

Efficiency of five RNA extraction protocols for *Grammostola actaeon* (Pocock, 1903) small spinneret tissue samples

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Abstract. Systemic optimization of RNA extraction protocols in non-model arachnids is indispensable for gene expression studies, including transcriptome sequencing and analyses. Spinnerets of the Neotropical tarantula *Grammostola actaeon* (Pocock, 1903) (family Theraphosidae) were used to evaluate the performance of two RNA extraction reagents and three commercially available kits for isolating total RNA from small eukaryotic tissue samples. Total RNA was extracted from spinneret tissues, using two samples for each method. We used the commercially available reagents TRIzol and NucleoZOL and the RNA extraction kits NucleoSpin and NucleoSpin XS (Macherey Nagel) and Total RNA Purification Kit (Norgen Biotek Corp). Quantification using Qubit revealed that NucleoSpin, Norgen, and NucleoSpin XS resulted in the highest RNA yields respectively, while Nanodrop analysis ranked Norgen, TRIzol, NucleoSpin, NucleoSpin XS in descending order. Bioanalyzer analysis indicated that Nucleospin, and NucleoSpin XS delivered the best results for our samples. While each method successfully yielded sufficient RNA for RNA-seq experiments, variations in RNA quality among methods indicate differences in their suitability for specific applications. Our data provide further evidence that RNA integrity number (RIN)-based assessments in *G. actaeon* may not be reliable for evaluating RNA quality due to a widespread occurrence of the ‘gap deletion’ phenomenon in arthropods. RNA from species with 28S rRNA collapsed can yield high-quality transcriptomes, suggesting that current RIN-based assessments may not be reliable for evaluating RNA quality in many non-model invertebrates. Overall, differences in results of commercially available RNA extraction reagents/kits should be considered when selecting the most appropriate RNA extraction method for gene expression analysis.

Keywords: Mygalomorphs, RNA quality, spiders, tissue RNA isolation

Resumo. A otimização sistêmica de protocolos de extração de RNA em aracnídeos não-modelos é indispensável para estudos de expressão gênica, incluindo sequenciamento e análises de transcriptomas. Fiandeiras de *Grammostola actaeon* (Pocock, 1903) foram utilizadas para avaliar o desempenho de dois reagentes de extração de RNA e três kits comercialmente disponíveis para isolar RNA total de pequenas amostras de tecido eucariótico. O RNA total foi extraído de tecidos de fiandeiras, utilizando duas amostras para cada método. Utilizamos reagentes TRIzol e NucleoZOL, bem como Macherey Nagel (MN) NucleoSpin, MN NucleoSpin XS e Norgen Biotek Corp. A quantificação usando Qubit revelou que NucleoSpin, Norgen e NucleoSpin XS resultaram nos maiores rendimentos de RNA, respectivamente, enquanto a análise Nanodrop classificou Norgen, TRIzol, NucleoSpin, NucleoSpin XS em ordem decrescente. A análise do Bioanalyzer indicou que o MN Nucleospin e o NucleoSpin XS forneceram os melhores resultados para nossas amostras. Embora cada método tenha produzido RNA suficiente para experimentos de RNA-seq, variações na qualidade do RNA entre os métodos indicam diferenças em sua adequação para aplicações específicas. Nossos dados fornecem evidências adicionais de que avaliações baseadas no número de integridade do RNA (RIN) em *Grammostola actaeon* podem não ser confiáveis para avaliar a qualidade do RNA devido ao fenômeno de perda do intervalo (‘gap deletion’) em artrópodes. O RNA de espécies com 28S rRNA colapsado pode produzir transcriptomas de alta qualidade, sugerindo que as avaliações atuais baseadas em RIN podem não ser confiáveis para avaliar a qualidade do RNA em muitos invertebrados não-modelo. No geral, as diferenças nos resultados dos reagentes/kits de extração de RNA disponíveis comercialmente devem ser consideradas ao selecionar o método de extração de RNA mais apropriado para análise de expressão gênica.

Palavras-chave: Aranhas, Caranguejeiras, Isolamento de RNA tecidual, Qualidade de RNA.

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RNA extraction is necessary for several molecular applications, including RT-PCR, qPCR, RNA sequencing (RNA-seq) or other high throughput gene expression analyses, where the quality of RNA directly influences the accuracy and reliability of results (Sambrook & Russell 2001; Wang et al. 2009; Conesa et al. 2016). At present, RNA-seq and transcriptomic analyses are used extensively in systematics and zoological research, with widespread use in phylogenomics and studies exploring the molecular evolution of genes and gene pathways, or gene dynamics in an evolutionary and developmental context.

A crucial step that precedes transcriptomic analyses is the extraction of high-quality RNA. RNA extraction is a fundamental process that determines the success of subsequent transcriptome

sequencing, as it directly impacts the integrity, purity, and yield of RNA. Without this, the generation of reliable transcriptomic data is not feasible. For phylogenomic and gene expression studies alike, extracting RNA of sufficient quality is essential, as it ensures that the sequenced transcripts accurately reflect the organism’s gene expression patterns. Efficient RNA extraction becomes especially challenging in arachnid research due to their small body sizes and the limited amount of tissue often available for analysis, making optimized extraction protocols a critical component of these studies.

In the context of arachnid research, transcriptome data is already well established in phylogenomics for offering insights into the evolutionary relationships among this diverse group of animals. For example, Foley et al. (2019) used transcriptome data to construct a robust phylogeny of deep theraphosid clades, which

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not only revealed novel phylogenetic relationships but also provided new perspectives on the evolution of urticating setae. Similarly, Baker et al. (2020) applied transcriptomic data to resolve the phylogeny of the mite harvestman family Pettalidae. Their study highlighted the impact of Gondwanan vicariance on the evolutionary history of these arachnids, underscoring the power of transcriptomics to uncover historical biogeographic patterns and to provide a clearer understanding of lineage diversification. Both examples illustrate the utility of transcriptomic data for enhancing our comprehension of arachnid phylogeny and evolution.

Additionally, transcriptomic data have become increasingly valuable for gene expression studies in Arachnida. For example, Sharma et al. (2014) used transcriptomic data to investigate the expression of Hox genes, revealing important patterns that clarify the evolution of body segmentation in chelicerates. Similarly, Mariano-Martins et al. (2022) examined developmental genes involved in the evolution of morphological novelties using spiders as their model. These studies highlight how transcriptomic data can provide insights into the molecular mechanisms underlying the development and evolutionary adaptations of specialized structures in arachnids. In this context, the RNA extraction step is vital to isolate good quality RNA needed for subsequent RNAseq and transcriptomic analyses to study the molecular mechanisms of development, which allows for a deeper understanding of specialized structures in arachnids.

Araneae, an order within the class Arachnida (Chelicerata), comprises over 53,000 described species of spider across 134 families (Regier et al. 2010; World Spider Catalog 2025). Among these diverse clades, the infraorder Mygalomorphae is notable for containing tarantula-like spiders. Within this group, *Grammostola* Simon, 1892, is a Neotropical genus in the family Theraphosidae, with 20 described species (World Spider Catalog 2025). Molecular studies on the tarantula genus *Grammostola* have focused on DNA barcoding to enhance taxonomic clarity and provide insights into evolutionary relationships within closely related species (Montes de Oca et al. 2016; Pittella et al. 2023). Additionally, studies have been conducted into the genetic basis of venom composition (Kimura et al. 2012; Borges et al. 2016).

Despite the limited financial resources, tools, methods, and references available to study non-model organisms overall, arachnids are emerging as important systems for tackling questions across biology, including, for example, pattern formation, branching morphogenesis, and regeneration (Lo-Man-Hung 2024). Normally, research protocols are developed in laboratory animals in culture, which are housed and maintained in strict and controlled conditions. Setting up a new colony of a non-model organism can be financially costly, time-consuming and is not always successful (Russell et al. 2017; Andersen & Winter 2019). Consequently, optimizing RNA extraction protocols for target samples, particularly when they are scarce, becomes indispensable for molecular research.

However, obtaining high-quality RNA for gene expression analyses presents numerous challenges. These include nuclease activity, the presence of various contaminating agents, sampling time, ambient temperature, and the time spent extracting the tissue sampled and the efficiency of extraction methods, especially when working with small tissue samples (<15 mg) (Gauthier et al. 1997; Gonzalez et al. 1999; Holland et al. 2003; Triant & Whitehead 2009; Hrdlickova et al. 2017). Additionally, when selecting the best method to fit the experiment, it is important to consider the capacity to retrieve the necessary amount of total

RNA and the capability to recover RNA that meets specific quality standards. RNA for gene expression experiments, for example, must be free of proteins, contaminants, and nucleases (Tavares et al. 2011).

In this study, we evaluated the performance of five RNA extraction protocols designed for small samples (with a kit average mass specification of <15 mg) to extract 5 µg of spinneret tissue from juveniles (15 days after reaching the third instar) of *Grammostola actaeon* (Pocock, 1903), a tarantula species with long, clearly visible spinnerets, and a good candidate for studies in development and regeneration of spinnerets. The RNA extraction protocols included two using commercially available reagents, TRIzol (Invitrogen) and NucleoZOL (Macherey-Nagel), and three commercial RNA extraction kits, NucleoSpin (Macherey-Nagel), NucleoSpin XS (Macherey-Nagel), and Total RNA Purification Kit (Norgen Biotek Corp.). Our aim was to determine the best extraction method to efficiently isolate total RNA of high quality and quantity from small spider tissue samples.

METHODS

Tissue samples.—Collections of *G. actaeon* were carried out in two campaigns, in the São Joaquim National Park, Urubici, Santa Catarina, Brazil, license ICMBio 86700.

Spinneret tip (about 2–3 mm from last segment of the spinneret) tissue samples of 5 µg were collected from live juveniles. Animals were immobilized on a watch glass with ice, and their spinnerets were cleaned with ethanol on a swab and cut with microsurgical scissors. The procedure was not fatal for any of the spiders used in this study. To prevent infection, a plug of liquid paraffin was smeared externally on the cut site. The tissues were then flash-frozen in liquid nitrogen and stored at -80°C until RNA isolation.

RNA extraction.—Samples were randomly allocated for RNA isolation with each of five extraction protocols that include the commercially available reagents (TRIzol and NucleoZOL) and kits (NucleoSpin, NucleoSpin XS and Total RNA Purification Kit Norgen Biotek Corp.), resulting in duplicates for each extraction protocol. A detailed description of each selected kit is provided in Table 1.

All spiders used in the study were from the same cocoon and at the same developmental stage, which helped to minimize individual variation that could affect RNA yield. For tissue normalization, we ensured consistency by using 5 µg of tissue per sample, carefully dissected to maintain uniformity.

For each of the kits tested, tissues were homogenized using a pestle in the manufacturer-supplied homogenization/lysis buffer. Manufacturer protocols were followed with one exception: to achieve a higher RNA concentration, elution was performed twice with 20 µL of RNase-free H₂O each time, followed by centrifugation at 11,000 × g for 1 minute, resulting in a total volume of 40 µL. In the extraction performed with TRIzol and NucleoZOL, the RNA pellets were resuspended in 40 µL of RNase-free H₂O.

Determination of RNA quantity and quality.—RNA was quantified by measuring total RNA concentration using a Qubit® RNA Assay Kit with the Qubit 2.0 Fluorometer. Qubit uses an RNA-binding dye for spectrophotometrical quantification of RNA concentration in the solution. The greater the absorption of light at

Table 1.—Description of the five RNA extraction methods selected for comparison.

Sample code	Kit/Reagent name	General description	Specifications*
T1 and T2	TRIzol (Invitrogen)	Acid-guanidinium-phenol based reagent	Tissue Mass: ≤ 50 –100 mg Minimum Elution Volume: 40–80 μ L
N1 and N2	NucleoZOL (Macherey-Nagel)	A combination of phenol with guanidinium thiocyanate	Tissue Mass: ≤ 50 mg Minimum Elution Volume: 60 μ L
U1 and U2	NucleoSpin (Macherey-Nagel)	Spin column-based method combining guanidine-isothiocyanate lysis with genomic DNA digestion by DNase I treatment and the silica-membrane technology	Tissue Mass: ≤ 30 mg Minimum Elution Volume: 60 μ L
O1 and O2	Total RNA Purification Kits (Norgen Biotek Corp)	Resin-based columns, without the use of phenol or chloroform, lysing with Buffer RL (contains guanidinium salts) with genomic DNA digestion by DNase I treatment	Tissue Mass: ≤ 50 mg Minimum Elution Volume: 50 μ L
XS1 and XS2	NucleoSpin XS Macherey-Nagel	Spin column-based method combining guanidine-isothiocyanate lysis with genomic DNA digestion by DNase I treatment and the silica-membrane technology	Tissue Mass: ≤ 30 mg Minimum Elution Volume: 60 μ L

*These are the specifications of the kit/reagent manufacturer describing the minimum and maximum mass recommended for their use, and the recommended elution volume. In our experiments, we used 5 μ g of tissue and an elution volume of 40 μ L of RNase-free H₂O for all kits/reagents.

the 260 nm wavelength, the higher the concentration (Haque et al. 2003; Bruijns et al. 2022), independent of contaminants.

The total RNA concentration and $A_{260}:A_{280}$ sample ratios were determined using a NanoDrop 2000 spectrophotometer (Thermo Scientific - NanoDrop products, Wilmington, Delaware, USA). Using NanoDrop, the level of sample contamination can be assessed by measuring the absorbance ratios at 260 and 280 nm ($A_{260}:A_{280}$). This ratio reflects the extent of contamination from proteins and other impurities, as nucleic acids absorb at 260 nm, while proteins and other impurities absorb at 280 nm (Becker et al. 2010). Total RNA samples with $A_{260}:A_{280}$ values higher than 1.8 are regarded as suitable for subsequent molecular analyses (Becker et al. 2010).

RNA integrity number (RIN) was assessed using an RNA Pico 6000 assay on the Bioanalyzer 2100 (Agilent Technologies, Santa Clara, California, USA). In this equipment, RNA samples are separated within the channels of microfabricated chips based on their molecular weight and are subsequently detected using laser-induced fluorescence detection (Mueller et al. 2000). Data are presented in an easy-to-read format consisting of electropherograms and a gel-like image (Figs. 1 & 2). Whereas the Bioanalyzer can help assess the presence of contaminants in RNA extraction samples (Mueller et al. 2000), and only consumes only 1 μ L of RNA preparations, in some cases, the usage of ribosomal ratio for RNA quality assessment can show weak correlation with RNA integrity (Miller et al. 2004).

RESULTS AND DISCUSSION

RNA purification.—All five RNA isolation methods yielded RNA in processed samples, but the different RNA isolation methods exhibited different yields of RNA as measured with the Qubit 2.0 Fluorometer or the NanoDrop Lite Spectrophotometer (Table 2). Nevertheless, it must be noted that the sample size ($n = 2$ for each approach) represents a limitation of this study, as it may influence the variability of the results and restrict the generalizability of the findings.

According to the Qubit measurements, the kits, NucleoSpin, Norgen, and NucleoSpin XS generally retrieved the highest yields, ranging from 1.54 to 2.75 ng/ μ L, except XS1 (0.446 ng/ μ L and O1 (0.886 ng/ μ L) (Table 2). In contrast, both reagents,

NucleoZOL and TRIzol, yielded the lowest concentrations of RNA, ranging from 0.389 to 0.557 ng/ μ L (Table 2).

Nanodrop measurements to quantify total RNA concentration and the ratio of absorbance at 260 and 280 nm, indicated that the Norgen kit exhibited the highest RNA concentration (27.2 and 55.4 ng/ μ L), and was followed by NucleoSpin and NucleoSpin XS (12.2 to 22.5 ng/ μ L) (Table 2). TRIzol and NucleoZOL had the lowest RNA concentrations (less than 2.5 ng/ μ L) (Table 2). NucleoSpin XS had $A_{260}:A_{280}$ values higher than 1.8 for both samples (Table 2). TRIzol and NucleoZOL had high $A_{260}:A_{280}$ ratios, with samples ranging between 1.73 – 2.10, but they recovered very low RNA concentrations, indicating that the higher ratios found in TRIzol and NucleoZOL were likely related to other impurities. This suggests they were not capable of isolating sufficiently pure RNA.

Both Qubit and Nanodrop are important tools for nucleic acid quantification, but their differences in principles, sensitivity, specificity, and sample preparation must be considered when interpreting results (Bruijns et al. 2022). Qubit can measure low nucleic acid concentrations with accuracy, while Nanodrop's accuracy may be affected by contaminants that absorb light (Bruijns et al. 2022). Qubit requires specific sample preparation steps, like dilution and dye addition, whereas Nanodrop needs

Table 2.—Metrics obtained using Qubit and Nanodrop. RNA Integrity Number (RIN) values. N1 and N2 (NucleoZOL); U1 and U2 (NucleoSpin); XS1 and XS2 (NucleoSpin XS); O1 and O2 (Norgen); T1 and T2 (TRIzol). N/A = Agilent's warning that the RIN may not be reliable.

Sample code	Qubit		Nanodrop				RIN
	ng/ μ L	Total RNA (μ g)	ng/ μ L	Total RNA (μ g)	260/280	260/230	
T1	0.557	0.022	1.7	0.06	1.73	0.06	N/A
T2	0.280	0.011	1.1	0.04	2.10	0.02	N/A
N1	0.700	0.028	2.3	0.92	1.73	0.35	N/A
N2	0.389	0.016	1.2	0.04	1.28	0.06	7
U1	2.75	0.110	12.5	0.50	1.47	0.14	N/A
U2	2.71	0.108	22.5	0.90	1.42	0.51	N/A
O1	0.886	0.035	27.2	0.90	1.33	0.82	N/A
O2	1.88	0.075	55.4	2.21	1.23	1.15	5.80
XS1	0.446	0.017	10.8	0.43	1.84	0.28	N/A
XS2	1.54	0.061	12.2	0.48	1.92	0.07	N/A

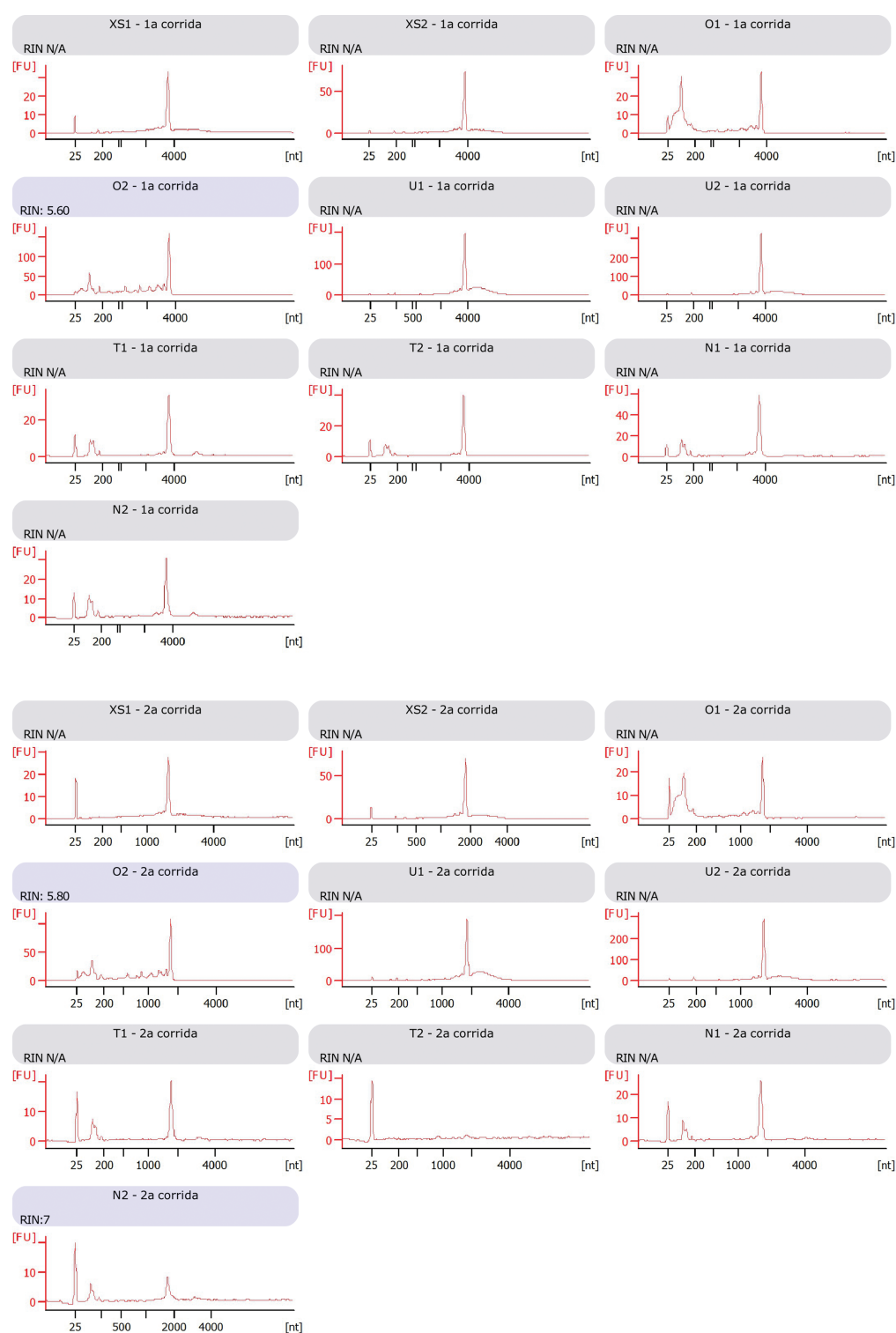


Figure 1.—Electropherograms obtained with the Agilent Bioanalyzer 2100. “1a corrida” (first run, above) and “2a corrida” (second run, below). RNA samples: N1 and N2 (NucleoZOL); U1 and U2 (NucleoSpin); XS1 and XS 2 (NucleoSpin XS); O1 and O2 (Norgen); T1 and T2 (TRIzol).

minimal preparation. Nanodrop’s measurements can be skewed by impurities absorbing light at nucleic acid wavelengths, unlike Qubit, which is less affected due to its dye’s specificity (Bruijns et al. 2022).

Spider RNA samples have no or low detectable RINs.—Using the Agilent Bioanalyzer 2100, most of the samples had no

detectable RIN (RNA integrity number values), except for samples extracted with Norgen (O2 = RIN: 5.60) and NucleoZOL (N2 = RIN: 7). Nucleospin and NucleoSpin XS obtained the best results for our samples (Fig. 1).

Traditionally, RNA quality is assessed using the RIN; however, this metric, designed around model bacterial and vertebrate

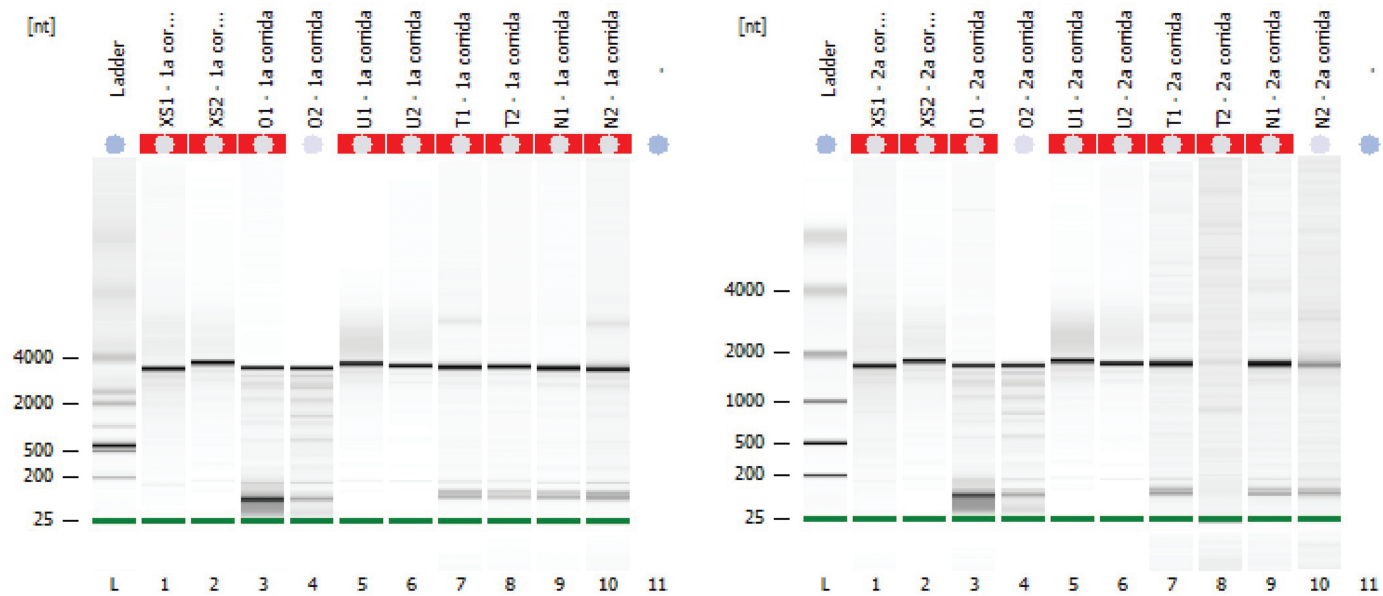


Figure 2.—A representation of gel images produced during RNA quality checks performed via Bioanalyzer 2100. Total RNAs from spinnerets of *Grammostola actaeon*. ‘1a corrida’ (first run) and ‘2a corrida’ (second run). The RNA samples extracted by N1 and N2 (NucleoZOL); U1 and U2 (NucleoSpin); XS1 and XS 2 (NucleoSpin XS); O1 and O2 (Norgen); T1 and T2 (TRIzol).

organisms, does not accurately reflect RNA quality in some invertebrates, mostly arthropods, due to a process called gap deletion (Sambrook & Russell 2001; Mueller et al. 2004; Opitz et al. 2010). This process involves the 28S ribosomal RNA (rRNA) subunit collapsing into two smaller fragments, often appearing as a single band similar to the 18S rRNA, which can be misinterpreted as degradation (Ishikawa & Newburgh 1972; Ware et al. 1985; Fujiwara & Ishikawa 1986; Sun et al. 2012). DeLeo et al. (2018) sampled more than half of the major lineages within Arthropoda to determine if this phenomenon is widespread or a methodological error, finding that approximately 90% of the sampled species displayed this 28S rRNA collapse. Remarkably, this form of denaturation can occur even at low temperatures, challenging the notion that it represents true degradation (DeLeo et al. 2018). Moreover, RNA from species with this type of 28S rRNA alteration can yield high-quality transcriptomes, suggesting that current RIN-based assessments may not be reliable for evaluating RNA quality in many non-model invertebrates (DeLeo et al. 2018). Despite the limitations in spider RIN assessment, the quantity, purity and integrity of the isolated RNA was visualized in Bioanalyzer electropherograms (Fig. 2). The RNA bands were clear and complete. Taken together, total RNA concentrations obtained from NucleoSpin XS and NucleoSpin were higher than the other methods, and the bands were clear and intact, despite the lack of RIN values for these samples; this aligns with the conclusions of DeLeo et al. (2018) regarding the use of RIN in non-model invertebrates.

Concentration of recovered nucleic acid varies across extraction methods.—Nucleic acid extraction performance at low concentrations varies depending on the method used. RNA extraction techniques involving acid guanidinium thiocyanate–phenol–chloroform extraction requires phase precipitation and separation, which can lead to the loss of small sample quantities in the absence of an RNA carrier (e.g., glycogen) and can also cause nucleic acid shearing or degradation if not properly executed

(Chomczynski & Sacchi 2006). NucleoZOL and TRIzol require careful phase separation and alcohol precipitation, which can result in the loss of material, especially small amounts of samples. On the other hand, kits include nucleic acid-binding matrices (like silica membranes or magnetic beads), optimized buffers, and enzymes that help maximize nucleic acids recovery and minimize degradation. Kits also include steps that specifically stabilize and protect nucleic acids, reducing the chance of loss (Tan & Yiap 2009). The column-based purification methods often used in kits can more effectively concentrate nucleic acids, improving the recovery of low-concentration samples (Tan & Yiap 2009). This variability in RNA extraction efficiency is evident in our study, where both Qubit and Nanodrop measurements revealed differing RNA concentrations depending on the method used, with kit-based methods generally yielding higher total RNA (Table 2).

CONCLUSION

In general, all tested RNA extraction methods successfully yielded the necessary amount of RNA for RNA-seq experiments, meeting the quantitative requirements. However, despite the small sample sizes, discernible variations emerged in the quality of the extracted RNA across and within the methods, indicating potential discrepancies in their efficacy for obtaining high-quality RNA suitable for specific applications. These differences underscore the importance of extraction methods, as they can impact downstream analyses, particularly gene expression studies.

Qubit measurements indicated that NucleoSpin, Norgen, and NucleoSpin XS kits yielded the highest concentrations of nucleic acids. Nanodrop results further supported these observations, with Norgen demonstrating the highest concentrations, followed by NucleoSpin and NucleoSpin XS. Bioanalyzer confirmed that NucleoSpin and NucleoSpin XS provided the best results for RNA quality and integrity. Taken together, these

results led us to choose the NucleoSpin RNA extraction kit for our next experiments, for its consistently good results in the assessed metrics.

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