DOI: 10.5433/1679-0359.2017v38n4p2201

# High-quality total RNA isolation from melon (*Cucumis melo* L.) fruits rich in polysaccharides

# Isolamento de RNA total de alta qualidade a partir de frutos de melão (*Cucumis melo* L.) contendo altos níveis de polissacarídeos

Gabrielle Silveira de Campos[1]; Ricardo Antônio Ayub[2*]; Rafael Mazer Etto[3]; Carolina Weigert Galvão[4]; Marília Aparecida Stroka[5]; Juliana Inaba[3]

## Abstract

Melon, a member of the family Cucurbitaceae, is the fourth most important fruit in the world market and, on a volume basis, is Brazil's main fresh fruit export. Many molecular techniques used to understand the maturation of these fruits require high concentrations of highly purified RNA. However, melons are rich in polyphenolic compounds and polysaccharides, which interfere with RNA extraction. This study aimed to determine the most appropriate method for total RNA extraction from melon fruits. Six extraction buffers were tested: T1) guanidine thiocyanate/phenol/chloroform; T2) sodium azide/β-mercaptoethanol; T3) phenol/guanidine thiocyanate; T4) CTAB/PVP/β-mercaptoethanol; T5) SDS/sodium perchlorate/PVP/β-mercaptoethanol, and T6) sarkosyl/PVP/guanidine thiocyanate, using the AxyPrep™ Multisource Total RNA Miniprep Kit. The best method for extracting RNA from both mature and green fruit was based on the SDS/PVP/β-mercaptoethanol buffer, because it rapidly generated a high quality and quantity of material. In general, higher amounts of RNA were obtained from green than mature fruits, probably due to the lower concentration of polysaccharides and water. The purified material can be used as a template in molecular techniques, such as microarrays, RT-PCR, and in the construction of cDNA and RNA-seq data.
**Key words**: RNA extraction methods. RNA quality. Melon.

## Resumo

O melão pertencente à família Cucurbitaceae é o quarto fruto mais importante no mercado mundial e a fruta mais exportada pelo Brasil. Muitas técnicas moleculares utilizadas para compreender a maturação destes frutos requerem o uso de RNA altamente purificado e em alta concentração. Entretanto, o elevado nível de compostos polifenólicos e polissacarídeos nos frutos tornam a extração de RNA um desafio. Este trabalho teve por objetivo determinar o método mais adequado para extração de RNA total em frutos de melão. Seis diferentes tampões de extração foram testados: T1) tiocianato de guanidina/fenol/clorofórmio; T2) azida de sódio/β-mercaptoetanol; T3) fenol/tiocianato de guanidina, T4) CTAB/PVP/β-mercaptoetanol, T5) SDS/perclorato de sódio/PVP/β-mercaptoetanol e T6) sarcosil/PVP/tiocianato de guanidina associado com AxyPrep TM Multisource Total RNA Miniprep Kit. O melhor método para extração do RNA tanto de fruto verde quanto maduro foi o baseado no tampão SDS/

[1] Discente, Curso de Graduação em Farmácia, UEPG, Ponta Grossa, PR, Brasil. E-mail: gabriellesilveiradecampos@yahoo.com.br
[2] Prof. Associado, Universidade Estadual de Ponta Grossa, UEPG, Departamento de Fitotecnia, Ponta Grossa, PR, Brasil. E-mail: rayub@uepg.br
[3] Profs., Adjunto, UEPG, Departamento de Química, Ponta Grossa, PR, Brasil. E-mail: mazeretto@uepg.br; juinaba@hotmail.com
[4] Profª Adjunta, UEPG, Departamento de Biologia Estrutural, Molecular e Genética, Ponta Grossa, PR, Brasil. E-mail: carolwgalvao@yahoo.com.br
[5] Discente, Curso de Graduação em Agronomia, UEPG, Ponta Grossa, PR, Brasil. E-mail: mariliastroka.agro@hotmail.com
[*] Author for correspondence

Received: Mar. 17, 2016 - Approved: Apr. 19, 2017

PVP/β-mercaptoetanol, por gerar material íntegro e de qualidade em grande quantidade, associado à rapidez de execução. Em geral, maiores quantidades de RNA foram obtidas a partir de frutos verdes, provavelmente devido à baixa concentração de polissacarídeos e água. O material purificado poderá ser utilizado como molde em técnicas de estudos moleculares como microarrays, RT-PCR e bibliotecas de cDNA e RNAseq, pelo qual foi testado.
**Palavras-chave:** Metodologias de extração de RNA. Qualidade do RNA. Melão.

Melon (*Cucumis melo* L.) belongs to the family Cucurbitaceae. It is the fourth most important fruit in the world market and, in terms of volume, is Brazil's main fresh fruit export (ANUÁRIO BRASILEIRO DA FRUTICULTURA, 2015). The taste and quality of the fruit depend on the physiological and biochemical variations that occur during its development and maturation (NUNEZ-PALENIUS et al., 2008). These physiological changes are the result of the transcriptional modulation of several genes. Thus, their understanding requires the use of molecular biology techniques, such as microarray assays, subtractive hybridization and RNA-Seq, which necessitate the extraction of high-quality total RNA. However, the isolation of total RNA from some plants is difficult due to the presence of high concentrations of secondary metabolites, such as polysaccharides and polyphenolic compounds, which may precipitate or interact with ribonucleic acids (ZAMBONI et al., 2008). Strawberry and grape tissues are rich in polyphenolic compounds and polysaccharides, therefore, the isolation of high-quality RNA in acceptable yields, particularly from their flesh tissues, is often a challenge (CHRISTOU et al., 2014; VASANTHAIAH et al., 2008).

Although there are many RNA protocols specific to plants with a high content of phenolic compounds, they typically involve an extensive preparation time and do not apply to all types of fruits (KALINOWSKA et al., 2012). In addition, for plants rich in secondary metabolites, isolation of the RNA using commercial kits is often unsatisfactory and does not produce acceptable yields. In particular, the isolation and purification of RNA from fruits is hindered by the presence of abundant compounds (polysaccharides, polyphenols, proteins and genomic DNA contamination) that interact with nucleic acids, forming insoluble co-precipitates (CHRISTOU et al., 2014). RNA isolation also depends on other critical factors, such as the inactivation of endogenous and exogenous RNAs that promote the degradation of the purified material. Many methods for RNA isolation use strong denaturants, such as guanidine salts to simultaneously disrupt cellular activity, solubilize their components and denature RNAs, which affect the quality of the process (MACKENZIE et al., 1997). Furthermore, despite the numerous RNA isolation protocols that exist, there is no universal method for all species of plants or plant organs. Even identical tissues, at different stages of development, may require a specific RNA isolation protocol due to their variable chemical compositions (SHARMA et al., 2003).

In this context, the current study evaluated six methods of RNA extraction, comparing the integrity, quantity and quality of the total RNA extracted from two cultivars of *C. melo* L. var. Inodorus. (Eldorado 300 and Pele de Sapo cultivars) cultivated in Ponta Grossa, Paraná, Brazil. The fruits were harvested in green and ripe stages, cut, immediately frozen in liquid nitrogen and stored at –80 °C. The glassware, porcelain and stainless steel materials were pre-sterilized at 180 °C for 6 h and the plastic materials treated with diethylpyrocarbonate (DEPC) and autoclaved.

The six methods of RNA extraction were as follows:

1) Guanidine thiocyanate/phenol/chloroform: this method is used for animal, plant and bacterial cells. An aliquot (0.1 g) of crushed tissue was treated using the TRIzol® Plus RNA Purification Kit buffer (Invitrogen, Carlsbad/California/USA), according to the manufacturer's recommendations.

2) Sodium azide/β-mercaptoethanol: this method applies to plant tissues rich in polyphenolic compounds and starch. An aliquot (0.1 g) of crushed tissue was used in combination with the Concert Plant RNA Reagent (Invitrogen), according to the manufacturer's recommendations.

3) Phenol/guanidine thiocyanate: a method used for animal, plant and bacterial cells. An aliquot (0.1 g) of triturated tissue was used in combination with the TriPure Isolation Reagent Kit (Sigma-Aldrich, St. Louis/Missouri/USA) buffer, according to the manufacturer's recommendations.

4) Cetyltrimethylammonium bromide (CTAB)/polyvinylpyrrolidone (PVP)/β-mercaptoethanol: method indicated for strawberry tissue (*Fragaria × ananassa*), according to Yu et al. (2012) (3% CTAB, 100 mM Tris-HCl, pH 8.0, 1.4 M NaCl, 20 mM EDTA, 5% PVP e 1% β-mercaptoetanol) were added to a sterile tube containing 700 µl of extraction buffer (PVP and 1% β-mercaptoethanol), homogenized and incubated at 60 °C for 10 min. The sample was centrifuged at 15,700 g for 5 min. The supernatant was collected and an equal volume of chloroform:isoamyl alcohol (24:1) was added, homogenized for 1 min and centrifuged at 15,500 g, at room temperature, for 10 min. The supernatant was again collected, treated with an equal volume of chloroform, and homogenized and centrifuged under the same conditions. The supernatant was transferred to a tube with an equal volume of 4 M LiCl and precipitated at 4 °C for 4 h. The sample was then centrifuged at 15,500 g, at 4 °C, for 15 min, the supernatant washed with 75% ethanol and separated by centrifugation at 6000 g for 5 min. This procedure was repeated and the precipitate was dried in a vacuum pump and then eluted in 30 µl RNase-free MilliQ water.

5) Sodium dodecyl sulfate (SDS)/sodium perchlorate/PVP/β-mercaptoethanol: indicated for grape tissues (*Vitis vinifera* L. cv. Shiraz), as described by Boss et al. (1996), with slight modifications. A 4-g aliquot of tissue was crushed in liquid nitrogen and transferred to a tube with 16 mL of the extraction buffer (0.3 M Tris-HCl, pH 8.3, 2% PEG 4000, 5 M sodium perchlorate, 1% SDS, 8.5% PVP, 1% β-mercaptoethanol), homogenized and incubated at room temperature for 30 min. The solution was transferred to a column containing cotton and centrifuged at 200 g, at 4 °C, for 15 min. The eluted fraction was treated with 2.5 volumes of ethanol at –20 °C, for 40 min, followed by centrifugation at 7700 g, at 4 °C, for 25 min. The precipitate was washed with 70% ethanol, dried under vacuum and resuspended in 1 mL Tris-EDTA (0.1 mM Tris/1 mM EDTA, pH 7.6) and 0.2% (v $v^{-1}$) β-mercaptoethanol. Then, it was washed three times with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) and a volume of chloroform:isoamyl alcohol solution (24:1), followed by centrifugation at 3200 g for 5 min and 12,000 g for 2 min. The RNA was treated with 0.1 volumes of 3 M sodium acetate solution and 2.5 volumes of 95% ethanol and incubated at –20 °C, for 20 min. It was then centrifuged at 12,000 g at 4 °C for 10 min, washed with 1 ml of 70% ethanol and dried under vacuum. The precipitate was resuspended in 100 µL of RNAse-free water.

6) Sarkosyl/PVP/guanidine thiocyanate, in combination with the AxyPrep™ Multisource Total RNA Miniprep Kit (Axygen, Corning/New York/USA). This method is indicated for woody plants with tissues rich in polysaccharides and polyphenolic compounds (MACKENZIE et al., 1997): A 1.1 g aliquot of tissue was crushed in liquid nitrogen and added to 14 mL of extraction buffer containing 4 M guanidine isothiocyanate, 0.2 M sodium acetate, pH 5, 0.25 mM EDTA, 5% (m $v^{-1}$) PVP 40 and 1% (m $v^{-1}$) β-mercaptoethanol. Then, 100 µL of 20% sarkosyl solution was added. The mixture was incubated in a water bath at 65 °C for 5 min, and then added to a 30-mL cotton-containing column. The sample was centrifuged at 156 g at 4 °C for 15 min and the supernatant treated with 2.5 volumes of absolute ethanol at –20 °C for 20 min. The sample was centrifuged at 3220 g, at

4 °C for 20 min, the pellet washed with 500 µl of 70% ethanol, centrifuged at 3220 g for 10 min and resuspended in 500 µl Tris-EDTA buffer, containing 0.2% β-mercaptoethanol. The solution was treated with 500 µL phenol:chloroform:isoamyl alcohol (25:24:1) and centrifuged at 4500 g for 2 min. The liquid phase was again treated and centrifuged at 4500 g for 4 min. The upper layer was removed and treated with 0.1 volume of 3 M sodium acetate, pH 5.2 and 2.5 volumes of absolute ethanol at –20 °C for 12 h. Thereafter, the sample was centrifuged at 7700 g, at 4 °C, for 15 min and the precipitate was dried under vacuum for 1 h and resuspended in 100 µL of RNAse-free water. Finally, the material was purified using the AxyPrep™ Multisource RNA total Miniprep Kit, as per the manufacturer's recommendations.

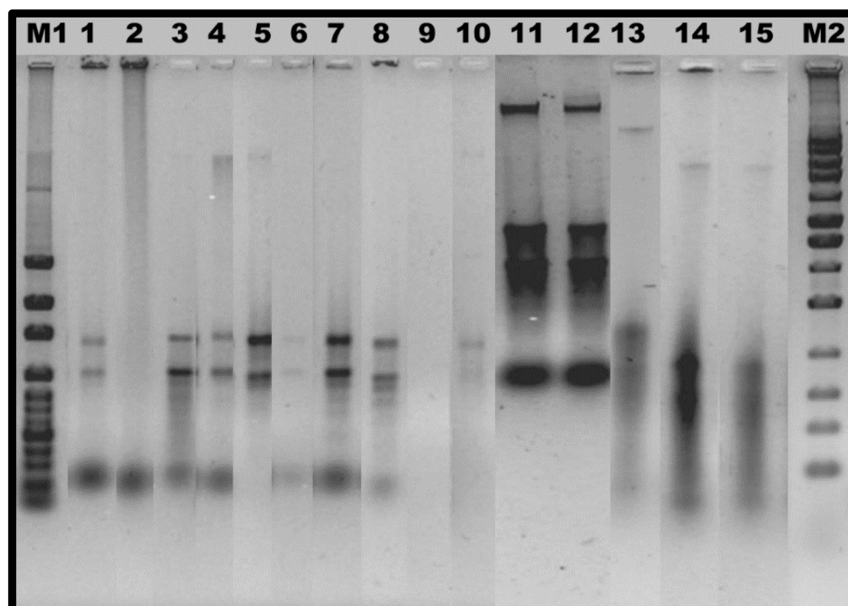
After extraction, the integrity, amount and quality of purified total RNA were analyzed. The integrity was evaluated by electrophoresis in 1.0% agarose gel, run at 60 V for 1 h. Afterward, the agarose gel was stained with ethidium bromide (5 µl $mL^{-1}$) for 20 min, exposed to ultraviolet (UV) light and digitalized by photodocumentation (Vilber Lourmat, Collégien/France). The concentration of the ribonucleic acids was measured by fluorimetry (Qubit, Invitrogen) and the purity of the extracted total RNA was determined by spectrophotometry, through absorbance ratios $A_{260\ nm}/A_{280\ nm}$ and $A_{260\ nm}/A_{230\ nm}$ (NanoVue, GE Healthcare Life Sciences/ São Paulo/Brazil). An $A_{260\ nm}/A_{280\ nm}$ ratio below 2.0, suggests that purified RNA may be contaminated with proteins, phenolic compounds or other contaminants with UV absorption at 280 nm. An $A_{260\ nm}/A_{230\ nm}$ ratio below 2.0, suggests contamination with carbohydrates, phenolic compounds, EDTA and other contaminants with a UV absorption at 230 nm (MANNING, 1991; LOGEMANN et al., 1987). Table 1 shows the results obtained from the total RNA extraction. The extraction efficiency was determined by dividing the mass of total RNA obtained from the extraction (ng) by the mass of the fruit sample (g).

**Table 1.** Quantification of the total RNA extracted from melons with the efficiency calculated from the fresh mass of the sample in (g).

| Method/ Runtime (h) | Cultivar | Stage | Concentration (ng µ$L^{-1}$) | Efficiency (ng RNA $g^{-1}$ fruit) | $A_{260\ nm/280\ nm}$ | $A_{260\ nm/230\ nm}$ | Corresponding sample number in Figure 1 |
|---|---|---|---|---|---|---|---|
| T1) TRIzol (4 h) | Eldorado | Ripe | 140.0 | 28000 | 1.712 | 0.232 | 1 |
| | Pele de Sapo | Ripe | <20.0 | <4000 | 1.644 | 0.329 | 2 |
| T2) Concert Plant (4 h) | Eldorado | Green | 85.2 | 25560 | 2.029 | 4.347 | 3 |
| | | Ripe | 44.8 | 13440 | 2.095 | 0.338 | 4 |
| | Pele de Sapo | Ripe | 83.2 | 24960 | 2.5 | 0.323 | 5 |
| T3) TriPure (4 h) | Eldorado | Green | 78.4 | 15680 | 1.654 | 0.328 | 6 |
| | | Ripe | 130.0 | 26000 | 1.84 | 0.138 | 7 |
| | Pele de Sapo | Ripe | 59.6 | 11920 | 1.784 | 0.282 | 8 |
| T4) Yu et al. (2012) (6 h) | Eldorado | Ripe | <20.0 | <6000 | 1.533 | 0.268 | 9 |
| | Pele de Sapo | Ripe | 7.46 | 2238 | 1.74 | 0.108 | 10 |
| T5) Boss et al. (1996) (8 h) | Eldorado | Green | 184.0 | 4600 | 1.898 | 2.251 | 11 |
| | | Ripe | 133.0 | 3325 | 1.994 | 1.697 | 12 |
| T6) MacKenzie et al. (1997) (13 h) | Eldorado | Green | 584.0 | 37163 | 2.071 | 2.012 | 13 |
| | | Ripe | 110.0 | 7000 | 2.165 | 2.115 | 14 |
| | Pele de Sapo | Ripe | 79.8 | 5078.2 | 1.947 | 0.847 | 15 |

The best method for extracting melon RNA was the T5 method, using SDS/sodium perchlorate/PVP/β-mercaptoethanol. The presence of sodium perchlorate was shown to be important in removing SDS and complexing with contaminating proteins (WILCOCKSON, 1973), improving the purity of the extracted RNA. The quality of the extracted material can be observed in Figure 1 (gel bands corresponding to samples 14 and 15), where the bands corresponding to the 18S and 28S RNA are evident. The T3 isolation method, based on phenol/guanidine thiocyanate, also showed good results. The presence of phenol, a denaturing organic solvent, separated the RNA from the contaminating proteins. However, the presence of phenol in the composition can damage the poly-A tail of the extracted mRNA (AZEVEDO et al., 2003), being a possible cause of low $A_{260\ nm}/A_{230\ nm}$ ratio (Table 1), impairing the use of this material in molecular techniques, such as RT-PCR.

**Figure 1.** Evaluation by agarose (1%) gel electrophoresis of the integrity of total RNA extracted from Eldorado (ELD) and Pele de Sapo (PS) melons at the mature (M) and green (V) developmental stages, using six methods of extraction. Sample no: 1) TRIzol, ELD-M; 2) TRIzol, PS-M; 3) Concert Plant, ELD-V; 4) Concert Plant, ELD-M; 5) Concert Plant, PS-M; 6) TriPure, ELD-V; 7) TriPure, ELD-M; 8) TriPure, PS-M; 9) Yu et al. (2012), ELD-M; 10) Yu et al. (2012), PS-M; 11) Boss et al. (1996), ELD-V; 12) Boss et al. (1996), ELD-M; 13) Mackenzie et al. (1997), ELD-V; 14) Mackenzie et al. (1997), ELD-M; 15) Mackenzie et al. (1997), PS-M; M1, molecular weight marker of 100 bp, and M2, molecular weight marker of 1 Kb (Axygen, Corning/New York/USA).




The T4 method resulted in a low RNA concentration and a low efficiency (Figure 1, gel bands corresponding to samples 9 and 10). The T6 method, which involved the AxyPrep™ Multisource Total RNA Miniprep Kit, showed a moderate total RNA extraction efficiency (between 7000−3700 ng). However, ribonucleic acids were degraded (Figure 1, gel bands corresponding to samples 11, 12 and 13). This is among the most widely used methods for RNA extraction, due to its simplicity and efficiency (RAMCHANDRA; STURM, 2010; CORBACHO et al., 2013; CHEN et al., 2016; ZHANG et al., 2016). However, in our study, the extracted material presented a low $A_{260\ nm}/A_{230\ nm}$ ratio, indicating the presence of contaminating polysaccharides or polyphenols in both the green and mature fruits. Another critical factor is the high cost of the reagent, which makes RNA extraction

from large samples very expensive (DINKINS et al., 2010).

Regarding the efficiency, the best protocols were T2, T3 and T6 in the green stage of the Eldorado cultivar but the T2 and T3 methods resulted in very low $A_{260\ nm}/A_{230\ nm}$ ratios (Table 1). The best absorbance ratios were obtained with the T5 and T6 methods. However, when comparing the electrophoresis gel results, the T5 method was the best because it did not present degraded RNA (Figure 1, gel bands corresponding to samples 14 and 15), unlike the T6 method (Figure 1, gel bands corresponding to samples 11, 12 and 13).

Thus, the T5 method, originally designed for RNA extraction from grapes, is also indicated for the extraction of total RNA from *C. melo* L., at both stages of maturation, using a large sample of fruit (4 g) and generating an acceptable yield of high-quality RNA. The method used by Boss et al. (1996) was slightly modified, regarding incubation time (from 20 min to 40 min) and centrifugation intensity (from 7700 g/25 min to 3220 g/15 min), during the ethanol precipitation step.

Considering the maturation stages of the melons, the green stages had the highest concentrations of total RNA (ng $\mu L^{-1}$), probably because the immature fruits have lower amounts of sugars and water (NUNEZ-PALENIUS et al., 2008). Hence, the results show that it is possible to purify high-quality RNA from melon fruits. Also, because of the high quality and large amount of purified RNA, the T5 method can be used in molecular studies of gene expression, such as RT-PCR, microarrays, and preparation of cDNA and RNA-seq data. Thus, the following conclusions can be drawn from this study:

1. In general, higher amounts of RNA were obtained from green (immature) fruits. The lower content of polysaccharides and water in these fruits compared to the mature fruits, may have favored the efficiency of RNA purification from the immature melons.

2. The reduced number of steps required for RNA extraction using commercial kits, may be responsible for their increased integrity.

3. The best method for extracting total RNA from *C. melo* in the green and mature stage was SDS/PVP/β-mercaptoethanol (BOSS et al., 1996), which produced total RNA of good integrity and in acceptable quantity, as tested by RNA-seq analyzes (data not shown).

## Acknowledgements

The authors express their gratitude to the CNPq, Capes and Araucária Foundation of the State of Paraná for their financial support.

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