CHAPTER 2

EXPERIMENTAL

2.1 Apparatus

1) Micropipettes: 100 µL, 1000 µL and 10 mL Brand, Germany
2) GF/A filter papers, Whatman International Ltd., England
3) 10, 50, 100, 250, 1000 mL volumetric flask, Duran, Germany
4) 50, 100, 250 mL beaker, scott Duran, Germany
5) 0.45 µm disposable syringe filter cellulose acetate, Chrom Tech, U.S.A.
6) 25 mL PP volumnetric flask, Vit Lab, Germany
7) 10, 5 mL graduate pipette, Witeg, Germany
8) 2000 mL volumetric flask, Technico, England
9) 1000, 2000 mL bottle, Duran, Germany
10) Plastic bag (zip log)
11) Plastic pots
12) Light Microscope, Model CX21FS1, Olympus Co., Japan

2.2 Chemicals and Materials

1) Nitrate (NO_3^-) standard solution 1000 ppm, Merck, Germany
2) Nitrite (NO_2^-) standard solution 1000 ppm, Merck, Germany
3) Sulfuric acid (H_2SO_4), 95-97%, Merck, Germany
4) Nitric acid (HNO_3), 65%, Merck, Germany

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5) Sodium carbonate anhydrous (Na_2CO_3), 99.9%, Scharlau, Spain
6) Sodium hydrogen carbonate ($NaHCO_3$), 99.7%, Scharlau, Spain
7) Milli Q Water, Chemistry Department, Chiang Mai University.
8) Copper powder
9) Deionized water (conductivity < 0.15 mS/m), Chemistry Department, Chiang Mai University
10) Salfanin O solution (1%w/v)
11) Soil, obtained from high content of humus and organic matter

2.3 Equipments

1) Ultrasonicator, P 300 H, Elma, Germany
2) Ultrasonicator, T 490DH, Elma, Germany
3) Oven, model 100-800, Memmert, Germany
4) Analytical balance, AB304-S, Mettler Toledo, Switzerland
5) Chamber, consisting of
    a) Gas analyzer, 350-XL, Testo, Germany
    b) Thermometer
    c) Vacuum pump, Rocker 100-800, Memmert, Germany
    d) Barometer
    e) Minifan, Super mute BB-816, China
    f) Hygrometer
    g) Desicator

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2.4 Instruments

1) Ion chromatograph, Model 2.733.0020, Metrohm, Switzerland, consisting of

a) 838 Advanced Sample Processor

b) Anion guard column, Metrosep A Supp 4/5 Guard

c) Anion separation column, Metrosep A supp5 250/4.0 mm

d) Anion self-generating Supperssor

e) Injection valve, Model C 2-2346DK

f) Output, MagIC Net 2.6 program

2.5 The Selected Plants for NO_2 Absorption

Two plant species, namely dumb cane (*Dieffenbachia seguine*) and little prayer plant (*Calathea vaginata* Petersen) are common ornamental plants in Thailand and can grow well indoor. The scientific classification of dumb cane and little prayer plants are shown in Table 2.1.

2.5.1 Dumb cane (*Dieffenbachia seguine* (Jacq.) Schott)

Dumb cane is a very common ornamental plant. The leaf of dumb cane is broad and has white streaks or speckles on a green background. Some leaves are green or cream-colored with a green border. The height can be from 60 cm up to 3 meters, while newer hybrids are more compact (about 30 - 60 cm height) as shown in Figure 2.1(a).

This plant originated in tropical America and is a popular ornamental plant used in home and office decoration. There are 25-30 species, but the two main species are *Dieffenbachia seguine* and *Dieffenbachia pincta* (McGovern, 2000). Dumb cane is

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a foliage plant, which can grow indoor. It grows well in bright, indirect sun-light and needs low amounts of water. It grows well in drained soil (Missouri Botanical Garden, 2013).

2.5.2 Little prayer plant (*Calathea vaginata* Petersen)

Little prayer plant (Figure 2.1(b)) is in the family of *Marantaceae*. It was first found in 1985 in Brazil, which is distributed from Bahia to Rio de Janeiro (World Checklist of selected plant families, 1889). It belongs to genus *Calathea*, which includes some of the most beautiful and striking tropical foliage plants in the world. *Calathea* species generally have boldly marked, upright, oblong leaves in a dazzling array of colors held on long, upright stalks. This plant can grow in light shade or dappled light indoors. There are many varieties of *Calathea*. such as *C. makoyana*, *C. allouia*, *C. zebrine* and *C. petersenii* etc. It has features purplish coloring on the undersides of leaves, with white and green on top. It is also known as the peacock plant. *C. zebrina*. (the zebra plant) has green markings on the leaf top and purple leaf undersides. *C. vaginata* has plain leaves, but displays upright orange-red flowers and *C. ornata*. has a reddish marking on leaf tops with purple undersides. *Calathea* has a reputation as a greenhouse plant because it grows best in warm, humid and bright stable conditions, but not direct sunlight (VanZile, 2013).

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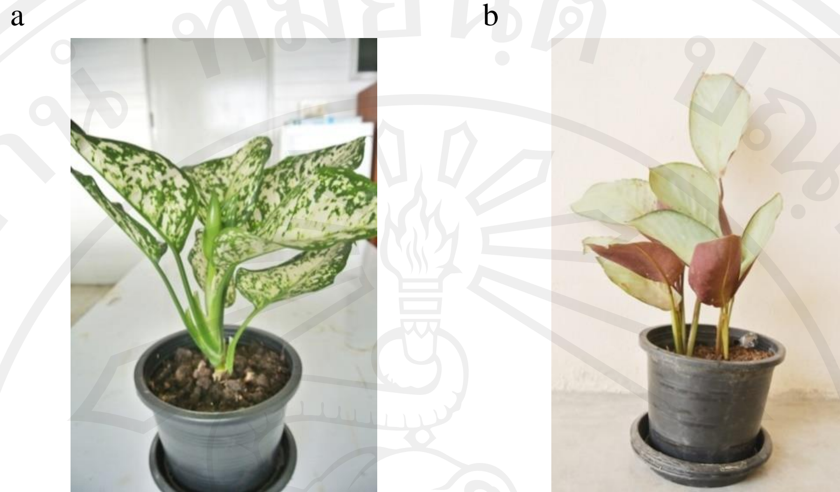

Figure 2.1 Sample plants: a) dumb cane and b) little prayer plant

Table 2.1 Scientific classification of dumb cane and little prayer plant (USDA, 2012; ITIS, 2010.)

Scientific Classification	dumb cane	little prayer plant
Kingdom	Plantae	Plantae
Subkingdom	Tracheobionta	Tracheophyta
Superdivision	Spermatophyta	Spermatophyta
Division	Magnoliophyta	Magnoliophyta
Class	Liliopsida	Liliopsida
Subclass	Arecidae	Zingiberidae
Order	Arales	Zingiberales
Family	Araceae	Marantaceae
Genus	*Dieffenbachia* Schott	*Calathea*
Species	*Dieffenbachia sequine* (Jacq.) Schott	*Calathea vaginata* Petersen

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2.6 Experimental Chamber

A chamber was constructed for testing the capability of selected plants on NO_2 absorption. The schematic diagram of the NO_2 experimental chamber is shown in Figure 2.2. The chamber was made from acrylic plates (thickness 0.4 cm) in order to minimize NO_2 absorption on the surface. The size of the chamber was 0.5 m (*W*)×0.5 m (*L*)×0.6 m (*H*) accounting for a total of 0.15 m^3. The chamber was equipped with a NO_2 sensor (Testo 350-XL, Germany), a thermometer, a barometer, a mini-fan and a hygrometer. The gas analyzer is the electrochemical cell, which create an output signal that is selective as well as proportional to the concentration of the gases in the combustion stream (ETV, 2013). An NO_2 generator and a pump (flow rate 0.037 m^3 /min) were also installed. NO_2 was generated by dissolving copper powder in nitric acid contained in a beaker inside a desiccators according to the following reaction (Boudreaux, 2013)

$$Cu(s) + 4HNO_3(aq) \longrightarrow Cu(NO_3)_2(aq) + 2NO_2(g) + 2H_2O(g) \qquad \textbf{Eq. 2.1}$$

NO_2 was pumped into the chamber and it was diluted with air until a concentration of approximately 20 ppm and 40 ppm was reached. Concentration levels were measured with a gas analyzer every 30 minutes over 8 hours. The temperature inside the chamber was kept at 28 ± 2^oC, while the relative humidity was recorded at $67\pm2\%$. The plant was placed inside the chamber in order to test for the capability of NO_2. The NO_2 exposure chamber system constructed was then used to test for the capability of selected plants under controlled conditions of NO_2 concentration, temperature and humidity.

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Before the experiment, plants in plastic bags from the nursery were transferred to the new pots containing soil with a high content of humus and organic matter. They were left indoor with an appropriate amount of indirect sunlight for about 1 week and were watered every 2 days. After that, each plant was put inside the chamber for further experimentation. Each experimental condition was repeated 3 times using new plants. Before being tested, the leaves of each plant were cleaned with water to remove the dirt.

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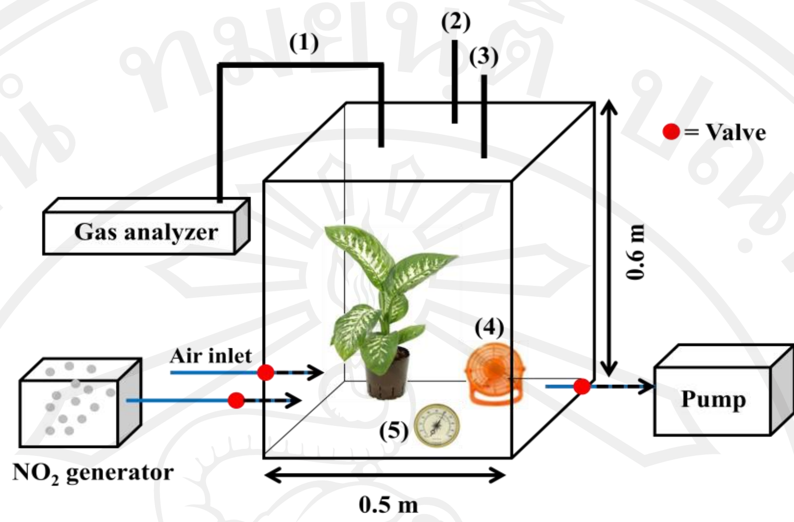
(a)



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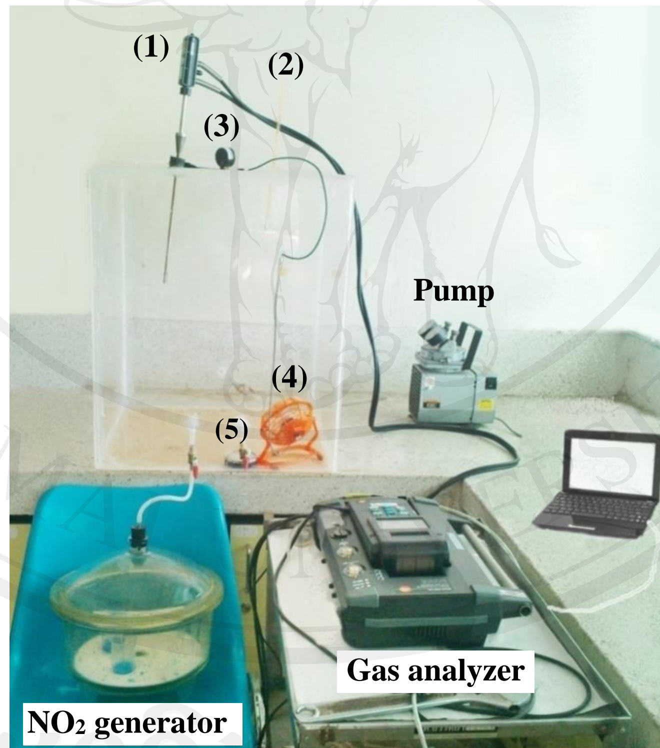

Figure 2.2 Nitrogen dioxide exposure chamber systems

a) schematic diagram of the system

b) picture of the chamber equipped with (1) NO_2 sensor (2) thermometer (3) barometer (4) minifan (5) hygrometer

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2.7 Testing of NO_2 Absorption by the Selected Plants in a Closed System

In order to test for the capability of plants for NO_2 absorption from the air in the closed system, 4 conditions were set up as shown in Figure 2.3. These conditions comprised 1) a control set; the chamber without a plant, 2) soil; a pot containing soil without a plant, 3) soil and plant; a pot containing soil and a plant and 4) plant; a pot containing soil and a plant with a plastic bag covering the pot to prevent gas absorption through the soil media.

Dumb cane was tested in the chamber with NO_2 concentration levels beginning at 20 and 40 ppm for 8 hours (9.00 am - 5.00 pm). Three replications were carried out for each concentration, with the plant being replaced by a new one each test. After exposure, leaves of tested plants were observed for morphological changes and extracted for ion analysis by comparing with leaves unexposed to NO_2. The same experiment was carried out for little prayer plant. However, only 40 ppm NO_2 was tested as the preceding experiment with the dumb cane indicated more meaningful results as this NO_2 concentration.

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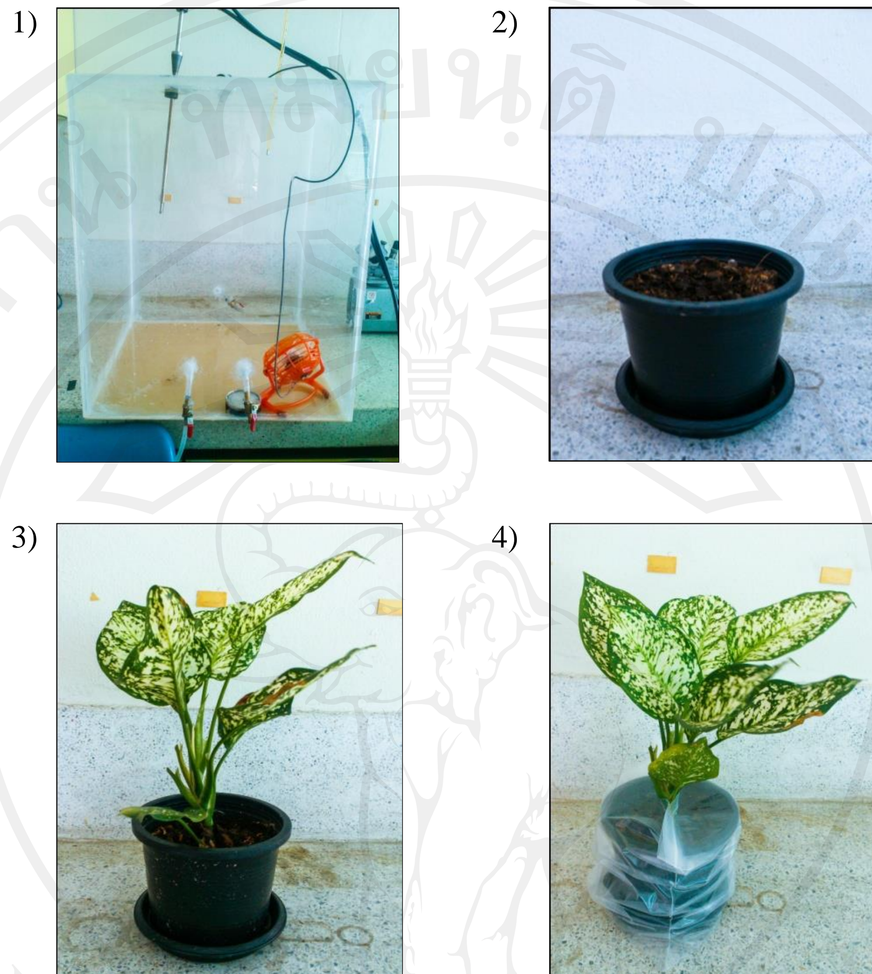

Figure 2.3 For conditions of the experiments for testing capacity of plants on NO_2 absorption in the sealed chamber

2.8 Physical Analysis of Leaves

2.8.1 Measurement of leaf area

Individual leaf area of each of the selected plants was determined by Image J 2X program (version 14.3). Each leaf was photographed on a white background with clear scale (Figure 2.4). The leaf area was calculated from the pixel value of a defined region (Ferreira and Rasband, 2010) and was converted from the actual pixel on the picture into the unit of millimeter (mm).

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(a) (b)

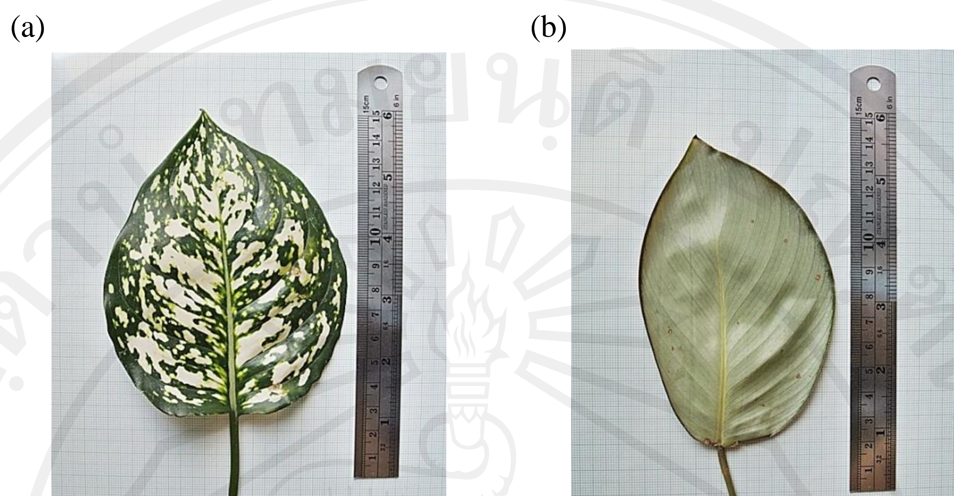

Figure 2.4 Image of leaf on a white background: a) dumb cane and b) little prayer plant

2.8.2 Morphological changes

Morphology of plants was observed before and after exposure to NO_2; the differences of plants, i.e. texture, color and healthy (% leaf necrosis) of leaves, were observed. In the case of dumb cane, peduncle was also observed

2.8.3 Anatomical changes

Free-hand sectioning refers to the process of making slices of tissues by hand using a razor blade. The advantage of this technique is that it is fast and easy. Fresh sections may be placed directly into stain, fixative, staining solution, or just immediately viewed. The disadvantages are variable section thickness or sections that are just too thick to see through under the microscope. Both of these defects hinder microscopic investigation. Interference contrast microscopy is especially degraded by thick sections. The most important avoid single-edge blade in half for safer cutting too and wet the blade with water before cutting tissue so that the section floats onto the blade rather than being compressed. Razor blades dull quickly therefore it is

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necessary to replace them frequently. Tissue samples are usually cut directly onto a wetted microscope slide. Relatively large tissues (>2 mm) can be supported by the forefinger with the finger nail or a coverslip used as a guide for the blade. The sections are cut with smooth strokes, starting with a thicker cut at the beginning microscope; somewhere near the end of the taper will be a portion of appropriate thickness (Ruzin, 1951).

The process of staining sections or whole tissues is as much art as science. Staining is a result of synergy between the added stain, frequently an added metal salt (mordant), length of time in the stain, stain “differentiation” by additional components, the amount of destaining (if appropriate), temperature of staining (for “reactive” dyes), and, of course, the tissue itself. Some dyes work best when overstained and subsequently washed from the tissues to reveal cellular structure (a regressive dye). Other dyes stain the tissue slowly enough so that the progression of staining may be watched overstaining, and loss of cellular detail, is almost imposible. Progressive dyes, on the other hand, are quite unforgiving. Once overstrained, tissue detail usually is lost permanently, (O’Brien and McCully, 1981). Among the myriad chemical stains available for histological investigation, plant microscopists have tended to limit their use to just a few-Safranin, Fast Green and Hematoxylin being the most prominent.

Anatomies of plant i.e., tissue, shape and color of plants were observed under microscope before and after exposure to NO_2 by free-hand section method. Size of stomata and guard cell were also observed.

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2.8.4 Measurement of number of stomata

In this work, leaf stoma of each plant type was measured for its density. Leaf sample sections of tissues were obtained using a razor blade and stained Safranin O (Ruszin, 1999). The steps taken include the following:

1) Fresh leaf samples (n=3) were directly peel off epidermis layer.
2) Drop the thin tissue section into a drop of water.
3) Sections from water were brought and stained in Safranin O 1 – 2 drops for 10 minutes. Excess stain was washed out for a few moments with DI water.
4) The section was fixed with glycerine.
5) The coverslip was mounted and the coverslip was sealed with nail polish.
6) The microscope slide was examined under a light microscope to at least 400x.
7) The stomta were counted in one microscopic field at 400x and the guard cell and stomata sizes were measured using an ocular micrometer.
8) The stoma densities were calculated in sq. mm from the average number/400x microscopic field.

2.8.5 Measurement of sizes of stomata

The sizes of stomata were calculated through a light microscope by measuring the guard cells and stoma size (Figure 2.5). The total stomatal number per leaf was estimated in terms of “absolute stomatal number”. The absolute stomatal number was estimated by multiplying an average stoma density in mm^2 with the leaf area (Gupta, 1961). Leaf stomata were observed under microscope. Stomata were stained with

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Safanin-O and counted. The relationship between NO_2 absorption and number of stomata for each plant (n=3) was investigated by ANOVA.

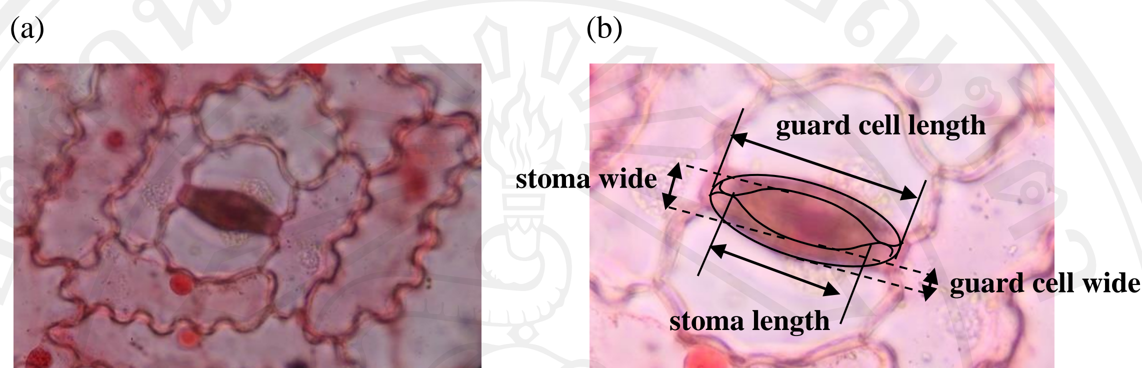

Figure 2.5 Sample of dumb cane photograph from microscope: a) stoma and b) size of guard cell and stoma

2.9 Analysis for Ion Content in Plant Leaves by Ion Chromatography

Ion chromatograph (882 Compact IC plus, Metrohm, Switzerland Figure 2.6) was used for the determination of anion (NO_2^- and NO_3^-) in and on plant leaves. The analysis conditions of the ion chromatograph are detailed in Table 2.2.

Figure 2.6 Ion chromatograph (Metrohm) for analysis of ion composition of leaf samples

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Table 2.2 Conditions of ion chromatograph for anion analysis (Sillapapiromsuk, 2013)

Analysis item	Anions
Eluent	3.2 mM Na_2CO_3/1.0 mM $NaHCO_3$
Guard column	Metrosep A Supp 4/5
Analysis column	Metrosep A Supp5 250/4.0 mm (250×4.0 mm)
Carrier material	Polyvinyl alcohol with quarternary ammonium groups
Suppressor	Anion self-regenerating suppressor with DI water/100mM H_2SO_4
Particle size	5 µm
Max. pressure	15 MPa
Flow rate	0.70 mL/min
Temperature	20-60 ºC
pH range	3-12
Injection loop	20.0 µL
Detector	Conductivity

2.9.1 Preparation of calibration curves and eluents

a) Calibration curve preparation

Before analysis of ion concentrations, a calibration curve for individual ion (NO_2^- and NO_3^-) species with 5-8 point of standard concentrations was constructed. The commercial individual ion standard solution (1000 µg/mL) from Merck Company was used for stock solution. Mixed anions standard solution (10 µg/mL) was prepared by pipetting 5.0 mL of the stock solution of each anion into a 50.0 mL volumetric flask and adjusting the volume with deionized water.

Working ranges of mixed standard solutions were 0.02-1.0 µg/mL. They were each injected onto the IC column under optimum conditions. The working standard solutions were freshly prepared form mixed stock standard solution by

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dilution every analysis day. Anion mixed standard was prepared in a polypropylene volumetric flask to avoid any problem associated with ion exchange. Then a calibration curve of each ion was drawn by plotting the peak areas against ion concentrations.

b) Preparation of mobile phase for ion analysis

A mixture of 1.70 mM $NaHCO_3$ / 1.8 mM Na_2CO_3 solution was used as an eluent for Metrosep A Supp5 250/4.0 mm column. The eluent stock solution was prepared by dissolving 1.4280 g sodium hydrogen carbonate ($NaHCO_3$) and 1.9085 g sodium carbonate anhydrous (Na_2CO_3) in deionized water and the resultant solution was diluted to 100.0 mL. This stock solution was then used to prepare a solution of 1.70 mM $NaHCO_3$ / 1.80 mM Na_2CO_3 by pipetting 10.0 mL of it into 1000 mL volumetric flask and the final volume was adjusted using milli Q water.

2.9.2 Limit of detection (LOD) and limit of quantification (LOQ) of IC for NO_2^- and NO_3^- analysis

The LOD is the lowest concentration of the analyte that can be detected with a given degree of confidence. LOQ is a parameter for quantitative assays for low levels of compounds in the sample matrices and it is used particularly for determination of products or low levels of active constituents in a product.

The LOD and LOQ were checked by injecting 10 times of the lowest concentration of mixed anions standard (0.06 µg/ml of NO_3^- and 0.02 µg/ml of NO_2^-) into the ion chromatographic system under the optimum conditions. The LOD was obtained from 3 times, while LOQ was obtained from 10 times, of standard deviation (SD) from measurement of standard solution (USEPA, 2010).

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2.9.3 Preparation of plant leaves for extraction and analysis for ion contents

After exposure of plants to NO_2 in the chamber, NO_2 can adsorb on plant and absorb through plant leaves. NO_2 can change into NO_2^- and NO_3^- when it react with water. Therefore both ions were analysed from leaves of the selected plants.

Three leaves were randomly selected from one individual plant (for both exposed- and unexposed plants) and cut from its stalk. Prior to use, leaves of plants were measured for leaf area. In the case of exposed plants, only the 40 ppm NO_2 condition was selected for this experiment.

In order to determine NO_2 adsorption on plant leaves, wash method was applied. A selected leaf was put in a 250 ml beaker. Then 60 ml of DIW was added. It was shaken for about 2 minutes to allow water to wash out any dust attached on the leaf surface. The leaf was taken out for further experiment, while water was filtered through a cellulose acetate membrane packed inside a syringe filter and ready for ion analysis.

To determine NO_2 absorption, the washed leaf was further tested. The leaf was cut into small pieces by cleaned scissors (Figure 2.7(a)) and into a 250 mL beaker and then extracted with 60 ml de-ionized water and covered with paraffin film prior to extraction by using an ultrasonicator (Figure 2.7 (b)) with the power effective of 380 Watt (P 300 H, Elma, Germany) for 30 minutes. The solution was filtered through a syringe filter containing a cellulose acetate membrane. The solution was then analyzed by IC.

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(a) (b)

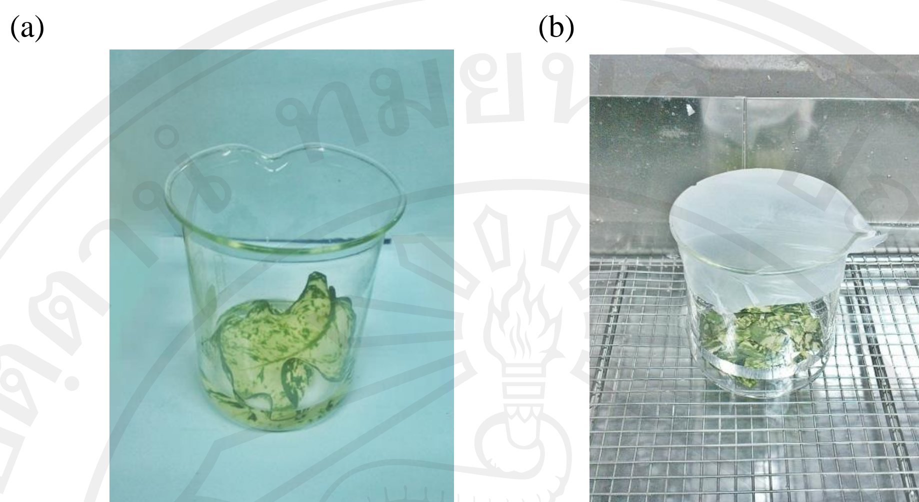

Figure 2.7 Preparation for determination of a) NO_2 adsorbed on plant leaf by wash method and b) NO_2 absorbed through plant leaf by extraction method

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