Optimisation of the DNA extraction protocol in *Vaccinium meridionale* Swartz

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Thesis Submitted for the Degree of Biologist

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Dedicatoria

A mis padres, *María Judith Joya Rondano* y *Onecimo Caviedes González*, mi hermano, *Ángel Gabriel Caviedes Joya* y mi abuela, *Alina Raquel Rondano Beleño*, quienes con su amor y compromiso han sido el fundamento sobre el que he construido cada uno de mis logros. Gracias por creer en mí, por alentarme a seguir adelante y por enseñarme que el esfuerzo y la constancia son el camino más honesto hacia los sueños.

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Resumen

Título: Optimización de la extracción de ADN en *Vaccinium meridionale* Swartz [*]

Autor: María Andrea Caviedes Joya[**]

Palabras Clave: *Vaccinium meridionale*, extracción de ADN, biología molecular vegetal, optimización de protocolos, metabolitos secundarios.

Descripción: *Vaccinium meridionale* es una especie de la familia Ericaceae con una importante relevancia económica y para la salud, y recientemente ha despertado un creciente interés en su estudio. Sin embargo, la extracción de ADN de alta calidad en esta especie representa un desafío debido a sus altos niveles de polisacáridos y compuestos fenólicos. Este estudio tuvo como objetivo optimizar un protocolo de extracción de ADN para *V. meridionale*. Se compararon tres protocolos de extracción de ADN y, posteriormente, se evaluó la eficiencia del protocolo optimizado bajo diferentes tiempos de almacenamiento y métodos de conservación de las muestras en campo. La calidad y cantidad del ADN se evaluaron mediante electroforesis en gel de agarosa, un espectrofotómetro NanoDrop One y fluorometría Qubit 4.

El protocolo optimizado, que incluye un tampón de prelavado a base de sorbitol y un tampón de extracción con altas concentraciones de β-mercaptoetanol, NaCl y CTAB, produjo una mayor calidad y cantidad de ADN en comparación con un kit comercial y el protocolo de Doyle (1991). Además, se obtuvo ADN de alta calidad en los diferentes métodos de conservación en campo y tiempos de almacenamiento evaluados. Estos resultados sugieren que el protocolo optimizado propuesto en este estudio constituye un método confiable y eficaz para la extracción de ADN en *V. meridionale*, facilitando futuros estudios genéticos en esta especie.

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[*] Trabajo de Grado

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Abstract

Title: Optimisation of the DNA extraction protocol in *Vaccinium meridionale* Swartz [*]

Author: María Andrea Caviedes Joya[**]

Key Words: *Vaccinium meridionale*, DNA extraction, plant molecular biology, protocol optimisation, secondary metabolites.

Description: *Vaccinium meridionale* is a species in the family Ericaceae with significant economic and health-related importance and has recently attracted increased interest in its study. However, extracting high-quality DNA from this species is challenging due to its high levels of polysaccharides and phenolic compounds. This study aimed to optimise a DNA extraction protocol for *V. meridionale*. Three DNA extraction protocols were compared, and the efficiency of the optimised protocol was further evaluated under different sample storage time and preservation methods in field. DNA quality and quantity were assessed using agarose gel electrophoresis, a NanoDrop One spectrophotometer, and Qubit 4 fluorometry. The optimised protocol, which includes a sorbitol-based pre-wash buffer and an extraction buffer with high concentrations of β-mercaptoethanol, NaCl, and CTAB, yielded higher DNA quality and quantity than a commercial kit and the Doyle (1991) protocol. Additionally, high-quality DNA was obtained across the different preservation methods in field and storage time evaluated. These results suggested that the optimised protocol proposed in this study is a reliable and effective method for DNA extraction in *V. meridionale*, facilitating future genetic studies in this species.

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[*] Degree Work

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Prefacio

Este documento corresponde al trabajo final de investigación de pregrado para optar al título de Bióloga, y fue elaborado siguiendo los lineamientos establecidos por la Biblioteca de la Universidad Industrial de Santander (UIS).

La investigación surge de la necesidad de contar con un protocolo de extracción de ADN confiable y reproducible para *Vaccinium meridionale*, una especie de alto interés científico y regional cuyo estudio molecular ha sido limitado, en parte, por los desafíos técnicos asociados a su composición química.

Cabe señalar que, a la fecha de entrega de este documento a la Biblioteca de la Universidad Industrial de Santander, el artículo científico derivado de esta investigación, titulado ″Comparative evaluation of DNA extraction methods for *Vaccinium meridionale* Swartz under different preservation and storage conditions″, se encuentra en proceso de revisión por pares en la revista *Molecular Biology Reports*.

Introduction

The genus *Vaccinium* belongs to the Ericaceae family and comprises more than 450 species recognised for their high concentrations of bioactive compounds, such as polyphenols, anthocyanins, and flavonoids (Ramos-Polo et al., 2025). One species within this genus is *Vaccinium meridionale* Swartz, commonly known as agraz or mortiño in Colombia, where it is mainly distributed in the departments of Boyacá, Magdalena, Antioquia, Santander, and Cundinamarca, at altitudes ranging from 2,000–3,200 m.a.s.l. (Magnitskiy, 2023), and also in Ecuador, Peru, and Venezuela (Becerra et al., 2022).

V. meridionale shares similarities with other widely studied berries within the genus, such as bilberry (*Vaccinium myrtillus*) and Andean blueberry (*Vaccinium floribundum*), which confer high nutritional value and strong potential as a functional food due to their anthocyanin and antioxidant content (Garzón et al., 2010; Medina-Cano et al., 2023).

The characterisation and analysis of genetic diversity in promising plant species are essential for understanding population variability and identifying genotypes of interest for conservation and breeding programmes (Mesfer ALshamrani et al., 2022), where molecular tools play a key role. For this purpose, obtaining intact, high-quality DNA is a critical prerequisite for downstream molecular analyses (Attikora et al., 2024). The presence of secondary metabolites, such as polyphenols, polysaccharides, and tannins, has been shown to affect DNA quality negatively, as these compounds can bind to DNA during cell lysis, leading to degradation and contamination, and can inhibit Taq polymerase during PCR (Arruda et al., 2017). Additionally, variables such as preservation method in field and storage time may influence DNA integrity, although their effect is closely related to the extraction protocol used (Attikora et al., 2024; Mitchell et al., 2023; Varma et al., 2007).

Therefore, it is necessary to evaluate these factors within an optimised DNA extraction protocol for *V. meridionale*. This study aimed to standardise a DNA extraction protocol for *V. meridionale*, based on modifications of the method described by Azmat (Azmat et al., 2012) and further adapted by Sepúlveda (Sepúlveda et al., 2025). Additionally, its efficiency was evaluated in comparison with the Doyle protocol (Doyle, 1991) and a commercial DNA extraction kit (InviSorb Spin Plant Mini Kit). The effect of storage time (fresh and stored samples) and preservation methods in field (liquid nitrogen and silica gel) on DNA quality and yield was also assessed.

1. Objectives

1.1 General Objective

Standardize the modified DNA extraction protocol in agraz (*Vaccinium meridionale*).

1.2 Specific Objectives

Identify the DNA extraction protocol with the highest efficiency in terms of DNA quantity and quality obtained between the modified Azmat (2012) protocol and the Doyle and Doyle (1990) protocol in *Vaccinium meridionale*.

Compare the effectiveness of the modified Azmat (2012) protocol with two commercial DNA extraction kits for obtaining DNA from *Vaccinium meridionale*.

Determine the most favorable preservation conditions (liquid nitrogen or silica gel) and sample origin (commercial or wild) for obtaining high-quality DNA using the modified Azmat (2012) protocol in *Vaccinium meridionale*.

2. Materials and Methods

2.1 Biological material

The biological material consisted of young, healthy leaves of *V. meridionale*, free from damage caused by insects, pathogens, or mechanical factors. Samples were collected in compliance with Amendment 31, under the access contract for genetic resources and their derivatives No. 121 (22 January 2016), signed between the Ministry of Environment and Sustainable Development of Colombia and the Universidad Nacional de Colombia, as well as contract No. 338-RGE403 (15 June 2022) signed between the same Ministry and the Universidad Industrial de Santander.

2.1.1 Fresh samples.

Samples were collected from wild populations located in the municipalities of Guaca and Tona (Santander, Colombia), obtaining 10 samples per municipality from different individuals. Half of the samples collected from each municipality were preserved in liquid nitrogen, while the remaining samples were stored in silica gel.

2.1.2 Stored samples.

A total of 10 samples collected in 2022 from the municipalities of Guaca and Tona (Santander, Colombia) were used. The plant material was initially preserved in liquid nitrogen and subsequently stored for two years at −80 °C in the Laboratory of Genetics and Biotechnology at the Universidad Industrial de Santander.

2.1.3 Control.

As a positive control, leaf samples of *Lippia origanoides* were obtained from the local market of San Francisco (Bucaramanga, Colombia). This species was selected because it has been previously studied in the laboratory and consistently yields DNA of optimal quality and quantity regardless of the extraction method used. Moreover, DNA from this species has been successfully extracted in previous studies using the Doyle protocol (Feijó et al., 2022; Frattini et al., 2023), without reported issues and enabling its use in downstream genetic analyses.

2.2 DNA extraction protocols

Three DNA extraction methods were used for *V. meridionale*: a commercial kit (InviSorb Spin Plant Mini Kit), the Doyle protocol (Doyle, 1991), and the optimised DNA extraction protocol developed in this study. A total of 30 extractions were performed using both protocols, whereas 26 extractions were carried out using the optimised protocol.

2.2.1 Optimised DNA extraction protocol for V. meridionale.

2.1.2.1 Reagents.

- *Pre-wash buffer:* 0.35 M sorbitol, 10 mM Tris-HCl (pH 8.4), 5 mM EDTA (pH 8.4), 2% (w/v) polyvinylpyrrolidone (PVP40), and 1% (v/v) β-mercaptoethanol.
- *Extraction buffer:* 100 mM Tris-HCl (pH 8.4), 20 mM EDTA (pH 8.4), 3 M NaCl, 4% (v/v) β-mercaptoethanol, 0.04 g/mL CTAB, and 0.025 g/mL polyvinylpyrrolidone (PVP40).
- *TE buffer:* 10 mM Tris-HCl (pH 8.4) and 1 mM EDTA (pH 8.4).

2.1.2.2 Procedure.

- *Cell lysis and initial precipitation:* Approximately 1 g of young leaves was weighed and ground in a mortar with liquid nitrogen. The homogenized material was transferred to a 50 mL Falcon tube and mixed with 10 mL of pre-wash buffer by inversion. Tubes were centrifuged at 5,000 x g for 5 min at room temperature, and the supernatant was discarded. Subsequently, 10 mL of preheat extraction buffer (60 °C) and 10 µL of proteinase K (1 mg/mL) were added, and samples were incubated in a water bath with agitation at the same temperature for 4 h. After incubation, an equal volume (~ 10 mL) of chloroform-isoamyl alcohol (24:1, v/v) was added, and the mixture was centrifuged at 5,200 x g for 15 min at room temperature. The aqueous phase was carefully transferred to a new 50 mL Falcon tube, and 10 mL of cold isopropanol (-20 °C) was added. Tubes were then stored at -20 °C overnight.

- *DNA washing and resuspension:* Samples were centrifuged at 8,500 x g for 10 min at 4 °C, and the supernatant was discarded. The DNA pellet was washed with 10 mL of cold 70% ethanol, followed by centrifugation at 10 000 x g for 10 min at 4 °C. This washing step was repeated twice. Prior to each centrifugation, pellets were gently detached from the tube walls. After removing the supernatant, the pellet was air-dried by inversion for 1-2 h until a translucent appearance was observed. DNA was then resuspended in 200 µL of TE buffer and transferred to a 1.5 mL Eppendorf tube, followed by the addition of 2.5 µL of RNAse (10 mg/mL) per 200 µL of sample. Tubes were incubated at 37 °C for 1 h in a thermoblock (Thermo Scientific, MA, USA).

2.3 DNA quality and quantity assessment

2.3.1 Electrophoresis.

DNA presence and quality were evaluated for all extractions using 0.8% agarose gel electrophoresis prepared with 1x TBE buffer (Tris-borate-EDTA). SYBR Safe was used as an intercalating agent at a concentration of 0.02 µL/mL. Electrophoresis was performed at 90 V for

1 h, and gels were visualised using an Enduro GDS II gel documentation system (Labnet International, NJ, USA).

2.3.2 Spectrophotometry.

DNA quality was assessed using a NanoDrop One spectrophotometer (Thermo Scientific, MA, USA) by measuring the A260/A280 and A260/A230 ratios.

2.3.3 Fluorometry.

DNA concentration was measured using a Qubit 4 fluorometer (Invitrogen, CA, USA) with the 1x dsDNA HS Working Solution Kit.

DNA extraction yield per 100 mg of initial sample was calculated using the formula $Y = (X \times V) / m$, where X is the DNA concentration, V is the volume of TE buffer used for resuspension, and m is the initial sample mass.

2.3.4 Downstream applications.

The functional quality of the extracted DNA was evaluated for downstream molecular applications by PCR using the markers matk3, rbcL1, and ITS under the conditions described in Table 1. PCR reactions were prepared with 7.5 µL of Go Taq Green Master Mix 2x, 0.6 µL of each primer (10 µM), 4.3 µL of sterile distilled water, and 2 µL (0.01 – 2.32 ng/µL) of DNA sample.

PCR products were analysed by 0.8% agarose gel electrophoresis using 1x TBE buffer, run at 90 V for 1 h, and visualised with the Enduro GDS II system.

Table 1

Primers used and PCR conditions

Gen	Primers		Sequence (5' - 3')	PCR conditions	References
matk3	F	matK 2.1a	ATCCATCTGGAAATCTTAGTTC	***Denaturing:*** 94°C/3min ***Annealing:*** 35 cycles, 94°C/45s; 54°C/45s; 72°C/45s ***Extending:*** 72°C/10min	(Vu et al., 2024)
	R	matK 5	GTTCTAGCACAAGAAAGTCG		
rbcL1	F	rbcLa-F	ATGTCACCACAAACAGAGACTAAAG	***Denaturing***: 94°C/3min ***Annealing:*** 35 cycles, 94°C/45s; 54°C/45s; 72°C/45s ***Extending:*** 72°C/10min	(Vu et al., 2024)
	R	rbcLa-R	GTAAAATCAAGTCCACCRCG		
ITS	F	ITS 5a	CCTTATCATTTAGAGGAAGGAG	***Denaturing:*** 94°C/5 min ***Annealing:*** 35 cycles, 94°C/45s; 50°C/45s; 72°C, 1min ***Extending:*** 72°C/10 min	(Paksoy et al., 2022)
	R	ITS 4	TCCTCCGCTTATTGATATGC		

2.3.5 Data analysis.

The data were analysed using R software (version 4.5.2). Statistical tests were conducted with the rstatix package (Alboukadel, 2023) while data visualizations were generated using ggplot2 (Wickham, 2016). The Shapiro-Wilk test indicated that none of the datasets met the normality assumption ($p < 0.05$); therefore, non-parametric statistical test (Mann-Whitney test and Kruskal – Wallis) were applied.

DNA concentration (ng/µL) obtained using NanoDrop One and Qubit 4 was analysed independently. DNA quality was evaluated based on A260/A280 and A260/A230 ratios.

The Mann-Whitney U test was used to compare DNA concentration and quality according to storage time and preservation method in field. Additionally, the Kruskal – Wallis test was applied to evaluate differences among DNA extraction protocols, followed by Dunn's post hoc test. Statistical significance was considered at $p < 0.05$.

3. Results

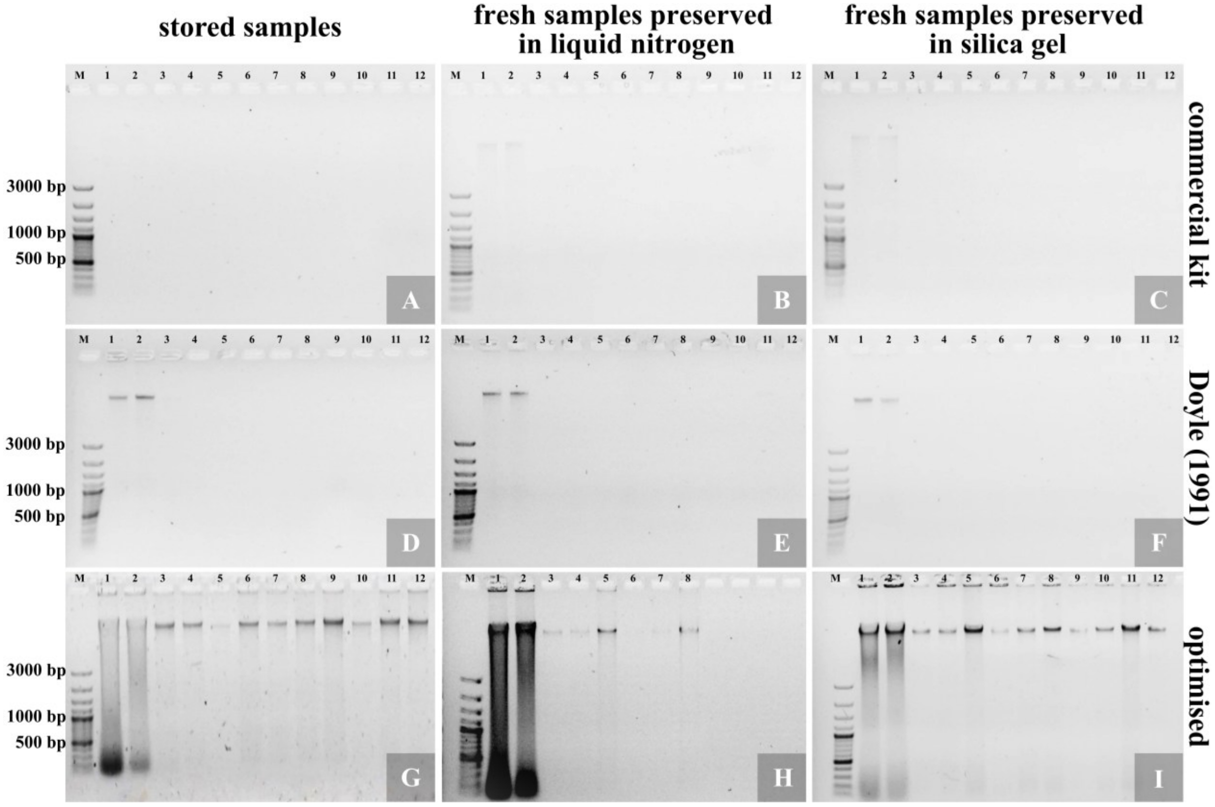
3.1 DNA extraction protocols

Agarose gel electrophoresis showed that genomic DNA from *V. meridionale* extracted using the InviSorb Spin Plant Mini Kit (Figure 1. A–C) and the Doyle protocol (Figure 1. D–F) produced no visible bands, whereas clear bands were observed for *L. origanoides*.

In contrast, the optimised protocol yielded intense, well-defined bands in most samples, corresponding to high-molecular-weight DNA (>3,000 bp) (Figure 1. G–I). Samples preserved in liquid nitrogen (Figure 1. H) showed lower band intensity, although molecular weight remained comparable.

Figure 1

Agarose gel electrophoresis for total DNA evaluation



Note. Genomic DNA was obtained using the commercial InviSorb Spin Plant Mini Kit (A-C), the Doyle extraction protocol (D-F), and the optimised protocol (G-I). V. meridionale samples correspond to stored samples (A, D, G) or fresh samples preserved in liquid nitrogen (B, E, H) or silica gel (C, F, I). Lanes correspond to the molecular marker (M), *L. origanoides* (1-2), and *V. meridionale* (3-12).

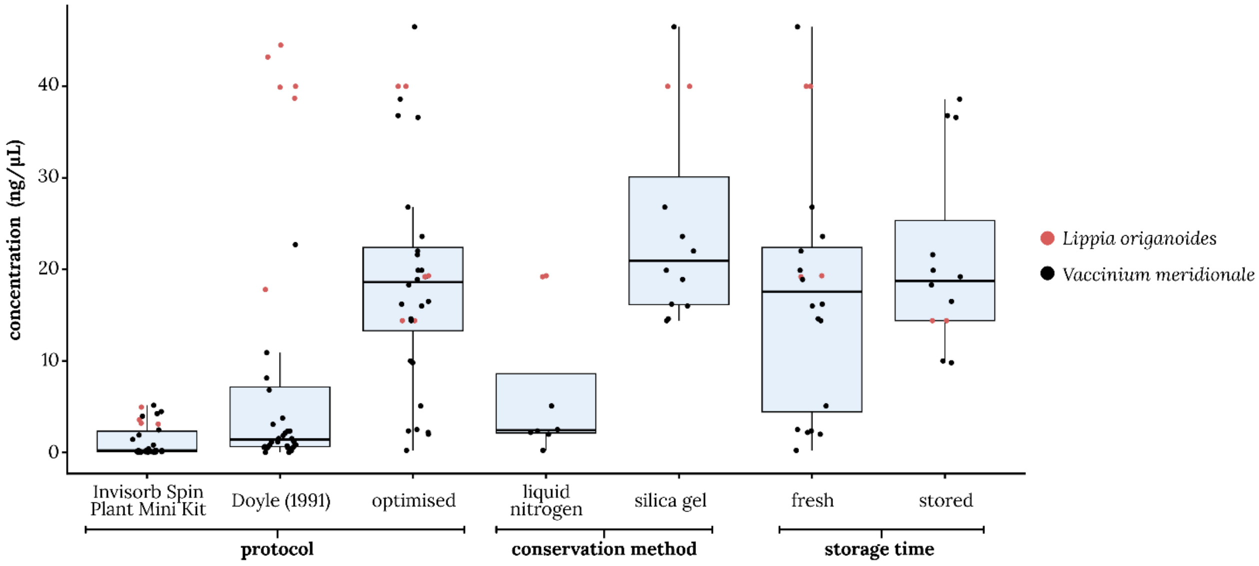
3.1.1 DNA yield

The optimised protocol yielded 13.44 µg/100 mg (NanoDrop One) and 0.354 µg/100 mg (Qubit 4), exceeding the yields obtained with the commercial InviSorb Spin Plant Mini Kit (0.19 and 0.09 µg/100 mg, respectively) and the Doyle protocol (0.22 and 0.053 µg/100 mg, respectively) (Figure 2 and Figure 3).

The Kruskal – Wallis test revealed significant differences in DNA concentration among all evaluated extraction protocols for both NanoDrop One ($p = 2{,}86 \times 10^{-14}$) and Qubit 4 ($p = 2{,}18 \times 10^{-11}$) (Table 2).

Figure 2

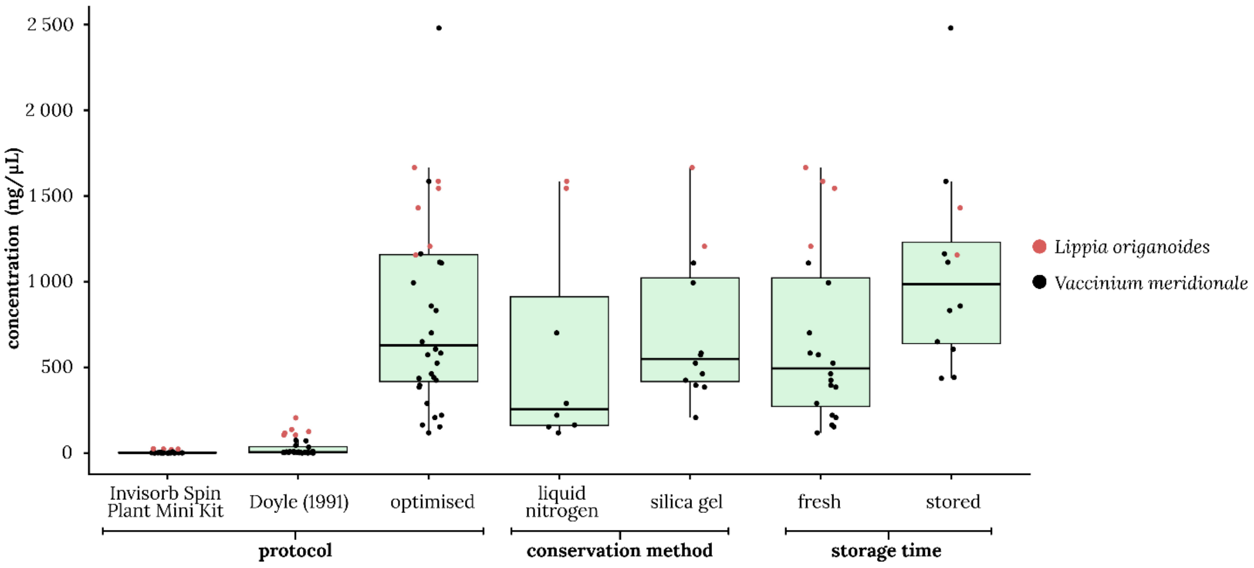
Distribution of DNA concentrations (ng/µL) measured using Qubit 4.



Note. Concentrations obtained from the three evaluated DNA extraction protocols are shown. For the optimised protocol, concentrations are presented according to preservation method in field (liquid nitrogen and silica gel) and storage time (fresh and stored samples).

Figure 3

Distribution of DNA concentrations (ng/µL) measured using NanoDrop One.



Note. Concentrations obtained from the three evaluated DNA extraction protocols are shown. For the optimised protocol, concentrations are presented according to preservation method in field (liquid nitrogen and silica gel) and storage time (fresh and stored samples).

3.1.2 DNA quality

The quality of DNA obtained with the optimised protocol, based on the A260/A280 ratio, did not show statistically significant differences compared with the commercial InviSorb Spin Plant Mini Kit, according to Dunn’s post hoc test. Similarly, no significant differences were observed between the Doyle protocol and the commercial kit for the A260/A230 ratio.

On average, the optimised protocol yielded A260/A280 and A260/A230 ratios of 1.78 and 1.58, respectively, which were higher than those obtained with the other two protocols. The distribution of these values is shown in Figure 4. Overall, the optimised protocol showed higher values and lower variability in both ratios, particularly for A260/A280.

Figure 4

Distribution of A260/A230 and A260/A280 ratios according to the DNA extraction protocol used for V. meridionale and L. origanoides.

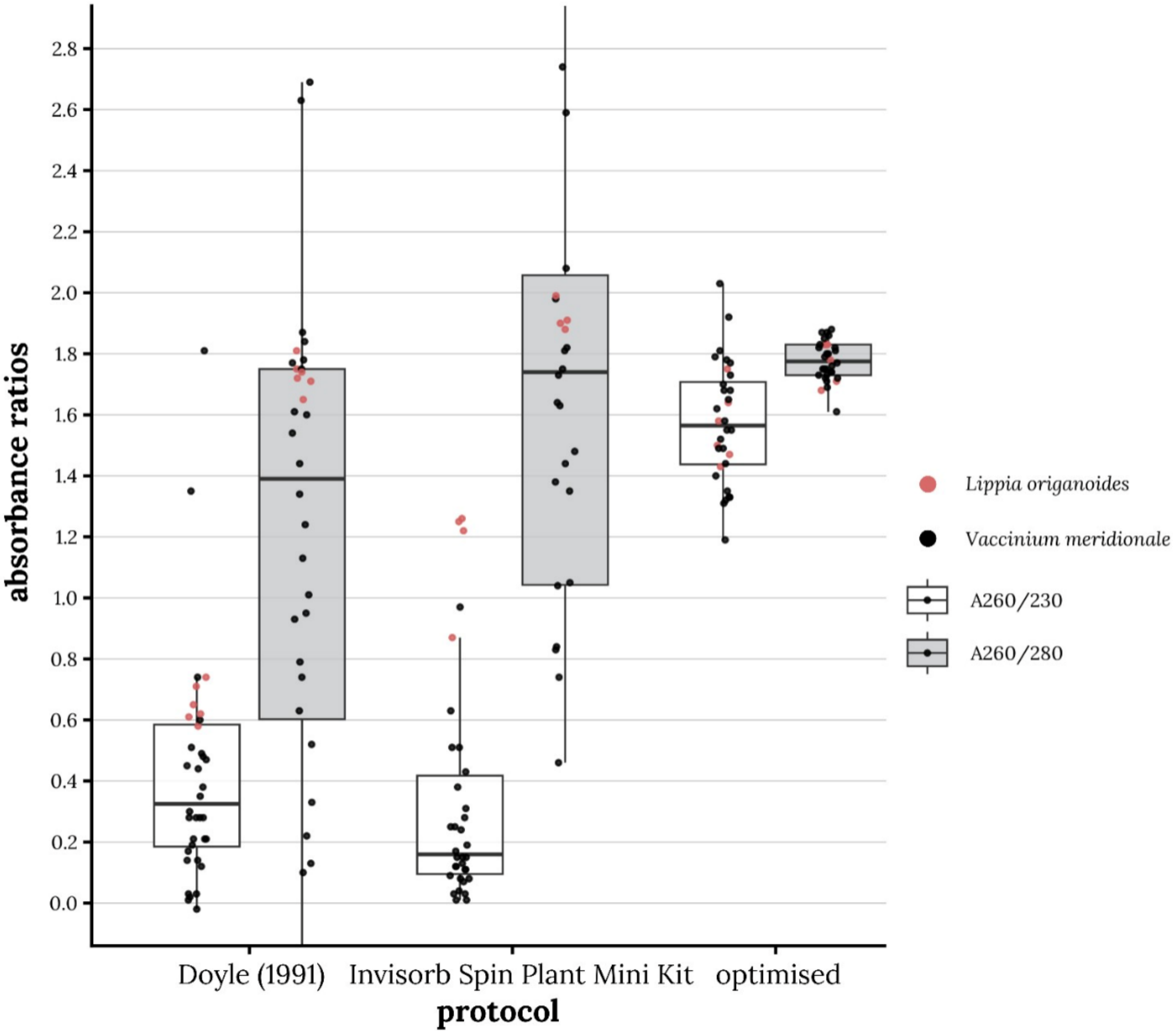


Table 2

Comparison among DNA extraction protocols.

QUANTITY									
		NanoDrop One				**Qubit 4**			
Protocol	**N**	**x̄ ± SE**	**Y±SE**	**H**	**p**	**x̄±SE**	**Y ± SE**	**H**	**P**
Commercial kit	30	1.92±1.45^{a}	0.19±0.14	62.37	2.86 × 10^{-14}	0.91±1.53^{a}	0.091±0.15	46.45	2.18 × 10^{-11}
Doyle (1991)	30	11.07±19.26^{b}	0.22±0.39			2.63±4.53^{b}	0.053±0.091		
Optimised	26	671.8±518.05^{c}	13.44±10.36			17.7±12.2^{c}	0.354±0.24		

QUALITY							
Protocol	**N**	**A260/A280 ±SE**	**H**	**p**	**A260/A230 ± SE**	**H**	**p**
Commercial kit	30	0.42±5.65^{a}	12.5	1.91*10^{-3}	0.22±0.21^{a}	52.1	4.97*10^{-12}
Doyle (1991)	30	0.78±1.99^{b}			0.37±0.38^{a}		
Optimised	26	1.78±0.06^{a}			1.58±0.20^{b}		

Note. N = number of extraction; x̄ = mean DNA concentration (ng/µL); Y = yield based on the mean (µg/100 mg); H = Kruskal – Wallis statistic; SE = standard deviation; p = p-value. Means sharing the same letter are not significantly different according to Dunn's post hoc test.

3.2 Effect of preservation method in field and storage time

3.2.1 Preservation method in field

Samples preserved in silica gel showed higher average DNA yield than those preserved in liquid nitrogen (NanoDrop: 11.31 vs 5.49; Qubit: 0.438 vs 0.04). The Mann-Whitney U test indicated that these differences were statistically significant (NanoDrop: p = 0.034; Qubit: p = 0.001).

Significant differences were also observed in both DNA quality ratios across preservation methods in field. Average DNA quality values were slightly higher for samples stored in silica gel compared with those preserved in liquid nitrogen (Table 3.).

Table 3.

Comparison between preservation methods in field of fresh samples.

QUANTITY

		NanoDrop One				**Qubit 4**			
Preservation method	**N**	**x̄ ± SE**	**Y**	**W**	**p**	**x̄ ± SE**	**Y**	**W**	**p**
Liquid nitrogen	6	274.48±217.49	5.49±4.35	50	0.034	2.39±1.56	0.04±0.03	60	0.001
Silica gel	10	565.75±279.03	11.31±5.58			21.89±9.56	0.438±0.19		

QUALITY

Preservation method	**N**	**A260/A280 ± SE**	**W**	**p**	**A260/A230 ± SE**	**W**	**P**
Liquid nitrogen	6	1.71±0.05	0	0.00136	1.39±0.13	3.5	0.00477
Silica gel	10	1.83±0.03			1.67±0.13		

Note. N = number of extractions; x̄ = mean DNA concentration (ng/µL); Y = DNA yield based on the mean (µg/100 mg); W = Mann–Whitney U statistic; SE = standard deviation; p = p-value.

3.2.2 Storage time

Based on Qubit 4 measurements and the Mann-Whitney U test, no significant differences were observed in DNA concentration between fresh and stored samples ($p = 0.102$). However, the NanoDrop One measurement indicated significant differences ($p = 0.00224$). Stored samples (NanoDrop = 20.33; Qubit = 0.45) showed a higher average yield compared with fresh samples (NanoDrop = 9.13; Qubit = 0.29).

Regarding DNA quality, no statistically significant differences were observed for either of the evaluated ratios ($p > 0.05$) (Table 4).

Table 4

Comparison according to storage time.

QUANTITY									
		NanoDrop One				**Qubit 4**			
Storage time	**N**	**x̄ ± SE**	**Y ± SE**	**W**	**p**	**x̄ ± SE**	**Y ± SE**	**W**	**p**
Stored	10	1016.36±625.86	20.33±12.52	24	$2.24*10^{-3}$	22.73±10.81	0.45±0.21	48.5	0.102
Fresh	16	456.52±289.29	9.13±5.78			14.57±12.27	0.29±0.24		
QUALITY									
Storage time	**N**	**A260/A280 ± SE**	**W**		p	**A260/A230 ± SE**	**W**	**p**	
Stored	10	1.75±0.03	1008		0.129	1.6±0.25	770	0.528	
Fresh	16	1.79±0.07				1.56±0.19			

Note. N = number of extractions; x̄ = mean DNA concentration (ng/µL); Y = DNA yield based on the mean (µg/100 mg); W = Mann–Whitney U statistic; SE = standard deviation; p = p-value.

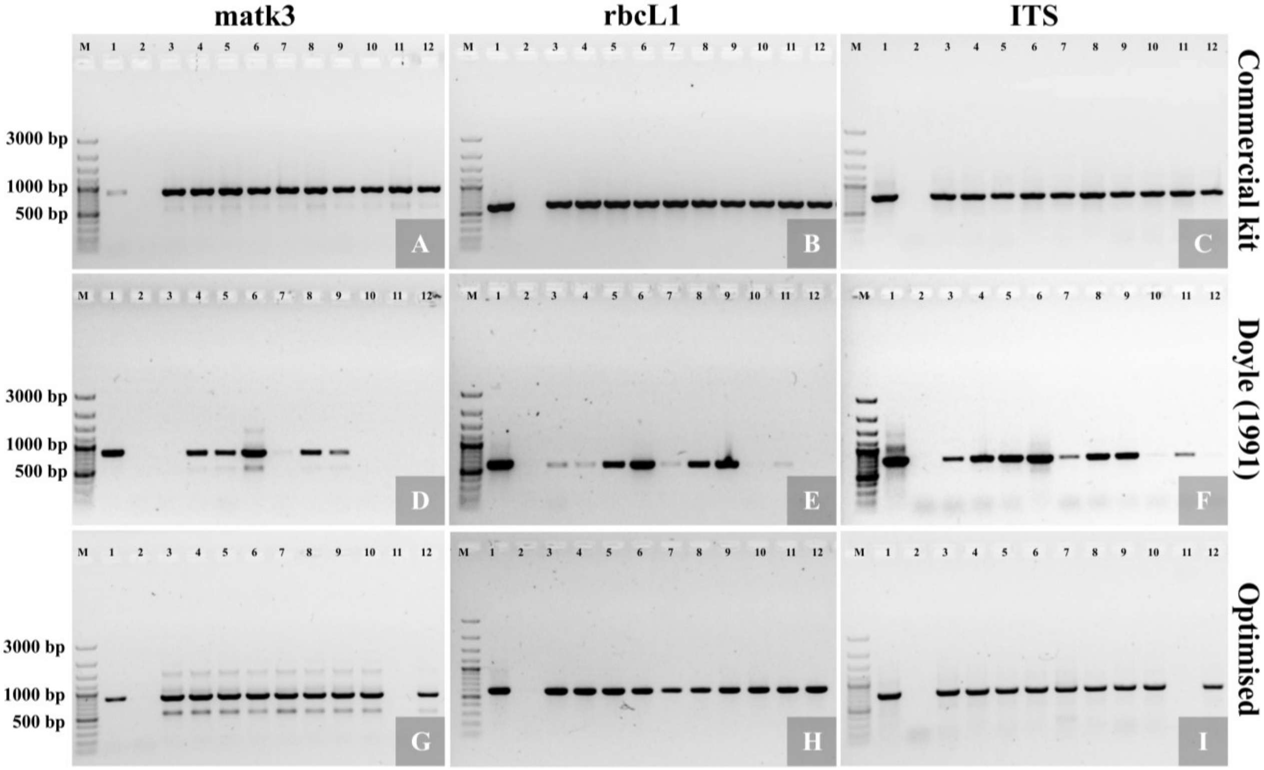
3.3 PCR-based quality assessment

The matk3, rbcL1, and ITS regions were successfully amplified regardless of preservation method in field or storage time. Some samples extracted using the Doyle protocol failed to amplify, whereas most samples obtained with the commercial kit and the optimised protocol showed successful amplification.

Nevertheless, a 1:20 dilution of all DNA samples obtained using the optimised protocol was required to achieve efficient amplification (Figure 5).

Figure 5

PCR-based assessment of DNA quality



Note. Amplified DNA fragments obtained using the commercial InviSorb Spin Plant Mini Kit (A–B), the Doyle (1991) extraction protocol (D–F), and the optimised protocol (G–I). Amplified products of the matk3 (A, D, G), rbcL1 (B, E, H), and ITS (C, F, I) genes are shown. Lanes correspond to the molecular marker (M), L. origanoides (1), negative control (2), and V. meridionale (3–12).

4. Discussion

The results demonstrate that the optimised protocol provides superior performance for genomic DNA extraction from *V. meridionale* compared with the Doyle protocol and a commercial kit. The method yielded higher DNA concentration and quality, produced high-molecular-weight DNA, and enabled successful PCR amplification. These findings indicate that the protocol improves DNA recovery efficiency across different preservation methods and storage conditions.

Both the Doyle protocol and the commercial InviSorb Spin Plant Mini Kit showed low performance for *V. meridionale* samples. Agarose gel visualisation (Figure 1. A-F) and the obtained concentrations (Table 2) indicate limited DNA recovery using these methods. In contrast, the optimised protocol yielded DNA with higher integrity, as reflected in well-defined bands without smearing (Figure 1. G-1), as well as higher concentrations (Table 2). These results further support the improved DNA recovery efficiency achieved with the optimised protocol (Figure 1; Table 2).

The A260/A280 ratio is commonly used to assess protein contamination in DNA samples, with an optimal range of 1.8-2.0 (Lopez et al., 2025). The values obtained with the optimised protocol were comparable to those from the commercial InviSorb Spin Plant Mini Kit, suggesting that both methods are effective at removing proteins from *V. meridionale* samples. In contrast, lower values were observed with the Doyle protocol (Table 2). Notably, the low variability observed in this ratio for the optimised protocol suggests greater reproducibility than that of the other evaluated methods (Figure 4; Table 2).

Although the optimal A260/A230 ratio is generally considered to be between 2.0 and 2.2 as an indicator of effective removal of phenolic compounds and polysaccharides (Lopez et al., 2025), several studies in recalcitrant species, such as *Coffea canephora* (Castilla Valdés et al., 2024), *Eucalyptus* spp. (Inglis et al., 2018), and *Rosa roxburghii* (Ren et al., 2025), have shown that lower values may still be compatible with downstream molecular applications such as PCR and Illumina sequencing. This is consistent with the present study, where even samples with low A260/A280 and A260/A230 ratios were successfully amplified (Figure 5)

These findings suggest that all three protocols can be used for plant DNA extraction; however, their efficiency decreases in species with high levels of phenolic compounds, such as *V. meridionale* (Arruda et al., 2017), which contains at least twice the phenolic concentration compared with other species of the same genus, such as *V. macrocarpon* and *V. corymbosum* (Česonienė & Daubaras, 2016; Garzón et al., 2010; Ramos-Polo et al., 2025; Vargas-Ramella et al., 2021).

High concentrations of phenolic compounds can negatively affect DNA quality and yield (Heikrujam et al., 2020). During tissue maceration, phenolic compounds may oxidise to form quinones, which can cause DNA fragmentation and covalent binding to DNA (Heikrujam et al., 2020; Schenk et al., 2023). Furthermore, residual phenols may interfere with downstream applications, such as PCR, by inhibiting or denaturing DNA polymerase, thereby compromising amplification efficiency (Swari et al., 2023). Beyond PCR, polyphenols pose significant risks for next-generation sequencing (NGS) by inhibiting critical enzymes during library construction, such as those used for end-repair and adapter ligation (Rossini et al., 2025). In third-generation platforms like Oxford Nanopore, DNA-polyphenol complexes can physically and irreversibly block nanopores, leading to a precipitous decline in available pores and the generation of reads significantly shorter than expected (Pearman et al., 2024). This behaviour may explain the low

A260/A230 ratios and DNA concentrations observed in samples extracted using the commercial kit and the Doyle protocol (Figure 1 and Figure 5).

To mitigate the effects of secondary metabolites on DNA quality, the optimised protocol incorporates two buffers. The pre-wash buffer contains 0.35 M sorbitol, which removes phenolic compounds dissolved in the cytosol prior to cell lysis, allowing their elimination after mild centrifugation and before DNA exposure (Ren et al., 2025). In addition, both the pre-wash and extraction buffers contain PVP40 (polyvinylpyrrolidone), which forms hydrogen bonds with phenolic compounds, preventing their oxidation and interaction with DNA (Lee et al., 2021). These findings suggest that the proposed protocol may be applicable to other plant species with high levels of secondary metabolites, where DNA extraction remains challenging.

Additionally, the extraction buffer in the optimised protocol contains 3.8% (v/v) more β-mercaptoethanol than the Doyle protocol. This reagent, like PVP40, prevents phenolic oxidation and contributes to nuclease inactivation by disrupting disulfide bonds in these enzymes, thereby preserving DNA integrity (Russo et al., 2022). Previous studies have shown that higher concentrations of this reagent result in improved DNA yield and quality (Silva, 2010).

In addition to phenolic compounds, *V. meridionale* is characterised by a high polysaccharide content, particularly in the form of dietary fibre, compared with other species of the same genus (Escobar-Barranco et al., 2025). These macromolecules may also hinder the extraction of high-quality DNA, as they tend to co-precipitate with DNA during alcohol precipitation steps, which may explain the decrease in A260/A230 ratios, given that polysaccharides absorb light at wavelengths near 230 nm (Russo et al., 2022).

Like phenolic compounds, polysaccharides can reduce the efficiency of downstream molecular applications. Their co-precipitation with DNA may result in highly viscous pellets that hinder resuspension and inhibit DNA polymerase activity (Buljević et al., 2025). To reduce polysaccharide contamination, the extraction buffer in the optimised protocol, as in the Doyle (1991) method, contains NaCl and CTAB; however, their concentrations were doubled. Increased NaCl concentration enhances polysaccharide solubility in alcohol, reducing co-precipitation with DNA during purification steps and thereby lowering the risk of contamination (Krishnan et al., 2024; Saługa, 2020). In addition, CTAB participates in membrane lysis and forms complexes with polysaccharides and proteins, which can be removed during chloroform–isoamyl alcohol (24:1) extraction (Heikrujam et al., 2020).

Overall, the results suggest that the efficiency of the optimised protocol stems from adjusting the chemical environment during extraction, thereby limiting interactions between DNA and plant secondary metabolites. This allows preservation of DNA structural integrity and improves extraction yield. The inhibition of oxidative reactions and the prevention of DNA–metabolite complex formation appear to be critical in species with high levels of phenolic compounds and polysaccharides, such as *V. meridionale*.

Significant differences were observed between samples stored in silica gel and those preserved in liquid nitrogen, in both concentration and quality (Table 3.), suggesting that the preservation method in field may influence DNA yield and quality. However, no studies have conclusively demonstrated that silica gel preservation is superior to liquid nitrogen; rather, both methods have yielded successful results (Quiñones et al., 2024; Varma et al., 2007).

Stored samples, which were preserved in liquid nitrogen, showed quality and yield values comparable to those of fresh samples, regardless of preservation method in field, except for DNA concentration measured with the NanoDrop One, where significant differences were observed (Table 4). Some authors have noted that spectrophotometric quantification may overestimate

DNA concentration because it detects any molecule that absorbs at 260 nm. In contrast, fluorometric methods such as Qubit 4 use DNA-specific dyes, providing more selective and accurate measurements (Bruijns et al., 2022; Versmessen et al., 2024).

Overall, the results indicate that the optimised protocol enables the extraction of high-quality DNA regardless of preservation method in field or storage time, as demonstrated by successful PCR amplification (Figure 5). This highlights the robustness and reproducibility of the protocol and supports its application in molecular studies of *V. meridionale*, facilitating future research on genetic diversity, conservation programmes, and breeding strategies for this species.

5. Conclusion

The optimised DNA extraction protocol enabled the recovery of high-quality genomic DNA from *V. meridionale*, outperforming the Doyle protocol and a commercial kit. The extracted DNA was suitable for PCR amplification. Silica gel preservation is supported as a practical and cost-effective alternative to liquid nitrogen. Overall, the protocol demonstrates robustness, reliability, and reproducibility, with strong potential for application in *V. meridionale* and other plant species rich in secondary metabolites.

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