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Short communication

Monomeric C_{18} chromatographic method for the liquid chromatographic determination of lipophilic antioxidants in plants

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Abstract

Reversed-phase liquid chromatography was used to determine lipophilic antioxidants in plants using two monomeric C_{18} columns operated at 30 °C and 4 °C, with a column-switching technique and acetonitrile-methanol gradient elution. The chromatograms were extracted at different wavelengths using a UV diode array detector (DAD). A wide range of plant antioxidants, including nine carotenoids (neoxanthin, violaxanthin, lutein, zeaxanthin, β-cryptoxanthin, lycopene, canthaxanthin, α-carotene and β-carotene) together with all-*trans*-retinol, capsaicin, dihydrocapsaicin, chlorophyll *a*, and chlorophyll *b* can be separated within 50 min. Fluorometric detection was applied to quantify trace amounts of six vitamin E analogues (α-, δ- and γ-tocopherols and tocotrienols). The detection limits were 0.2–0.4 μg/g for various xanthophylls and 0.04–0.10 μg/g for vitamin E analogues.

Keywords: Vitamins; Antioxidants; Column switching

1. Introduction

Vegetables and fruits contain a wide spectrum of lipophilic antioxidants such as carotenoids, tocopherols and tocotrienols. So far, there is no chromatographic method for their simultaneous determination. Several techniques such as C_{30} reversed-phase LC coupled to mass spectrometric detection and nuclear magnetic resonance have been developed recently for carotenoids [1–3]; however, they are costly and can be problematic when tocotrienols have to be determined simultaneously.

We have recently developed a C_{18}-based method for the determination of various antioxidants in human plasma [4]. However, this method was not suitable for plant antioxidants as the matrix is generally more complex than human plasma. We now describe a column-switching approach using gradient elution for the separation of a wide range of plant lipophilic antioxidants, including capsaicin, chlorophylls, major carotenoids and six vitamin E analogues.

2. Material and methods

2.1. Reagents and chemicals

All reagents and chemicals were of the highest quality available from various sources. Tetrahyrofuran, *n*-hexane, methanol, *n*-butanol, ethyl acetate, acetone and acetonitrile (HPLC/Spectro grade) were purchased from Tedia (Fairfield, OH, USA). Absolute ethanol, butylated hydroxytoluene (BHT) and disodium sulphate (analytical grade) were obtained from Merck (Darmstadt, Germany). Capsaicin, dihydrocapsaicin, chlorophyll *a*, chlorophyll *b*, all-*trans*-retinol, α-tocopherol, δ-tocopherol, γ-tocopherol, tocopherol acetate, β-carotene and lycopene were purchased from Sigma Chemical (St. Louis, MO, USA). Lutein, zeaxanthin, canthaxanthin and β-cryptoxanthin were kind

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gifts from BASF (Ludwigshafen, Germany). Neoxanthin and violaxanthin were from DHI Water and Environment Agency (Agern Alle, Hoersholm, Denmark). Echinenone, capsorubin, capsanthin and α-carotene were from CaroteNature (Lupsingen, Switzerland). Retinol acetate was from Supelco (Bellefonte, PA, USA). Stock solutions of each standard were prepared individually with appropriate solvents as described earlier [4]. The stock solution of ethanol-BHT (0.02%, w/v) was prepared once in a fortnight. The internal standard (IS) solution containing retinol acetate (4 μg/ml) as IS1 for all-*trans*-retinol, tocopherol acetate (400 μg/ml) as IS2 for capsaicin, dihydrocapsaicin and vitamin E vitamers, and echinenone (8 μg/ml) as IS3 for chlorophylls and carotenoids quantification; as prepared weekly in ethanol-BHT. For calibration, 100 μl of the IS solution were mixed with 100 μl of standard mixtures of various concentrations.

2.2. Samples preparation

10–20 g samples were weighed and frozen in liquid nitrogen. The samples were then freeze-dried for 2–4 days, depending on their moisture content. Under dim lighting, the lyophilized sample was ground into powder with a mortar and stored at −70 °C until analysis. In an amber micro-centrifuge tube A, 5–15 mg fine powder of an individual sample was added to approximately 20 mg of disodium sulphate and 100 μl each of acetonitrile and the IS solution prepared in ethanol-BHT. For spiked sample (5 ± 0.5 mg of the fine powder of alfalfa), 100 μl of a mixed standard solution were used for the extraction. The sample was then placed in a Vortemp™ vortexing incubator (UniEquip, Munich, Germany) with the shaking speed set at 1,350 rpm and the temperature at 25 °C. After shaking for 5 min, the sample was centrifuged at 15,000 × *g* for 1 min and 160 μl of the supernatant were transferred to another amber micro-centrifuge tube B. The sample in tube A was mixed with 100 μl of butanol–ethyl acetate (1:1, v/v) and 500 μl of *n*-hexane. After 5 min of shaking the sample was mixed with 200 μl of water and centrifuged at 15,000 × *g* for 2 min. 600 μl of supernatant were then transferred to another amber micro-centrifuge tube C. Using a Visidry™ and Visprep™ drying device (24-port Model, Supelco, Bellefone, PA, USA), the supernatant was evaporated to dryness under a stream of nitrogen for about 20 min. The residue was reconstituted using 40 μl of THF and then mixed thoroughly with the 160 μl of supernatant in tube B which was collected earlier. 20 μl of the extract was used for the HPLC determination using an autosampler maintained at 20 °C. For reproducibility and recovery studies,

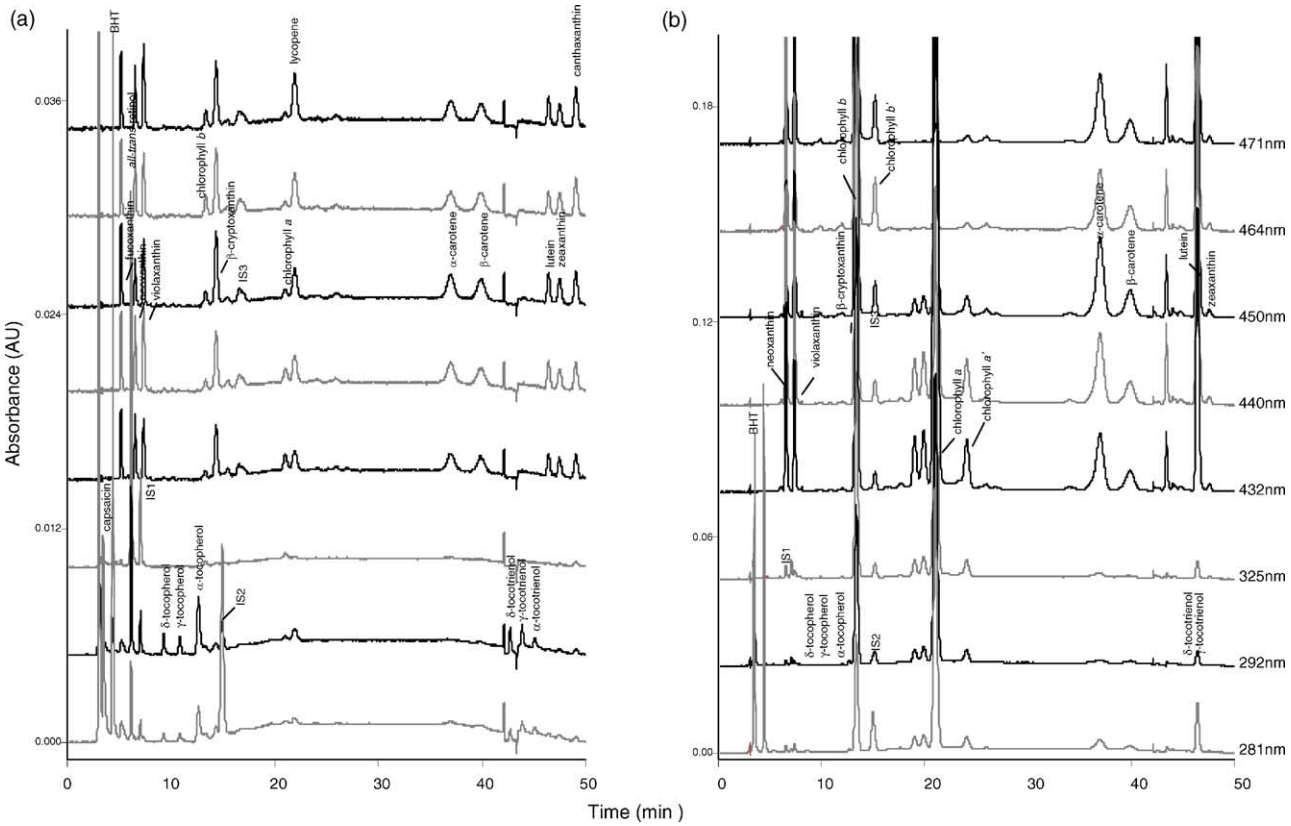

Fig. 1. Chromatograms extracted at various wavelengths. (a) A mixed standards solution added with equal volume of IS solution (IS1, 4 μg/ml and IS3, 8 μg/ml). The initial concentrations of this solution was 4 μg/ml of neoxanthin; 5 μg/ml of violaxanthin and all-*trans*-retinol; 10 μg/ml each of α-carotene, β-cryptoxanthin, zeaxanthin and canthaxanthin; 25 μg/ml of lycopene; 50 μg/ml of β-carotene; 100 μg/ml each of lutein and chlorophyll *b* and 200 μg/ml of chlorophyll *a*. (b) A plant sample extracted from 6.3 mg of lyophilized pandan leaf (*Pandanus amaryllifolius*).

the assays were carried out using the alfalfa blank and spiked samples four times a day for four consecutive days.

2.3. Equipment and conditions

The LC system was a Waters (Milford, MA, USA) Alliance 2695 Separation Module, a Model 996 diode-array UV detector (DAD) and a Model 2475 fluorescence detector. An Agilent (Ringoes, NJ, USA) Zorbax SB-C_{18} column (5 μm, 150 mm × 4.6 mm i.d.) used as column-1 was protected by a guard cartridge (Jour Guard C_{18}, VICI, Schenkon, Switzerland) and maintained at 30 °C. A Whatman (Maidstone, UK) replaceable Partisphere 5 C_{18} cartridge (5 μm, 110 mm × 4.7 mm i.d.) used as column-2 was chilled to 4 °C in a thermostated cuvette controlled by a temperature regulator (Model 832, Gilson, Villier le Bel, France). Gradient elution was performed at 1 ml/min with an initial condition of 80% of acetonitrile and 20% of methanol for 10 min. This was followed by a linear gradient to 60% of acetonitrile and 40% of methanol at 17 min (hold, 17–40 min). The system was then returned to the initial condition from 40 to 42 min and the run was terminated at 50 min. A 2-position six-port Synergi™ fluid processor (Models AVO-6082, Phenomenex, Torrance, CA, USA) was used for column switching. Chromatograms were extracted as follows: 325 nm for all-*trans*-retinol; 440 nm for neoxanthin and violaxanthin; 450 nm for fucoxanthin, β-cryptoxanthin, lutein, zeaxanthin, α- and β-carotenes; 471 nm for capsanthin, capsorubin, lycopene and canthaxanthin; 432 nm for chlorophyll *a*; 464 nm for chlorophyll *b*; 281 nm for capsaicin and dihydrocapsaicin. Tocopherols and the tocotrienols were quantified by fluorimetric detection with excitation and emission wavelengths set at 296 nm and 330 nm, respectively. Data acquisition was performed with the Waters Empower™ software.

3. Results and discussion

3.1. Chromatography

The first phase of separation (0–8 min) was to elute the very polar compounds (capsaicin, dihydrocapsaicin, all-*trans*-retinol, IS1, neoxanthin, capsorubin and violaxanthin) with 80% of acetonitrile and 20% of methanol on both column-1 (Zobax C_{18}, 30 °C) and column-2 (Partisphere 5 C_{18}, 4 °C). During the second phase, the non-polar compounds (tocopherols, β-cryptoxanthin, chlorophylls and lycopene and carotenes) were separated from column-1 (30 °C) with a linear decrease of acetonitrile to 60% from 8 to 40 min. The less polar compounds (capsathin, tocotrienols, lutein, zeaxanthin and canthaxanthin) which were being retained on column-2 were then eluted with the initial mobile phase, from 42 to 50 min.

Fig. 1a and b show LC-UV traces using UV for a standard mixture and a common tropical plant, pandan leaf, respectively. Fig. 2a and b show the corresponding assays using fluorescence detection. The detailed detection conditions are

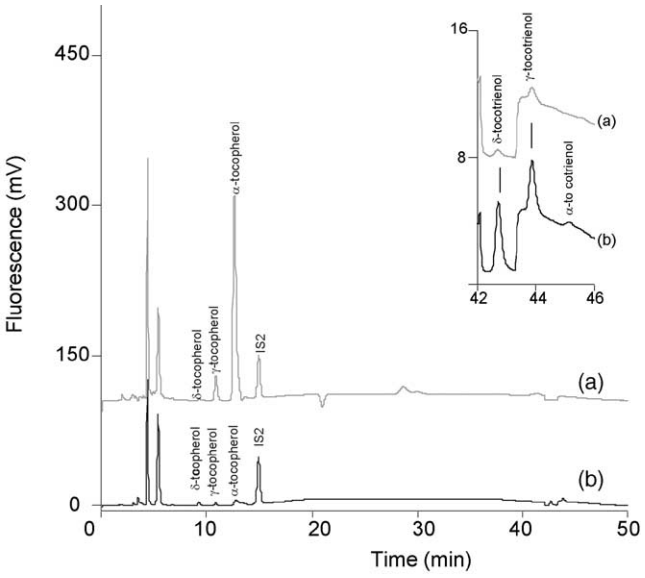

Fig. 2. Chromatograms obtained using fluorescence detection for tocopherol and tocotrienol (insert) analysis. (a) A mixed standards solution containing 1 μg/ml each of δ- and γ-forms of tocotrienol and tocopherols, 5 μg/ml of α-tocotrienol, 10 μg/ml of α-tocopherol and equal volume of IS solution (IS2, 400 μg/ml). (b) The same pandan leaf (*Pandanus amaryllifolius*) extract as in Fig. 1b.

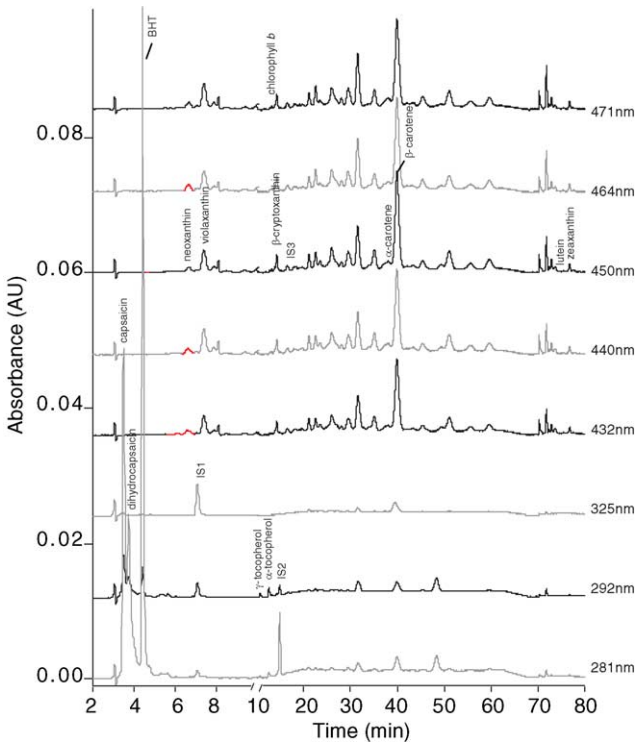

Fig. 3. Chromatograms obtained with 15.1 mg lyophilized Thai bird chili, showing 80 min run-time necessary due to presence of several unknown carotenoids in red *Capsicum annuum*. Capsaicin (3.4 min), dihydrocapsaicin (3.7 min), capsorubin (6.6 min) and capsanthin (71.6 min) were quantified as 1292, 626, 0.1 and 1 μg/g of fresh Thai bird chili, respectively.

Table 1
Analytical performance data

Compounds	Concentration (μg/g)		Recovery (%)
	Alfalfa	Added	
Capsaicin	<0.1	300	95
Dihydrocapsaicin	<0.2	300	85
All-*trans*-retinol	<0.2	8	95
Neoxanthin	1.5	6	95
Violaxanthin	4.2	8	95
Chlorophyll *b*	30.9	150	94
β-Cryptoxanthin	<0.1	15	86
Chlorophyll *a*	55.4	300	93
Lycopene	<0.1	38	90
α-Carotene	<0.1	15	90
β-Carotene	10.1	75	93
Lutein	24.9	150	84
Zeaxanthin	1.4	15	98
Canthaxanthin	0.4	15	88
δ-Tocopherol	0.03	3	89
γ-Tocopherol	0.25	3	103
α-Tocopherol	5.99	30	93
δ-Tocotrienol	0.05	3	90
γ-Tocotrienol	0.06	3	94
α-Tocotrienol	<0.02	15	88

included in Section 2.3. The retention times of all analytes were reproducible with coefficients of variation <0.7%.

3.2. Selectivity and sensitivity

As shown in Fig. 1, using the proposed gradient condition, chlorophyll *b* (λ_{max} 464 nm) was well separated from β-cryptoxanthin (450 nm), while chlorophyll *a* (λ_{max} 432 nm) was eluted before lycopene (471 nm). The peaks appearing at 15.2 min and 24.1 min had spectra identical to chlorophyll *b* and chlorophyll *a*, respectively. Fig. 3 shows that both capsaicin and dihydrocapsaicin (λ_{max} at 227 and 281 nm) are highly polar and were eluted before BHT. The peaks eluted at 6.6 and 71.6 min were identified as capsorubin (λ_{max} 478 nm) and capsanthin (λ_{max} at 472 nm), respectively. Although these non-polar carotenoid esters could be detected if the separation time is extended from 40 min to about 70 min, subsequent studies showed that these esters are rare, thus a chromatographic run-time of 50 min is generally sufficient for routine assays. The UV detection limits (signal/noise >3) for the various analytes are listed in Table 1. Based on the proposed extraction method, carotenoids can be detected down to 0.2 μg/g, capsaicin of about 1–2 μg/g and chlorophylls as low as 2–4 μg/g, with the use of 5 mg of lyophilized sample.

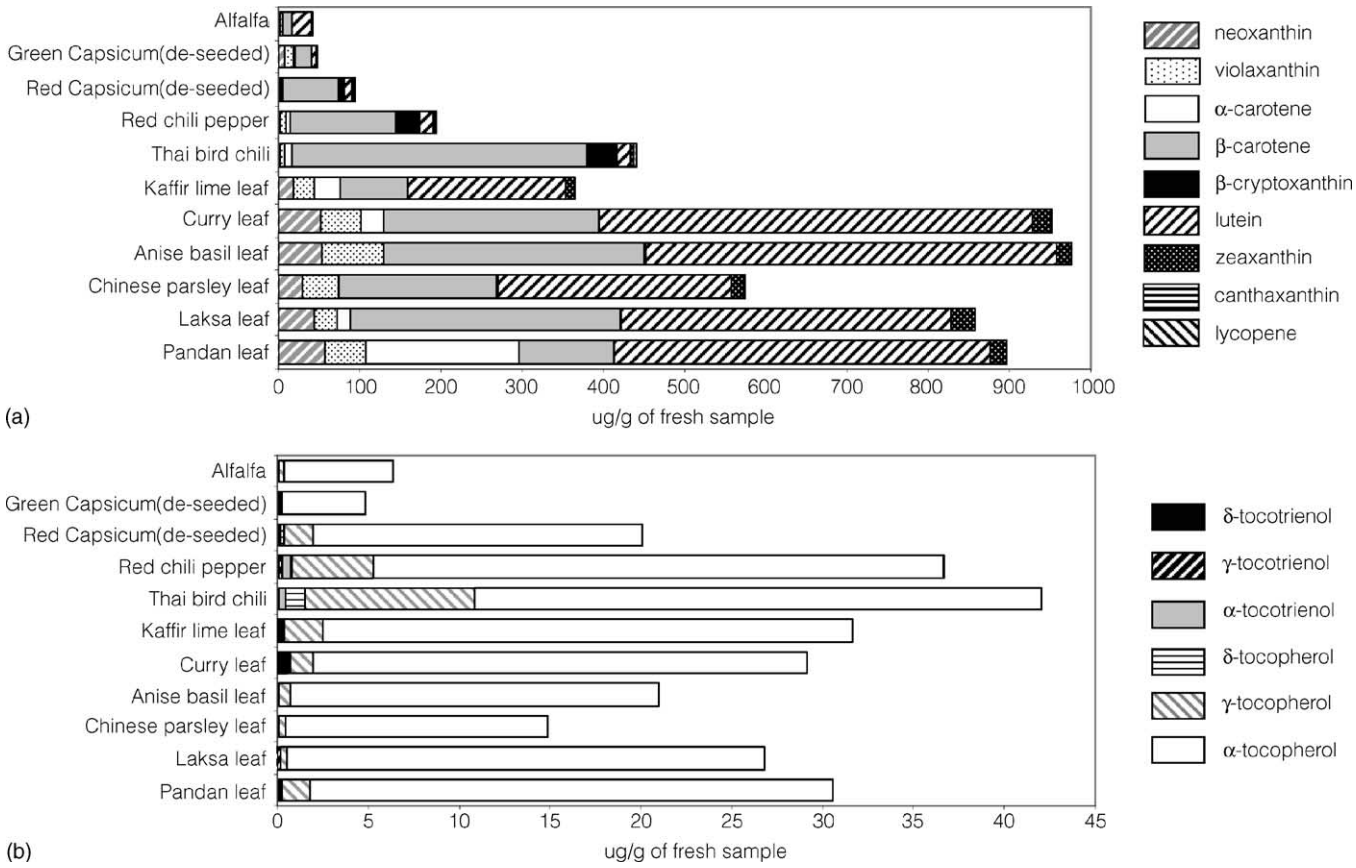

Fig. 4. (a) Various carotenoids and (b) vitamin E analogues concentrations (μg/g of fresh weight) quantified from 11 plant samples.

The detection sensitivity of fluorometric detection is known to be higher than that of UV spectrometry for tocopherols and tocotrienols [5]. With the proposed method, approximately 0.2 μg/g of α-tocopherol, 0.1 μg/g of α-tocotrienol, and as low as 0.04 μg/g of the other vitamin E analogues, could be quantified when using 5 mg of the lyophilized plant sample compared to over 100 mg in the case of UV detection (Table 1). Thus fluorometric detection is the method of choice for measuring these two groups of antioxidant.

3.3. Sample preparation and application

Light, heat and oxidative agents are known to cause degradation of antioxidants during sample preparation. Preparation using saponification is known to be tedious and time consuming and loss of carotenoids during saponification is of concern [6]. In the present study, a simple extraction procedure that avoided the saponification step was developed. The proposed procedure involved freeze-drying of the sample prior to grinding of dry samples into fine powder and subsequent solvent extraction. Recoveries of over 80% of could be achieved if acetonitrile was used as the first extraction solvent, followed by five extractions using *n*-hexane. This extraction procedure is time- and cost-effective, as a batch of 24 samples could be processed in 2 h. Using the proposed procedures, the recoveries of all compounds of interest were found to be highly reproducible: the within-day and between-day CVs were generally <12% ($n = 4$). The recoveries of spiked standards were generally >88%, as shown in Table 1.

The proposed method was used to analyse three Western and eight tropical plants. All the plants studied contain a small amount of all-*trans*-retinol. Interestingly, many tropical herbs were noted to contain substantial amounts of carotenoids such as neoxanthin (ranged, 1–58 μg/g), violaxanthin (7–76 μg/g), β-carotene (83–364 μg/g), lutein (16–533 μg/g) and zeaxanthin (3–29 μg/g). Among the samples analyzed, curry leaves (*Murraya koenigii*), anise basil leaves (*Ocimum basilicum*) and pandan leaves (*Pandanus amaryllifolius*) were found to contain about eight-fold more total carotenoids than alfalfa and bell capsicum. α-Tocopherol is the main component (>90%) of the vitamin E family in most of the plants studied (ranged, 5–31 μg/g of fresh plant) followed by γ-tocopherol (1–22%); and tocotrienols were generally present at trace levels (Fig. 4).

In summary, this paper shows that by using an LC column-switching technique and gradient elution, effective separation of tocopherols, tocotrienols, various carotenoids, capsaicins as well as chlorophylls can be performed using two monomeric C_{18}-based silica columns. This method was used to study several plants, and the overall findings suggest that the method is reliable and can be used for a variety of sample types.

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