

# Factors Affecting the Extraction of Intact Ribonucleic Acid from Plant Tissues Containing Interfering Phenolic Compounds

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## ABSTRACT

Using conventional methods it is impossible to extract RNA as uncomplexed intact molecules from the leaves of grapevines (*Vitis vinifera* L.) and from a number of woody perennial species that contain high levels of reactive phenolic compounds. A procedure involving the use of high concentrations of the chaotropic agent sodium perchlorate prevents the binding of phenolic compounds to RNA during extraction. Analyses of the phenolics present in plant tissues used in these experiments indicate that there is a poor correlation between the total phenolic content and the complexing of RNA. However, qualitative analyses suggest that proanthocyanidins are involved in the tanning of RNA during conventional extractions.

Many higher plants contain reactive phenolic compounds which form chemical bonds with both proteins and nucleic acids when tissues are homogenized. These reactions have been most closely studied with proteins because the complexing of phenolic compounds to enzymes usually leads to lowered enzyme activities. Loomis (9) has reviewed the problems of phenolic and quinone interactions with plant enzymes and lists many methods which help to overcome them. The techniques used for proteins are often not effective in preventing the formation of nucleic acid-phenolic complexes, and because of this studies of plant nucleic acids have been largely restricted to a relatively few herbaceous higher plants along with a number of algae and fungi. Many of the plants from which it is difficult to extract RNA are of economic importance, such as grapevines, avocados, bananas, and hops, and nucleic acid investigations are required for studies of their virus and viroid status.

This paper describes a modified procedure by which RNA can be extracted from plant tissues containing high concentrations of reactive phenolic compounds. Leaf material of the grapevine (*Vitis vinifera* L.) was mainly used in this study but observations were also made on a range of other woody and herbaceous plants.

## MATERIALS AND METHODS

**Plant Material.** Grapevines (*V. vinifera* L. cv. Cabernet sauvignon) were grown in a glasshouse. Spinach (*Spinacia oleracea* L.) plants were grown in liquid culture (11). Other plants were grown either in the field or in a glasshouse.

**Modified RNA Extraction Procedure.** Sixteen ml of extraction medium (5 M sodium perchlorate, 0.4 M tris, 5% [w/v] SDS, 0.05 M NaCl, 0.05 M disodium EDTA, and 8.5% [w/v] insoluble PVP) were added to 0.8 g of tissue which was homogenized using a Polytron blender. All steps were carried out at 0 to 4 C. The homogenate was centrifuged at 30,000g for 10 min in a swingout rotor (Beckman SW 27) and the

supernatant was removed from between the small pellet and a large raft formed by undissolved SDS and PVP, mixed with 2 volumes of ethanol, and stored at -10 C for 1 hr. The contaminated RNA precipitate was pelleted at 5,000g for 5 min and the pellet resuspended in 6 ml of aqueous phase containing 8.5% [w/v] insoluble PVP, and 6 ml of 50/50 phenol phase-chloroform (aqueous and phenol phases as described by Loening and Ingle [8]). The mixture was shaken vigorously and centrifuged at 10,000g for 10 min, after which the aqueous phase was removed by aspiration and re-extracted with phenol-chloroform. The RNA was then precipitated from the aqueous phase using ethanol, dissolved in 0.15 M sodium acetate-0.5% [w/v] SDS, reprecipitated with ethanol, and finally washed twice with 80% ethanol containing 0.5% [w/v] SDS. The RNA was then resuspended and fractionated in polyacrylamide gels or was further purified by passage through a Sephadex G-100 column.

**Gel Electrophoresis.** RNA was fractionated in 2.4% [w/v] polyacrylamide gels in the presence of 1 mM disodium EDTA following the methods of Loening (7). Electrophoresis was a 5 mamp/gel and unless otherwise stated was for 2 hr. RNA concentrations were estimated by comparison of the absorbance at 260 nm with yeast RNA standard. Gels were scanned at 260 nm using a Joyce Loeb Chromoscan.

**Extraction of Total Phenolics.** Samples (0.5 g) of plant tissue were homogenized in 15 ml of 50% [v/v] aqueous methanol. Fifteen ml of *n*-hexane were added and the mixture was shaken vigorously for 30 min. The samples were then centrifuged and the methanolic phase was removed and its UV absorbance profile measured.

**Proanthocyanidin Extraction and Identification.** The extraction followed the basic method of Bate-Smith (1) and Bate-Smith and Lerner (2). Samples of tissue (1 g) were homogenized with 6 ml of 2 N HCl and the homogenate was heated in a boiling water bath for 20 min. The samples were cooled and centrifuged at 3,000g for 5 min and 1 ml of the supernatant was thoroughly mixed with 0.5 ml of isoamyl alcohol. When the two phases separated, the color of the upper isoamyl alcohol layer was noted. Samples of the colored isoamyl alcohol were spotted onto thin layer plates (Merck DC-Plastikrolle cellulose) and fractionated using Forestal solvent (30:3:10, glacial acetic acid-concentrated HCl-water) along with authentic cyanidin (kindly supplied by Dr. T. C. Somers).

## RESULTS

**Conventional RNA Extractions.** When grapevine leaves were extracted by the widely used method B of Loening and Ingle (8), the RNA produced was grossly contaminated by interfering substances when compared with spinach leaf RNA extracted by the same procedure (Fig. 1). This method employs a phenol denaturation of proteins followed by alcohol precipitations of RNA. A large number of adjustments were made to this basic technique (including the use of insoluble PVP, 2-mercaptoetha-

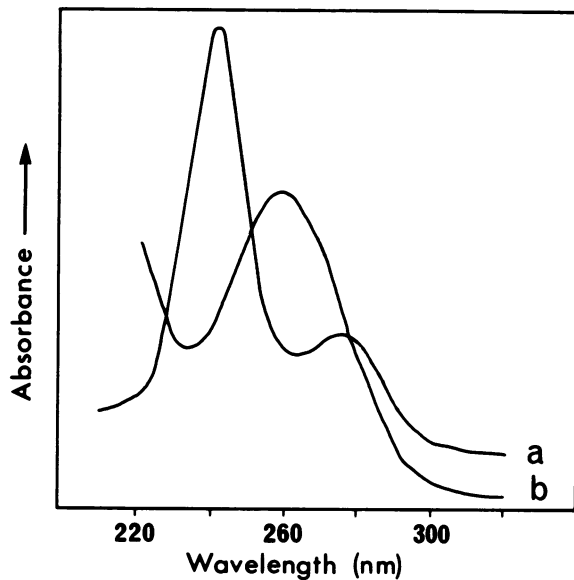


FIG. 1. Absorbance profiles of samples extracted by the Loening and Ingle (8) method from grapevine leaves (a) and spinach leaves (b) and resuspended in tris-phosphate-EDTA buffer (7).

nol, sodium diethyldithiocarbamate, cysteine, and DTT) but none significantly improved the purity of the grapevine RNA extracted. Samples of the grapevine leaf RNA could not be successfully fractionated in 2.4% [w/v] polyacrylamide gels because the RNA was complexed to phenolic material. Several other RNA extraction procedures were applied to grapevine leaves including the high pH method of Tanne *et al.* (15), the dimethylsulfoxide method of Gusta and Runeckles (3), the protease treatment of Randles (10), and the diethylpyrocarbonate procedure of Solymosy *et al.* (14). None of these techniques successfully prevented the complexing of RNA to phenolic material.

**RNA Extraction Using a Chaotropic Agent.** The only methods used which extracted grapevine leaf RNA that was not complexed to phenolics were those that employed chaotropic agents, such as sodium perchlorate. The method of Wilcockson (16) produced RNA which was heavily contaminated with phenolic compounds (Fig. 2a). However, this RNA could be separated in 2.4% [w/v] polyacrylamide gels into various ribosomal and soluble RNA fractions, although phenolic contamination was still observed in gel UV absorbance ( $E_{260}$ ) traces (Fig. 3a). A large proportion of the free phenolic compounds contaminating the sample was removed by using insoluble PVP (9) and a further quantity by re-extracting the sample with phenol-chloroform (see under "Materials and Methods" for full procedure). Using this method, intact RNA could be extracted and contaminating phenolic material (Fig. 2b) was not observed in 2.4% [w/v] polyacrylamide gel scans (Fig. 3b). The remaining phenolic material could be removed by passage of the RNA through a Sephadex G-100 column (Fig. 2c).

**Quantitative and Qualitative Yield of Modified Procedure.**

With spinach leaves the yield of RNA extracted by the modified procedure (without Sephadex fractionation) was approximately 45% of that extracted by the Loening and Ingle (8) method, and 24% of that extracted by the Smillie and Krotkov (13) procedure. It is suggested that the lower yield may be caused by the loss of RNA trapped in the large insoluble PVP/SDS rafts although it is possible that some RNA may be lost because of incomplete deproteinization during homogenization in the perchlorate medium. However, when the temperature of homogenization was raised from ice-cold to 20 C to increase the amount of SDS in solution, the yields of RNA were not increased.

RNA was extracted by the modified procedure and by the Loening and Ingle (8) method from young leaves of four plants which do not contain high levels of interfering phenolic compounds (*Spinacia oleracea*, *Citrullus lanatus*, *Chrysanthemum indica*, and *Chenopodium quinoa*) and the samples were fractionated in 2.4% (w/v) polyacrylamide gels. No qualitative differences could be found between the RNA samples extracted by the two methods in their relative proportions of chloroplast and cytoplasmic rRNA and in the 4S and 5S fraction.

The new method produces RNA which can be fractionated in 2.4% [w/v] polyacrylamide gels from leaves or fruits of a range of plants which do not yield pure RNA following the Loening and Ingle (8) extraction (*e.g.* *Eucalyptus sp.*, *Humulus lupulus*, *Acacia pycnantha* and young *Persea americana* leaves and the fruit peel of *Musa sapientum*). See Figure 4 for a selection of gel traces and Table I for trivial plant names.

**Phenolic Compounds That Interfere with Conventional RNA Extractions.** A series of experiments was carried out in order to identify the phenolic compounds that cause problems during the extraction of RNA from certain plant tissues. Ten plant tissues were tested for the effectiveness of RNA extraction by the Loening and Ingle (8) method and their total phenolic content was estimated. No correlation existed between the ease of RNA extraction and the total phenolic contents of these tissues, suggesting that specific phenolic compounds might be involved in complexing with RNA. One possible group of complexing agents is the "condensed tannins" which are polymeric polyhydroxyflavonols. These compounds are proanthocyanidins (4) which, when heated with dilute HCl, produce anthocyanidins.

Table I shows the results of proanthocyanidin tests performed on 14 plant tissues. The anthocyanidin compounds were identified by comparison of their mobilities on TLC plates with authentic cyanidin. There is good correlation between the formation of a deep crimson anthocyanidin solution and the ineffectiveness of the Loening and Ingle (8) method to extract uncomplexed RNA. Tissues which yielded only a pink-red or pale gray-green color could be successfully extracted by the Loening and Ingle (8) method to yield uncontaminated RNA,

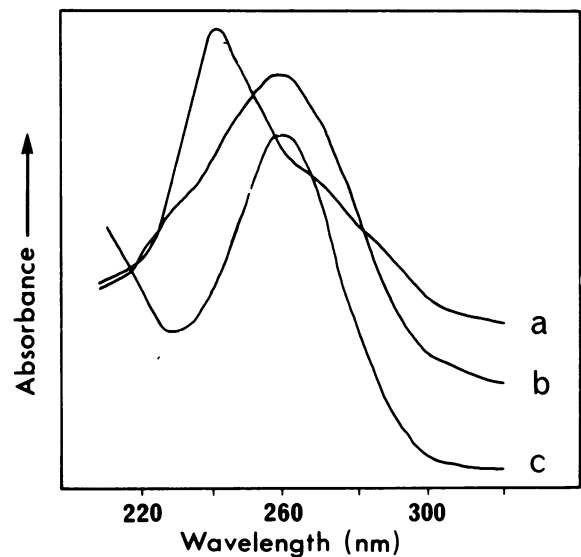


FIG. 2. Absorbance profiles of RNA samples extracted (a) by the Wilcockson (16) method; (b) by the procedure described under "Materials and Methods" without Sephadex gel filtration; and (c) as (b) but with further purification by passage of RNA through a Sephadex G-100 column. All three RNA samples were resuspended in tris-phosphate-EDTA buffer (7).

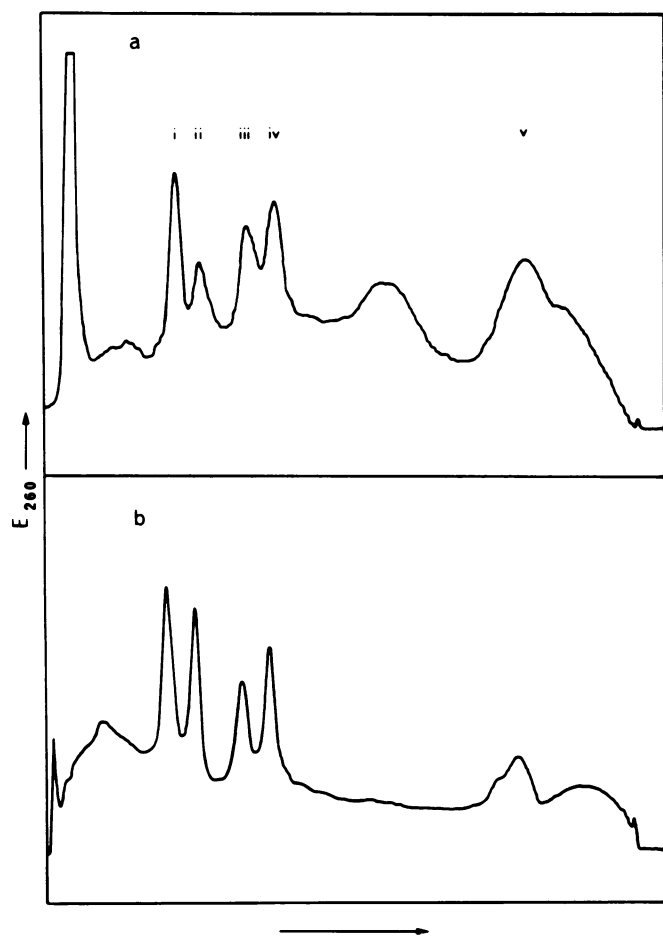


FIG. 3. Absorbance profiles ( $E_{260}$ ) of 2.4% (w/v) polyacrylamide gels. Samples were grapevine leaf RNA (a) extracted by the method of Wilcockson (16) and (b) extracted as under "Materials and Methods" (without Sephadex gel filtration). RNA species (i)  $1.3 \times 10^6$ ; (ii)  $1.1 \times 10^6$ ; (iii)  $0.7 \times 10^6$ ; (iv)  $0.56 \times 10^6$  mol wt ribosomal RNAs; and (v) 4S and 5S RNA. Trace (a) also shows a fraction of phenolic material in the center of the gel.

whereas tissues yielding deep crimson colors were not readily extractable by this procedure.

Samples of extracted RNA were also tested for the presence of proanthocyanidins. Grapevine leaf RNA extracted by the method of Loening and Ingle (8) produced a deep crimson solution and fractionation of this sample on TLC plates indicated the presence of both cyanidin and delphinidin. Some of the procyanidin and prodelphinidin present in grapevine leaves is carried through this RNA extraction procedure, almost certainly as condensed tannins. When viewed under UV light the TLC plate of the grapevine leaf RNA sample had a fluorescent spot corresponding to one of two spots noted in grapevine leaf sample plates. This was possibly a cinnamic acid derivative. Grapevine leaf RNA samples extracted by the new method did not contain proanthocyanidins.

### DISCUSSION

The procedure described here allows extraction of uncontaminated, intact RNA from a number of plant tissues that do not yield pure RNA with any of the previously described extraction methods. Our results suggest that proanthocyanidins, when present in tissues in high concentrations, complex with RNA following homogenization in the absence of chaotropic agents. The proanthocyanidins are almost certainly condensed tannins (4) and are common in woody plants but not normally found in

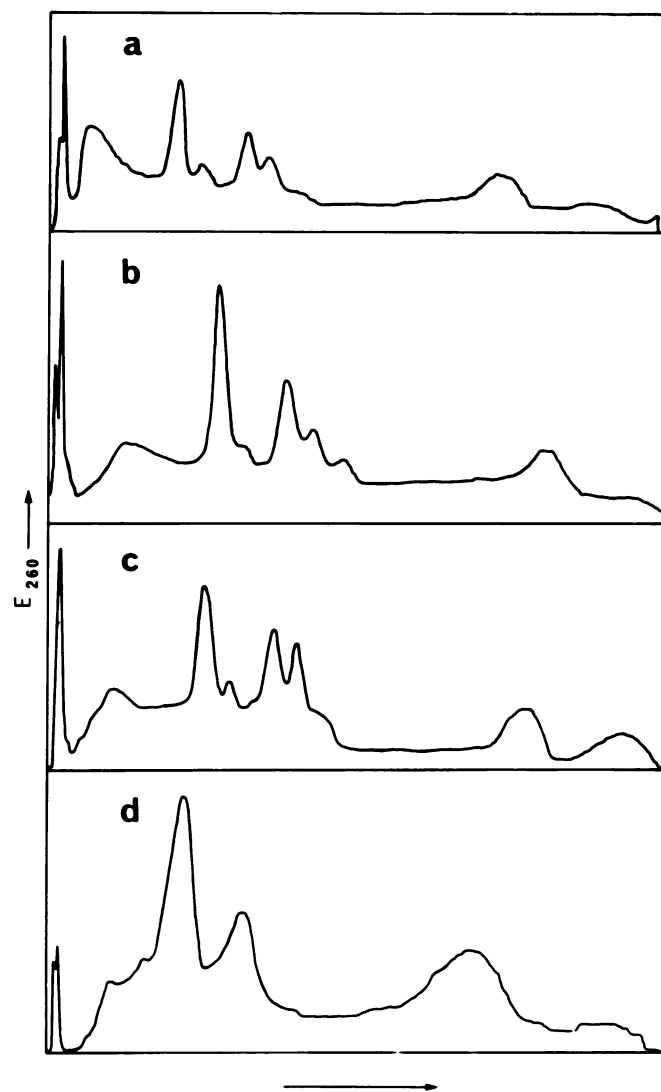


FIG. 4. Absorbance profiles ( $E_{260}$ ) of 2.4% (w/v) polyacrylamide gels. RNA was extracted as under "Materials and Methods" (without Sephadex gel filtration) from (a) *Humulus* leaves; (b) eucalyptus leaves; (c) acacia leaves; and (d) banana fruit peel. Electrophoresis was carried out for various periods and hence the mobilities of fractions are not necessarily comparable between gels.

herbaceous species (1). Our results do not preclude the possibility that other phenolic species may also be involved in complexing with nucleic acids.

Perchlorate and thiocyanate ions are known to be highly chaotropic (5, 12). In RNA extractions from grapevine leaves sodium thiocyanate could be substituted for sodium perchlorate whereas sodium sulfate, which is not chaotropic, was ineffective. The action of chaotropic agents is thought to be related to their effect on the structure and lipophilicity of water which, in turn, induces changes in the properties of macromolecules. Chaotropic agents are known to effect the dissociation and solubilization of antigen-antibody complexes, the denaturation of proteins, and the depolymerization of protein polymers (5, 6). It is suggested that during the early stages of the extraction procedure described here, phenolic compounds are prevented from binding to nucleic acids by the action of chaotropic agents.

This RNA extraction procedure is now being applied to various grapevine stocks in an effort to isolate certain viral and viroid RNAs.

TABLE I

Tissue	RNA extraction by Loening & Ingle method	Colouration following proanthocyan- idin test	Anthocyanidins identified
<u>Eucalyptus torquata</u> X <u>woodwardii</u> leaf	-	Deep crimson	Cyan. Delph.
<u>Acacia pycnantha</u> (Wattle) leaf	-	" "	Cyan. Delph.
<u>Persea americana</u> (avocado) young leaf	-	" "	Cyan.
<u>Cocos nucifera</u> (coconut) frond	<sup>1</sup> +	" "	Cyan. Rosin.(?)
<u>Vitis vinifera</u> (grape- vine) leaf	-	" "	Cyan. Delph.
<u>Humulus lupulus</u> (hops) leaf	-	" "	Cyan. Delph.
<u>Musa sapientum</u> (banana) fruit peel	-	Red	Cyan.
<u>Camellia sinensis</u> (tea) leaf	+	Brown/red	N.R.
<u>Persea americana</u> (avocado) mature leaf	+	Pink/red	Cyan.
<u>Malus sylvestris</u> (apple) var. Granny Smith fruit peel	<sup>2</sup> +	" "	Cyan.
<u>Lycopersicon esculentum</u> (tomato) leaf	+	Brown	-
<u>Citrullus lanatus</u> (watermelon) leaf	+	Light grey/ green	-
<u>Chenopodium quinoa</u> leaf	+	" " "	-
<u>Chrysanthemum indica</u> leaf	+	" " "	-
<u>Spinacia oleracea</u> (spinach) leaf	+	" " "	-

RNA extraction by the Loening and Ingle (ref.8) method is noted as possible (+) or not possible (-), depending upon whether ribosomal RNA species could be separated into distinct fractions in 2.4% (w/v) polyacrylamide gels. Anthocyanidins were identified by comparing their mobilities with authentic cyanidin on thin-layer plates using Forestal solvent. Cyan. = cyanidin; Delph. = delphinidin; Rosin. = rosinidin; N.R. = not run.

<sup>1</sup> The ratio of the UV absorbances of the rRNA species to the 4S and 5S fraction suggested that a great deal of the rRNA was lost during extraction.

<sup>2</sup> A large amount of UV-absorbing material remained at the top of the gel.

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#### LITERATURE CITED

- BATE-SMITH EC 1953 Colour reactions of flowers attributed to (a) flavonols and (b) carotenoid oxides. *J Exp Bot* 4: 1-9
- BATE-SMITH EC, NH LERNER 1954 Leucoanthocyanins. 2. Systematic distribution of leucoanthocyanins in leaves. *Biochem J* 58: 126-132
- GUSTA LV, VC RUNBECKLES 1972 The isolation of nucleic acid from woody tissue using dimethyl sulfoxide. *Can J Bot* 50: 1085-1090
- HASLAM E 1975 Natural proanthocyanidins. In JB Harborne, TJ Mabry, H Mabry, eds, *The Flavonoids*. Chapman and Hall, London pp 505-559
- HATEFI Y, WG HANSTEIN 1969 Solubilisation of particulate proteins and non-electrolytes by chaotropic agents. *Proc Nat Acad Sci USA* 62: 1129-1136

6. HIPPEL PH, K-Y, WONG 1964 Neutral salts: the generality of their effects on the stability of macromolecular conformations. *Science* 145: 577-580
7. LOENING UE 1968 The fractionation of high molecular weight RNA. *In* I Smith, ed, *Chromatographic and Electrophoretic Techniques*, Vol. 2. Pitman Press, Bath pp 437-442
8. LOENING UE, J INGLE 1967 Diversity of RNA components in green plant tissues. *Nature* 215: 363-367
9. LOOMIS WD 1974 Overcoming problems of phenolics and quinones in the isolation of plant enzymes and organelles. *Methods Enzymol* 31: 528-544
10. RANGLES JW 1975 Association of two ribonucleic acid species with Cadang-Cadang disease of coconut palm. *Phytopathology* 65: 163-167
11. SAURER W, JV POSSINGHAM 1970 Studies on the growth of spinach leaves (*Spinacia oleracea*). *J Exp Bot* 21: 151-158
12. SAWYER WH, J PUCKRIDGE 1973 The dissociation of proteins by chaotropic salts. *J Biol Chem* 248: 8429-8433
13. SMILLIE RM, G KROTKOV 1960 The estimation of nucleic acids in some algae and higher plants. *Can J Bot* 38: 31-49
14. SOLMOSY F, G LAZAR, G BAGI 1970 An improved version of the diethyl pyrocarbonate method for extracting ribosomal ribonucleic acids. *Anal Biochem* 38: 40-45
15. TANNE E, I SELA, I HARPAZ 1974 Transmission of grapevine leafroll virus to herbaceous plants. *Phytopathol Z* 80: 176-180
16. WILCOCKSON J 1973 The use of sodium perchlorate in deproteinisation during the preparation of nucleic acids. *Biochem J* 135: 559-561