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Use of ferric thiocyanate derivatization for quantification of polysorbate 80 in high concentration protein formulations

Nimesh Savjani, Eugene Babcock, Hui Koon Khor, Anil Raghani *

Process and Product Development, Amgen, Inc. One Amgen Center Drive, Thousand Oaks, CA 91320, USA

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ABSTRACT

Quantitation of polysorbate 80 in high protein formulation using solid-phase extraction (SPE) followed by derivatization with cobalt thiocyanate and UV measurement of the complex at 620 nm resulted in lower recovery of polysorbate 80. Dilution of protein samples with water improved the recovery of polysorbate, however, it compromised the sensitivity of the method when cobalt thiocyanate was used for derivatization. The presented work discusses an evaluation of alternative approaches for increasing the sensitivity of the quantitation method for polysorbate using ferric thiocyanate and molybdenum thiocyanate. Ferric thiocyanate complex of polysorbate 80 exhibited the highest sensitivity among the metals thiocyanate evaluated in the current work. The improvement in the sensitivity through derivatization with ferric thiocyanate allowed 10-fold dilution of a 140 mg mL^{-1} protein sample without affecting the recovery or compromising the sensitivity of polysorbate 80 quantitation, indicating that this methodology could be used as an alternate approach for the quantitation of polysorbate 80 in high concentration protein formulations.

Stability of ferric thiocynate and cobalt thiocyanate complex was also studied. When these complexes were allowed to equilibrate for 1 h between an organic layer containing polysorbate/thiocynate complex and an aqueous layer containing un-reacted metal thiocyanate, it resulted in the most reproducible UV absorbance measurements.

The SPE method for quantification of polysorbate 80 using ferric thiocyanate for derivatization provided accuracy (% spike recovery) within 107%, reproducibility (%relative standard deviation) less than 11.7%. The method is linear from 0.0001 to 0.008% polysorbate 80 concentrations in the formulations with protein formulations as high as140 mg mL^{-1}.

1. Introduction

Proteins in general are prone to physical and chemical degradation that often result into aggregation to form multimers and/or chemical modifications such as deamidation, oxidation, etc. Controlling the levels of protein aggregation in biotherapeutics is crucial for the product quality and safety of patients. To stabilize proteins, they are commonly formulated in excipients containing surfactants such as polysorbate 20 and polysorbate 80, commercialized as Tween 20™ and Tween 80™ [1]. Presence of polysorbate 20 in protein formulations was shown to reduce shear during manufacturing of monoclonal antibody products [2]. Controlling the levels of polysorbate in protein formulation within a target level during manufacturing of protein products is essential. Most protein formulations contain polysorbates from 0.006 to 0.1% (w/v). It becomes a challenge to measure polysorbate at such low levels owing to the fact that these surfactants do not have a strong UV absorbing chromophore, thus cannot be quantified using spectrophotometric methods. Numerous analytical methods employing various detection techniques have been reported for the accurate quantitation of polysorbates. These methods include reversed phase HPLC using condensation nucleation light scatting [3], evaporative light scattering [4], and charge aerosol detection [5]. Acid hydrolysis of polysorbate followed by HPLC determination of the free lauric acid was also used as an indirect measurement of polysorbates [6].

For the measurement of polysorbates in protein formulations, most methods require removal of the protein from the sample to eliminate interference from the protein. Some of the commonly used methods for trace quantitation of polysorbate in complex matrices uses extraction of polysorbate using liquid–liquid extraction (LLE) [7,8]. LLE methods are quite labor intensive and therefore, less preferable for routine testing [9]. Recently, the extraction of polysorbate 80 from therapeutic formulations using solid phase extraction (SPE), followed by evaporative light scattering detection was reported [10]. In our laboratory, polysorbate 20 and polysorbate 80 in the formulations

* Corresponding author. Tel.: +1 805 447 0668; fax: +1 805 375 3378.
E-mail address: araghani@amgen.com (A. Raghani).

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containing high protein concentration are separated using SPE, followed by derivatization with cobalt–thiocyanate (CoSCN) and the quantitation of the polysorbate–thiocyanate complex using UV spectrophotometry [11]. UV measurement of derivatized polysorbate 80 was preferred due to general availability of UV spectrophotometers in most testing laboratories. For high protein concentration formulations, we encountered an issue of a decrease in the recovery of polysorbate when using the cobalt thiocyanate derivatization and UV spectrophotometry measurement. The loss of the polysorbate from the protein solution was attributed to the binding of polysorbate to protein [12,13]. A solution to improve the recovery was dilution of the sample with water, which likely reduced the protein–polysorbate interaction. Dilution of the samples, however, compromised the sensitivity of the cobalt thiocynate method. These observations led us to evaluate alternative approaches to increase the sensitivity for the quantitation of polysorbate 80 in the diluted protein samples.

Molybdenum(V) was reported to be selectively extracted in the presence of tungsten(III) using polysorbate 80 [14]. In this paper we report an investigation of polysorbate–thiocyanate complexes of other metals such as iron and molybdenum and their UV spectrophotometric measurement for the determination of the surfactant in protein containing samples. The objective of the method development was to improve the sensitivity of the existing cobalt thiocyanate method for trace quantitation of polysorbate 80. In addition, the goal of the current work was to develop an accurate method that provides consistent measurements of polysorbate in the diluted high protein formulations to facilitate specification testing for the release of protein product lots.

2. Material and methods

The procedure for extraction of polysorbate 80 from protein samples was adopted from that developed by Kim and Qiu [11]. Cobalt thiocyanate solution was prepared by dissolving 15 g of cobalt nitrate hexahydrate, 50 g of ammonium thiocyanate and 25 g of sodium chloride in 250 mL water. Similarly, ferric thiocyanate was prepared by dissolving 2.7 g of iron(III) chloride hexahydrate and 3.0 g of ammonium thiocyanate into 100 mL of water. Molybdenum thiocyanate was prepared by dissolving 2.7 g of molybdenum(V) chloride and 3.0 g of ammonium thiocyanate into 100 mL of water. Polysorbate used in these studies was purchased from Avantor Performance Materials (Phillipsburg, NJ, USA). Polysorbate 80 standards were prepared from 0.0001 to 0.01% (w/v) by serial dilution of a 0.1% (w/v) stock solution with water.

Solid phase extraction cartridges used in this procedure were Oasis HLB with 1 mL bed volume purchased from Waters (Milford, MA, USA). These cartridges were conditioned with methanol and water prior to loading the samples. The SPE cartridges were attached to a vacuum manifold to elute the solutions used in the extraction procedure. 1 mL of protein samples were loaded to the conditioned SPE cartridges. Protein from the SPE was eluted with 3 M guanidine hydrochloride solution, followed by a water wash to remove any residual guanidine hydrochloride. Polysorbate 80 retained by the SPE resin was eluted with 1 mL acetonitrile. The extract containing polysorbate in acetonitrile was dried under nitrogen. To the dried extraction containing polysorbate 80, 1 mL of aqueous metal thiocyanate was added to form a polysorbate/thiocyanate complex. Excess metal thiocyanate was then removed by liquid–liquid extraction with 1 mL of methylene chloride that contained derivatized polysorbate 80 and the unreacted ferric thiocyanate remained in the aqueous layer. Concentration of polysorbate/thiocyanate complex in the methylene chloride layer was measured using UV spectrophotometer, Cary 50 (Agilent, DE, USA) at the specified wavelengths. UV spectra were also collected from 200 to 800 nm for polysorbate/thiocyanate complexes of the metals.

Concentration of polysorbate 80 was determined by linear regression analysis of the calibration curve plotted for the standards. A monoclonal antibody product at >120 mg mL^{-1} protein concentration formulated with polysorbate 80 targeted at 0.01% (w/v) was used in the present studies. Pre-formulated protein samples without the presence of polysorbate 80 were used to determine spike recovery of the method.

3. Results and discussion

3.1. Loss of polysorbate 80 in high protein concentration formulation

In our laboratory, polysorbate 80 in monoclonal antibody products is routinely quantitated using SPE extraction followed by cobalt thiocyanate derivatization [11]. However, when this analytical method was employed to a monoclonal antibody product with a protein concentration greater than 120 mg mL^{-1}, recovery of the targeted polysorbate 80 was rather unsatisfactory (58%), as provided in Table 1. Upon dilution of the samples with water, spike recovery significantly improved with increasing dilution, as evident from the data provided in this table. While there was no significant difference in the observed spike recoveries between 4-fold versus 5-fold dilution, a dilution factor for 5-fold was chosen in order to ensure the robustness of the method. The range for the specification for polysorbate 80 in this drug product was 50 to 150% of the target PS80 concentration in the product. Therefore, the calibration curve needed to be adjusted to 25 to 200% of the target polysorbate 80 concentration so that it was wide enough to encompass the specification range (refer to Table 1). However, 5-fold dilution of the sample resulted in a lower UV absorbance of polysorbate/cobalt thiocyanate complex such that it was near the limit of quantitation of 0.0005% (w/v) of the method. In order to increase the sensitivity to allow the measurement of polysorbate 80 below 0.0005% (w/v), alternative approaches were evaluated for the situation when a dilution factor of 5-fold or greater became necessary.

3.2. Evaluation of other metal thiocyanate complexes

Polysorbate 80 forms a stable complex with molybdenum thiocyanate relative to its tungsten thiocyanate complex [14]. The stable molybdenum thiocyanate complex was, therefore,

Table 1
Improvement of polysorbate 80 recovery with sample dilution.

Dilution factor	Spike recovery (%)	Target[a] polysorbate 80 concentration, % (w/v)	Dilution factor[b] adjusted specification range, % (w/v)	Standard curve range[c]
No dilution	58	0.0100	0.0500–0.0150	0.00250–0.0200
2	79	0.00500	0.00250–0.00750	0.00125–0.0100
4	90	0.00250	0.00125–0.00375	0.000625–0.00500
5	90	0.002000	0.00100–0.00300	0.000500–0.00400

[a] Target concentration is the expected concentration of polysorbate 80 in the product after dilution factor.
[b] Dilution factor adjusted specification range is specification range of 50 to 150% of the target PS80 concentration divided by the dilution factor.
[c] Standard curve range is 25 to 200% of the target PS80 concentration.

quantitatively removed using liquid extraction with chloroform. Similarly, ferric thiocyanate was used for the quantitation of polysorbate 20 in water [15]. Ferric thiocyanate was also employed for derivatization of poloxomers and poloxamines, surfactants used in biological samples [16]. To explore the sensitivity of these metal thiocyanates, polysorbate 80 solutions with concentrations ranging from 0.00005 to 0.01% (w/v) were derivatized. Polysorbate 80/metal thiocyanate complexes were extracted following liquid–liquid extraction using methylene chloride while employing the same procedure described for the cobalt thiocynate complex of polysorbate 80. As evident from the spectra compared in Fig. 1, the cobalt thiocyanate/polysorbate complex had two maxima for absorbance, one at 320 nm and the other at 620 nm. Similarly, molybdenum thiocyanate complex had a maximum at 470 nm which was higher than the other maximum at 327 nm. Ferric thiocyanate complex had a single maximum absorbance at 510 nm. For 0.001% polysorbate 80 solution, ferric thiocyanate had the highest absorbance with 0.011 absorbance units (510 nm), followed by cobalt thiocyanate with 0.0097 (320 nm), molybdenum thiocyanate with 0.0060 (470 nm) and 0.0037 (327 nm), and finally cobalt thiocyanate with 0.0011 (620 nm). Recall that the existing method using derivatization with cobalt thiocynate employed UV absorbance measurement at 620 nm. UV measurement at 620 nm rather than 320 nm for the cobalt thiocyanate complex was originally selected to minimize interference from the other components of the samples based on the work reported by Kim et al. [11]. However, an SPE procedure usually offers a high degree of specificity towards an analyte of interest (polysorbate 80 in this case) and derivatization reaction between polyoxythethylene group and cobalt thiocyanate also offers another degree of specificity, therefore, it is likely that under these two specific conditions, the interference from the sample matrix component could be minimal. In order to confirm whether there would be any matrix interference, we evaluated the feasibility of improving the sensitivity for the quantification of polysorbate 80/cobalt thiocyanate complex at 310 nm. Derivatization of polysorbate 80 with the ferric thiocyanate complex and UV absorbance measurement at 510 nm exhibited as much as ten times higher UV absorbance than that observed for the cobalt thiocyanate complex at 620 nm.

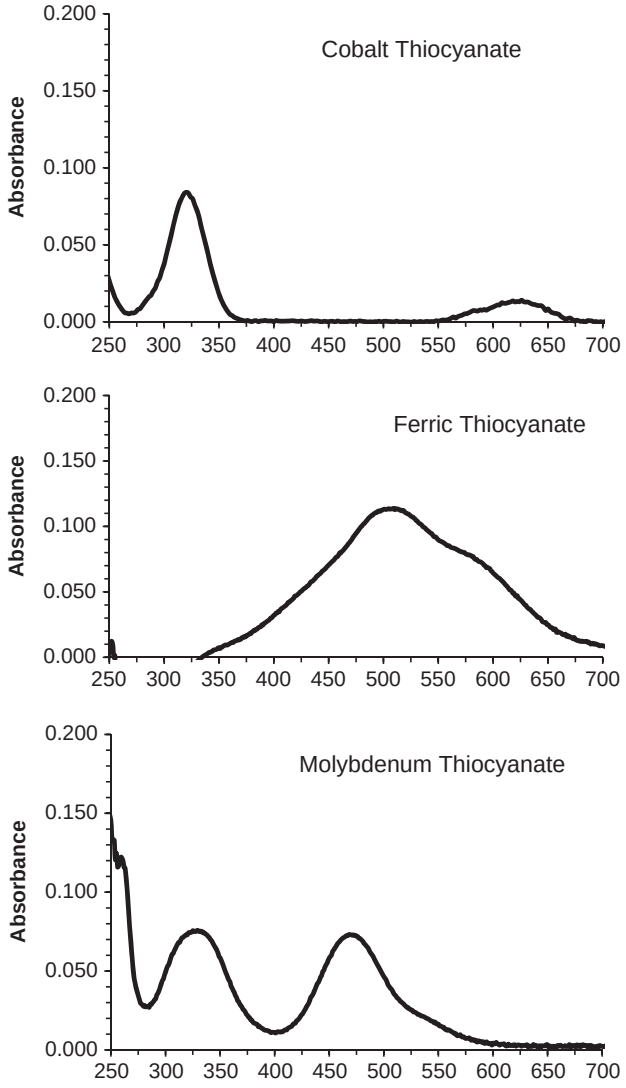

Fig. 1. Comparison of UV–visible spectra of cobalt, ferric and molybdenum complexes of 0.001% polysorbate 80.

Sensitivity of the thiocyanate complexes of the metals based on the slope of the standard curve in Table 2, were in the order (from the highest to the lowest) as Fe (510 nm) > Co (320 nm) > Mo (327 nm) > Mo (470) > Co (620 nm). In all cases, each metal demonstrated linearity for polysorbate concentration in the range of 0.0001 to 0.008%, as evident from the correlation of determination > 0.99 provided in Table 2. Since the goal of the current study was to increase the sensitivity, further evaluation for optimum condition for polysorbate 80 quantitation continued with the two metal complexes, cobalt thiocyanate at 320 nm and ferric thiocyanate at 510 nm, that showed the highest absorbance values for a given polysorbate 80 concentration.

3.2.1. Stability of cobalt and ferric thiocyanate complexes of polysorbate 80

Reproducibilities of the absorbance of ferric and cobalt thiocyanate complexes were studied by observing the stability of the corresponding complexes. One of the goals of the study was to find a sampling condition after the separation of two layers where the changes in the absorbance values could be minimized to attain reproducible polysorbate 80 measurements.

The stability of the polysorbate 80/metal thiocyanate complex at room temperature under ambient light was assessed by measuring the absorbance of the complex repeatedly in a UV spectrophotometric cuvette over the duration of 12 min. The dependence of the stability was determined for various concentrations of polysorbate 80 and also wait time (0, 1 and 2 h after phase separation before the organic layer is sampled). When the cobalt thiocyanate complex was removed immediately after the

Table 2
Comparison of standard curve parameters of metal thiocyanate complexes of polysorbate 80 from 0.0001 to 0.008% polysorbate 80.

Characteristics	Cobalt thiocyanate (620 nm)	Cobalt thiocyanate (320 nm)	Molybdenum thiocyanate (470 nm)	Molybdenum thiocyanate (327 nm)	Ferric thiocyanate (510 nm)
Coefficient of determination	0.9984	0.9989	0.9996	0.9996	0.9993
Intercept	−0.0032	−0.0173	−0.0098	−0.0148	−0.0191
Slope	18.685	117.806	85.429	92.673	140.175

separation of the two phases, a consistent decrease in absorbance of the complex at 320 nm was observed, as shown Fig. 2 (0 h panel). In this figure, absorbance values were normalized to the absorbance at 0 min to determine the relative change in the absorbance readings. Interestingly, the decrease in the absorbance was more pronounced at lower polysorbate concentration relative to the absorbance decrease observed at higher polysorbate concentration levels. For example, at polysorbate concentration of 0.0005% (w/v), the absorbance of cobalt thiocyanate complex reduced to approximately 60% in a matter of 12 min. Such a variation in the absorbance readings would results in imprecise quantitation of polysorbate 80. On the other hand, when the cobalt thiocyanate complex was allowed to equilibrate for 1 h before the organic layer was sampled for absorbance measurement, a decline in the absorbance measurement was at a much lesser extent compared to that observed when the organic layer was sampled immediately after the phase separation (Fig. 2, 1 h versus 0 h). Further improvement in the stability of the complex was observed when the organic layer was sampled after 2 h of phase separation, in which case, the absorbance for the lowest concentration (0.0005% (w/v)) of polysorbate 80 studied declined only to 95% after 12 min relative to the initial absorbance value at 0 min. Stability of the complex longer than 2 h after the phase separation was not studied because for routine testing a wait time longer than 2 h is not preferable.

Stability of the ferric thiocyanate complex of polysorbate 80 was also studied in a similar manner. As observed with the cobalt thiocynate complex, absorbance of the ferric thiocyanate complex of polysorbate at 0.0005% declined rapidly when sampled immediately after the phase separation, as shown in Fig. 3 (0 h). However, for the ferric thiocyanate complex of polysorbate 80 with concentrations higher than 0.0005%, the decline in absorbance was not as much as it was observed for the cobalt thiocyanate complex (refer to Fig. 2). On the other hand, when ferric thiocynate complex was allowed to equilibrate for 1 h, the complex of polysorbate 80 at concentrations ranging from 0.0005 to 0.008% remained within 97 to 110% of the initial absorbance reading. When the ferric thiocyanate complex was allowed to equilibrate longer than 2 h, consistent increase in the absorbance was observed for the complex of polysorbate 80 with lower (0.0005%) concentration. The observed stability of the ferric thiocyanate complex over 1 h is consistent with the stability reported by Ivsic et al. [17]. The difference in the stability of the cobalt and the ferric thiocyanate complexes of polysorbate 80 could be due to the difference in the rate of reaction to reach equilibrium when forming the complexes.

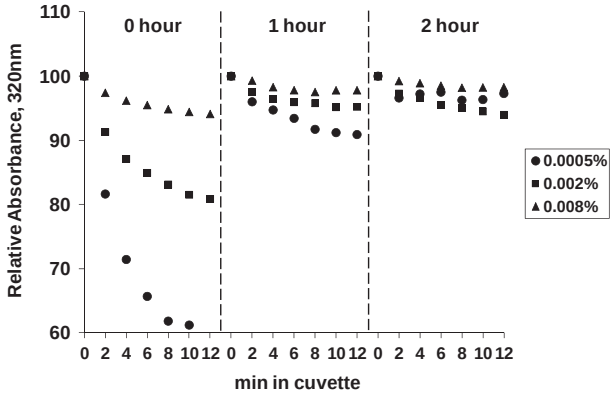

Fig. 2. Stability of cobalt thiocyanate complex of polysorbate 80 at 320 nm. 0, 1 and 2 h times indicate the wait time after phase separation time before the organic layer is sampled.

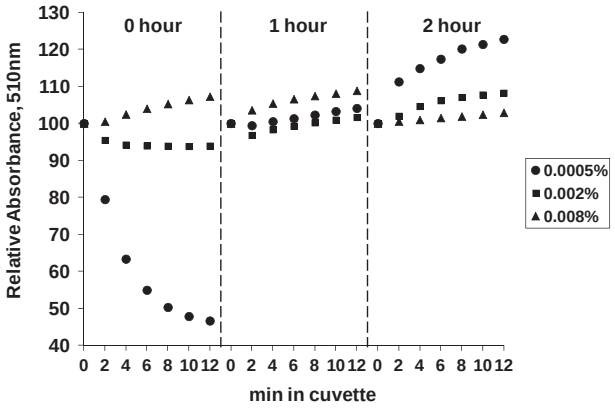

Fig. 3. Stability of ferric thiocyanate complex of polysorbate 80 at 510 nm. 0, 1 and 2 h times indicate the wait time after phase separation time before the organic layer is sampled.

Table 3
Recovery of polysorbate 80 from 140 mg mL^{-1} monoclonal antibody product derivatized with ferric thiocyanate and cobalt thiocyante.

Dilution factor	Target polysorbate 80 concentration, % (w/v)	Spike recovery (%)	
		Ferric thiocyanate, 510 nm	Cobalt thiocyanate, 320 nm
1	0.01000	59	48
2	0.00500	95	102
4	0.00250	96	105
5	0.00200	94	102
8	0.00125	87	107
10	0.00100	107	112

3.3. Determination of polysorbate 80 in high protein formulation using ferric thiocyanate complex

To demonstrate the suitability of the ferric thiocynate derivatization procedure as an alternative method a monoclonal antibody drug product containing 0.01% polysorbate 80 with a protein concentration of 140 mg mL^{-1} was analyzed. The goal of this experiment was to evaluate whether the dilution of the sample to a factor in excess of 5-fold would provide an acceptable recovery and sensitivity for the measurement of polysorbate in such a high protein concentration sample. Samples were diluted as much as 10-fold with water, followed by SPE for extraction of polysorbate, derivatization with ferric thiocyanate and measurement of the absorbance at 510 nm. Recovery data for polysorbate extracted from protein solution are reported in Table 3. Recoveries between 87 to 107% were observed when the samples were diluted as much as 10-fold with water, indicating that derivatization with ferric thiocyanate allows accurate quantitation of low levels of polysorbate in the diluted samples.

For the purpose of comparison, recovery experiments were also repeated for the determination of polysorbate and the cobalt thiocyanate complex with the absorbance measurement at 320 nm. Comparison of the recovery data for polysorbate derivatized with ferric thiocyanate and cobalt thiocyanate indicated that both were suitable for accurate quantitation of polysorbate 80 as low as 0.00025% in the 10-fold diluted protein samples. However, derivatization with the ferric thiocyanate offered higher sensitivity, as represented by its higher slope (Table 2), relative to the sensitivity provided by derivatization with cobalt thiocyanate and UV absorbance at 320 nm. Based on the relative mean square method with signal-to-noise ratio of 10, limit of quantitation (LOQ) for polysorbate 80 derivatized with ferric thiocyanate and UV

measurement at 510 nm was 0.000046%. On the other hand, the LOQ of polysorbate 80 when it was derivatized with cobalt thiocynate and UV absorbance measurement at 320 nm was 0.00021%, indicating that the ferric thiocyanate complex offered more than 4 times higher sensitivity than that provided by the cobalt thiocyanate complex. The SPE method using ferric thiocyanate for derivatization of polysorbate 80 had percent relative standard deviation from 5.3 to 11.7% for the polysorbate concentration percent spike recovery under 107% over the linearity range of 0.0001% to 0.008%.

The presence of degradants of polysorbate is reported to affect the quantitative results of polysorbate because the degradants may not be derivatized by the thiocynate complex [18]. The effect of the presence of polysorbate degradants on the quantitation using the present method will be evaluated in the future. The application of the presented methodology to polysorbate 20 is underway in our laboratory.

4. Conclusions

The sensitivity of polysorbate 80 quantitation was significantly improved when it was derivatized with ferric thiocyanate. Derivatization with ferric thiocyanate was able to quantitate polysorbate 80 as low as 0.0001% (w/v), which was 10 times lower compared to the original method utilizing derivatization with cobalt thiocyanate and measurement at 620 nm. Furthermore, the improvement in the sensitivity through derivatization with ferric thiocyanate allowed 10-fold dilution of a 140 mg mL^{-1} protein sample without affecting the recovery of polysorbate 80. In terms of reproducibility as determined by stability, polysorbate/ferric thiocyanate measured at 510 nm have good stability when sampled between 1 and 2 h after phase separation.

During the original method development absorbance at 320 nm of polysorbate/cobalt thiocyanate was not selected due to the possibility of sample matrix interference [11]. In this paper, sample matrix interference was not an issue and it yielded the second most sensitive approach after ferric thiocyanate with similar accuracy and reproducibility. Side by side analysis of experimental data indicates that the ferric thiocyanate approach has more than 4 times higher sensitivity than cobalt thiocyanate (320 nm).

Ferric thiocyanate yielded the highest sensitivity with good accuracy and consistent absorbance measurements when the organic layer containing PS80/ferric thiocyanate complex was sampled after 1 h of wait time. The ferric thiocyanate method is suitable for the photometric determination of polysorbate 80 in high protein concentration samples where the samples could be diluted with the dilution factor as high as 10-fold without sacrificing the recovery of the extracted polysorbate 80 from the protein samples.

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