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Application News

An Analytical Method of Paralytic Shellfish Toxins Using a Triple Quadrupole Mass Spectrometer

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User Benefits

- It is possible to simultaneously analyze for 12 toxins (saxitoxin [STX], decarbamoylsaxitoxin [dcSTX], neosaxitoxin [NEO], decarbamoylneosaxitoxin [dcNEO], gonyautoxins [GTX1 to 4], protogonyautoxins [C1 and C2], and decarbamoylgonyautoxins [dcGTX2, dcGTX3]) using an LC-MS/MS.
- Rapid analysis can be achieved within 15 minutes with a simple LC system.

■ Introduction

Shellfish poisoning mainly occurs when bivalves (scallops, clams, oysters, etc.) feed on toxin-containing marine dinoflagellates and these toxins accumulate in the body of the bivalve. Eating these poisonous shellfish causes four syndromes termed diarrheal shellfish poisoning, paralytic shellfish poisoning, neurotoxic shellfish poisoning, and amnesic shellfish poisoning. In Japan, the syndromes of principal concern are paralytic shellfish poisoning and diarrheal shellfish poisoning, each of which is caused by several chemical groups of toxins.

Safety standards and regulatory limits for shellfish toxins in Japan are established based on the Food Sanitation Act (Shokuan Notice No. 0306-1, dated March 6, 2015). Regulatory levels for the toxic potency or toxic dose of the edible parts of bivalves is 4 mouse units/g or below for paralytic shellfish toxins and 0.16 mg of okadaic acid equivalent/kg or below for diarrheal shellfish toxins. This regulatory level for paralytic shellfish toxins is equivalent to 800 µg of STX·2HCl equivalent/kg, the regulatory limit cited in CODEX CXS 292-2008 and Regulation EC No. 853/2004. Paralytic shellfish toxins are currently tested for using mouse toxicity tests, but with Shokuanki Notice No. 0306-3, dated March 6, 2015, testing for diarrheal shellfish toxins is already moving to an instrumental analysis that uses LC-MS/MS. In addition, given the current status of regulations in other countries, Japan's Ministry of Agriculture, Forestry and Fisheries has cooperated with other institutions to validate instrumental methods for paralytic shellfish toxin testing and is now working on establishing guidelines for these methods.

Internationally, paralytic shellfish toxin levels are tested by methods that include the liquid chromatography post-column oxidation and fluorescence detection method (J. AOAC Int. 2011, 94, 1154–1176), recognized by The International Ship Security Certificate (ISSC) and used by Canada, parts of the USA, Norway, and other countries, and the liquid chromatography pre-column oxidation and fluorescence detection method (AOAC 2005.06), which became an Official Reference Method of the EU in January 2019 and is used by the UK, Ireland, Portugal, New Zealand, and other countries. Due to the complexity of the HPLC systems and analytical operations needed to implement these methods, an ultra high-speed LC-MS/MS method has recently been developed that uses hydrophilic interaction liquid chromatography (HILIC).[1)2)] In 2018, 21 institutions participated in an international collaborative validation of this method, and the LC-MS/MS instrumental method is now considered a valid method alongside the existing HPLC method.

This article describes a case study in which toxin-containing scallop samples were analyzed using the hydrophilic interaction liquid chromatography LC-MS/MS method.

■ Structure of Saxitoxin

The general structure of saxitoxin (STX) is shown in Fig. 1.

Numerous analogs of saxitoxin have been discovered, including analogs with a hydroxyl group at R1, sulfate ester groups at R2 and R3, and analogs with sulfonated structures at the carbamoyl nitrogen on R4 side chains.

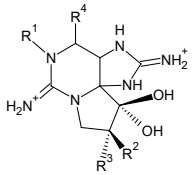

Fig. 1 General Structure of STX

Table 1 lists the functional groups at sites R1 to R4 and the Toxicity Equivalency Factor (TEF) of each paralytic shellfish toxin. Total toxicity is calculated by multiplying the molar concentration of each detected toxin by its TEF and summing the resulting values.

Due to difficulty procuring standard reference materials for STX, dcNEO, dcGTX2, and dcGTX3, calculations for these compounds were performed using the calibration curves for a similar toxin for which a standard reference was available. The "Calibrant" column in Table 1 shows the calibration curve toxin used to calculate values for each compound.

Table 1 Functional Groups and TEF of Paralytic Shellfish Toxins

Group	Analog	R1	R2	R3	R4	TEF	Calibrant
C toxins	C1	H	H	OSO_3^-	$COCONHSO_3^-$	0.01	C1
	C2	H	OSO_3^-	H	$COCONHSO_3^-$	0.1	C2
	C3	OH	H	OSO_3^-	$COCONHSO_3^-$	0.02	
	C4	OH	OSO_3^-	H	$COCONHSO_3^-$	0.1	
GTXs	dcGTX2	H	H	OSO_3^-	CH_2OH	0.2	GTX2
	dcGTX3	H	OSO_3^-	H	CH_2OH	0.4	GTX3
	GTX1	OH	H	OSO_3^-	$COCONH_2$	1.0	GTX1
	GTX2	H	H	OSO_3^-	$COCONH_2$	0.4	GTX2
	GTX3	H	OSO_3^-	H	$COCONH_2$	0.6	GTX3
	GTX4	OH	OSO_3^-	H	$COCONH_2$	0.7	GTX4
	GTX5	H	H	H	$COCONHSO_3^-$	0.1	
	GTX6	OH	H	H	$COCONHSO_3^-$	0.1	
STXs	dcSTX	H	H	H	CH_2OH	1.0	dcSTX
	STX	H	H	H	$COCONH_2$	1.0	dcSTX
	NEO	OH	H	H	$COCONH_2$	1.0	NEO
	dcNEO	OH	H	H	CH_2OH	0.4	NEO

■ Analytical Conditions

LC and MS analytical conditions are shown in Table 2.

Table 2 Analytical Conditions

[HPLC conditions] (Nexera™)

Column : Hydrophilic Interaction (HILIC) Column/Amide* (100 mm × 2.1 mm, 1.7 µm)

Mobile Phases : A) 0.015 % formic acid + 0.015 % ammonium water
B) 0.01 % formic acid in acetonitrile: water = 7:3

Column Temp. : 60 °C

Injection Volume : 2 µL

Time Program :

Time (min)	Flow rate (mL/min)	A.Conc (%)	B.Conc (%)
5.00	0.4	2	98
8.50	0.4	50	50
10.00	0.6	50	50
10.10	0.6	2	98
15.00	0.6	2	98

[MS conditions] (LCMS™-8060NX)

Ionization : ESI Positive & Negative

Interface Voltage : +1 kV & -1 kV

Ion Focus Voltage : +2 kV & -2 kV

Nebulizing Gas Flow : 3 L/min

Heating Gas Flow : 15 L/min

Drying Gas Flow : 3 L/min

IF/DL/HB Temp. : 300/250/400 °C

CID Gas Pressure : 270 kPa

ESI Probe Position : +4 mm

* See References

■ MRM Transitions

The MRM transitions used for analysis are shown in Table 3. These settings were used to perform simultaneous detection in positive and negative modes.

Table 3 MRM Transitions

Analog	Ret. Time (min)	Polarity	MRM Transition 1	Collision Energy (V)	MRM Transition 2	Collision Energy (V)
STX	8.94	+	300.1>204.1	-20	300.1>138.0	-20
NEO	8.95	+	316.1>126.1	-24	316.1>220.1	-22
dcSTX	8.94	+	257.1>126.1	-22	257.1>220.1	-35
dcNEO	9.06	+	273.1>126.1	-20	273.1>225.1	-20
dcGTX2	7.08	-	351.1>164.0	20	351.1>333.1	20
dcGTX3	7.66	+	353.1>255.1	-20		
GTX1	7.11	-	410.1>367.1	18	410.1>349.1	20
GTX2	6.80	-	394.1>351.1	19	394.1>333.1	22
GTX3	7.65	+	396.1>298.1	-18		
GTX4	7.76	+	412.1>314.1	-18		
C1	3.68	-	474.1>122.0	33	474.1>351.1	25
C2	4.33	+	396.1>298.1	-23		

■ Sample Preparation

The sample preparation protocol is shown in Fig. 2. Two extraction methods were used and compared: acetic acid extraction and hydrochloric acid extraction.

The acetic acid extraction method is used for simultaneous analysis of paralytic shellfish toxins and tetrodotoxin (pufferfish toxin) and the hydrochloric acid method is used for mouse toxicity testing in Japan.

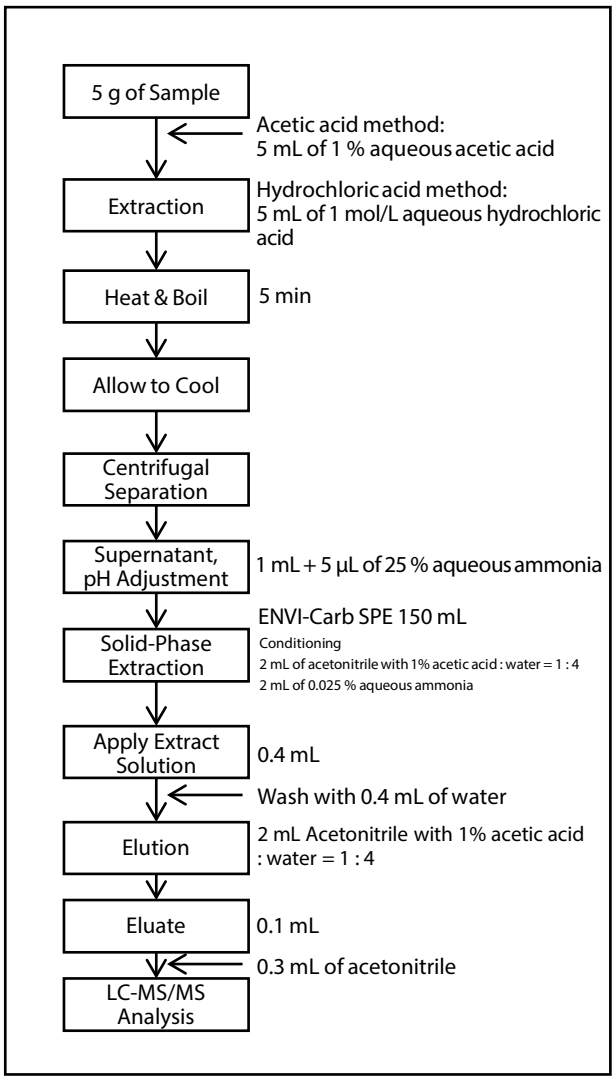

Fig. 2 Sample Preparation Protocol

■ Chromatograms of Saxitoxin Analog (STXs)

Fig. 3 shows a typical example of MRM chromatograms of the toxins extracted from scallops with the acetic acid extraction method. The highest-concentration toxin in the samples was GTX2 followed by STX.

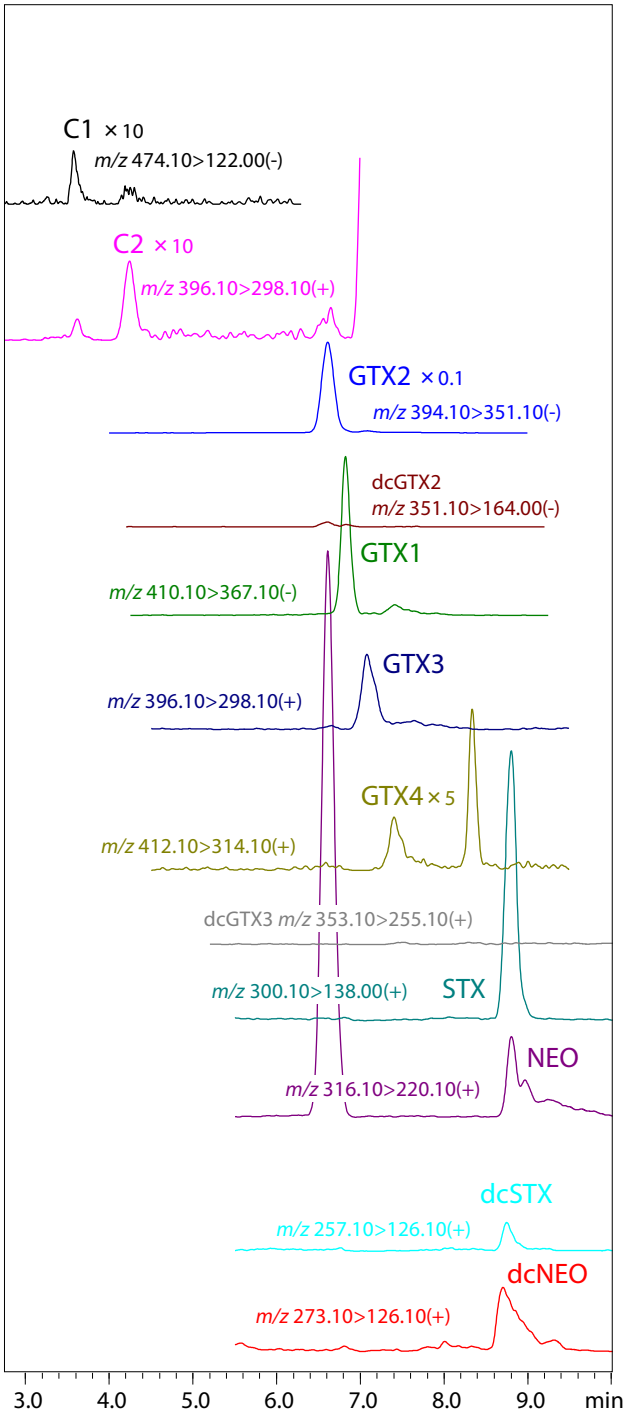

Fig. 3 MRM Chromatograms of STXs in Scallops

■ Calibration Curves

Fig. 4 shows the calibration curve for each toxin in Fig. 3. The minimum concentration points in the calibration curves were in the range 0.39 to 3.09 ng/mL, equivalent to a concentration range of 0.0004 to 0.0031 mg/kg. This is well below the limit of detection of 0.01 to 0.1 mg/kg cited by CODEX CXS 292-2008.

In this article, toxins for which standard reference materials were not available were quantitated using the calibration curves of toxins for which standards were available (See Table 1). STX was quantitated using the calibration curve of dcSTX, dcNEO using the calibration curve of NEO, dcGTX2 using the calibration curve of GTX2, and dcGTX3 using the calibration curve of GTX3.

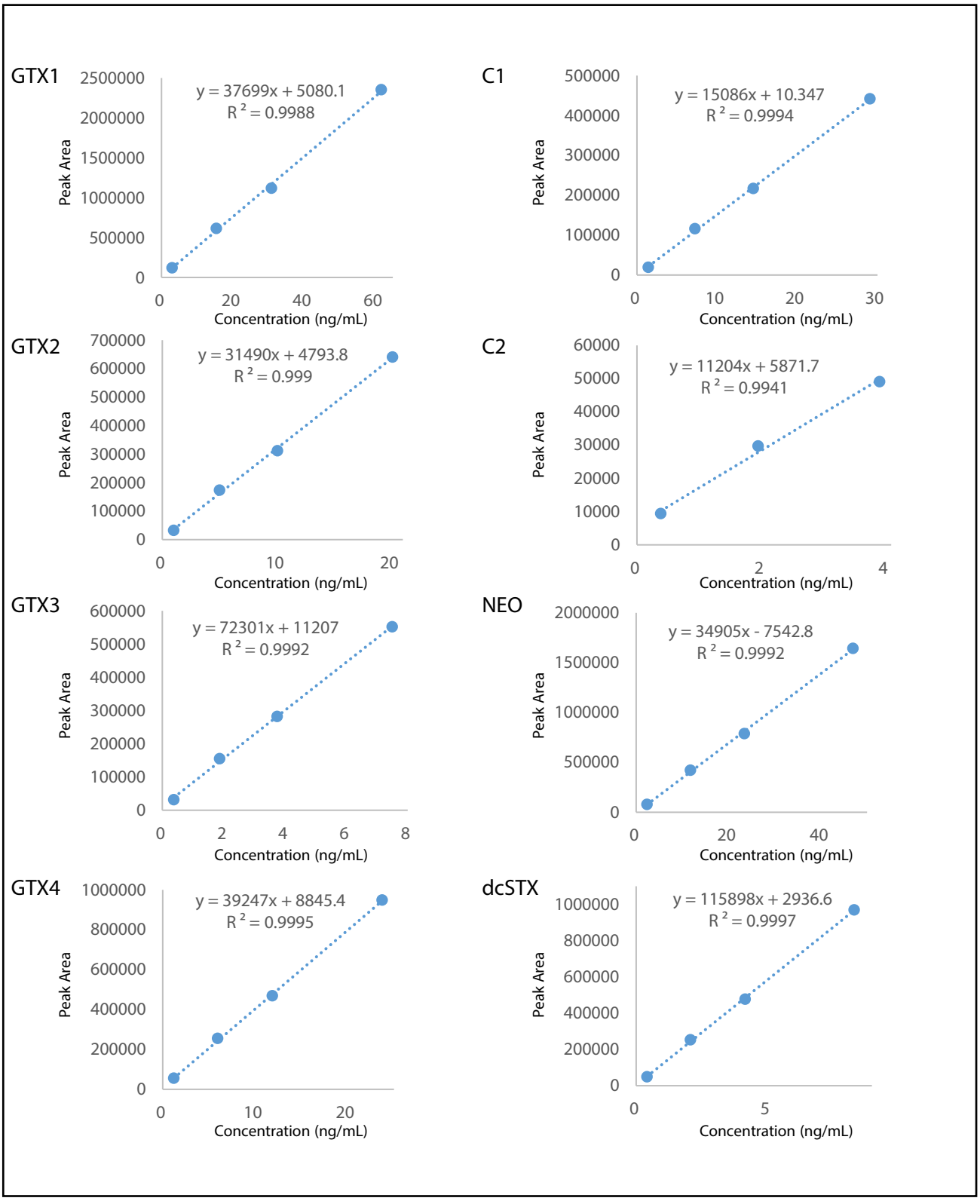

Fig. 4 Calibration Curves for Each Analyte

■ Quantitative Results

STX and STX analogs were detected in all five toxin-containing scallop samples (samples A to E). Quantitative results are shown in Fig. 5. The chromatograms shown in Fig. 3 are taken from the analysis of sample A.

Of the two sample preparation methods used, quantitative results tended to be lower after the hydrochloric acid extraction method that is used in mouse toxicity testing. Possible reasons for this difference include improper pH adjustment of the extract solution, lower recovery from solid phase extraction, and matrix effects when using the hydrochloric acid extraction method.

From these results, it was confirmed that acetic acid extraction was suitable as a sample preparation method for the LC-MS/MS method.

Quantitative values were calculated using "quantitative amount of toxin (ng of STX equivalent/g) = quantitative amount of toxin (ng/g) × TEF × molecular weight of saxitoxin dihydrochloride ÷ molecular weight of toxin."

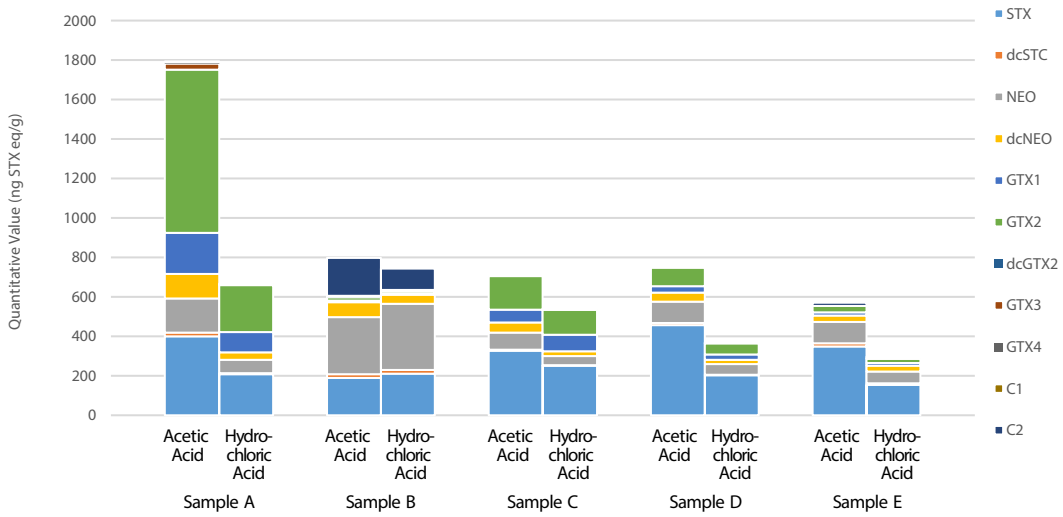

Fig. 5 Comparison of Quantitative Results for Each Sample

■ Conclusion

In place of mouse toxicity testing, this article presents an instrumental method of analyzing paralytic shellfish toxins that uses an LC-MS/MS method with hydrophilic interaction liquid chromatography (HILIC). This instrumental method uses a simple LC system and detects toxins within 10 minutes with a high degree of quantitative sensitivity.

We confirmed that the acetic acid extraction method was better suited to preparing samples from scallops for this LC-MS/MS method than the hydrochloric acid extraction method.

In October 2020, the EU issued a draft SPS notification through the WTO. ePing (epingalert.org)

In the near future, exports to the EU will be required to use only alternative methods that do not require the use of animals for the detection of paralytic shellfish poison [Paralytic Shellfish Poison (PSP)] in accordance with (EU) 2019 627.

In addition, it is desirable to establish an environment in which all molecular reference materials can be purchased at each testing site in the future when the instrumental analysis methods might be announced in Japan.

References

1) Boundy M. et al., J. Chromatography A, 1387, 1-12 (2015).
2) A. D. Turner et al., *J. AOAC Int.*, **98**, 3, 609-621 (2015).
3) A. D. Turner et al., *J. AOAC Int.*, **103**, 2, 533-562 (2020).

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