# OTIMIZAÇÃO DA EXTRAÇÃO E CROMATOGRAFIA PARA DETECÇÃO DE ÁCIDO ABSCÍSICO SOB SALINIDADE

Ianah de Sousa Braga Marcilon[1], Isabelle Mary Costa Pereira[2], Luan Victor Maia[2], Andel Cabral Nery[1], Carolina da Silva Evaristo[3], Humberto Henrique de Carvalho[4]

**RESUMO:** Dada a importância do ácido abscísico (ABA), sua quantificação é essencial para compreender como sinais ambientais alteram suas concentrações nos tecidos. A cromatografia líquida de alta eficiência (CLAE) oferece método confiável e quimicamente estável, garantindo integridade e precisão dos dados. Assim, este trabalho objetivou padronizar a preparação de amostras e as condições cromatográficas para quantificação de ABA em biomassa foliar reduzida de diferentes espécies sob estresse salino. Plantas de *Solanum lycopersicum*, *Vigna unguiculata* e *Zea mays* foram cultivadas em casa de vegetação e tratadas com 100 mM de NaCl por sete dias. As folhas foram coletadas e submetidas a diferentes métodos de extração, solventes e estratégias de ressuspensão. Os extratos foram analisados por CLAE com variações na fase móvel e vazão. O milho apresentou maior rendimento e pico mais nítido, apesar da interferência da matriz. O método mostrou forte linearidade ($R^2$ = 0,9818) e tendência de aumento de ABA sob estresse salino. Solvente, fase móvel, fluxo e idade da amostra influenciaram o desempenho. No entanto, ainda são necessárias análises adicionais para maior reprodutibilidade.

**PALAVRAS-CHAVE:** ABA, HPLC, Estresse salino.

# OPTIMIZATION OF EXTRACTION AND CHROMATOGRAPHIC PROCEDURES FOR DETECTION OF ABSCISIC ACID UNDER SALINITY

**ABSTRACT:** Given the importance of abscisic acid (ABA), its quantification is essential to understanding how environmental signals alter its concentrations in tissues. High-performance liquid chromatography (HPLC) offers a reliable and chemically stable method, ensuring data

[1] Graduação em biotecnologia – UFC; Graduando; ianahmarcilon@gmail.com; andelcabralnery@gmail.com;
[2] PPG Bioquímica – UFC; Mestrado; isabellemcpereira@gmail.com; maialuansax@gmail.com;
[3] Graduação em agronomia – UFC; carolinadasilvaevaristo@alu.ufc.br;
[4] Professor do departamento de Bioquímica e Biologia Molecular – UFC; humberto.carvalho@ufc.br;

integrity and accuracy. Therefore, this study aimed to standardize sample preparation and chromatographic conditions for ABA quantification in reduced leaf biomass of different species under salt stress. *Solanum lycopersicum*, *Vigna unguiculata*, and *Zea mays* plants were grown in a greenhouse and treated with 100 mM NaCl for seven days. The leaves were collected and subjected to different extraction methods, solvents, and resuspension strategies. The extracts were analyzed by HPLC with variations in the mobile phase and flow rate. Corn showed higher yield and a sharper peak, despite matrix interference. The method showed strong linearity ($R^2$ = 0.9818) and a tendency for ABA to increase under salt stress. Solvent, mobile phase, flow rate, and sample age influenced performance. However, further analysis is still needed for greater reproducibility.

**KEYWORDS:** ABA, HPLC, Salt stress.

## INTRODUCTION

The accumulation of salt in the soil significantly reduces the water potential around plant roots, negatively affecting water uptake and overall plant development (Acosta-Motos et al., 2017; Zhu, 2016). This osmotic stress, combined with ionic toxicity caused by excess sodium and chloride ions, results in cellular damage, impaired metabolism, and reduced crop yields. To cope with these adverse conditions, plants activate a complex set of physiological and molecular responses aimed at maintaining homeostasis and enhancing tolerance (Tiwari et al., 2017). These responses include modulation of gene expression patterns (Yoshida et al., 2014), biosynthesis of protective primary and secondary metabolites (Batista et al., 2019), and dynamic regulation of phytohormones, which serve as central signaling molecules and biomarkers in stress adaptation (Gupta et al., 2020). Abscisic acid (ABA) is a key phytohormone that plays an essential role in mediating plant responses to salinity and drought stress. It regulates stomatal closure to reduce transpiration and water loss, thus maintaining plant water status and ionic balance under challenging environmental conditions (Haverroth et al., 2023). Moreover, ABA modulates the expression of stress-responsive genes and coordinates metabolic adjustments that help plants tolerate salinity. Given its importance, accurate quantification of endogenous ABA is critical for understanding how environmental cues influence hormonal signaling and plant adaptive mechanisms (Verslues, 2016). Despite advances in detection methods, quantifying ABA remains challenging due to its low concentration in plant tissues, chemical instability, and interference from complex plant

matrices. Conventional methods such as GC-MS and ELISA, while sensitive, require expensive equipment and reagents, limiting their accessibility and scalability (Bordin et al., 2015). High-performance liquid chromatography (HPLC), particularly using C18 reversed-phase columns, offers a practical alternative due to its balance of sensitivity, specificity, and cost-effectiveness, along with compatibility for thermolabile compounds like ABA (Kim et al., 2019). A significant challenge in ABA quantification is analyzing samples with limited biomass, such as small leaf tissues from certain species or developmental stages (Wang et al., 2023). This necessitates optimization of both extraction protocols and chromatographic conditions to maximize recovery, minimize degradation, and reduce matrix interference. Key factors include solvent selection, sample preparation steps, mobile phase composition, flow rate, and detection wavelength, all of which influence analytical performance. Therefore, this study aimed to develop and standardize a reliable method for ABA extraction and quantification by HPLC in small leaf samples from various plant species subjected to salt stress. The methodology focuses on preserving ABA stability during extraction and analysis while improving sensitivity and reproducibility. The optimized protocol intends to facilitate ABA quantification in physiological and biochemical research, contributing to a better understanding of plant stress responses and advancing agricultural practices focused on crop resilience.

## MATERIAL E MÉTODOS

The experiment was carried out in a greenhouse at the Plant Physiology Laboratory (LabFiVe), located at the Federal University of Ceará (UFC), in Fortaleza, Ceará, Brazil, under controlled conditions, using vermiculite as substrate and a hydroponic system supplied with Clark's nutrient solution (1975). Three plant species were evaluated: *Solanum lycopersicum* L. (tomato), *Vigna unguiculata* L. (cowpea), and *Zea mays* L. (maize). All plants were subjected to salinity stress by adding 100 mM NaCl to the nutrient solution for a continuous period of seven days. After this period, fresh leaf samples were collected for subsequent ABA extraction and quantification. The collected leaves were processed immediately or stored under conditions that preserved their chemical integrity until analysis.

Samples were first ground in liquid nitrogen to obtain a fine and homogeneous powder, preventing ABA degradation during handling. For each extraction, 0.25 g or 0.5 g of the powdered material was weighed into pre-labeled 2 mL microtubes. To each sample, 1,500 µL of HPLC-grade organic solvent was added to facilitate efficient phytohormone extraction. At

this stage, three solvent systems were tested: pure acetonitrile, pure methanol, and 80% methanol diluted in ultrapure water. After solvent addition, each sample was homogenized using a vortex mixer to ensure complete interaction between the plant tissue and the extraction solvent. The mixtures were then centrifuged, and the resulting supernatants were carefully transferred to vials containing inserts for chromatographic analysis. Before injection, additional resuspension conditions for the concentrated extracts were evaluated, including the use of methanol, 80% methanol, acetonitrile, and 60% acetonitrile.

Chromatographic analyses were performed using a high-performance liquid chromatography (HPLC) system to evaluate the effect of different operational parameters on ABA detection. Flow rates of 0.1, 0.4, 0.6, and 1.0 mL $min^{-1}$ were tested, as well as different mobile phase compositions: methanol + acetonitrile and acidified water + acetonitrile. Each sample was injected at a volume of 30 µL. For ABA quantification, a standard calibration curve was prepared using analytical-grade (+)-Abscisic acid (Supelco) at concentrations ranging from 0.5 to 250 µg $mL^{-1}$. Each concentration point was analyzed under the same chromatographic conditions as the plant extracts to ensure consistency in quantification. Among the three species tested, maize was selected for the final analyses because it yielded the highest ABA extraction efficiency and presented the most clearly resolved chromatographic peaks. In this phase, four biological replicates were analyzed for each treatment.

## RESULTS AND DISCUSSION

Samples from different plant species were extracted using the previously described combination of methods. The resulting chromatograms revealed notable differences in the chromatographic response for ABA separation, both before and after co-injection with the abscisic acid (ABA) standard. In leaf samples of cowpea, the initial chromatographic profile appeared confusing, with multiple peaks outside the expected retention time. Following co-injection with the standard, a peak consistent with ABA was detected. However, it remained overlapped with interfering signals, possibly due to the immaturity of the leaf tissue used. In tomato, a small peak was observed at the retention time corresponding to the ABA standard, but with very low intensity. After co-injection, this peak exhibited a marked increase in signal, confirming the presence of ABA, at low concentrations. Among the tested species, maize showed the most favorable chromatographic behavior, with a clearly distinguishable peak at the expected retention time (Figure 1).

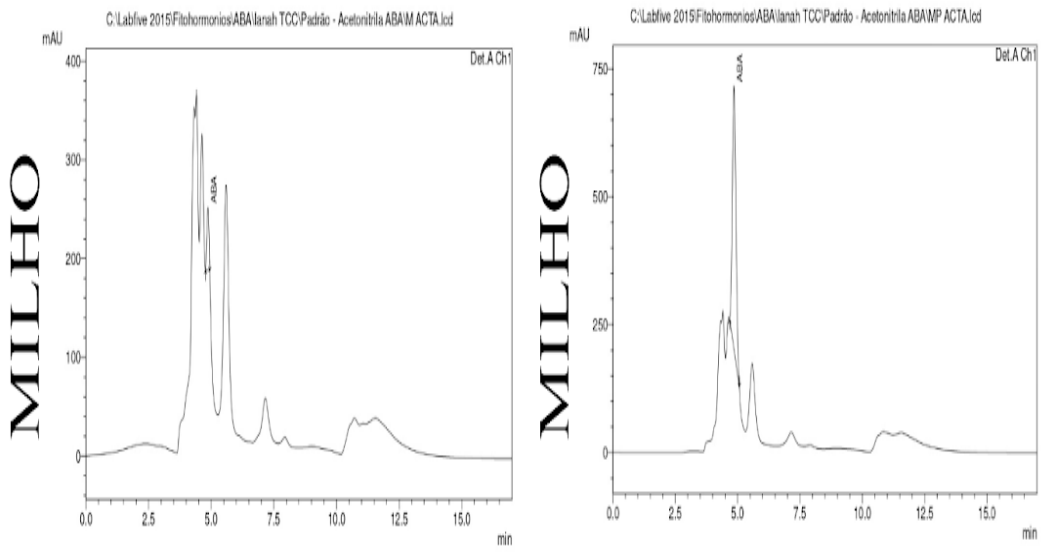


**Figura 1.** The chromatogram of Maize without standard and ABA standart

The standard calibration curve demonstrated a clear and proportional relationship between the known concentrations of ABA, ranging from 0 to 25 µg/mL, and the corresponding chromatographic peak areas obtained for each standard sample (Figure 2). As ABA concentration increased, the peak area also increased progressively and consistently, with a stable retention time of approximately 4.8 minutes across all injections. The resulting regression line followed the equation Y = 226951x + 121618 and yielded a determination coefficient ($R^2$) of 0.9818, which indicates a strong linear correlation and minimal deviation between the observed and expected values. This level of linearity demonstrates that the method is not only analytically sound but also highly reliable for quantitative purposes. The robustness of the curve allows accurate calculation of ABA concentration in plant samples based on peak area, even in low-concentration contexts. Furthermore, the high $R^2$ reinforces the reproducibility of the method and suggests that the system's sensitivity and stability are suitable for routine phytohormone analysis under similar conditions. Collectively, these results support the use of this approach for consistent ABA quantification in different species, provided that matrix effects are accounted for in sample preparation (Figure 3).

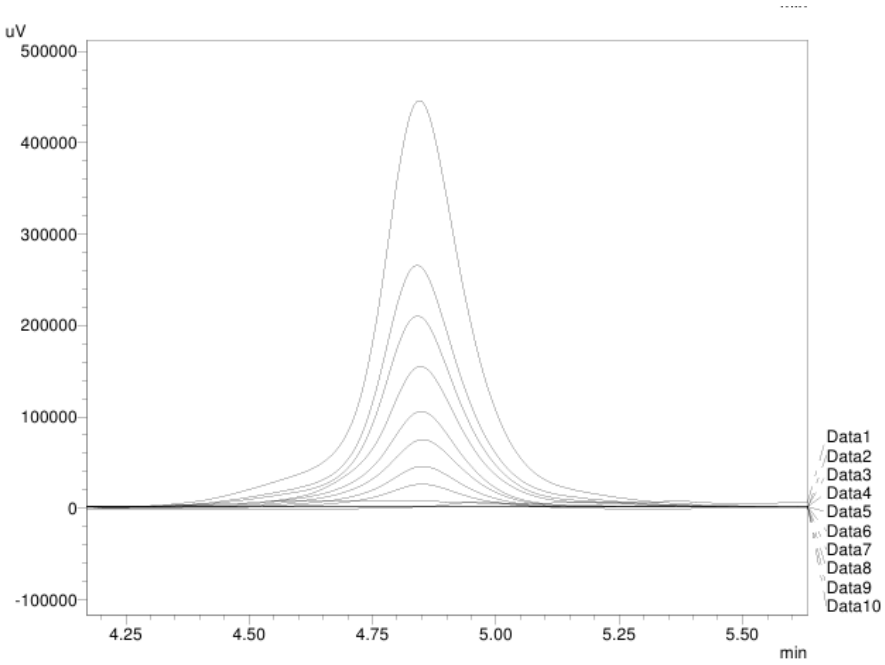


**Figura 2.** Overlaid chromatograms of ABA standard solutions at different concentrations.

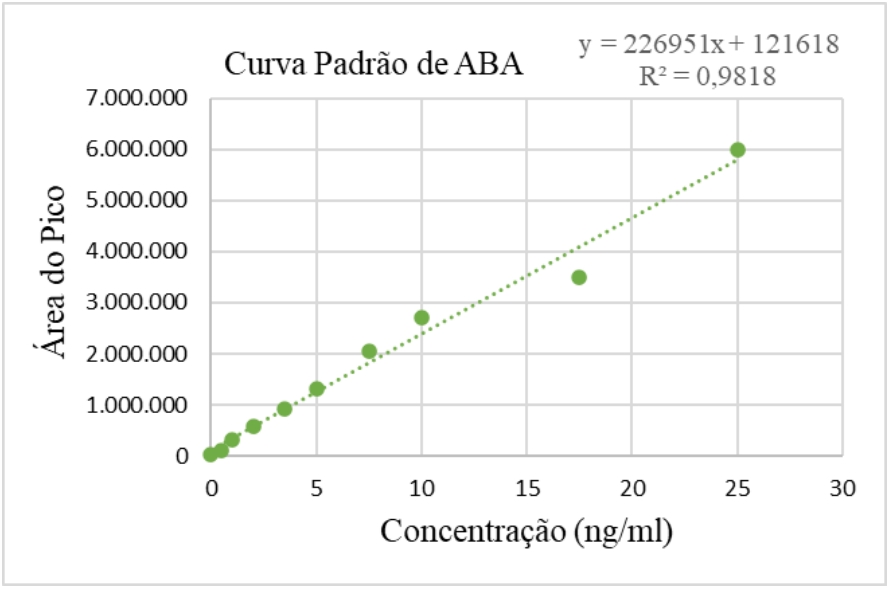


**Figura 3.** Graphical representation of the ABA standard curve.

These findings reinforce that the observed differences in chromatographic behavior among species are influenced not only by ABA content but also by matrix effects that vary according to leaf composition and developmental stage. The clear peak resolution in maize supports its selection as a model for testing extraction and chromatographic conditions, while the less favorable results in cowpea and tomato highlight the challenges of analyzing species

with higher background interference. This comparison provides a basis for optimizing analytical parameters and adapting the method to different plant matrices in future applications.

## CONCLUSION

This study aimed to optimize an efficient method for abscisic acid (ABA) quantification across different plant species by standardizing extraction procedures and chromatographic conditions. Solvent selection, mobile phase composition, flow rate, and sample age proved to be critical variables influencing analytical performance and data consistency. Among the tested species, maize leaves produced the most consistent results, with well-resolved chromatographic peaks and a trend toward increased ABA levels under salt stress, although these differences were not statistically significant. These findings indicate that the proposed protocol is suitable for applications involving limited plant material and is particularly effective in species with physiological traits similar to maize. Although the standard curve demonstrated strong linearity ($R^2 = 0.9818$), the variability observed among biological replicates suggests the need for further investigations aimed at minimizing matrix interference and refining sample preparation steps to achieve greater consistency in the results.

## REFERÊNCIAS BIBLIOGRÁFICAS

ACOSTA-MOTOS, J. R. et al. PLANT RESPONSES TO SALT STRESS: ADAPTIVE MECHANISMS. **AGRONOMY**, BASEL, V. 7, N. 1, P. 18, 2017.

BATISTA, V. C. V. et al. SALICYLIC ACID MODULATES PRIMARY AND VOLATILE METABOLITES TO ALLEVIATE SALT STRESS INDUCED PHOTOSYNTHESIS IMPAIRMENT ON MEDICINAL PLANT EGLETES VISCOSA. **ENVIRONMENTAL AND EXPERIMENTAL BOTANY**, V. 167, P. 103870, 2019.

BORDIN, D. et al. ESTRATÉGIAS ANALÍTICAS APLICADAS À QUANTIFICAÇÃO DE HORMÔNIOS VEGETAIS. **QUÍMICA NOVA**, V. 38, N. 9, P. 1229–1237, 2015.

CLARK, R. B. CHARACTERIZATION OF PHOSPHATASE OF INTACT MAIZE ROOTS. **JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY**, V. 23, N. 3, P. 458–460, 1975.

GUPTA, A. et al. ROLE OF PHYTOHORMONES AND PLANT GROWTH-PROMOTING RHIZOBACTERIA IN STRESS MANAGEMENT. **PLANT CELL REPORTS**, V. 39, N. 12, P. 1553–1572, 2020.

HAVERROTH, M. et al. ESTRESSES AMBIENTAIS E SUAS INFLUÊNCIAS NA FISIOLOGIA VEGETAL: UMA ABORDAGEM SOBRE ESTRESSES HÍDRICOS E TÉRMICOS. **REVISTA AGROPECUÁRIA CATARINENSE**, V. 36, N. 2, P. 41–46, 2023.

KIM, J. et al. **ADVANCES IN DERIVATIZATION TECHNIQUES FOR GC–MS-BASED METABOLOMICS**. TRAC – TRENDS IN ANALYTICAL CHEMISTRY, V. 118, P. 706–716, 2019.

TIWARI, S. et al. STRESS RESPONSIVE MICRORNAS IN PLANTS: ROLES IN STRESS REGULATION AND ADAPTATION. **FRONTIERS IN PLANT SCIENCE**, V. 8, 2017.

VERSLUES, P. E. ABA AND CYTOKININS: CHALLENGE AND OPPORTUNITY FOR PLANT STRESS RESEARCH. **PLANT MOLECULAR BIOLOGY**, V. 91, P. 629–640, 2016.

WANG, Y. et al. MINIATURIZED ANALYTICAL SYSTEMS FOR PLANT HORMONE QUANTIFICATION. **PLANT METHODS**, V. 19, P. 56–66, 2023.

YOSHIDA, T. et al. ABA-DEPENDENT AND ABA-INDEPENDENT SIGNALING IN RESPONSE TO OSMOTIC STRESS IN PLANTS. **CURRENT OPINION IN PLANT BIOLOGY**, V. 21, P. 133–139, 2014.

ZHU, J. K. ABIOTIC STRESS SIGNALING AND RESPONSES IN PLANTS. **CELL**, V. 167, N. 2, P. 313–324, 2016.