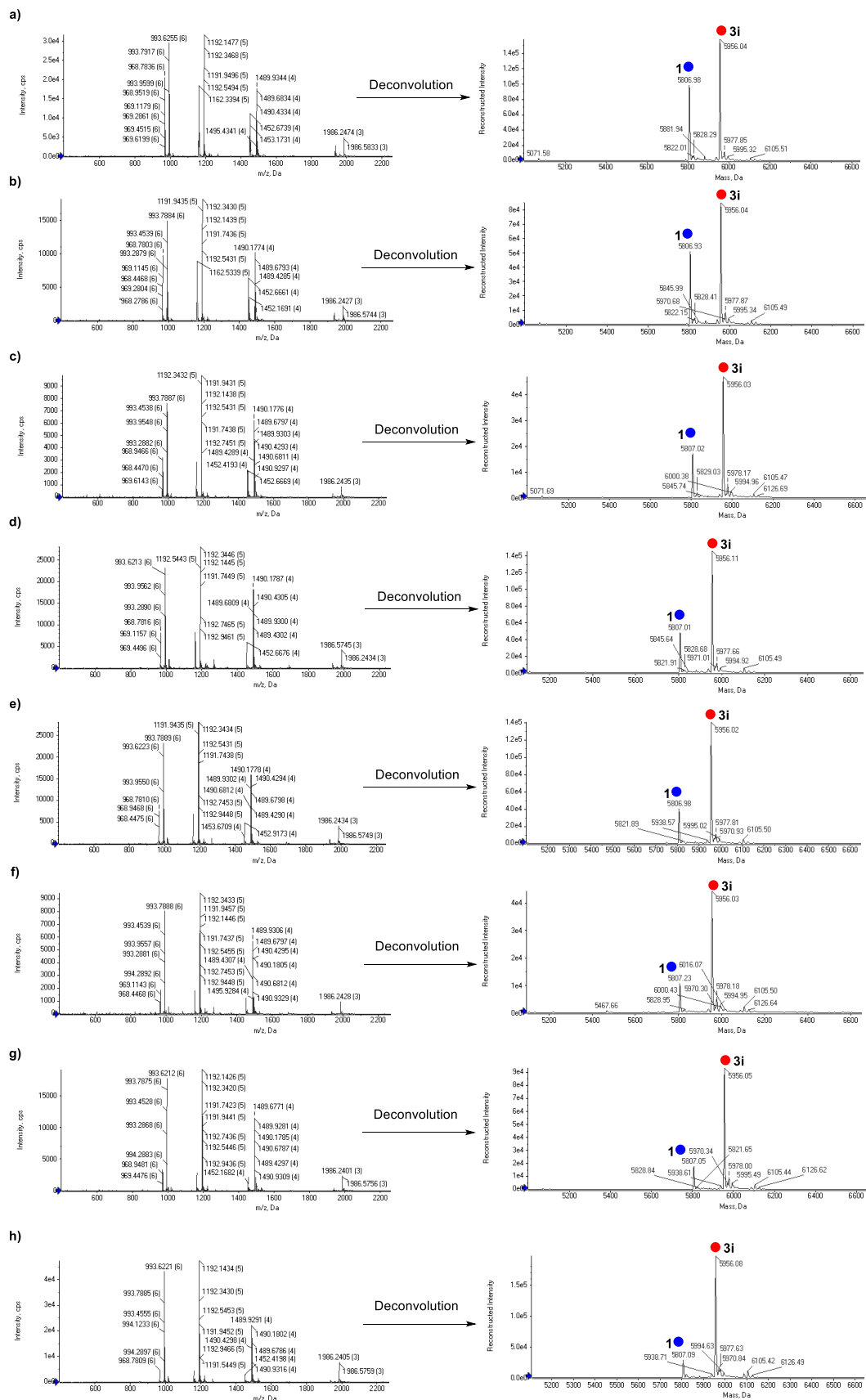
Replicate MS data Fig. 30. Replicate 3: Charged and deconvoluted MS spectra for insulin (1) after reaction with 2h for kinetics at different time intervals (40-120 min). (a) 40 min, (b) 45 min, (c) 50 min, (d) 55 min, (e) 60 min, (f) 75 min, (g) 90 min, and (h) 120 min.

1.9 Kinetics experiments: N-Gly bioconjugation of insulin with 2i



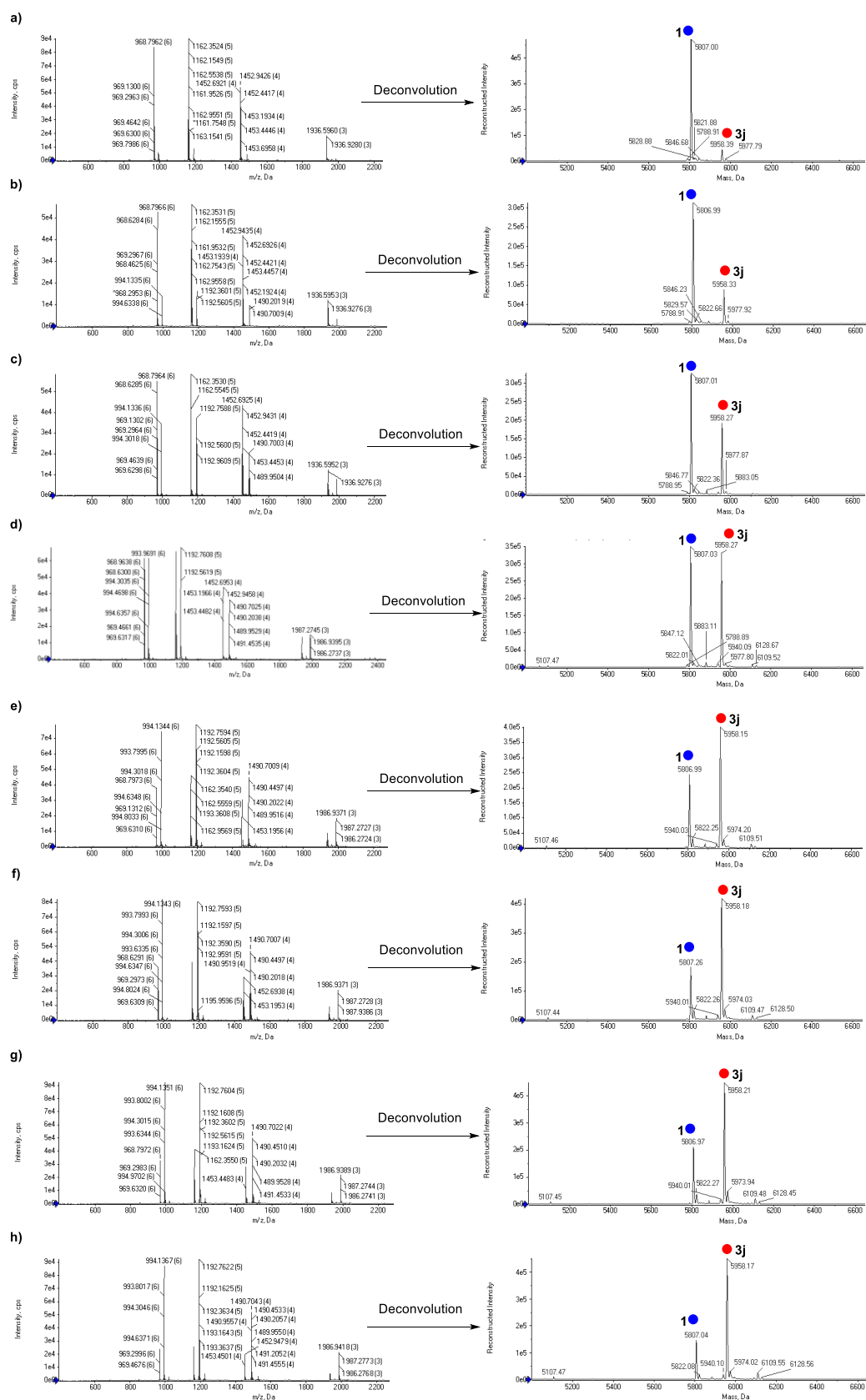


Replicate MS data Fig. 33. Replicate 3: Charged and deconvoluted MS spectra for insulin (**1**) after reaction with **2i** for kinetics at different time intervals (2-35 min). (a) 2 min, (b) 5 min, (c) 10 min, (d) 15 min, (e) 20 min, (f) 25 min, (g) 30 min, and (h) 35 min.

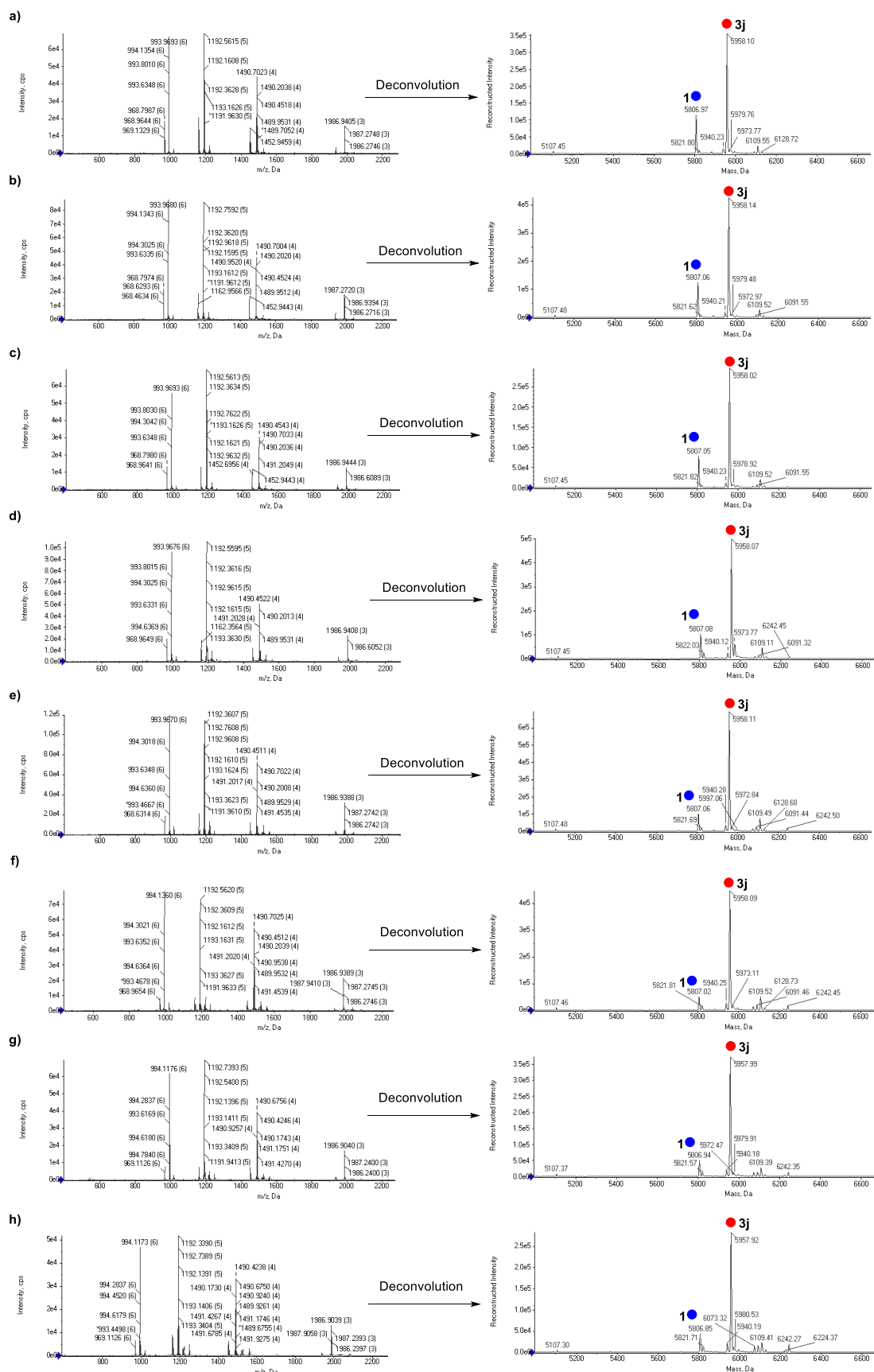


Replicate MS data Fig. 34. Replicate 3: Charged and deconvoluted MS spectra for insulin (**1**) after reaction with **2i** for kinetics at different time intervals (40-120 min). (a) 40 min, (b) 45 min, (c) 50 min, (d) 55 min, (e) 60 min, (f) 75 min, (g) 90 min, and (h) 120 min.

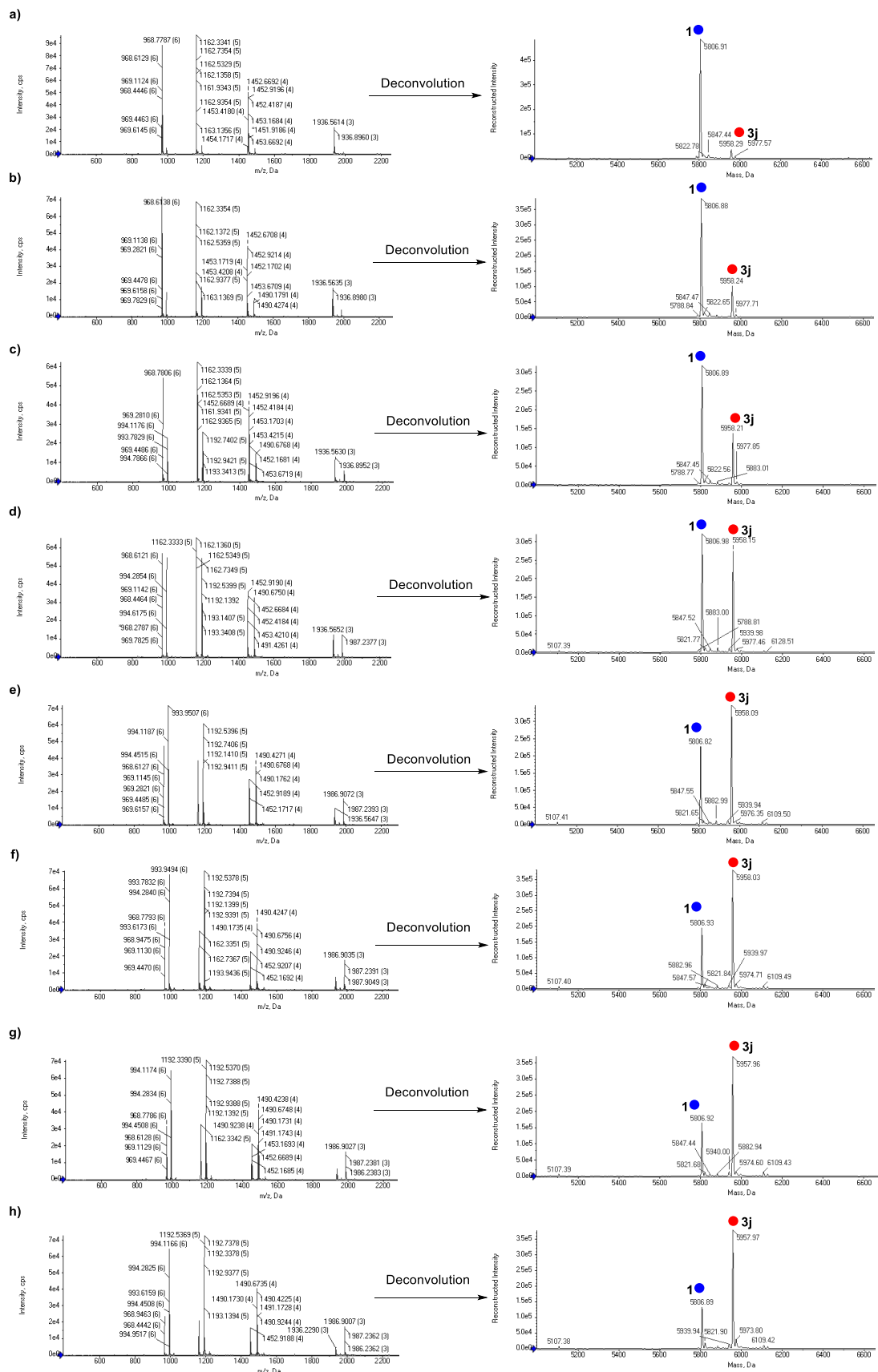
1.10 Kinetics experiments: N-Gly bioconjugation of insulin with 2j



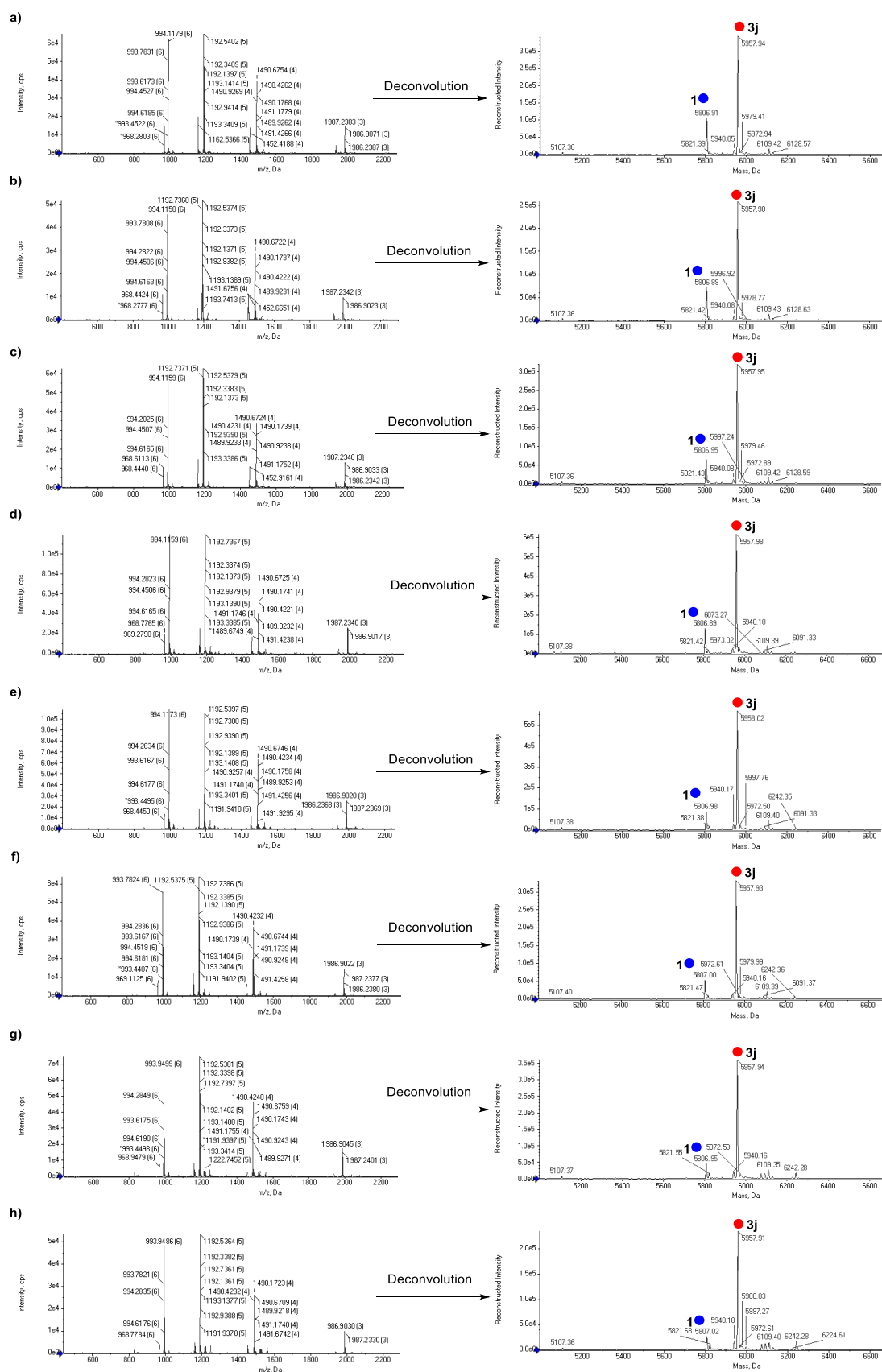
Replicate MS data Fig. 35. Replicate 2: Charged and deconvoluted MS spectra for insulin (**1**) after reaction with **2j** for kinetics at different time intervals (2-35 min). (a) 2 min, (b) 5 min, (c) 10 min, (d) 15 min, (e) 20 min, (f) 25 min, (g) 30 min, and (h) 35 min.



Replicate MS data Fig. 36. Replicate 2: Charged and deconvoluted MS spectra for insulin (**1**) after reaction with **2j** for kinetics at different time intervals (40-120 min). (a) 40 min, (b) 45 min, (c) 50 min, (d) 55 min, (e) 60 min, (f) 75 min, (g) 90 min, and (h) 120 min.

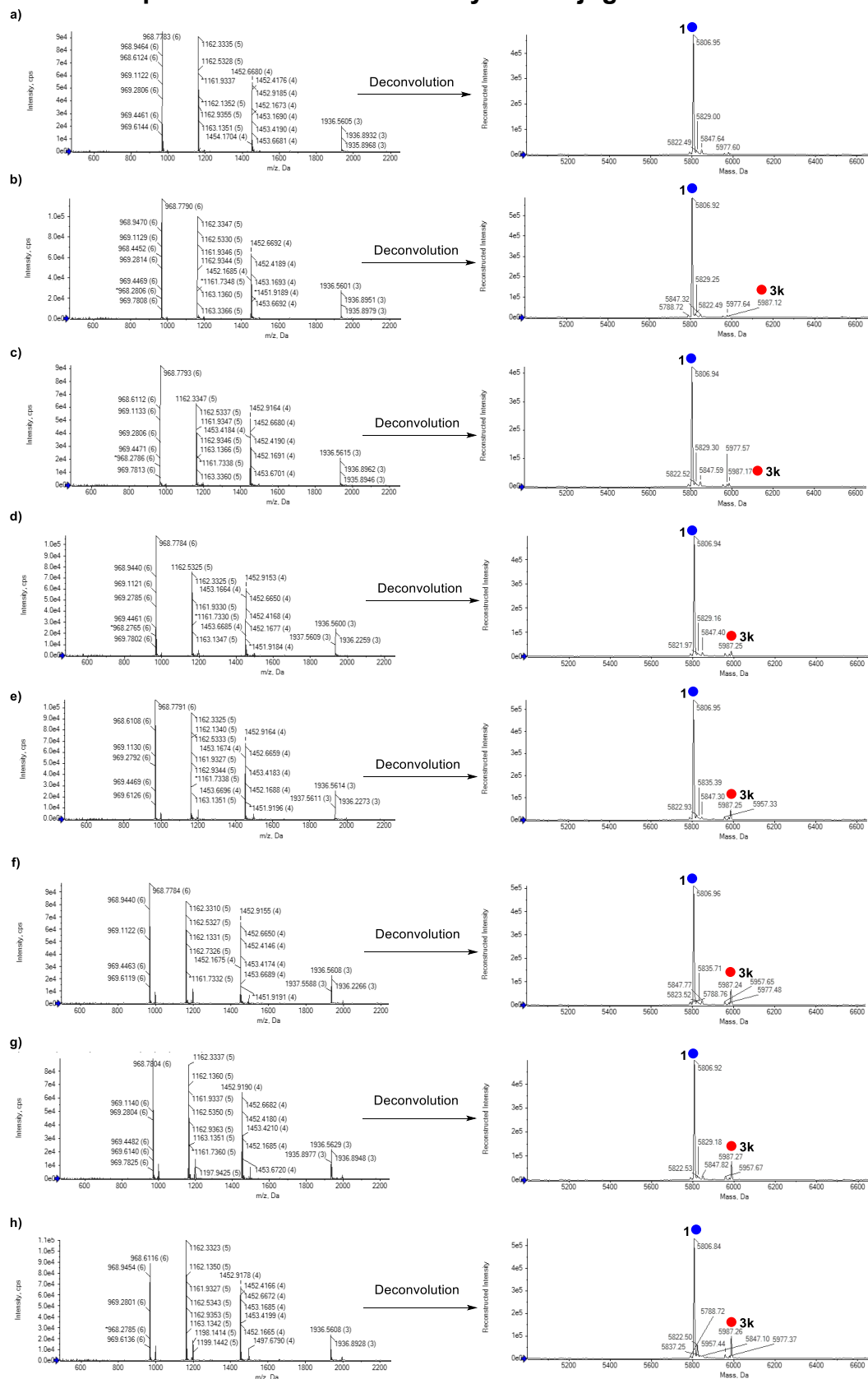


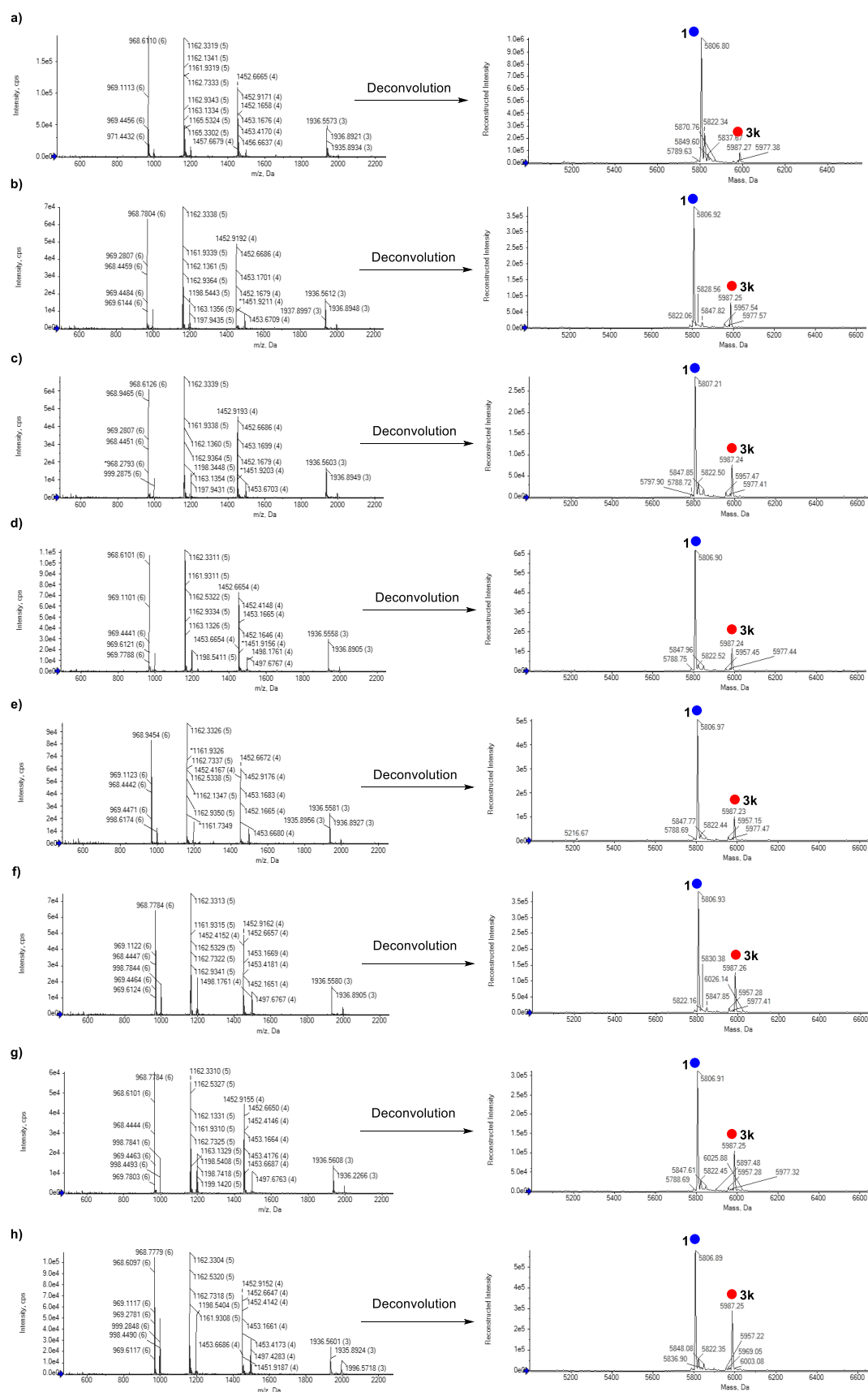
Replicate MS data Fig. 37. Replicate 3: Charged and deconvoluted MS spectra for insulin (1) after reaction with 2j for kinetics at different time intervals (2-35 min). (a) 2 min, (b) 5 min, (c) 10 min, (d) 15 min, (e) 20 min, (f) 25 min, (g) 30 min, and (h) 35 min.



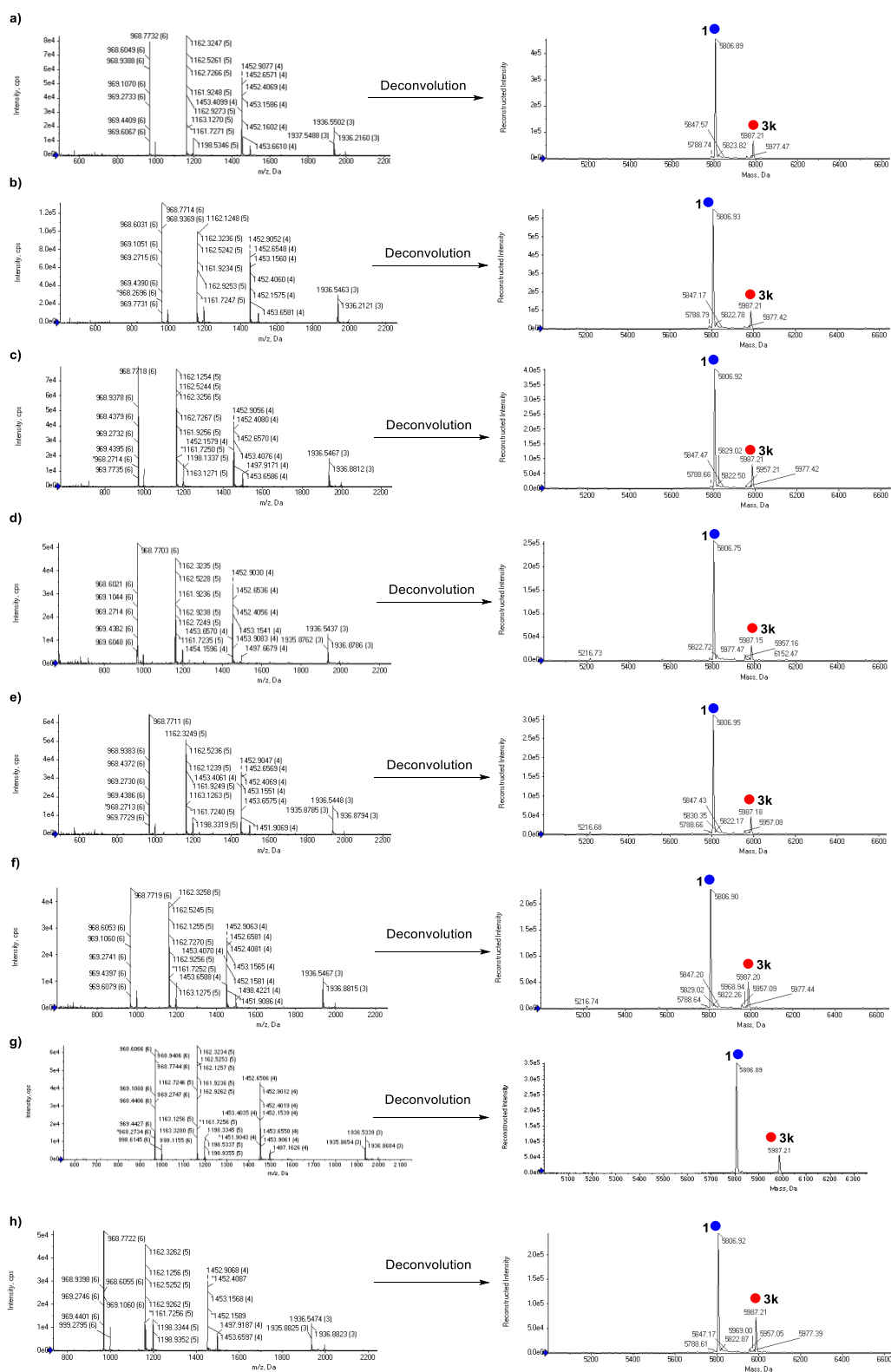
Replicate MS data Fig. 38. Replicate 3: Charged and deconvoluted MS spectra for insulin (1) after reaction with 2j for kinetics at different time intervals (40-120 min). (a) 40 min, (b) 45 min, (c) 50 min, (d) 55 min, (e) 60 min, (f) 75 min, (g) 90 min, and (h) 120 min.

Replicate MS data Fig. 39. Replicate 2: Charged and deconvoluted MS spectra for insulin (**1**) after reaction with **2k** for kinetics at different time intervals (2-35 min). (a) 2 min, (b) 5 min, (c) 10 min, (d) 15 min, (e) 20 min, (f) 25 min, (g) 30 min, and (h) 35 min.



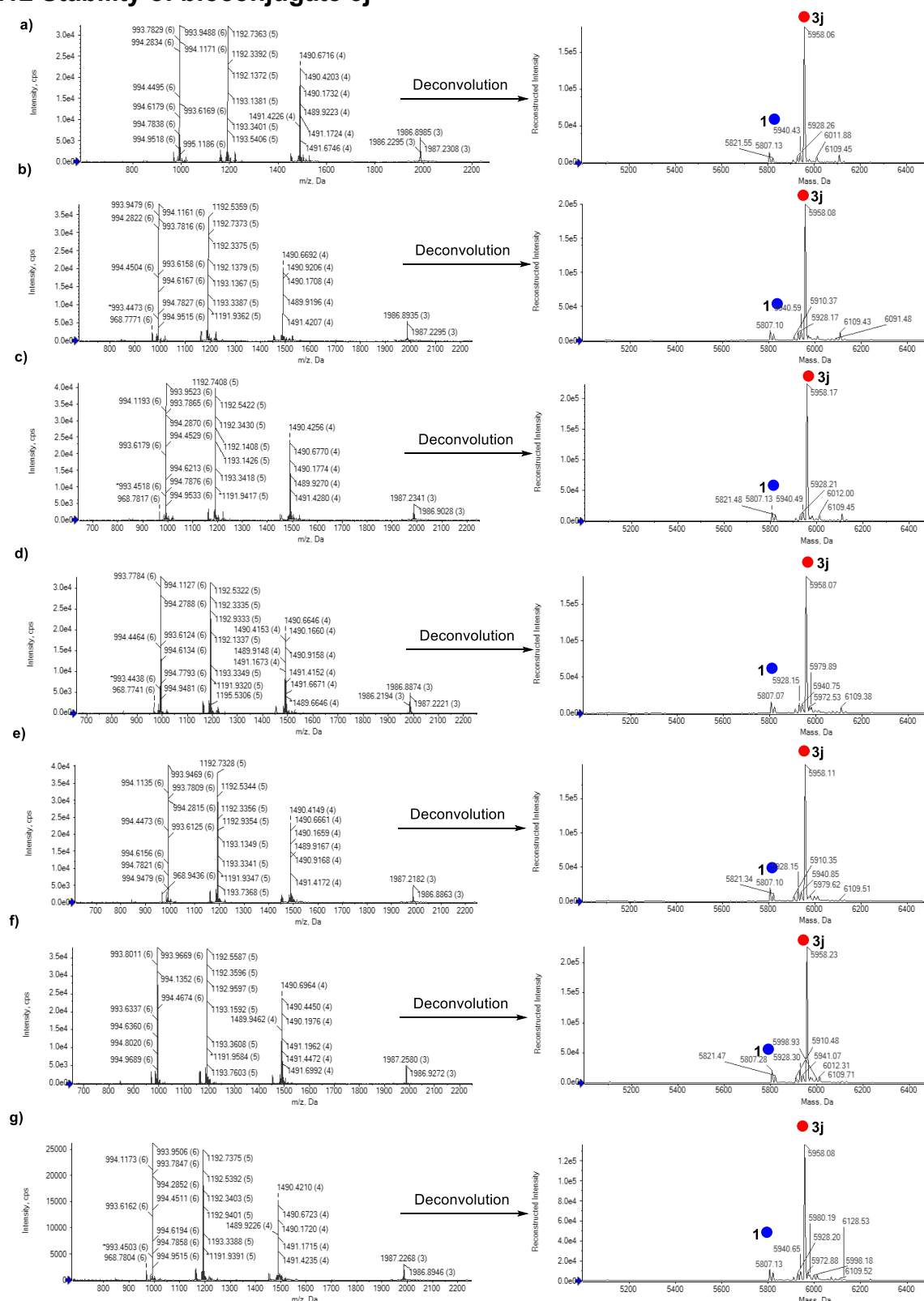


Replicate MS data Fig. 40. Replicate 2: Charged and deconvoluted MS spectra for insulin (**1**) after reaction with **2k** for kinetics at different time intervals (40-120 min). (a) 40 min, (b) 45 min, (c) 50 min, (d) 55 min, (e) 60 min, (f) 75 min, (g) 90 min, and (h) 120 min.

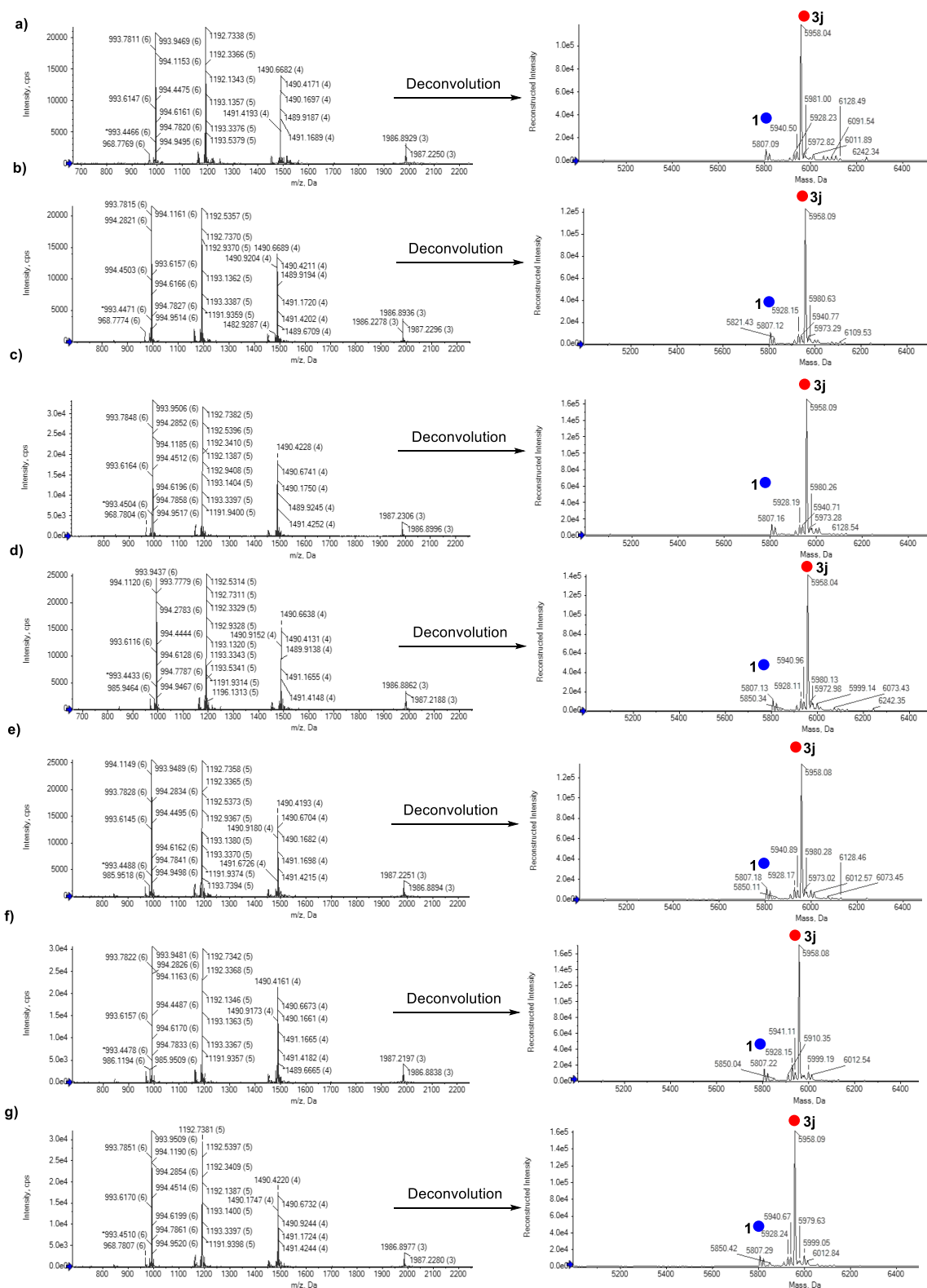


Replicate MS data Fig. 42. Replicate 3: Charged and deconvoluted MS spectra for insulin (1) after reaction with 2k for kinetics at different time intervals (40-120 min). (a) 40 min, (b) 45 min, (c) 50 min, (d) 55 min, (e) 60 min, (f) 75 min, (g) 90 min, and (h) 120 min.

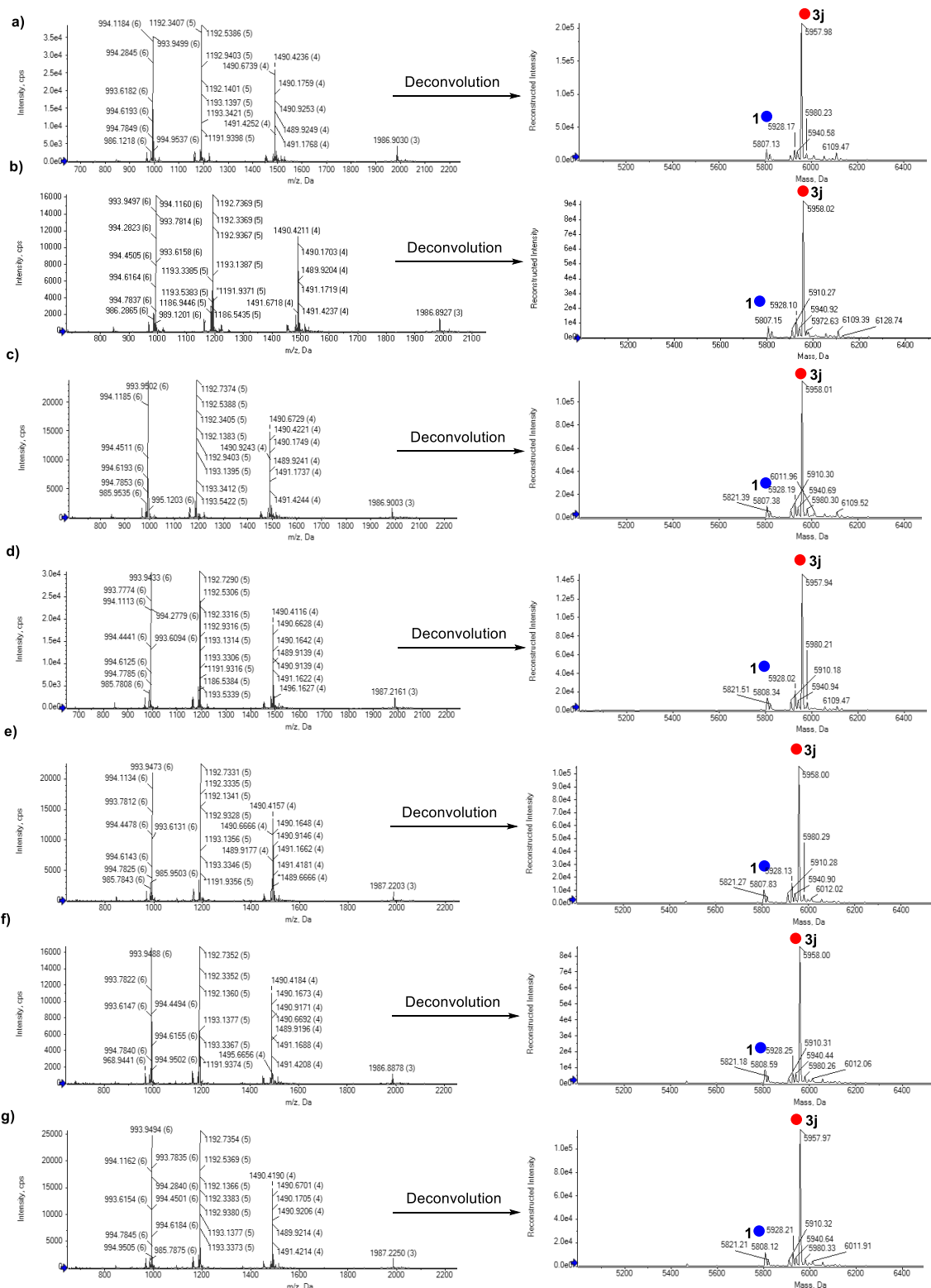
1.12 Stability of bioconjugate 3j



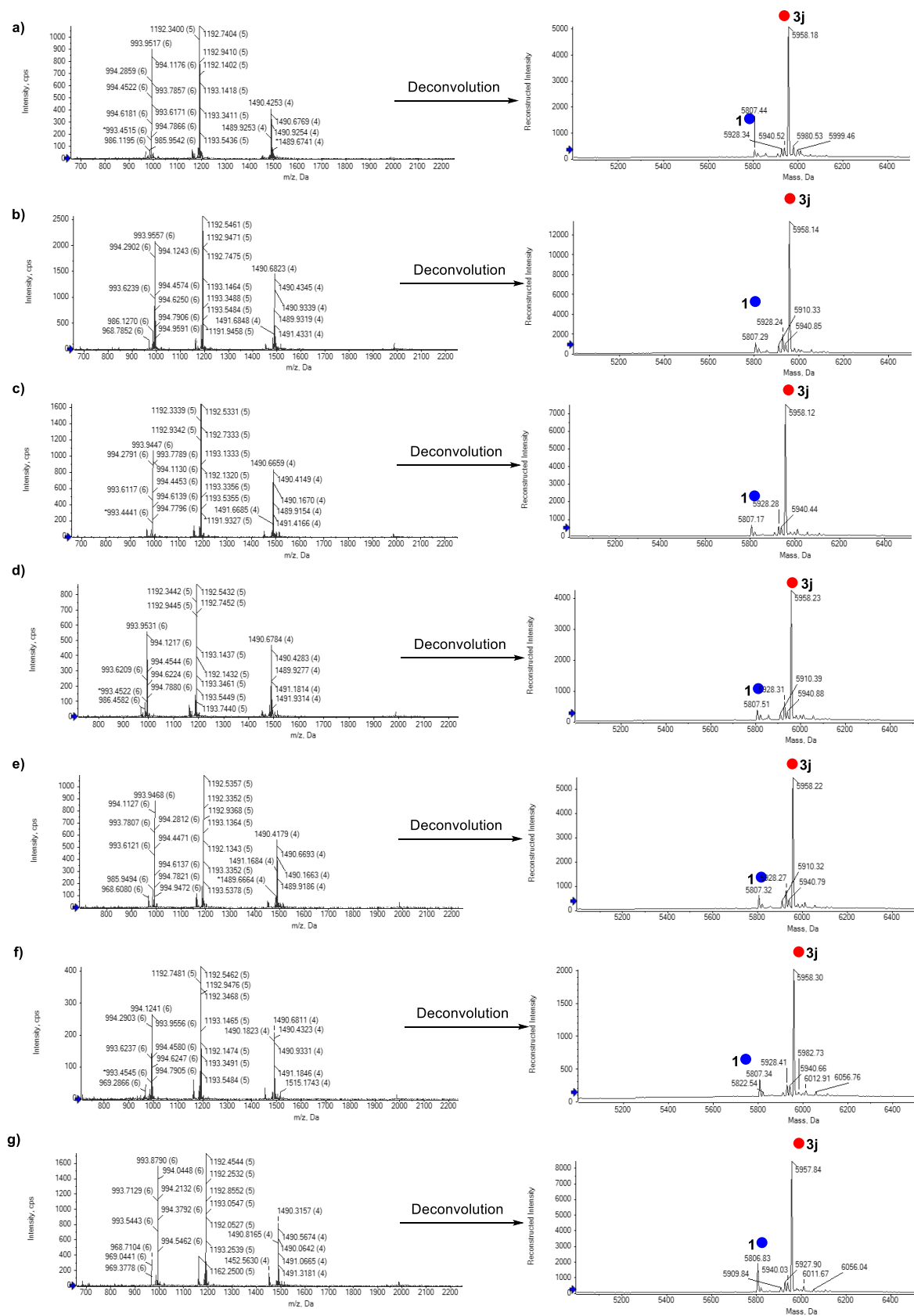
Replicate MS data Fig. 43. Replicate 2: Charged and deconvoluted MS spectra for stability of insulin bioconjugate 3j in water at 4 °C for (a) day 1, (b) day 2, (c) day 3, (d) day 4, (e) day 5, (f) day 6, and (g) day 7.



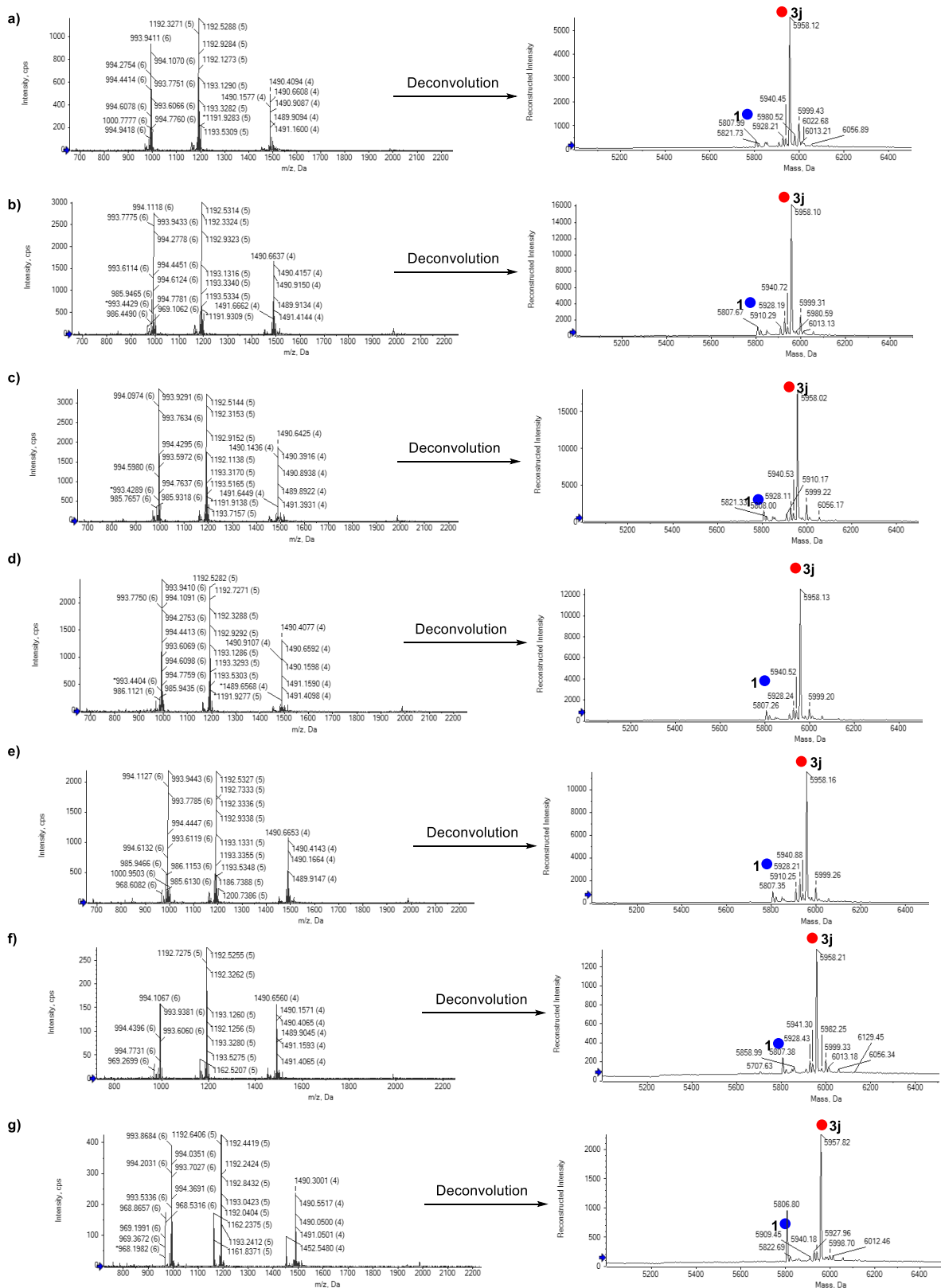
Replicate MS data Fig. 44. Replicate 2: Charged and deconvoluted MS spectra for stability of insulin bioconjugate **3j** in water at 25 °C for (a) day 1, (b) day 2, (c) day 3, (d) day 4, (e) day 5, (f) day 6, and (g) day 7.



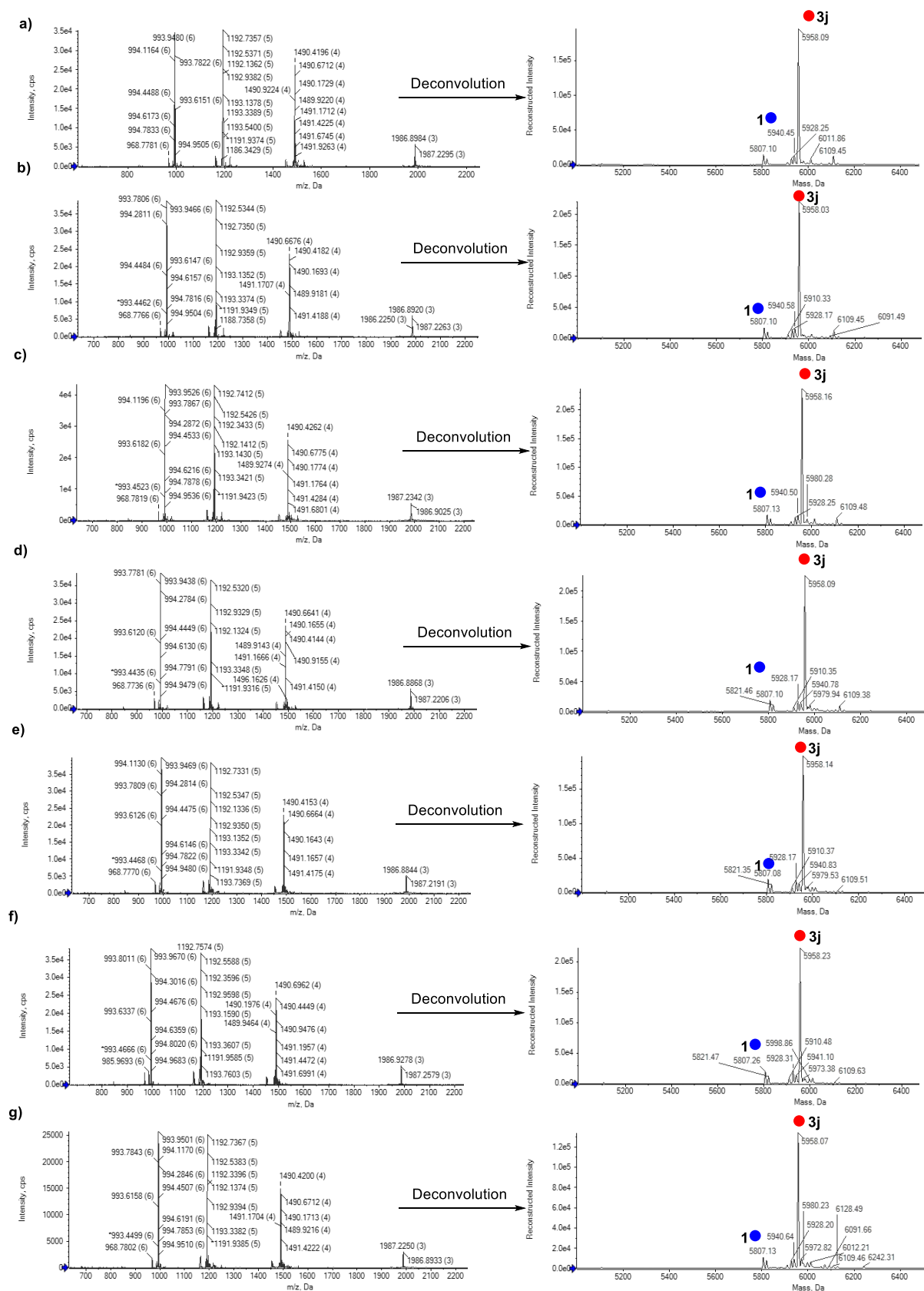
Replicate MS data Fig. 45. Replicate 2: Charged and deconvoluted MS spectra for stability of insulin bioconjugate **3j** in PBS at 4 °C for (a) day 1, (b) day 2, (c) day 3, (d) day 4, (e) day 5, (f) day 6, and (g) day 7.



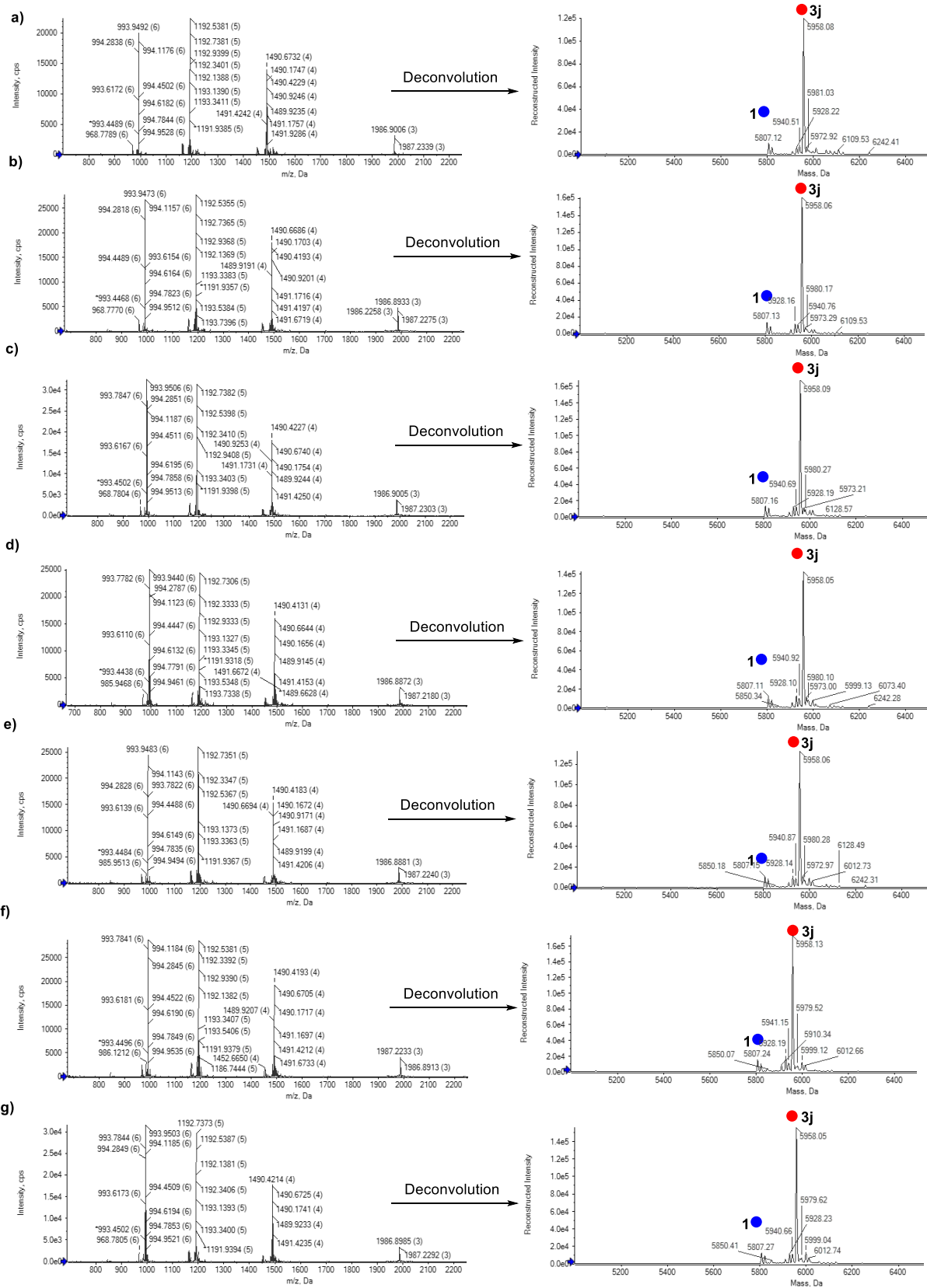
Replicate MS data Fig. 47. Replicate 2: Charged and deconvoluted MS spectra for stability of insulin bioconjugate **3j** in 10% human serum at 4 °C for (a) day 1, (b) day 2, (c) day 3, (d) day 4, (e) day 5, (f) day 6, and (g) day 7.



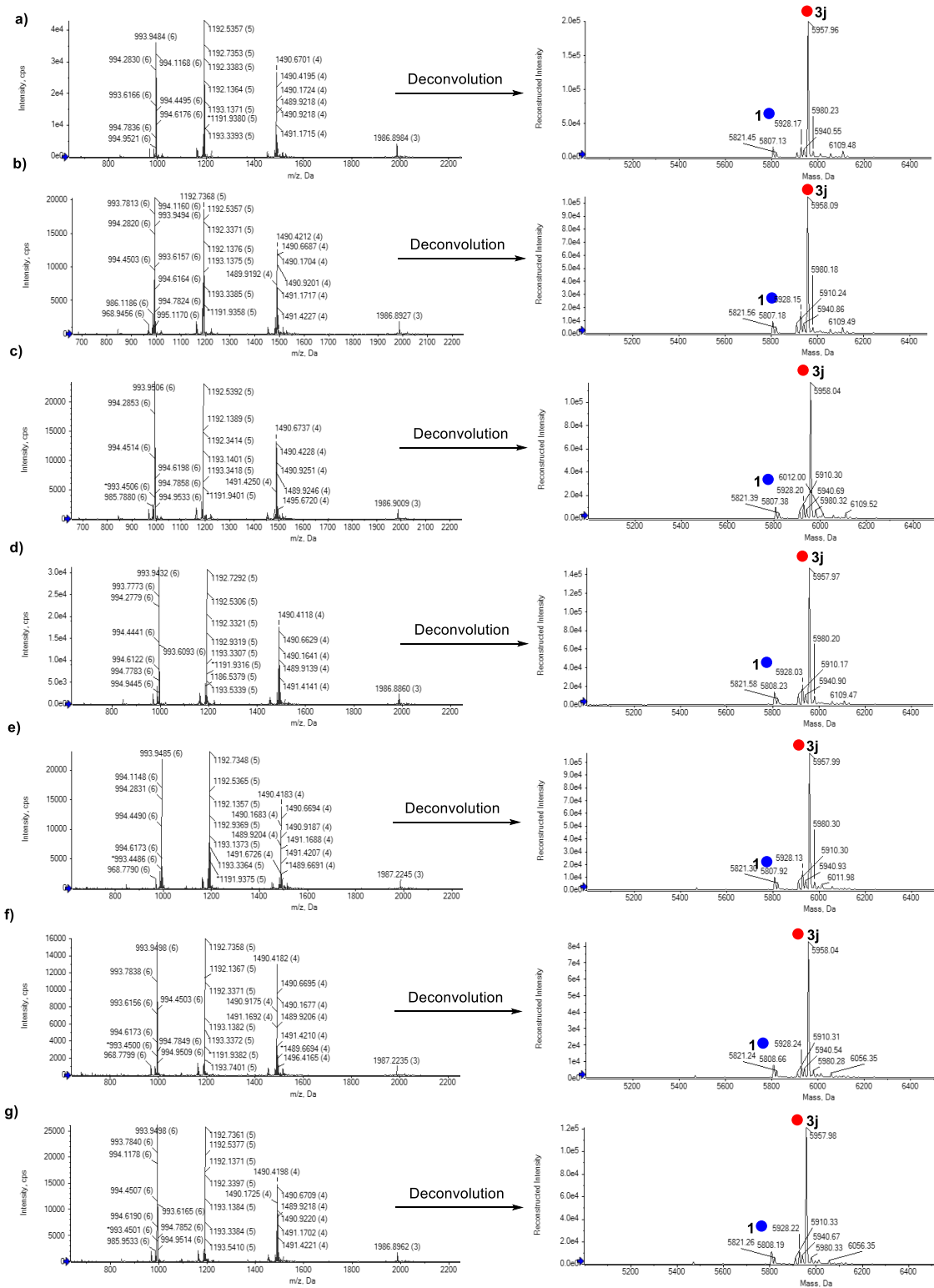
Replicate MS data Fig. 48. Replicate 2: Charged and deconvoluted MS spectra for stability of insulin bioconjugate **3j** in 10% human serum at 25 °C for (a) day 1, (b) day 2, (c) day 3, (d) day 4, (e) day 5, (f) day 6, and (g) day 7.



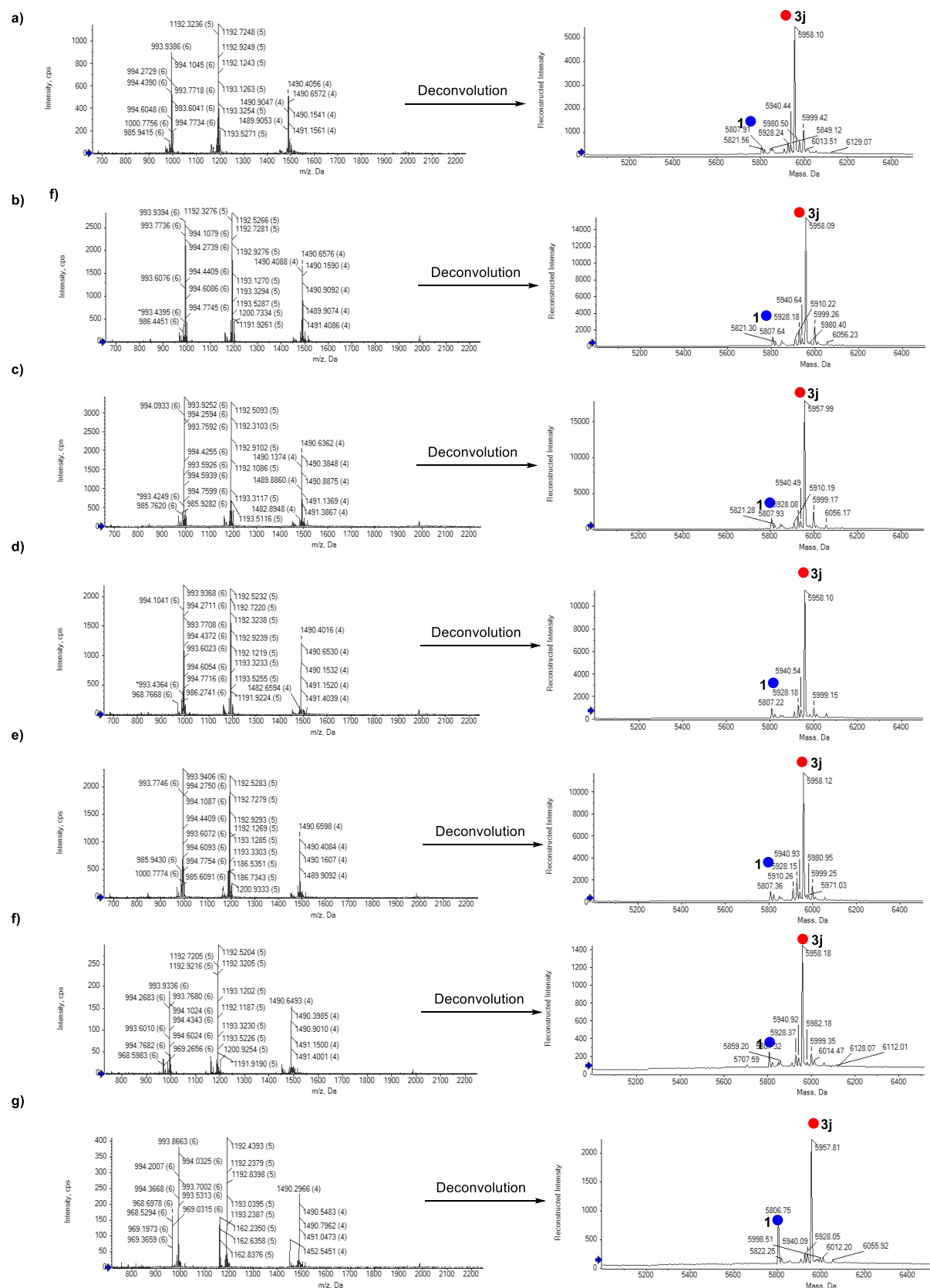
Replicate MS data Fig. 49. Replicate 3: Charged and deconvoluted MS spectra for stability of insulin bioconjugate **3j** in water at 4 °C for (a) day 1, (b) day 2, (c) day 3, (d) day 4, (e) day 5, (f) day 6, and (g) day 7.



Replicate MS data Fig. 50. Replicate 3: Charged and deconvoluted MS spectra for stability of insulin bioconjugate **3j** in water at 25 °C for (a) day 1, (b) day 2, (c) day 3, (d) day 4, (e) day 5, (f) day 6, and (g) day 7.



Replicate MS data Fig. 51. Replicate 3: Charged and deconvoluted MS spectra for stability of insulin bioconjugate **3j** in PBS at 4 °C for (a) day 1, (b) day 2, (c) day 3, (d) day 4, (e) day 5, (f) day 6, and (g) day 7.



Replicate MS data Fig. 54. Replicate 3: Charged and deconvoluted MS spectra for stability of insulin bioconjugate **3j** in 10% human serum at 25 °C for (a) day 1, (b) day 2, (c) day 3, (d) day 4, (e) day 5, (f) day 6, and (g) day 7.