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# Extraction and chromatography of carotenoids from pumpkin

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## Abstract

Vitamin A deficiency is a health problem in Southeast Asia that can be corrected by feeding orange fruits and vegetables such as mango. Pumpkin is a traditional Korean food that is easy to store and is already believed to have health benefits. We extracted carotenoids from pumpkin by liquid–liquid extraction and by supercritical fluid extraction. We measured carotenoids by reversed-phase chromatography with diode array detection. The major carotenoid in pumpkin (>80%) is β-carotene, with lesser amounts of lutein, lycopene, α-carotene and cis-β-carotene. Pumpkin is a rich source of β-carotene and might be useful for preventing Vitamin A deficiency.
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## 1. Introduction

Carotenoids are a major source of Vitamin A, which is necessary for normal eyesight, growth, and embryonic development [1–3]. Vitamin A deficiency is a common cause of blindness and infant mortality [2,3], and a major health problem in Southeast Asia, Africa, and parts of South and Central America [2,3]. Currently, Vitamin A deficiency is usually treated with commercial Vitamin A supplements [4,5]. However, there are ongoing public health initiatives that seek to prevent Vitamin A deficiency by feeding pregnant women and children at risk the bright orange or dark green fruits and vegetables that are rich sources of carotenoids [6–13].

Carotenoids appear to be difficult to absorb from the green leafy vegetables that were first used for food supplementation programs [5], but absorption appears to be much better from yellow and orange fruits and vegetables [6–13]. The most common successful interventions have used mangos [7,9,11], which are popular and nourishing, but spoil easily and have a short season during the Spring and Summer. Pumpkin might also be an excellent source of provitamin A carotenoids for preventing Vitamin A deficiency because it is known to contain carotenoids, is easy to store and transport, and it is readily available in the Fall and Winter. Pumpkin is a traditional Korean food that is widely eaten during the Fall [14–20]. It is one of the foods recommended for postpartum women in Korea [16,17]. It is believed to have health benefits and is often eaten with fat, which aids carotenoid absorption [16–20]. Therefore, it should be a useful source of provitamin A carotenoids in Southeast Asia.

Most of the carotenoids in pumpkin have not been identified or measured, although preliminary data is available from a few spectrophotometric and chromatographic studies [21–24]. The major reason for this is that to our knowledge, no methods have been developed to quantitatively extract carotenoids or fat-soluble vitamins from pumpkin. The recommended method for extracting carotenes from freeze-dried vegetables [25] is labor-intensive, uses toxic solvents, and requires overnight saponification. Although saponification gives excellent extraction efficiencies and is useful for quantitative measurements of total carotenoids, it is less successful for determining individual carotenoids because carotenoids are degraded and isomerized by saponification conditions. Developing a simpler, faster, less toxic method for routine measurements of carotenoids in pumpkin would be useful for scientists and public health workers attempting to use fruits and vegetables to combat Vitamin A deficiency. We

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extracted carotenoids from pumpkin by liquid–liquid extraction and by supercritical fluid extraction. We compared the effects of temperature, pressure, and modifier concentration on supercritical extraction of freeze-dried pumpkin. We measured the carotenoid composition of pumpkin by a previously developed reversed-phase chromatography using diode array detection [26]. To our knowledge, this is the first report of supercritical fluid extraction of carotenoids in pumpkin or squash.

## 2. Experimental

### 2.1. Chemicals

All solvents used were HPLC or reagent grade. Reagent grade chemicals were purchased from Sigma Chemical Company (St. Louis, MO, USA), Fisher Scientific (Pittsburgh, PA, USA) and J.T. Baker Chemical Company (Phillipsburg, NJ, USA). Retinyl palmitate (>95% purity), lutein (xanthophylls from alfalfa, 85% purity), $\alpha$-carotene (>90% purity), lycopene (>95% purity) and $\beta$-carotene (>97% purity) standards were from Sigma. Supercritical grade $CO_2$ was purchased from AirPak (Sacramento, CA, USA).

### 2.2. Pumpkin samples

We analyzed samples from fully ripe Asian (*Curcurbita moschata*) pumpkin. Samples of pumpkin were prepared by peeling and seeding the ripened pumpkin, and chopping the pumpkin flesh into small pieces. Approximately 800 g of pumpkin flesh was then freeze-dried to constant mass (for approximately 24 h). *C. moschata* freeze-dried samples were prepared in Daegu, South Korea, packaged and shipped by Federal Express on dry ice to our laboratory in Davis, California, USA. The freeze-dried pumpkin lost approximately 90% of its mass (for a yield of 10.2%, w/w).

### 2.3. Preparation of solvents and HPLC standards

Our HPLC method is based on a previously published method [26]. Mobile phase solvents were made by mixing acetonitrile, tetrahydrofuran and methanol, then adding 1% ammonium sulfate in HPLC grade water and 0.0005% $\beta$-hydroxytoluene as an antioxidant. Solvent A contained acetonitrile–tetrahydrofuran–methanol–1% aqueous ammonium sulfate solution (85:5:5:5, v/v/v/v), and solvent B contained acetonitrile–tetrahydrofuran–methanol–1% aqueous ammonium sulfate solution (55:35:5:5, v/v/v/v). Each solvent was prepared fresh before use. HPLC standards were prepared by dissolving approximately 1 mg standard into 10 mL toluene–ethanol (1:99, v/v). Standards were prepared by mass, and stored in the dark at 4 °C for up to two weeks before use.

### 2.4. Equipment

Liquid chromatography was run on an Agilent 1100 gradient chromatograph with a binary pump, degasser, refrigerated autosampler, column heater, and diode array detection. Chromatographic analysis was run and interpreted with a Chemstation for LC 3D revision A.08.03 (847) for Agilent Technologies, running on a HP Kayak XM600 computer with Windows NT (Hewlett-Packard GmbH, Chemical Analysis Group, Waldbronn, Germany). Supercritical fluid extraction was with an ISCO model 356024-chamber Automated Supercritical Fluid Extractor with dual 260D pumps (ISCO, Lincoln, NE, USA).

### 2.5. Sample preparation by liquid–liquid extraction

Homogeneous freeze-dried pumpkin samples (0.1 g) were mixed with 1 mL 95% ethanol containing 0.05% BHT to denature proteins, vortexed for 60 s, then mixed with an equal volume of hexane. The hexane layer was removed and saved in 12 mm × 75 mm borosilicate test tubes, and the extraction repeated three times. Each hexane extract was dried under a stream of nitrogen. The extracts were resuspended in 100 μL solvent B. Samples and standards were stable under these conditions for up to one week. After extraction, evaporation and resuspension, we typically ended with a sample volume of 100 μL.

### 2.6. Sample preparation by supercritical fluid extraction

Homogeneous freeze-dried pumpkin samples (0.1 g) were mixed with 5 g Hydromatrix. Carotenoids were extracted with supercritical fluid with supercritical grade $CO_2$. We analyzed the effect of time, temperature (40, 80 °C), pressure (31, 35, 50 mPa), static and dynamic extraction times and modifier on the solvating power and selectivity of the supercritical fluid $CO_2$. The supercritical fluid modifiers tested were denatured ethyl alcohol (190 proof alcohol, four parts methyl isobutyl ketone and one part kerosene); and HPLC grade methanol. We tested 0%, 10% and 30% modifier concentrations. Static extraction times tested were 1, 10 and 30 min.

The carbon dioxide flow rate was kept constant at 2 mL/min. The effect of temperature was measured first, then pressure at the best temperature, then percent modifier at the best temperature and pressure, then finally the effect of static extraction time. Each sample was extracted three times at the specified temperature, pressure, and modifier concentration and each extraction was analyzed separately by chromatography, to test for the completeness and uniformity of the extraction method. We analyzed three samples at each condition.

### 2.7. Chromatography

We measured the carotenoid composition of pumpkin by reversed-phase liquid chromatography using diode array detection. Chromatography was run as a gradient at 1.0 mL/min

with a Prodigy 5μ C$_{18}$ ODS 3 100 A pore 250 mm × 4.6 mm reversed-phase column (Phenomenex, Torrance, CA, USA). Total run time was 48 min. The gradient changed from 5% to 95% solvent B, as follows: 0–10.0 min, 5% solvent B; 10.0–29.0 min increasing linearly to 95% solvent B; 29.0–35.9 min, maintaining 95% solvent B; 35.9–36.0 min, abruptly decreasing to 60% solvent B; maintaining 60% solvent B from 36.0 to 44.9 min; then abruptly decreasing to 5% solvent B at 45 min. The column re-equilibrated with 5% solvent B from 45.0 to 48.0 min. Serum carotenoid concentrations were measured by diode array detection set at 452 nm.

Standards for β-carotene were analyzed after every fifth sample. Most peaks were identified by comparing retention times with those of the corresponding standards, and inspecting diode array three dimensional plots scans (run from 250 to 500 nm wavelength) for wavelength maxima. Peaks that did not have standards (such as zeaxanthin) were identified by their spectra and chromatographic characteristics (e.g., zeaxanthin is known to migrate closely with lutein under almost all chromatographic conditions).

### 2.8. Data analyses

Data were evaluated by simple statistics (mean, standard deviation) and histograms were drawn and analyzed with Sigmaplot 2000 (SPSS Inc., Chicago, IL, USA).

## 3. Results

A chromatograph of carotenoids from *C. mochata* is shown in Fig. 1. The chromatography conditions described measure lutein, lycopene, cryptoxanthin, α-carotene, β-carotene and *cis*-β-carotene in pumpkin. These carotenoids, as well as most other unidentified carotenoid isoforms, were base-line separated. The major carotenoid identified by both extraction methods was β-carotene (Table 1). There also were small percentages of β-carotene, *cis*-β-carotene, cryptoxanthin, lutein, lycopene, and several unidentified carotenoids.
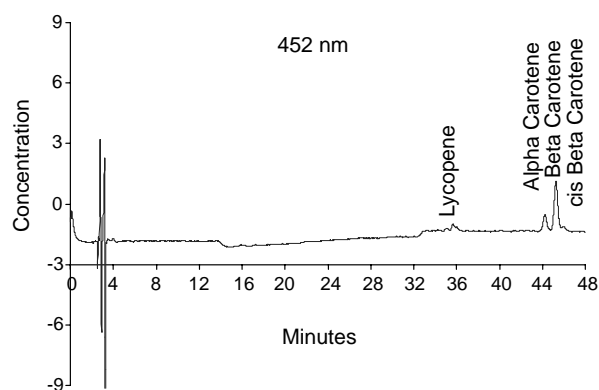


Fig. 1. Typical chromatograph of *C. moschata* at 452 nm. The following peaks were identified: peak 1, lutein; peak 2, cryptoxanthin; peak 3, lycopene; peak 4, α-carotene; peak 5, β-carotene; peak 6, *cis*-β-carotene.

Table 1
Carotenoids present in pumpkin measured by comparison to external standards[a]

| Carotenoid I.D. | %Carotenoid, mean (R.S.D.) | |
|---|---|---|
| | Standard method; Table 4 | Table 4 method, except 30 min static extraction |
| β-Carotene | 79 (0.02) | 55 (0.07) |
| Cryptoxanthin | 1 (0.06) | 2 (0.10) |
| α-Carotene | 10 (0.03) | 12 (0.04) |
| *Cis*-β-carotene | Trace | Trace |
| Lutein | 2 (0.04) | 3 (0.05) |
| Lycopene | 5 (0.10) | 3 (0.08) |
| Unknown | 3 (0.11) | 25 (0.12) |

[a] Standard method, $n = 6$; 30 min static extraction method, $n = 3$.

Our liquid–liquid extraction did not give complete carotenoid recoveries even after three extractions with hexane. However, a comparison of the extractions from each sample showed that the carotenoids were uniformly extracted, i.e., there were the same percentages of β-carotene, α-carotene, cryptoxanthin, lutein, lycopene, and *cis*-β-carotene in the first and last extractions.

Supercritical fluid extraction recovery and selectivity depended on temperature, pressure, and percent modifier. Best recoveries were from the highest temperature investigated (80 °C; Table 2). Pressure appeared to have a lesser effect, and most consistent results were obtained at 31 mPa (Table 2). Extraction efficiency was improved greatly by using a modifier, with methanol giving more complete extractions than ethanol (Table 3). While 30% modifier gave somewhat better recoveries than 10% modifier, the use of this much modifier negates much of the advantages of supercritical fluid extraction compared to liquid–liquid extraction, and results in an unwieldy and dilute extract. Increasing static extraction times from 1 to 30 min increased the recovery of β-carotene by approximately 11%. However, it decreased the percentage of β-carotene extracted from the pumpkin (Table 1). Thus, β-carotene constituted about 79% of the total carotenoids extracted with 1 min static extraction, but only 55% of the total carotenoids extracted with 30 min static extraction. Although some of this difference was because the lutein and cryptoxanthin peaks increased, most of the difference resulted from the appearance of a variety of poorly shaped peaks that did not migrate with any of our carotenoid standards. We be-

Table 2
Effect of temperature and pressure on recovery of carotenoids from pumpkin by supercritical fluid extraction[a]

| Temperature (°C) | Pressure (mPa) | Recovery (peak area units), mean (R.S.D.) |
|---|---|---|
| 40 | 30 | 63 (0.13) |
| 60 | 30 | 85 (0.12) |
| 80 | 30 | 99 (0.11) |
| 80 | 31 | 107 (0.05) |
| 80 | 35 | 103 (0.09) |
| 80 | 50 | 109 (0.11) |

[a] $n = 2$.

Table 3
Effect of modifier and time of static extraction on recovery of carotenoids from pumpkin by supercritical fluid extraction[a]

| Modifier | Static extraction (min) | Recovery (peak area units), mean (R.S.D.) |
|---|---|---|
| %Methanol | | |
| 0 | 1 | 85 (0.20) |
| 10 | 1 | 126 (0.05) |
| 30 | 1 | 132 (0.04) |
| 0 | 10 | 88 (0.06) |
| 10 | 10 | 126 (0.09) |
| 10 | 30 | 139 (0.07) |
| %Ethanol | | |
| 10 | 1 | 112 (0.05) |
| 30 | 1 | 125 (0.11) |

[a] $n = 3$.

Table 4
Supercritical fluid extraction method

| | |
|---|---|
| Static extraction temperature (°C) | 80 |
| Dynamic extraction temperature (°C) | 80 |
| Restrictor temperature (°C) | 60 |
| Collection temperature (°C) | 5 |
| Static extraction time (min) | 1 |
| Dynamic extraction time (min) | 40 |
| Restrictor flow rate (mL/min) | 2 |
| Solvent added to sample (mL) | 2 |
| Percent modifier (%methanol) | 10 |
| Pressure (mPa) | 31 |

lieve these new peaks are carotenoid isomers and degradation products that formed during the static extraction products and were not present in the pumpkin tissue originally.

The supercritical fluid extraction method that gave us our best results is shown in Table 4. Reproducibility for our supercritical fluid extraction-LC method was very good ($n = 6$; R.S.D. $= 0.02$ for β-carotene, R.S.D. $= 0.04–0.11$ for carotenoids present in lesser concentrations; Table 1).

## 4. Discussion

There are three common types of pumpkin world-wide: *Curcurbita pepo*, *Curcurbita maxima*, and *C. moschata*, but differences between varieties are not great [23]. All can be similar in size, shape, and color, although miniature pumpkins tend to be *C. pepo* and giant pumpkins tend to be *C. maxima* varieties. *C. moschata* is the most commonly eaten variety of pumpkin in both Asia and the United States.

The chromatography method we developed for these analyses is based on a method for measuring fat-soluble vitamins in buccal epithelial cells [27], which was modified in our laboratory for human plasma [26]. The major carotenoid in pumpkin is β-carotene, with small amounts of α-carotene, lutein, lycopene, and trace amounts of cryptoxanthin and *cis*-β-carotene.

There are only a few previous reports of the carotenoid content of pumpkin [23,24]. These studies also show that

pumpkin is a rich source of β-carotene. However, they report greater percentages of α-carotene (31–33%) and unidentified carotenoids (5–33%) than we do. The reasons for these differences are not known, and could be real differences in carotenoid composition. However, the large percentage of unidentified carotenoids, and the increased 'α-carotene' concentration is similar to our results when we used prolonged static extraction and thus they might be degradation products caused by the extraction process.

Simple liquid–liquid and supercritical fluid extraction methods have been developed that give excellent recoveries for carotenoids and fat-soluble vitamins from meat, oils, and vitamin supplements [28–31]. Carotenoid extraction from vegetables is much more difficult. The official liquid–liquid extraction of carotenoids from dried vegetables uses harsh conditions that result in carotenoid degradation [25]. Laboratories have often used milder liquid–liquid extraction conditions for vegetables [23,24], but our use of these methods suggest that they did not obtain complete recoveries.

Supercritical fluid extraction is less toxic and less labor-intensive than liquid–liquid extractions. Extraction recovery depends on the vegetable matrix, but has sometimes been excellent especially for β-carotene [28,32–34]. Our method did not produce complete recovery, but recovery was similar or better than milder liquid–liquid extraction methods ([23,24], this paper).

Extraction depended on temperature, pressure, and modifier: our best results were obtained at the highest temperature and moderate pressure (Table 2). Using an organic modifier was essential to obtain good recovery, but 10% modifier gave essentially the same recovery as 30% modifier and provided a more concentrated sample (Table 3). These results are similar to those from studies of the extraction of lycopene (an acyclic carotenoid) from tomato waste [35–37]. Prolonged extraction times resulted in an improvement in total carotenoid recovery, but much greater isomerizations and degradation (Tables 1 and 3). Our final method is shown in Table 4.

Our results show that pumpkin is a rich source of carotenoids, especially in β-carotene and other carotenoids that are good precursors of Vitamin A. Our results suggest that this food might be very useful for public health interventions seeking to prevent Vitamin A deficiency.

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