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Elimination of Color Quench in Liquid Scintillation Counting of ^{14}C-Carotenoids[1]

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In attempting to count ^{14}C-carotenoids using liquid scintillation spectrometers a high degree of color quench occurs. This is due to absorption of the light emitted by the phosphor. The carotenoids have absorption maxima in the range of emission of the phosphor; thus color quenching by these compounds is severe.

Color quench has been observed in other systems and various methods have been developed to determine the amount of quench. Peng (1) plotted quench curves using internal standards and varying amounts of quenching materials, which permitted extrapolation to zero quench. DeBersaques (2) and Ross and Yerrick (3) related quench to absorbance of the colored material. The first author found a linear relationship between quench and absorbance to about 50% quench. The latter authors obtained quench coefficients for a three-component system. Using these coefficients and absorbancies at three wavelengths they were able to calculate the amount of quench. For more complicated systems a computer would be needed. Recently Ross (4) applied a modified method of internal standardization: by counting the sample without the standard, followed by recounting with the standard in the solution, color quench could be determined.

These methods are not completely satisfactory since color quench is often so severe that valid counting statistics cannot be obtained at lower levels of radioactivity.

Means of increasing counting efficiencies in color quench samples have been explored. Helf *et al.* (5) used organic wavelength shifters to shift the emission spectrum to a range of less absorption. This increased efficiency without destroying the colored material, but the range of applica-

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bility is limited. Destruction of chromophores has been useful in the cases of blood (6), urine (7), and chlorophyll and carotene (8–12).

Anderson and Porter (8) and Beeler and Porter (9) decolorized carotenoids by hydrogenation over PtO_2 catalysts. Yokoyama *et al.* (10) decolorized by exposure to ultraviolet light. Krzeminski and Quackenbush (11) exposed the dried carotenes to oxygen until colorless. Shneour *et al.* (12) used chlorine water to decolorize carotene and chlorophyll in amounts of 0.001 mg per reaction vial.

Most of the above methods require several manipulations or extended time. A method was sought which would permit counting of at least 1 mg of carotenoid per counting vial with good efficiency and a minimum of manipulation and time. The results obtained indicate the desirability of decolorizing carotenes with benzoyl peroxide and correcting for quench using external standardization.

MATERIALS AND METHODS

Scintillation-grade and analytical reagent-grade chemicals were used when available. The individual carotenoids were obtained from tomatoes by the method of Purcell *et al.* (13).

The scintillation medium was prepared by dissolving 4.0 gm of 2,5-diphenyloxazole (PPO) and 0.05 gm of 1,4-bis-2-(5-phenyloxazolyl)-benzene (POPOP) in 500 ml of toluene. Ten milliliters of this solution was mixed with the material to be counted and sufficient toluene added to give a total volume of 20 ml. The solution was then placed in the refrigerated compartment of the spectrometer and allowed to reach temperature equilibrium before counting. The internal standard used was ^{14}C-toluene giving about 7860 disintegrations per minute (dpm). In addition, a sealed standard obtained from the National Bureau of Standards (^{14}C-toluene, 49,700 $\pm$ 1% dpm) was used to calculate absolute counting efficiency.

Counting was done with a Packard model 3002[3] liquid scintillation spectrometer equipped with automatic external standardization. The high voltage was adjusted to give optimum counting on a tritium standard at 50% gain in the red channel with the window set at 50–1000. The following settings were used throughout this study: (*a*) gain in the red channel was 8% with discriminator settings 50–1000 for counting ^{14}C; (*b*) green channel gain settings were 8% with discriminator setting of 700–∞ for counting external standard.

The calibration curve is constructed by counting a series of solutions

[3] Use of trade names of specific materials does not constitute a recommendation by the U. S. Department of Agriculture to the exclusion of others which may also be available.

containing known quantities of quencher and a constant amount of internal standard. From the counts per minute (cpm) given by each solution, counting efficiency can be calculated. A plot of this efficiency against the external standard count (ES) will give a curve such as the one shown in Fig. 1. By comparing ES of the unknown sample with the

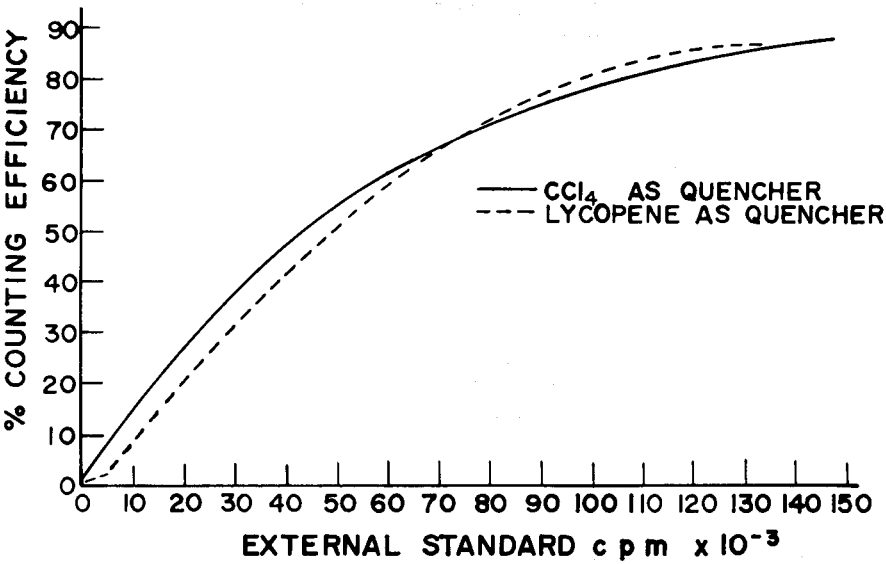

FIG. 1. Quenching curves for chemical quencher (CCl_4) and color quencher (lycopene).

calibration curve, the counting efficiency and dpm can be obtained. The curves in Fig. 1 are for a chemical quenching agent (CCl_4) and a color quenching agent (lycopene). It should be noted that, at higher counting efficiencies, both curves give approximately the same results.

Four systems for decolorization were studied. These were the reduction of the carotenoids by a modification of hydrogenation system developed by Brown and Brown (14) (System A), decolorization by an irradiated benzoyl peroxide solution (System B), treatment with an acidic solution of *N*-bromosuccinimide (System C), and agitating the carotenoid solution with chlorine water (12) (System D). In all tests, 0.5 ml of toluene containing 1 mg of the carotene was used as substrate.

System A. To a small separatory funnel was added 0.2 ml of 0.01 *M* chloroplatinic acid solution (in isopropyl alcohol), 0.3 ml of 0.1 *M* sodium borohydride solution (isopropyl alcohol), about 2 mg of activated charcoal and 2 drops of glacial acetic acid. The lycopene solution was

then added, followed by 0.5 ml of 2.0 *M* sodium borohydride in 1.0 *M* aqueous sodium hydroxide. After the reaction was completed (clear epiphase), the solution was washed several times with water and dried for 5 min with sodium sulfate. The clear solution was removed, the funnel was rinsed with toluene, and the washings and solution were combined and diluted to 9.0 ml with toluene. Ten milliliters of the phosphor solution, and 1 ml of ^{14}C-toluene (7860 dpm) were added to low-potassium screw-cap vials and counted. These volume proportions were followed in Systems B and C, and D.

System B. Varying quantities (0.1–0.6 ml) of freshly prepared benzoyl peroxide solution (10% in toluene) were added to the substrate solution. A fine stream of high-purity nitrogen was passed into the solution for 5 min. The solution was stoppered and placed in direct sunlight until bleaching had occurred (about 1.5 hr for lycopene and 2.5 hr for β-carotene). The decolorized solution was then diluted to 9.0 ml or shaken with 0.5 ml of 10% aqueous $FeSO_4$ solution, agitated with sodium sulfate, and then diluted to 9.0 ml and counted as in the preceding system.

System C. One-tenth milliliter of a freshly prepared saturated solution of *N*-bromosuccinimide in glacial acetic acid was added to the substrate solution. Reaction was instantaneous, giving a yellow color. This solution was diluted to 9.0 ml and counted as previously described.

System D. One milligram of lycopene in toluene was bleached with chlorine water according to the procedure described by Schneour *et al.* (12). Since 5.0 ml of chlorine water was needed for decolorization, a separation of layers was required followed by drying over sodium sulfate before the sample could be prepared for counting.

RESULTS AND DISCUSSION

The severity of color quench by carotenoids can be noted in Table 1. The counting efficiency of ^{14}C-toluene in the presence of 1 mg of lycopene in 20 ml is about 0.5%. It may also be noted in Fig. 1 that the external standard curves for color quench and chemical quench deviate appreciably with severe quench. The color quench due to β- and ζ-carotenes appears to be at least as severe as with lycopene (Table 2).

Reduction of nonlabeled lycopene by Brown hydrogenation (System A) increased the counting efficiency from 0.5% to over 80%, as indicated by both internal and external standards. When radioactive lycopene was reduced by this method, about 19% loss of counts occurred (Table 3), even with careful handling. This loss is attributed to manipulation of small volumes.

Lycopene mixed with benzoyl peroxide (System B) was decolorized in about 1.5 hr in sunlight. β-Carotene samples required 2.5 to 3 hr for

TABLE 1

COUNTING EFFICIENCES OF ^{14}C-TOULENE IN THE PRESENCE OF LYCOPENE DECOLORIZED BY FOUR SYSTEMS[a]

Treatment	cpm (^{14}C)	cpm (ES)	% E_{is}	% E_{es}	dpm (calc.)
^{14}C standard, 7860 dpm	6760	141,600	86.0	86.0	7850
Lycopene, 1 mg + 7860 dpm	43	1,364	0.51	3.0	1430
System A					
Brown hydrogenation	6372	111,700	81.0	80.5	7910
Overnight	6450	117,800	82.0	82.0	7860
System B					
Benzoyl peroxide					
0.4 ml	6100	101,00	77.5	77.0	7920
Overnight					
0.2 ml	6279	112,300	79.8	80.5	7800
0.4 ml	6162	102,100	78.4	77.5	7940
Benzoyl peroxide-Fe^{+2}					
0.1 ml	6443	115,600	82.0	81.5	7910
0.2 ml	6329	104,200	80.5	78.0	8100
0.6 ml	5945	88,300	75.6	73.5	8080
Overnight					
0.1 ml	6455	114,400	82.0	81.0	7980
0.2 ml	6255	102,800	79.5	77.5	8070
0.6 ml	5881	87,800	74.8	73.0	8050
Benzoyl peroxide					
Degassed					
0.1 ml	6812	132,400	86.6	85.0	8000
0.2 ml	6532	116,200	83.0	82.0	7960
0.6 ml	5960	88,400	75.8	73.5	8100
7 days 0.1 ml	6519	119,200	83.0	82.5	7900
0.2 ml	6451	115,100	82.0	81.5	7900
0.6 ml	5886	87,400	75.0	73.0	8060
System C					
N-Bromosuccinimide					
0.1 ml, 8% in HAc	3929	47,400	50.0	54.0	7280
Overnight					
0.1 ml	4534	57,300	58.2	60.0	7820
0.2 ml	6258	110,200	79.5	80.0	8000
0.4 ml	6165	101.200	78.4	71.0	7560
System D[b]					
Chlorine water	6597	132,100	71.4	85.0	7720
Overnight	6508	132,700	70.6	85.5	7660

[a] Unless otherwise noted, counting was done on same day samples were prepared.
[b] 9230 dpm of ^{14}C-lycopene added.

complete decolorization. The efficiency was increased to nearly 80%. Addition of ferrous ion after the reaction caused another slight increase. When the reaction mixtures were treated with CO_2 or N_2 resulting effi-

TABLE 2

COMPARISON OF COUNTING EFFICIENCIES OF DECOLORIZED AND UNTREATED CAROTENOIDS[a]

	Untreated				Decolorized			
	cpm (^{14}C)	cpm (ES)	% E_{es}	dpm	cpm (^{14}C)	cpm (ES)	% E_{es}	dpm
Phytoene	103,307	140,375	86.0	119,800	100,560	128,830	85.0	118,300
β-Carotene	257	437	1.0	25,700	66,894	107,785	79.0	84,680
ζ-Carotene	6,586	32,125	41.0	16,000	15,318	120,278	83.0	18,460
Neurosporene	143	5,767	10.0	1,430	4,145	118,303	82.0	5,050
γ-Carotene	349	10,713	16.0	2,180	4,365	127,828	84.0	5,200
Lycopene	26	274	1.0	260	7,689	123,446	83.0	9,260

[a] These ^{14}C-labeled carotenoids were obtained from tomatoes treated with labeled mevalonic acid.

TABLE 3
LOSS OF ACTIVITY OF ^{14}C-LYCOPENE[a] DUE TO ANALYTICAL MANIPULATION

Treatment	cpm (^{14}C)	cpm (ES)	% E_{es}	dpm (calc.)	% loss of activity
System A	6349	134,494	85.5	7420	19.4
System B	7689	123,400	83.3	9230	—
System D	6597	132,100	85.5	7720	17.4

[a] 9230 dpm of lucopene was decolorized by each system.

ciencies as high as 85% were achieved. The addition of ferrous ion did not increase counting efficiency of degassed solutions, so this addition was eliminated in subsequent studies.

No transfers, washings, or filtration are required so there is no loss due to manipulation. Since this method gave as good efficiency as any method and the highest recovery of a label, the results were used to calculate the level of radioactivity of the lycopene.

N-Bromosuccinimide (System C) decolorized the solution instantaneously. It appears that an excess of this reagent causes quenching as expected from the halogen content, making quantitative addition critical.

Chlorine water (System D) decolorized very rapidly and completely, but a water layer was formed when sufficient reagent was added to completely decolorize 1 mg of lycopene. The counting efficiency as determined by external standard is high (85%), but it appears some counts were lost in removal of the water after decolorization was completed (Table 3).

Most of this work was done with lycopene. It was found that comparable results were obtained with other carotenes. Results of decolorization of lycopene, β-carotene, ζ-carotene, neurosporene, and γ-carotene with benzoyl peroxide are shown in Table 2. It is seen that the efficiencies by external standardization are all about the same as for lycopene.

During the course of this work, it was noted that various manipulations such as filtering, transferring, and washing with immiscible liquids all cause detectable loss of counts when attempting to keep the volumes small. Washing toluene solutions with water is cumbersome, since emulsions form easily and break very slowly. One of the advantages of decolorization with benzoyl peroxide is that it can be carried out in toluene solutions in the scintillation tube without requiring transfer to other vessels or solvents. The escape of volatile radioactivity was checked by evaporating toluene from solution containing benzoyl peroxide and labeled lycopene with a stream of nitrogen and collecting the toluene in a liquid nitrogen trap. No radioactivity was found in the distillate.

An interesting sidelight to this study was the performance of the automatic external standard. This is a relatively new development and it

appears to be quite useful. If one examines Table 1, it is seen that, after decolorization, the counting efficiency as calculated from the added internal standard (E_{is}) compared quite well with the efficiency as determined by the automatic external standard (E_{es}). E_{is} is usually within 1 to 0.5% or less of E_{es}, well within manipulative error. Hence it is no longer necessary to determine counting efficiency by the tedious process of counting the sample, adding an internal standard, and recounting.

When benzoyl peroxide is used as the decolorizing agent, counting efficiencies of around 81% can be assumed. For instruments not equipped with external standardization, this assumption will result in a possible error of 2–3% in counting efficiency. An error of this magnitude can be tolerated in some cases, thereby saving the investigator the extra steps involved in internal standardization. The decolorization apparently does not interfere with the channels ratio method of quench correction.

SUMMARY

Methods of eliminating color quench in measuring radioactivity of carotenes by liquid scintillation spectrometry were studied. Solutions containing internal ^{14}C standards and 1 mg of lycopene or beta carotene were counted with about 0.5% efficiency. The pigments were decolorized in a counting vial using benzoyl peroxide and light. After 1–3 hours of decolorization of the pigments, counting efficiencies of over 80% were obtained in PPO-POPOP phosphor. Similar results were obtained in counting ^{14}C carotenes using an external standard to determine efficiency.

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