Certificate of Analysis

NRC·CNRC Certified Reference Material

CRM-AZA-Mus (Lot# 200603)

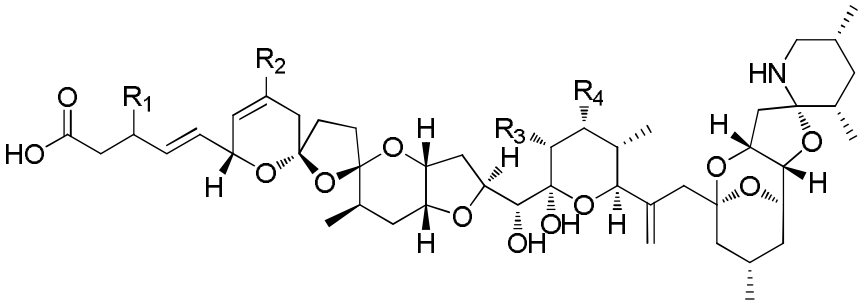
Mussel Tissue Certified Reference Material for Azaspiracids

Azaspiracids (AZAs) are a group of marine biotoxins containing both carboxyl and amine functional groups (Figure 1). AZAs are produced by the small dinoflagellate *Azadinium spinosum* [1] and have been shown to accumulate to high levels in a variety of shellfish species [2]. Human consumption of shellfish contaminated with AZAs causes poisoning with symptoms resembling those of diarrhetic shellfish poisoning [3]. The maximum permissible total level of AZA1, -2 and -3 in shellfish is currently 0.16 mg/kg in whole tissue [4]. CRM-AZA-Mus is a certified reference material (CRM) prepared from naturally contaminated mussel tissues (*Mytilus edulis*) containing certified concentrations of AZA1, -2 and -3 (Table 1). CRM-AZA-Mus is designed to assist the analyst in assessing entire analytical methods used to monitor AZA toxins in shellfish tissues. Each bottle contains approximately 8 g of mussel homogenate with AZAs at levels appropriate for analytical testing.

Table 1: Certified concentration values for CRM-AZA-Mus

Compound	AZA1	AZA2	AZA3
Certified value (mg/kg or µg/g)	1.16 ± 0.10	0.273 ± 0.024	0.211 ± 0.023

Expiry date: 1 year from date of sale.
Storage conditions: -12 ˚C or below



	R^1	R^2	R^3	R^4	$[M+H]^+$ m/z
AZA1	H	H	CH_3	H	842.5
AZA2	H	CH_3	CH_3	H	856.5
AZA3	H	H	H	H	828.5
AZA4	OH	H	H	H	844.5
AZA5	H	H	H	OH	844.5
AZA6	H	CH_3	H	H	842.5
AZA7	OH	H	CH_3	H	858.5
AZA8	H	H	CH_3	OH	858.5
AZA9	OH	CH_3	H	H	858.5
AZA10	H	CH_3	H	OH	858.5
AZA11	OH	CH_3	CH_3	H	872.5
AZA12	H	CH_3	CH_3	OH	872.5

Figure 1: Structures and *m/z* values for the $[M+H]^+$ ions of selected AZA analogues

Intended Use

CRM-AZA-Mus is a matrix CRM intended to test the accuracy of entire analytical methods or to assist in the development of new analytical methods. This may include, but is not limited to, sample extraction procedures and liquid chromatography-mass spectrometry (LC-MS) detection methods. CRM-AZA-Mus is also valuable for testing separation methods because it contains several related AZAs in addition to the certified analogues (Figure 1). The attached chromatograms (Figures 2 - 4) illustrate the AZA profile in CRM-AZA-Mus.

Preparation of the matrix CRM

Several goals were established for this CRM. First, since most AZA monitoring is performed on whole mussel tissues, it was important that the CRM represent this matrix as closely as possible. Therefore an homogenised liquid slurry of whole mussel tissues was chosen. Second, since problems have been observed with thermal stabilisation of AZA materials [5], it was decided that the tissue would be stabilised by gamma irradiation [6]. Third, it was important to have the AZAs in the CRM at levels sufficient to allow accurate certification, while maintaining a concentration that is appropriate for end users.

Mussels (*Mytilus edulis*) naturally contaminated with AZAs were retrieved from Bruckless Bay on the Northwest coast of Ireland following a toxic event in 2005. The mussels were steam cooked and shucked prior to shipment to the NRC. A large scale homogenate was prepared using approximately 6.5 kg of the contaminated mussels and 13 kg of pre-cooked uncontaminated mussels harvested in Prince Edward Island, Canada. The materials were combined by multiple passes through a Comitrol food cutter. Double distilled water was added to bring the water content in the final homogenate to approximately 85%. Stabilisers (ethoxyquin, oxytetracycline, erythromycin and ampicillin) were added to the homogenate at concentrations of 0.02% (*w/w*). During the bottle filling process, the mussel homogenate was continuously mixed and flushed with nitrogen. Using a peristaltic pump 8 g aliquots of the homogenate were dispensed into nitrogen-flushed 8-mL polypropylene vials. Immediately after filling, the vials were flushed with nitrogen and heat sealed using trilaminate polypropylene film closures. The seals were trimmed, inspected and bottle caps were attached. The bottles were frozen and then transported under dry-ice for sterilisation by gamma-irradiation. An average dose of 17 kGy was applied across the entire CRM-AZA-Mus fill series. Following gamma-irradiation the bottles were individually heat sealed in 100 × 165 mm trilaminate pouches.

Homogeneity

Homogeneity was assessed following an approach described by van der Veen *et al.* [7]. To test between bottle homogeneity, 15 units selected from across the entire fill series of CRM-AZA-Mus were tested by LC-MS [8]. To examine within-bottle homogeneity, four sub-samples were homogeneously taken from a single bottle of CRM-AZA-Mus after thorough mixing (see section Instructions for use). Extraction and LC-MS analysis of the homogeneity samples was carried out under repeatability conditions. An uncertainty due to variation between bottles of CRM-AZA-Mus was derived for inclusion in the final combined uncertainty of CRM-AZA-Mus.

Stability Studies

An isochronous short-term stability study on CRM-AZA-Mus demonstrated good stability of AZA1, -2 and -3 at +18 °C over two weeks. Therefore, there is limited risk of degradation during shipping and transport of the CRM. AZA1, -2 and -3 had good long-term stability in CRM-AZA-Mus at -12 °C, with no significant changes in measured concentrations over a 12 month period at this condition. An uncertainty associated with the long-term stability assessment was determined for inclusion in the final combined uncertainty of CRM-AZA-Mus.

Certified Value

The certified values for AZA1, -2 and -3 (Table 1) were obtained using a combination of two separate extraction and quantitation approaches [8]. The first approach utilised an exhaustive liquid-solid extraction method accompanied with standard addition to compensate for matrix effects in LC-MS analysis. In the second approach CRM-AZA-Mus was extracted using an exhaustive matrix solid phase dispersion (MSPD) method with matrix matched calibration (MMC) to account for matrix effects in LC-MS analysis. The spiking solution for standard addition was prepared using AZA calibration solutions supplied by Biotoxin Metrology: CRM-AZA1, -AZA2 and -AZA3. For MMC calibrant CRMs were spiked into a “blank” mussel tissue (CRM-Zero-Mus) extract prepared with the same MSPD method applied to CRM-AZA-Mus.

It is important to note that the certified values for AZA1, -2 and -3 were calculated as a sum of the main peak and a structural isomer for each analogue, which elutes later with the mobile phase-column system that was used for our measurements (see Figure 2). With some more commonly used mobile phase-column systems (see Figure 3), the isomers may not be resolved from the main toxin analogue. Equimolar response factors were used when combining the peak areas of the main AZA and its isomer. Isomer peak

areas corresponded to approximately 10% for the total peak of AZA1 and AZA2, and approximately 7% for the total peak area of AZA3 (data not shown).

Uncertainty

The overall uncertainty estimate (U_{CRM}) for CRM-AZA-Mus includes uncertainties associated with batch characterization (u_{char}), between-bottle variation (u_{hom}), and instability during long-term storage (u_{stab}) [9]. These components can be combined as:

$$U_{CRM} = k\sqrt{u_{char}^2 + u_{hom}^2 + u_{stab}^2}$$

where *k* is the coverage factor for a 95% confidence level (= 2).

All sources of error related to characterization of CRM-AZA-Mus were considered. The certified values of AZA1, -2 and -3 were based on the combination of two analytical approaches. The uncertainty from homogeneity (u_{hom}) is an important component of the overall uncertainty because CRM-AZA-Mus is a matrix material. The uncertainty from long-term stability (u_{stab}) was calculated based on a one year shelf-life for CRM-AZA-Mus, while uncertainty due to short-term stability was not considered. A coverage factor of 2 was applied to the combined uncertainty resulting in the final standard uncertainty values (Table 2). A detailed description of the uncertainty approach applied in the certification of CRM-AZA-Mus is available upon request from Biotoxin Metrology.

Table 2: Uncertainty components for certified values in CRM-AZA-Mus

Uncertainties	AZA1	AZA2	AZA3
$u_{characterization}$	0.029	0.0081	0.0087
$u_{homogeneity}$	0.040	0.0058	0.0071
$u_{stability}$	0.014	0.0064	0.0013
combined uncertainty (k=2)	0.10	0.024	0.023

Characterization of Additional Toxins in CRM-AZA-Mus

CRM-AZA-Mus contains small amounts of several other known AZA analogues (Figure 4) and information values for their levels are provided in Table 3. Additional AZA analogues were detected at trace levels but are not reported here. Other known toxins from the okadaic acid and dinophysistoxin (OA and DTXs), yessotoxin (YTX) and spirolide (SPX) groups are present in CRM-AZA-Mus at low levels (Table 4). Acyl esters of the okadaic acid group toxins, several other spirolides toxins and pinnatoxin-G were also detected at trace levels.

Table 3: Information values for additional AZA analogues present in CRM-AZA-Mus.

Analogue	$[M+H]^+$	RRT**	mg/kg*
AZA4	844.5	0.77	0.17
AZA5	844.5	0.83	0.04
AZA6	842.5	0.97	0.09
AZA7	858.5	0.83	0.02
AZA8	858.5	0.86	0.03
AZA9	858.5	0.81	0.04
AZA10	858.5	0.87	0.02

* These concentrations are not certified.
** Relative retention times (RRT) relative to AZA1 using LC conditions in Figure 4.

Table 4: Information values for additional lipophilic toxins present in CRM-AZA-Mus.

Toxin	$[M+H]^+$	mg/kg*
OA	805.5	0.08 (0.13)**
DTX2	805.5	0.01 (0.01)**
YTX	1143.5	0.01
SPX C	706.5	0.01
13-desMe-SPX C	692.5	trace

* These concentrations are not certified
** Values in parentheses are following base hydrolysis to show ester concentration

Storage Instructions

CRM-AZA-Mus should be stored in a freezer (-12 °C or below) to ensure stability.

Expiry

If stored unopened at the recommended conditions (section Storage Instructions), the certified concentration of the CRM is valid for 1 year from the date of sale**.**

Instructions for Use

Each bottle should be allowed to warm to room temperature prior to opening and the contents thoroughly mixed by vortexing for a minimum of 2 min. Each bottle contains approximately 8 g (± 0.5 g) of tissue. The mass of homogenate is not certified. Remove hermetic seal carefully using a knife or a blade, mix contents thoroughly with a spatula and measure the required sample recording the weight

on an analytical balance. It is not recommended that bottles be refrozen between sub-sampling, so all sub-samples should be taken following the initial defrosting. A minimum sub-sample size of 2 g is recommended. Once tissues have been weighed accurately into an extraction vessel they can be treated accordingly using in-house methods for AZA analysis.

Safety Instructions

AZAs are potent marine biotoxins and can cause stomach cramps, nausea, vomiting, diarrhea and headaches if ingested in sufficient quantities [10]. Only qualified personnel should handle CRM-AZA-Mus and appropriate disposal methods should be used for sample extracts. Gloves and eye protection should be worn when handling CRM-AZA-Mus. A safety data sheet (SDS) is available for CRM-AZA-Mus.

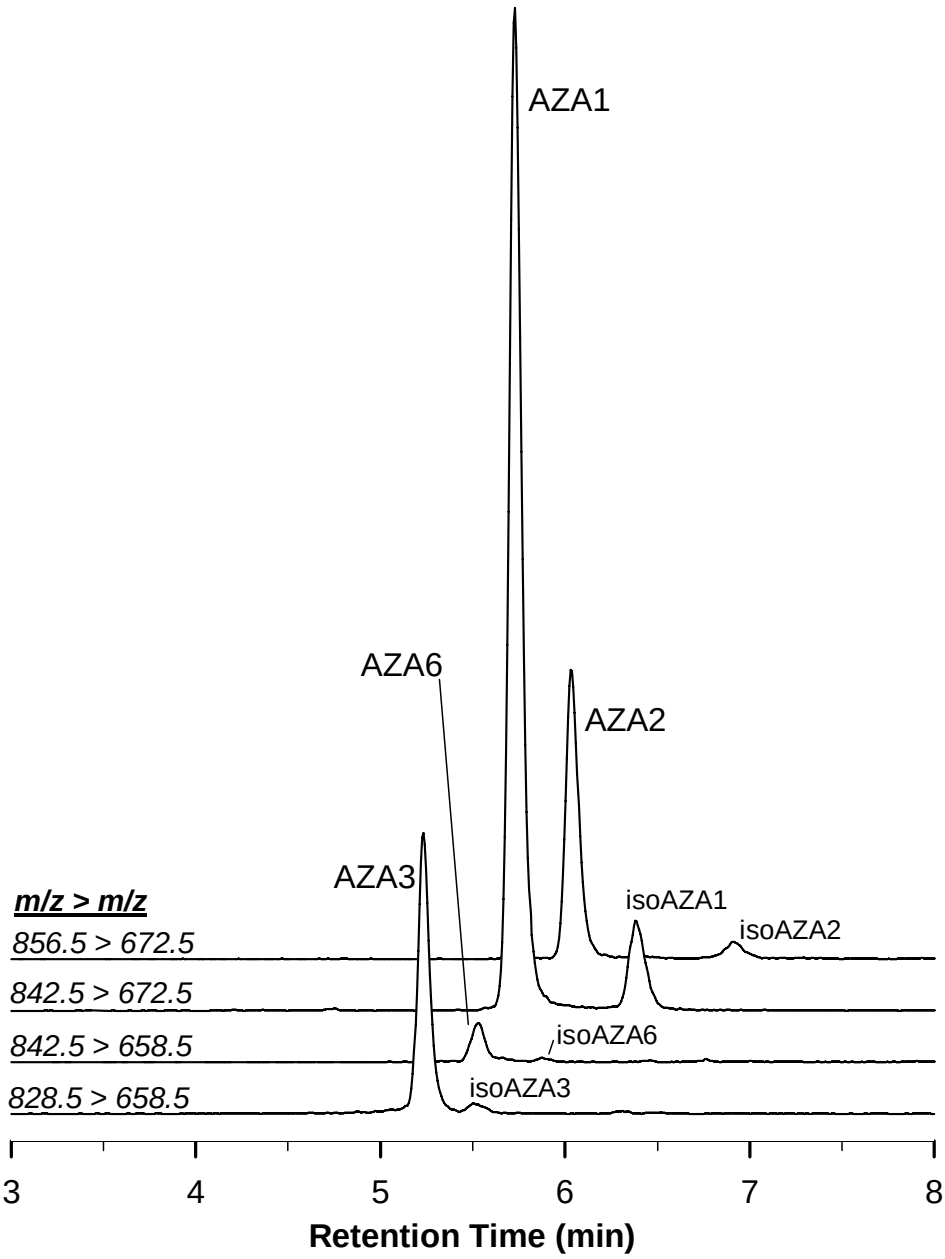


Figure 2: Analysis of CRM-AZA-Mus for AZA1, -2, -3 and -6 by LC-MS. Conditions: Luna C18(2) column (2.1 × 50 mm, 2.5 µm); mobile phase: 5 mM ammonium acetate (pH 6.8) in both deionised water (A) and acetonitrile (B); gradient: 25 -100% B over 5 min, 350 µL/min at +15 °C; injection volume: 5 µL.

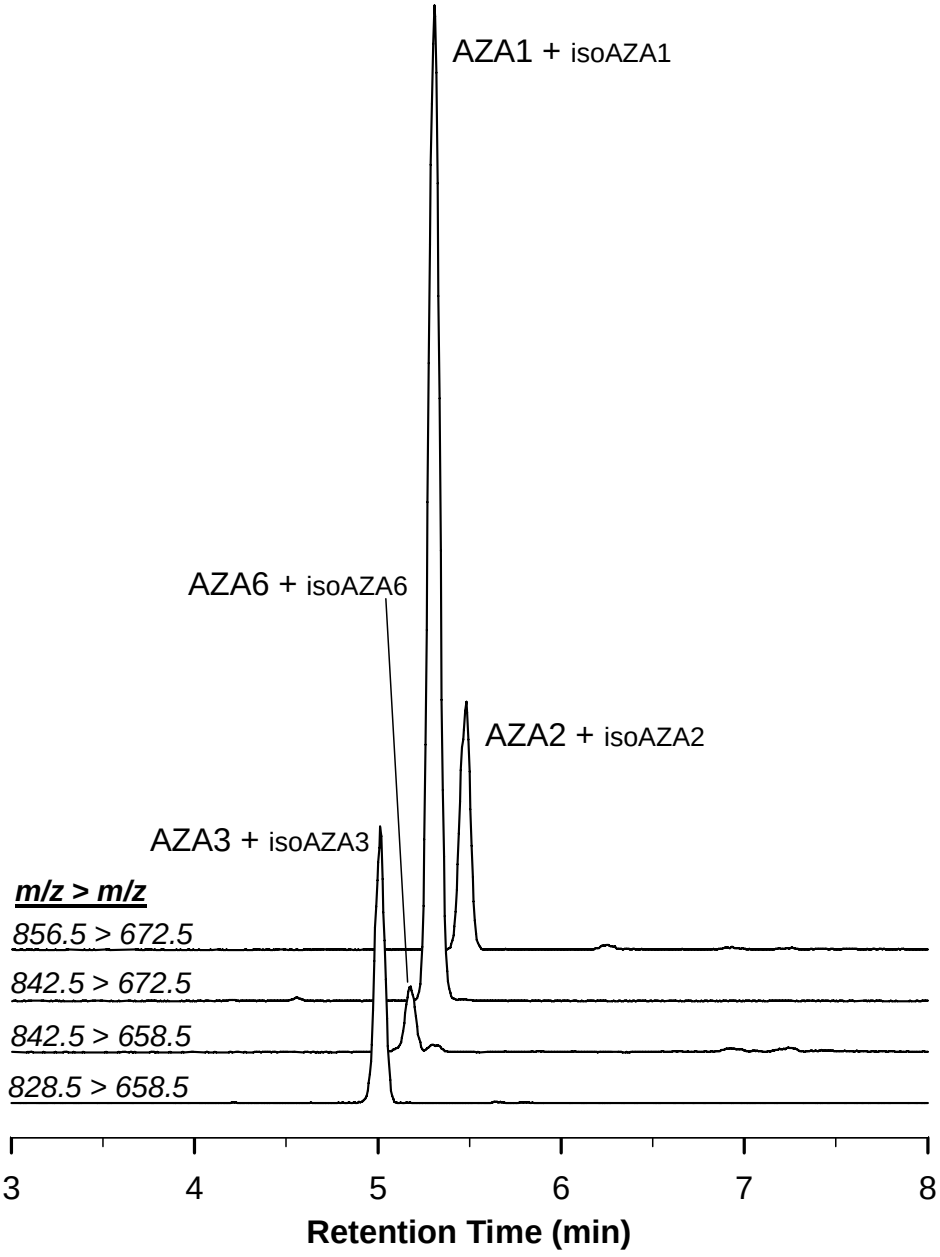


Figure 3: Analysis of CRM-AZA-Mus for AZA1, -2, -3 and -6 by LC-MS. Conditions: Luna C18(2) column (2.1 × 50 mm, 2.5 µm); mobile phase: 2 mM ammonium formate and 50 mM formic acid (pH 2.3) in both deionised water (A) and acetonitrile (B); gradient: 25 -100% B over 5 min, 300 µL/min at +20 °C; injection volume: 5 µL.

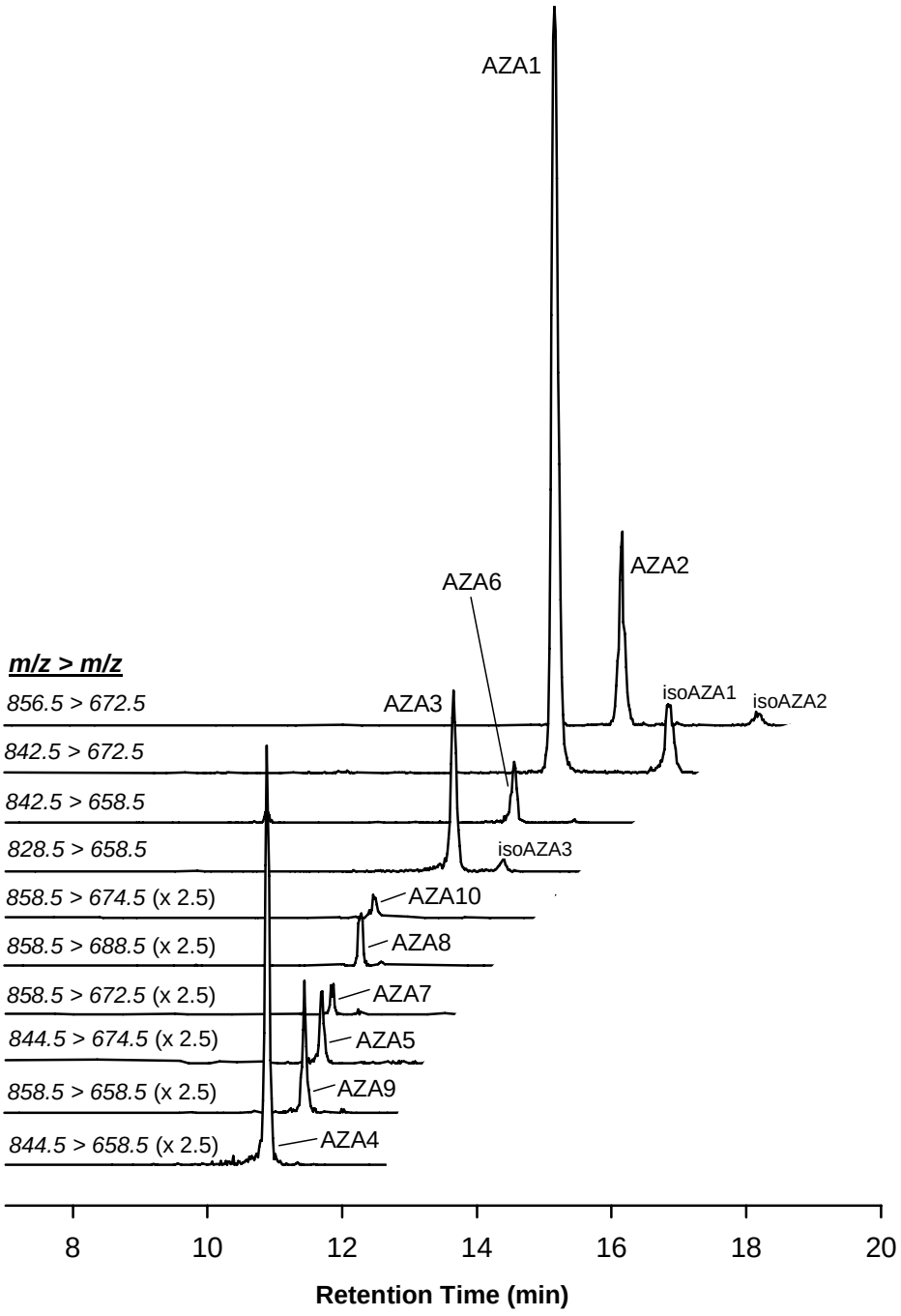


Figure 4: Additional AZA analogues present in CRM-AZA-Mus. LC-MS conditions: Luna C18(2) (2.1 × 100 mm, 2.5 µm); mobile phase: 5 mM ammonium acetate (pH 6.8) in both deionised water (A) and acetonitrile (B); gradient: 25 -100% B over 20 min, 250 µL/min at +20 °C; injection volume: 5 µL.

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Signed **:** 

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