"Our investigation...included comparisons of UV detection versus fluorescence, normal phase chromatography versus reverse phase..."

Vitamin A includes retinol as well as pro-vitamin A beta carotene. Beta carotene has one sixth of the vitamin activity as an equivalent retinol. The chemical structure of retinol allows for the possibility of sixteen cis-trans isomers in addition to the all-trans form. Only three of the cis-isomers are free from stearic hindrance and occur most commonly in nature, the most common being 13-cis-retinol. Beta carotene occurs predominantly in the all-trans form, but also has many cis-isomers. Although it has not been clearly defined which forms of retinol and beta carotene are to be measured as vitamin A, in our lab we include all trans- and 13-cis-retinol as well as all beta carotene in a vitamin A determination. The following article discusses HPLC methodology used in our lab to measure vitamin A.

Methodology

Our investigation of an HPLC method to measure retinol included comparisons of UV detection versus fluorescence, normal phase chromatography versus reverse phase, as well as sample preparation considerations. Optimum detector options and sample preparation schemes related directly to the chromatographic conditions employed.

In our studies, samples and an all-trans-retinol standard were saponified overnight at room temperature in 10% ethanolic potassium hydroxide that contained 2% pyrogallol as an antioxidant. Following saponification, the solution was diluted 1:1 with water and a 5.0 mL aliquot removed. Retinol was extracted from the 5.0 mL aliquot with 2 x 5.0 mL hexane, and the extracts were pooled.

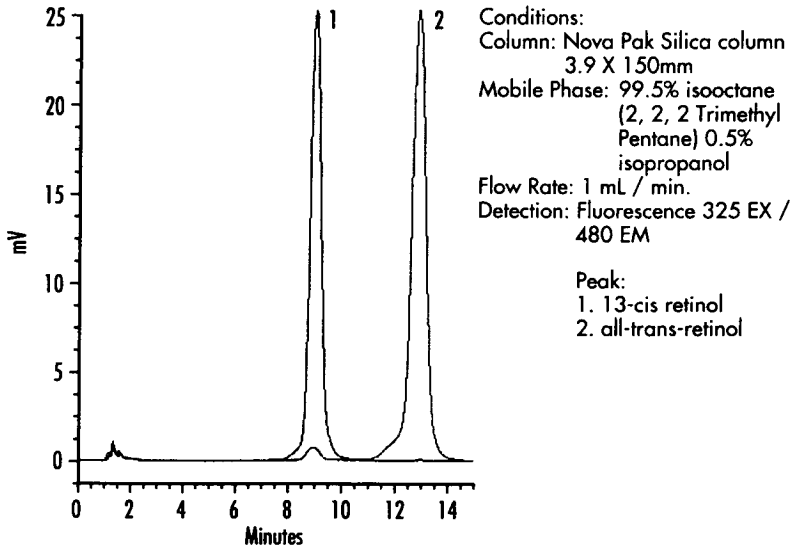

Figure 1. Normal phase chromatography on a Nova-Pak silica column fully resolves 13-cis-retinol from all-trans-retinol, enabling accurate quantitation of each.

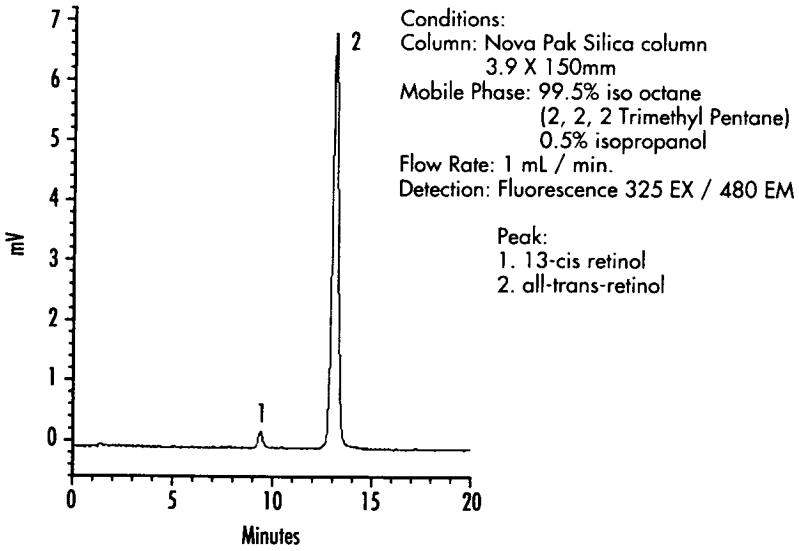

***Figure 2.** Normal phase chromatography using fluorescence detection at 325nm excitation / 480nm emission produces the necessary sensitivity and selectivity to quantitate all-trans-retinol and 13-cis-retinol in RTE cereal.*

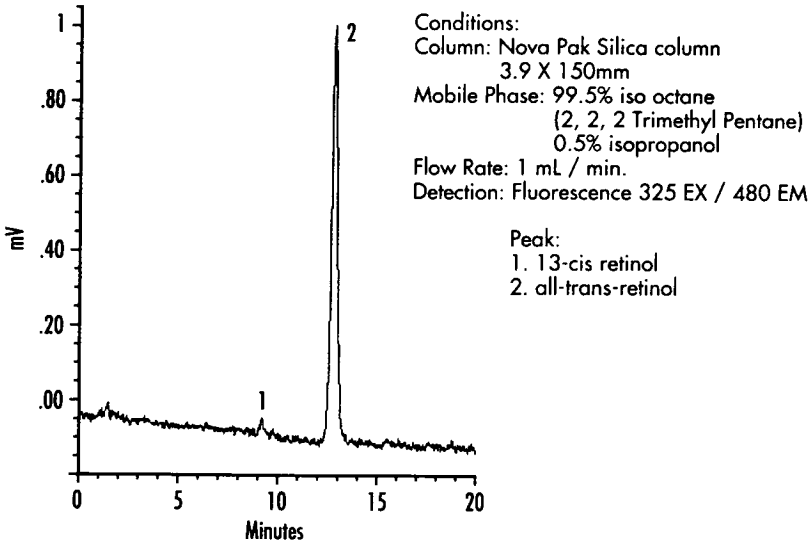

***Figure 3.** Normal phase chromatography using fluorescence detection at 325nm excitation / 480nm emission produces the necessary sensitivity and selectivity to quantitate all-trans-retinol and 13-cis-retinol in whole milk.*

Discussion and Results

Normal phase chromatography on a Nova-Pak® silica column fully resolves 13-cis-retinol from all-trans-retinol, enabling accurate quantitation of each (Figure 1). A hexane or hexane/ether extract of a saponified sample is readily miscible with the hydrophobic mobile phase allowing direct injection. Another advantage to the miscibility between sample diluent and mobile phase is that larger injection volumes can be administered, putting more mass of analyte onto the column which allows quantitation at lower levels. It was found that in normal phase eluent conditions using isooctane and isopropanol, fluorescence detection at 325nm excitation/480nm emission gave nearly 30% more signal-to-noise response than UV absorbance at 325nm. This added sensitivity along with the advantage of greater specificity makes fluorescence the detector of choice for retinol under these normal phase conditions. Figures 2 and 3 are chromatograms of retinol in a RTE cereal and whole milk using the conditions described, demonstrating the required sensitivity and selectivity to quantitate all-trans-retinol as well as 13-cis-retinol.

HPLC in a reverse phase mode has also been successfully used to measure retinol. Many chromatographers are more comfortable running reverse phase conditions as they are

commonly perceived to be more rugged. Relative detector response in the methanol/ water reverse phase eluent is opposite of what is observed in the iso-octane/ isopropanol normal phase eluent. UV absorbance of retinol at 325nm has nearly double the signal-to-noise response as fluorescence at 325nm excitation/ 480nm emission under reverse phase conditions. In our studies, we compromised the greater specificity of fluorescence to gain the sensitivity of UV absorbance. A major difference in the selectivity of a reverse phase system using a Nova-Pak C_{18} column is that 13-cis-retinol does not resolve from

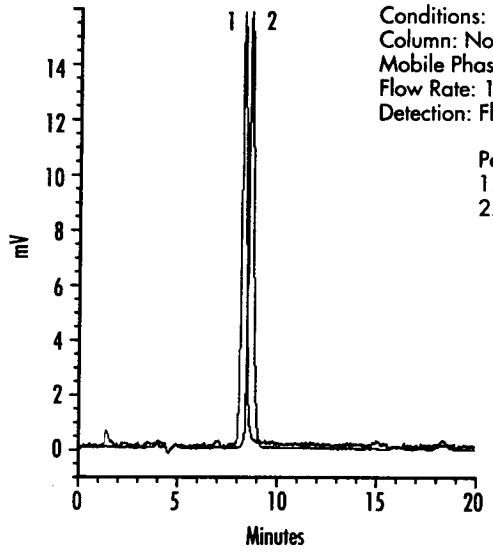

***Figure 4.** Reverse phase chromatography does not resolve 13-cis-retinol from all-trans-retinol.*

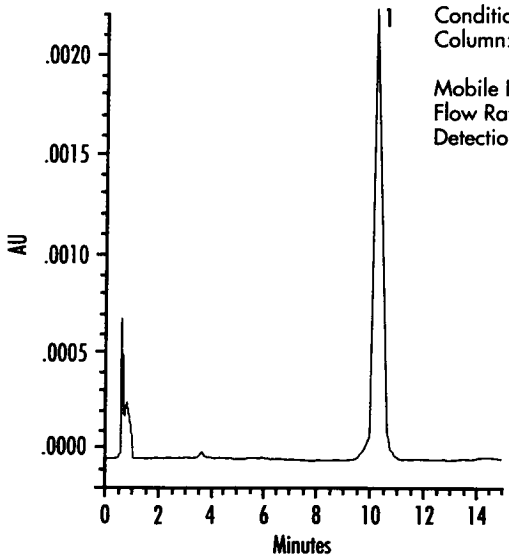

***Figure 5.** Reverse phase chromatography using UV/Vis detection at 325nm quantitates the two isomers as a single peak.*

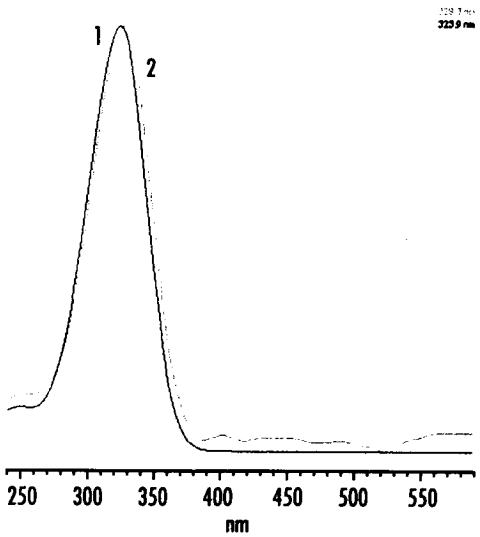

***Figure 6.** The Waters 996 Photodiode Array Detector can distinguish between 13-cis and all-trans-retinol even though there are only minor differences in their absorbance spectra.*

The LC Module 1 and Millennium 2010 Chromatography Manager provide an integrated, reliable and flexible system for routine vitamin A analysis.

all-trans-retinol (Figure 4). This was initially perceived as a disadvantage because the two isomers cannot be quantitated separately. However, 13-cis-retinol absorbs 92% as much UV light at 325nm as all-trans-retinol and usually occurs as a relatively minor component. Also, we assume that 13-cis-retinol will be quantitated as having the equivalent vitamin A activity as all-trans-retinol.

For these reasons our lab is confident in quantitating the two isomers as a single peak (Figure 5).

When new or unknown products are analyzed, it is often valuable to be able to identify individual isomers. The Waters 996 Photodiode Array Detector can distinguish between 13-cis and all-trans-retinol by the minor differences in their absorbance spectra. The detector has a true optical resolution of 1.2nm, enabling accurate identification of either isomer even though their spectral maximas differ by only 3nm (Figure 6).

An advantage of employing reverse phase chromatography to determine retinol is that beta carotene can also be analyzed on the same Nova-Pak C_{18} column, with a different sample extraction procedure and mobile phase. Conditions for beta carotene, including a different mobile phase and UV/Vis wavelength (450nm), can be switched to automatically allowing unattended, sequential analysis of both of the required analytes for vitamin A labeling. Waters Millennium® Chromatography Manager software enables easy importation of values for retinol and beta carotene into a spreadsheet, such as Excel®, where the necessary calculations can be performed to report results in retinol equivalents for a total vitamin A measurement.