common foods that are readily available. The niacin supplement from coffee might be important to certain individuals.

The absence of pellagra in some areas of the world, where the diet appears to be low in niacin and good quality protein, has not been satisfactorily explained. There are other areas where pellagra (not necessarily simple niacin deficiency) is not uncommon.

Other Vitamins. Values for eight B vitamins, other than niacin, are given in Table IV. Except for thiamine, the vitamins survive the roasting process well, considering the high temperatures employed. Assays on beverage prepared from the roasted coffee indicate that the vitamins are easily extracted. The vitamins are present in measurable amounts, although at rather low levels in relation to dietary requirements.

Minerals. Moderate levels of extractable calcium and iron, and low levels of sodium and fluorine were present in roasted coffee (Table V).

Acknowledgment

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Literature Cited

(1) Assoc. Offic. Agr. Chemists, Washington, D.C., "Official Methods of Analysis," 8th ed., p. 415, 1955.
(2) *Ibid.*, first action, p. 830.
(3) Assoc. Vitamin Chemists, "Methods of Vitamin Assay," 2nd ed., p. 111, Interscience, New York, 1951.
(4) Atkins, L., Schultz, A. S., Williams, W. L., Frey, C. N., *Ind. Eng. Chem., Anal. Ed.* **15,** 141 (1943).
(5) Barton-Wright, E. C., *Biochem. J.* **38,** 314 (1944).
(6) Bills, C. E., McDonald, F. C., Niedermeier, W., Schwartz, M. C., *J. Am. Dietet. Assoc.* **25,** 304 (1949).
(7) Cravioto, R. O., Guzman, G. J., Suarez, M. L., *Ciencia (Mex.)* **15,** 24 (1955).
(8) Horowitz, N. H., Beadle, G. W., *J. Biol. Chem.* **150,** 325 (1943).
(9) Hughes, E. B., Smith, R. F., *J. Soc. Chem. Ind.* **65,** 284 (1946).
(10) Ingols, R. S., Murray, P. E., *Anal. Chem.* **21,** 525 (1949).
(11) Krehl, W. A., Teply, L. J., Elvehjem, C. A., *Science* **101,** 283 (1945).
(12) Moores, R. G., Greninger, D. M., *Anal. Chem.* **23,** 327 (1951).
(13) National Academy of Sciences, National Research Council, Washington, D. C., "Recommended Dietary Allowances," Pub. 302, rev., 1953.
(14) Pharmacopeia of the United States of America, 15th rev., p. 461, Mack Printing Co., Easton, Pa., 1955.
(15) *Ibid.*, p. 885.
(16) *Ibid.*, p. 912.
(17) Psychological Corp., New York, "The Beverage Biography Studies," winter 1956.
(18) Sandell, E. D., "Colorimetric Determination of Traces of Metals," 2nd ed., p. 378, Interscience, New York, 1950.
(19) Sauberlich, H. F., *J. Biol. Chem.* **181,** 467 (1949).
(20) Skeggs, H. R., Wright, L. D., *Ibid.*, **156,** 21 (1944).
(21) Slotta, K. H., Neisser, K., *Ber.* **72B,** 133 (1939).
(22) Squibb, R. L., Institute of Nutrition of Central America and Panama, Guatemala, private communication.
(23) Teply, L. J., Elvehjem, C. A., Abstracts, 107th Meeting, ACS, April 1944.
(24) Teply, L. J., Krehl, W. A., Elvehjem, C. A., *Arch. Biochem.* **6,** 139 (1945).

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FORAGE ANALYSIS

Effect of Cold Storage on the Fluoride Content of Alfalfa

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The tediousness of determinations of fluoride in vegetation samples, coupled with a frequent need to gather a large number of samples in a short time, usually requires storage of samples prior to analysis. Tests using alfalfa samples were conducted to determine the effect of storage at 0° F. for 4 to 6 weeks on fluoride content. Stored samples show more variation than unstored samples, but no consistent change in fluoride content. The variation can be reduced by adding lime to stored samples. Samples stored in a tight container do not gain or lose moisture consistently.

THE SPREAD OF INDUSTRIES into agricultural areas in recent years has produced an increased concern over the contamination of vegetation by fluorides. Although the toxic effects of fluorides to vegetation create some concern, a more important consideration is the effect of fluoride contamination upon animals eating the vegetation as forage. This forage problem makes a rather extensive vegetation sampling and chemical analysis program a necessity in evaluating the degree of any air pollution by fluorides. Because the analytical procedures involved in the fluoride determinations of biological materials are time-consuming, samples must often be stored for varying periods before they can be analyzed. Another complicating factor arises when large numbers of samples arrive at the laboratory in a short period of time. Alfalfa samples as used throughout this paper refer to green alfalfa and not to hay made from alfalfa.

These studies were conducted to determine whether any errors were being introduced into the fluoride determinations by the storage of samples. The objectives of these tests were to ascertain if alfalfa samples could be placed in cold storage without first weighing, liming, and taking a moisture aliquot; any change in percentage of moisture in the samples after deep freeze storage, and the effect of cold storage on both limed and unlimed alfalfa samples as compared to a control group. Alfalfa was studied because of its availability, its sensitivity to the intake of air-borne fluorides, and its general importance as an agricultural product.

Procedure

In preparing alfalfa for deep-freeze storage 20 grams of the material, which had been detruncated in the field at the time of collection into approximately

3/4-inch lengths, were placed in a tightly closed plastic container (polyethylene or polystyrene). Approximately 1 gram of fluoride-free lime was added to the 20-gram aliquot to fix any volatile fluorides. A second aliquot was weighed out, usually about 10 grams, for use in determining the percentage of moisture in the sample. The moisture aliquot was dried to constant weight at 221° F. The aliquot to be stored for subsequent chemical analysis was placed in cold storage at 0° F. A series of tests was carried out to see if the sample could be stored satisfactorily without liming or previous weighing and omit unnecessary work. Tests of this type were reported by Miller and coworkers (*1*), who found no change in forage samples stored without lime at 0° F. for 90 days. It was decided to repeat the study on a more extensive scale and to use alfalfa rather than pasture grass.

All analyses were carried out using the method of Willard and Winter (*4*) with modifications as suggested by Remmert and Parks (*3*). The sampling and analysis and all steps in the procedure were checked and standardized in the manner described by Nichol and coworkers (*2*).

Phase 1 of the study consisted of gathering duplicate alfalfa samples from 118 sample sites. One aliquot of the material from 80 of these sites was stored at 0° F. upon arrival at the laboratory and without weighing, liming, or taking a moisture aliquot. The other aliquot from each of these 80 sites was started in the analytical circuit in the usual manner at this same time. The remaining 38 pairs of samples were placed immediately in the analytical circuit and were analyzed in the usual manner.

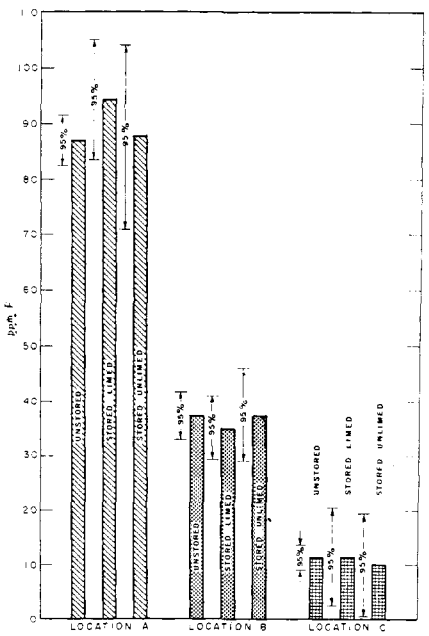

Figure 1. Effects of storage at 0° F. for 4 weeks upon fluoride content of alfalfa samples

Table I. Statistical Analysis of Stored and Unstored Samples

	Stored Samples	*Unstored Samples*
Number of pairs	80	38
Average % deviation	+0.58	+0.59
Standard deviation of % deviation	9.0	5.5

In approximately 6 weeks, the 80 samples in cold storage were removed and prepared for analysis. A 20-gram sample was weighed out, limed, and sent through the analytical circuit, and a moisture aliquot was taken.

Phase 2 of the study used replicates started immediately in the analytical circuit as controls and was carried out at a later date. A large quantity of alfalfa was collected at three sites of different fluoride content. Twenty-four replicate samples from each of the three sites were prepared: eight prepared for immediate analysis, eight weighed and placed in suitable containers at 0° F. without lime, and eight placed in cold storage with lime added (approximately 1 gram). Moisture aliquots were taken immediately. The samples were kept in storage at 0° F. for 4 weeks and then analyzed.

Results

The results of phase 1 are based upon a study of the percentage of variation in fluoride content from the average value, with the freezer-held sample compared with its previously analyzed duplicate. The usual duplicates were compared with each other. The expression which was analyzed statistically was $(X_1 - X_2)/\bar{X}$, where X_1 and X_2 are the two samples in the pair and $\bar{X}$ the average. These data are shown in Table I. The variations shown by the 38-sample pairs were used as a guide to determine the effect, if any, of unlimed storage on the samples.

It is evident that there was no over-all difference between the two groups of samples on the basis of average per cent deviation either from each other or from a theoretical value of 0% deviation. However, the standard deviations of the two groups show that there is significantly more variation within the deep freeze duplicates than there is within the other set of duplicates. This difference, based upon a statistical F test, is significant at the 1% level. These standard deviations indicate that 90% of the control duplicates came within 11% of the average value and that in the freezer samples 90% of the samples are within 18% of the average value.

Variations in moisture content can result in variations in apparent fluoride concentrations. In this test the average difference between the aliquots where one had been stored prior to the taking of the moisture determination was 0.01%. In the 38 duplicate samples where both were analyzed at the same time, the average difference in moisture was the same, 0.01%, essentially 0. The individual results were studied to determine whether those samples which differed most in moisture also differed in fluoride content. No connection was apparent from the data. The stored samples showed more variability in moisture values than did the unstored duplicate samples although no net change was indicated.

The experiment lacked at least one important feature—the deep freeze storage was not carried out on aliquots of the same sample material which was run in the usual duplicate manner. This tends to limit the sensitivity of the experiment and to require larger variations to show significance than if the freezer and normal replicates had been aliquots from the same sample site. As the experiment showed significant variation even with this limitation, the conclusions still seem to be valid.

The second storage test described as phase 2 was carried out because there might be some question as to the applicability of the unstored duplicate variability between the stored and unstored samples, and also to determine whether the addition of lime to freezer-stored samples was advantageous.

The results of this test covering the three treatments of samples from three areas of different fluoride content are shown in Figure 1. The 95% confidence limits as calculated on the basis of 2 standard deviations are indicated. These data show that the following conclusions can be drawn from phase 2: The storage of alfalfa samples either with or without lime does not produce an over-all change in fluoride content, the storage of samples increases the sample variation, the increased sample variation brought about by storage is greater for unlimed samples than it is for samples stored with lime. Thus, the results of this test are comparable with those of the first.

Conclusions

If there had been a loss of fluoride upon storage, it could probably have been assumed that some of the more volatile materials had been lost by the sample. However, the results show no consistent change in fluoride content, only increased variation between replicate samples. At present, there is no ready explanation for this particular result. The consistency between the two phases of the study which were carried out several months apart would seem to rule out fortuitous analytical or sampling errors.

The results of these tests indicate that alfalfa samples can be stored at 0° F. prior to analysis and that the preparation for cold storage should consist of weighing, liming, and the taking of a moisture aliquot. The sample container should be sealed. Although all stored samples exhibit more variation in fluoride content than those samples analyzed soon after collection, the addition of lime to stored samples will reduce the variation. The increased variability can be obviated by carrying out replicate analyses on the stored samples. By standard statistical procedures it can be shown that two replicate analyses of stored material reduce the probable uncertainty by 30%, while a sample value based upon four replicates will have only half the probable uncertainty of a single analysis of the same material.

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Literature Cited

(1) Miller, V. L., Allmendinger, D. F., Johnson, F., Polley, D., J. AGR. FOOD CHEM. **1,** 526 (1953).
(2) Nichol, A. A., Benedict, H. M., Byrne, J. L., McCarty, C. P., Air Pollution Control Assoc. Annual Meeting, Buffalo, N. Y., Paper 56-23, May 1956.
(3) Remmert, L. F., Parks, T. D., *Anal. Chem.* **25,** 450 (1953).
(4) Willard, H. H., Winter, O. B., *Ind. Eng. Chem., Anal. Ed.* **5,** 7 (1933).

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ANTIOXIDANT DETERMINATION

Determination of 2,6-Di-*tert*-butyl-*p*-cresol in Edible Fats by Ultraviolet Spectrophotometry

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2,6-Di-*tert*-butyl-p-cresol is a very effective agent for inhibiting oxidation in organic substances, including edible fats. To maintain effective control over food processing operations and ensure adherence to governmental regulations, a quantitative method was developed for determining 20 to 200 p.p.m. (0.002 to 0.02%) in lards in the presence of other allowable preservatives, including butylated hydroxyanisole, nordihydroguaiaretic acid, propyl gallate, citric acid, monoisopropyl citrate, and phosphoric acid. The lard sample is dissolved in cyclohexane and percolated through a chromatographic column packed with 100-mesh silicic acid. By washing the column with successive portions of cyclohexane, 2,6-di-*tert*-butyl-p-cresol is selectively removed from the adsorbent and recovered in the column filtrates. The filtrates are subsequently analyzed by ultraviolet spectrophotometry, and the amount is calculated from absorbance measurements made at a wave length of 284 mμ. This procedure has been tested with synthetic formulations of commercial brand lards and a hydrogenated vegetable oil shortening containing this additive. Recoveries consistently better than 93% can be obtained.

A VERY EFFECTIVE OXIDATION INHIBITOR for a wide variety of organic substances, including rubber, petroleum products, and plastics, is 2,6-di-*tert*-butyl-*p*-cresol (DBPC, Koppers Co. registered trade-mark). Recently, by approval of the Meat Inspection Division, U. S. Department of Agriculture (*7*), its use has been extended to edible fats, notably lards, where prior studies have shown that the development of rancidity can be delayed effectively through the use of as little as 100 p.p.m. (0.01 weight %) of this compound.

To maintain effective production control over lard processing operations employing this antioxidant and to ensure adherence to governmental regulations, a quantitative method for the analysis of commercial products was needed. Moreover, because mixtures of preservatives are currently employed in the stabilization of lard, this method should be capable of determining 20 to 200 p.p.m. of 2,6-di-*tert*-butyl-*p*-cresol in lards in the presence of other allowable preservatives. According to present regulations, the latter could include butylated hydroxyanisole (BHA), nordihydroguaiaretic acid (NDGA), propyl gallate, citric acid, monoisopropyl citrate, and phosphoric acid.

A survey of the existing literature revealed that Chapman (*2*) and Chapman and Mahon (*3*) had developed selective solvent extraction procedures for the separation and quantitative analysis of mixtures of propyl gallate, butylated hydroxyanisole, nordihydroguaiaretic acid, and naturally occurring tocopherols in vegetable fats and oils. According to their methods, the differential solubilities of these compounds in water, ethyl alcohol, and petroleum ether were utilized to provide extracts which could be analyzed by suitable colorimetric procedures. A similar procedure was adapted by Austin to the analysis of 2,6-di-*tert*-butyl-*p*-cresol in fats and oils (*1*), and as this method appeared to be suited to the problem, it was tested in these laboratories using lards containing accurately known quantities of 2,6-di-*tert*-butyl-*p*-cresol. Unfortunately, the results obtained were variable, quantitative recoveries could not be obtained, and the elapsed time required was so long that the method seemed unsuitable for the ultimate demands to be placed on it.

Subsequent attempts were made to apply other existing procedures for the determination of 2,6-di-*tert*-butyl-*p*-cresol in various materials to its determination in lards. In general, these procedures depended on the use of visual colorimetric methods which, while adequate for the original purpose, proved to be insensitive to the small quantities