Direct Determination of Water-Soluble Vitamins by Circular Dichroism

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Circular dichroism spectropolarimetric detection has been applied to the direct determination of vitamins B_2, B_{12}, and C in a variety of pharmaceutical products, frozen fruit juice concentrates, and fresh apples and green peppers. The assays were performed without preconcentration, derivatization, or chromatographic separation steps. Potential interferences are discussed. Results are excellent for B_2 and C but less reliable for B_{12}.

INTRODUCTION

Vitamin C (L-ascorbic acid), vitamin B_2 (riboflavin), and vitamin B_{12} (cyanocobalamin) are classified as water-soluble vitamins. They function as coenzyme or oxidation inhibiting agents in metabolic pathways. Their roles in biochemical processes make them essential nutrients, best obtained from foodstuffs, but a large number of commercial products are available as vitamin supplements. The proliferation of these products and the vitamin fortification of deficient foodstuffs have prompted the search for accurate and efficient methods for quality control. Present USP procedures (*U.S. Pharmacopeia*, 1990) rely on titrimetry (for ascorbic acid), fluorometry (for riboflavin), and spectrometry (for cyanocobalamin). In every case extensive sample separation and workup are required to eliminate the host of interferences commonly found in the complex matrices of pharmaceutical formulations and foodstuffs.

Spectrophotometric and polarographic methods have been described by Hashmi (1973). The preparation and determination of vitamin C samples have been summarized in a variety of resources (Pachla and Reynolds, 1985), and determinations of B_{12} by colorimetric, electrochemical, radioassay, microbiological, and enzymatic assay techniques have been reviewed by Kirschbaum (1981). Potentiometry, flow injection analysis, and a number of spectrophotometric techniques have all been used from time to time for the determination of ascorbic acid. Fluorescence detection (Barray et al., 1986) is still preferred for the determination of riboflavin. Radioassay and microbiological procedures for cyanocobalamin are rapidly being replaced with HPLC techniques (Hudson et al., 1984).

HPLC has become increasingly more important in determinations of vitamins, especially for the simultaneous determinations of the individual components of multivitamin mixtures (Wittmer and Haney, 1979; DeLeenheer et al., 1985). It is clear that a number of problems exist with methodology, e.g., incompatibility between columns and stabilizing compounds, coelution of compounds, and the need for dual detection systems to measure the widely disparate concentrations of vitamins in multiple mixtures. Quick and easy but very reliable methods are still a high priority in the search for vitamin assay procedures.

Although B_2, B_{12}, and C vitamins are optically active, few references exist in the literature in which chiroptical detection has been applied as an assay procedure. Polarimetric detection would require the complete separation of the vitamins from each other and from all other optically active compounds. It could only be used therefore as a detector for HPLC. As such it has no special advantage over absorption or refractive index detection. Circular dichroism (CD) spectra of cyanocobalamin and some structural analogues were characterized some 20 years ago (Firth et al., 1967) but never used for analyses. Each spectrum is entirely unique, and the analogues are readily distinguishable one from the other. A recent application does describe the use of a combined fluorescence–CD detector in the determination of riboflavin, but only laboratory samples were analyzed (Christensen and Yeung, 1989). We have had a number of recent successes where CD has been used to determine the principal chiroptical analytes in solutions of real pharmaceutical mixtures, e.g., the β-lactams (Purdie and Swallows, 1987) and cinchona alkaloids (Han and Purdie, 1986), and in extracts of organic materials such as marijuana (Han and Purdie, 1985), opium (Han and Purdie, 1986), and tobacco (Atkinson et al., 1984), penicillin broth (Purdie and Swallows, 1987), and *Rauwolfia serpentina* (Swallows and Purdie, 1989). Optical activity and absorption are both required for CD activity. This double requirement makes the method so selective that complex mixtures can be analyzed without chromatographic separations (Purdie and Swallows, 1989). Even enantiomeric distinction and optical purity determination are possible using CD detection. This has been successfully demonstrated for mixtures of D- and L-cocaine (Swallows, 1989).

In this work the CD spectra for the three vitamins in dilute aqueous solutions have been characterized and the data used for their analytical determination in commercial vitamin preparations. Vitamin C contents of some liquid and solid food samples were also measured. None of these determinations involved a chromatographic separation or prolonged workup.

EXPERIMENTAL PROCEDURES

Materials and Samples. The following compounds were purchased as standard reference materials for CD spectral characterization and were used without purification: L-ascorbic acid (vitamin C), L-dehydroascorbic acid, and L-ascorbic acid 6-palmitate (Aldrich Chemical Co.); riboflavin, cyanocobalamin, D-ascorbic acid, L-malic acid, (−)-quinic acid, and (−)-shikimic acid (Sigma Chemical Co.). Commercially distributed products in tablet form were purchased from a local pharmacy and consisted of Marquee Vitamin C (Fleming Co.), Nature Made Vitamin B_2 and B_{12} (Nature Made Nutritional Products), One-A-Day Maximum Formula multivitamin (Miles Laboratories, Inc.), Regal-Natal 1 mg + iron multivitamin (Regal Laboratories, Inc.), Thera-vites M multivitamin (Natural Wealth), and Nature Made Stress B-complex multivitamin (Nature Made). Minute

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Table I. Preparation of Samples for Data Collection

vitamin	product	wt/vol taken	dissolved in	diluted by/with (centrifugation)
C	Marquee	2–5 mg	50 mL of EDTA	1:5 with EDTA
B_2	Nature Made	entire tablet	100 mL of buffer (15 min)	1:5 with buffer
B_{12}	Nature Made	entire tablet	10 mL of buffer (reduced light)	1:5 with buffer (15 min)
C	One-A-Day	2–5 mg	50 mL of EDTA (15 min)	1:5 with EDTA
	Thera-vites (outer coat removed)	2–5 mg (from 3 tablets)	50 mL of EDTA (15 min)	1:5 with EDTA
	Regal-Natal	2–5 mg (1 tablet)	50 mL of EDTA (15 min)	1:5 with EDTA
B's and C	Stress B	200 mg	25 mL of buffer	1:5 with buffer for B; 1:1000 with EDTA for C
C	juice products	0.5–2 mL	10 mL of EDTA	1:10 with EDTA (15 min for orange)
	Kool-Aid	50 mg	25 mL of EDTA	1:10 with EDTA
	fresh fruit (pureed)	slice	25 mL of EDTA (15 min)	1:10 with EDTA

Maid orange juice concentrate, Welch's cranberry juice concentrate, TV grapefruit and apple juice concentrates, Gerber apple juice prepared for pregnant women, unsweetened cherry Kool-Aid drink mix, and whole green peppers and apples were obtained from local groceries.

Solutions. All solutions that contained vitamin C and/or its analogues or possible interferences were prepared using a solution of 5.5×10^{-5} M EDTA (Aldrich) dissolved in a pH 5.4 buffer. The presence of EDTA retards the autoxidation of ascorbic acid to dehydroascorbic acid (Lau et al., 1986) long enough to gather calibration and analysis data. The CD signal quality varied with pH and was optimum in signal to noise ratio at pH 5.4. Solutions of riboflavin and cyanocobalamin standards and samples were prepared using a pH 4.8 buffer, which was selected because cyanocobalamin is most stable at that acidity. Solutions of B_{12} were handled in subdued lighting.

Tablets of the pharmaceutical products were powdered and homogenized in an agate mortar. Samples were either whole individual tablets or pooled quantities from as many as three tablets. The actual weights taken and the dilutions that were made to have the concentrations fall within the linear response range of the CD instrument varied from sample to sample and were dependent upon the amounts of vitamins present in the original sample. These preparations are described in Table I. For some of these the weights required were only 2–5 mg taken from samples that may have weighed almost a gram. In other instances entire tablets were extracted. The different sampling procedures are reflected in the relative imprecisions of the analyses. The apple and green pepper slices that were taken weighed around 20–50 g. Each was added to 20–30 mL of the EDTA solutions and pureed in a blender. The puree was centrifuged at low speed for approximately 15 min. A known aliquot of the supernate was taken and diluted to the mark in a volumetric flask with the EDTA solution (Table I).

Instrumentation. CD is most easily understood if it is thought of as a modified form of absorbance spectrophotometry in which the *differences* in absorbances between two coincident circularly polarized beams of light that pass simultaneously through the sample are measured as a function of wavelength. The Beer Lambert law is obeyed and analytical determinations are done in precisely the same way as they are for absorbance detection. The experimentally measured quantity is the ellipticity ψ, usually expressed in units of millidegrees, and the CD equivalent to the molar absorbance is the molar ellipticity Θ_M [which has units of deg/(M cm)]. CD spectra quite typically show both positive and negative "absorption bands".

CD spectra were measured using a Jasco 500A automatic recording spectropolarimeter (manufactured by the Japan Scientific Co. Inc., Easton, MD) with an IF-500 accessory interfacing the instrument to a personal computer for data collection and manipulation. Sensitivity, scan rates, and repeat functions were selected that gave the optimum signal to noise ratios for each of the three vitamins and the possible interferences. Calibration of the sensitivity scale was done daily using a 0.5% androsterone solution in dioxane as recommended by the manufacturer. Because of its carcinogenic properties, dioxane should be handled with the appropriate care. Blank spectra were obtained using the appropriate buffer solutions. Wavelength ranges were selected such that a significant lead in was measured prior to the first CD band to establish the instrument baseline. Calibration curves were prepared for solutions of all of the standards. A solution path length of 1 cm was used for all measurements.

RESULTS AND DISCUSSION

CD spectra for the three vitamins in aqueous buffers are shown in Figure 1. Spectra are not shown for the other standard reference materials, but the molar ellipticity values Θ_M and wavelengths of maximum CD signals are included with the data for the three vitamins in Table II. The positive and negative values indicate that CD bands can be of opposite polarity, a phenomenon that is useful for analytical discriminations.

At the dilutions described in Table I, neither of the B vitamins has a measurable signal at wavelengths less than 300 nm, so neither is an interference in the determination of vitamin C in multiple vitamin preparations. Vitamin C is never an interference in the determination of either B vitamin. Some spectral overlap does exist for the B vitamins in the 330–370-nm range which might complicate the determination of both in a B mixture. B_{12} can, however, be determined with equal or better precision at 430 nm, and concentrations calculated from these data could be used to correct the overlap at 342 nm, the maximum for B_2. In CD spectra of mixtures, the experimental ellipticities are additive. From the Beers law equation the contribution from each component in a 1 cm path length cell is given by the product of the molar ellipticity times the molar concentration. The very low value for the molar ellipticity for B_2 compared to that for B_{12} is more than offset by the very large differences in their molar concentrations in typical B vitamins, so much so that B_2 can be determined at 342 nm without interference from B_{12}.

For the purposes of evaluating the quality of the results from the CD analyses the figures have been compared with labeled values for tablet forms and literature values for food products (Souci et al., 1981). While there is good reason to question these comparisons, there is some justification in that in many instances the *U.S Pharmacopeia* (1990) has stated that, when describing validated methods for assaying tablets, the actual contents typically differ from the labeled contents by only as little as ±10%. If the CD results are found to lie within this rather broad range, then there is good reason to believe in the validity of the method. The experimental emphasis in this work was on CD detection with no sample separation. It was not our intention to offer procedures that are already completely developed for routine applications but only to demonstrate that there is an excellent basis for further study and further development. This development would require more stringent comparisons to be made with the titrimeteric and chromatographic methods that are already

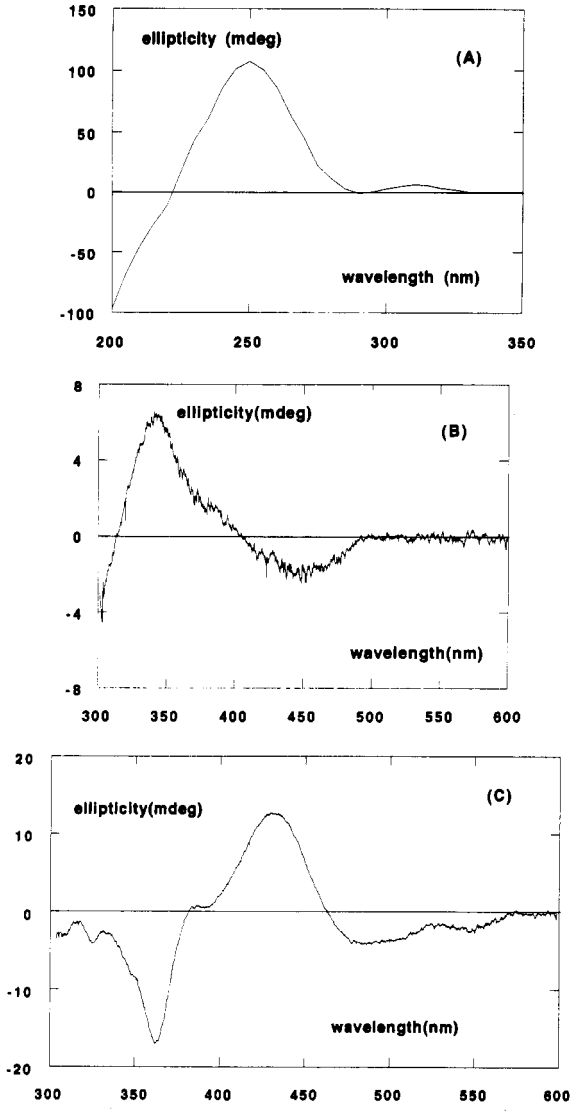

Figure 1. CD spectra of (A) 1.98×10^{-4} M L-ascorbic acid in EDTA/pH 5.4 aqueous buffer, (B) 1.76×10^{-4} M riboflavin in pH 4.8 aqueous buffer, and (C) 2.74×10^{-5} M cyanocobalamin in pH 4.8 aqueous buffer.

Table II. CD Spectral Data for the Vitamins and Other Reference Standards

compound	solvent	θ_M, deg/(M cm)	wavelength maxima, nm
ascorbic acid	EDTA/5.4	−3	285
		+105	251
riboflavin	4.8 buffer	−11	440
		+32	342
cyanocobalamin	4.8 buffer	−117	500
		+437	430
		−587	363
L-dehydroascorbic	EDTA/5.4	+69	232
D-isoascorbic acid	EDTA/5.4	+134	241
		−99	203
L-malic acid	EDTA/5.4	+71	207
(−)-quinic acid	EDTA/5.4	+15	203
(−)-shikimic acid	EDTA/5.4	−301	206

approved by the industry. The results provided here seem to substantiate the feasibility of using CD detection for the routine analysis of water-soluble vitamins.

Vitamin C. Problems associated with vitamin C determinations have been described in earlier work (Cooke and Moxon, 1982). In solution the oxidation products are L-dehydroascorbic and ultimately 2,3-diketogulonic acid. The oxidation is catalyzed by metal ions (Weissberger and LuValle, 1944). The rate of the reaction is such that oxidation either has to be complete or has to be retarded to measure the CD spectra. There is no advantage to choosing to measure the L-dehydroascorbic acid spectrum because it is less intense and is moved to shorter wavelengths where interferences from other related compounds may be more serious. Instead, EDTA was added to retard the oxidation process.

Average values and imprecisions in the results for the calculated contents that are reported in Table III, column four, are based upon the total data compiled for each complete set of samples listed in column three. Distinctions have not been made between units that are separate samples vs pooled samples. Imprecisions that are relatively high are probably indicative of the lack of homogeneity among tablets. Results for calculations of vitamin C for 12 independent samples taken from three different tablets of the Marquee product show good precision and are in excellent agreement with the labeled content. However, for measurements on One-A-Day, Thera-vites M, and Regal-Natal multivitamins, the measured values are not within an anticipated ±10% limit of the labeled amounts. Part of the reason may well be related to the nonhomogeneity of the units and the fact that very small weighed quantities (2–5 mg) were taken. For samples where three tablets were combined, i.e., for One-A-Day and Thera-vites M, the imprecisions improved to ±5.1 and ±8.0%, respectively. There is also, however, an apparent trend toward a greater percent imprecision in the measured amounts of vitamin C as the iron(II) content increases from 18 to 27 to 65 mg, respectively, for the three products. Increasing the EDTA concentration by a factor of 20 did not improve the results. Improvements might be accomplished by using other complexing agents or by precipitating the metal. If iron is not present in a multivitamin mixture, i.e., the Nature Made Stress tablets, both the very good correspondence between measured and labeled quantities and the excellent precision are restored.

Vitamin C is abundant in fruits, fruit juices, and vegetables. Aqueous extracts of these contain a host of substances, some of which are potential interferences to CD detection, for example, L-dehydroascorbic acid and D-isoascorbic acid. The latter is commonly used as an inexpensive antioxidant, as is ascorbic acid 6-palmitate. The palmitate has no CD spectrum. Three others that we considered are L-malic, (−)-quinic, and (−)-shikimic acids. These five compounds we have presumed to be representative of all water-soluble interferences. All five have spectra that maximize at shorter wavelengths (Table II) and should not present a problem unless the quantity is very large relative to vitamin C. No distortion of the vitamin C spectrum by the matrix was observed for extracts of either unsweetened cherry Kool-Aid or TV grapefruit frozen concentrate. Determinations were simple and straightforward. N, the number of samples measured, and the results of the assays are given in Table III.

CD bands for the five acid analogues are broad, and if concentrations are high, there could be significant intensity at longer wavelengths away from the maximum. This is illustrated for Gerber's apple juice in Figure 2. Similar spectra are observed for TV apple juice, Welch's cranberry juice, and aqueous extracts of fresh apples. Rather than consider all possibilities for the band that maximizes at 210 nm, we treated it as if it were due entirely to L-malic acid. A simple curve-fitting program was used to quan-

Table III. Assays of Vitamins in Pharmaceutical and Fruit Products

sample (vitamin)	labeled content, mg/unit	sample data	calcd content mg/unit	calcd content % of tablet[a]
Nature Made (B_2)	25	6 separate tablets (N = 4–6)	23.7 ± 1.7	4.8 ± 0.32
Nature made (B_{12})	0.25	4 separate tablets (N = 2)	0.36 ± 0.02[b]	0.07 ± 0.003
	0.25	10 separate tablets (N = 2)	0.36 ± 0.02[c]	0.07 ± 0.002
Marquee (C)	250	3 separate tablets (N = 4)	243 ± 8	72.2 ± 3
One-A-Day (C) (multivitamin + Fe)	60	3 separate tablets (N = 4–6) 3 combined tablets (N = 5)	63.9 ● 9.5	5.5 ± 0.8
Thera-vites M (C) (multivitamin + Fe)	120	3 combined tablets (N = 3–5) 6 separate tablets (N = 3–5)	108.4 ± 12	13.7 ± 1.3
Regal-Natal (C) (multivitamin + Fe)	90	3 separate tablets (N = 3–5)	79.7 ± 18	4.9 ± 1.2
Nature Made Stress				
(B_2)	15	6 separate tablets (N = 2)	16.8 ± 1.5	1.5 ± 0.08
(B_{12})	0.12	6 separate tablets (N = 2)	0	
(C)	600	6 separate tablets (N = 2)	591 ± 20	52.7 ± 1.7
Kool-Aid (C)	48	entire package (N = 10)	42.3 ± 3.0	
TV grapefruit (C)	NA	entire package (N = 5)	273.9 ± 11.4	
TV apple juice (C)	120	entire package (N = 5)	72	
Minute Maid (C) orange juice	288	entire package (N = 5)	272.5 ± 3.5	
Welch's (C) cranberry juice	NA	entire package (N = 7)	404.6 ± 4.3	
Gerber apple juice (C) (pregnant women)	96	entire package (N = 8)	100.4 ± 3.6	
apple slice (C)	NA	30–50-g slices (peeled) (N = 5)	8.9 ± 0.5 mg/slice (0.02% by weight)	
green pepper (C)	NA	30–60-g slices (2 peppers/4 slices)	17.4 ± 0.5 mg/slice (0.14% by weight)	

[a] Used to normalize among tablets of different mass. [b,c] Measurements 6 months apart.

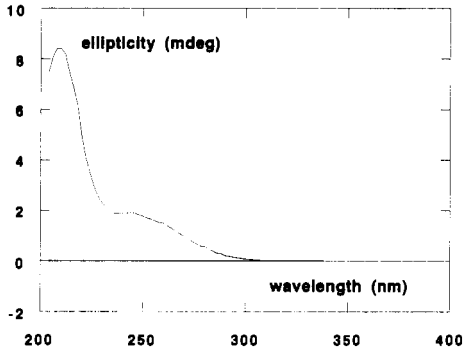

Figure 2. CD spectrum of the Gerber apple juice for pregnant women diluted with EDTA/pH 5.4 buffer. Band with 210-nm maximum is attributed to L-malic acid.

titate both analytes. The ratio of malic to ascorbic acid was calculated to be 98:2 for TV apple juice and 91:9 for Gerber apple juice and the fresh apple extract. These ratios are in good agreement with that obtained using the average values of 506 and 11 mg/100 g quoted by (Souci et al., 1981) for malic acid and vitamin C in apples. The spectrum for cranberry juice was somewhat noisier due to the presence of natural pigments in the extract, yet it was still possible to determine both analytes. The ratio found was 85:15. Results for the percent by mass of vitamin C in slices of fresh apples and fresh green peppers (Table III) are in excellent agreement with typical literature values, i.e., 0.012% for apples and 0.04% for green peppers (Souci et al., 1981).

The CD spectra of EDTA solutions of Minute Maid orange juice showed an additional positive band with a maximum at 340 nm and another, relatively narrow, negative band with a 235-nm maximum. These are entirely new bands and their sources have not been identified. Using data measured at 251 nm, the vitamin C was determined as before. The long-wavelength band is out of range to interfere with the assay at 251 nm, and from the correspondence between the measured and labeled values (Table III), there appears to be little interference from the negative band with the 235-nm maximum.

B Vitamins. Riboflavin is usually determined using fluorescence detection. This seemingly would be required only when it is necessary to reach very low limits of detection. In quality control applications the amounts of B_2 are quite high and alternative detectors which have fewer experimental limitations than fluorescence could be employed. The CD spectrum of B_2 is relatively weak, which is to be expected since the chiral center, the saccharide moiety, and the chromophore, the aromatic system, are separated by a saturated carbon atom. Close juxtaposition of these functional groups is essential for strong chiroptical response. Nevertheless, the CD spectrum is of sufficient intensity to allow for the direct determination of B_2 in aqueous extracts of commercial products. Results obtained for the determination of B_2 in the single-vitamin Nature Made product (four to six samples from each of six different tablets) and in the multivitamin Nature Made Stress tablets (two samples from each of six separate tablets) are within our adopted limits of ±10% of the labeled amounts (Table III) and would therefore be considered to be satisfactory. Imprecisions in the figures are again perhaps indicative of nonhomogeneity of the separate units.

The poorest correspondence between the measured and labeled content for any of the determinations was obtained for B_{12}. As a recommended precaution, extractions and sample preparations were carried out in subdued lighting

to reduce photodecomposition. There was no evidence for any distortion of the CD spectrum of the standard material by the matrix, yet the figures obtained for the assays are very high for the Nature Made single-vitamin product and zero for the multivitamin Nature Made Stress tablets. The fact that the spectrum of the extract matches exactly the spectrum of the standard eliminates from consideration the following analogues: dicyanocobinamide, vinylcyanocobinamide, aquo-, hydroxo-, ethyl-, and selenocyanatocobalamin, all of which were characterized by Firth (Firth et al., 1967). Assays on the single-vitamin product were repeated after 6 months, and the same result was obtained. As a rough check of the validity of the CD result, the absorption spectra for the standard and for the extract of the single-vitamin tablet were compared. There was some evidence for the presence of other absorbers in the extract but not at every absorption maximum. The figure obtained using absorbance data at points where the two spectra corresponded was 0.40 mg, i.e., also considerably higher than the labeled value. One possible source of error that could account for anywhere from 7 to 36% of the difference between the measured and labeled figures is the extent of water of crystallization. One mole of recrystallized B_{12} could contain as much as 22 mol of water (Kirschbaum, 1981). The actual amount present depends upon the recrystallization conditions. These are not known for either the standard or the sample. At present we have no explanation for the assay figure of zero for B_{12} in the multivitamin sample other than its shelf life was reduced by some catalytic factor.

SUMMARY

The results of these studies are further confirmation that CD has considerable potential for applications in quality assurance testing of pharmaceuticals and food products. Assays can be done routinely by a simple spectral measurement after a direct extraction. Sample preconcentration, derivatization, or chromatographic separation steps, all of which serve to substantially reduce the sample turnaround time, are not part of the experimental procedure. An even greater reduction is accomplished if the qualitative identity of the sample is well-known; in these instances flow systems could be arranged that have single-wavelength CD detection. The direct analysis of chiroptical species in whole food products is particularly interesting, and numerous other applications can be conceived. The CD spectrum for the C-glycoside riboflavin, weak though it may be, might be an indication that other glycosides, such as the nucleotides and saponins (for which we have preliminary information), and flavones can also be determined directly. Some monosaccharides such as fructose and sorbose have already been characterized (Hayward and Angyal, 1977) and are easily amenable to quantitation.

One legitimate and critical concern about the conduct of this study is that the analytical results were compared with labeled contents and not compared directly with data for similar matrices using the accepted methodologies, e.g., HPLC. We consider ourselves experts in the use of CD as an analytical detector and have neither the expertise nor the HPLC equipment at our disposal with which to make these comparisons. The point was made that the purpose of the study was to demonstrate the feasibility of the CD detection method and not to provide completely developed procedures for routine analysis. This purpose has been fulfilled.

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Registry No. Vitamin C, 50-81-7; vitamin B_{12}, 68-19-9; riboflavin, 83-88-5.