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Determination of Boron Content Using a Simple and Rapid Miniaturized Curcumin Assay

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[Abstract] To determine boron quantity in soil, water and biological samples, several protocols are available. Colorimetric assays are the simplest and cheapest methods which can be used to determine boron concentration. However, published protocols do not give straightforward guidance for beginners to adopt these protocols for routine use in the laboratory. Based on a previously published available procedure, we present a detailed and modified version of a curcumin based colorimetric protocol to determine boron concentration extracted from any sample. Our modified protocol is able to determine up to 0.2 nmole of Boron in a sample volume of 300µl.

Keywords: Boron, Curcumin method, Protoplasts, *Arabidopsis*, Yeast

[Background] Boron (B) can be quantified using spectrometric and colorimetric methods. Inductively coupled plasma mass spectrometry (ICP-MS) is the most sensitive method currently available having a detection limit of 0.01 mg/L (Kmiecik *et al*., 2016) but requires a sample volume of 5 ml. However, this technique requires sophisticated and expensive equipment which is not affordable for smaller laboratories. Alternatively, colorimetric based assays using curcumin or Azomethine-H dyes can be used for the routine analysis of boron in all laboratories with access to a spectrophotomer that can measure absorbance at 550 nm. Bingham (1982) reported that the curcumin assay is more efficient than the Azomethine-H based assay. Therefore, we present a modified version of simple and rapid curcumin assay to quantify B based on a protocol originally published by Wimmer and Goldbach (1999). We have used this protocol to determine the intracellular boron in yeast cells and Arabidopsis protoplasts. Additionally, this protocol can be used to study the uptake, root to shoot translocation-mechanisms of Boron in plants.

Materials and Reagents

1. Falcon tube
2. 1.5 ml tubes
3. Boric acid (H_3BO_3) (Sigma, catalog number: 15663)
4. 0.1 N hydrochloric acid (HCl)
5. Concentrated sulfuric acid (H_2SO_4) (Fisher Scientific, catalog number: 10294300)
6. Concentrated acetic acid (Fisher Scientific, catalog number: 10304980)
7. 2-ethyl-1,3-hexanediol (Acros organics, catalog number: 118512500)
8. Chloroform (Fisher Scientific, catalog number: 10122190)

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9. Curcumin (Alfa Aesar, catalog number: B2157)
10. Methyl-Isobutyl-Ketone (MIBK) (VMR Chemicals, catalog number: MK624704)
11. Extraction solution (see Recipes)
12. Acid mixture (see Recipes)
13. Curcumin solution (see Recipes)

Equipment

1. Centrifuge
2. Microplate reader (CLARIOstar®)
3. 96-Well UV Microplate (Thermo Scientific™, catalog number: 11670352)

Procedure

1. Harvest the yeast/protoplasts samples by centrifugation and resuspend the pellet in 400 µl of Milli-Q water. Heat the samples at 90 °C for 30 min and vortex to release intracellular B in to solution. Centrifuge at maximum speed and collect 300 µl of supernatant for B quantification
   Note: We recommend that users test the assay with their own buffers/solvents if they do not use water to solubilise their samples.
2. Prepare standards containing 0, 0.02, 0.05, 0.10, 0.19, 0.39, 0.77 mg boric acid/L using Milli-Q water in a Falcon tube (Refer Table 1).

Table1. Boric acid standards presented in different units

Boric acid µM	Boric acid mg/l	nmole B/300 µl
0.00	0.00	0.0
0.39	0.02	0.1
0.78	0.05	0.2
1.56	0.10	0.5
3.13	0.19	0.9
6.25	0.39	1.9
12.50	0.77	3.7

3. Aliquot 300 µl (which is equivalent to 0.1, 0.2, 0.5, 0.9, 1.9, 3.7 nmole B) of each standard and samples in to 1.5 ml tubes.
4. Acidify the samples and standards by adding 100 µl of 0.1 N HCl. Mix well by vortex and incubate for 5 min at room temperature.
   Note: From step 3 onwards perform all the reactions in chemical fume hood.
5. Add 70 µl extraction solution (see Recipes), mix vigorously by vortexing for 30 sec. After 2 min repeat the vortex for another 30 sec.

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6. Centrifuge the samples at maximum speed for 5 min to facilitate clear separation of organic phase.
7. Pipette 50 µl of lower organic phase (Figure 1A) into a new 1.5 ml tube containing 200 µl of acid mix (see Recipes) and mix well by vortexing.

   Notes:

   a. *Be aware that the lower phase contains chloroform and that chloroform leaks out from plastic pipette tips. To avoid pipetting errors, make sure that the tip is tightly attached to pipette and transfer liquids quickly.*
   b. *The mixture of sulphuric acid and acetic acid is a viscous solution. Therefore, care must be taken while pipetting to avoid pipetting errors and hazards to users from corrosive solutions. We recommend to cut the tip of the 1 ml tip and pipette slowly in a fume hood.*

1. Add 250 µl of curcumin solution (see Recipes) and shake well until it forms homogenous dark purple colour (Figure 1B).
2. Briefly centrifuge the tubes and incubate the reaction mixture at room temperature for 1 h.
3. After 1 h stop the reaction by adding 500 µl of Milli-Q water and mix well by inverting the tubes.
4. Briefly centrifuge the tubes to facilitate clear phase separation.
5. Carefully pipette 200 µl of upper phase (Figure 1C) into 96-well UV Microplate and measure the absorbance at 550 nm using a microplate reader.

   Note: Choloroform in the reaction mixture melts many types of plastic microplates and intereferes in absorbance reading. Therefore, it is very important to use microplasts resistant to organic solvents (for example, Thermo Scientific™ 96-Well UV Microplate (Product code: 11670352) or a quartz microplate).
6. Plot the absorbance against quantity of B and prepare the calibration curve as shown in Figure 2.
7. Use the obtained calibration curve to determine the quantity of B in unknown samples.

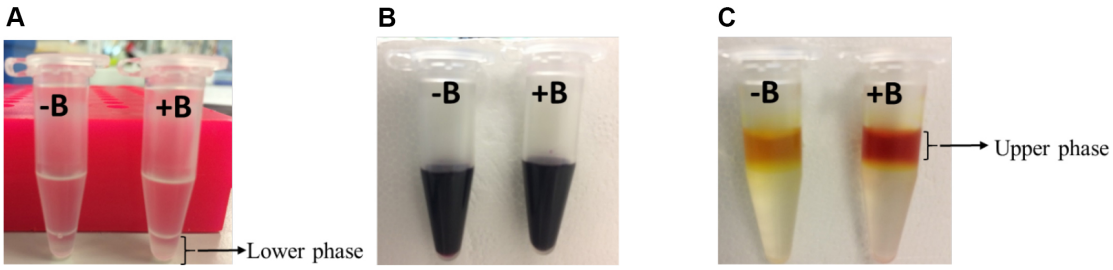

Figure 1. Steps showing phase separation and colour development in the absence (-B) or presence (+B) of boron

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Data analysis

To obtain calibrartion curve, plot the abosrbance values (A_{550nm}) against quantity of boron (nmole B) using a spreadsheet.

nmole B	A550nm
0	0
0.1	0.02
0.2	0.05
0.5	0.10
0.9	0.20
1.9	0.37
3.7	0.76

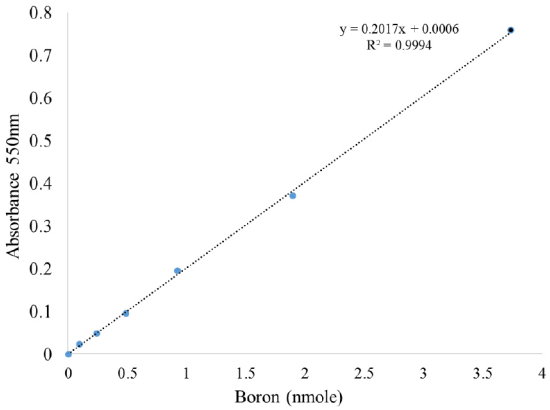

Figure 2. Calibration curve for the determination of boron concentration. Absorbance measured at 550 nm.

Recipes

1. Extraction solution
   Dissolve 2-ethyl-1,3-hexanediol 10% (v/v) in chloroform
2. Acid mixture
   Mix sulphuric acid (conc.) and acetic acid (conc.) in 1:1 (v/v) ratio in a Falcon tube
3. Curcumin solution
   Dissolve curcumin (2 mg/ml) powder in MIBK

Acknowledgments

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Authors declares no conflict of interest.

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