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# Supercritical fluid extraction and reversed-phase liquid chromatography methods for vitamin A and β-carotene Heterogeneous distribution of vitamin A in the liver

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**Abstract**

We developed supercritical fluid procedures for extracting vitamin A and β-carotene from vitamin supplements and calf liver tissue. The SF extracts could be injected onto an HPLC column without further pretreatment. Samples were analysed by RP–HPLC using diode array detection or by spectrophotometry. Recoveries were very good. SF extracts from a vitamin preparation of uniform composition had an R.S.D. of 4%. Extracts from calf liver supplements were predictably more heterogeneous. The SF extraction method is less labor intensive then traditional liquid–liquid procedures for extracting vitamin A and carotenoids from tissues.

*Keywords:* Liver; Sample preparation; Vitamins; Carotenes

## 1. Introduction

Vitamin A is a fat soluble vitamin that is essential for normal human development, growth, hormonal functions and eyesight [1]. Vitamin A is an umbrella description for retinol and the retinyl esters. Approximately 95% of vitamin A is stored in the liver. Almost all liver vitamin A is in the form of the retinyl palmitate ester, with only a few percent as retinol [1]. Vitamin A in blood is usually retinol, with a few percent as retinyl palmitate and smaller percentages as other esters. Measurements of vitamin A in blood and other body fluids that are simpler to collect are of little value in most individuals, because concentrations of vitamin A in blood and body fluids respond to tissue reserves only under conditions of extreme deficiency or toxicity [1]. Individual liver concentrations of vitamin A can vary widely (from almost 0 to over 1000 mg/kg) without engendering symptoms or serum concentration changes warning of risk for toxicity or deficiency.

Liver samples are difficult to obtain, so many less invasive tests have been invented in an attempt to estimate vitamin A status before the damage of toxicity or deficiency has occurred [2–4]. None of these tests have been proven to be completely satisfactory over the full range of vitamin A status, so new tests are still being invented and compared to vitamin A estimates in liver biopsies. Thus, measuring vitamin A in liver is of scientific and public health interest.

Carotenoids, such as β-carotene, are fat soluble nutrients that appear to be useful in the antioxidant defence system and for prevention of degenerative diseases such as cancer and cataract formation [5]. In addition, β-carotene is a precursor for vitamin A in

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the human body, and serves as the main source of vitamin A for most of the world's population [1,5]. Thus, it is desirable to measure concentrations of β-carotene in vitamin supplements and foods.

Unfortunately, it has been difficult to measure tissue concentrations of vitamin A. The difficulty lies not so much in the analysis itself, as in the sample preparation required before analysis. Vitamin A (and the carotenoids) are typically analysed by reversed-phase HPLC. Many methods have been described for measuring these analytes, but most are similar to each other. Most methods described use RP-HPLC on $C_{18}$ columns; combinations of standard solvents such as methanol, acetonitrile, water and tetrahydrofuran; and UV detection [6–8]. Most of these methods provide good separation and measurement of fat soluble vitamins and carotenoids in 20 to 40 min. However, solid materials (tissues, foods and vitamin supplements) require extensive preparation before they can be analysed by HPLC. Traditional methods use homogenization, exhaustive grinding with a solid matrix, multiple liquid–liquid extractions, filtration, evaporation, then resuspension in buffer [9,10]. Typically these extraction methods require at least 30 min of labor per sample. Recently, manual labor has been decreased by the use of proteases and other digestive enzymes, but these steps increase the cost and total time spent per sample [11].

We have developed new supercritical fluid extraction (SFE) methods for vitamin A from calf liver tissue and β-carotene from vitamin supplements. Both methods use $CO_2$ without a modifier, so there are no waste disposal issues. Supercritical $CO_2$ is less viscous than liquid, so less grinding and homogenization of the samples are needed.

Other studies, using liquid–liquid extraction [12,13] or visualization with gold chloride [14], have reported that vitamin A is distributed heterogeneously throughout the liver. This is a problem, because vitamin A concentrations in liver biopsies serve as the standard to which all new non-invasive tests of vitamin A status are compared. If vitamin A is distributed heterogeneously in the liver, then the liver biopsy sample itself could be a poor estimate of vitamin A status, resulting in artificially bad results for the non-invasive tests. Could heterogeneity in the liver be so extreme that a good quality liver sample would result in a false diagnosis of vitamin A toxicity or deficiency? At the other extreme, could these reports of the heterogeneity of vitamin A in the liver be essentially an artifact of labor intensive, time consuming extraction or visualization techniques? We used these new SFE methods to estimate the uniformity of distribution of vitamin A in liver. We compared these results to the uniformity of distribution of β-carotene from a commercial vitamin supplement. We hypothesized that our results would show that vitamin A concentrations in liver samples were indeed heterogeneous, but that this heterogeneity would not be so great as to actually effect most diagnoses of vitamin A toxicity or deficiency.

## 2. Experimental

### 2.1. Chemicals

All solvents used were SFC, HPLC or reagent grade. Hydromatrix was purchased from Varian (Harbor City, CA, USA). All gases (nitrogen, analytical and SFC grade $CO_2$ gases with dip tubes) were supplied from Altair (San Leandro, CA, USA). Retinyl palmitate, retinol, and β-carotene standards were from Sigma (St. Louis, MO, USA). Dry carotene beadlets were a gift of Hoffmann-La Roche (Nutley, NJ, USA). Two lots of beadlets were used: most commonly lot 0093009 from 1995, but also lot 011605 from 1992. The dry carotene beadlets had a water-dispersable matrix of gelatin, sucrose, food starch, and peanut oil; with ascorbyl palmitate and DL-α-tocopherol as antioxidants. They contained 10% (w/w) β-carotene. Calf liver was purchased from local supermarkets.

### 2.2. Apparatus

SFE was with a Prepmaster coupled to an Accutrap (Suprex, Pittsburgh, PA, USA). HPLC was with a gradient system coupled to an autosampler and diode array detector (System Gold version 8.1, Beckman, Fullerton, CA, USA). Spectrophotometery was done with a Spectronic Genesys 5 (Milton Roy, Rochester, NY, USA).

### 2.3. Sample preparation

β-Carotene beadlets were stored at room temperature (20°C) under gold lights before analysis. Calf liver was purchased, wrapped in paper, then carried approximately 4 blocks to the laboratory. After arrival, liver samples were frozen at −70°C. Liver samples were thawed at room temperature under gold lights before use.

One calf liver was divided into eight equal portions of approximately 10 g each. Each portion was homogenized for 3 min, then blended. Each individual portion was then divided into two equivalent portions, placed in 15 ml centrifuges and quick frozen to −70°C. One portion, chosen randomly, was transported by car to the Western Regional Research Center in Albany, CA, USA for analysis and will be reported in a separate publication. The other portion was analysed for vitamin A at the Western Human Nutrition Research Center.

### 2.4. Liquid–liquid extraction

Aliquots of dry carotene beadlets (0.15 mg) were weighed, then ground exhaustively (approximately 10 min) in a mortar and pestle. Hydromatrix (0.75 g) was mixed with the beadlets, then 5 ml methanol–chloroform (4:1) was added. The sample was mixed by vortexing for 1 min, centrifuged for 20 min at 2500 *g*, then the methanol–chloroform layer was transferred to a 50-ml centrifuge tube. This extraction was repeated twice. The combined extract was filtered through a paper filter, then evaporated under a stream of nitrogen to a volume of approximately 2 ml. The sample was then transferred into an amber glass vial with a Pasteur pipet and evaporated to dryness under nitrogen. The pellet left was dissolved in 200 μl HPLC solvent.

Liver samples were prepared for analysis as follows. Representative 0.75 g portions of liver tissue were weighed and transferred to a mortar. Approximately 2.4 g anhydrous sodium sulfate was added to each sample and ground exhaustively to a free-flowing powder. Five ml methanol–chloroform (4:1) was added, and the sample ground further. The methanol–chloroform layer was decanted off onto a paper filter, into a 50 ml volumetric flask. This extraction was repeated 3 times, and the extracts combined. A 2-ml aliquot was evaporated under a stream of nitrogen, and the residue redissolved in 200 μl HPLC solvent.

### 2.5. Supercritical fluid extraction

Sample size was 0.7 mg dry carotene beadlets mixed with 6 g white quartz sand, or 0.15 g minced liver mixed with 0.75 g Hydromatrix packed into a 5 ml sample container. β-Carotene beadlet samples were eluted with SFC grade $CO_2$ at 31 mPA, with 1 min static extraction followed by 40 min dynamic extraction at 2 ml/min flow-rate at 40°C. Liver samples were eluted with SFC grade $CO_2$ at 31 mPA, with 1 minute static extraction followed by 40-min dynamic extraction at 2 ml/min flow-rate at 80°C. Extracts were eluted into 1.5 ml hexane (restrictor temperature 60°C, eluted at 1.5 ml/min).

### 2.6. Spectrophotometry

Measurements of vitamin A and β-carotene concentrations were on neat samples read at 325 nm for vitamin A and 452 nm for β-carotene. Samples were also measured with spectrophotometry scans; scanned from 300 to 400 nm for vitamin A and 400 to 500 nm for β-carotene.

### 2.7. Chromatography

For HPLC, 30-μl aliquots were injected onto a reversed-phase HPLC column (8×4.6 cm ODS $C_{18}$ column with 3-μm packing, plus guard column; Beckman, Fullerton, CA, USA). Samples were eluted with a gradient of acetonitrile–methanol–tetrahydrofuran–ammonium acetate–BHT as previously described [7].

## 3. Results

A typical spectrum of a calf liver extract by SFE is shown in Fig. 1. Spectrum from extracts prepared by liquid–liquid extraction were very similar. A typical spectrum of β-carotene beadlets extracted by SFE is shown in Fig. 2. The spectrum from liquid–liquid extraction appeared almost identical. A typical chro-

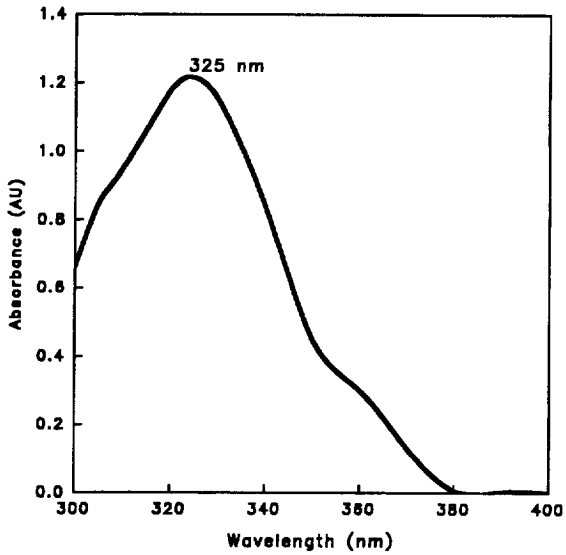


Fig. 1. Spectrum of an extract of calf liver. The UV maximum of vitamin A is 325 nm. Extracts prepared by SFE and by liquid–liquid methods gave very similar or identical spectrum.

matogram for calf-liver extracted by SFE is shown in Fig. 3. Again, the chromatogram of the liquid–liquid extraction is almost identical. Chromatograms for

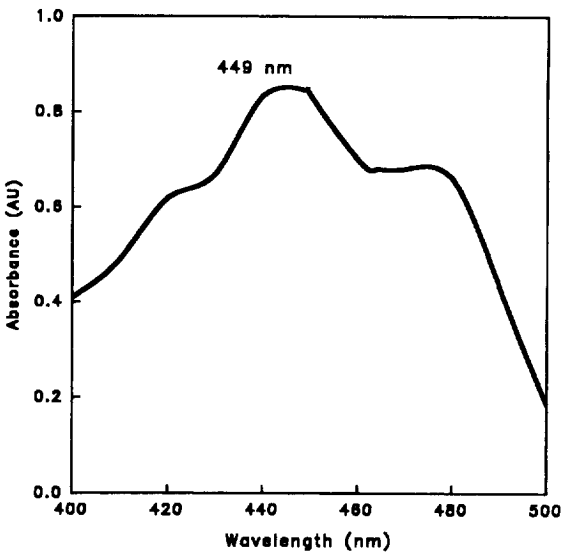


Fig. 2. Spectrum of β-carotene beadlets. The UV maximum of β-carotene is 466 nm (in chloroform). Spectrum from extracts prepared by SFE or liquid–liquid were very similar or identical.

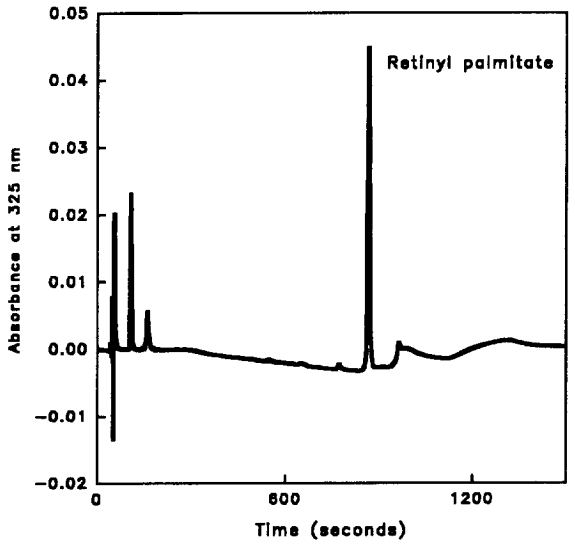


Fig. 3. Chromatogram of a calf liver, extracted by SFE at 325 nm. For methods see Section 2.5. Chromatograms of calf liver extracted by liquid–liquid were very similar.

β-carotene beadlets prepared by the different extraction methods were very similar (data not shown).

### *3.1. Reproducibility and uniformity of β-carotene beadlet sample extractions*

Extraction and HPLC resulted in recoveries of ≥90% for β-carotene by SFE or liquid–liquid extraction. Supplements had uniform content, as expected. Relative standard deviation (R.S.D.; for extraction and chromatography, $n=9$) was 4% for samples from lot 0093009 of beadlets. R.S.D. was greater for the second, older lot (011605, obtained in April 1992). R.S.D. for the old lot ($n=5$) was 16%, probably indicating sample degradation.

### *3.2. Reproducibility and uniformity of liver sample extraction*

Total extraction of calf liver by SFE compared favourably to extraction by liquid; SFE resulted in approximately 108% (±10) of the vitamin A recovered by liquid–liquid extraction. Reproducibility was good, but not as good as for new β-carotene beadlets (mean R.S.D. of 8%, $n=8$). Vitamin A content in samples from the same calf liver were less

Table 1
Reproducibility of vitamin A extracted with SFE from one calf liver divided into eight equal portions

| Sample | Absorbance at 325 nm | | |
|---|---|---|---|
| | Mean | S.D. | R.S.D. (%) |
| 1 | 1.14 | 0.15 | 13 |
| 2 | 1.31 | 0.10 | 7.6 |
| 3 | 1.25 | 0.11 | 8.8 |
| 4 | 0.87 | 0.01 | 0.1 |
| 5 | 1.56 | 0.10 | 6.4 |
| 6 | 1.19 | 0.10 | 8.4 |
| 7 | 1.33 | 0.12 | 9.0 |
| 8 | 0.98 | 0.11 | 11 |
| Mean of test results | 1.20 | 0.10 | 8.3 |
| Mean of all samples tested (total liver) | 1.20 | 0.21 | 18 |

S.D.=standard deviation; R.S.D.=relative standard deviation.

uniform than β-carotene beadlets, as would be expected (R.S.D. of 18%, $n=8$; Table 1). However, the uniformity of liver vitamin A from a single liver was much greater then the difference in vitamin A content between different calf livers (R.S.D. of 62%, $n=6$; Table 2).

## 4. Conclusions

The SFE conditions we derived for extracting β-carotene from β-carotene beadlets were similar to SFE conditions previously reported for extracting β-carotene from leaves and vegetables [15]. The SFE conditions we found were best for extracting vitamin A from liver tissue are similar to those previously found for extracting fat from meat [16,17]. It was also similar to the method previously developed for extracting lipid and cholesterol from guinea pig livers by Drs. Talwinder Kahlon and Faye Chow, Western Regional Research Center, Albany, CA, USA; except that they add ethanol to the SFE with a modifier pump. Sample preparation with supercritical fluid extraction was somewhat faster and much less labor intensive than traditional methods using liquid–liquid extraction.

Table 2
Variability of vitamin A content of six calf livers extracted with SFE

| Liver | Absorbance at 325 nm | | |
|---|---|---|---|
| | Mean | S.D. | R.S.D. (%) |
| 1 | 1.43 | 0.17 | 12 |
| 2 | 0.19 | 0.21 | 110 |
| 3 | 1.20 | 0.21 | 18 |
| 4 | 0.56 | 0.33 | 59 |
| 5 | 1.91 | 0.17 | 8.9 |
| 6 | 0.76 | 0.12 | 16 |
| Mean of livers tested | 1.01 | 0.63 | 62 |

Recoveries of β-carotene from the β-carotene beadlets were similar, or somewhat higher, for SFE than for liquid–liquid extraction. Recoveries of vitamin A from calf-liver were also similar or higher than for liquid–liquid extraction. However, recoveries of vitamin A from hamster liver by our SFE method were not as good as from liquid–liquid extraction, or by the SFE method (with ethanol as modifier) developed by Talwinder Kahlon's group at WRRC. These results —which will be described in more detail elsewhere— suggest that an organic modifier is needed for some animal tissues, especially tough, stringy tissues.

Reproducibility was excellent for new β-carotene beadlet preparations and good for calf liver samples. Our results with SF extraction confirm previous reports of the heterogeneity of vitamin A distribution in calf liver. This heterogeneity is evident even in our purchased samples; where blood vessels, scar tissue, fibrous tissue and discolorations were removed prior to purchase or processing. We were unable to detect any visible difference in the quality or consistency of our calf liver samples that could account for differences in vitamin A distribution in the sample. Thus, our results also confirm that liver biopsy samples are likely to yield poor estimates of vitamin A status. Using these estimates as a "gold standard" for non-invasive estimates of vitamin A status may be essential, but will be problematic. However, our results suggest that errors resulting from liver heterogeneity —at least in a good quality liver sample— are smaller than the differences between liver vitamin A concentrations in different livers. Thus these errors are not so great as to cause a significant number of false diagnoses of vitamin A toxicity or deficiency in animals (and presumably people) with normal liver stores.

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