

# A Nonsaponification Method for the Determination of Carotenoids, Retinoids, and Tocopherols in Solid Human Tissues<sup>1</sup>

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## Abstract

There has been an increasing interest in the measurement of carotenoids, retinoids, and tocopherols in human tissues because some of these micronutrients have been shown to have chemopreventive activity. Since clinical tissue samples obtained for analysis are usually small in quantity, a sensitive analytical procedure that can simultaneously measure all the micronutrients of interest in one small piece of human tissue is necessary. Moreover, some solid tissues, such as skin, are very difficult to homogenize unless they are first saponified in alcoholic KOH, but the saponification often causes substantial destruction of some micronutrients. Thus, a nonsaponification procedure using collagenase to facilitate homogenization was developed. Solid tissues are first incubated in a collagenase solution, homogenized, then incubated in a protease solution, followed by precipitation of tissue proteins and extraction with hexane. Collagenase digestion facilitates homogenization, and protease digestion increases the extractable amounts of the micronutrients from certain tissue samples. In this study, the recovery and precision (coefficient of variation) of the new procedure was determined. In addition, the extracted amounts of seven carotenoids, two retinoids, and two tocopherols from human skin, cervical/ovarian tissue, as well as sarcoma and kidney tumors using the new procedure and a commonly applied saponification procedure were compared.

## Introduction

Epidemiological and experimental data have suggested that some carotenoids, retinoids, and  $\alpha$ -tocopherol may have chemopreventive activity against certain types of human cancer (1-12). These micronutrients may exert their actions at a specific tissue site or sites where cancer develops. Thus, their concentrations in the target tissue(s) may be critical for the chemopreventive activity (13). In

order to define the role of the micronutrients in cancer prevention, their concentrations in the target tissue(s) of normal subjects, subjects at high risk for cancer, and cancer patients need to be quantified and compared.

Due to a lack of suitable analytical procedures, however, as well as difficulties in obtaining solid human tissues on a routine basis, there have been few detailed, systematic studies on the concentrations of these micronutrients in human tissues. Previous publications on skin carotenoids and retinoids, for example, have usually reported only retinol, carotene, or total carotenoids (14-16).

For the extraction of skin micronutrients for analysis, a commonly applied procedure has been saponification of tissue in KOH-methanol, followed by homogenization and then extraction with hexane or other solvents. The extract is then analyzed spectrophotometrically for total carotenoids (14) or by HPLC<sup>3</sup> for individual carotenoids (15, 16). The saponification is necessary because human skin is very difficult to homogenize. However, the saponification often causes destructions of certain micronutrients (17-19). Our previous study indicated that saponification of human buccal mucosal cells with KOH-methanol (5%, w/v) resulted in a substantial loss of  $\alpha$ -tocopherol, retinyl palmitate, and lycopene when compared to the values obtained by using a nonsaponification procedure (19).

Kaplan *et al.* (30) have recently reported the concentrations of zeaxanthin,  $\beta$ -cryptoxanthin, lycopene,  $\alpha$ -carotene, and  $\beta$ -carotene in the liver, pancreas, kidney, adrenal glands, spleen, heart, testes, thyroid, ovary, and fat of human autopsies. The tissues were directly homogenized in an EDTA solution containing ascorbic acid. A similar homogenization procedure used by us produced satisfactory results in most mouse tissues (21); however, this procedure did not always produce satisfactory results in human skin.

In the present study, a nonsaponification procedure for solid tissues was developed. Human skin and various tissues were first incubated in a collagenase solution, then homogenized. The homogenates were immediately subjected to protease digestion, followed by precipitation of tissue proteins and extraction with hexane. The new procedure not only allows solid tissues to be homogenized effectively but also retains all the micronutrients of interest for HPLC analysis. This procedure simultaneously measures lutein, zeaxanthin,  $\beta$ -cryptoxanthin,

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<sup>3</sup> The abbreviations used are: HPLC, high-performance liquid chromatography; BHT, butylated hydroxytoluene; SDS, sodium dodecyl sulfate; PBS, phosphate-buffered saline.

Table 1 Comparison of extracted amounts of major carotenoids, retinoids, and tocopherols in facial skin and cervical/ovarian tissues using three homogenization procedures

Micronutrients	Procedures		
	KOH-methanol (ng/g wet weight)	Collagenase (ng/g wet weight)	Collagenase/protease (ng/g wet weight)
<b>Facial skin</b>			
Lutein	14.57 ± 0.22 <sup>a</sup>	16.34 ± 0.22	16.87 ± 1.10
Lycopene	115.12 ± 4.47	93.60 ± 4.32	134.27 ± 0.25
α-Carotene	8.12 ± 1.13	7.94 ± 1.37	15.71 ± 1.58
β-Carotene	58.21 ± 3.81	47.97 ± 3.95	69.36 ± 4.06
Retinol	— <sup>b</sup>	29.78 ± 2.78	51.77 ± 3.69
Retinyl palmitate	Trace	31.98 ± 0.79	53.72 ± 5.63
α-Tocopherol	4,049 ± 351	8,325 ± 636	10,942 ± 75
γ-Tocopherol	1,497 ± 90	2,847 ± 244	2,742 ± 258
<b>Cervical/ovarian tissue</b>			
Lutein	14.75 ± 2.16	21.95 ± 0.71	25.43 ± 2.34
Lycopene	97.27 ± 3.17	103.36 ± 10.15	180.57 ± 10.96
α-Carotene	22.20 ± 1.52	35.86 ± 2.40	55.77 ± 1.27
β-Carotene	51.43 ± 2.15	74.91 ± 4.06	120.73 ± 1.34
Retinol	— <sup>b</sup>	63.55 ± 3.88	77.97 ± 1.13
Retinyl palmitate	None	Trace	Trace
α-Tocopherol	1,845 ± 122	4,955 ± 48	6,787 ± 73
γ-Tocopherol	819 ± 96	1,468 ± 99	2,169 ± 163

<sup>a</sup> Mean ± SD of two aliquot values.

<sup>b</sup> Retinol could not be identified due to the presence of a large peak generated by the saponification.

lycopene, α-carotene, β-carotene, *cis*-β-carotene, retinol, retinyl palmitate, α-tocopherol, and γ-tocopherol<sup>4</sup> in a small piece of human tissue.

## Materials and Methods

**Chemicals.** α-Carotene (Type V), β-carotene (Type IV), retinol, retinyl palmitate, BHT, collagenase (Type IV), and protease (Type XXV) were purchased from Sigma Chemical Co. (St. Louis, MO). The collagenase was from *Colostriidium histolyticum* containing 460 collagen digestion units/mg solid. The protease (pronase E) was from *Streptomyces griseus* containing 5 casein digestion units/mg solid. Prior to use, retinol and β-carotene were purified according to the methods recommended by the National Institute of Standards and Technology.<sup>5</sup> α-Tocopherol and γ-tocopherol were purchased from Eastman Kodak (Rochester, NY). Lutein, zeaxanthin, and lycopene were kindly provided by Amoco Technology Company (Naperville, IL). β-Cryptoxanthin was a gift from Hoffmann-La Roche, Inc. (Nutley, NJ). SDS was purchased from BioRad (Rochester, NY). Acetonitrile, methanol, tetrahydrofuran (nonspectro with 250 ppm BHT), and hexane were purchased from Burdick and Jackson (Muskegon, MI). Ethanol was purchased from Midwest Grain Product Co. (Weston, MO), and ammonium acetate was purchased from JT Baker, Inc. (Phillipsburg, NJ). All aqueous and organic solvents were passed through a 0.2-μm filter prior to use.

**Biological Samples.** All of the biological samples were provided by local clinics. Human skin was obtained from adult females who had undergone cosmetic facial surgery. Normal and precancerous cervical and ovarian tis-

ues were obtained from patients who had undergone hysterectomy or biopsies. Kidney tumors and abdominal sarcoma were surgically removed from cancer patients. All of the tissues were cleaned and washed with PBS. Any visible adipose tissue was removed. The tissues were cut into pieces, weighed, and stored at -80°C. For the development of the homogenization procedure, tissues were cut into small pieces, finely minced, and pooled, then aliquoted into about 50 mg/tube and stored at -80°C until use. All the sample cleaning and handling were carried out under dim red light.

**Preparation of Extraction Solvents, Mobile Phase, and HPLC Standards.** Collagenase solution and protease solution were prepared by adding 10 ml of cold PBS to 500 mg of collagenase powder and 200 mg of protease powder respectively. They were gently vortex-mixed until the powders dissolved. The solutions were stored at -20°C until used.

KOH-methanol-BHT solution was prepared by adding 100 ml of methanol to 5 g of KOH tablets and was stir-mixed until the tablets dissolved. Prior to use, BHT was added to make the BHT concentration 0.1% (w/v).

Preparations of solutions for extraction, HPLC analysis, and constructions of standard calibration curves as well as verification of our quantitation have been described previously (19).

**Homogenization and Extraction Procedures.** Frozen tissues were first allowed to thaw at room temperature. For homogenization, two kinds of tubes were used, depending upon the amount of tissue available. For homogenizing a larger piece of tissue, usually 100 to 200 mg, 17 x 100 mm polypropylene test tubes (Falcon 2059; Becton Dickson, Lincoln Park, NJ) were used. To 100 mg of tissue a very small amount of BHT crystal (1–2 mg), 560 μl of PBS, and 70 μl of collagenase solution (50 mg/ml) were added. The samples were incubated at 37°C for 1 h. After the incubation, they were homogenized on ice with a polytron tissue homogenizer (model PT 10/35;

<sup>4</sup> This procedure cannot separate γ-tocopherol and β-tocopherol. Thus, the γ-tocopherol values reported in this paper may contain some β-tocopherol.

<sup>5</sup> Neal Craft, personal communication.

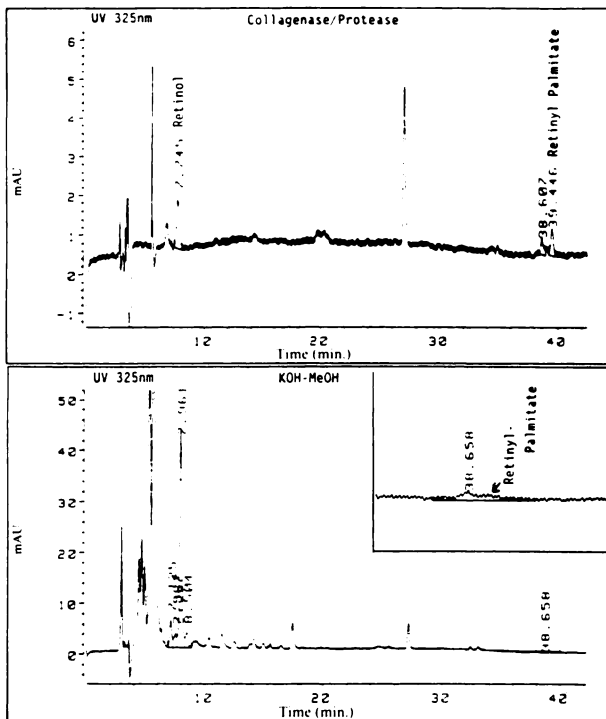


Fig. 1. Comparison of HPLC chromatograms (325 nm) of pooled human skin extracted with the collagenase/protease method and the KOH-methanol method.

Brinkmann Instruments, Westbury, NY). To the whole homogenate, 70  $\mu$ l of protease solution (20 mg/ml) were added and then were vortex-mixed and incubated at 37°C for 30 min.

For extraction, a 0.25-ml aliquot of the digested homogenates was pipeted into a 1.5-ml microcentrifuge tube (Intermountain Scientific Corp., Bountiful, UT), and 250  $\mu$ l of a SDS-ethanol-BHT solution (19) were added and vortex-mixed. The sample was then extracted twice with 500  $\mu$ l of hexane containing 0.1% BHT as described (19). When the amount of micronutrients in the tissue was too low to be accurately measured, two or more aliquots were extracted, and all of the hexane layers were combined and dried. The dried extract was stored at -20°C until HPLC analysis.

To homogenize a small piece of tissue weighing 50 mg or less, the tissue was placed directly into a 2-ml microcentrifuge tube (Intermountain Scientific Corp.). A very small amount (1–2 mg) of BHT crystal, 280  $\mu$ l of PBS, and 35  $\mu$ l of collagenase solution (50 mg/ml) were then added. The sample was vortex-mixed and incubated at 37°C for 1 h. After the incubation, the sample was homogenized on ice with a hand-held polytron tissue homogenizer (model PT 1200; Brinkmann Instruments), then 35  $\mu$ l of protease solution (20 mg/ml) were added. After mixing, the sample was incubated at 37°C for 30 min. Following the second incubation, 350  $\mu$ l of a SDS-ethanol-BHT solution were added. The sample was then extracted and analyzed with HPLC as previously described (19).

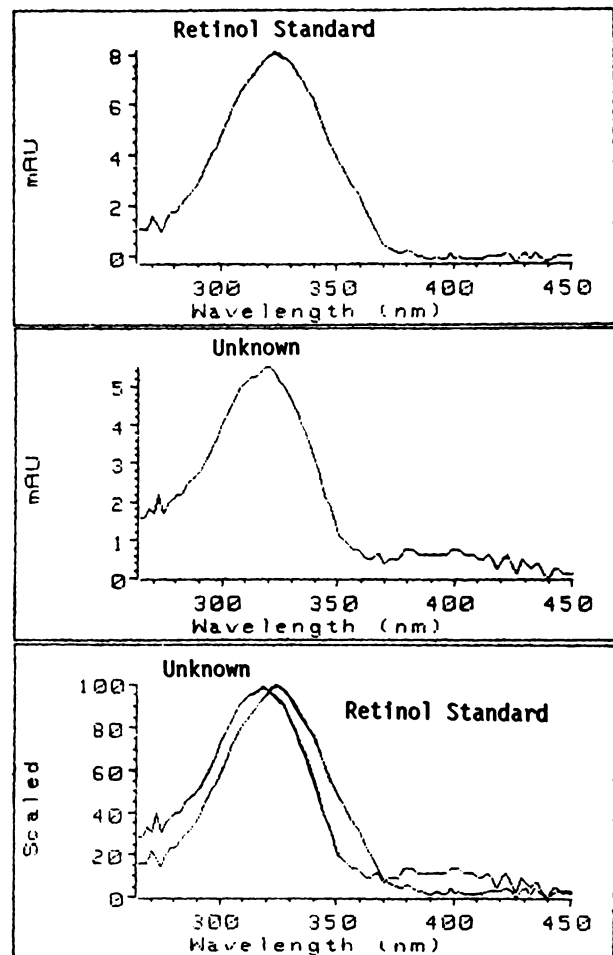


Fig. 2. Absorption spectra of standard retinol and an unknown peak generated by the KOH-methanol saponification of human skin.

**Recovery and Precision of the Assay.** To determine the recovery of the assay, facial skin and cervical tissue were homogenized as described. The tissue homogenates were then aliquoted into 0.25 ml each. They were spiked with or without a standard solution consisting of lutein, lycopene,  $\beta$ -carotene, retinol, retinyl palmitate, and  $\alpha$ -tocopherol in triplicate at three different concentrations. All of the samples were then extracted and analyzed.

The precision (coefficient of variation) of the assay during a 1-month period was determined by weekly extraction and analysis of the micronutrients in the above aliquoted tissue homogenates by the new procedure.

## Results and Discussion

There has been an increasing interest in recent years in the measurement of carotenoids, retinoids, and tocopherols in solid human tissues from which cancer develops. One major problem facing the investigators is the size of the clinical samples obtained for analysis, which is usually very small—50 mg or less in our experience. Therefore, for the measurement of tissue micronutrients, it is necessary to have a sensitive and reproducible analytical procedure which is capable of simultaneously measuring

Table 2 Comparison of extracted amounts of micronutrients in sarcoma and kidney tumors using two homogenization procedures

Micronutrients	Procedures	
	Collagenase (ng/g wet weight)	Collagenase/protease (ng/g wet weight)
<b>Sarcoma</b>		
Lutein	103.02 ± 4.64 <sup>a</sup>	124.66 ± 4.06
Zeaxanthin	17.08 ± 0.95	18.86 ± 1.61
β-Cryptoxanthin	29.47 ± 1.93	31.44 ± 1.53
Lycopene	168.66 ± 11.61	213.75 ± 12.12
α-Carotene	10.48 ± 1.34	11.08 ± 0.70
β-Carotene	31.63 ± 2.01	36.71 ± 2.96
cis-β-Carotene	12.88 ± 3.39	12.18 ± 1.44
Retinol	31.06 ± 2.86	34.41 ± 0.83
Retinyl palmitate	None	None
α-Tocopherol	14,542 ± 1,371	15,714 ± 973
γ-Tocopherol	5,301 ± 663	5,413 ± 306
<b>Kidney tumor</b>		
Lutein	51.22 ± 1.63	48.09 ± 1.59
Zeaxanthin	11.35 ± 0.49	11.35 ± 0.41
β-Cryptoxanthin	Present <sup>b</sup>	Present <sup>b</sup>
Lycopene	745.65 ± 10.00	827.54 ± 68.01
α-Carotene	8.89 ± 0.18	8.93 ± 0.49
β-Carotene	19.09 ± 0.60	20.89 ± 1.20
cis-β-Carotene	5.01 ± 0.84	6.35 ± 0.67
Retinol	32.30 ± 1.96	32.15 ± 2.89
Retinyl palmitate	104.01 ± 4.43	99.58 ± 9.64
α-Tocopherol	51,118 ± 1,423	51,904 ± 1,433
γ-Tocopherol	3,947 ± 59	4,152 ± 385

<sup>a</sup> Mean ± SD of two aliquot values.

<sup>b</sup> β-Cryptoxanthin could not be accurately quantified due to the presence of an interference peak in the kidney tumor.

all of the micronutrients of interest from a single piece of obtained tissue. Such a simultaneous measurement also allows investigators to determine whether any interactions among the micronutrients exist in a dosing study. It has been reported that chronic oral administration of β-carotene caused a significant reduction in the concentration of α-tocopherol in the plasma of humans, mice, and rats (22, 23).

For the extraction of micronutrients for HPLC analysis, tissues need to be homogenized first. As shown in Table 1, the commonly applied KOH-methanol saponification method caused the substantial destruction of retinyl palmitate, tocopherols, and to a lesser extent, carotenoids; thus, this commonly used procedure has its limitations. The KOH-methanol saponification also produced many unknown peaks with retention times shorter than 20 min, which were mostly detected at 325 nm and 300 nm. Fig. 1 shows the comparison of HPLC chromatograms of pooled human skin extracted with the present procedure and the KOH-methanol procedure monitored at 325 nm. One new peak generated by the saponification had a retention time approximately 0.2 min longer and a peak area much larger than for retinol (Fig. 1). As a result, the presence of retinol in the saponified samples could not be ascertained. The spectra of the unknown peak and the standard retinol peak were compared using the photodiode array detector (Fig. 2). The two peaks had similar spectra, but the spectrum of the unknown shifted to the left. Because of the similarity in the spectrum, the unknown peak was probably a retinoid.

In search of an alternative method, we investigated several homogenization procedures, including mechani-

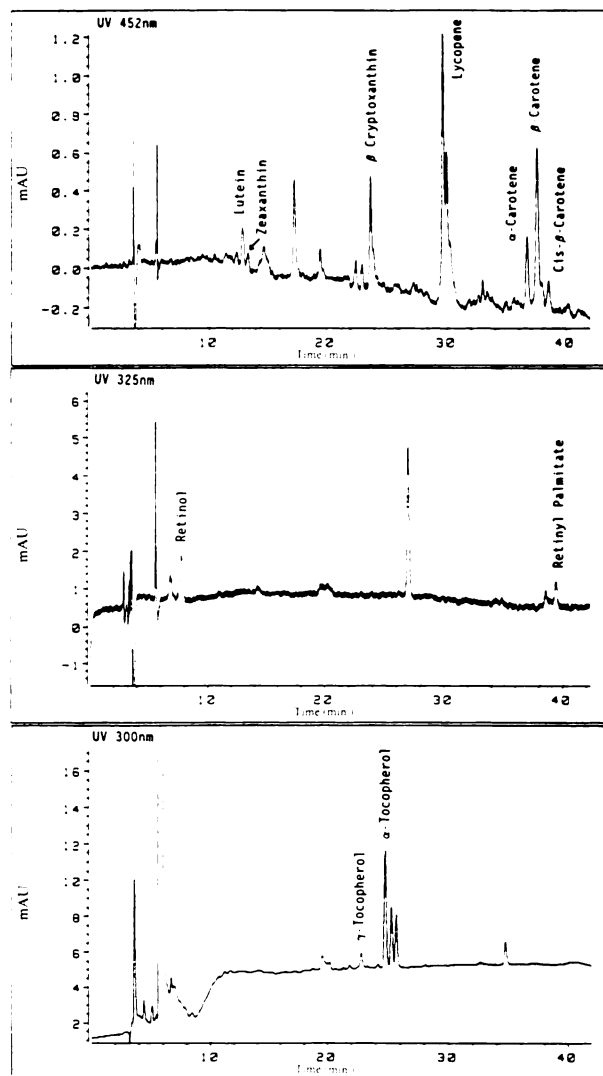


Fig. 3. Representative HPLC chromatograms of carotenoids, retinoids, and tocopherols extracted from pooled human skin.

cal grinding of frozen tissues, homogenization of tissues in an EDTA-ascorbic acid solution, and enzymatic treatments of tissues prior to homogenization. Mechanical grinding produced a satisfactory tissue powder; however, transferring the powder into a homogenization tube was a problem. The transfer often resulted in a substantial loss of precious samples. Thus, this method was not practical for a small piece of tissue. As for the homogenization in an EDTA-ascorbic acid solution, grinding of skin and connective tissues was a problem, even when using a high-power homogenizer. Therefore, an alternative procedure of treating tissues with collagenase prior to homogenization was investigated. Following collagenase digestion at 37°C for 1 h, the tissues, including skin, were much easier to homogenize. The collagenase concentration of approximately 5 mg/ml and the incubation time of 1 h at 37°C were chosen because this combination optimized the subsequent homogenization.

In our previous study of human buccal mucosal cells, the recovery of carotenoids was increased by incubating

**Table 3** Recovery of micronutrient standards spiked to pooled human skin and cervical tissue homogenates

Standards	Amount spiked (ng/g wet weight)	Recovery	
		Skin (%)	Cervical tissue (%)
Lutein	23.88	96.52 <sup>a</sup>	93.99
	58.43	95.38	94.91
	122.72	92.42	93.16
		94.77 ± 2.12 <sup>b</sup>	94.02 ± 0.88
Lycopene	52.12	84.72	83.19
	128.74	84.19	87.51
	260.29	86.86	85.84
		85.26 ± 1.41	85.51 ± 2.18
β-Carotene	42.78	88.09	92.41
	100.47	88.52	92.18
	208.94	86.48	86.39
		87.69 ± 1.08	90.33 ± 3.41
Retinol	27.83	100.43	96.20
	69.34	98.72	95.96
	137.80	96.60	96.14
		98.58 ± 1.92	96.10 ± 0.12
Retinyl palmitate	40.30	87.78	88.90
	100.17	88.92	86.84
	206.91	88.16	88.98
		88.29 ± 0.58	88.24 ± 1.21
α-Tocopherol	2,800.33	101.83	96.79
	6,736.39	96.07	99.75
	14,002.64	95.69	96.36
		97.86 ± 3.44	97.63 ± 1.85

<sup>a</sup> Average of three spiked samples.

<sup>b</sup> Mean ± SD of 9 spiked samples.

the cells in a protease solution prior to extraction (19). Thus, the homogenates of skin, cervical/ovarian tissue, and two tumors were calculated with protease at 37°C for 30 min following collagenase digestion. As shown in Table 1, protease digestion further increased the concentration of carotenoids, retinoids, and tocopherols in both skin and cervical/ovarian tissue; however, such an increase in the concentration by protease was not observed in all of the cervical tissue samples analyzed.

Also, as shown in Table 2, in sarcoma and kidney tumors, the same protease digestion only increased the concentration of carotenoids slightly, and the concentration of retinoids and tocopherols was not improved at all. Thus, it appears that the effect of protease digestion cannot be generalized. Since protease digestion was not deleterious, and in some cases increased the extractable amounts of micronutrients in tissues and tumors, protease digestion has been incorporated into our routine assay procedure for various clinical samples.

Fig. 3 shows representative HPLC chromatograms of carotenoids, retinoids, and tocopherols extracted from pooled human skin using the new procedure. The profile of the micronutrients seen in the skin is very similar to that in plasma (19), with the exception that retinyl palmitate was detected in the skin at a relatively high concentration in relation to retinol. Lycopene, β-carotene, and tocopherols were the predominant micronutrients, while zeaxanthin was the least concentrated. Lycopene was also detected as a cluster of two isomers.

Table 3 shows the recovery of spiked standards in the skin and cervical tissue homogenates. The recovery

**Table 4** Coefficients of variation for the analysis of pooled skin and cervical tissue homogenates during a 1-month period

Micronutrients	Concentration (ng/g wet weight)	Coefficient of variation (%)
Pooled skin homogenate		
Lutein	6.68 ± 0.55 <sup>a</sup>	8.23
Zeaxanthin	Trace	
β-Cryptoxanthin	30.26 ± 2.73	9.02
Lycopene	25.58 ± 2.21	8.65
α-Carotene	7.85 ± 0.62	7.90
β-Carotene	49.14 ± 2.73	5.56
cis-β-Carotene	10.66 ± 0.91	8.54
Retinol	29.94 ± 2.26	7.55
Retinyl palmitate	30.94 ± 1.65	5.33
α-Tocopherol	4,207.84 ± 198.19	4.71
γ-Tocopherol	1,019.22 ± 79.88	7.84
Pooled cervical tissue homogenate		
Lutein	14.41 ± 1.24	8.60
Zeaxanthin	Trace	
β-Cryptoxanthin	11.93 ± 1.04	8.72
Lycopene	130.50 ± 9.65	7.40
α-Carotene	13.13 ± 1.05	7.99
β-Carotene	66.24 ± 5.09	7.68
cis-β-carotene	15.84 ± 1.44	9.09
Retinol	23.86 ± 1.17	4.90
Retinyl palmitate	41.96 ± 1.89	4.50
α-Tocopherol	8,886.68 ± 406.74	4.58
γ-Tocopherol	2,363.96 ± 166.37	7.04

<sup>a</sup> Mean ± SD of 10 aliquot values (2 aliquots analyzed/week).

of the micronutrients ranged from 85.3% to 98.6% in the skin and from 85.5% to 97.6% in the cervical tissue, indicating that the recovery was satisfactory.

Table 4 shows the coefficients of variation of our assay during a 1-month period. Only a trace amount of zeaxanthin was detected; thus, this micronutrient was not quantified. The coefficients of variation for the other 10 micronutrients was less than 10%. Such results were in general agreement with our previous findings in human buccal mucosal cells (19).

As stated, saponification with alcoholic KOH causes substantial destruction of some micronutrients in the tissues. A new nonsaponification procedure was therefore developed. This procedure, coupled with our established extraction and HPLC procedure, is able to simultaneously measure seven carotenoids, two retinoids, and two tocopherols in one small piece of human tissue weighing 50 mg or less. In our laboratory, the new procedure has been routinely used in skin, cervix, muscle, heart, and various tumor samples except subcutaneous fat, which contained too much oil. The fatty extract of the fat tissue could not completely dissolve in mobil phase for HPLC analysis. A new method using lipase is under investigation. With the nonsaponification procedure, the concentrations of the 11 micronutrients in paired plasma and skin biopsies from patients with actinic keratoses have been successfully determined. The results will be reported shortly.

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