



**Fig. 1** Change in the apparent acidity from solution to lyophile measured via change of the color of an indicator (photos), quantified by measuring ionization extent of the indicator in solution and lyophile (diffuse reflectance visible spectra), and expressed as the Hammett acidity function,  $H_x$  (the numbers in the graph). The change in color from solution to lyophile reflects significant change in the extent of protonation of the indicator and therefore major shift in the apparent acidity during freeze-drying. Visible diffuse reflectance spectra of solution and freeze-dried formulation show a major shift in the protonation of an indicator, from partially protonated in solution (pH 3.2) to completely protonated in the freeze-dried state ( $H_x < 1.0$ )

## Phosphate Buffer

Phosphate buffer is widely used and has been extensively studied in the pharmaceutical field. As early as the 1950s, Van den Berg conducted pioneering work to describe the effect of freezing on phosphate buffer system under equilibrium conditions [49]. Van den Berg showed that the pH and composition of maximally freeze-concentrated solutions can be defined by the eutectic point and is independent of both the initial pH and buffer concentration for sodium phosphate buffer and potassium phosphate buffer. An important finding was that the pH at the eutectic point is very different for sodium and potassium phosphate buffers with pH of 3.6 and 7.5, respectively [50]. This result has significant implication on protein stability during freezing, because independent of initial solution pH, under equilibrium freezing conditions, the unfrozen portion of phosphate buffer may approach pH 3.6 for sodium phosphate. Experimental studies have shown that freezing of 0.1 M sodium phosphate buffer resulted in a significant decrease in pH from 7.4 to 3.4 [27]. The pH behavior becomes even more complex for mixed solutions of sodium/potassium phosphates, where ten eutectic points ranging from 3.3 to 9.3 have been reported to exist. The pH change in this mixed system is influenced by both the sodium and potassium concentration and the ratio of pH of the initial solution, so the pH drop may be closer to 5.3 [27].