Results and Discussion

Yeast performance on calcium and ammonium SSL over the 10-day test periods reflects reasonably good control for this type of substrate when one considers that a blend of hardwood and softwood, usually 1 to 1, was used in most of the cooks. The higher the proportion of hardwood in such mixtures, the greater the content of xylose and organic acids, and the longer the retention time of yeast in the fermentor; however, yeast yield (based on sugar) is enhanced by the additional conversion of the nonsugar carbon compounds. Preliminary oxygen-uptake measurements in the Warburg microrespirometer showed yeast activity on hardwood liquors from 70 to 75% of that on corresponding hexose-rich softwood liquors. Oxygen-uptake rates remain about the same when the hexose-pentose in hardwood-softwood mixtures is about 1 to 1 at equivalent sugar concentrations. These rates closely parallel those observed for pure sugar solutions and their mixtures.

Amino acid values obtained by microbial assay were higher than those by the Moore-Stein method (*5, 6*) for five of the nine amino acids considered essential for man (Table II). In general, good agreement is shown by the two methods, and neither assay procedure detected a change in amino acid composition of yeast grown on calcium SSL and then shifted to ammonium SSL substrates.

Similarly, prolonged propagation of *C. utilis* on ammonia-base liquor (Table III) produced food yeast of uniform amino acid composition. Again, the first nine compounds listed are those essential for man. The values for six of them are higher than those listed previously for the corresponding amino acids assayed chromatographically. In addition, the range of values for all amino acids is surprisingly narrow over the 6-month observation period.

While these assays have affirmed the high quality and uniformity of yeast protein following drastic changes in fermentation substrates, they also allow updating of product information (*1, 16*), and offer reliable data useful in nutritional projects elsewhere (*11, 13, 17*).

The fresh data on the folic acid content of food yeasts were obtained after the Food and Drug Administration removed dried yeasts from the GRAS list. Folic acid values (Table IV) accumulated for both types of dried yeast—*Candida utilis* and *Saccharomyces cerevisiae*—are essentially similar, but much lower than recorded by the earlier literature. Both kinds of yeast contain roughly only half as much folic acid as reported (*4, 9, 17*).

Values for free folic acid (Table V) reveal a consistently higher *Candida* food yeast content, about threefold greater than levels found in primary-grown *Saccharomyces* food yeast. Since the older data were inadequate (*4*), no meaningful conclusions can be drawn from comparisons with them.

Both kinds of yeast, however, contain folic acid levels well within the limits established by a recent Food Additives Order (*7*). This order provides that dried yeast (*C. utilis, S. cerevisiae*, or *S. fragilis*) may be safely used in food (as a flavoring), provided the total folic acid content of the yeast does not exceed 40 μg. per gram of yeast [approximately 8 μg. of free folic acid (pteroylglutamic acid) per gram of yeast]. In special dietary food usage, the limit is 400 μg. of folic acid per day (100 μg. of free folic acid per adult per day).

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NITRATE IN PLANT MATERIAL

Determination of Nitrate in Silages and Forages

H. G. WISEMAN and W. C. JACOBSON

Dairy Cattle Research Branch, Animal Husbandry Research Division, Agricultural Research Center, Beltsville, Md.

Methods for the determination of nitrate in plant material have assumed increased interest with growing reports of nitrate toxicity in livestock (*1, 3, 5, 6, 8, 15*). The number and variety of available analytical methods, according to Morris and Gonzales-Mas (*10*), may be an indication that no single method is completely satisfactory.

Papenhagen (*12*) noted the limitations of colorimetric methods in respect to interferences by nitrites, chlorides, and organic and inorganic material. These criticisms applied wholly or in part to methods which involved nitration, oxidation, or reduction properties of the nitrate ion.

Methods which utilize the reduction of nitrate to ammonia require a separate analysis or removal of ammonia originally present. An error in either component analysis may give an amplified error in the estimate of nitrogen.

The color reaction of nitrates with ferrous sulfate is the basis of a method by Swann and Adams (*16*), which was modified by Morris and Gonzales-Mas

A method for the determination of nitrate in plant material has been devised whereby plant extracts are clarified by use of charcoal-Celite columns. Monosaccharides, sulfates, chlorides, and ammonium salts are eluted by water while nitrate is retained. Nitrate is eluted by sodium bicarbonate solution. Dried aliquots of nitrate are treated with salicylic acid in concentrated sulfuric acid. The nitrated salicylic acid is made alkaline and the resulting yellow color is measured at 414 mμ in terms of nitrate ion. Interfering nitrite is quantitatively removed by treatment with sulfanilic acid before clarification.

(*10*) to measure nitrates in forage crops. These workers noted the interference by β-nitropropionic acid which occurs in certain legumes.

The microbiological reduction of nitrates to nitrites, and determination of the latter by diazotization with sulfanilic acid and coupling with 1-naphthylamine, have been described by Garner *et al.* (*7*). Nelson and coworkers (*11*) similarly determined nitrate after reduction to nitrite by zinc. Woolley *et al.* (*18*) modified the latter method to determine nitrate in plant material. These workers discussed the critical effect of traces of copper ion on the reduction of nitrate.

A method for the determination of nitrate based on the nitration of 2,4-xylenol has been devised by Blom and Treschow (*2*), and adapted to determination of nitrate in meat products by McVey (*9*).

Buckett, Duffield, and Milton (*4*) have modified the xylenol method for use in soil analysis. In alkaline solution, the nitrated compound yields a yellow color which is measured spectrophotometrically in terms of nitrate ion.

In the method here reported, nitrate is converted to nitrosalicylic acid, which is rendered alkaline and measured spectrophotometrically. The utility of charcoal columns for the separation of nitrate from nitrite, chloride, sulfate, ammonium salts, and other plant materials is described.

Materials and Reagents

Darco G-60, Atlas Powder Co., Wilmington, Del.

Celite 545, Johns-Manville, digested overnight with concentrated hydrochloric acid, washed, and air-dried at 100° C.

Celite 535, Johns-Manville.

Salicylic acid–sulfuric acid, SS reagent, 10% (w./v.) freshly prepared from ACS quality reagents.

Adsorbent mixture, Darco G-60 + Celite 545, 1 to 2 by weight, thoroughly mixed.

Eluting alkali, sodium bicarbonate, 0.1% (w./v.), ACS quality.

Sodium hydroxide solution, 30% (w./v.), ACS quality.

Extracting solution, 0.6*N* orthophosphoric acid containing 4% sodium sulfate (w./v.), ACS quality.

Apparatus

Chromatograph tube (Corning Glass Co., Catalog LG-1, No. 38460 or equivalent), 20-mm. diameter, 400-mm. length, with 29/42 standard taper (female) joint at base of tube body. Fritted disk, coarse porosity, sealed into top of lower (male) member of joint. Delivery stem of lower joint provided with rubber stopper to seat in neck of suction flask.

Suction flask, 500-ml. side tube coupled by Tygon sleeve to glass adapter (5-cm. length) packed with glass wool; reduced end of adapter connected to vacuum line.

Separatory funnel, 500-ml., stem provided with rubber stopper to seat over chromatograph tube.

Experimental

Sample Preparation. Samples to be stored before analysis are treated as follows. Twenty-five grams of plant material are tamped into a specimen jar of smallest convenient size—capacity (ounces) = 0.15 × dry matter (%). Generally, fresh green plant material (dry matter < 26%), may be stored in 4-ounce jars, while wilted material (dry matter < 53%) requires 8-ounce jars, etc. Analysis of ground hay or oven-dried material may proceed with 5-gram samples in 4-ounce jars. Forty milliliters of extracting solution are added, and the jars are capped and stored in a refrigerator until receipt of a dry matter determination. Additional extracting solution is added to adjust the ratio of dry matter to liquid to 1 gram per 16 ml. A small crystal of thymol is added as a preservative.

After 3 days of soaking, the liquor is drained into a beaker, slurried with approximately 5 grams of Celite 535, and filtered by pressure through a 3-cm. column of Celite 535.

Alternatively, extracts may be prepared by comminution of 25 grams of sample with 250 ml. of extraction solution for 5 minutes in a Waring Blendor provided with a Polytron head. The ratio of dry matter to liquid is calculated after receipt of the dry matter determination.

Chromatographic Procedure. A circular piece of filter paper, No. 42 Whatman or equivalent, is placed over the filter plate of the chromatograph tube. Celite 545 is added above the filter to give a column height of approximately 25 mm. This Celite bed is leveled by gentle tamping and suction is applied while 25 grams of adsorbent mixture are added. The top surface of the adsorbent is leveled by gentle tamping and then capped with a 25-mm. section of Celite 545.

The separatory funnel is seated over the chromatograph tube. Suction is applied with the separatory stopcock closed. Then 80 ml. of distilled water, approximately the volume necessary to saturate the dry column, are added to the separatory funnel and admitted to the column. Suction is interrupted when the water level approaches the top of the adsorbent column. The separatory funnel is removed.

An aliquot of the plant extract (5 to 20 ml.) is added and drawn into the column along with successive rinsings. Five-hundred milliliters of distilled water are added to the separatory funnel, from which approximately 30 ml. are added to the column. The funnel is then seated over the column, stopcock opened, and vacuum applied to draw water through the column. Thereby, monosaccharides, chlorides, sulfates, etc., are washed through the column while nitrate is retained. Vacuum is interrupted when the water level is slightly above the top of the packed column, and the filtrate is discarded.

In the same manner, 500 ml. of eluting alkali are drawn through the column to elute nitrate. A preliminary volume of the filtrate (ca. 50 ml.) is safely discarded before nitrate begins to emerge from the column. Filtration is resumed to elute the nitrate and is terminated when all of the eluting liquid has been drawn into the column. The volume of filtrate is adjusted to 500 ml. with distilled water.

Color Development. A suitable aliquot of the eluate (1 to 50 ml.), according to the nitrate content, is pipetted into a 150-ml. beaker and brought to dryness over a steam bath. Three milliliters of SS reagent are added to the cooled beaker and allowed to stand for 1 hour; a short glass stirring rod is used to loosen and speed solution of any salt crust. Ten milliliters of water are slowly added from a pipet to layer over the SS reagent. The beaker contents are cautiously swirled and then allowed to cool. Twenty milliliters of sodium hydroxide, 30% (w./v.), are added slowly to layer over the beaker contents, which are then gradually mixed and allowed to cool in the dark to avoid photochemical change.

The resulting yellow color is measured spectrophotometrically at 414 mμ. Nitrate is estimated by reference to calibration curves obtained on 25- to 100-μg. quantities of nitrate ion measured from a standard solution of potassium nitrate.

Table I. Chromatographic Recovery of Nitrate Ion Added at Three Levels to Charcoal-Celite Columns as Potassium Nitrate in 0.6*N* Phosphoric Acid

	Nitrate Recovered, Mg. NO_3^-, at Each Level		
	Level 1, 1.000 mg., 6 columns	Level 2, 5.000 mg., 5 columns	Level 13, 10.000 mg., 6 columns
	1.013	4.850	9.820
	1.009	4.893	9.820
	1.003	4.969	9.820
	0.993	4.925	9.860
	1.000	4.955	9.970
	1.019		9.970
Av.	1.0062	4.919	9.877
Av. recovery, %	100.62	98.38	98.77

Table II. Nitrate Found in Orchardgrass and Corn Plant

Comparison of 0.6N phosphoric acid with 0.6N sulfuric acid as an agent for both extractive and chromatographic use

	Nitrate Found, P.P.M. (Dry Basis), for Extractant		
	0.6N phosphoric acid	0.6N sulfuric acid	0.6N phosphoric acid–4% sodium sulfate
	Orchardgrass S3		
	13,940	15,110	
	13,940	15,070	
	14,070	15,110	
	13,980	15,200	
Mean	13,980	15,120	
S. E.	±30	±28	
	Orchardgrass S7		
		13,750	14,610
		13,940	14,480
		13,940	14,390
		14,030	14,390
Mean		13,920	14,460
S. E.		±66	±66
	Corn Plant		
	10,070	10,090	10,300
	10,070	10,090	10,300
	9,900	9,960	10,320
	9,940	10,020	10,290
Mean	9,995	10,040	10,302
S. E.	45	33	8

Table III. Nitrate Found in Orchardgrass Silage by Immediate Extraction and by Soaking Procedures

	Nitrate Found, NO_3^-, P.P.M., Dry	
	Immediate extraction (6 samples)	Soaking procedure (6 samples)
	3750	3670
	3930	3680
	3810	3540
	3840	3370[a]
	3810	3690
	3660	3680
Mean	3793	3652
S. E.	39	28

[a] Omitted in av. cf. Pierce and Haenisch (*13*).

Results and Discussion

The choice of reagent, salicylic acid, was in deference to its long acceptance for the determination of nitrate in the modified Kjeldahl procedure. Scovell (*14*) in 1887 first proposed the use of excess salicylic acid for quantitative conversion of nitrate to nitrosalicylic acid and since then this reagent has been widely used (*17*).

The absorption curve of salicylic acid in alkaline solution exhibits a peak at 414 mμ. Solutions prepared by treatment of known amounts of nitrate with SS reagent, as described, show essentially linear absorption in respect to concentration of nitrate. As measured in this laboratory, the absorbance for 100 μg. in the reaction volume was 0.663 at 414 mμ. Absorbance measurements for data reported herein were made by use of a Beckman spectrophotometer, Model DU, with slit openings of 0.04 mm. Absorbance corrections for the reagent blank were made for the data presented herein, and generally were approximately 0.010.

The extractant, 0.6*N* phosphoric acid–4% sodium sulfate, was chosen after earlier tests had been made with 0.6*N* solutions of phosphoric and sulfuric acids. These tests were made, initially, to determine if standard solutions of potassium nitrate in either of these two acids could be added to charcoal columns and the nitrate be quantitatively recovered. Subsequent tests measured the relative suitability of these acids for the complete procedure—that is, for extraction as well as for chromatographic performance.

Thus, experiments confined to chromatographic recovery consistently showed small losses, sometimes up to 5%, when potassium nitrate solutions in 0.6*N* sulfuric acid were chromatographed. Solutions of nitrate in 0.6*N* phosphoric acid, however, gave essentially complete recovery, as shown in Table I. The average recovery from 17 columns was 99.4%. In the tests, columns were charged at three levels of nitrate ion—1, 5, and 10 mg. Aliquots of chromatographed nitrate solutions and equivalent aliquots of standard nitrate solutions were analyzed and their relative absorbances compared as a measure of recovery.

Thus, at the 1-mg. level, six aliquots (100 μg. each) of standard nitrate had an average absorbance of 0.675 (S.E. = 0.002) against 0.679 (S.E. = 0.003), the average for equivalent aliquots for six columns. At the 5-mg. level, five aliquots (50 μg. each) of standard nitrate averaged 0.326 (S.E. = 0.002) against 0.321 (S.E. = 0.002) for equivalent aliquots from five columns. Similarly, at the 10-mg. level, six standards (100 μg. each) averaged 0.662 (S.E. = 0.001) against 0.654 (S.E. = 0.001) for six chromatographed samples.

In Table II analyses are listed to compare the use of 0.6*N* solutions of sulfuric and phosphoric acid for the complete procedure. Samples S3 and S7 were duplicate field samples of orchardgrass as ensiled. Analyses were made on the ground oven-dried samples. The results for sample S3 indicate that the extraction of nitrate from orchardgrass by 0.6*N* phosphoric acid is incomplete—less than by 0.6*N* sulfuric acid. However, 0.6*N* phosphoric acid–4% sodium sulfate gave extracts (sample S7) which analyzed 3.9% higher in nitrates than extracts by 0.6*N* sulfuric acid—i.e., 14,460 against 13,920 p.p.m. This difference may reflect the slightly higher chromatographic recoveries noted earlier for standard solutions of nitrate in 0.6*N* phosphoric acid rather than in 0.6*N* sulfuric acid.

The differences noted in Table II cannot be attributed to blank differences brought about by charring action of the SS reagent on organic matter conceivably present in the eluate. Char blanks made by treating the dried aliquots with concentrated sulfuric acid in place of SS reagent were negligible in absorbance (<0.001) for analyses corresponding to samples S3 and S7.

Also shown in Table II are analyses for dried corn plant made on the same day with each of the three extractants. Here, results are slightly highest for phosphoric acid–sodium sulfate extracts.

The presence of sodium sulfate may improve the extractability of nitrate by phosphoric acid by providing sulfate ion to displace nitrate ion from plant constituents. An alternative or supplementary role of sodium sulfate may be suppression of emulsions. Thus, Celite-filtered extracts of fresh plant material by 0.6*N* phosphoric acid are strikingly turbid when sodium sulfate is not included. Dried plant material, on the other hand, does not present this problem. Extracts of either fresh or dried material by 0.6*N* sulfuric acid or by 0.6*N* phosphoric acid–4% sodium sulfate are clear.

The two methods of extraction—i.e., by the immediate or by the soaking procedure—are compared in Table III. Twelve 25-gram samples from one basket of orchardgrass were analyzed for nitrate—six samples by each method. Immediate extraction yielded 3.9% more nitrate than extraction by soaking.

Nitrite, when present, is retained by charcoal-Celite columns and is eluted

Table IV. Recovery of Nitrate Added to Plant Materials

	Orchardgrass		Orchardgrass Silage		Corn Plant	
	Control	Control + NO_3^-	Control	Control + NO_3^-	Control	Control + NO_3^-
A_{414} samples	0.185	0.768	0.034	0.600	0.257	0.806
	0.192	0.763	0.027	0.607	0.265	0.832
	0.192	0.776	0.026	0.601	0.266	0.848
	0.186	0.766	0.031	0.611	0.266	0.822
	0.196	0.779	0.029	0.607	0.240	0.858
	0.183	0.775	0.031	0.592	0.247	0.872
Mean	0.189	0.771	0.030	0.603	0.257	0.840
S.D.	0.005	0.006	0.003	0.007	0.011	0.024
A_{414} recovery	0.582		0.573		0.583	
A_{414} calibration	0.603		0.596		0.594	
Recovery ± S.D., %	96.5 ± 1.3		96.1 ± 1.2		98.2 ± 4.6	

Table V. Recovery of Nitrate from Nitrate-Nitrite Solutions Treated with Sulfanilic Acid (SA) before Chromatography

(4.75 mg. nitrate, 4.93 mg. nitrite + 40 mg. SA chromatographed)

Column	Nitrate Found, (NO_3^-), Mg.
1	4.65
2	4.64
3	4.71
Mean	4.67
Mean recovery, %	98.3

Table VI. Nitrate (NO_3^-) Disappearance from High-Nitrate Forage Stored in Farm and Quart-Jar Silos

Storage Time, Days	Farm Silo		Quart-Jar Silos: Chopped Forage		Quart-Jar Silos: Ground Forage	
	pH	Nitrate, p.p.m.	pH	Nitrate, p.p.m.	pH	Nitrate, p.p.m.
0	...	8240	...	8160	...	8160
3	4.30	6570	5.57	6660	4.77	1070 (13%)[a]
39	3.90	6520	3.99	4250	3.88	1180
90	4.84	600 (7.35%)[a]	4.59	1140	3.96	2090 (26%)[a]

[a] % of initial nitrate content.

along with nitrate by sodium bicarbonate solution. Nitrite reacts with SS reagent to yield approximately one sixth the absorbance of an equal weight of nitrate. This reaction presumably involves oxidation of nitrite to nitrate (*4*).

However, no significant amount of nitrite was found in silages in this laboratory, a condition also noted elsewhere (*7*, *18*). The need for removal of interfering nitrite does arise in studies of silage gases. Thus, nitric oxide evolved from silage, when mixed with air and trapped in alkali, is converted to a large extent to nitrite as well as nitrate.

Nitrite is removed from nitrate by treatment with sulfanilic acid before chromatography, as shown in Table IV. Here, three 10-ml. aliquots of nitrate (4.75 mg.) were chromatographed directly as reference; three additional 10-ml. aliquots were each mixed with 15 ml. of nitrite (4.93 mg. of nitrite ion) and 10 ml. of sulfanilic acid (0.4% in 0.6*N* phosphoric acid) and then chromatographed. The results shown in Table IV indicate effective removal of nitrites by this procedure.

Table V lists recoveries of nitrate added to plant materials before extraction. Each result represents a completely independent analysis of plant material extracted with 250 ml. of 0.6*N* phosphoric acid–4% sodium sulfate. In the case of fortified samples, 50 ml. of this volume of extractant were added as a solution of potassium nitrate (125 mg. of NO_3^-) in the same medium. Analyses were made on 25-gram samples of chopped material: fresh orchardgrass, orchardgrass silage, and fresh corn plant. Aliquots of the potassium nitrate standard were also analyzed for calibration absorbances.

Results in Table V indicate 96.1 to 98.2% recovery of nitrate added to samples before extraction. Larger analytical variation shown for corn plant may be associated with the relatively heterogeneous composition of the chopped samples.

Data in Table VI suggest that toxic levels of nitrate may be removed by the ensiling process. Although Barnett (*1*) has suggested that nitrate is somewhat removed by silage fermentation, over 90% of the nitrate may occasionally be removed. Thus, in the farm silo, noted in Table VI, over 92% of the nitrate was removed—a major disappearance occurring with rise in pH. Analyses listed for quart-jar silos were made on individual jars of silage. The ideal conditions for maximum removal of nitrate in production of good silage require further study.

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