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FULL PAPER

Validation of Ultra High Pressure Liquid Chromatography (UHPLC) with Pre-column Derivatization Method for Quantitative Analysis of Histamine in Fish and Fishery Products

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ABSTRACT

A pre-column derivatization-ultra high performance liquid chromatographic (UHPLC) method is described for the determination of histamine in fish and fishery products. The homogenized samples were extracted with trichloroacetic acid (TCA) solution and derivatized with o-phthaldialdehyde. Histamine was separated using reversed-phase column and determined using UHPLC with fluorescence detection. The linear calibration range was 10 to 60 µg/mL with a correlation coefficient of 0.9993. Good recoveries were observed for the histamine under investigation at all spiking levels, and average recoveries were higher than 89% with a precision smaller than 8.46%. The detection and quantification limit were 2.7 and 8.3 µg/g, respectively. The uncertainty was estimated to be ± 0.45. The performance of the proposed method was checked with a proficiency test sample from the Food Analysis Performance Assessment Scheme (FAPAS) as external quality control; the resulting z-score was -0.2, which was found within acceptable range of $-2 \leq z \leq 2$. The results indicated that this HPLC method was reliable, sensitive, reproducible, and practical for the routine analysis of histamine in fish and fishery products.

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1. INTRODUCTION

According to FAO's Food Safety and Quality, Scombrotoxin Fish Poisoning (SFP), often called "histamine poisoning," is caused by ingestion of certain marine fish species containing high levels of histamine and possibly other biogenic amines (FAO 2013). These biogenic amines occur naturally in food particularly in fish by microbial decarboxylation of amino acid when fish are subjected to temperature abuse during or after harvest. Biogenic amines like histamine, putrescine, cadaverine, tyramine, and agmatine are formed from histidine, ornithine, lysine, tyrosine, and arginine, respectively. The bacteria that are considered to be involved in biogenic amines (i.e. *Enterococcus; Staphylococcus; Pseudomonas; Aeromonas*; *Campylobacter* and other Lactic acid bacteria) are present in the aquatic environment of the fish and naturally present in the normal microflora of live flesh. Due to death, the defense mechanism of fish is no longer capable of inhibiting bacterial growth; hence histamine forming bacteria may grow in the muscle tissue (Olajos 2015).

Several methods to analyze biogenic amines in food have been described so far, including colorimetric, fluorometric, enzyme-linked immunosorbent assay (ELISA), and chromatographic methods (Peng et al. 2008). Among these methods, chromatographic technique is one of the most frequently used techniques. Although gas chromatography (GC) with flame ionization detection (FID), electron capture detection (ECD), or mass spectrometry detection (MSD) were employed for the analysis of histamine, these methods suffered from complex matrix effects and sample carryover (Jana et al. 2002). On the contrary, HPLC coupled with different detectors provided better sensitivity, selectivity, and reliability for the complex sample analysis (i.e. fish matrix). HPLC with

ultraviolet detection has low sensitivity for histamine detection. Consequently, pre-, post-, and on-column derivatization methods are generally used to improve the detection sensitivity by using fluorescence detection (FLD). Among these three derivatization methods, the post- and on-column methods result in broader peaks of derivatives than the pre-column method due to mixing process of the analyte and reagent in the derivatization reaction device (Peng et al. 2008).

The method routinely used for histamine involves extraction with methanol or trichloroacetic acid, subsequent ion-exchange chromatography, and a chemical reaction with dansyl chloride, fluorescamine, or o-phthaldialdehyde (OPA) under defined conditions to measure the resulting fluorescence reaction products (Latorre-Moratalla et al. 2009). Dansyl chloride forms derivatives with limited stability and is itself sensitive to light. Moreover, the reaction is time-consuming even under heating. The reaction between fluorescamine and histamine, on the other hand, is not recommended due to the instability and complexity of the product; however, the reaction time is only few minutes. As the reaction between OPA and histamine completes within a minute in the presence of 3-Mercaptopropionic acid at room temperature, pre-column derivatization with OPA is commonly adapted for HPLC-FLD analysis of histamine. However, critical conditions have to be controlled to eliminate the influence of the relative instability of the derivatives (Papavergou et al. 2012).

For consumer protection, fish importing countries have regulations and varying limits for histamine in fish and fishery products. According to European Union (EC) No 2073/2005, histamine in fishery products must not exceed the following established maximum level shown in Table 1.

Indicated in the EU standard, the analytical reference method to be used for histamine analysis is HPLC. Therefore, this study was conducted to evaluate the UHPLC method for quantitative determination of histamine. The objective was to establish a rapid, sensitive, and reproducible analysis method and to validate it in terms of selectivity, linearity, repeatability, reproducibility, and accuracy. If the method achieved the acceptance criteria set by the Association of Official Analytical Chemist (AOAC), CODEX Alimentarius, and Food Analysis Performance Assessment (FAPAS) for all parameters of performance characteristics, then this method can be declared as fit for its intended purpose.

2. MATERIALS AND METHODS

2.1. Reagents and Chemicals

HPLC-grade acetonitrile was purchased from RCI Labscan (Bangkok, Thailand). Histamine dihydrochloride (≥99%), o-phthaldialdehyde (≥99%), and 3-mercaptopropionic acid (≥99%) were purchased from Sigma-Aldrich (Missouri, USA). All other chemicals and solvents were analytical reagent grade and ultrapure water (18.2MΩcm) produced by a Milli-Q water system (Millipore, Massachusetts, USA).

2.2. Equipment

The UHPLC equipment used was Shimadzu Nexera x2 LC system (Kyoto, Japan), including a quaternary pump and fluorescence detector (excitation: 330 nm, emission: 440 nm). A personal computer equipped with a Shimadzu LabSolution program to process chromatographic data. An auto-sampler with 10 µL injection valve and a 250 x 4.6 mm internal diameter, 5 µm particles C18 column (GL Sciences, Tokyo, Japan) thermostated to 40°C were used for injection and separation of analytes.

2.3. Preparation of standard solutions and derivatization reagent

Histamine stock solution (1000 mg/L) was prepared by dissolving 16 mg of the compound in 10 mL of 100 mM hydrochloric acid. OPA derivatization

Table 1. Histamine levels in fish and fishery products by the European Committee (EU 2073/2005)

Designation of products	Regulated Limits (mg/kg wet weight)
Raw fish	>100 mg/kg
Salted fish for species belonging to the Scomboidae and Clupeidae families	>200 mg/kg
Fishery products undergoing enzyme maturation treatment in brine/fish sauce	400 mg/kg

reagent was prepared by dissolving 10 mg in 10 mL borate buffer (100 mM, adjusted to pH 9.2 by 1 M sodium hydroxide). Mercaptopropionic acid solution was prepared by diluting 10 µL of the compound in 10 mL borate buffer, pH 9.2. All solutions were freshly prepared.

2.4. Sample preparation and extraction procedures

Fresh frozen raw yellowfin tuna (*Thunnus albacares*) samples were carefully cut opened using a knife to remove the organs (e.g., kidney and liver). Samples were homogenized into uniform particle size before analysis using Retsch homogenizer. For extraction, 1 g of homogenized sample was weighed and extracted with 10 mL 0.5M TCA. The mixture was filtered with Whatman 40 filter paper and made up to 25 mL with 100 mM borate buffer, pH 9.2.

2.5. Derivatization and chromatographic separation

Pre-column derivatization was done by adding 225 µL mercaptopropionic acid and 110 µL OPA solution to 37.5 µL standard solution or sample extracts. The mixtures were mixed for 2 minutes prior to UHPLC determination. A linear gradient program was implemented following the conditions listed in Table 2.

2.6. Validation design

A full internal laboratory validation procedures were followed according to EURACHEM Guide to Method Validation, 2014. The action plan requires that the following performance characteristics must be treated and evaluated: (a) selectivity, (b) linearity, (c) precision – repeatability and reproducibility, (d) accuracy – spiking with known concentration of histamine, (e) limit of detection, (f) limit of quantification, and (g) measurement of uncertainty. Evaluation of relevant performance characteristics is shown in Table 3.

Furthermore, participation in proficiency test (canned fish, T27189) from Food Analysis Performance Assessment Scheme (FAPAS) was also conducted to further assess the reliability and accuracy of the validated analytical method.

3. RESULTS AND DISCUSSION

3.1. Selectivity

Selectivity is recommended to express whether a method can determine the target analyte (histamine) under certain chromatographic conditions in the presence of other components with similar behavior. In the chromatographic method, particularly HPLC, selectivity is based on the separation process called separation selectivity or separation factor (EURACHEM Guide for Method Validation, 2014). This selectivity indicates how strongly the result is influenced by other compounds present in the sample. The result of validation presented as chromatogram (Figure 1) shows that there is no peak detected in sample matrix as compared to the matrix spiked with 60 µg/mL histamine, which means the method is selective for histamine and no interfering compounds eluting near histamine was observed.

Table 2. Chromatographic conditions for the sensitive analysis of histamine

UHPLC Separation Conditions		
LC Pump Mode	Low Pressure Gradient	
Mobile Phase	A: Acetonitrile B: 0.1 M Acetate Buffer (pH 4.7 at 25°C)	
Detector Wavelength	Ex: 330 nm Em: 440 nm	
Column Oven Temperature	40°C	
Flow Rate	1.5 mL/min	
Injection Volume	10 µL	
Gradient Program	Time (min)	B concentration (%)
	0	85
	19.22	70
	47.02	60
	47.08	85
	64.66	85

Table 3. Validation design and performance characteristics evaluated

Performance Characteristics to be Evaluated	Procedure	*n* (No. of Determinations)
Linearity, Working Range	Establish linearity by plotting the analytical response versus the standard concentration	At least 4 standard concentrations plus 1 reagent blank
Precision (Repeatability)	Analyze spiked sample matrix (10 µg/mL) by the same analyst in the same laboratory and equipment	10 independent measurements
Intermediate Precision (Reproducibility)	Analyze spiked sample matrix (10 µg/mL) by the same analyst in the same laboratory and equipment but different time scale	10 independent measurements
Accuracy	Analyze blank sample matrix and spike with a low, mid and high standard (10, 30 and 60 µg/mL)	10 independent measurements
LOD and LOQ	Analyze spiked sample matrix	10 independent measurements

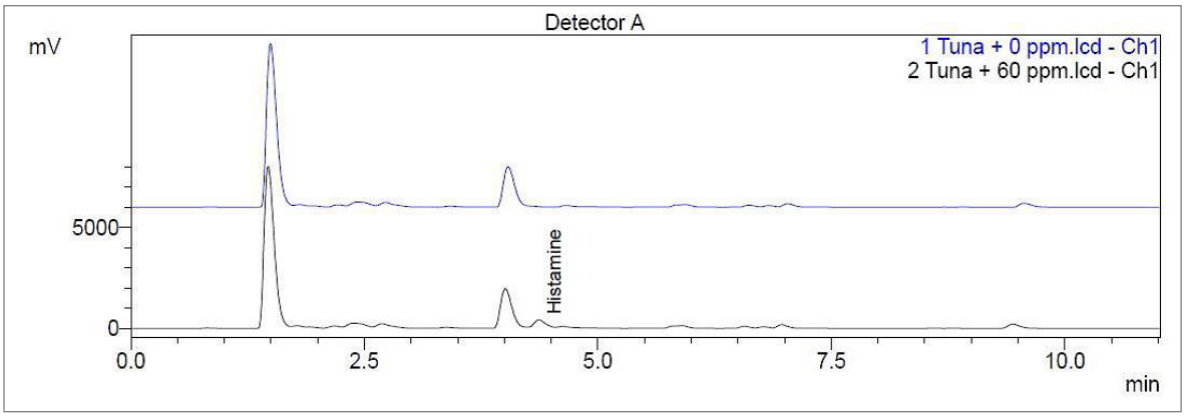

Figure 1. Chromatogram of blank sample matrix (blue) and sample matrix fortified with 60 µg/mL histamine (black)

3.2. Linearity and working range

Linearity or calibration curve was evaluated by regression analysis and least square method. The method of calibration used was standard addition to account for the selectivity. Calibration for histamine was done by standard addition method which means a matrix blank sample was spiked with 4 levels of increasing standard concentrations (10, 20, 40, and 60 µg/mL). Figure 2 shows the external calibration curve generated for two successive days. A linear curve was found between peak area and histamine concentration with good correlation coefficient (R2 = 0.9999). The results passed the acceptance criteria based on the AOAC Peer-Verified Methods Program which is ≥ 0.990.

3.3. Precision – repeatability and reproducibility

Precision describes the closeness between independent results under one specific chromatographic condition. This parameter is usually expressed by statistical parameters that describe the spread of results, typically the standard deviation (or relative standard deviation), calculated from results obtained by carrying out replicate measurements of spiked samples (n = 10).

Precision can be determined as repeatability and reproducibility. Repeatability is defined by EURACHEM as the variability in results when a measurement is performed by a single analyst and the same equipment on a short time scale, while intermediate precision or reproducibility measures the variability in results between two different days of analysis.

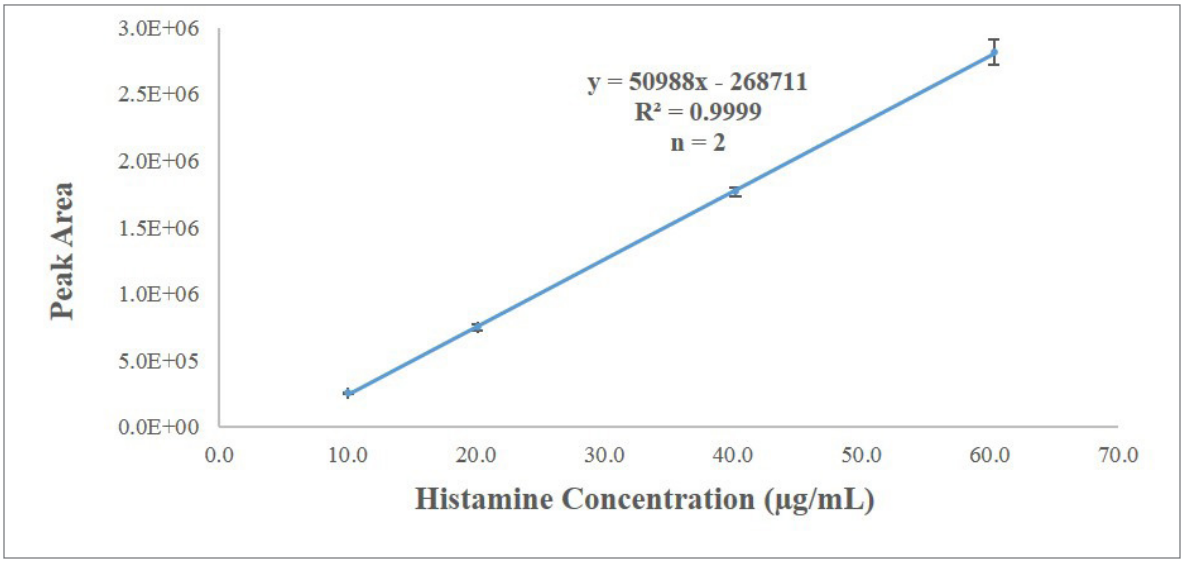

Figure 2. External standard calibration plot for histamine

Results of repeatability and intermediate precision (average and relative standard deviation for day 1 and day 2 analysis) for low-spiked samples (10 µg/mL) are shown in Table 4. Furthermore, paired t-test was done to evaluate reproducibility. Results showed a significant difference ($p < 0.05$) between day 1 and day 2. This could be attributed to the preparation of standards being added in the sample. Moreover, due to thawing process, an increase in histamine levels can be observed. Nevertheless, relative standard deviation for both repeatability, for day 1 and day 2, and reproducibility passed the acceptance criterion which are 11% and 16%, respectively (Table 8).

3.4. Accuracy – recovery

According to EURACHEM, measurement 'accuracy' expresses the closeness of a single result to a reference value. In this validation, accuracy in terms of recovery was evaluated by means of spiking the sample matrix with three different concentration levels of standard (10, 30, and 60 µg/mL). Recovery is an important step as it provides the analyst knowledge about the efficiency of the procedure. It was calculated by comparing the peak area of the fortified sample with that of the standard.

Satisfactory recoveries were observed at all spiking levels of histamine. Data from these experiments are reported in Table 5. The mean recoveries obtained are 97.52, 89.68, and 89.75% for low, mid, and high spike, respectively, which complied with the acceptance criteria of 80-110% (Table 8).

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

As part of the validation study, the limit of detection (LOD) and limit of quantification (LOQ) were determined. LOD is basically defined as the lowest concentration that can be detected but not necessarily quantified. To evaluate LOD, 10 replicate of blank samples (matrix contains no detectable analyte) spiked with the lowest standard concentration, 10 µg/mL, was performed (Table 6) and LOD was calculated using equation 3.1. The LOD value was determined to be 2.4 µg/g histamine.

$$LOD = \frac{(3 \times SD \text{ of spiked sample})}{(\text{Slope of the line})} \qquad (3.1)$$

On the other hand, LOQ is the lowest level of analyte that can be detected and quantified with acceptable performance. 'Acceptable performance' according to EURACHEM is considered by different guidelines, which include precision and trueness. LOQ is calculated by most conventions to be the analyte concentration corresponding to the obtained LOD multiplied by a factor, kQ. The IUPAC default value for kQ is 3.3. It can also be calculated using equation 3.2. The limit of quantification for this method was computed to be 8.0 µg/g histamine.

$$LOQ = \frac{(10 \times SD \text{ of spiked sample})}{(\text{Slope of the line})} \qquad (3.2)$$

Table 4. Precision studies data

Day	Spiked Concentration (µg/mL)	Recovered analyte conc. (µg/mL)	RSD (%)	
			Repeatability	Reproducibility
1	10	9.8000 ± 0.8295	8.46	6.94
2	10	10.9036 ± 0.5875	5.39	

Table 5. Accuracy studies data

Actual concentration (µg/mL)	Calculated concentration (µg/mL)	Recovery percentage	RSD (%)
10	9.8000 ± 0.8295	97.52 ± 8.25	8.46
30	27.0352 ± 1.9432	89.68 ± 6.45	7.19
60	54.1148 ± 5.6047	89.75 ± 9.30	10.36

Table 6. Peak area of sample spiked with 10 µg/mL histamine

Sample	Peak Area
Spiked Sample 1	238302
Spiked Sample 2	230758
Spiked Sample 3	237299
Spiked Sample 4	271236
Spiked Sample 5	317286
Spiked Sample 6	327677
Spiked Sample 7	297896
Spiked Sample 8	297896
Spiked Sample 9	216218
Spiked Sample 10	239837
Average	**260063**
$SD_{Spikedsample}$	**40655**

3.6. Measurement uncertainty

The measurement of uncertainty is calculated using the guidelines for quantifying uncertainty in the analytical measurement of EURACHEM Guide. The measurand is identified as the concentration of Histamine (µg/g) in fish. Figure 3 shows the possible factors that can contribute to the uncertainty of measurement in the analysis, whereas Table 7 shows the summary of uncertainty sources or uncertainty budget. Calculated uncertainty for the analysis is ± 0.45.

3.7. Proficiency test (PT)

According to ISO/IEC 17043:2010, proficiency test is a method used to demonstrate competency and validate a laboratory's measurement process by comparing its results to the results of a reference laboratory and other participant laboratories. Furthermore, it also enables the laboratory to validate the traceability of standards and assess the ability of laboratory personnel.

Proficiency testing schemes operate by providing participating laboratories with test samples for analysis. Laboratories analyze the test samples according to their routine procedures and report the results to the organizer. Most schemes convert the participant's result into a 'z-score.' This score reflects the difference between the participant's result and the accepted true value. Moreover, the consensus of participating laboratories may be calculated to determine satisfactory performance. Scores falling between -2

and 2 indicates a satisfactory result.

A proficiency testing from the Food Analysis Performance Assessment Scheme (FAPAS) was performed to evaluate further the validated method. The assigned value for this PT round was derived from the consensus of the results submitted by the participants, and it was calculated to be 153 mg/kg, as received. With this method, the laboratory generated a result of 150.8 mg/kg and has a z-score of -0.2, indicating that the proposed method is fit-for-purpose.

4. CONCLUSION

The reverse-phase ultra high pressure liquid chromatography method with fluorescence detection after pre-column derivatization with o-phthaldialdehyde was validated in this study. A summary of results for the different performance characteristics and their corresponding acceptance criteria is shown in Table 8. Measurement Uncertainty was calculated to be ± 0.45. All parameters passed the acceptance criteria set by AOAC Peer-Verified Method Program and the CODEX Alimentarius. In addition, the proposed method was confirmed through the participation of proficiency testing from FAPAS. The results (150.8 mg/kg) agreed well with the assigned value (153 mg/kg). Overall, this UHPLC method was reliable, sensitive, reproducible, and practical for the routine analysis of histamine in fish and fishery products.

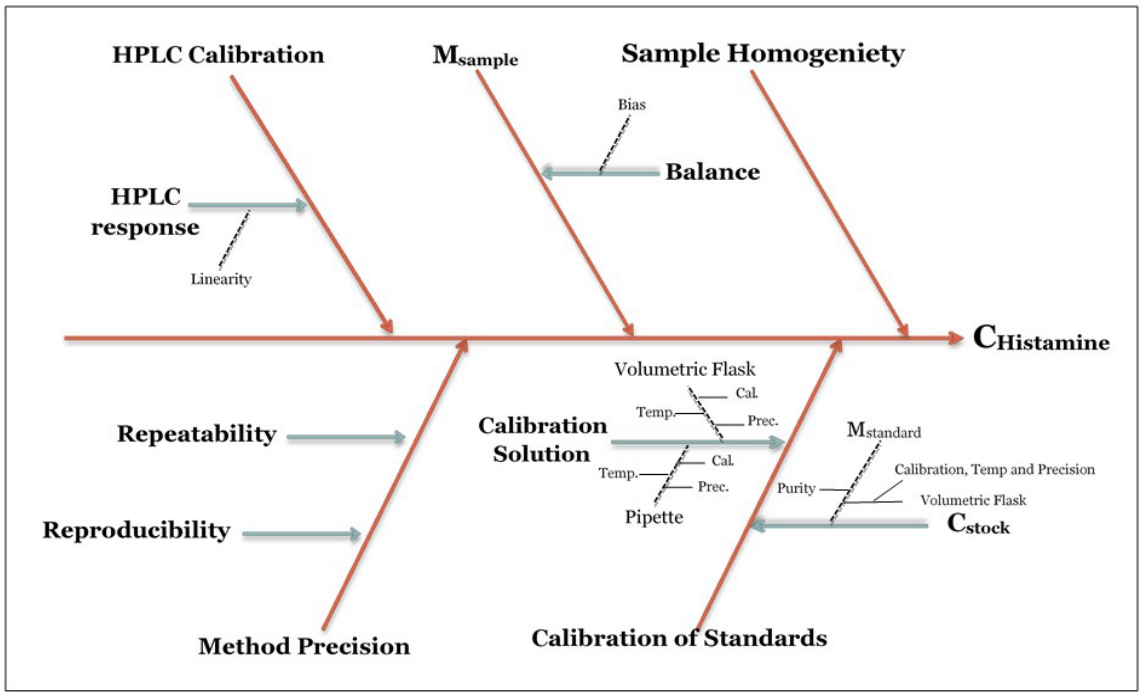

Figure 3. Cause and effect diagram for measurement uncertainty in the analysis of histamine

Table 7. Summary of uncertainty sources or uncertainty budget

Source of uncertainty	Value	Standard uncertainty	Relative standard Uncertainty
Mass sample	5.0446	0.0004031	0.0000799
Mass standard	0.1681	0.0004031	0.0023977
Calibration curve	1	0.86822	0.86822
Volumetric Flask	100	0.012250	0.000123
Volumetric Flask	25	0.006124	0.002499
Pipettor	0.1	0.00197	0.019698
Pipettor	1	0.00347	0.00347
Method Precision		0.450739171	

Table 8. Summary of the method parameters established in the validation

Performance Characteristics	Results	Acceptance Criteria
Linearity	Day 1 – 0.9993 Day 2 – 0.9997	≥ 0.990[a]
Precision (Repeatability)	8.46% (Day 1) 5.39% (Day 2)	11% RSD[a]
Intermediate Precision (Reproducibility)	6.94%	16% RSD[a]
Accuracy	97.52% (10 µg/mL) 89.68% (30 µg/mL) 89.75% (60 µg/mL)	80 – 110%[a]
LOD	2.4 µg/g	< 10 µg/g[b]
LOQ	8.0 µg/g	< 20 µg/g[b]

[a] AOAC Peer-Verified Method
[b] CODEX Alimentarius

Reproducibility of this method may differ from other laboratories due to several factors such as different liquid chromatography techniques (i.e. UHPLC and HPLC), internal diameter, and length of flow lines, column dimensions, and sensitivity of the equipment. Optimization of parameters and mobile phase composition could also be done to enhance run time.

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