Evaluating Analytical Procedures for Quantifying Ammonium in Leaf Tissue

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Abstract. The effects of different methods of sample preparation, extraction, and storage on the recovery of the combined pool of ammonia plus ammonium ($\text{NH}_3 + \text{NH}_4^+$) from 'Washington' navel orange leaves previously incubated in solutions of increasing NH_4Cl concentrations were assessed. Procedures and instruments for quantifying $\text{NH}_3 + \text{NH}_4^+$ were tested for their sensitivity, reproducibility, and freedom from interference by amino acids. Reliable recoveries of $\text{NH}_3 + \text{NH}_4^+$, free from amino acid interference, were obtained with oven-dried (60C) 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 $\text{NH}_3 + \text{NH}_4^+$ 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 -20C of fresh or oven-dried leaf samples in 10% TCA before or after homogenization and filtration did not alter $\text{NH}_3 + \text{NH}_4^+$ levels, whereas storage of these samples at 4C increased $\text{NH}_3 + \text{NH}_4^+$ levels.

Concentrations of the combined pool of ammonia plus ammonium ($\text{NH}_3 + \text{NH}_4^+$) 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 $\text{NH}_3 + \text{NH}_4^+$ 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 $\text{NH}_3 + \text{NH}_4^+$ accumulates in plant tissues in response to abiotic stresses. For example, Frota and Tucker (1978) reported that bean (*Phaseolus vulgaris*) plants receiving ($^{15}\text{NH}_4$) SO_4 accumulated $^{15}\text{NH}_4^+$ when the plants were subjected to salinity or water-deficit stress. Subsequently, Nilsen and Muller (1981) reported that ammonium accumulated in the roots of *Lotus scoparius* during water-deficit stress, and Flores and Galston (1982) added a note in proof that free ammonium titers increased in oat seedlings during wilting. Accumulation of NH_4^+ during mineral nutrient deficiencies of several plant species (Rabe and Lovatt, 1986; Feng and Barker, 1992a, 1992b), low-temperature and water-deficit stress of citrus cultivars (Lovatt et al., 1988a, 1988b), and salinity stress of

muskmelon (Adler and Wilcox, 1988) has also been demonstrated.

Methods currently used for quantifying $\text{NH}_3 + \text{NH}_4^+$ 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 (Harrow 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 $\text{NH}_3 + \text{NH}_4^+$ 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 $\text{NH}_3 + \text{NH}_4^+$ from citrus leaves incubated in solutions of increasing concentrations of NH_4Cl to provide a range of tissue $\text{NH}_3 + \text{NH}_4^+$ concentrations.

Materials and Methods

Chemicals

Trichloroacetic acid (TCA) was purchased from Fisher Scientific (Pittsburgh). Amino acid standards (Sigma UltraPure, 99% by TLC; $\text{NH}_4^+ < 0.05\%$) and all other chemicals used in this study

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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 $\text{NH}_3 + \text{NH}_4^+$ 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* x *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 60C 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 x 14 x 14-cm, 5.5-liter) containing aerated NH_4Cl 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 30C under continuous illumination (PFD of 3 10 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$). At the end of 48 h the leaves were collected, washed thoroughly, blot-dried, cut into pieces (5 x 5 mm, midvein removed) and analyzed as either i) fresh tissue or ii) after being oven-dried at 60C 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 $\text{NH}_3 + \text{NH}_4^+$ 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 calorimetrically 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 $\text{NH}_3 + \text{NH}_4^+$ was measured in a 200-mg oven-dried sample. The sample was extracted in 50 ml 1 N KCl by shaking in a reciprocal shaker for 30 min and filtered through Whatman no. 1 filter paper. The assay was linear for concentrations of ammonium between 0 and 100 $\mu\text{g}\cdot\text{ml}^{-1}$. Sample filtrates were diluted with 1 N KCl to give values in this range.

Salicylate method. The method of Nelson (1983) for the analysis of soil ammonia by reaction with salicylate to form an emerald

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-mg 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 37C and cooled to room temperature, and absorbance at 667 nm was determined. The $A_{667\text{nm}}$ was linear for $\text{NH}_3 + \text{NH}_4^+$ concentrations between 0 and 0.5 $\mu\text{g}\cdot\text{ml}^{-1}$. 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 undigested 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 biiodate 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 biiodate 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):

$$\mu\text{g NH}_4\text{-N/g} = (0.2 \text{ mg nitrogen/ml acid}) \times \text{ml titrant KH}(\text{IO}_3)_2 / \text{sample weight (g)}.$$

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

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 ($\text{ml} \cdot \text{min}^{-1}$) were used: sample, 1.32; base, 5.2; and absorbing solution, 1.32. The assay is linear for ammonium concentrations between 0 and $100 \mu\text{g} \cdot \text{ml}^{-1}$. 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 $\text{NH}_3 + \text{NH}_4^+$ 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 $10,000 \times g$ 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 $\text{NH}_3 + \text{NH}_4^+$ 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 $10,000 \times g$ 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 (4°C) and analyzed for $\text{NH}_3 + \text{NH}_4^+$ at the end of 1 week.

In addition, 1 g of fresh 'Washington' navel orange leaves (midvein removed) was cut into pieces ($5 \times 5 \text{ mm}$) 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 quantify the combined pool of $\text{NH}_3 + \text{NH}_4^+$ as either NH_3 or NH_4^+ . For comparison, all results are presented as $\mu\text{g} \text{NH}_4^+/\text{g}$ fresh or dry weight tissue.

Results

$\text{NH}_3 + \text{NH}_4^+$ content of a series of oven-dried standard leaf samples prepared by incubating leaves of the 'Washington' navel orange in NH_4Cl 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 $\text{NH}_3 + \text{NH}_4^+$ concentrations optimal for each method, the Technicon method was insensitive at the lower concentrations of NH_4^+ , and the salicylate method was insensitive at lower and higher concen-

trations of NH_4^+ . The $\text{NH}_3 + \text{NH}_4^+$ 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 ($P \leq 0.05$). No difference between the $\text{NH}_3 + \text{NH}_4^+$ content of the control leaves incubated in distilled water and that of leaves incubated in 12.5 mM NH_4Cl for 48 h was detected by any method, but all methods approximated $2500 \mu\text{g} \text{NH}_3 + \text{NH}_4^+/\text{g}$ dry weight for the 200 mM NH_4Cl treatment, except the salicylate method, by which only $1307 \pm 24 \mu\text{g} \text{NH}_3 + \text{NH}_4^+/\text{g}$ dry weight was detected.

Detection of amino acids as $\text{NH}_3 + \text{NH}_4^+$ by five analytic methods. Only the two procedures using electrical conductance did not detect amino acids as $\text{NH}_3 + \text{NH}_4^+$ (Table 1). Results obtained with the Technicon autoanalyzer procedure were the most severely contaminated, detecting a net difference of $6168 \mu\text{g} \cdot \text{g}^{-1}$ dry weight (386-fold) more $\text{NH}_3 + \text{NH}_4^+$ than the least compromised method, the Wescan ammonia analyzer ($16 \mu\text{g} \cdot \text{g}^{-1}$). By the salicylate method, $1453 \mu\text{g} \cdot \text{g}^{-1}$ dry weight (92-fold) more $\text{NH}_3 + \text{NH}_4^+$ was detected compared to the Wescan ammonia analyzer; for the distillation the difference was only $172 \mu\text{g} \cdot \text{g}^{-1}$ dry weight.

Effect of leaf preparation method on the concentration of $\text{NH}_3 + \text{NH}_4^+$ determined. Regardless of the method of $\text{NH}_3 + \text{NH}_4^+$ analysis used, i.e., electrical conductance (Table 2) or distillation (Table 3), greater concentrations of $\text{NH}_3 + \text{NH}_4^+$ 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 $\text{NH}_3 + \text{NH}_4^+$ 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 $\text{NH}_3 + \text{NH}_4^+$ content of the leaf per gram fresh weight.

Effect of extractant and extraction procedure on leaf $\text{NH}_3 + \text{NH}_4^+$ content. The $\text{NH}_3 + \text{NH}_4^+$ content of leaves incubated in NH_4Cl 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. $\text{NH}_3 + \text{NH}_4^+$ content of citrus leaves previously incubated in NH_4Cl solutions from 0 to 200 mM for 48 h determined by six analytical procedures. Vertical bars at given concentrations of NH_4Cl represent LSD at $P \leq 0.05$.

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 $\text{NH}_3 + \text{NH}_4^+$ was detected in leaves incubated in low concentrations of NH_4Cl (0 to 50 mM) when they were extracted by shaking in KCl. Even at greater concentrations of NH_4Cl (100 and 200 mM), lower amounts of $\text{NH}_3 + \text{NH}_4^+$ 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 $\text{NH}_3 + \text{NH}_4^+$ than extraction in 2% acetic acid by shaking for 10 min (Fig 2).

Effect of sample processing on $\text{NH}_3 + \text{NH}_4^+$ content. The $\text{NH}_3 + \text{NH}_4^+$ content of fresh leaves cut into 5×5 -mm pieces and stored in 10% TCA at 4C for 1 week increased 100%, whereas the $\text{NH}_3 + \text{NH}_4^+$ content of the same sample stored in 10% TCA at -20C for 1 week was not affected (Table 4). In contrast, storage of the filtered homogenate of fresh leaves for 1 week at 4C resulted in only a 50%

Table 1. Amino acids detected as $\text{NH}_3 + \text{NH}_4^+$ by five analytic methods.

Method	Individual amino acids detected as $\text{NH}_3 + \text{NH}_4^+$ ($\mu\text{g NH}_4^+/\text{g dry wt}$)	Total $\text{NH}_3 + \text{NH}_4^+$ detected from the mixture
Technicon	Glu+Gln	4800
	Asn	946
	Citrulline	322
	Urea	114
Salicylate	Serine	773
	Lysine	192
	Ornithine	119
	Proline	101
	Glycine	86
	Citrulline	86
	Arginine	43
Distillation	Arginine	104
	Asn	44
	Urea	36
	Glu+Gln	5
Wescan ammonia analyzer		
(2% acetic acid)	Glu+Gln	8
	Ornithine	2
	Asn	1
	Citrulline	1
Alltech nitrogen analyzer		
(10% TCA)	Glu+Gln	3
	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 $\mu\text{g}\cdot\text{g}^{-1}$ dry wt leaf tissue) which accumulated $\text{NH}_3 + \text{NH}_4^+$ and contained 3.5 times the concentration of total free amino acids as leaves of healthy, nonstressed seedlings (Rabe and Lovatt, 1984). Amino acids ($\mu\text{g}\cdot\text{g}^{-1}$ 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 $\text{NH}_3 + \text{NH}_4^+$ content. Storage of the filtered homogenate at -20C for 1 week did not affect $\text{NH}_3 + \text{NH}_4^+$ content. The storage of ground oven-dried orange leaf tissue in 10% TCA for 1 week at 4C resulted in a 20% increase in $\text{NH}_3 + \text{NH}_4^+$ content; freezing at -20C for 1 week did not affect the $\text{NH}_3 + \text{NH}_4^+$ concentration of the samples (Table 5). Storage of the filtered homogenate of oven-dried leaf samples at 4 or -20C resulted in a slight (12%) increase in leaf $\text{NH}_3 + \text{NH}_4^+$ concentration.

Discussion

Only the two procedures by which $\text{NH}_3 + \text{NH}_4^+$ 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 $\text{NH}_3 + \text{NH}_4^+$ for the leaves incubated with 0 to 200 mM NH_4Cl , 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 $\text{NH}_3 + \text{NH}_4^+$ increased (Rabe and Lovatt, 1984). The detection of significant amounts of several amino acids as $\text{NH}_3 + \text{NH}_4^+$ by the Technicon procedure suggests that the high recovery of leaf- $\text{NH}_3 + \text{NH}_4^+$ 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 $\text{NH}_3 + \text{NH}_4^+$ 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 NH_4Cl , detected a significant number of amino acids as $\text{NH}_3 + \text{NH}_4^+$. However, due to the lack of sensitivity of this method, the total amount of $\text{NH}_3 + \text{NH}_4^+$ 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 $\text{NH}_3 + \text{NH}_4^+$ to accumulate also compromised the salicylate method. Because this procedure was linear for $\text{NH}_3 + \text{NH}_4^+$ concentrations up to only $0.5 \mu\text{g}\cdot\text{ml}^{-1}$, 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 $\text{NH}_3 + \text{NH}_4^+$ concentrations up to $100 \mu\text{g}\cdot\text{m}^{-1}$. Detection of amino acids as $\text{NH}_3 + \text{NH}_4^+$ by the distillation procedure was lower than the Technicon or the salicylate procedures. Bremner (1965) reported that distillation with NaOH caused decomposition of some organic N to NH_4^+ ; use of MgO 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 $\text{NH}_3 + \text{NH}_4^+$ content^a

Species	Leaf preparation method		
	Fresh	Freeze-dried	Oven-dried
	$\mu\text{g NH}_4^+/\text{g dry wt}$		
<i>Citrus limon</i>	46.0 $\pm$ 0	54.8 $\pm$ 3.9	85.6 $\pm$ 1.6
<i>Citrus sinensis</i>	28.8 $\pm$ 0	46.2 $\pm$ 8.3	106.7 $\pm$ 0

^aIn each case the leaf tissue was extracted by homogenization for one minute in 10 ml 10% TCA with a Polytron tissue homogenizer. $\text{NH}_3 + \text{NH}_4^+$ was quantified by electrical conductance using a Wescan ammonia analyzer. Data are the means $\pm$ SE for three separate experiments.

Table 3. Effect of different methods of leaf preparation on $\text{NH}_3 + \text{NH}_4^+$ content of 'Washington' navel orange leaves previously incubated in NH_4Cl solutions from 0 to 200 mM for 48 h².

NH_4Cl treatment (mM)	Leaf preparation method			Significance ^y
	Fresh	Freeze-dried	Oven-dried	
	$\mu\text{g NH}_4^+/\text{g dry wt}$			
0 (Control)	278 b	400 a	440 a	**
6.5	406 b	460 b	580 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	****

²Detached citrus leaves were incubated in NH_4Cl 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. $\text{NH}_3 + \text{NH}_4^+$ 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 $P \leq 0.05$. Means within a horizontal row followed by different letters are significant at $P \leq 0.01$ (**), 0.001 (***), and 0.0001 (****), respectively.

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 $\text{NH}_3 + \text{NH}_4^+$ 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 $\text{NH}_3 + \text{NH}_4^+$ 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 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 $\text{NH}_3 + \text{NH}_4^+$ extracted. These need to be optimized for each plant species and tissue being analyzed over a range of $\text{NH}_3 + \text{NH}_4^+$ 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 (Labanuskas 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 5 ml 10% TCA or after homogenization in 10 ml 10% TCA and filtration without any effect on the $\text{NH}_3 + \text{NH}_4^+$ content of the sample.

In conclusion, the method of quantifying $\text{NH}_3 + \text{NH}_4^+$ based on differences in electrical conductance between the sample and the reference cell developed by Carlson (1978) and incorporated into the Wescan ammonia analyzer and its successor, the Alltech inorganic nitrogen analyzer, is the most reliable analytic procedure of the four we tested. Interference from amino acids was minimal and significantly (10-fold) lower than the next-best method, distillation (researchers with limited budgets or only occasional analyses might find the distillation method acceptable, especially after replacing NaOH with MgO in the distillation). The conductance method is linear for ammonium concentrations between 0 and 100

$\mu\text{g}\cdot\text{ml}^{-1}$; the results are highly reproducible as evidenced by the small standard errors from three separate experiments. The method is compatible with different extractants (1 N KCl, 2% acetic acid, and 10% TCA). The best recoveries of $\text{NH}_3 + \text{NH}_4^+$ were obtained with oven-dried (60°C) leaves ground to pass through a 40-mesh screen extracted by shaking in 2% acetic acid or by homogenization in 10% TCA. The latter extracting procedure can be used with fresh and dried (freeze-dried or oven-dried at 60°C) leaf tissue and requires only 1 min of homogenization, facilitating the processing many samples. Of additional advantage, fresh or oven-dried samples can be stored at -20°C for one week in 10% TCA before homogenization or after homogenization and filtration without affecting the $\text{NH}_3 + \text{NH}_4^+$ 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

Fig. 2. Effect of extraction on $\text{NH}_3 + \text{NH}_4^+$ content of oven-dried leaves of 'Washington' navel orange previously incubated in NH_4Cl solutions from 0 to 200 mM for 48 h. $\text{NH}_3 + \text{NH}_4^+$ was determined by electrical conductance using Wescan ammonia analyzer. Data points with different letters at given concentrations of NH_4Cl represent significance at $P \leq 0.05$ by Duncan's multiple range test.

Table 4. Effect of interruption of sample processing and storage for 1 week on $\text{NH}_3 + \text{NH}_4^+$ content of fresh 'Washington' navel orange leaves

Treatment [†]	Storage temp for 1 week	$\mu\text{g NH}_4^+/\text{g}$ fresh wt $\pm$ SE
Extracted (1 g/10 ml 10% TCA), analyzed immediately (control)	Not stored	16.0 $\pm$ 0.8
Not extracted (1 g/5 ml 10% TCA)	4 C	30.6 $\pm$ 1.6
Not extracted (1 g/5 ml 10% TCA)	-20C	16.6 $\pm$ 0.8
Extracted (1 g/10 ml 10% TCA), filtered	4 C	23.2 $\pm$ 0.8
Extracted (1 g/10 ml 10% TCA), filtered	-20C	16.6 $\pm$ 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 -20C for 1 week or analyzed immediately. See Materials and Methods for details. $\text{NH}_3 + \text{NH}_4^+$ was quantified by electrical conductance using an Alltech inorganic nitrogen analyzer. Data are the means $\pm$ SE for two separate experiments.

Table 5. Effect of interruption of sample processing and storage for 1 week on leaf $\text{NH}_3 + \text{NH}_4^+$ content of oven-dried 'Washington' navel orange leaves.

Treatment [‡]	Storage temp for 1 week	$\mu\text{g NH}_4^+/\text{g}$ dry wt $\pm$ SE
Extracted (1 g/10 ml 10% TCA), analyzed immediately (control)	Not stored	229 $\pm$ 77
Not extracted (0.5 g/5 ml 10% TCA)	4C	274 $\pm$ 4
Not extracted (0.5 g/5 ml 10% TCA)	-20C	235 $\pm$ 4
Extracted (0.5 g/10 ml 10% TCA), filtered	4C	257 $\pm$ 12
Extracted (0.5 g/10 ml 10% TCA), filtered	-20C	245 $\pm$ 6

[‡]Leaves, collected and washed as described in Materials and Methods, were oven-dried at 60C 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 -20C for 1 week. See Materials and Methods for details. $\text{NH}_3 + \text{NH}_4^+$ was quantified by electrical conductance using an Alltech inorganic nitrogen analyzer. Data are the means $\pm$ SE for two separate experiments.

and puke nucleotides.

Given the results of this study, researchers and diagnostic laboratories must examine carefully the methods they use to measure $\text{NH}_3 + \text{NH}_4^+$ 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.

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