Antioxidant Activity of Amino Acids Bound to Trolox-C®

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ABSTRACT

Various amino acids, selected for their potential antioxidant activity, were covalently attached to 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox-C), a lower homolog of vitamin E that has great antioxidant effectiveness. The resulting Troloxyl-amino acids (T-AA) had greater antioxidant effectiveness than Trolox-C in a linoleate emulsion system oxidized by hemoglobin. Troloxyl-tryptophan-methyl ester and Troloxyl-methionine-methyl ester were the most effective T-AA evaluated in the linoleate emulsion. However, butylated hydroxyanisole (BHA), butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT) and α-tocopherol were more antioxidant than any T-AA in the emulsion system. In a Schaal oven test at 45 C, Trolox-C was by by far the most effective antioxidant evaluated in corn oil. BHT and Troloxyl-cysteine had significant antioxidant activity in corn oil, but no other T-AA was antioxidant in corn oil. In butter oil, Trolox-C again had the highest antioxidant activity, and BHA and BHT were also highly antioxidant. All T-AA had antioxidant activity in butter oil, with Troloxyl-methionine and Troloxyl-cysteine having the greatest antioxidant effectiveness. The T-AA of highest antioxidant activity were hydrolyzed by chymotrypsin and/or trypsin, in vitro.

INTRODUCTION

The empirical search is increasing for suitable antioxidants among natural products. α-Tocopherol, citric acid and ascorbic acid are already added to some foods to retard lipid oxidation. Other natural products have also been proposed for use as antioxidants including amino acids, protein hydrolyzates and proteins (1), and enzymes such as superoxide dismutase, catalase and glutathione peroxidase (2). Other natural materials have the ability to scavenge free-radicals and may function as antioxidants as a result. In addition, many substances have potential antioxidant properties other than scavenging of free radicals. Methionine, for instance, can decompose preformed peroxides, a potential antioxidant effect (3). Compounds that scavenge reactive species of oxygen involved in the initiation of lipid oxidation may also function as antioxidants in foods (4). For example, tryptophan can react with singlet oxygen, which is presumably involved in the initiation of lipid oxidation (4).

Mixtures of suitable compounds, each with a particular antioxidant activity, may be of great antioxidant effectiveness as a result of the multifunctional antioxidant activity. Many commercial antioxidant preparations consist of more than one antioxidant to take advantage of the synergism exhibited between certain antioxidants. The preparation and evaluation of a series of multifunctional antioxidant compounds are reported in this paper. The multifunctional antioxidants prepared were not mixtures of individual antioxidants but were single compounds designed to contain several antioxidant functionalities. Various amino acids, selected for their potential antioxidant activity, were covalently linked to 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox-C), a lower homolog of vitamin E with substantial antioxidant activity (5,6). The resulting Troloxyl-amino acids (T-AA) have several functional groups that possess antioxidant activity. As a result, the T-AA may have greater antioxidant activity than Trolox-C alone. In addition, the compounds consisted of a natural product (the amino acid) and an antioxidant based on a natural product (Trolox-C).

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EXPERIMENTAL

Materials

L-Tryptophan, L-cysteine, L-methionine, butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), D-α-tocopherol (type I), chymotrypsin (bovine, type II) and trypsin (bovine, type III) were obtained from Sigma Chem. Co., St. Louis, MO. Other reagents and their sources included: N,N'-dicyclohexylcarbodiimide (DCC, redistilled) and 1-hydroxybenzotriazole hydrate (HOBT) (Aldrich Chem. Co., Milwaukee, WI); L-histidine-HCl (Schwarz-Mann, Orangeburg, NY); Linoleic acid methyl ester 75% (U.S. Biochem. Corp., Cleveland, OH); pepsin, porcine, B grade (Calbiochem, LaJolla, CA); pancreatin, trypsin 4X pancreatin (Nutri. Biochem. Co., Cleveland, OH); and 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox-C) (a gift from Hoffmann-LaRoche, Inc., Nutley, NJ, or from Burdick and Jackson Labs., Inc., Muskegon, MI). All other chemicals were reagent grade and only distilled, deionized water was used.

Analytical Procedures

Nuclear magnetic resonance (NMR) spectra were recorded using a Varian EM390 spectrometer with tetramethylsilane as the internal standard. Mass spectra were obtained with an AEI-MS9-DS50 spectrometer operated at 70 eV. Brinkman precoated plates (0.25 mm, Silica Gel G) were used for thin layer chromatography (TLC). Elemental analyses were performed by Galbraith Laboratories, Knoxville, TN.

Synthesis of Troloxyl-Amino Acids (T-AA)

Trolox-C and an amino acid methyl ester (7) were coupled using the carbodiimide DCC and a catalyst (HOBT) (7). The resulting Troloxyl-amino acid-methyl ester was washed and then purified by silica gel column chromatography. T-AA were prepared by saponification of the corresponding methyl esters (7). Elemental analyses, mass spectra, TLC and NMR data supported the assigned structures (7). Because Trolox-C is a racemic mixture, all T-AA were diastereoisomeric and thus obtained as oils.

Antioxidant Activity in Linoleate Emulsion

T-AA were evaluated for antioxidant activity in a linoleate emulsion to which hemoglobin was added as prooxidant (8). Antioxidant activity was expressed as a protective index (PI), defined as:

$$PI = \frac{\text{oxidation of emulsion with antioxidant (min)}}{\text{oxidation of control emulsion (min)}}$$

Oxidation times of replicate emulsions had a coefficient of variation of 1.3% (8).

Antioxidant Activity in Edible Oils

T-AA were evaluated for antioxidant activity in refined, deodorized corn oil (Kraft, Inc., Glenview, IL) in a Schaal oven test (9). Corn oil (35 g) containing 0.02% (w/w) antioxidant was incubated in a 50-ml beaker with a glass

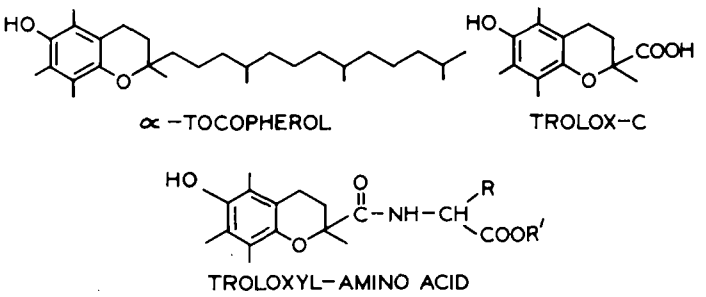

FIG. 1. Structures of α-Tocopherol, Trolox-C and Troloxyl-amino acid compounds. R = side-chain of amino acid; R' = H or CH_3.

coverslip at 45 C. Corn oil (1 g) was analyzed as necessary for peroxide content (10). T-AA were also evaluated for antioxidant activity in anhydrous butter oil (Level Valley Dairy, West Bend, WI) in a Schaal thin layer oven test (5). Butter oil (1 g) containing 0.02% (w/w) antioxidant was incubated in a 30-ml beaker with a glass coverslip at 45 C. Butter oil (1 g) was analyzed as necessary for peroxide content (10).

Enzymatic Hydrolysis

T-AA were treated with pepsin, trypsin, chymotrypsin and pancreatin to determine if the amide bond linking Trolox-C to the amino acid was hydrolyzable by proteolytic enzymes (7,11). TLC was used to monitor the treatment.

RESULTS AND DISCUSSION

Trolox-C is a lower homolog of α-tocopherol (vitamin E), differing by having a carboxyl group in place of the isoprenoid side-chain of vitamin E (Fig. 1). The phenolic hydroxyl group of Trolox-C should serve as a free radical scavenger and reduce superoxide anion and hydroxyl radical as well. In addition, the chroman nucleus is an excellent scavenger for singlet oxygen (12). As a result, Trolox-C should be antioxidant, and has been shown to be an extremely effective antioxidant in both vegetable oils and animal fats (5,6). In Schaal thin layer oven and active oxygen method (AOM) tests, Trolox-C had greater antioxidant activity than many commercial food antioxidants such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT) and α-tocopherol (5,6). In addition, Trolox-C is relatively nontoxic with an LD_{50} similar to that of BHA and BHT (6,13). However, Trolox-C has not been approved for food use anywhere. The manufacturer (Hoffmann-LaRoche) has no plans to seek approval, because of unfavorable market considerations and not because of technical shortcomings with Trolox-C (14).

Various amino acids were covalently attached to Trolox-C through the formation of an amide bond between the free carboxyl group of Trolox-C and the free amino group of the amino acid (Fig. 1). The amino acids attached to Trolox-C were selected for their potential antioxidant activity (discussed in next section). The resulting T-AA might have greater antioxidant effectiveness than Trolox-C alone because of the additional antioxidant functionality supplied by the amino acid.

Covalently linked T-AA were used rather than a mixture of Trolox-C and amino acid for solubility reasons. T-AA may be surface-active (because Trolox-C is lipophilic and the amino acids are hydrophilic) and therefore orient at the oil/water or oil/air interface where they would presumably by most effective. Because Trolox-C is lipophilic and the amino acids are hydrophilic, a mixture of the two would not equally partition in a lipid emulsion. However, T-AA generally are lipophilic and thus partition into the oil phase of an emulsion where they would presumably be most effective.

ANTIOXIDANT ACTIVITY OF TROLOXYL-AMINO ACIDS IN THE LINOLEATE EMULSION

Antioxidant Effectiveness of Troloxyl-Methionine

Methionine has been shown to have antioxidant activity in both model emulsion systems and in edible oils (15,16). In the presence of oxidizing methyl linoleate, a free radical was observed in methionine (17), indicating the abstraction of hydrogen from methionine by the oxidizing lipid. In addition, the thioether of methionine can decompose preformed hydroperoxides by a nonradical mechanism, preventing the initiation of radical chains by homolytic scission of the hydroperoxides (3).

In the linoleate emulsion system, Troloxyl-methionine-methyl ester (T-M-OMe) had much greater antioxidant effectiveness than Trolox-C or than a mixture of Trolox-C and methionine-methyl ester (Table I). Troloxyl-methionine (T-M-OH) was also a more effective antioxidant than either Trolox-C or a mixture of Trolox-C and methionine, but was

TABLE I

Antioxidant Effectiveness of Troloxyl-Amino Acids in a Linoleate Emulsion

Antioxidant	Protective Index[a] (10^{-3} M) pH 6.7	pH 7.2
α-Tocopherol	19.7	–[b]
BHA	11.5	–[b]
BHT	51.2	–[b]
Trolox-C	1.78	1.94
Troloxyl-Met-OMe	6.99	10.14
Methionine-OMe	0.93	1.00
Trolox-C + Met-OMe	1.87	1.74
Troloxyl-Met-OH	2.69	3.20
Methionine	0.95	1.00
Trolox-C + Met	1.88	1.93
Troloxyl-Trp-OMe	11.24	13.71
Tryptophan-OMe	.49	.46
Trolox-C + Trp-OMe	.72	.62
Troloxyl-Trp-OH	7.16	8.31
Tryptophan	.88	.81
Trolox-C + Trp	1.83	2.03
Troloxyl-His-OMe	2.11	3.18
Histidine-OMe	1.02	.94
Trolox-C + His-OMe	2.02	2.31
Troloxyl-His-OH	1.09	1.24
Histidine	.96	.97
Trolox-C + His	1.83	2.18
Troloxyl-Cys-OH	2.85	4.89
Cysteine	14.6	24.3
Trolox-C + Cys	3.67	3.21

[a]Protective index is defined in Experimental. Values are averages of at least two determinations.

[b]Not measured.

not as effective as T-M-OMe (Table I). Both T-M-OMe and T-M-OH had greater antioxidant activity at pH 7.2 than at pH 6.7 in the linoleate emulsion.

Antioxidant Effectiveness of Troloxyl-Tryptophan

Tryptophan has been shown to have antioxidant activity in both model emulsion systems and in edible oils (16,18). In the presence of oxidizing methyl linoleate, a free radical was observed in tryptophan (17), indicating the abstraction of hydrogen from tryptophan by the oxidizing lipid. In addition, the indole nucleus of tryptophan should serve as a scavenger for singlet oxygen (4).

In the linoleate emulsion system, Troloxyl-tryptophan-methyl ester (T-T-OMe) had much greater antioxidant effectiveness than either Trolox-C or than a mixture of Trolox-C and tryptophan-methyl ester (Trp-OMe) (Table I). At pH 7.2, the protective index of T-T-OMe was 7-times greater than that of Trolox-C. However, Trp-OMe was highly prooxidant in the linoleate emulsion system, with a protective index less than 0.5 at either pH 6.7 or 7.2. Trp-OMe was so strongly prooxidant that even the mixture of Trolox-C and Trp-OMe was prooxidant. Troloxyl-tryptophan (T-T-OH) was also highly antioxidant in the linoleate emulsion system but was not quite as effective as T-T-OMe (Table I). Tryptophan was prooxidant, but less so than Trp-OMe, and the mixture of Trolox-C and tryptophan had about the same antioxidant activity as Trolox-C alone. Both T-T-OMe and T-T-OH had greater antioxidant activity at pH 7.2 than at pH 6.7. The prooxidant activity of tryptophan may be related to its ability to photogenerate superoxide anion (19).

Antioxidant Effectiveness of Troloxyl-Histidine

Histidine has been shown to have antioxidant activity in both model emulsion systems and in edible oils (15,16, 18,20). The imidazole group of histidine may coordinate with the iron in heme-proteins, decreasing the great prooxidant effect of heme-proteins. In addition, the imidazole has a secondary amine capable of oxidation to a stable nitroxide radical, and imidazole is highly reactive with hydroxyl radicals which probably have reaction characteristics similar to alkoxy radicals formed during lipid oxidation (20). In the presence of oxidizing methyl linoleate, a free radical was observed in histidine (17), indicating the abstraction of hydrogen from histidine by the oxidizing lipid.

In the linoleate emulsion system, Troloxyl-histidine-methyl ester (T-H-OMe) had only slightly greater antioxidant activity than Trolox-C or than a mixture of Trolox-C and histidine-methyl esters (Table I). However, Troloxyl-histidine (T-H-OH) had less antioxidant activity than Trolox-C or than a mixture of Trolox-C and histidine. T-H-OH was only weakly antioxidant. Both T-H-OMe and T-H-OH had greater antioxidant activity at pH 7.2 than at pH 6.7.

Antioxidant Effectiveness of Troloxyl-Cysteine

Cysteine has been shown to have antioxidant activity in both model emulsion systems and in edible oils (8,15, 18). Thiols are well-known as free radical scavengers, and thus as antioxidants, in biological and other systems (21). Sulfhydryl groups are able to donate their labile hydrogen to free radicals (21) and should be able to reduce superoxide anion and hydroxyl radical. In the presence of autoxidizing methyl linoleate, a free radical was observed in cysteine (17), indicating abstraction of hydrogen from cysteine by the oxidizing lipid. Gardner et al. (22) recently reported that cysteine reacts with linoleic acid hydroperoxide to yield several fatty acid-cysteine adducts. In addition, it has been reported that thiols react with superoxide anion (23). Cysteine may also ligate copper in metallo-proteins, providing yet another mechanism for antioxidant activity.

In the linoleate emulsion system, cysteine was highly antioxidant and was comparable to BHA, BHT and α-tocopherol in antioxidant effectiveness (Table I). Cysteine was the only amino acid (of 15 tested) with antioxidant activity in the emulsion system (8). However, a mixture of Trolox-C and cysteine, or the covalently linked Troloxyl-cysteine (T-C-OH) adduct, had much less antioxidant activity than cysteine alone (Table I). The mixture of Trolox-C and cysteine was comparable to T-C-OH in antioxidant activity. T-C-OH was more antioxidant at pH 7.2 than a mixture of Trolox-C and cysteine, but less antioxidant at pH 6.7. T-C-OH had 2.5-times the antioxidant effectiveness of Trolox-C at pH 7.2 in the linoleate emulsion.

Evaluation of Troloxyl-Amino Acids in the Linoleate Emulsion: Summary

At pH 6.7, the methionine and tryptophan derivatives of Trolox-C were more effective antioxidants in the linoleate emulsion system than either Trolox-C or a mixture of

TABLE II

Antioxidant Effectiveness of Troloxyl-Amino Acids in Corn Oil[a]

Compound	Relative molar concentration[b]	Days to reach peroxide value of 70 meq/kg[c]	Increase in protection (d)
Control	–	44	–
BHA	2.95	45	+ 1
BHT	2.41	64	+20
Trolox-C	2.12	137	+93
α-Tocopherol	1.00	39	- 5
Troloxyl-Met-OMe	1.34	44	0
Troloxyl-Met-OH	1.39	47	+ 3
Troloxyl-Trp-OMe	1.18	42	- 2
Troloxyl-Trp-OH	1.22	40	- 4
Troloxyl-His-OMe	1.32	39	- 5
Troloxyl-His-OH	1.37	43	- 1
Troloxyl-Cys-OH	1.50	53	+ 9

[a]Schaal oven test, 45 C. Compounds at 0.02% (w/w) of oil.

[b]Based on α-tocopherol (MW 531) = 1.00.

[c]Initial peroxide value = 0.9.

Trolox-C and the respective amino acid (Table I). T-T-OMe was particularly effective and had about the same antioxidant activity as BHA. However, BHT and α-tocopherol were more antioxidant than any T-AA at pH 6.7.

All T-AA had greater antioxidant effectiveness at pH 7.2 than pH 6.7, and were more effective in the linoleate emulsion than Trolox-C or than a mixture of Trolox-C and the respective amino acid (Table I, except T-H-OH). T-T-OMe and T-M-OMe were the most effective of the T-AA evaluated in the emulsion system at pH 7.2.

The methyl esters of the T-AA had greater antioxidant activity in the linoleate emulsion than the corresponding free acids. This difference may reflect the increased solubility of the methyl esters in the oil phase of the emulsion, and may also explain the low antioxidant activity observed for Trolox-C relative to BHA or BHT. Scott et al. (24) reported that esterification of Trolox-C resulted in increased antioxidant activity in a safflower oil emulsion catalyzed by hemoglobin, which may partially explain the lesser activity of Trolox-C relative to T-AA in the linoleate emulsion. The greater hydrophilicity of Troloxyl-histidine compounds relative to other T-AA may explain, in part, their low antioxidant activity in spite of the antioxidant potential of histidine. However, cysteine was highly antioxidant in the linoleate emulsion, and it is a hydrophilic compound. The role of the solubility of a compound on its antioxidant activity in the linoleate emulsion deserves further study.

The results presented in Table I are for an antioxidant concentration of 10^{-3} M in the emulsion, which represents ca. 0.40% (w/w) T-AA in the oil phase of the emulsion. Although this amount greatly exceeds the 0.02% (w/w) limit for antioxidants in oils permitted by the Food and Drug Administration, this amount was necessary to observe a large antioxidant effect. For instance, the protective index of BHA, BHT and α-tocopherol ranged from 1.65-1.77 at 10^{-4} M (pH 6.7) whereas the protective index of T-T-OMe, T-M-OMe and Trolox-C was 1.53, 1.45 and 1.16, respectively (pH 6.7, 10^{-4} M).

ANTIOXIDANT ACTIVITY OF TROLOXYL-AMINO ACIDS IN EDIBLE OILS

The T-AA were evaluted for antioxidant effectiveness in two edible oils using the Schaal oven test method (9). Results from Schaal oven tests better predict the effectiveness of antioxidants in shelf storage tests of edible oils than do results from the linoleate emulsion (9). Antioxidants were evaluated in butter oil using a Schaal thin layer oven test (5) to increase the rate of oxidation of butter oil, which is relatively stable to oxidation. Antioxidants were also evaluated in corn oil in a Schaal oven test. Antioxidants were dissolved in the oils at 0.02% (w/w) of the oil, the limit permitted by the Food and Drug Administration. Because the antioxidants vary in molecular weight, the relative molar amount of antioxidants in the oil range from 1.00 to 2.95 for α-tocopherol and BHA, respectively (Table II).

By far the most effective antioxidant in corn oil was Trolox-C, which supported previous results indicating that Trolox-C was more effective than other commercial antioxidants (5,6) (Table II). The only other antioxidants with substantial antioxidant activity in corn oil were BHT and T-C-OH. BHA, α-tocopherol and the other T-AA had insignificant antioxidant activity in corn oil.

Unlike the results in corn oil, all antioxidants evaluated in butter oil slowed the rate of oxidation (Table III). The most effective antioxidants were BHA, BHT and Trolox-C. Among the T-AA, T-M-OH and T-C-OH had the greatest antioxidant activity. Unlike the results in the linoleate emulsion, the methyl esters of the T-AA did not have greater antioxidant activity than the corresponding free acids.

TABLE III

Antioxidant Effectiveness of Troloxyl-Amino Acids in Anhydrous Butter Oil[a]

Compound	Days to reach peroxide value[b] meq/kg		Increase in protection[d] (days)
	20	70	
Control	37	43	
BHA	265	290	247
BHT	276	293	250
α-Tocopherol	42	75	32
Trolox-C	>267[c]	>267[c]	>224
Troloxyl-Met-OMe	59	76	33
Troloxyl-Met-OH	137	147	104
Troloxyl-Trp-OMe	64	79	36
Troloxyl-Trp-OH	86	97	54
Troloxyl-His-OMe	76	86	43
Troloxyl-His-OH	54	63	20
Troloxyl-Cys-OH	142	153	110

[a]Schaal thin layer oven test, 45 C. Compounds at 0.02% (w/w) of oil. See Table II for relative molar concentration.

[b]Initial peroxide value = 0.0.

[c]On day 267, peroxide value = 9 (Trolox-C), 14 (BHT), and 21 (BHA).

[d]To reach peroxide value of 70.

ENZYMATIC HYDROLYSIS OF TROLOXYL-AMINO ACIDS

T-AA were treated with various proteolytic enzymes in vitro to determine whether the amide bond linking Trolox-C to the amino acid was susceptible to enzymatic hydrolysis. Hydrolysis of the amide bond would result in the release of the relatively nontoxic amino acid and Trolox-C that made up the T-AA. Those T-AA with greatest antioxidant activity in the linoleate emulsion system, corn oil and butter oil were hydrolyzed by at least one of the enzyme treatments. T-M-OMe was hydrolyzed by chymotrypsin or pancreatin, T-M-OH and T-T-OMe by chymotrypsin (treatment with trypsin or pancreatin was not evaluated), and T-C-OH by chymotrypsin, trypsin, or pancreatin. The three other T-AA were not hydrolyzed by any of the four enzyme treatments.

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Hexane Losses in Solvent Extracted Soya Meal: Measurement by Gas Chromatography and Brief Evaluation

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ABSTRACT

Hexane losses in solvent extraction plants arise from a variety of causes. With the constantly increasing price of hexane, it has become imperative to identify the major loss areas and quantify losses in these areas. Hexane lost in meal leaving the desolventizer-toaster (D-T) is an obvious choice for investigation. A method has been developed for determining relatively high levels of hexane in hot meal leaving the D-T. The method was designed to minimize the loss of solvent by evaporation between the time of sampling and the actual measurement by gas chromatography (GC). Examination of the results confirmed that losses of hexane as a result of inadequate stripping of the meal in the D-T formed a very significant part (10–40%) of total hexane losses.

INTRODUCTION

A modified volatilization procedure has been described (1) which overcomes some of the possible deficiencies in earlier gas chromatography (GC) methods developed by Dupuy and Fore for determining residual hexane in meal (2, 3). In essence, all these methods have been developed to provide a further degree of quality control over the final product meal.

There still exists, however, a need for a method which will determine hexane in meal in the 500–4,000 ppm level actually encountered in practice when solvent extraction plants are being run at or above rated capacity. The loss of hexane from the hot, moist meal leaving the D-T is minimized in the method described here by the following procedures: (a) initial sampling is done rapidly into a container which is sealed when full; (b) solvent extraction with cyclohexane, using relatively large aliquots (50 g meal to 100 ml solvent) is used to extract the hexane. (For complete extraction, a 24-hr steeping period was found to be necessary). A further feature of the method, apart from its relative simplicity, is that no modifications are required to GC equipment.

EXPERIMENTAL PROCEDURE

Sampling

A 1-kg plastic container equipped with screw-top lid was inserted into a connecting chute of the meal conveyor system leaving the D-T and was closed as soon as it was full. Meal sampled in this way contained 13–14% moisture and all hexane measurements were subsequently related to

TABLE I

Recovery Data

Recovery (%)	Initial spiking (ml)	Known additions (ml)	Peak Height (adjusted for cyclohexane impurities) Line counts
	50	0	4.5
95		250	30
		500	58
		750	86
	100	0	8
97		50	14.2
		100	19.2
		150	23.0
		175	25.0
		200	27.5
	150	0	13.5
97		50	17.0
		100	21.1
		150	25.2
		200	30.4
	200	0	16.0
95		50	20.2
		100	24.7
		150	30.0
		200	35.8