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Collaborative study: determination of retinol and carotene by high-performance liquid chromatography

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Seventeen laboratories participated in the analysis of 10 products for carotene and retinol by high-performance liquid chromatography. Test materials were saponified and the nonsaponifiables were extracted with petroleum ether. The extract was injected into the liquid chromatograph for determination of carotene at 450 nm/436 nm, using a C18 reversed-phase column with a mobile phase of acetonitrile:methylene chloride:methanol:water (70:20:8:2, v/v). Retinol was determined at 325 nm/313 nm by using a C18 reversed-phase column with a mobile phase of methanol:water (90:10, v/v). The biological activities of retinol and carotene expressed in international units were summed to give total activity. The repeatability (within laboratory) coefficients of variation ranged from 3.46–15.65%, and the reproducibility (among laboratories) coefficients of variation ranged from 5.34–15.77%. © 1997 Published by Elsevier Science Ltd. All rights reserved

INTRODUCTION

The aim of this study was to develop a rapid high-performance liquid chromatographic (HPLC) method of analysis for retinol and carotene in various commodities for regulatory purposes. The colorimetric method for retinol and the spectrophotometric method for carotene in *Official Methods of Analysis* of the AOAC (1990) require experience in column chromatography partitioning for successful performance. Numerous HPLC methods have been published that quantitatively separate retinol and carotene from various foods and animal feeds (Ball, 1988; Quackenbush & Smallidge, 1986), fruits and vegetables (Bureau & Bushway, 1986; Wills *et al.*, 1988), dietary fats (Heinonen *et al.*, 1988), and milk and infant formula (Thompson & Duval, 1989), but results appear to be method-dependent. The collaborative study reported here was undertaken (a) to determine if the column packing type used influences the results significantly; and (b) to recommend specific parameters for an HPLC reversed-phase method.

MATERIALS AND METHOD

Samples and reagents

Cereal product, powered infant formula, chicken feed, pureed carrots, vegetable juice, low fat milk, baby food (squash), beta-carotene capsules and retinol capsules were purchased from a local supermarket. Vitamin A acetate capsules (obtained from the United States Pharmacopeia (USP), Rockville, MD, USA), beta-carotene standard, butylated hydroxytoluene (BHT) and reagent grade ascorbic acid (obtained from Sigma Chemical Company, St. Louis, MO, USA) were sent along with the samples to collaborators. Potassium hydroxide solution (50%), ethanol, petroleum ether, anhydrous sodium sulfate, isopropanol, hexane, methanol, acetonitrile, methylene chloride, distilled water and phenolphthalein (1%) were provided by the collaborators.

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Sample preparation

The foods were composited, and a test portion was weighed or accurately measured into an Erlenmeyer or round-bottom flask to contain 14–16 I.U. of retinol/ml after final dilution of the extract if the concentration was known; a retinol concentration of 14–16 I.U./ml, based on the label claim, controlled the size of the test portion. When the concentration was not known, ≥20 g of composite was weighed. In the case of capsules/tablets, two to five capsules/tablets were used.

Saponification and extraction

To this test portion were added 200 ml of ethanol, 0.5 g of ascorbic acid and 50 ml of potassium hydroxide solution; the mixture was refluxed for 60 min with an air condenser and then cooled to room temperature. The

contents of the flask were quantitatively transferred to a 1 liter separatory funnel, the flask was rinsed with 100 ml of water, followed by 50 ml of ethanol, and the rinsings were added to the funnel. A 200 ml portion of petroleum ether was added to the separatory funnel, the funnel was inverted and vented, and then shaken vigorously with occasional venting, and the layers were allowed to separate. Any emulsion that occurred was broken by adding ethanol. The bottom layer was drained into a second separatory funnel and 200 ml of petroleum ether was added; the mixture was extracted a second time, the layers were allowed to separate and the aqueous (bottom) layer was drained into a waste receptacle. The ether extracts were combined in the first separatory funnel, the second funnel was rinsed with petroleum ether and the rinsings were added to the first funnel. Then 400 ml of distilled water was added to the ether extract, the funnel was not shaken, the layers were allowed to separate and the water was drained to waste. Again 400 ml of distilled water was added, the funnel was swirled or shaken, lightly at first, the layers were allowed to separate and the water layer was discarded. The ether extract was repeatedly washed with water until the water was neutral to 1% phenolphthalein solution (no visible pink color). The extracted ether was filtered through anhydrous sodium sulfate into a beaker or round-bottom flask, and the funnel was rinsed with an additional 50 ml of petroleum ether. The ether extract was evaporated to dryness on a steam bath by a nitrogen jet or on a rotary evaporator. The residue was immediately dissolved in hexane, and the solution was transferred quantitatively with hexane into a volumetric flask and diluted to volume with hexane.

Preparation of retinol reference standard solution

Five USP vitamin A capsules were placed in a round-bottom flask, and 50 ml of distilled water, 150 ml of ethanol and 25 ml of potassium hydroxide solution were added. An air condenser was attached to the flask and the mixture was refluxed for 30 min, cooled to room temperature and quantitatively transferred to a 500 ml separatory funnel by rinsing with water and then ethanol. The mixture was extracted twice with 100 ml of petroleum ether; the ether extracts were combined and washed with water until the water waste was neutral to phenolphthalein indicator (no pink color was observed). The extract was dried by passing it through 65 g of anhydrous sodium sulfate, followed by 50 ml of petroleum ether to quantitatively elute retinol. BHT (5–10 mg) was added, and the petroleum ether extract was evaporated to 50 ml in a rotary evaporator or with a nitrogen jet on a water bath/steam bath; the 50 ml was quantitatively transferred to a 100 ml volumetric flask and diluted to volume with petroleum ether (stock standard solution). A 1 ml aliquot of the stock standard solution was placed in a 100 ml volumetric flask, the solution was evaporated to dryness by a nitrogen jet, the residue was dissolved in hexane and the solution was diluted to volume with hexane; the flask was labeled "working standard solution of retinol for HPLC use". The exact concentration of the working standard solution was determined by taking another 1 ml aliquot of the stock standard solution, evaporating the solution to dryness with the nitrogen jet, dissolving the residue in isopropanol and diluting the solution to volume with isopropanol. The absorbance was measured by a scanning UV/Vis spectrophotometer at 325 nm, and the concentration was calculated by the formula: retinol, I.U./ml = Abs. 325×18.3.

Preparation of carotene reference standard solution

A 5 mg test portion of beta-carotene was weighed into a 250 ml volumetric flask and dissolved in hexane. A few drops of chloroform were added to completely solubilize the beta-carotene, and the solution was diluted to volume with hexane and labeled "stock standard solution of carotene". Then 10 ml of stock standard solution was pipetted into a 50 ml volumetric flask and diluted to volume with hexane; the solution was labeled "working standard solution of carotene". The absorbance of the working standard solution was measured by a scanning UV/Vis spectrophotometer at 450 nm. The concentration was calculated as follows: carotene, μg/ml = Abs. 450×4.17.

HPLC conditions

The HPLC system consisted of pump, injector, UV/Vis detector, chart recorder or data acquisition system and a 10 μm ODS–C18 column, 4 mm i.d. × 250 mm long. For retinol, HPLC conditions were as follows: C18 ODS column, 10 μm, 150 Å, (15 nm) pore size, 4 mm i.d. × 250 mm; mobile phase, methanol:water (90:10, v/v); flow rate, 1.5 ml/min; chart speed, 0.5 cm/min; detector at 325 or 313 nm; range, 0.05 (AUFS) (or range that gives a peak height between 50 and 75% full scale deflection for injected reference standard solution). For carotene, HPLC conditions were as follows: C18 ODS column, 10 μm, 4 mm i.d. × 250 mm; mobile phase, acetonitrile:chloride:methanol:water (70:20:8:2, v/v); flow rate, 3 ml/min or 1 ml/min; chart speed, 0.5 cm/min; detector at 450 or 436 nm; range, 0.02 (AUFS) (or range that gives a peak height between 50 and 75% full scale deflection for injected reference standard solution).

Two chromatograms were submitted with the study for carotene analysis. A C18 column packing with a pore size of 150 Å (15 nm) or less required a flow rate of 3 ml/min to give optimum retention time with good resolution; a C18 column packing with a pore size of 300 Å (30 nm) required a flow rate of 1 ml/min. This information was provided for the benefit of collaborators who might have had only one of the column packings.

A scanning UV/Vis spectrophotometer was used to determine the concentration of the reference standard. A rotary evaporator or a nitrogen jet was used to evaporate the petroleum ether.

AOAC chemical method, 974.29, 15th edn. (1990)

The test portion is saponified and extracted with petroleum ether, and the unsaponifiable material is passed through an alumina column. Carotene is eluted from the column first with 16% ether in petroleum ether; then retinol is eluted with 25% ether in petroleum ether.

Calculation/expression of results

For retinol, a 5–10 μl aliquot of reference standard solution was injected, followed by a 5–10 μl aliquot of extract, and peak height or area was used for quantitation as follows: I.U. = (area of extract peak/area of standard solution peak) × (μl of standard solution injected/μl of extract injected) × (concentration of standard solution) × (dilution factor) × (1/weight of test portion).

For carotene, a 5–10 μl aliquot of reference standard solution was injected, followed by a 5–10 μl aliquot of

Table 1. Results for determination of carotene (I.U./g)[a] by HPLC

Lab.	Cereal	Powered infant formula	Pureed carrots	V-8 juice	Baby food squash A	Baby food squash B	β-Carotene capsule
1	14.63	1.59	53.91	6.18	13.02	11.68	12766
2	28.90[b]	1.73	80.00	9.16	15.58	14.96	10050
4	22.23	0.00[b]	37.35	14.88	8.18	8.75	9969
5	17.20	0.83[b]	7.18	7.27	4.42	5.99	10090
7	22.30	3.00[b]	78.90	9.40	13.80	15.00	12290
8	42.00[b]	1.00[b]	80.40	7.30	19.90	19.10	11507
9	19.40	1.70	77.80	13.10	16.40	21.10	10796
11	45.80[c]	1.61	223.16	14.95	22.91	23.31	9428
12	19.54	0.00[c]	61.13	7.65	12.94	11.30	11666
13	13.18	1.32	119.81	11.75	16.73	14.76	11885
15	19.00	1.62	98.28	11.64	18.10	21.99	8787
16	22.60	1.30	63.70	5.55	19.30	19.50	12716
18	17.90	0.00[c]	55.90	7.32	19.40	11.40	13591
19	3.50[b]	1.25[b]	45.70	6.70	15.44	14.88	8272
20	17.93	1.32	47.83	6.49	16.25	17.17	9554
21	21.76	0.00[c]	64.87	4.91	13.77	12.00	10546
23	22.30	1.70	88.30	10.10	23.40	21.30	11885

[a]One I.U. = 0.6 μg.
[b]Apparent outlier (invalid value resulting from the use of too small a test portion).
[c]Outlier.
For cereal: Mean 19.228; S.D. 3.043. For powdered infant formula: Mean 1.571; S.D. 0.170.

Table 2. Results for determination of retinol (I.U./g)[a] by HPLC

Lab.	Cereal	Powdered infant formula	Chicken feed	Low fat milk	Retinol capsule
1	198.37[b]	16.95	3.58	4.32	12485
2	107.43[c]	17.80	2.40	2.64	10002
4	133.64	20.18	2.60	2.32	9969
5	125.25	17.20	0.00	1.91	11361
7	176.00	20.30	4.30	2.50	10666
8	222.00[b]	22.00[b]	1.00	3.00	10625
9	122.60	18.10	2.18	2.54	9658
11	123.52	18.30	35.20	3.90	10056
12	271.28[b]	26.34[c]	2.52	4.48	12682
13	155.81	10.04[c]	2.16	2.85	8529
15	178.80	18.00	4.73	2.96	12668
16	286.00[b]	19.30	5.79	2.72	14504
18	166.40	14.10[c]	2.70	3.91	7897
19	231.00[b]	22.80[b]	1.72	2.71	15831
20	100.58[c]	9.25[c]	1.85	3.30	5663
21	286.06[b]	27.54[c]	4.48	5.10	15723
23	168.00	15.90	1.90	2.44	7541

[a]One I.U. = 0.3 μg.
[b]Apparent outlier (invalid value resulting from the use of too small a test portion).
[c]Outlier.
For cereal: Mean 150.00; S.D. 23.530. For powdered infant formula: Mean 18.203; S.D. 1.160.

Table 3. Total activity (I.U./g)[a] by HPLC

Lab.	Cereal	Powdered infant formula	Chicken feed	Pureed carrots	V-8 juice	Low fat milk	Baby food squash A	Baby food squash B	β-Carotene capsule	Retinol capsule
1	213.00[b]	18.54	3.58[b]	53.91[b]	6.18	4.32[b]	13.02	11.66[b]	12766	12485
2	136.33[b]	19.53	2.40	80.00	9.16[b]	2.64	15.58	14.96	10050	10002
4	155.87	20.18	2.60	37.35[b]	14.88[b]	2.32	8.13[b]	8.75[b]	9969	9969
5	142.25[b]	18.03	0.00[b]	7.18[b]	7.27	1.91[b]	4.42[b]	5.99[b]	10090	11361
7	198.30	23.30[b]	4.30[b]	78.90	9.40[b]	2.50	13.80	15.00	12290	10666
8	264.00[b]	23.00	1.00[b]	80.40	7.30	3.00	19.90[b]	19.10	11507	10625
9	142.00[b]	19.80	2.18	77.80	13.10[b]	2.54	16.40	21.10	10796	9658
11	169.32	19.91	35.20[b]	233.16[b]	14.95[b]	3.90[b]	22.91[b]	23.31[b]	9428	10056
12	290.82[b]	26.34[b]	2.52	61.13	7.65	4.48[b]	12.94[b]	11.30[b]	11666	12682
13	168.99[b]	11.36[b]	2.16	119.81[b]	11.75[b]	2.85	16.73	14.76	11885	8529[b]
15	197.80	19.62	4.73[b]	98.28[b]	11.64[b]	2.96	18.10	21.99[b]	8787[b]	12668
16	308.60[b]	20.62	5.79[b]	63.70	11.55[b]	2.72	19.30	19.50	12716	14504[b]
18	184.30	14.10[b]	2.70	55.90[b]	7.32	3.90	19.40	11.40[b]	13591[b]	7897[b]
19	234.50[b]	24.05[b]	1.72[b]	45.70[b]	6.70	2.71	15.44	14.88	8272[b]	15831[b]
20	118.51[b]	10.57[b]	1.85[b]	47.83	7.49	3.30[b]	16.25	17.17	9554	5663[b]
21	307.82[b]	27.54[b]	4.48[b]	64.87	4.91[b]	5.10[b]	13.77	12.00[b]	10546	15723[b]
23	190.30	17.60	1.90[b]	88.30	10.10[b]	2.44	23.40[b]	21.30[b]	11885	7541[b]
Mean	185.314	19.374	2.427	74.388	6.987	2.688	16.163	17.53	11035	11017
SD	17.445	0.670	0.222	9.81	0.534	0.230	2.517	2.763	1138	1199

[a]Sum of retinol and carotene.
[b]Outlier.

extract; peak height or area was used for quantitation as follows: I.U. of vitamin A as carotene = (area of extract peak/area of standard solution peak) × (μl of standard solution injected/μl of extract injected) × (concentration of standard solution) × (dilution factor) × (1/0.6) × (1/weight of test portion).

Total activity

I.U. retinol + I.U. carotene = total activity

For samples that contained either retinol or carotene, the calculated activity in I.U represented the total activity. For samples that contained both carotene and retinol, activities in I.U. units were calculated separately and summed.

Statistical analysis

Data were purged of outliers by the conventional 'IUPAC-87' outlier-screening procedure (Horwitz, 1988), using both the Grubbs test (for extreme within-laboratory values) and the Cochran test (for extreme within-laboratory variances). The Horwitz formula represents historically based norms for the reproducibility coefficient of variation as a function of concentration.

RESULTS AND DISCUSSION

The results of the analyses are shown in Tables 1–3. Mean values were based on non-outliers. When both beta-carotene and retinol were present and either one was an outlier, the total activity value was considered an outlier and was not used in the statistical analysis. Many apparent 'outliers' were invalid data resulting from the use of amounts smaller than the specified test portion, which provided insufficient analyte for measurement. When the test portions analyzed by the collaborators were less than 5 g, the results were outliers. Table 4 shows the percent relative difference between the official AOAC chemical method and the HPLC method. The official AOAC method gave higher values.

Figure 1 shows the descriptive Horwitz plots (Horwitz *et al.*, 1980). Figure 2 shows the typical chromatograms of carotene and retinol.

The use of some C18 packings with a pore size of 75–125 Å (7.5–12.5 nm) results in splitting of the retinol peak; when this happens, the analyst must increase the mobile phase concentration of methanol:water to (95:5, v/v) or use a packing of 150 Å (15 nm) pore size.

Table 4. Comparison of mean values by HPLC with AOAC chemical method

Commodity	HPLC (I.U./g/cap)	AOAC[a] (I.U./g/cap)	RD[b]
Cereal	185.314	242.20	−23.49
Powdered infant food	19.374	20.140	−3.81
Chicken feed	2.427	2.320	+4.61
Pureed carrots	74.388	120.000	−38.01
V-8 juice	6.987	12.570	−44.42
Low fat milk	2.688	3.540	−24.07
Baby food squash A	16.163	17.610	−8.22
Baby food squash B	17.530	17.120	+2.39
β-carotene capsule	11035	11867	−7.01
Retinol capsule	11017	11796	−6.60

[a]Lab. 24 performed the AOAC chemical analysis.
[b]RD (relative difference) = 100 × (method value-AOAC value)/AOAC value.

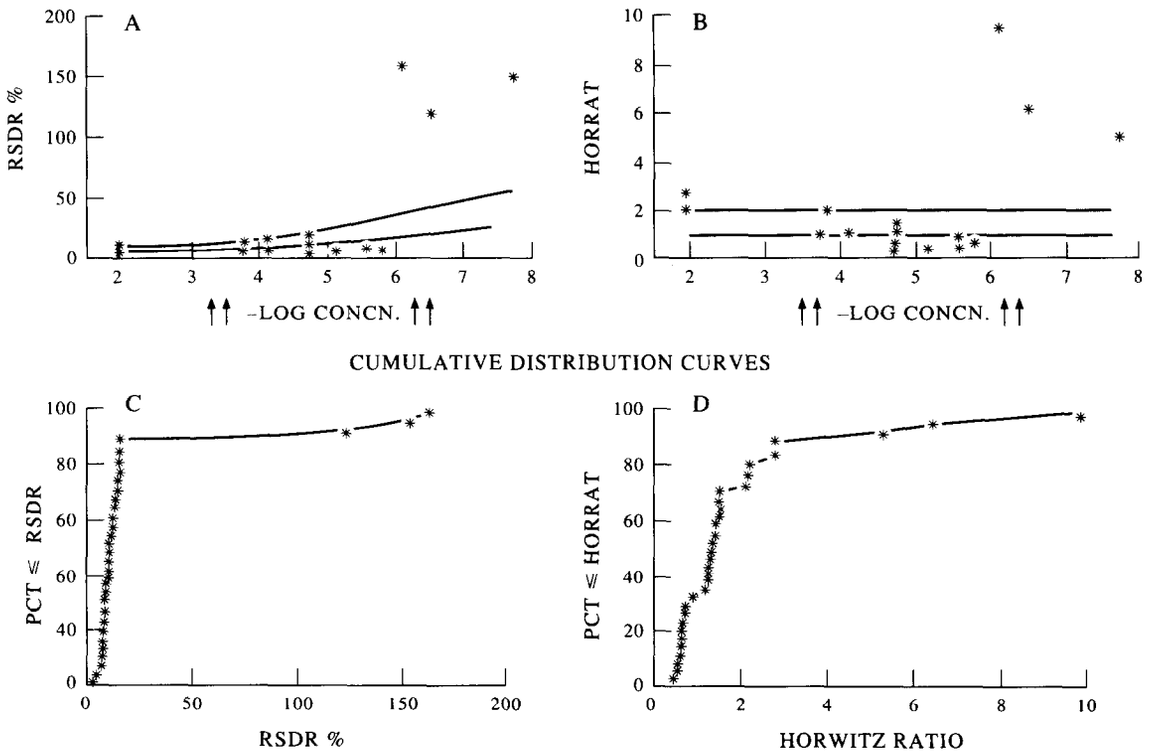

Fig. 1. Horwitz plots. Horwitz formula represents historically based norms for the reproducibility coefficient of variation as a function of concentration.

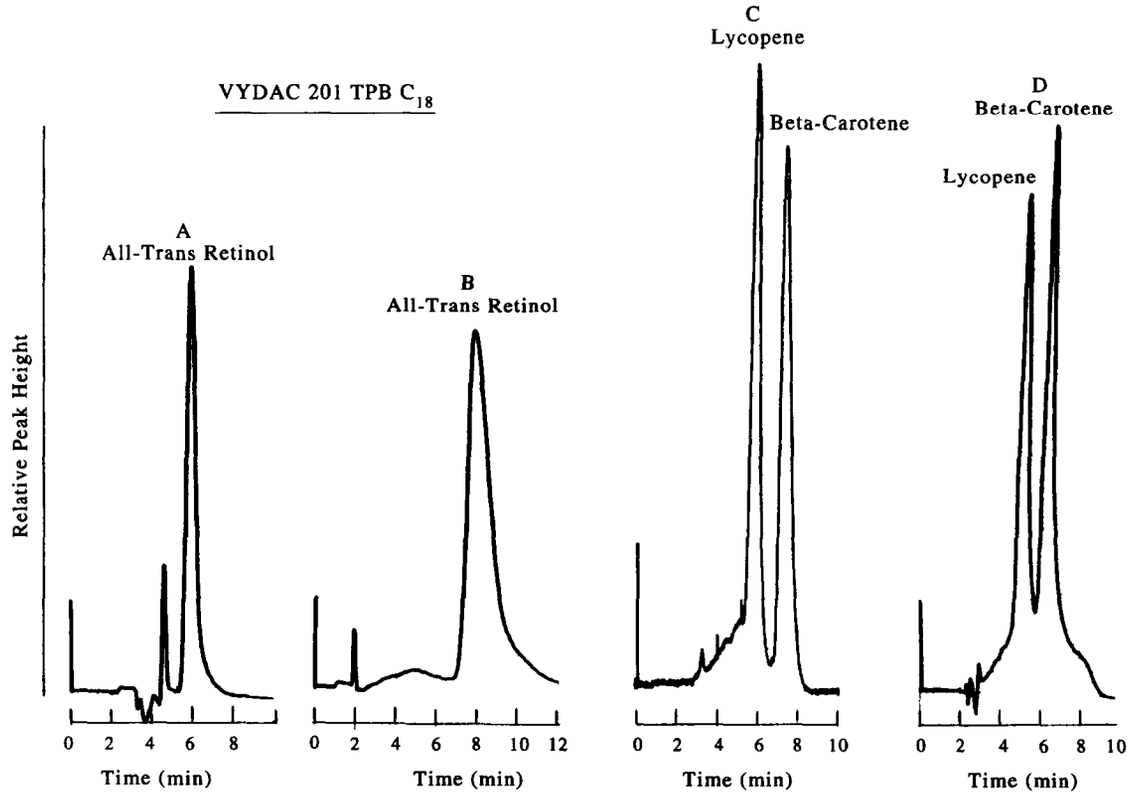

Fig. 2. Chromatograms of all-*trans* retinol, beta-carotene and lycopene. A. All-*trans* retinol in hexane at 325 nm. B. All-*trans* retinol in methanol at 325 nm. C. Lycopene and beta-carotene in hexane at 436 nm. D. Lycopene and beta-carotene in hexane at 450 nm.

CONCLUSION

A C18 packing of 5–10 μm particle size and 300 Å (30 nm) pore size and a flow rate of 1 ml/min are prescribed for carotene determination. A C18 packing of less than 300 Å (30 nm) pore size generates too much solvent waste at 3 ml/min and is not cost-effective. A C18 packing of 5–10 μm particle size and 150 Å (15 nm) pore size and a flow rate of 1.5 ml/min are prescribed for retinol determination. A flow rate of 1.5 ml/min decreases the retention time a little but retains good resolution for retinol determination. The weight of the test portion used for analysis must not be less than 5 g to ensure homogeneity.

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