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Method Development and Validation using UV Spectrophotometry for *Nigella sativa* Oil Microparticles Quantification

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ABSTRACT

Nigella sativa oil (NSO) has been exploited for medical purposes for many generations. The fabrication of microparticles containing NSO intended for sustained release was done to be used in treating osteomyelitis. Method in quantifying NSO using UV-spectroscopy was developed and validated. Linearity shown a good correlation coefficient with the values higher than 0.995, both for actual and different analysts. The LOD and LOQ values were recorded to be 2.89 µg/mL and 8.75 µg/mL respectively. In addition, the highest %RSD values for the intermediate and repeatability studies were 0.970% and 0.445% which suggested the method was precise. The percentage recovery for 4 known concentrations gave the range between 98.16% to 99.39%, indicating the high accuracy of the method. The parameters analyzed in this study were in accordance with ICH Q2 (R1) guidelines.

INTRODUCTION

Belongs to the family of Ranunculaceae, *Nigella sativa* is an annual flowering plants cultivated mostly in Mediterranean countries and Middle East (Shrivastava *et al.*, 2011). Other than normally being used as a spice in foods, the plant also is very famous in treating many acute and chronic diseases such as diarrhoea, dyspepsia, indigestion, obesity and dyspnoea (Gilani *et al.*, 2004). Having such a low toxicity level, the seeds extract of *N. sativa* is reported to have even wider spectrum of medical properties such as anti-cancerous, antioxidant, anti microbial and anti diabetic effect (Shrivastava *et al.*, 2011).

The potential of this plant of wonders is enormously exploited over the time. Known to be as one of the prophetic medicines and the emerging demands in utilizing natural products to replace the synthetic drugs, *N. sativa* has become amongst the top candidates selected by researchers as a natural product in medical field (Paarakh, 2009).

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Most of the previous studies reported the utilization of common high end instruments i.e. HPLC and GC-MS in quantifying the *N. sativa* seeds extract (Velho *et al.*, 2011; Avula *et al.*, 2010; Lupidi *et al.*, 2010). However, these two common quantifying methods are relatively expensive and time consuming. The utilization of UV-spectrophotometry as the measuring tool in quantifying the compounds is believed to reduce the time consumption and it is a reasonably cheap procedure. UV-spectrophotometry is one of the most commonest technique in pharmaceutical analysis. The amount of ultraviolet as well as the visible light which are being absorbed by the material or drug substances can easily be quantified and analysed (Behera *et al.*, 2012). The simplicity, rapid and moderately specific characteristics of UV-spectrophotometry in analysing small amount of substance have become one of the preferable choices amongst the scientists.

Beer-Lambert law plays a major role in understanding the basic concept of UV-spectroscopy. The law stated that the intensity of light beam passes through a transparent cell containing an absorbing solution will have a directly proportional interaction between the concentration of the solution and the intensity of the beam detected (Behera *et al.*, 2012). The law commonly is being mathematically expressed as follow:

$$A = \varepsilon lc$$

Where, A = absorbance value

ε = absorptivity constant

l = the constant length of light beam passes through

c = the solute concentration

It is reported that *N. sativa* oil extracted from the seeds contains more than 80% of unsaturated fatty acids mainly from linoleic and oleic acids (Gharby *et al.,* 2014). The composition of NSO has been investigated by many researchers mostly by using HPLC and GC. Although the composition of NSO has been widely studied based on the data obtained using HPLC and GC, there has been very limited use on UV spectroscopy in evaluating the crude extract of the NSO. However in 2014, Salmani et al. reported that the UV-vis spectra of thymoquinone can be characterized by a prominent peak around 254-257 nm. Thymoquinone, thymol, thymohydroquinone, dithymoquinone and many others are among the active compounds isolated from the seed of N. sativa which responsible in providing the therapeutic properties (Zribi *et al.,* 2014). Hydroquinone is described to give another peak around 290 nm but no distinguished peak is observed at 294 nm, although the same wavelength was used in other study to detect thymoquinone (Salmani *et al.,* 2014). For thin layer chromatography, thymohydroquinone and thymoquinone give a typical fluorescence of the droplets under UV and blue light excitation which may be attributed to the presence of phenolic and other aromatic compounds (Botnick *et al.,* 2012). In this study, cold-pressed *N. sativa* oil extract was obtained commercially from Pakistan with a valid certificate of analysis to be used in fabricating microparticles. This microparticles fabrication was formulated to give a sustained release of *N. sativa* oil over a period of time. The sustained release microparticles were intended to be used together with PLGA-gentamicin microparticles as a fusion treatment for osteomyelitis. One of the conventional treatments of osteomyelitis is by administrating a chain of gentamicin-poly (methyl methacrylate) (PMMA) beads on the site of the infectious tissue. These PMMA beads come in 7 mm in diameter with 4.5 mg of loaded gentamicin will be removed from the patient under secondary invasive surgery after 10 to 14 days of implantation (Neut *et al.,* 2001).

Although intravenous injection of antibiotics is considered as one of the treatments for osteomyelitis, it has to couple with long term indwelling catheter and local implantation containing antibiotics to overcome the problem with poor blood flow to the infected area (Brin *et al.,* 2008). On top of that, patient compliance is one of the major issues discussed when it comes to the requirement to undergo secondary invasive surgery. Microencapsulation method is proven to give slow sustained release of drugs and active pharmaceutical ingredients as long as up to 107 days in the case of timolol (Bertram *et al.,* 2009). The idea of having *Nigella sativa* oil (NSO) to be encapsulated by using poly(lactic-co-glycolic acid) (PLGA) is believed to provide sustained release of NSO over a period of time. Coupled with the slow released of gentamicin, this fusion is postulated to be used to treat osteomyelitis without having to undergo the secondary surgery. By adopting the single-emulsion-solvent-evaporation method, NSO was successfully encapsulated using PLGA. This study utilized the UV-spectroscopy to determine the quantity of NSO for post-fabrication of NSO microparticles. As mentioned earlier, this method will provide a cheap, fast and reliable way in quantifying NSO and this will open to many possibilities in exploiting the wonders of this plant.

MATERIALS

N. sativa Oil, Chemicals and Reagent

The crude cold pressed *N. sativa* oil extract was purchased commercially (Hemani, Pakistan). and it came together with the certificate of analysis produced by the company. Analytical grade chloroform and ethanol (Merck, Germany) and PVA with molecular weight approximately 115 kDa (BDH Laboratory Supplies, England) was used. Purasorb® PLGA with three different intrinsic viscosities; 0.2 dL/g (14 kDa), 0.4 dL/g (34 kDa) and 1.0 dL/g (100 kDa) were purchased (Purac, Netherland).

Instrument

The analysis was done using single beam UV-spectrophotometry module (U-1900 Toshiba, Japan) with wavelength scanned from 600 nm to 190 nm. The silica quartz square cell cuvette (Optima, Japan) with transmittance of 51.4±0.3% at wavelength 545 nm was used throughout the whole experiment.

EXPERIMENTAL METHODS

Fabrication of NSO Microparticles

The fabrication process was adopted and modified from Ismail *et al.,* (2012). Briefly, the designated PLGA was dissolved in 2 ml chloroform and 100 mg of stock of NSO was added. The ratio of PLGA molecular weights (MW) and the blends were used for the respected formulations (Table 1).

Table 1: The blends of PLGA intrinsic viscosity and the ratio used for each formulations respectively. The amount of NSO used for each formulations is 100 mg.

Formulation	Intrinsic Viscosity (dL/g)	PLGA Ratio
F1	0.2/0.4	50:50
F2	0.2/1.0	50:50
F3	0.4/1.0	50:50
F4	0.2/0.4	25:75
F5	0.2/0.4	75:25
F6	0.2/1.0	25:75
F7	0.2/1.0	75:25
F8	0.4/1.0	25:75
F9	0.4/1.0	75:25

The emulsion was homogenized by using ultra sonic processor (QSonica, USA; 3 cycles for 3 seconds each at 50 MHz). The chloroform was evaporated by continuous stirring for 3 hours. Aqueous solution containing PVA 1% w/v was used to stabilize the microparticles fabricated during the evaporation process. The emulsion underwent centrifugation and washing process to collect the particles before they were lyophilized under

reduced pressure. The interaction between the type of PLGA used and the molecular weight blends was evaluated based on the particle size distributions and the encapsulation efficiency.

Particle Size Distribution

The size distribution of the particles was measured by suspending some of the particles in deionised water. The method was adopted from Ismail *et al.,* (2012) and the measurement was carried out by using Laser Particle Size Analyzer (Bettersize Instruments, China). The measurement of each formulation was carried out in triplicate.

Encapsulation Efficiency

The lyophilized particles were re-dissolved in chloroform (1 ml) by using end-to-end shaker for 1 hour. Another 1 ml of ice cold ethanol was added to precipitate the PLGA before proceed with centrifugation (14000 rpm, 10 mins). The supernatant was collected and subjected for NSO quantification using UV-spectrophotometry. The quantification was done in triplicate for each formulation.

METHOD DEVELOPMENT AND VALIDATION

Preparation of Stock Sample

Chloroform and ethanol were mixed well with 1:1 ratio. 20 g of *N. sativa* oil was accurately weighted and dissolved in 100 ml of the co-solvents to become the stock (20% w/v) and was diluted accordingly whenever required. The PVA 1% w/v was prepared by dissolving in ultra pure water.

Specificity

The wavelength scanning for NSO was performed at the initial experiment from 600 nm up to 190 nm. This was done to determine the possible wavelength detection for NSO with the highest absorbance value. The best wavelength was chosen and the same wavelength would then be used to detect NSO whenever required. The same wavelength scanning process was done for PLGA to ensure the specificity of the method. For post microparticle fabrication, the extracted NSO based on the formulations was again subjected to UV wavelength scan to check for any changes as compared to the pre fabrication peaks.

Linearity and Standard Curve

6 different known concentrations of NSO diluted from the stock were subjected to specific UV wavelength obtained from the wavelength scan previously done. The known concentrations versus absorbance graph was constructed (Figure 2). The standard curve $y=mx+c$ was obtained, with y is the absorbance value, *m* is the slope of the regression line and *c* is the value of intersection of the line with the *y-a*xis. The concentration of analyte is represented by the value of *x*. Correlation coefficient R^2 of the standard curve was obtained from the software of the UV-spectroscopy module. All standard curves constructed in this experiment would have R^2 not lesser than 0.995 as the acceptance criteria (Rajitha *et al.,* 2011; Rajesh *et al.,* 2014).

Accuracy and Precision

Accuracy was calculated by subjecting 4 known concentrations of NSO at 271 nm and the actual concentrations obtained by the module were recorded. Each concentration was measured in triplicate. The precision was determined by considering the deviation of absorbance value obtained for each concentration. The percentage recovery should not be less than 98% and more than 102% with %RSD value is smaller than 2.0% to meet the acceptance criteria (Rajitha *et al.,* 2011).

Limit of Detection and Limit of Quantification.

Limit of detection (LOD) and limit of quantification (LOQ) were obtained by subjecting blank samples with the same wavelength used for *N. sativa* oil. The formulae used to calculate the LOD and LOQ are:

LOD: $\frac{3.3\ x\ S.D}{m}$ .....Formula 1

LOQ: $\frac{10\ x\ S.D}{m}$ .....Formula 2

whereby S.D is the standard deviation of the absorbance for blanks and *m* is the slope of the linear regression (Iqbal *et al.,* 2013). The determination of LOD and LOQ were done in triplicate.

Robustness

The robustness of the method was validated by constructing the standard curve using single solvent system (Chloroform). By using 6 known concentrations, the same UV-spectrophotoscopy module was used to construct the curve. Furthermore, another analyst was assigned to construct another standard curve and the R^2 was recorded. The absorbance of each known concentration was measured in triplicate.

RESULTS AND DISCUSSION

Particle Size Distribution and Encapsulation Efficiency

All formulation for NSO microparticles shown the average size distribution from 3.343 µm (±0.124) to 4.523 µm (±1.028). Although the size of microparticles was not our top priority since they were intended to be used for local implantation, but the distribution for all the formulation seemed to be homogenously dispersed (Table 2). Encapsulation efficiency (EE) of *N. sativa* microparticles was determined by using the method adopted from Ismail et al. (2012). The EE of the microparticles was presented in Figure 1. The readings were taken with the average value of n=3. The EE was determined by subjecting the absorbance values obtained to the standard curve constructed previously. The response surface plots were constructed to evaluate the interaction between the formulations and the responses (Figure 2). Based on the plots, the interactions between

the ratio of PLGA molecular weights and the blends can be interpreted and predicted depending on the desired outcome.

The highest EE of the NSO microparticles could be achieved by employing 0.2/0.4 MW blends with the ratio of 50:50 for both PLGA molecular weights. In addition, the MW blends of 0.4/1.0 with the ratio of 25:75 should be employed to get the smallest size distributions of the microparticles.

Table 2: Average size distribution of NSO microparticles (n=3) according to the formulations.

Formulation	Size Distribution (µm)	S.D
F1	3.343	±0.124
F2	3.493	±0.118
F3	3.543	±0.110
F4	3.930	±0.020
F5	3.353	±0.146
F6	4.523	±1.028
F7	4.127	±0.549
F8	3.650	±0.265
F9	3.577	±0.140

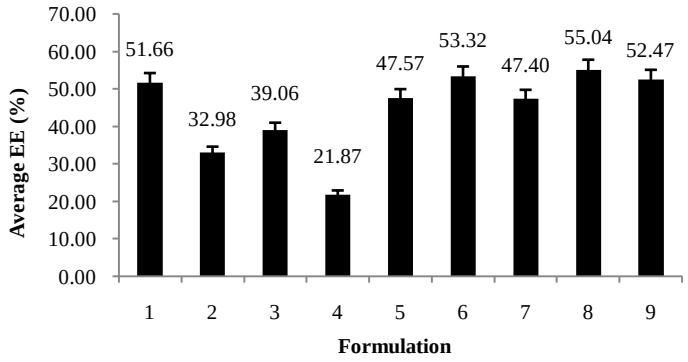

Fig. 1: Encapsulation efficiency of *N. sativa* oil microparticles done in triplicate.

Specificity

The selection of specific wavelength obtained from the wavelength scanning was chosen to be at 271 nm. The selection of wavelength depends on the solubility and the nature of the sample the highest peak for detection using UV spectrophotometry was 271 nm as shown in Figure 3. Another 2 best peaks were also being tested (221.5 nm and 216.5 nm) by constructing linearity.

However, the correlation coefficient, R^2 of both wavelengths showed undesired values of 0.043 and 0.275 respectively. The peak for PLGA however was detected below 250 nm which made 271 nm is specific wavelength for *N. sativa* oil.

The peaks for the post fabrication wavelength scan showed no changes in all nine formulations of the NSO microparticles as shown in Figure 4. The NSO extracted from the microparticles indicated that no changes were observed chemically as the highest peak was still observed at 271 nm. The fabrication process used for all the nine formulations was proven to encapsulate NSO without altering the oil chemically.

Linearity and Standard Curve

The standard curve (Figure 5) was constructed using 6 different concentrations of NSO at wavelength of 271 nm. The response obtained from the UV-spectroscopy of NSO was found to be in linear form and the equation obtained was denoted as $y = 0.448x + 0.015$ with the value of R^2 of 0.998.

The readings for 6 different known concentrations were taken in triplicate for each concentrations. The average of each point would increase the reliability of the obtained standard curve. In addition, chloroform was used as the solvent in order to reduce the variability of the readings caused by the solvent volatility. Despite having dichloromethane (DCM) as one of the regular solvents used to prepare PLGA microparticles (Mohamed *et al.*, 2006), DCM seemed to have a very high volatility as compared to chloroform. The significant difference within and between the readings for DCM was higher as compared to chloroform, hence would increase the inconsistency of the readings.

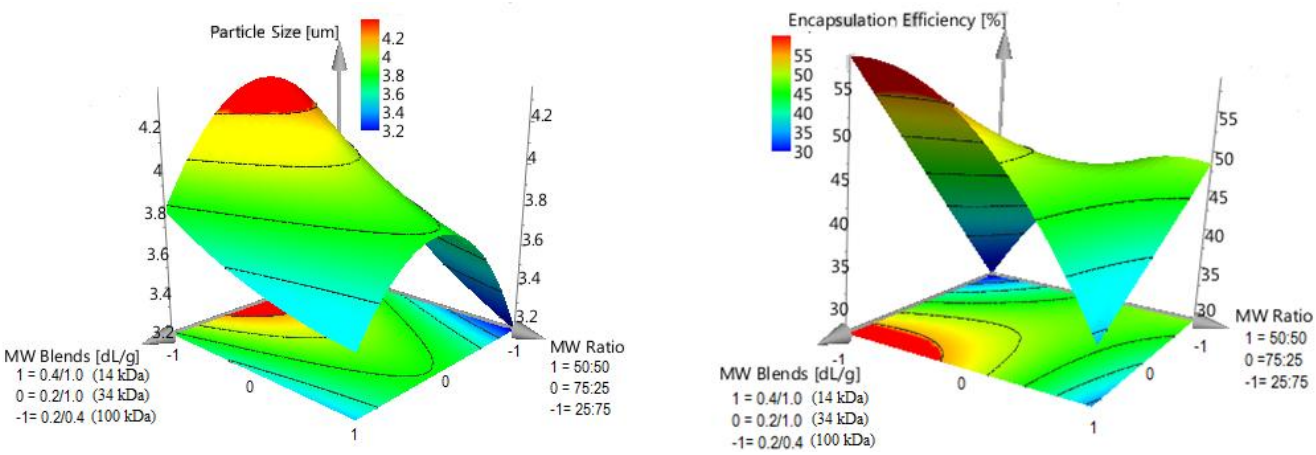

Fig. 2: Response surface plots for particle size distribution (left) and encapsulation efficiency (right) of NSO based on MW blends and MW ratio.

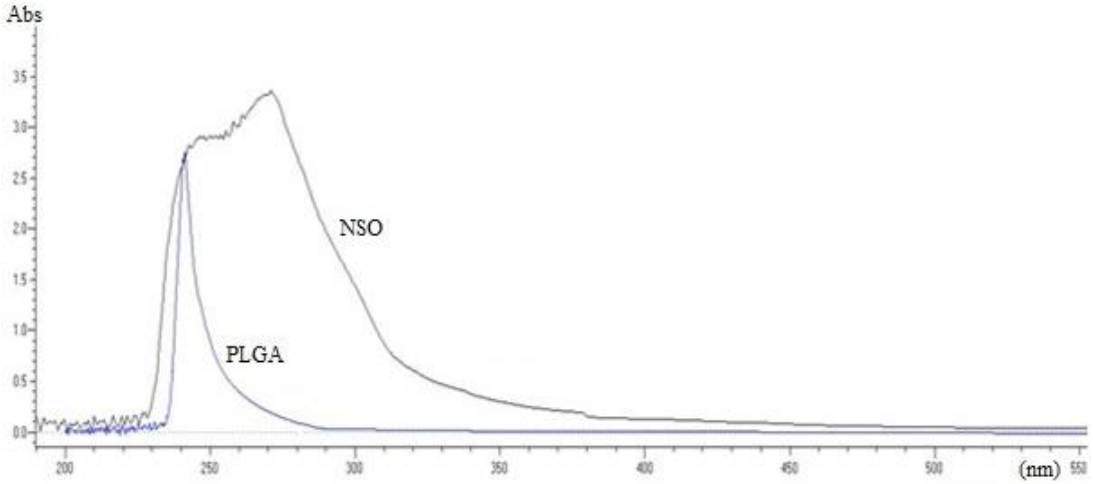

Fig. 3: The highest detection of *N. sativa* oil using spectrophotometry at 271 nm (black) and the peak for PLGA (blue).

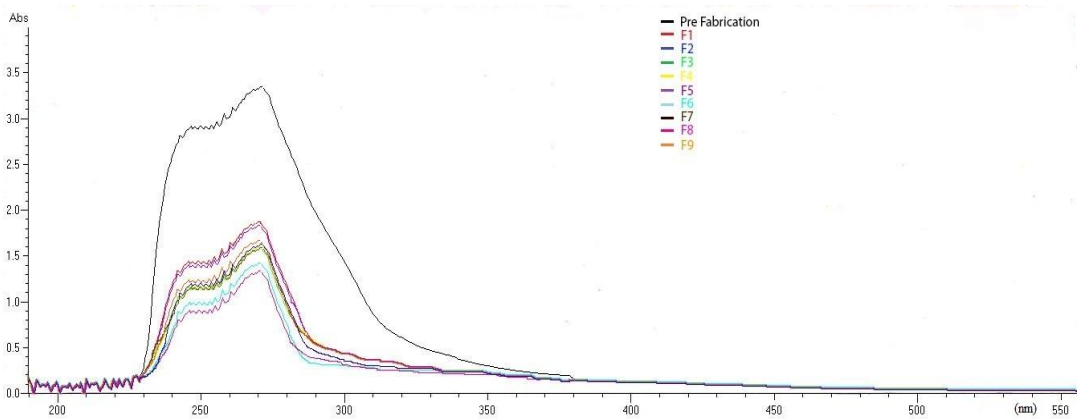

Fig. 4: The wavelength scan of NSO for post fabrication microparticles based on the formulations used.

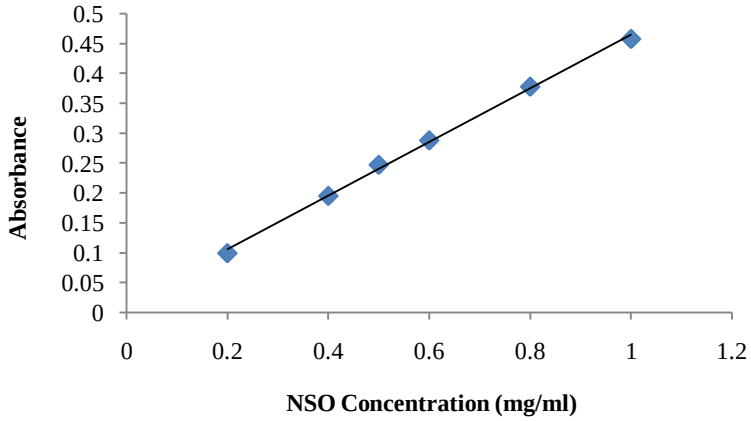

Fig. 5: The standard curve or linear regression of *N. sativa* oil using spectrophotometry.

Accuracy and Precision

Table 3 showed the precision data obtained from the experimental condition in 3 different days denoted as Day 1, Day 2 and Day 3. The quantification of NSO based on this method was found to be precise as the %RSD values for each concentration for Day 1, Day 2 and Day 3 were less than 2.0%. Almost similar finding was reported in method development for paracetamol quantification using UV-spectroscopy (Behera *et al.*, 2012). This intermediate precision study showed the reliability of the method in quantifying NSO using a cheap and simple approach. Similar finding was observed for repeatability study (Table 4) where the % RSD values obtained for every run were less than 2.0%. For accuracy study, it was not possible to measure the absorbance values of NSO at 100% concentration and more.

This was due to the transparency of the solution containing NSO dropped significantly as the solute increased. The absorbance value increased more than 2 and this would drop the detection capacity (transmittance) below 0.1%. Hence, the same

concentrations were used (0.2, 0.3, 0.7 and 0.9 mg/ml) to evaluate the accuracy of the developed method (Table 5). The result indicated that the method was accurate in quantifying NSO as the best recoveries (98.16-99.39%) of the spiked NSO were obtained with %RSD was not more than 2.0%.

Table 3: Interday precision of N. sativa oil recorded using UV-spectrophotometry.

Concentration (mg/ml)	Average Actual Concentration (mg/ml) (n=3)	S.D	R^2 Value of Standard Curve	Precision (% RSD)
Day 1:			0.998	
0.2	0.176	0.001		0.571
0.3	0.273	0.001		0.366
0.7	0.721	0.001		0.139
0.9	0.929	0.002		0.215
Day 2:			0.999	
0.2	0.218	0.001		0.459
0.3	0.294	0.002		0.680
0.7	0.703	0.007		0.970
0.9	0.892	0.005		0.561
Day 3:			0.997	
0.2	0.206	0.001		0.485
0.3	0.277	0.002		0.722
0.7	0.676	0.002		0.295
0.9	0.854	0.002		0.234

Table 4: Intraday precision of N. sativa oil determined by UV-spectrophotometry

Concentration (mg/ml)	Average Actual Concentration (mg/ml) (n=3)	S.D	R^2 Value of Standard Curve	Precision (% RSD)
1st Run:			0.997	
0.2	0.199	0.0002		0.117
0.3	0.292	0.001		0.337
0.7	0.644	0.0004		0.069
0.9	0.854	0.002		0.229
2nd Run:			0.997	
0.2	0.193	0.0002		0.085
0.3	0.305	0.0005		0.177
0.7	0.683	0.0004		0.058
0.9	0.868	0.0003		0.04
3rd Run:			0.997	
0.2	0.217	0.001		0.445
0.3	0.312	0.0001		0.041
0.7	0.68	0.0004		0.066
0.9	0.889	0.0021		0.236

Table 5: Data evaluation of accuracy study for NSO using UV-spectroscopy.

Nominal Concentration (mg/ml)	Actual Concentration (mg/ml)	% Recovery	Average % Recovery	S.D	% RSD
0.2	0.199	99.26	99.39	0.117	0.117
	0.199	99.41			
	0.199	99.49			
0.3	0.295	98.46	98.16	0.522	0.531
	0.293	97.56			
	0.295	98.46			
0.7	0.699	99.96	98.74	1.054	1.067
	0.686	98.05			
	0.688	98.22			
0.9	0.894	99.37	99.07	0.522	0.526
	0.894	99.37			
	0.886	98.45			

Limit of Detection (LOD) and Limit of Quantification (LOQ)

LOD was calculated by subjecting the blank solution to 271 nm to get the background noise. Under the experimental conditions, the LOD value was 2.89 µg/mL and LOQ was obtained with the value of 8.75 µg/mL, calculated based on the formulae mentioned earlier. The slope of the standard curve and the standard deviation for both LOD and LOQ were 4.325 and 0.0038 respectively.

Robustness

The standard curve constructed using single-solvent system was plotted (Figure 6). The standard curve was observed to give the R^2 of 0.996 with the equation of $y = 0.222x - 0.003$. The robustness of any developed method should be tested by deliberately varying the method parameters. These variations should be considered in improving the method in future if they affect the outcomes significantly. There should be precautionary statements which need to be included in the procedure of analytical conditions to avoid any inaccuracies.

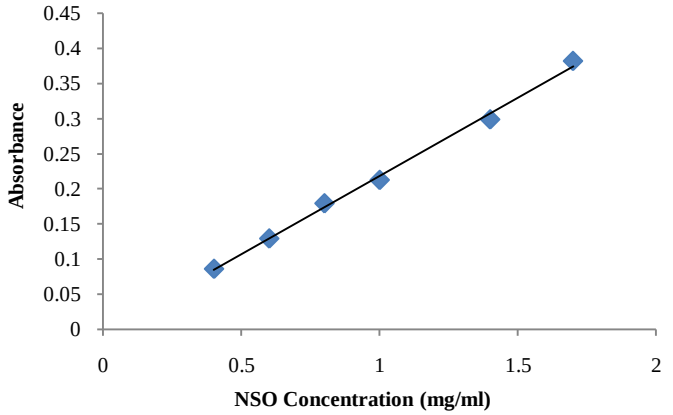

Fig. 6: The single-solvent system of NSO standard curve was constructed using chloroform.

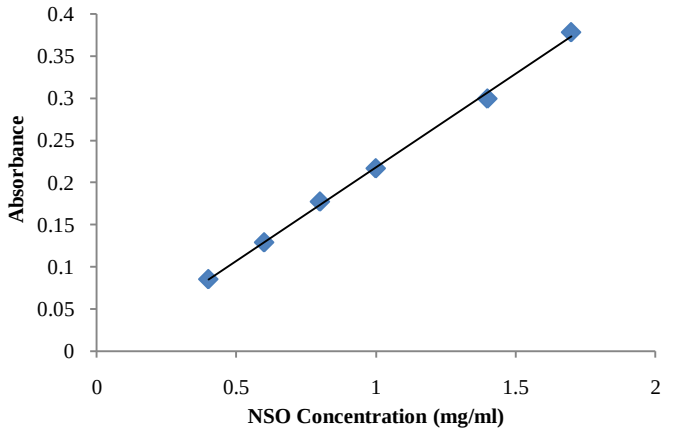

Figure 7: The standard curve of NSO constructed by different analyst.

In addition, the standard curve constructed by different analyst showed the value of R^2 0.998 with the equation of $y = 0.221\,x - 0.003$ with 6 different points of known concentrations (Figure 7). This clearly shown that the analytical method in quantifying NSO would be concluded as robust. The deliberated variation in the assay preparation solution was not affecting the analytical system and was in accordance with the actual test preparation. In addition, the correlation coefficient obtained from

different analyst also shown the value not less than 0.995 similarly observed with the actual analyst.

CONCLUSION

The present method development in quantifying NSO using UV-spectroscopy was validated based on the basic requirements stated under ICH Q2 (R1) guidelines. All the acceptance criteria were fulfilled and it could be concluded that this analytical method was precise, linear, specific and robust to be used in accordance to the purpose of quantifying NSO.

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