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A DNA extraction protocol for Bois de ronde (*Erythroxylum sideroxyloides*)

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Abstract

Four DNA extraction protocols were compared for Bois de ronde *(Erythroxylum sideroxyloides)*, a medicinal plant endemic to Mauritius and Réunion Island. Preliminary tests by spectrophotometry and Agarose Gel Electrophoresis (AGE) showed that yield and purity of the DNA were unsatisfactory using the four protocols. Subsequent modifications of the Križman *et al.* (2006) protocol and the CTAB protocol allowed RAPD-PCR DNA amplification and digestion. Results using these protocols showed heavy contamination of the DNA with polysaccharides and polyphenols.

An optimized protocol based on that of Križman *et al.* (2006), Murray and Thompson (1980), and Saghai-Maroof *et al.* (1984) was devised. The yield of DNA obtained was 29.2µg/g of leaf material.The $A_{260/280}$ and $A_{260/230}$ ratios, 2.33 and 2.69 respectively, indicated that the quality of DNA obtained was good.

Keywords: Genomic DNA extraction, *Erythroxylum sideroxyloides*, Bois de ronde, Erythroxylaceae, activated charcoal, medicinal.

**For correspondences and reprints*

Abbreviations
DNA, Deoxyribonucleic Acid; **dNTP**, deoxyribonucleotide Triphosphate; ***Eco*RI**, *Escherichia coli* RI endonuclease; **g,** gram; **M**, Molar; **RAPD**, Random Amplified Polymorphic DNA; **RNA**, Ribonucleic Acid; **RNAse**, Ribonuclease; **Taq**, *Thermus aquaticus;* **w/v**, weight by volume; **v/v**, volume by volume.

1. INTRODUCTION

Bois de ronde *(Erythroxylum sideroxyloides)*, member of the Erythroxylaceae family (or coca family), is a species for which no optimized DNA extraction protocol has been worked out to date. It is a medicinal shrub that may reach a height of up to 4m and is endemic to Mauritius and Réunion Island. This Erythroxylum species has elliptical leaves, being dark green above and yellow green underneath. *E. sideroxyloides* is not of a threatened status, and can be found in the drier areas surrounding the Central Plateau, such as the Corps de Gardes and Bambous Mountain in Mauritius. (Gurib-Fakim & Brenler, 2004).

Due to the growing interest of modern medicine for ethno-medicine, an optimized DNA extraction protocol for *E. sideroxyloides* might be of potential importance, especially for molecular studies. According to a study done by Gurib-Fakim and coworkers in 2005, *E. sideroxyloides* is very interesting in terms of biological activity, being of 'anti-infective potential'. Decoctions of this plant's leaves and stem can be used against fever, as a diuretic (Gurib-Fakim *et al.*, 2005) and against renal stones (Gurib-Fakim, 2004).

According to Soobrattee *et al.* (2008), Mauritian endemic plants contain significant levels of phenolics, and *E. sideroxyloides* is one of them. Furthermore, being part of the Medicinal and Aromatic Plants (MAPS), the shrub is also expected to contain exceptionally high levels of polysaccharides, hydrocolloids and many other secondary metabolites, which may interfere in DNA isolation (Padmalatha and Prasad, 2006). Preliminary experiments, revealed high polysaccharide content and confirmed the presence of polyphenols, which are two main DNA contaminants. Polyphenolics are capable of permanently binding to DNA, giving it a brown color upon oxidation and rendering it useless for most molecular research (Katterman and Shattuck, 1983). These compounds are prevented from binding to DNA by the addition of antioxidants, of which some examples can be ascorbic acid, citric acid, sodium sulphite, β-mercaptoethanol and BSA (Dawson and Magee, 1995; Clark, 1997). Polyvinyl Pyrrolidone (PVP) and activated charcoal can also be used as they adsorb polyphenolics (Križman *et al.,* 2006). Polysaccharides can be tackled either by destarching (Puchooa, 2004) or by using salts, such as sodium chloride at high concentrations to prevent co-precipitation with DNA (Ribeiro and Lovato, 2007). According to Murray and Thompson (1980), NaCl concentrations above 0.5M favor CTAB complex formation with polysaccharides, which then precipitate out. A salt concentration of up to 6M of NaCl has been used by Aljanabi and co-workers (Aljanabi *et al.*, 1999*)*.

Genomic DNA is often required in reasonable amounts and purity for molecular studies (Cheng *et al.*, 2003) and its isolation is often one of the most tedious parts preceding further analysis of the genetic material (Puchooa, 2004). In fact, the relative purity and amount of DNA depend upon the type of studies to be done. For example, DNA required for PCR amplification may be as little as that from a single cell (Das, 2005), whereas DNA of high molecular mass and purity is required for the construction of genomic DNA libraries (Clark, 1997).

Four different protocols were used to extract the genomic DNA of the 'Bois de ronde' in an attempt to identify the most promising one. The procedure was then optimized for *E. sideroxyloides* to target polysaccharides and phenolics more specifically, as they can be a major problem during DNA purification (Aljanabi *et al.*, 1999).

The use of the DNA obtained from the optimized DNA extraction protocol could then be used for obtaining the genetic fingerprint of the Bois de ronde species, adding up to the pool of information about potentially exploitable genetic resources found in Mauritius. The plant's genetic fingerprint can then be used for identification purposes. This information will be very handy in the preservation of Intellectual Property and the endemic status of the species to both Mauritius and Réunion Island. It could also aid in bioprospection purposes and help prevent cases of biopiracy.

2. MATERIALS & METHODS

2.1. Plant materials

Leaves were obtained from an *Erythroxylum sideroxyloides* shrub growing in the yard of the National Parks & Conservation Services (NPCS) at Curepipe. Uninfected leaves of the Bois de ronde species were chosen and kept in the dark (inside a dark plastic bag) and were transported, in-between moist towels, to the laboratory, washed and stored at -40°C.

2.2. DNA Extraction

Four DNA extraction protocols were chosen from published protocols, namely the Dellaporta (Wood and Hicks, 1983), CTAB (Clark, 1997), Križman *et al.* (2006) and the Deshmukh *et al.* (2007) to isolate *E. sideroxyloides* DNA. However, given the high levels of contamination for the unmodified protocols, these protocols were amended to obtain workable DNA. Anomalous phase separation and absence of DNA pelleting for the unmodified Dellaporta (Woods and Hicks, 1983) protocol suggested contamination, which was investigated by spectrophotometric analysis and HPLC. Finally, the CTAB (Clark, 1997) and the Križman *et al.* (2006) protocols were combined to produce an optimized DNA extraction protocol for *E. sideroxyloides*, which is described below.

2.3. Optimized DNA extraction protocol

1. Finely homogenize 300 mg of frozen fresh plant tissue with a mortar and pestle using liquid nitrogen followed by transferring of the powder to 9 mL of extraction buffer in a 15 mL corning tube.
2. After incubating at 55°C for 30 minutes with frequent agitation, cool the tubes to room temperature and dispense the contents equally to 6 microcentrifuge tubes (1.5 mL each).
3. Centrifuge the tubes at 13,000 rpm for 20minutes at room temperature and transfer the supernatant from each tube to two new tubes.
4. Add 1 volume of chloroform: isoamyl alcohol (4% IAA) to the each tube and vortex thoroughly.

5. Centrifuge the tubes at 13,000 rpm for 20minutes at room temperature and transfer the aqueous phase to new tubes.
6. Repeat steps 4 and 5.
7. Pool up collected supernatant to a 15 mL corning. Add sodium acetate (pH 5.2) to reach a concentration of 0.2M with respect to the collected supernatant, followed by addition of 1 volume of isopropanol and mixing of the contents by gentle inversion.
8. Leave the corning overnight at -20°C for precipitation.
9. Centrifuge the tube at 3000 rpm for 20 minutes and discard the supernatant before washing the pellet with 5 mL 76% ethanol.
10. After spinning the tube at 3000 rpm for 20 minutes, discard the supernatant and wash the pellet with 5 mL 70% ethanol.
11. Discard the supernatant, and then air-dry the pellet, before dissolving it in 200 µL of sterile distilled water.
12. Leave the DNA pellet to dissolve overnight at 4°C before treating with RNAse**.**
13. Store the DNA solution for short term at 4°C or at -20°C for long term storage.

2.3.1. Chemicals used for the optimized DNA extraction protocol.

- **Extraction buffer:** Tris-HCl pH8 (100mM), NaCl (2M), EDTA pH 8 (20mM), CTAB (2% w/v), 2% w/v PVP (Molecular weight 10, 000), activated charcoal (0.5% w/v), 20mM β-mercaptoethanol.
- Isopropanol, ethanol (70% and 76%) and isoamyl alcohol (4% v/v) in chloroform.
- 0.2M Sodium acetate
- RNAse.

2.4. Restriction digestion

1) Add 0.1- 4 µg of DNA to 2 µL of 10X of *Eco*RI restriction buffer.
2) Adjust the reaction volume to 18 µL with water.
3) Add *Eco*RI enzyme (1 to 5U per µg of DNA) and incubate the mixture for 1½ hours.
4) Stop the reaction by adding 3µL of gel loading buffer to 7µL of digest prior to loading on a 1.5% agarose gel.

2.5. RAPD-PCR parameters

Table 1: Reaction parameters of the RAPD-PCR.

Components	Concentrations		Required volumes
	Stock	**Required**	**1 tube**
Buffer	10X	1X	2.5 µL
$MgCl_2$	50 mM	1.5 mM	2 µL
dNTPs	2.5 mM	0.2 mM	2 µL
Water	-	-	15.05µL
Primer	10 µM	0.5 µM	1.25 µL
Taq polymerase	2 U	0.016 U	0.2 µL
DNA	25 ng/µL	50 ng	2 µL
Final volume	-	-	25 µL
Denaturation	94 ˚C for 30 seconds		
Primer annealing	35 ˚C for 30 seconds		
Primer extension	72 ˚C for 2 minutes		
Number of cycles	38 cycles		

Apparatus used: Spectrophotometer (Spectronic Genesys 5; Milton Roy Spectronic 1201); PCR Thermocycler (Peltier Thermal Cycler PTC 100®); HPLC system (Hewlett Packard™).

3. RESULTS

	Modified Dellaporta protocol	Modified Križman *et al.* (2006) protocol	Modified CTAB protocol	Optimized protocol
DNA yield per gram of leaf	105.83 µg	76.67 µg	49 µg	29.17 µg
A_{260}/A_{280}	1.34	1.15	0.73	2.33
A_{260}/A_{230}	0.65	1.92	1.44	2.69

Table 2: Yield and purity of genomic DNA from modified protocols after RNAse treatment.

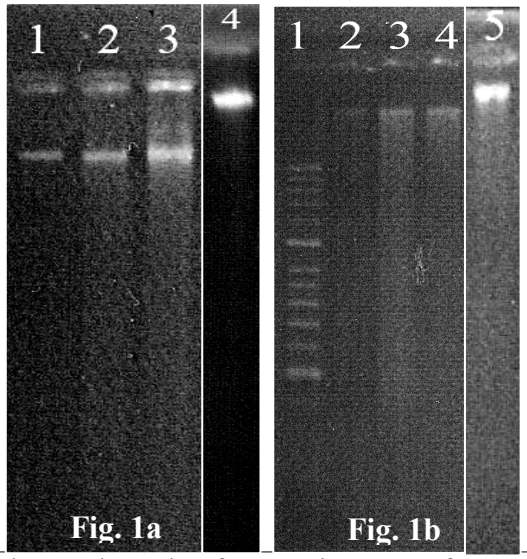

Figure 1 a. Gel electrophoresis of genomic DNA after RNA elimination. **Lane: 1.** Modified Dellaporta protocol; **2.** Modified Križman *et al.* (2006) protocol; **3.** Modified CTAB protocol; **4.** Optimized protocol. **Figure 1 b.** Agarose gel electrophoresis of EcoRI-digested genomic DNA. **Lane: 1.** Hyperladder II; **2.** Modified Dellaporta protocol; **3.** Modified Križman *et al.* (2006) protocol; **4.** Modified CTAB protocol; **5.** Optimized protocol.

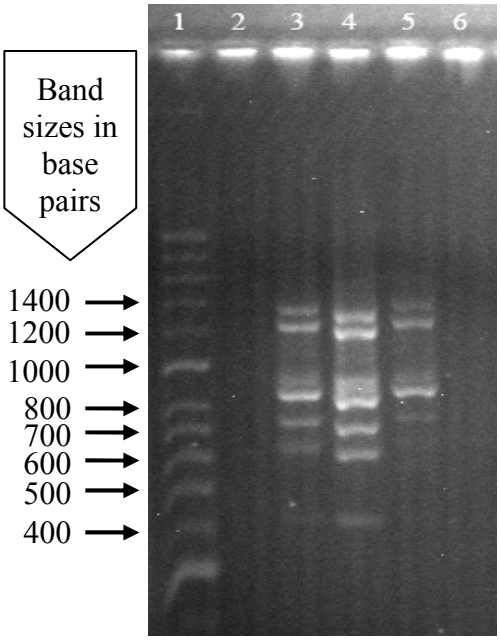

Figure 2. RAPD amplification using the OPA 18 primer. **Lane: 1.** Hyperladder II; **2.** Modified Dellaporta protocol; **3** Modified Križman *et al.* (2006) protocol; **4.** Modified CTAB protocol; **5.** Optimized protocol; **6.** Control.

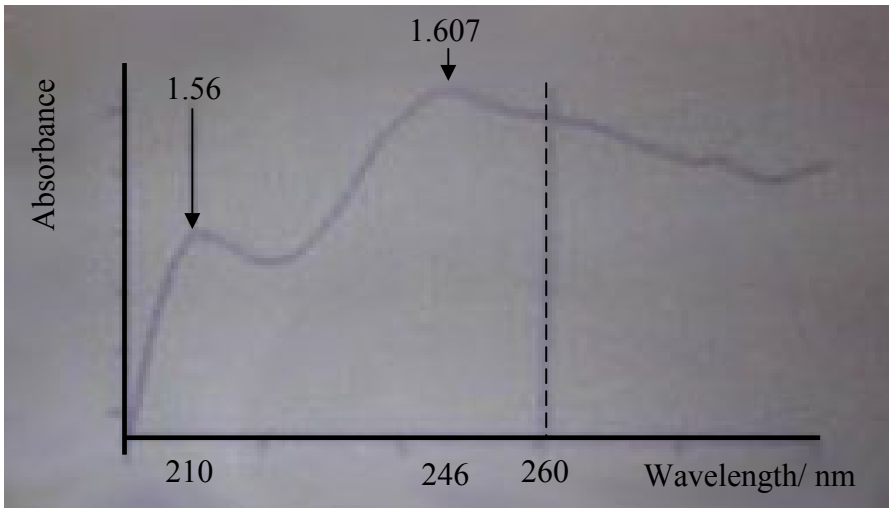

Figure 3. *Absorption peaks from the Dellaporta sample.*

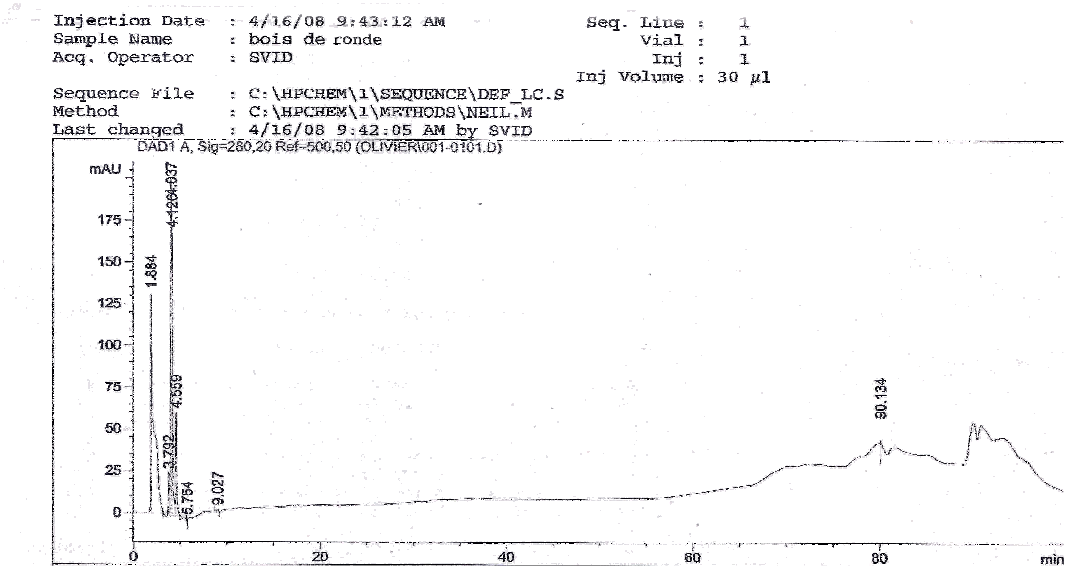

Figure 4. *Gradient HPLC output for the Dellaporta DNA sample, measured at 280nm.*

4. DISCUSSION

As mentioned by Padmalatha and Prasad (2006), several MAPS contain exceptionally high levels of plant-derived contaminants. Preliminary DNA extractions from the *E. sideroxyloides* species showed high levels of polyphenols and polysaccharides as indicated by $A_{260/280}$ and $A_{260/230}$ ratios, which were lower than 1.8. A countermeasure therefore against the ubiquitous green plant contaminants (Vermerris and Nicholson, 2006) was a natural polysaccharide depletion procedure consisting of a 48-hour destarching (Puchooa and Khoyratty, 2004) and the use of young leaf tissues - these have smaller vacuoles and therefore lower secondary metabolite content, facilitating DNA isolation. Tissues were stored frozen and freeze-thaw cycles were avoided by storing the DNA at 4°C to limit damage of high molecular weight DNA (Wilson and Walker, 1994).

All four original protocols used gave low performances in terms of DNA yield and purity. Modifications of the protocols improved DNA quality in some cases, but the $A_{260/230}$ and the $A_{260/280}$ ratios were not consistent in each case (Table 2). While the yield was in general quite low, that from the modified Dellaporta protocol was highest (105.83 µg/ g of leaf material), but showed high levels of contamination. An $A_{260/280}$ ratio of 1.34 denoted contamination by proteins and polyphenols, while the $A_{260/230}$ ratio of 0.65 indicated heavy contamination by polysaccharides. The low purity may explain the lack of amplification (Figure 2). Nevertheless, the DNA was digestible by *Eco*RI (Figure 1b). On the other hand, the original Dellaporta (Wood and Hicks, 1983) protocol did not yield a DNA pellet, but an immiscible pinkish lower phase in isopropanol. After decanting the upper isopropanol phase,

the lower phase constituents was dissolved in water and found fully miscible. An analysis of maximum absorbances using the Spectronic Genesys 5 spectrophotometer showed two peaks away from the 260nm wavelength (Figure 3) and denoted heavy DNA contamination. Further investigation by gradient HPLC for a 100-minute run at 280nm indicated the presence of polyphenols by two sharp peaks after 1.88 and 4.13 minutes (Figure 4). A possible explanation for the phase separation might therefore have been a covalent association of DNA with polyphenols (Aljanabi *et al.,* 1999) and the latter being involved in anomalous re-association kinetics (Merlo and Kemp, 1976) with DNA.

Out of the four protocols tested, one designed for *Terminalia* species (Deshmukh *et al.*, 2007) was discarded due to significant loss of the already low amount of DNA during several initial washing steps.

The modified Križman *et al.* (2006) protocol consisted in pooling up 6 tubes for a total weight of 300mg and in carrying out two chloroform isoamyl alcohol (CIA) extractions to increase the amount of DNA and decrease the amount of protein contaminants respectively. However, the yield was still low (76.67µg/g leaf material), the $A_{260/280}$ did not improve, but the $A_{260/230}$ was satisfactory, denoting heavy protein contamination and low polysaccharide levels respectively. Addition of 20mM β-mercaptoethanol and increasing amount of PVP to 2% improved the $A_{260/280}$ to 2, but showed heavy polysaccharide contamination.

Modifications of the CTAB protocol (Clark, 1997) were not completely satisfactory, giving low DNA yields with heavy polyphenol contamination, but with $A_{260/230}$ ratios consistently above 1. The latter ratio was probably a result of the use of 0.2M sodium acetate coupled with two washing steps in ethanol.

Owing to the performance of the modified Križman *et al.* (2006) protocol for polyphenol and protein removal and the modified CTAB protocol for its persistence in polysaccharide removal, these two protocols were merged to improve DNA quality despite the expected low yields. The use of 20mM β-mercaptoethanol, 2% PVP (molecular weight 10, 000), coupled to two CIA extractions, of 0.2M sodium acetate and two washing steps in ethanol adequately removed polyphenols, proteins and polysaccharides as shown by the absorbance values in Table 2. Despite the absorbance values above 2, the DNA was found RNA-free after an AGE (Figure 1a), digestible by *Eco*RI (Figure 1b) and amplifiable by RAPD (Figure 2).

5. CONCLUSIONS

The optimized protocol is the first one that has been designed for *Erythroxylum sideroxyloides*, the quality of which has been shown sufficient for PCR amplification and restriction digestion. The DNA thus obtained can be amenable to further molecular studies, such as genetic fingerprinting, in which case the DNA profile may serve for identification purposes and help protect the endemic status of the shrub among the Mascarene pharmacopoeia.

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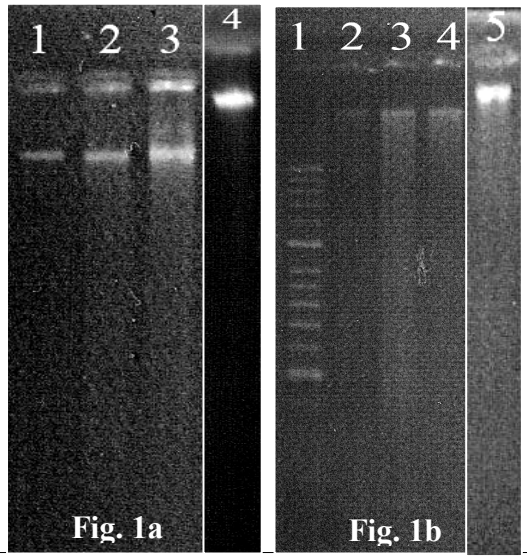
Illustrations



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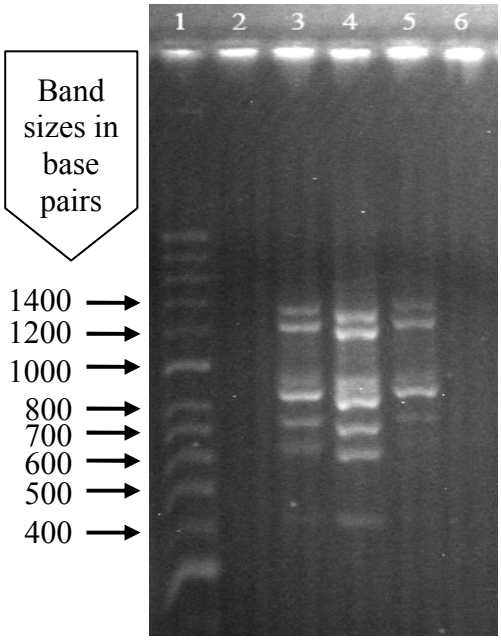

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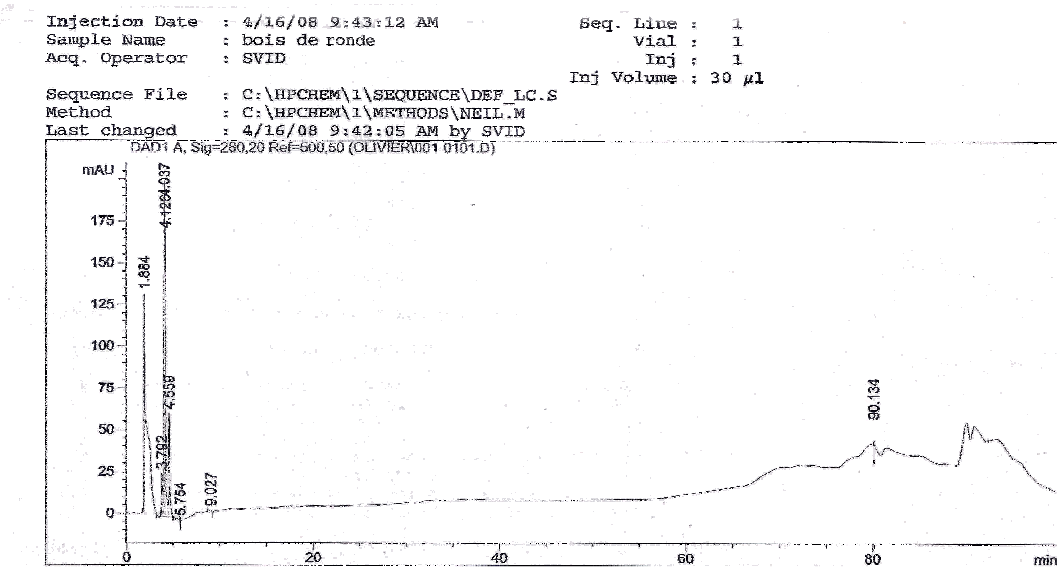
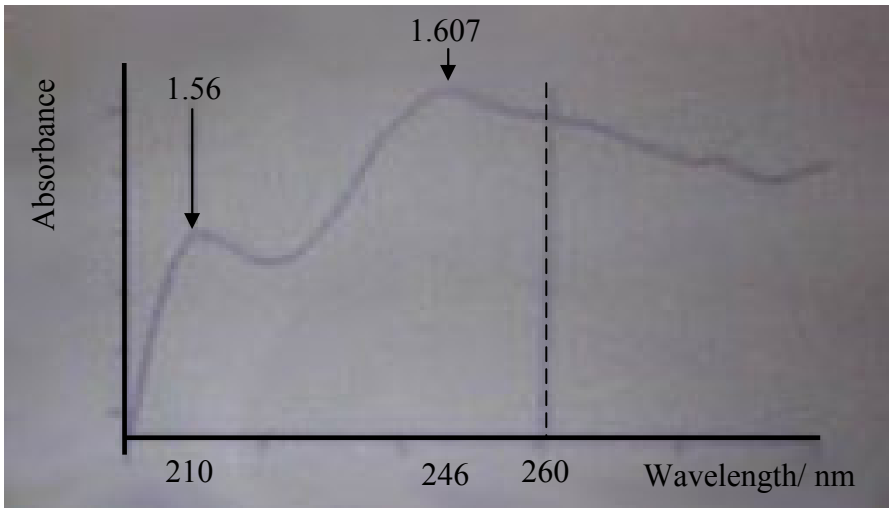


Figure 4. Gradient HPLC output for the Dellaporta DNA sample, measured at 280nm.



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