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METHODOLOGY

Determination of Cystine as Cysteic Acid after Low Voltage Paper Electrophoresis

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A procedure for the chemical determination of cystine includes as basic steps: oxidation of the samples with performic acid, hydrolysis with HCl, paper electrophoresis, ninhydrin reaction, elution, and colorimetric determination. This procedure when applied to a variety of food samples yielded accurate and reproducible results. With two electrophoresis cells as many as 12 samples can be assayed simultaneously.

Because carbohydrates destroy cystine during acid hydrolysis (*1*) the direct chemical determination of cystine is not reliable. Microbiological assays have been found to yield erroneous values because a short period of hydrolysis gave results that were too high; prolonged hydrolysis, results that were too low (*5*). Since cysteic acid is stable during acid hydrolysis, Schram, Moore, and Bigwood (*11*) recommended the oxidation of cystine to cysteic acid before hydrolysis and chemical assay. Methods for oxidation of cystine by performic acid have been improved to yield recoveries of 94% or more (*3, 8, 9*), but the subsequent separation from other amino acids by ion exchange is not easily adaptable to the simultaneous assay of several samples.

Hartel and Pleumeekers (*6*) used ion exchange paper chromatography for separating cysteic acid in wool hydrolyzates; in this laboratory a similar technique has been applied to foods after oxidation but with little success.

Mabry and Karam (*7*) reported that high voltage paper electrophoresis with a low ionic strength buffer separated cysteic acid from other amino acids. Low voltage paper electrophoresis was used by Diehl (*4*) for separating cysteic acid in wool hydrolyzates. In this laboratory, the latter method gave incomplete separation of cysteic acid from homocysteic acid and was not sensitive enough for the assay of food samples. The method described uses low voltage electrophoresis under conditions which permit the separation of homocysteic acid from cysteic acid and is sufficiently sensitive to be applied to the assay of food samples.

Experimental Procedure

Apparatus. Electrophoresis Cell. Durrum type paper electrophoresis cell (Spinco Model R) operated by a Spinco Duostat power supply.

Paper Strips. Whatman 3 MM paper strips 3.0 × 30.6 cm.

Paper Wicks. Grade EP 14, $2^2/_{32} \times 12^1/_2$ inches.

Spectrophotometer. Spectronic 20 with matched cuvettes of about 16-mm. inside diameter.

Evaporator. Craig rotary evaporator or similar.

Reagents. Performic Acid Reagent. Add 10 ml. of 30% H_2O_2 to 90 ml. of 88% formic acid. Allow the mixture to stand for 1 hour at room temperature. Cool to 0° C.

Hydrobromic Acid, 48% reagent grade HBr.

Sodium Hydroxide, 2*N* NaOH.

Hydrochloric Acid, 6*N* HCl.

Buffer Solution. To 700 ml. of H_2O add 30 ml. of formic acid and 120 ml. of glacial acetic acid. Dilute to 1 liter with H_2O.

Cysteic Acid Stock Standard Solution. Weigh 28 mg. of cysteic acid (equivalent to 20 mg. of cystine) and dilute to 100 ml. with H_2O.

Cysteic Acid Working Standard Solutions. Dilute 2, 3, 4, and 5 ml. of stock standard solution to 10 ml. with H_2O.

Ninhydrin Solution. 0.4% ninhydrin in ethyl alcohol containing 0.4% pyridine.

Copper Nitrate Solution. Mix 10 ml. of saturated copper nitrate solution with 0.2 ml. of concentrated nitric acid and make up to 1 liter with acetone.

Oxidation. This is essentially the method of Moore (*9*), in which some technical modifications have been incorporated. To a 125-ml. flat-bottomed flask with ground-glass joint add a sample containing about 1 mg. of cystine and 20 ml. of cold performic acid reagent and allow to stand at 0° C. for 4 hours with soluble proteins, or overnight with proteins that do not dissolve in the performic acid reagent. While swirling add 3.0 ml. of 48% HBr to the flask in the ice bath. Distill the free bromine at 40° C. under reduced pressure in a rotary evaporator and absorb the distillate into 25 ml. of 2*N* NaOH in the condenser. After the free bromine distillation is complete the last few milliliters of solution may be evaporated to near dryness at 60° C. without affecting the results. To minimize the tendency of HBr to condense in the stem of the rotary evaporator the level of water in the bath should reach the top of the reaction flask.

When the distillation is complete, raise the flask above the level of the condenser before the rotary evaporator is stopped, so that the condensation in the stem will run into the condenser

rather than back into the flask. Wipe the rim free from condensation. Alternatively, the whole stem of the condenser may be warmed to 60° C. or a high vacuum pump may be used with solid CO_2 in the condenser bath. The results are not affected by a small amount—e.g., 0.5 ml.—of residual HBr.

Hydrolysis. Add 30 ml. of 6 *N* HCl to the residue and hydrolyze by refluxing overnight.

Electrophoresis. Remove the HCl by distillation in the rotary evaporator, dissolve the residue in 25 ml. of water, and clarify by centrifuging. Place on a piece of Saran Wrap (Dow Chemical Co.) 50 µl. of the supernatant in two approximately equal drops 10 mm. apart. Bend the paper strip slightly in the center of its 30.6-cm. length and lower the bent part over the two spots on the Saran Wrap so that the spots are absorbed into a band. After 10 minutes apply another 50 µl. of samples to the same paper. To separate strips make a single 50-µl. application of standard solutions containing 40, 60, 80, and 100 µg. per ml. Add 500 ml. of buffer to each of the two chambers of the electrophoresis cell and allow the two horizontal paper wicks to become saturated. Attach the strips at both ends of the extensible rack and fold over the rack with glass rods in the electrophoresis cell so that the ends of the strips stick to the paper wicks. After closing the cell, allow the strips to saturate fully with buffer. It is possible to speed up saturation of the strips by adding buffer gradually by pipet to the bottom of the strips alternately and equally on each side and up to not more than 30 mm. from the apexes. Allow electrophoresis to proceed overnight at 350 volts. With the Duostat, two electrophoresis cells will accommodate 12 samples in addition to the four levels of standard.

Color Reaction. Remove the rack from the cell and place it, extended, in a fume hood for about 1 hour. Transfer the rack into a drying oven at 67° C. for about 1 hour. During the drying operation, it is preferable to place the rack at a 45° to 90° angle.

Add by pipet 1 ml. of ninhydrin solution in such a manner as to saturate the paper uniformly without excess, from the center to the anode end. After 5 minutes, repeat the procedure on the reverse side of the paper. Let stand 10 minutes in the fume hood before drying at 67° C. for 3 hours. Add 1 ml. of copper nitrate solution over the same portion of the paper and let stand in a semidarkened room for at least 30 minutes.

Remove the cysteic acid area from the strip by cutting uniformly wide bands (15 mm. was found satisfactory). Roll the cut band over its length, place in the bottom of a 16-mm. cuvette, and cover with 5 ml. of methanol. Swirl the tubes occasionally—e.g., every 15 minutes—and after 1 hour remove the strips. Make blanks by cutting a 15-mm. band about 5 mm. below the cysteic acid band towards the anode.

Read absorbance in 16-mm. matched cuvettes at 505 mµ, using a Spectronic 20 set at 0 absorbance with methanol.

Calculations. Correct absorbance by subtracting appropriate blanks. Plot a standard curve in micrograms per strip *vs.* absorbance. Read samples against a standard curve. If 100 µl. of sample solution was applied, the calculations are as follows:

$$\% \text{ cystine} = \frac{\mu\text{g. (read from curve)}}{\text{weight of sample in grams}} \times 0.0266$$

This factor of 0.0266 includes a correction based on 94% recovery of cystine as cysteic acid.

Results and Discussion

As shown in Table I, samples of casein in the absence of carbohydrates gave the same values whether hydrolyzed before or after oxidation, but in the presence of an equal amount of carbohydrate there was a considerable loss when hydrolysis preceded oxidation. This demonstrates that oxidation must precede hydrolysis in order to obtain full conversion of cystine to cysteic acid in the presence of carbohydrates.

The effect of residual HBr during heating and hydrolysis was tested by adding 1 ml. of 48% HBr to cysteic acid (equivalent to 1 mg. of cystine). In the presence of HBr, no cysteic acid was lost on heating at 70° C. for 1 hour and hydrolyzing with HCl for 18 hours.

For electrophoresis at 350 volts, a low ionic strength buffer (*7*) consisting of formic acid, glacial acetic acid, and distilled water in the ratio 30:120:850 gave a good separation of cysteic acid from other amino acids including homocysteic acid. This required 16 hours but resulted in no loss of time, since it could be done conveniently overnight. Another buffer (*12*) composed of formic acid, glacial acetic acid, and distilled water in the ratio 3.5:29:967.5, gave much more rapid migration but incomplete separation from homocysteic acid. Diehl's buffer (*4*) consisting of pyridine, acetic acid, and water in the ratio 10:100:890, also gave a rapid separation from other amino acids except homocysteic acid.

In a sample of fish that had been extracted with ethylene dichloride, homocysteic acid was found in an amount equivalent to about 20% of the cysteic acid present. Thus, depending on the processing conditions, foods may contain compounds which upon oxidation could yield homocysteic acid.

For optimum color development it was necessary to dry the strips at room temperature for 1 hour and at 67° C. for the same period before applying ninhydrin; after adding ninhydrin, the strips were dried 3 hours at 67° C. Air drying at this step decreased the color reaction, and higher temperatures resulted in high blanks and variable results. For optimal results, the ninhydrin solution must contain an equal amount of pyridine, which produced a slightly alkaline solution; furthermore, the solution was added in two applications rather than one of a doubly concentrated solution because the latter resulted in higher blanks. Figure 1 shows a linear plot of absorbance *vs.* cysteic acid expressed as micrograms of cystine. The intensity of absorbance varied at times

Table I. Effect of Sequence of Oxidation and Hydrolysis on Yield of Cysteic Acid from Casein (Expressed as % Cystine in Casein)

	Oxidation and Hydrolysis		Hydrolysis and Oxidation	
Chromatogram	No starch	50% starch	No starch	50% starch
1	0.305	0.307	0.310	0.236
2	0.309	0.319	0.309	0.239
3	0.320	0.311	0.306	0.245
4	0.309	0.310	0.310	0.231
Mean	0.311	0.312	0.309	0.238
S.D.	0.006	0.005	0.002	0.006

Table II. Assay of Cystine from a Hydrolyzate of Oxidized Casein and Recovery of Added Cysteic Acid

Test	% Cystine	% Recovery[a]
1	0.305	97.5
2	0.310	101.5
3	0.307	102.5
4	0.303	103.5
5	0.327	105.0
6	0.317	94.0
7	0.301	100.0
Mean	0.310	100.6
S.D.	0.009	3.8

[a] An amount of cysteic acid equivalent to half the cystine content was added to the hydrolyzate before electrophoresis. Recoveries represent difference from basic content.

Table III. Cystine in Samples of Rice with Added Portions of Fish Flour and Dry Milk

Sample	% Cystine		(Assay × 100)
	Expected[a]	Assay	(Expected)
Rice	0.178	0.178	100
Rice and 0.77% fish flour	0.182	0.183	100.5
Rice and 1.53% fish flour	0.188	0.176	93.6
Rice and 2.3% fish flour	0.193	0.186	96.4
Rice and 2% milk powder	0.180	0.182	101.1
Rice and 4% milk powder	0.181	0.184	101.7
Rice and 6% milk powder	0.183	0.185	101.1
Mean ± S.D.	...	...	99.2 ± 3.0

[a] Expected amount of cystine in mixtures was calculated from assay of constituents.

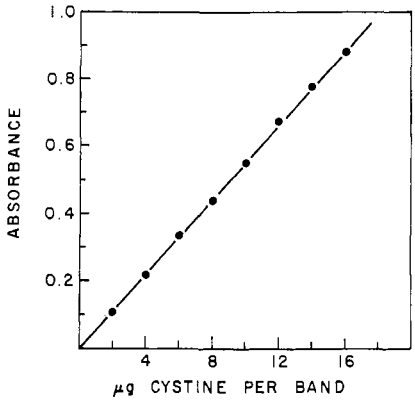

Figure 1. Absorbance of color produced by various amounts of cystine in 5 ml. of methanol as measured in 16-mm. cuvettes at 505 mμ

Table IV. Cystine in Various Foods (Gram %)

Food	Found	Lit. (10)
Casein	0.31	0.33
Promine[a]	1.09	0.97[a]
Whole egg (dry)	1.20	1.10
Yeast	0.56	0.55
Milk solids (fat-free)	0.26	0.32
Fish flour (alcohol extraction)	0.83	0.95
Fish flour (ethylene dichloride extraction)	0.61	...
Rice	0.18	0.10
Wheat	0.34	0.32

[a] Information on Promine obtained from Central Soya Lab. (2).

and the line did not always go through the origin, so that it was necessary to repeat the standard curve at four levels with each series of assays.

The reproducibility of the method was tested by assaying on 7 different days a hydrolyzate of oxidized casein with and without added cysteic acid in an amount equivalent to half that present in the sample. Table II shows that the method was reproducible and that recoveries were complete.

Rice was added to small portions of fish flour and dry milk and assayed (Table III). Taking the value for rice alone as 100%, the expected value due to addition of previously assayed fish flour and milk was calculated. Results were close to 100% of the expected value and the standard deviation was 3%. These data furnish additional proof of the reproducibility of the method.

Cystine contents of various types of foods are reported in Table IV and compared with data of Orr and Watt (*10*). Most values agree fairly well with those from the literature, but significantly higher values were obtained for rice; these are probably due to a difference in the cystine content of the samples of rice.

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LABELED HERBICIDES

Preparation and Stability of C^{14}-Labeled Trifluralin and Related Compounds

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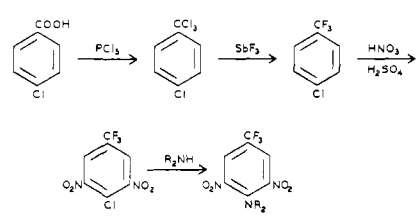
Trifluralin-ring-(Universally Labeled)-C^{14} was prepared by a five-step synthesis from 4-chlorobenzoic-ring-(U.L.)-C^{14} acid with an over-all radiocarbon yield of 31%. Trifluralin-$C^{14}F_3$ and trifluralin-propyl-1-C^{14} were prepared also. The methods developed for these syntheses were utilized also for the preparation of radiocarbon labeled benefin and of several *N*-monoalkyl analogs. Samples of C^{14}-labeled trifluralin and benefin were found to be radiochemically stable when stored in the dark as evaporated films. When in dilute heptane solution, exposure to ultraviolet light produced extensive photodecomposition.

TRIFLURALIN (Treflan, Elanco Products Co., *N*,*N*-dipropyl-2,6-dinitro-4-trifluoromethylaniline), is a very effective pre-emergence herbicide (*1*) for the control of both grasses and weeds. The increasing use of trifluralin has led to the need for material labeled with carbon-14 for use in metabolism (*3*), transport, and stability studies. To have the C^{14}-label in various positions in the molecule was desirable so that all possible routes of metabolism could be followed. Thus, trifluralin-trifluoromethyl-C^{14}, trifluralin-propyl-1-C^{14}, and trifluralin-ring-(U.L.)-C^{14} were prepared. The general reaction procedure was as follows:



Benefin (Balan, Elanco Products Co.), *N*-butyl-*N*-ethyl-2,6-dinitro-4-trifluoromethylaniline, is an analog of trifluralin which is also a valuable herbicide. As a part of this study, benefin-butyl-1-C^{14} and benefin-ring-(U.L.)-C^{14} were prepared by the same general procedure outlined above.

A number of related *N*-alkyl-2,6-dinitro-4-trifluoromethylanilines, *N*-methyl-C^{14}, *N*-ethyl-1-C^{14}, *N*-propyl-1-C^{14}, and *N*-butyl-1-C^{14}, were also prepared. In addition to these, the *N*-ethyl-, *N*-