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DETERMINING NEREISTOXIN RESIDUE IN PALM OIL MATRIX – AN EXTENSION TO THE METHOD FOR THIOSULTAP DISODIUM RESIDUE DETERMINATION

(Penentuan Sisabaki Nereistoxin Dalam Matriks Minyak Sawit – Penambahan Kepada Kaedah Penentuan Sisabaki Thiosultap Disodium)

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Abstract

This study aimed to incorporate the nereistoxin, a compound that is the major metabolite of all nereistoxin analogue pesticides, including thiosultap-disodium into the previously reported analytical method developed for thiosultap-disodium residue analysis in the palm oil matrix. All steps that have been developed previously for the determination of thiosultap-disodium involving liquid-liquid extraction and subsequently determination using liquid chromatograph triple quadrupole mass spectrometer (LC-MS/MS) were followed, except the omission of matrix-matched calibration curve preparation and this was substituted with the use of spiked calibration curve technique due to its better performance in overcoming the matrix effect. The performance of the analytical method was evaluated in-house and found to be satisfactory. Recoveries of the nereistoxin residue spiked at 12, 30, and 50 ng g^{-1} were at 85-95% with a relative standard deviation of below 9%. Three months of monitoring of method intermediate precision by two analysts gave a relative standard deviation value of 3.6% to 8.3% for the same spiking levels. The limit of detection (LOD) and limit of quantification (LOQ) were first estimated using the standard deviation of the calibration curve at a 95% confidence level and subsequently evaluated by actual experimental work. The values found were 5.0 ng g^{-1} and 12.0 ng g^{-1}.

Keywords: nereistoxin, palm oil, method development, dimehypo, spiked calibration

Abstrak

Kajian ini bertujuan untuk menambahkan sebatian nereistoxin, iaitu sebatian metabolit utama bagi kesemua racun perosak dari kelas analog nereistoxin termasuk thiosultap-disodium ke dalam kaedah analisa sisabaki thiosultap-disodium dalam matriks minyak sawit yang dibangunkan sebelum ini. Kesemua langkah pengolahan sampel yang telah dibangunkan sebelum ini bagi penentuan thiosultap-disodium melibatkan pengekstrakan fasa cecair-cecair lalu penentuan menggunakan kromatografi cecair spektrometri jisim caturkutub ganda tiga masih diikut, kecuali langkah persediaan keluk kalibrasi padanan-matriks telah digantikan dengan penggunaan teknik keluk kalibrasi pemakuan memandangkan prestasinya yang lebih bagus dalam mengatasi kesan dari matriks. Prestasi kaedah analitikal ini telah dinilai secara dalaman di makmal sendiri dan didapati ianya memuaskan. Nilai

perolehan semula sisabaki nereistoxin yang telah dipakukan pada 12, 30 and 50 ng g^{-1}, masing-masing adalah dalam lingkungan 85-95 % dengan nilai piawai relatif di bawah 9%. Pemantauan selama tiga bulan bagi menentukan kepersisan perantaraan kaedah oleh dua orang penganalisa berbeza memberikan nilai piawai relatif 3.6% ke 8.3% untuk kepekatan pemakuan yang sama. Had pengesanan dan had kuantitatif telah dianggarkan pada permulaan menggunakan sisihan piawai yang diperolehi daripada keluk kalibrasi pada tahap keyakinan 95% lalu pengesahan dilakukan melalui kajian secara eksperimen. Nilai yang diperolehi adalah 5.0 ng g^{-1} dan 12.0 ng g^{-1}, masing-masing.

Kata kunci: nereistoxin, minyak sawit, pembangunan kaedah, dimehypo, kalibrasi pemakuan

Introduction

Thiosultap-disodium or in its trade name dimehypo is an insecticide known for its usage in oil palm plantations for controlling foliage pests [1-3]. Hence, a study was carried out to develop a method for determining the residue of this insecticide in crude palm oil (CPO) as a proactive step in monitoring the possible pesticide residues that may be present in the local CPO [4]. The method reported was simple and effective involving an aqueous-oil liquid-liquid extraction step and omitting the need for sample clean-up, thus it is cost-effective and time-saving.

Thiosultap-disodium is a compound classified under the group of nereistoxin analogue insecticides. Compounds under this category shared the common chemical structure of their parent compound of nereistoxin (Figure 1). Upon ingestion by insects, nereistoxin analogue insecticides tend to be metabolised into nereistoxin or its uncyclized dithiol and subsequently act as the nicotinic acetylcholine receptor or ion channel complex in the central nervous system thus killing the insects [5,6]. A few other nereistoxin analogue insecticides examples are thiocyclam and Cartap (Figure 1).

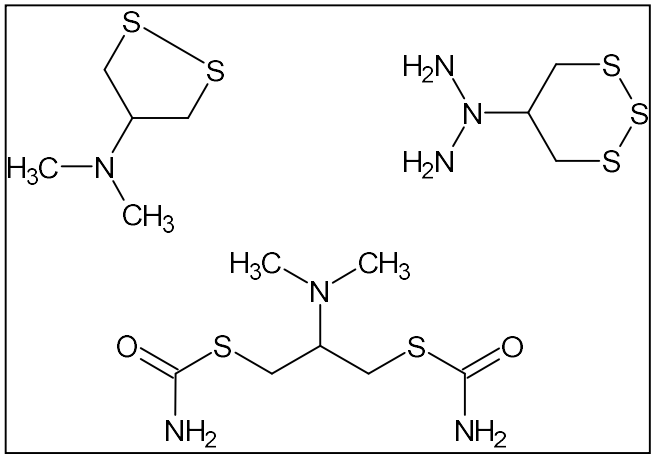

Figure 1. Chemical structure of nereistoxin (top left), thiocyclam (top right), and cartap (bottom)

Hence, it is obvious that the residue analysis method developed by Yeoh et.al. [4] in palm oil matrix for thiosultap-disodium alone is insufficient without the inclusion of determining the possible nereistoxin residue that might be present since the compound is likely to be formed as the primary metabolite in the action of the insecticide [7]. Therefore, this paper aims to re-examine the established workflow to incorporate nereistoxin as another compound of interest in order for the residue of both compounds can be determined in a single run. This article reports the performance of the analytical method to determine nereistoxin residue in palm oil matrix from the aspects of selectivity, linearity, recovery, and precision.

Materials and Methods

The performance of the previously developed analytical method for the thiosultap-disodium residue in handling the nereistoxin residue was evaluated through the analysis of control CPO samples spiked with the

nereistoxin standard. No major alteration was made to the previous method except for the inclusion of deuterated nereistoxin as an internal standard (IS).

Reagents and chemicals

All solvents used were at least of analytical reagent grade. Nereistoxin oxalate (96.1% purity) and nereistoxin oxalate D6 (99.5% purity) standard materials were obtained from Dr. Ehrenstorfer GmbH (Augsburg, Germany). Ultrapure water from Merck Millipore Direct Q® 8-UV-R water purification system (Darmstadt, Germany) was used throughout the experiments.

Blank crude palm oil (CPO) sample

CPO was obtained from a palm oil mill that processed fresh fruit bunches nearby Carey Island, Selangor, Malaysia with known records of pesticide applications. CPO obtained is considered blank upon confirmation with respective plantations in which no nereistoxin analogue insecticides have been used for at least the past 3 months before the harvesting. The CPO was filtered through anhydrous sodium sulphate (10% of blank oil weight) to ensure the sample used in method validation studies is dry and free from insoluble materials [8].

Standard solutions

Standard stock solutions of either native or isotopically labelled nereistoxin at the concentration of 100 µg mL^{-1} were prepared by dissolving 10.41 mg of nereistoxin and 10.05 mg of deuterated nereistoxin in 100 mL of methanol and stored in dark at -18°C. Working standard solutions of both compounds at 10 µg mL^{-1} were prepared by 10-fold dilution of the stock solution in methanol and stored in dark at -18°C. Prior to their use, all stocks or working standard solutions were kept for at least 1.5 h at ambient temperature.

Spiked calibration curve, linearity evaluation limit of detection (LOD) and quantification (LOQ)

Spiked calibration curves of 7 levels at 10, 20, 30, 50, 70, 90, and 100 ng g^{-1} were prepared by the addition of 5, 10, 15, 25, 35, 45, and 50 µL of nereistoxin working standard, respectively to 5 g portions of blank CPO in each level. An aliquot of 25 µL of the IS working standard was also added to each of the spiked CPO. The spiked samples obtained were then treated following the sample extraction procedures described in the later part of this section.

For linearity evaluation, it begins with the visual evaluation of the calibration curve in combination with the coefficient of determination, r^2 expression to see if a linear regression is an appropriate model for the curve. It was then followed by a residual plot that allows for graphical evaluation to observe the presence of any pattern as well as heteroscedasticity in the linear regression model.

LOD and LOQ methods were estimated from the calibration plotted curve followed by a recovery experiment that was performed at a spiking level close to LOQ for confirmation.

Recovery test samples preparation

300 g accurately weighed blank CPO was stirred in a dry and clean 500 mL beaker (tall form) with a controlled temperature at 60°C followed by the addition of 36 µl of nereistoxin stock solution (100 µg mL^{-1}). The mixture was then stirred continuously for another 40 min before the sub-sampling of spiked control samples. While stirring, 5 g of each of the control samples spiked with a nereistoxin standard at 12 ng g^{-1} of CPO were weighted. A total of 50 spiked control samples were obtained and stored in a freezer at -18°C in the dark. Samples were used in recovery and precision studies. The same procedures were repeated for the preparation of control samples spiked with nereistoxin at 30 ng g^{-1} and 50 ng g^{-1} of CPO.

Matrix effect evaluation

For matrix effect (% ME) evaluation, nereistoxin calibration curves at 6 concentration levels (10, 20, 30, 50, 70, and 100 ppb) prepared by three different techniques: neat solvent, matrix matched solution, and spiked calibration technique. The % of ME was calculated as follows:

$$\%ME = \left(1 - \frac{Slope\ of\ Calibration\ Curve\ Prepared\ in\ Solvent}{Slope\ of\ Calibration\ Curve\ Prepared\ in\ Matrix}\right) \times 100\% \quad (1)$$

Liquid chromatography triple quadrupole mass spectrometer (LC-MS/MS) analysis

LC-MS/MS analysis from Yeoh *et al.* [4] was performed on an AB Sciex QTRAP® 4500 (Foster City, CA; USA) triple quadrupole mass spectrometer coupled to an Eksigent Ekspert™ Ultra LC 100 system (Eksigent, Redwood City, CA, USA). Ionisation was in a positive mode at 5500 V and 550ºC with an electrospray probe. The Applied Biosystems Analyst 1.6 software was used to control the operation of the instrument and data acquisition or processing. Chromatography separation was achieved on a Phenomenex Kinetex® C18 100 Å 50 mm × 2.1 mm i.d., 2.6 µm particle size column, and coupled to a 2 mm × 2.1 mm i.d. Security Guard C18 column set at 40°C. The injection volume was 10 µl with a flow rate of 200 mL min^{-1}. The mobile phase is composed of methanol as the organic phase and an aqueous solution of ammonium acetate (10 mM, adjusted to pH 5.0). The following gradient elution profile was used; the initial conditions (95% aqueous phase) were maintained for 0.5 min before the organic eluent was increased to 95% in 5 min. After maintaining this high organic mobile phase for 5 min, the condition was brought back to the initial conditions in 0.5 min and kept the composition for 5 min before the next analysis, thus giving a total runtime of 16 min. The determination of nereistoxin residue was performed via multiple reaction monitoring (MRM) experiments with two precursor-daughter ion transitions. The detailed settings of the MRM experiment such as the declustering potential (DP), entrance potential (EP), collision energy (CE), and collision cell exit potentials (CXP) are summarised in Table 1. Examples of blank and nereistoxin standard chromatograms at 10 ng g^{-1} are shown in Figure 2. It can be observed that no significant interference was found within the window period of ± 30 s from the expected retention time for nereistoxin suggesting that selectivity was not a problem in this study.

Table 1. MRM parameters setting in nereistoxin and internal standard detection

Ion Transition	DP (Volt)	EP (Volt)	CE (Volt)	CXP (Volt)	Remark
150→105	20	7	22	5	Quantifying ion
150→61	20	7	31	51	Qualifying ion
156→105	39	9	18	13	Quantifying ion (IS)

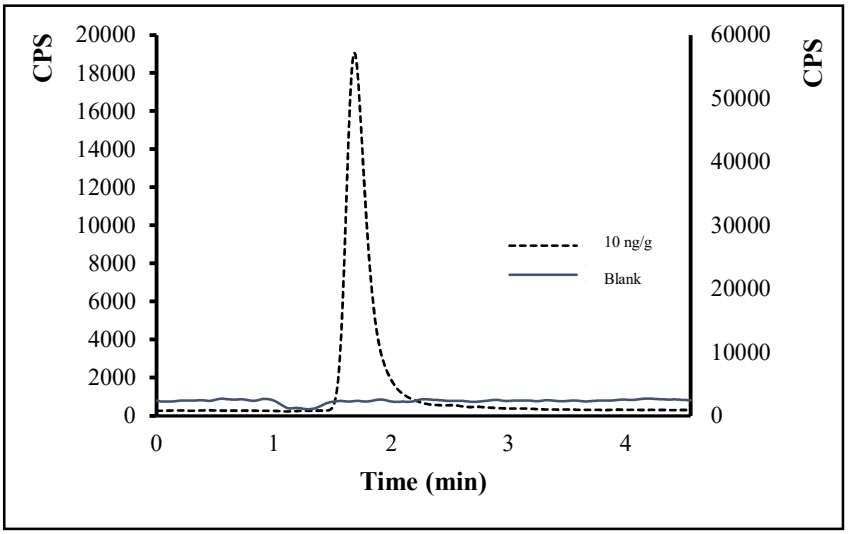

Figure 2. Overlay chromatograms of nereistoxin standard at 10 ng g-1 (dotted line and referring to right vertical axes) and blank extract (solid line and referring to left vertical axes)

Results and Discussion

Matrix effect and linearity

Figure 3 shows the calibration curves prepared using three different techniques: neat solvent, matrix matched solution, and spiked calibration technique. Results revealed that the nereistoxin signal was suffering from ~15% ion suppression due to the matrix effect. Although this was acceptable according to the SANTE guidelines [9], efforts were still made to examine the possibility of utilising other calibration techniques [10,11]. In this study, the spiked calibration technique is selected although preliminary data showed that the matrix-matched calibration technique is viable with sufficiently good linearity and recovery. Furthermore, it can be observed from Figure 3 that the spiked calibration curve is no different (%ME ~7.4%) from the calibration curve prepared in a neat solvent, thus suggesting that the spiked calibration technique is also viable for the quantification of nereistoxin residue in the palm oil matrix. Both % of ME shown by using matrix-matched and spiked calibration techniques were well within the analytical errors (± 20%) as stipulated in the SANTE guidelines [9].

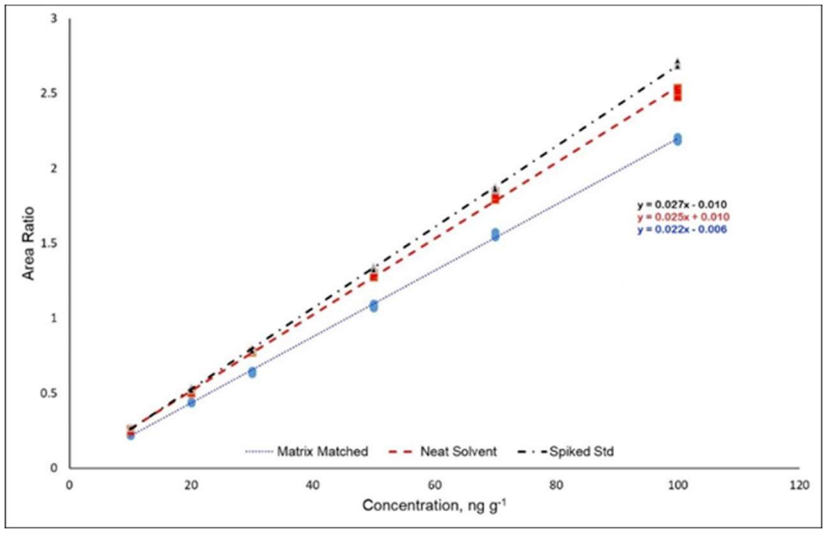

Figure 3. Comparison of calibration curves prepared in neat solvent, blank matrix extract and following spiked standard technique

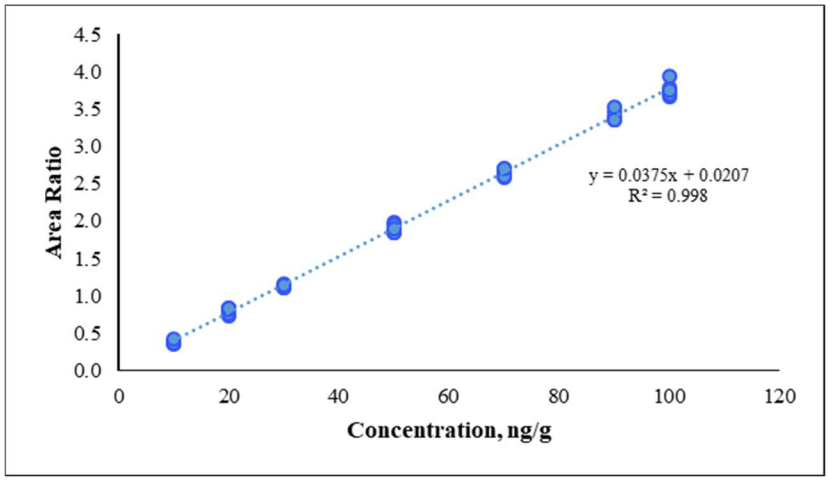

Figure 4. Nereistoxin calibration curve prepared according to the spiked standard technique

Figure 4 shows the representative spiked nereistoxin calibration curve ranging from 10 to 100 ng mL^{-1} (six injections per level) which were used for quantification in this study. It can be observed that nereistoxin responses were linear throughout the calibration range with a coefficient of determination (r^2) of 0.998. Plotting of Y residues against nereistoxin concentrations showed that the distribution of the errors was fairly randomised around the concentration axis except for some degree of increase in the standard deviation of measurements following the increase in the nereistoxin concentrations (Figure 5). This indicates the presence of heteroscedasticity in the linear regression model fitted for the calibration curve [12, 13].

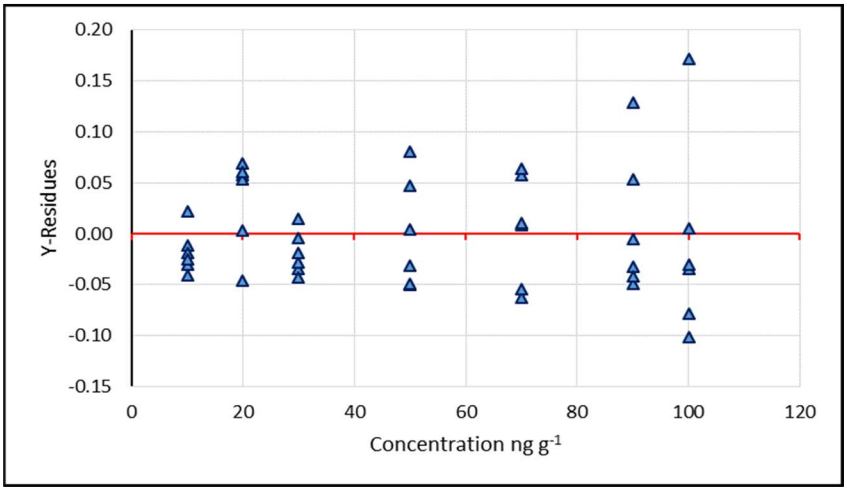

Figure 5. Residual plot for homoscedasticity evaluation

To ascertain the heteroscedasticity in the linear regression model used, the *F*-test was conducted by comparing the variances between the highest (100 ng g^{-1}) and the lowest (10 ng g^{-1}) calibration levels following Equation (2) [12-15].

$$F_{exp} = \frac{S_H^2}{S_L^2} \quad \text{and} \quad F_{tab}(f_1, f_2; 0.99) \qquad (2)$$

where F_{exp} is the experimental F value from the calibration data expressed as a ratio of the variance between the highest concentration level (S_H^2) and the lowest concentration level (S_L^2). The F_{exp} is then compared with the F_{tab} from the F-table at a 99% confidence level for $f_1 = f_2 = (n\text{-}1)$ degrees of freedom. The homoscedasticity test is passed when $F_{tab} > F_{exp}$.

Upon computation, the presence of heteroscedasticity in the calibration curve is confirmed whereby the experimental F-value (F_{exp} = 19.41) was found to be larger than the tabled F-value (F_{tab} = 10.97). Thus, a weighted linear regression model is used in this study for the quantitation of nereistoxin residue. Only a few weighting factors such as 1/X, $1/X^2$, ln X, 1/Y, $1/Y^2$, and ln Y are considered in this study depending on the built-in functions that are available in the data processing software bundled to the LC/MS/MS. The selection of a suitable weighting factor is based on the sum of absolute relative error (∑%RE) computed from each regression model for every weighting factor. A regression model that gives the lowest ∑%RE will be chosen for calibration curve construction.

Table 2 shows the regression parameters of the calibration curve generated for each weighing factor and the respective ∑%RE for the calibration data. The data show that the regression model with $1/Y^2$ as a weighting factor was chosen in this study.

Table 2. Regression parameters of the calibration curve generated for each weighing factor and the respective ∑%Re for the calibration data

Model	Weighting Factor	Slope	y-Intercept	Correlation Coefficient, r	∑%RE
1	None	0.03752	0.02073	0.99898	135.66
2	1/X	0.03763	0.01459	0.99906	131.36
3	$1/X^2$	0.03791	0.00657	0.99814	129.15
4	ln X	0.03749	0.02236	0.99880	136.80
5	1/Y	0.03764	0.01277	0.99909	129.83
6	$1/Y^2$	0.03788	0.00381	0.99830	126.85
7	ln Y	0.03763	0.01148	0.99878	128.80

Limit of detection (LOD) and limit of quantification (LOQ)

LOD and LOQ are crucial parts of method validation. In this study, LOD and LOQ were estimated from the standard deviation of the calibration curve and calculated according to Equations (3) and (4) [16-18]. The LOD and LOQ were estimated to be 5.0 ng g^{-1} and 15.0 ng g^{-1}, respectively at a 95% confidence level. The estimated LOQ was further verified through the conduct of recovery tests at 12.0 ng g^{-1}, a value close to the estimated LOQ. The results suggested that the estimated LOQ is appropriate in describing the performance of the developed method and the final LOQ was altered to 12.0 ng g^{-1} resulting from the acceptable recovery results obtained at this level of spiking.

$$LOD = 3.3 * \frac{\sigma}{s} \quad (3)$$

$$LOQ = 10 * \frac{\sigma}{s} \quad (4)$$

Where σ is the standard deviation and S is the slope of the calibration curve.

Recovery and precision

The performance of the developed method was further evaluated from the recovery study. For precision evaluation, two analysts of different analytical skills were accessed for 3 months, and the method repeatability and intermediate precision were recorded. From Table 3, it can be observed that nereistoxin recoveries throughout the assessment period were all within 80-95% with a relative standard deviation (RSD %) of < 10%. For intermediate precision evaluation, the recovery values of every measurement by both analysts across 3 months period were taken into consideration. The calculated RSD % represents the extent of error among those measurements including the factors of different analysts, different time, different reagent batches, and different spiking concentrations, of which were between 3.6% to 8.3%. Judging from the present data, the precision of the method is well accepted and far below the set value of 20% as stipulated in the SANTE guidelines [9].

Conclusions

This study demonstrates how an appropriate linear regression model (with or without weighting factor) in pesticide residue method development can be chosen based on the statistical analysis and, in brief, how the decision was made by using the incorporation of nereistoxin residue into the existing analytical method for thiosultap disodium residue in the palm oil matrix as an example. From the matrix effect evaluation, it was found that the analysis of nereistoxin residue in the palm oil matrix did not suffer much from the matrix effect and both matrix-matched, and spiked calibration curve techniques were viable and effective in quantifying the nereistoxin residue. With the introduction of the spiked calibration technique, the current method is found to be simple and effective as published in the initial method. Analysis of sodium thiosultap and nereistoxin residues in palm oil matrix can now be performed simultaneously.

Table 3. Recovery of nereistoxin residue (at different spiking levels and times of experiment by different analysts)

Spiking Level (ng g^{-1})	12 (Low)		30 (Medium)		50 (High)	
Analyst	**A**	**B**	**A**	**B**	**A**	**B**
Experiment period (Number of replicates)	**Period 1 (n= 7)**					
Average Recovery (%)	87.30	84.08	87.81	95.28	90.53	90.03
RSD (%)	6.83	8.71	8.49	7.53	4.78	4.90
Experiment period (Number of replicates)	**Period 2 (n= 7)**					
Average Recovery (%)	86.83	81.46	92.57	88.48	90.98	88.61
RSD (%)	6.05	6.83	5.35	6.62	4.86	1.92
Experiment period (Number of replicates)	**Period 3 (n= 8)**					
Average Recovery (%)	95.16	95.11	91.29	93.69	91.34	92.91
RSD (%)	3.18	4.4	1.26	2.12	1.56	2.10
	Intermediate Precision (3 Months)					
Average Recovery (%)	88.63		91.56		90.80	
RSD (%)	8.23		6.09		3.68	

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