Concentrations of the combined pool of ammonia plus ammonium (NH\textsubscript{4}+NH\textsubscript{3}) in plant tissues are determined with increasing frequency by researchers and commercial laboratories. Total nitrogen content of plant tissue is routinely used as the basis for nitrogen fertilization recommendations, analysis of the nutritional quality of forage crops, and in research. The standard method for measuring total nitrogen is that of Kjeldahl (Bremner, 1965), which is based on quantitation of ammonium in an acid-digested sample or its distillate. Development of ammoniacal and urea fertilizers for soil and foliar nitrogen fertilization of crops has increased the demand for analysis of NH\textsubscript{4}+NH\textsubscript{3} in plant tissue samples. Brown and Zhang (1993) assessed ammonium accumulation in leaves as a diagnostic measure of optimal nitrogen fertilization of nectarines and almonds.

In addition, an increasing number of reports in the literature document that NH\textsubscript{4}+NH\textsubscript{3} accumulates in plant tissues in response to abiotic stresses. For example, Fraot and Tucker (1978) reported that bean (Phaseolus vulgaris) plants receiving ('Washington' navel orange leaves previously incubated in solutions of increasing NH\textsubscript{4}Cl concentrations were assessed. Procedures and instruments for quantifying NH\textsubscript{4}+NH\textsubscript{3} were tested for their sensitivity, reproducibility, and freedom from interference by amino acids. Reliable recoveries of NH\textsubscript{4}+NH\textsubscript{3}, free from amino acid interference, were obtained with oven-dried (60°C) leaves ground to pass through a 40-mesh screen, extracted by homogenization in 10% TCA or by shaking in 2% acetic acid, and then filtered and analyzed on the basis of differences in electrical conductance between the sample and the reference cell. Methods measuring NH\textsubscript{4}+NH\textsubscript{3} in KCl extracts by reaction with salicylate-nitroprusside in the presence of hypochlorite were compromised by significant color formation due to amino acids. Using fresh or freeze-dried leaf samples resulted in lower recoveries than use of oven-dried samples. Storage at -20°C of fresh or oven-dried leaf samples in 10% TCA before or after homogenization and filtration did not alter NH\textsubscript{4}+NH\textsubscript{3} levels, whereas storage of these samples at 4°C increased NH\textsubscript{4}+NH\textsubscript{3} levels.

Methods currently used for quantifying NH\textsubscript{4}+NH\textsubscript{3} in plant tissues were adapted from those used in analyzing biological fluids, water, or soil extracts. Reaction of ammonia with phenol in the presence of an oxidizing agent such as hypochlorite to produce an intense blue color, first described by Berthelot (1859), has been used to quantify ammonia in urine, blood, and tissue fluids (Harrov et al., 1933; Van Slyke and Hiller, 1933). Lubochinsky and Zalta (1954) improved the sensitivity of the method by introducing sodium nitroprusside as a catalyst in the reaction between ammonia and phenol for determining ammonia in rat tissues and human plasma. The modified method was subsequently used for analyzing whole blood (McCullough, 1967) and soil extracts (Kempers, 1974; Selmer-Olsen, 1971). Reardon et al. (1966) replaced noxious phenol with salicylate, which yielded an emerald green color as ammonia at basic pH reacted with salicylate in the presence of hypochlorite. This method was then used to measure ammonia derived from urea in biological fluids (Seamy et al., 1967) animal feeds (Wall and Gehrke, 1975; Wall et al., 1975), and soil extracts (Nelson, 1983). Due to increased demand for ammonia quantitation in medicine and agriculture in recent years, instruments have been developed for the automated analysis many samples colorimetrically or by electrical conductance.

In this communication, we used common procedures reported in the literature in the manner in which they are routinely used for quantifying NH\textsubscript{4}+NH\textsubscript{3} and compared their sensitivity, reproducibility, and freedom from interference by amino acids. Reliability of these procedures was not known. The sensitivity of each method reported in the Materials and Methods was determined experimentally as part of this study. In addition, we report the effects of different methods of tissue preparation, extraction, and storage on the recovery of NH\textsubscript{4}+NH\textsubscript{3} from citrus leaves incubated in solutions of increasing concentrations of NH\textsubscript{4}Cl to provide a range of tissue NH\textsubscript{4}+NH\textsubscript{3} concentrations.

Materials and Methods

Chemicals

Trichloroacetic acid (TCA) was purchased from Fisher Scientific (Pittsburgh). Amino acid standards (Sigma UltraPure, 99% by TLC; NH\textsubscript{3} < 0.05%) and all other chemicals used in this study.
were purchased from Sigma Chemical Co. (St. Louis). All solutions were made with ammonia-free deionized water. Whatman no. 1 filter paper was routinely analyzed for NH$_4$ + NH$_3$, using the extractant in each analytical procedure described below. The respective extractants in each procedure were used to prepare the standards.

**Plant material**
Mature leaves were collected from 30-year-old ‘Washington’ navel orange (Citrus sinensis L. Osbeck) or lemon scions (Citrus limon L. Burm. f.) on Troyer citrange rootstock (C. sinensis × Poncirus trifoliata L. Raf.) located at the Univ. of California, Riverside, Agricultural Experiment Station. The leaves were washed with soapy water and rinsed thoroughly with distilled water. The leaf samples used in all subsequent analyses were from one of the following two groups.

**Group I.** Collected leaves were divided into three aliquots, which were analyzed as fresh tissue or after being oven-dried at 60°C for 72 h or freeze-dried. Dried samples were ground in a Wiley mill to pass through a 40-mesh screen.

**Group II.** The upper portion of the leaf was excised 5 mm above the petiole, and the excised portion was rolled and inserted vertically into holes in the lid of a polyurethane box (38 × 14 × 14-cm, 5.5-liter) containing aerated NH$_4$Cl solutions (0 to 200 mM) maintained at 5.5 liters by daily addition of distilled water to insure that the cut ends of the leaves were immersed in the solutions at all times. The boxes were placed in a growth chamber at 30°C under continuous illumination (PFD of 3 to 10 µmol·m$^{-2}$·s$^{-1}$). At the end of 48 h the leaves were collected, washed thoroughly, blot-dried, cut into pieces (5 × 5 mm, midvein removed) and analyzed as either i) fresh tissue or ii) after being oven-dried at 60°C for 72 h or freeze-dried and ground with a Wiley mill to pass through a 40-mesh screen.

**Quantitation of amino acid contamination**
To determine whether any of the methods falsely detected amino acids as ammonia or caused their degradation and release of ammonia, commercial ammonia-free amino acid standards were analyzed. The amino acids and concentrations tested were those previously quantified by Rabe and Lovatt (1984) when leaf NH$_4$ + NH$_3$ content increased in young fully expanded leaves of 7-month-old phosphorus-deficient rough lemon seedlings (Citrus limon) (concentration, in µg amino acid/g dry weight citrus leaves, in parentheses): glutamine (346), glutamate (348), arginine (6970), ornithine (6745), citrulline (7009), proline (20937), serine (17313), lysine (10837), glycine (1307), and asparagine (2996). Samples were diluted before analysis to represent the amount of dry leaf sample typically analyzed by each procedure. Dilutions were made with the extractant used in each method.

**Analytical procedures**

**Technicon autoanalyzer.** By this method (method no. 329-74; Technicon Industrial Systems, Tarrytown, N.Y.), ammonia is determined colorimetrically based on the reaction of sodium salicylate-sodium nitroprusside with ammonia in the presence of hypochlorite to form a blue color. The combined pool of NH$_4$ + NH$_3$ was measured in a 200-mg dry weight citrus leaves, in parentheses: glutamine (346), glutamate (348), arginine (6970), ornithine (6745), citrulline (7009), proline (20937), serine (17313), lysine (10837), glycine (1307), and asparagine (2996). Samples were diluted before analysis to represent the amount of dry leaf sample typically analyzed by each procedure. Dilutions were made with the extractant used in each method.

**Wescan ammonia analyzer and Alltech inorganic nitrogen analyzer.** The Wescan ammonia analyzer (model 360; Alltech Associates, Deerfield, Ill.) and Alltech inorganic nitrogen analyzer (model 380; Alltech Associates) use a strong base, 11.3% (w/v) potassium hydroxide plus 1% (w/v) diethylenetriamine-pentaacetic acid (DTPA) to maintain the pH of the sample between 11 and 13 to convert all the ammonium ion present in the sample to ammonia green color in the presence of sodium hypochlorite at pH 13 was modified by E. Rabe (Univ. of Stellenbosch, South Africa, personal communication) for analysis of ammonia in plant tissue. Rabe eliminated ethylenediaminetetraacetic acid (EDTA) from the reaction mixture, and leaf samples were extracted in 1 N KCl as in the Technicon method instead of 2 N KCl (Nelson, 1983). A 500-ng oven-dried leaf sample was extracted in 50 ml 1 N KCl by reciprocal shaking for 30 min. The homogenate was filtered through Whatman no. 1 filter paper, 2.5 ml of filtrate fraction was transferred to a 50-ml volumetric flask containing 2.5 ml 1 N KCl and mixed gently, and 4 ml of a solution of 78% (w/v) sodium salicylate and 0.10% (w/v) sodium nitroprusside was added. After mixing thoroughly, 13 ml of deionized water was added to the sample followed by the addition of 2 ml of buffer comprised of 3% (w/v) sodium hydroxide, 7.5% (w/v) sodium phosphate, and 0.7% (v/v) sodium hypochlorite, adjusted to pH 13 with NaOH. The solution was incubated for 30 min in a waterbath at 37°C and cooled to room temperature, and absorbance at 667 nm was determined. The $A_{667}$ was linear for NH$_4$ + NH$_3$ concentrations between 0 and 0.5 µg·ml$^{-1}$. Sample filtrates were diluted with 1 N KCl to give values in this range.

**Distillation method.** This method is a modification of the Kjeldahl method (Bremner, 1965). Sample digestion is eliminated. Ammonia in the digested sample is released by distillation at a basic pH using NaOH instead of MgO, collected in a standard acid, and quantified by titration with potassium bichromate rather than sulfuric acid (Barker and Volk, 1964).

One gram fresh weight of ‘Washington’ navel orange leaves was homogenized with a Polytron tissue homogenizer (PCU, Brinkman Instruments, Westbury, N.Y.) in 10 ml 1 N KCl containing 0.025 M CuSO$_4$ and centrifuged, and the resulting supernatant was filtered through Whatman no. 1 filter paper. The ammonia content of the filtrate was measured after about 36 h storage on ice. For comparison, 1 g oven-dried or freeze-dried leaf samples were extracted in 50 ml 1 N KCl containing 0.025 M CuSO$_4$ by shaking in a reciprocal shaker for 30 min. The samples were filtered through Whatman no. 1 filter paper, and the filtrate was transferred to a 100-ml Kjeldahl flask.

In each case, 10 ml 40% (w/v) NaOH was added slowly to the filtrate, and steam was allowed to pass through the samples. The heat generated by the steam passing through the distillation unit and the high pH maintained by the NaOH resulted in the formation of ammonia gas, which was subsequently condensed and collected in a 125-ml Erlenmeyer flask containing 10 ml 2% (w/v) boric acid-indicator solution. The boric acid-indicator solution was prepared by adding 6.7 ml 0.2% (w/v) methyl red in 95% ethanol and 3.3 ml 0.2% (w/v) methylene blue in 95% (v/v) ethanol to 2% boric acid solution at final volume of one liter. The purple color of the absorbing solution changed to green in the presence of ammonia. Complete distillation of a sample took 7 to 10 min. The distillate was titrated with 0.014 M potassium bichromate until the color reverted back to the original purple color. The volume of the titrant was recorded, and the ammonia in the sample was calculated (Barker and Volk, 1964):

$$\text{mg NH}_4\text{-N/g} = (0.2 \text{ mg nitrogen/ml acid}) \times \text{ml titrant KH(IO}_3 \}) / \text{sample weight (g)}.$$

J. Amer. Soc. Hort. Sci. 120(5):871-876. 1995.
gas, which diffuses across a membrane and dissolves in the absorbing solution [1% (w/v) boric acid or deionized water for the two instruments, respectively]. The difference in the electrical conductance between the sample and reference cell is proportional to the ammonium concentrations in the sample (Carlson, 1978). The following flow rates (ml·min⁻¹) were used: sample, 1.32; base, 5.2; and absorbing solution, 1.32. The assay is linear for ammonium concentrations between 0 and 100 µg·ml⁻¹. Sample filtrates were diluted with their respective extracts (see below) to give values in this range.

**Tissue extraction.** Extractants and procedures commonly used for quantitating NH₄⁺, NH₃⁺ by electrical conductance (Wescan and Alltech) were compared. A 50-mg (dry weight) oven-dried leaf sample was extracted in either i) 50 ml 2% (v/v) acetic acid or ii) 1 N KCl by shaking for 10 min with a reciprocal shaker. With a 50-mg (dry weight) sample, shaking for 10 min in either extractant gave maximum recovery. In addition, fresh (1 g fresh weight), freeze-dried (1 g dry weight), or oven-dried (1 g dry weight) leaf samples were homogenized for 1 min in 5 ml 10% (w/v) TCA with a Polytron tissue homogenizer. The probe was rinsed with 5 ml 10% TCA, which was added to the homogenate. The homogenate was centrifuged at 10,000×g at 4°C for 10 min and filtered through Whatman no. 1 filter paper.

**Sample storage.** In anticipation of analyzing many samples, we evaluated possible points at which sample processing could be interrupted and the temperature at which samples could be stored without any negative effect on NH₄⁺/NH₃⁺ content.

Three grams of fresh ‘Washington’ navel orange leaves (midvein removed) or 1.5 g of oven-dried leaves were homogenized for 1 min with a Polytron tissue homogenizer in 15 ml 10% TCA. The probe was rinsed with 15 ml 10% TCA, which was added to the homogenate. The homogenate was centrifuged at 10,000×g at 4°C for 10 min and filtered through Whatman no. 1 filter paper. The filtrate was divided into three aliquots, which were processed as follows: i) analyzed immediately; ii) stored in a freezer (–20°C) and analyzed at the end of 1 week; iii) stored in a refrigerator (–20°C) and analyzed for NH₄⁺/NH₃⁺ at the end of 1 week.

In addition, 1 g of fresh ‘Washington’ navel orange leaves (midvein removed) was cut into pieces (5 × 5 mm) or 500 mg of oven-dried leaf samples was suspended in 5 ml 10% TCA and stored at 4 or –20°C. After 1 week, the samples were homogenized with a tissue homogenizer and the probe was rinsed with 5 ml of 10% TCA, which was added to the homogenate. The homogenate was centrifuged and filtered, and the ammonium content of the filtered acid soluble supernatant fraction was determined.

**Presentation of data.** The five analytical procedures used in this study quantified the combined pool of NH₄⁺/NH₃⁺ as either NH₄⁺ or dry weight tissue. NH₄⁺/NH₃⁺ content of a series of oven-dried standard leaf samples prepared by incubating leaves of the ‘Washington’ navel orange in NH₄Cl solutions from 0 to 200 mM for 48 h was determined using five analytic procedures: Technicon autoanalyzer, salicylate method, distillation, Wescan ammonia analyzer, and Alltech inorganic nitrogen analyzer. The results obtained with all five procedures differed quantitatively (Fig. 1). Despite the fact that samples were diluted to give values within the range of NH₄⁺/NH₃⁺ concentrations optimal for each method, the Technicon method was insensitive at the lower concentrations of NH₄⁺, and the salicylate method was insensitive at lower and higher concentrations of NH₄⁺. The NH₄⁺/NH₃⁺ content of the leaves assessed by the distillation procedure was significantly 2-fold greater than the Wescan ammonia analyzer, which was 1.6 to 1.8-fold greater than the Alltech inorganic nitrogen analyzer (P ≤ 0.05). No difference between the NH₄⁺+ NH₃⁺ content of the control leaves incubated in distilled water and that of leaves incubated in 12.5 mM NH₄Cl for 48 h was detected by any method, but all methods approximated 2500 µg NH₄⁺/NH₃⁺ dry weight for the 200 mM NH₄Cl treatment, except the salicylate method, by which only 1307 ± 24 µg NH₄⁺/NH₃⁺ dry weight was detected.

**Detection of amino acids as NH₄⁺/NH₃⁺ by five analytic methods.** Only the two procedures using electrical conductance did not detect amino acids as NH₄⁺/NH₃⁺ (Table 1). Results obtained with the Technicon autoanalyzer procedure were the most severely contaminated, detecting a net difference of 6168 µg·g⁻¹ dry weight (386-fold) more NH₄⁺/NH₃⁺ than the least compromised method, the Wescan ammonia analyzer (16 µg·g⁻¹). By the salicylate method, 1453 µg·g⁻¹ dry weight (92-fold) more NH₄⁺/NH₃⁺ was detected compared to the Wescan ammonia analyzer; for the distillation the difference was only 172 µg·g⁻¹ dry weight.

**Effect of leaf preparation method on the concentration of NH₄⁺/NH₃⁺.** Regardless of the method of NH₄⁺/NH₃⁺ analysis used, i.e., electrical conductance (Table 2) or distillation (Table 3), greater concentrations of NH₄⁺/NH₃⁺ were detected in oven-dried leaf samples compared to aliquots of the same leaves analyzed after freeze drying or immediately upon collection as fresh samples. Leaf NH₄⁺/NH₃⁺ was found to be evenly distributed within the leaf; e.g., apical and basal halves of leaves had equal concentrations. The midvein, about 10% of the total fresh weight of citrus leaves, contributed <10% of the NH₄⁺/NH₃⁺ content of the leaf per gram fresh weight.

**Effect of extractant and extraction procedure on leaf NH₄⁺/NH₃⁺ content.** The NH₄⁺/NH₃⁺ content of leaves incubated in NH₄Cl solutions (0 to 200 mM) was determined by Wescan ammonia analyzer for a 50-mg oven-dried sample extracted in either 50 ml 2% acetic acid or 50 ml 1 N KCl by shaking with a

---

Fig. 1. NH₄⁺/NH₃⁺ content of citrus leaves previously incubated in NH₄Cl solutions from 0 to 200 mM for 48 h determined by six analytical procedures. Vertical bars at given concentrations of NH₄Cl represent 1SD at P ≤ 0.05.
reciprocal shaker for 10 min or for a 500-mg oven-dried sample extracted in 10 ml 10% TCA by homogenization with a Polytron tissue homogenizer. No NH₄⁺ + NH₃⁺ was detected in leaves incubated in low concentrations of NH₄Cl (0 to 50 mm) when they were extracted by shaking in KCl. Even at greater concentrations of NH₄Cl (100 and 200 mm), lower amounts of NH₄⁺ + NH₃⁺ were generally recovered by extraction in KCl than in 2% acetic acid or 10% TCA (Fig. 2). Leaves extracted in 10% TCA with a Polytron tissue homogenizer for about 1 min yielded significantly lower concentrations of NH₄⁺ + NH₃⁺ than extraction in 2% acetic acid by shaking for 10 min (Fig 2).

Effect of sample processing on NH₄⁺ + NH₃⁺ content. The NH₄⁺ + NH₃⁺ content of fresh leaves cut into 5 × 5-mm pieces and stored in 10% TCA at 4°C for 1 week increased 100%, whereas the NH₄⁺ + NH₃⁺ content of the same sample stored in 10% TCA at -20°C for 1 week was not affected (Table 4). In contrast, storage of the filtered homogenate of fresh leaves at 4°C in 10 ml 10% TCA by homogenization with a Polytron tissue homogenizer for about 1 min yielded significantly lower concentrations of NH₄⁺ + NH₃⁺ than extraction in 2% acetic acid by shaking for 10 min (Fig 2).

Table 1. Amino acids detected as NH₄⁺ + NH₃⁺ by five analytic methods.

| Method                  | Individual amino acids detected as NH₄⁺ + NH₃⁺ | Total NH₄⁺ + NH₃⁺ detected from the mixture (µg NH₄⁺ /g dry wt) |
|-------------------------|-----------------------------------------------|---------------------------------------------------------------|
| Technicon               | Glu+Gln                                       | 4800                                                          | 6184 |
|                         | Asn                                           | 946                                                           |
|                         | Citrulline                                    | 322                                                           |
|                         | Urea                                          | 114                                                           |
| Salicylate              | Serine                                        | 773                                                           | 1469 |
|                         | Lysine                                        | 192                                                           |
|                         | Ornithine                                     | 119                                                           |
|                         | Proline                                       | 101                                                           |
|                         | Glycine                                       | 86                                                            |
|                         | Citrulline                                    | 86                                                            |
|                         | Arginine                                      | 43                                                            |
| Distillation            | Arginine                                      | 104                                                           | 188  |
|                         | Asn                                           | 44                                                            |
|                         | Urea                                          | 36                                                            |
|                         | Glu+Gln                                       | 5                                                             |
| Wescan ammonia analyzer (2% acetic acid) | Glu+Gln                                      | 8                                                             | 16   |
|                         | Ornithine                                     | 2                                                             |
|                         | Asn                                           | 1                                                             |
|                         | Citrulline                                    | 1                                                             |
| Alltech nitrogen analyzer (10% TCA) | Glu+Gln                                      | 3                                                             | 19   |
|                         | Asn                                           | 3                                                             |
|                         | Arginine                                      | 3                                                             |
|                         | Lysine                                        | 2                                                             |

Commercial amino acid standards (Sigma, 99% pure by TLC) were prepared at concentrations equal to those found in young fully expanded leaves of 7-month-old phosphorus-deficient rough lemon (Citrus limon) seedlings (expressed as µg g⁻¹ dry wt leaf tissue) which accumulated NH₄⁺ + NH₃⁺ and contained 3.5 times the concentration of total free amino acids as leaves of healthy, nonstressed seedlings (Rabe and Lovatt, 1984). Amino acids (µg g⁻¹ dry wt) tested were glutamine (346), glutamate (348), arginine (6970), ornithine (6745), citrulline (7009), proline (20937), serine (17313), lysine (10837), glycine (1307), and asparagine (2996). Samples were diluted before analysis to represent the amount of dry leaf sample typically analyzed by each procedure. Amino acids listed in the table contributed the greatest proportion of the contamination. The data are the means of two separate experiments. The difference between replicates was a maximum of 20% for the Technicon method and a minimum of 7% for the Alltech method.

increase in leaf NH₄⁺ + NH₃⁺ content. Storage of the filtered homogenate at -20°C for 1 week did not affect NH₄⁺ + NH₃⁺ content. The storage of ground oven-dried orange leaf tissue in 10% TCA for 1 week at 4°C resulted in a 20% increase in NH₄⁺ + NH₃⁺ content; freezing at -20°C for 1 week did not affect the NH₄⁺ + NH₃⁺ concentration of the samples (Table 5). Storage of the filtered homogenate of oven-dried leaf samples at 4 or -20°C resulted in a slight (12%) increase in leaf NH₄⁺ + NH₃⁺ concentration.

Discussion

Only the two procedures by which NH₄⁺ + NH₃⁺ was quantified based on changes in electrical conductance (Wescan and Alltech) were free from interference by amino acids. The Technicon method, which consistently measured much greater levels of NH₄⁺ + NH₃⁺ for the leaves incubated with 0 to 200 mM NH₄Cl, detected a significant amount of Glu + Gln, Asn, and citrulline. These particular amino acids have been reported to increase in concentration as the concentration of NH₄Cl increased (Rabe and Lovatt, 1984). The detection of significant amounts of several amino acids as NH₄⁺ + NH₃⁺ by the Technicon procedure suggests that the high recovery of leaf-NH₄⁺ + NH₃⁺ by this method was due to direct interference from amino acids or to ammonium released by their decomposition. Burton et al. (1989) previously reported that an automated Technicon Industrial method, which uses the Berthelot reaction (indophenol procedure), detected amino acids as ammonium, whereas no significant interference occurred with steam distillation. Surprisingly, the salicylate method, which yielded lower NH₄⁺ + NH₃⁺ recoveries than the distillation method and Wescan ammonia analyzer (using 2% acetic acid as the extractant) for each of the leaf samples in the series from 0 to 200 mM NH₄Cl, detected a significant number of amino acids as NH₄⁺ + NH₃⁺. However, due to the lack of sensitivity of this method, the total amount of NH₄⁺ + NH₃⁺ detected was lower than that of the Technicon autoanalyzer. Most of the interference was from serine; nonetheless, amino acids (such as proline, arginine, and ornithine) known to accumulate in response to the same abiotic stresses that cause NH₄⁺ + NH₃⁺ to accumulate also compromised the salicylate method. Because this procedure was linear for NH₄⁺ + NH₃⁺ concentrations up to only 0.5 µg·ml⁻¹, most samples would need to be diluted to give values in this range. Subsequent expression of the data per gram fresh or dry weight would magnify errors compared to methods that are linear for NH₄⁺ + NH₃⁺; use of Mg0 minimized this result. Thus, it is possible that amino acid interference in the distillation procedure of Barker and Volk (1964) could be reduced if NaOH were replaced by MgO. Previous research provided 

Table 2. Effect of different methods of leaf preparation on NH₄⁺ + NH₃⁺ content.

| Species            | Fresh µg NH₄⁺ /g dry wt | Freeze-dried µg NH₄⁺ /g dry wt | Oven-dried µg NH₄⁺ /g dry wt |
|--------------------|-------------------------|--------------------------------|-------------------------------|
| Citrus limon       | 46.0 ± 0.0              | 54.8 ± 3.9                     | 85.6 ± 1.6                   |
| Citrus sinensis    | 28.8 ± 0.0              | 46.2 ± 8.3                     | 106.7 ± 0.0                  |

In each case the leaf tissue was extracted by homogenization for one minute in 10 ml 10% TCA with a Polytron tissue homogenizer. NH₄⁺ + NH₃⁺ was quantified by electrical conductance using a Wescan ammonia analyzer. Data are the means ± SE for three separate experiments.
evidence that air drying or oven drying soil samples affected the release of amino acids and increased the interference associated with calorimetric determination of ammonia (Monreal and McGill, 1985). Burton et al (1989) stated that amino acids were involved in producing higher estimates of ammonia by the indophenol method when soil samples were air-dried or oven-dried. The results of our research with leaf tissue are consistent with these reports. Greater concentrations of NH$_4^+$ were obtained with oven-dried vs. fresh or freeze-dried leaf samples, possibly due to degradation of amino acids in the tissue during drying at 60°C.

While there were differences in the estimates of leaf NH$_4^+$ + NH$_3$ content for acetic acid vs. TCA extracts of the same leaf samples, the differences are not necessarily due to the extractant because the extraction volume and the length of extraction were different as well. Greater (about 113% to 242%) recovery was because the extraction volume and the length of extraction were different as well. Greater (about 113% to 242%) recovery was obtained with the acetic acid procedure, suggesting that extracting a smaller sample (50 mg) by shaking in a larger volume of extractant (50 ml) for 10 min may be more effective than extracting 500 mg of tissue in 10 ml extractant by homogenization for only 1 min. Rigor of shaking, length of the stroke, and number of strokes per minute significantly affected the amount of NH$_4^+$ + NH$_3$ extracted. These need to be optimized for each plant species and tissue being analyzed over a range of NH$_4^+$ + NH$_3$ concentrations.

In addition, it is unlikely that either acid caused amino acid decomposition. Acid-soluble fractions are routinely used for recovery and quantitation of amino acids and other organic nitrogen compounds. In addition, the standard procedure for hydrolyzing protein to determine its amino acid content requires extracting oven-dried ground leaf tissue in 6 N HCl under vacuum at 110°C for 22 h (Labanauskas and Handy, 1971). When analyzing a large number of samples, fresh or oven-dried leaf samples can be stored at –20°C for 1 week in 10% TCA before homogenization or after homogenization and filtration without affecting the NH$_4^+$ + NH$_3$ content of the samples. Compatibility of the method with TCA is highly desirable, as the TCA acid-soluble fraction can be used concurrently for quantitative recovery and determination of leaf concentrations of amino acids, polyamines, and pyrimidine.

### Table 3. Effect of different methods of leaf preparation on NH$_4^+$ + NH$_3$ content of ‘Washington’ navel orange leaves previously incubated in NH$_4$Cl solutions from 0 to 200 mM for 48 h.

| NH$_4$Cl treatment (mM) | Leaf preparation method | µg NH$_4^+$/g dry wt | Significance |
|------------------------|-------------------------|----------------------|--------------|
|                        | Fresh                    | Freeze-dried         | Oven-dried   |             |
| 0 (Control)            | 278 b                    | 400 a                | 440 a        | **          |
| 6.5                    | 406 b                    | 460 b                | 589 a        | **          |
| 12.5                   | 426 c                    | 600 b                | 680 a        | ***         |
| 25.0                   | 592 b                    | 899 a                | 919 a        | ***         |
| 200.0                  | 3198 c                   | 5376 b               | 6037 a       | ****        |

Detached citrus leaves were incubated in NH$_4$Cl solutions from 0 to 200 mM, washed, oven-dried at 60°C for 72 h and ground to pass through a 40-mesh screen. See Materials and Methods for details. NH$_4^+$ + NH$_3$ was quantified using the distillation method. Means within a horizontal row with the same letter are not significantly different by Duncan’s multiple range test at P ≤ 0.05. Means within a horizontal row followed by different letters are significant at P ≤ 0.01 (**), 0.001 (***) , and 0.0001 (****), respectively.

![Fig. 2. Effect of extraction on NH$_4^+$ + NH$_3$ content of oven-dried leaves of ‘Washington’ navel orange previously incubated in NH$_4$Cl solutions from 0 to 200 mM for 48 h. NH$_4^+$ + NH$_3$ was determined by electrical conductance using Wescan ammonia analyzer. Data points with different letters at given concentrations of NH$_4$Cl represent significance at P ≤ 0.05 by Duncan’s multiple range test.](image)
and puke nucleotides.

Given the results of this study, researchers and diagnostic laboratories must examine carefully the methods they use to measure NH₄⁺ + NH₃ in plant tissue based on the accuracy required in the results relative to their intended purpose. Differences in sensitivity and interference from amino acids between methods should be considered when comparing data reported in the literature or interpreting threshold values, e.g., for ammonia toxicity.

### Literature Cited

| Author(s) | Title | Journal | Year |
|-----------|-------|---------|------|
| Adler, P.R. and G.E. Wilcox. | 1988. Influence of NaCl and N source on muskmelon growth and NO₃ and NH₄ accumulation. | HortScience | 23:765-766 |
| Barker, A.V. and R.J. Volk. | 1964. Determination of ammonium, amide, amino, and nitrate nitrogen in plants extracts by modified Kjeldahl method. | Anal. Chem. | 36:439-441 |
| Berthelot, P. | 1859. Violet d'aniline. | Reportoire de Chimie Applique6 | 58: 284 |
| Bremner, J.M. | 1965. Inorganic forms of nitrogen. | In: C.A. Black (ed.). Methods of soil analysis. | 2:499-504 |
| Feng, J. and A.V. Barker. | 1992b. Ethylene evolution and ammonium accumulation by nutrient-stressed tomato plants. | J. Plant Nutr. | 15:137-153 |
| Feng, J. and A.V. Barker. | 1992a. Ethylene evolution and ammonium accumulation by nutrient-stressed tomatoes grown with inhibitors of ethylene synthesis or action. | J. Plant Nutr. | 15:155-167 |
| Flares, H.E. and A.W. Galston. | 1982. Polymamines and plant stress: activation of putrescine biosynthesis by osmotic shock. | Science | 217:1259-1261 |
| Frola, J.N.E. and T.C. Tucker. | 1978. Salt and water stress influence nitrogen metabolism in red kidney beans. | Soil Sci. Soc. Am. J. | 42:743-746 |
| Harrow, B., I.M. Chaminel, and H. Wagreich. | 1933. The action of ammonia on phenols. | J. Amer. Soc. Hort. Sci. | 120(5):871-876, 1995 |

---

**Table 4. Effect of interruption of sample processing and storage for 1 week on NH₄⁺ + NH₃ content of fresh ‘Washington’ navel orange leaves**

| Treatment | Storage temp for 1 week | µg NH₄⁺/g fresh wt ± SE |
|-----------|-------------------------|-------------------------|
| Extracted (1 g/10 ml 10% TCA), analyzed immediately (control) | Not stored | 16.0 ± 0.8 |
| Not extracted (1 g/5 ml 10% TCA) | 4°C | 30.6 ± 1.6 |
| Not extracted (1 g/5 ml 10% TCA) | –20°C | 16.6 ± 0.8 |
| Extracted (1 g/10 ml 10% TCA), filtered | 4°C | 23.2 ± 0.8 |
| Extracted (1 g/10 ml 10% TCA), filtered | –20°C | 16.6 ± 0.8 |

Leaves collected and washed as described in Materials and Methods, were cut and stored or processed through homogenization and filtration and stored at 4 or –20°C for 1 week or analyzed immediately. See Materials and Methods for details. NH₄⁺ + NH₃ was quantified by electrical conductance using an Alltech inorganic nitrogen analyzer. Data are the means ±SE for two separate experiments.

**Table 5. Effect of interruption of sample processing and storage for 1 week on leaf NH₄⁺ + NH₃ content of oven-dried ‘Washington’ navel orange leaves**

| Treatment | Storage temp for 1 week | µg NH₄⁺/g dry wt ± SE |
|-----------|-------------------------|------------------------|
| Extracted (1 g/10 ml 10% TCA), analyzed immediately (control) | Not stored | 229 ± 77 |
| Not extracted (0.5 g/5 ml 10% TCA) | 4°C | 274 ± 4 |
| Not extracted (0.5 g/5 ml 10% TCA) | –20°C | 235 ± 4 |
| Extracted (0.5 g/10 ml 10% TCA), filtered | 4°C | 257 ± 12 |
| Extracted (0.5 g/10 ml 10% TCA), filtered | –20°C | 245 ± 6 |

Leaves, collected and washed as described in Materials and Methods, were oven-dried at 60°C for 72 h and ground to pass through a 40-mesh screen. Samples were stored or processed through homogenization and filtration and stored at 4 or –20°C for 1 week. See Materials and Methods for details. NH₄⁺ + NH₃ was quantified by electrical conductance using an Alltech inorganic nitrogen analyzer. Data are the means ±SE for two separate experiments.