compared to the conventional methodology. On the other hand, determination of monoenoic and dienoic fatty acid composition in seeds may possibly involve a 10–15% error. The major problem in this analysis arises from the weakness of olefinic carbon signals as well as some undesirable quantitative aspects of ^{13}C NMR spectroscopy (Shoolery and Jankowsky, 1973). An accurate quantitative compositional analysis and determination of total oil content in the seeds will, however, need a somewhat more elaborate procedure. It has been demonstrated that during the germination process catabolic breakdown of the storage carbohydrate into major polysaccharide components in seeds can be detected by ^{13}C NMR. The same technique can also be employed for the determination of the quantity of starch converted during malting or hydrolysis of barley (Diner and Elofson, 1977) or other grain seeds. Many aspects of the economically important areas of agriculture and food production that affect crop quality, such as starch aging, storage methods and processing conditions, would seem amenable to ^{13}C NMR technique.

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An Improved Method for Nitrite Extraction from Plants

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An improved technique for aqueous extraction of nitrite from plant tissues is described. Additions of methylene chloride ($MeCl_2$) and insoluble polyvinylpyrollidone (PVP) produced clear, aqueous nitrite-containing extracts. The $MeCl_2$/PVP addition increased recoveries of nitrite from all 15 plant species tested.

Nitrite is a naturally occurring metabolite in the process of nitrate assimilation by green plants. It is considered an "enzyme-bound", intermediate and is not easily detectable in healthy plants. In the past, nitrite analyses have been largely confined to soils tests and nitrate reductase assays. Recently, nitrite has been shown to accumulate in plant tissues as a result of herbicidal action (Klepper, 1974, 1975, 1976) and there has been considerable concern regarding nitrite and its role as a precursor in the formation of nitrosamines. Nitrite has been reported to accumulate in certain processed or raw foods when high nitrate content was coupled with improper handling or storage (Bassir and Maduagwu, 1978; Keating et al., 1973; Keybets et al., 1969). Also, recent studies of the effects of air pollution on plants have required tests for nitrite in vegetation (Zeewart, 1976). These new research approaches would be aided by a reliable nitrite test.

Losses of known quantities of nitrite have been reported upon extraction of plant tissues (Finke et al., 1977) or when free nitrite was in contact with green leaf tissue (Vanecko and Varner, 1965). Nitrite additions to tannic acid solutions, tea, or apple juice resulted in the disappearance of the added nitrite (Bogovski et al., 1972). Up to 100 ppm nitrite added to apple juice could not be detected but a darkening of the juice was associated with an oxidative change in the tannin content. They concluded that the tannins present in the juice reacted with the added nitrite.

The sulfanilamide/α-naphthylethylenediamine/HCl reagent is extremely sensitive to nitrite and can easily be used following extraction. The problem lies in recovery during extraction of nitrite from plant tissues. Nitrite is known to react with many compounds present in the green plant (Klepper, 1974). Upon extraction, both nitrite and reactive metabolites are released into an aqueous medium, permitting the destruction of nitrite during the extraction process. The result is a loss of nitrite before an analysis can be conducted.

I have developed an extraction method that achieves higher recovery of nitrite in plant tissues than ordinary aqueous extraction.

METHODS AND MATERIALS

Preparation of Insoluble Polyvinylpyrollidone (PVP). PVP was obtained from Sigma Chemical Co. Analysis indicated it contained the equivalent of more than 900 nmol of nitrite/g. Thus, the material had to be thoroughly washed before use (Klepper and Hageman, 1969). One hundred grams of PVP was placed in 3 L of 0.1 N HCl and stirred for 4 h. Nitrite quickly disappeared from the PVP. The PVP was strained through four layers of cheesecloth and resuspended in distilled, deionized H_2O. This water-washing procedure was repeated four times.

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The final slurry was brought to pH 6.0 (Anderson and Sowers, 1968) with 3 N KOH, and water was manually squeezed out through cheesecloth. The hydrated PVP preparation was stored in brown bottles in the refrigerator until time for use. One gram of hydrated PVP contained 0.2 g of dry PVP. The hydrated PVP was free of nitrite after preparation in this manner. A water slurry of PVP (20 g of PVP/100 mL of H_2O) was used throughout all experiments.

Tea Solution. Five grams of tea (Orange Pekoe) was added to 500 mL of boiling water. The resulting tea solution was filtered through six layers of cheesecloth and diluted to 1000 mL.

Extract Preparation. All plant extracts were prepared as follows: fresh plant tissue was weighed (0.25–0.5 g), placed in a test tube, and 10 mL of water or 10 mL of the PVP slurry added. Two milliliters of methylene chloride (99% pure, Fisher Scientific Co.) was added to the tubes containing the PVP slurry. These preparations were immediately homogenized with a Polytron homogenizer (Model PCU-2-110) for 10–20 s at speed 7. After homogenization, the solutions were centrifuged at 20000*g* for 5 min. In studies of nitrite loss and recovery in plant extracts, nitrite was added after the extracts were prepared. In recovery and loss experiments with intact tissues, nitrite was added before homogenization.

Analysis for Nitrite. After centrifugation, the aqueous, nitrite-containing portion of the plant extract/$MeCl_2$/PVP mixture was present in the centrifuge tube as the top layer of three layers. An aliquot (1–2 mL) of the upper portion was carefully taken directly from the centrifuge tube and the remainder discarded. The aliquot was added to 2 mL of the nitrite reagent and optical density determined at 540 nm (Klepper, 1974).

Techniques for Inducing Endogenous Nitrite. Two methods were used to induce plant tissues to accumulate endogenous nitrite. One procedure involved vacuum infiltration of the tissue with a 0.01 M KNO_3 solution and incubation in darkness under anaerobic conditions as in the in vivo assay for nitrate reductase (NR) activity (Klepper, 1974). The method was slightly modified by removing the medium after infiltration so that nitrite would not diffuse out into the medium but would remain in the tissue.

In the second procedure, leaves were floated on 2,4-dichlorophenoxyacetic acid (2,4-D; 200 ppm) or dinitrophenol (DNP; 100 ppm) for 20 min using a method previously described by Klepper (1976). Tissue was incubated in darkness under aerobic conditions.

Extracts of tissues treated in these two procedures were prepared as described above.

Data Replication and Analyses. All experiments have been performed at least twice. Values presented in all tables are means ± standard deviation (SD) of quadruplicate samples.

Plant Species Tested. The following plant species were used during the course of this research: apple, *Malus sylvestris* L.; avocado, *Persea americana* L.; dock, *Rumex crispus* L.; pennycress, *Thlaspi arvense* L.; pine, *Pinus strobus* L.; potato, *Solum tuberosum* L.; pumpkin, *Cucurbito pepo* L.; radish, *Raphanus sativus* L.; soybean, *Glycine max* L. (Merr.); spinach, *Spinacea oleracea* L.; strawberry, *Fragaria sp.* L.; sunflower, *Helianthus annuus* L.; sweet potato, *Ipomea batatas* L.; tea, *Camellia sinensis*; wheat, *Triticum aestivum* L.

RESULTS AND DISCUSSION

A tea solution was used to establish loss of nitrite and to attempt increased recovery by adding various combinations of compounds. Only those data which resulted in improved recoveries are presented. Developmental data are not shown. In these tests, the tea solution was extracted with the addition of $MeCl_2$ or PVP before nitrite was added to determine if the interfering substances had been removed. As shown in Table I the $MeCl_2$/PVP treatment allowed approximately 95% recovery of the nitrite compared to 43% when nitrite was added to untreated tea solution. This loss of nitrite in tea solutions occurred very rapidly as reported by Bogovski et al. (1972). Prolonged incubation of nitrite in the tea did not result in further loss.

Table I. Loss and Recovery of Nitrite Added to Tea Solutions

treatment	nmol of NO_2^- detected ($\bar{x}$ ± SD)[a]	% recov.
water	50.0 ± 0.3	100.0
tea solution	21.6 ± 0.7	43.2
tea solution + $MeCl_2$	26.0 ± 1.5	52.0
tea solution + $MeCl_2$ + PVP	47.4 ± 0.8	94.8

[a] 50 nmol of nitrite was added to each solution after extraction.

Table II. Loss and Recovery of Nitrite Added to Leaf Extracts of Five Plant Species

leaf extract	nmol of NO_2^- recovered ($\bar{x}$ ± SD)[a] H_2O	+ $MeCl_2$/PVP	% increase in recov.
pennycress	11.4 ± 0.2	18.2 ± 0.9	59.6
pine	11.8 ± 0.3	18.2 ± 0.5	54.4
pumpkin	11.1 ± 0.7	18.3 ± 0.7	64.8
sweet potato	13.9 ± 0.5	19.3 ± 0.4	38.8
avocado	6.0 ± 0.5	18.7 ± 1.1	211.6

[a] 20 nmol of nitrite was added to extracts after preparation.

Following initial testing with tea solutions, extracts were prepared from leaf tissues of five other plant species to test for nitrite recovery with and without the $MeCl_2$/PVP mixture. Two-milliliter aliquots (equal to 50 mg of green leaf tissue) of the plant extracts were placed in test tubes, and 20 nmol of nitrite was added. Nitrite content was immediately determined by the addition of 2 mL of the sulfanilamide/α-naphthylethylenediamine/HCl reagent. Recoveries ranged from 30% with aqueous avocado leaf extracts to 70% recovery with the sweet potato leaf extracts (Table II). By adding the $MeCl_2$/PVP, the mean recovery for all species was about 93%. These data demonstrate that the $MeCl_2$/PVP technique can remove certain substances, present in several plant species, that interfere with nitrite extraction.

In another experiment nitrite was added prior to homogenization of intact tissues. This procedure permitted the mixing of interfering substances and nitrite in an aqueous medium with later testing for loss and recovery. Nitrite (100 nmol) was homogenized with 0.25 g of leaf tissue. Apple leaf tissue lost approximately 68% of the added nitrite while soybean only lost 13 percent (Table III). The $MeCl_2$/PVP treatment resulted in increased recoveries for all treatments with a mean recovery of 89% over all species. Lowest recovery (69%) was obtained with apple leaf tissue which is known to be high in tannins and polyphenols. Extraction of nitrite from this tissue might have been more effective with an increased amount of PVP added to the extraction medium.

Data in Table IV show the recovery of endogenous nitrite that had been induced to accumulate as described in materials and methods. Five plant species were tested.

Table III. Loss and Recovery of Nitrite Added Before Extraction of Five Plant Species

leaf extract	nmol of NO_2^- recovered ($\bar{x}$ ± SD)[a] H_2O	+ $MeCl_2$/PVP	% increase in recov.
sweet potato	73.0 ± 2.2	98.6 ± 5.9	35.1
soybean	87.0 ± 5.4	105.8 ± 3.1	21.6
apple	32.0 ± 2.1	69.0 ± 2.1	115.6
spinach	62.6 ± 4.5	84.5 ± 2.1	34.9
sunflower	66.7 ± 1.5	87.6 ± 1.0	31.3

[a] 100 nmol of nitrite added before homogenization of leaf tissues.

Table IV. Effect of the Addition of $MeCl_2$/PVP on Recovery of Endogenous Nitrite

plant leaves	nmol of NO_2^- g fresh wt^{-1} ($\bar{x}$ ± SD) H_2O	+ $MeCl_2$/PVP	% increase in recov.
sunflower (2,4-D)[a]	15.7 ± 1.2	26.0 ± 6.6	65.6
apple (2,4-D)	42.2 ± 4.7	70.9 ± 5.1	68.0
spinach (in vivo NR)	122.0 ± 47.0	141.0 ± 18.3	15.6
dock (2,4-D)	83.3 ± 34.0	110.0 ± 11.8	32.1
strawberry (in vivo NR)	290.0 ± 28.5	384.0 ± 67.2	32.4

[a] Treatments for the induction of nitrite accumulation shown in parentheses.

Table V. Percent Increase in Recovery as Affected by Time of Incubation and Total Nitrite Content in Wheat Leaves

incubation time, min	nmol of NO_2^- detected, g fresh wt^{-1} ($\bar{x}$ ± SD)[a] H_2O	+ $MeCl_2$/PVP	% increase in recov.
30	252 ± 30.4	428 ± 32.6	69.8
60	946 ± 111.5	1213 ± 128.0	28.2
120	1430 ± 74.8	1683 ± 107.5	17.6

[a] Wheat leaves were treated with 100 ppm DNP.

Again, all $MeCl_2$/PVP treatments resulted in increased recovery of nitrite after extraction. These increases varied from nearly 16 to 68%. These data agree with those in Tables I, II, and III. This type of testing for recovery of endogenous nitrite closely duplicates normal analyses for nitrite.

Further testing for endogenous nitrite within wheat leaves was done in order to illustrate an important effect (Table V). As nitrite concentration within the leaf tissue increases, the percent increase in recovery decreases. Wheat leaves were treated with DNP and incubated in darkness to allow nitrite to accumulate (Klepper, 1976). All assays for nitrite were conducted using 0.25 g of leaves. At the end of 30 min, a 69.8% increase in recovery was shown. At 120 min only a 17.6% increase was shown but nitrite content had increased four- to sixfold during that time. In many tests with wheat with lower concentrations of nitrite (10–20 nmol, g fresh wt^{-1}), the addition of $MeCl_2$/PVP often increases recovery by 300–500% (data not shown).

Root and tuber tissue of four plant species were tested (Table VI). It appears that of the species chosen, none had a large amount of substances that interfere with nitrite extraction. Yet, the $MeCl_2$/PVP extraction still produced better recoveries than a simple water extraction.

Table VI. Loss and Recovery of Nitrite in Plant Root and Tuber Tissue

root and tuber tissue	nmol of NO_2^- recovered ($\bar{x}$ ± SD)[a] H_2O	+ $MeCl_2$/PVP	% increase in recov.
sweet potato	89.0 ± 6.6	96.6 ± 4.1	8.5
radish	83.1 ± 16.5	96.6 ± 1.3	16.2
potato	94.3 ± 3.6	98.3 ± 4.7	4.2
soybean	85.0 ± 2.1	98.7 ± 5.5	16.1

[a] 100 nmol of nitrite added to 1 mL of extract containing an equivalent of 0.050 g of fresh tissue.

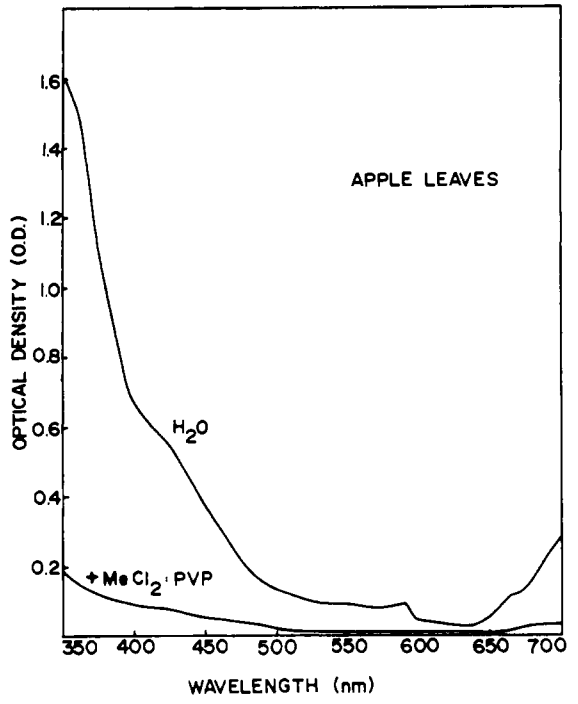

Figure 1. Absorption spectra of H_2O and $MeCl_2$/PVP extracts of apple leaves.

Spectrophotometric analyses of apple leaf extracts, water and $MeCl_2$/PVP treated, were conducted (Figure 1). The aqueous extracts were yellow and absorbed light primarily in the 650–700 and 350–500 nm ranges. The $MeCl_2$/PVP treated extract exhibited little absorbance from 500–750 nm and only a small amount from 350–500 nm as compared to the water extract.

In most extractions, when $MeCl_2$/PVP was used, the aqueous portion of the extract was almost colorless. Chlorophyll and other plant pigments were present in the $MeCl_2$ fraction at the bottom of the centrifuge tube due to their organic solubilities and the relatively high density of $MeCl_2$. By visual inspection some proteins also appeared to be denatured and precipitated by the $MeCl_2$ addition. The insoluble PVP bound polyphenols and tannins to remove them from solution. The result after centrifugation was a clear, aqueous, nitrite-containing supernatant above the PVP/$MeCl_2$ portion.

Evidence has been presented that the additions of $MeCl_2$ and insoluble PVP can protect nitrite from reactive substances during aqueous extractions for nitrite analyses. This technique is not prescribed to be a rigid procedure for all plant species, but should be subjected to variation and experimentation in the amount of $MeCl_2$ and PVP used dependent upon the plant species being tested.

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Overcoming the Nutritionally Harmful Effects of Tannin in Sorghum Grain by Treatment with Inexpensive Chemicals

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Treatment of high-tannin sorghum grain with moist, alkaline conditions was shown to substantially reduce the amount of tannin as measured by three chemical assays. Chicks fed a high-tannin grain (Savannah III), treated as the whole grain with dilute ammonium hydroxide for 30 days, showed 3-week weight gains and feed efficiencies which were statistically equivalent to those of chicks fed an untreated low-tannin control (RS-610). A shorter treatment of a ground high-tannin grain (BR-54) with a 0.5 M aqueous solution of K_2CO_3 resulted in a comparable improvement in weight gains and a substantial improvement in feed efficiencies. Treatment of the same grain with moisture and CaO gave an improvement of a lesser magnitude. Increases in available protein after treatments did not appear sufficient to account for the nutritional improvements.

Tannin in sorghum grain provides a natural protective system against bird depredation (McMillan et al., 1972), weathering (Harris and Burns, 1973), and preharvest germination (Harris and Burns, 1970). In areas where any of these stresses are severe, high-tannin genotypes generally produce higher yields and better quality grain. However, when fed to monogastric animals, high-tannin sorghum grain can result in considerably lower growth rate and feed efficiency than low tannin types (Featherston and Rogler, 1975). [The nutritional significance of tannin in ruminant diets is unclear, some reports indicating that it is harmful, e.g., McGinty (1969), Maxson et al. (1973), but others recommending addition of tannin to protect protein from microbial deamination, e.g., Dreidger and Hatfield (1972), Zelter et al. (1970). The balance of evidence seems to be no effect or a negative effect except at low N intake (Broster et al., 1978).] An economical treatment to neutralize the effect of the tannin would add greatly to the value and marketability of high-tannin grain. The impact could be especially important for certain developing nations which lie in geographical regions where high tannin sorghum genotypes must be grown to obtain satisfactory production.

Ammoniation of high-tannin grain (BR-54) by moistening whole grain with concentrated NH_4OH (28–30% NH_3) has been shown to decrease assayable tannin to approximately 30% of the original level and to greatly improve the growth rate and feed efficiency when fed to rats and chicks (Price et al., 1978a). In this investigation, we have examined the effectiveness of a milder treatment with dilute aqueous ammonia as a chemical detoxicant for high-tannin sorghum grain. We have also determined the effects of other aqueous alkalies on both the chemical assays and nutritional properties of high-tannin grain. In addition to BR-54, this study was broadened to also include another commercial high-tannin sorghum, Savannah III.

Table I. Comparison of Detoxification Treatments[a]

	% reduction in assayable tannin					
	days of treatment					
treatment	2	3	8	11	18	22
water		3		12	19	
conc. NH_4OH	59	70	84			94
2.5 M NH_4OH		53		70	87	
6 M NaOH	93	94	98			99
1 M NaOH	44	59	59			79
0.5 M NaOH		24		52	53	
0.2 M NaOH		38		34	25	
1 M K_2CO_3		68		79	90	

[a] One milliliter of solution mixed with 10 g of BR-54 (whole grain) and stored in a closed container at room temperature. Two-gram samples, removed at indicated times and air-dried overnight, were assayed for tannin by the vanillin assay and compared to the tannin content of the grain before the treatments were begun.

EXPERIMENTAL SECTION

Sorghum grain was supplied by Dr. John Axtell from grain grown at the Purdue University Agronomy Farm in 1975 (RS-610), 1976 (BR-54), and 1977 (Savannah III and

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