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Novel Procedure for the Extraction and Concentration of Carotenoid-Containing Chromoplasts from Selected Plant Systems

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Natural sources of carotenoids for nutraceutical use are desired by the food industry as a result of the increased production of convenience and other highly processed foods. As new physiological roles are discovered for some of the minor carotenoids that are found in only small amounts in present sources, the need for discovery of new sources will amplify. Thus, a method is needed that will effectively and gently concentrate carotenoids from potential new sources for subsequent identification and analysis. A procedure is presented by which carotenoid-containing tissue chromoplasts can be extracted and subsequently concentrated by precipitation, all in an aqueous milieu. The chromoplasts are extracted and solubilized with 0.3% sodium dodecyl sulfate (SDS) in water. The addition of a nominally equal volume of acetonitrile to the chromoplasts in SDS immediately precipitates the chromoplasts out of solution with generally >90% recovery. Carotenoids contained in the concentrated, still-intact chromoplasts can then be solubilized by organic solvent extraction for subsequent analysis. This methodology offers a means to effectively and gently concentrate carotenoids from fruit tissues where yields are often low (e.g., yellow watermelon).

KEYWORDS: Carotenoids; chromoplasts; lycopene; β-carotene; chromoplast extraction; SDS; chromoplast precipitation; acetonitrile

INTRODUCTION

A substantial number of studies have demonstrated an inverse relationship between the consumption of certain fruits and vegetables and the risk of cancer, cardiovascular disease, and UV damage to skin (*1*). This protective effect has been generally ascribed to the carotenoids contained in those foods (*2*), although high doses of carotenoids have been implicated in negative health effects in selected cases. Rather than preventing lung cancer among smokers, β-carotene supplements appear to substantially increase the risk of smokers dying from lung cancer (*3*). Recent studies suggest that >90% of the β-carotene, lutein, and lycopene contained in fruits and vegetables is available in the gut during the entire digestion process (*4*). In the contemporary food industry, food supplements have become more and more prominent as a result of the increased production of prepared, processed, and convenience foods. Carotenoids are one of the classes of compounds occupying an ever-increasing role as food supplements.

As more and more of the minor carotenoids receive attention for their potential health benefits and as less common sources are sought for these carotenoids, smaller and smaller quantities are encountered with which to work. Concentration from organic solvent extraction for these smaller quantities is frequently fraught with two pitfalls. First, extraordinary care must be exercised during solvent evaporation to prevent destruction of appreciable quantities of the carotenoids. Second, some of the more polar carotenoids have reduced solubility in the typically used nonpolar organic solvents such as hexane and are sometimes not fully extracted.

In a recent study with watermelon, we observed that the carotenoid-containing chromoplasts of watermelon could be solubilized in aqueous sodium dodecyl sulfate (SDS) (*5*). SDS proved to be moderately successful at extracting and solubilizing chromoplasts from other sources as well. However, some sources proved to be highly resistant to chromoplast extraction/solubilization by SDS, and only smaller proportions of the total carotenoids could be extracted, for example, processed tomato products. Anecdotally, it appeared that the "softer" fruits were more amenable to the extraction/solubilization of chromoplasts by SDS. The ability to solubilize the intact chromoplasts allows their separation from insoluble cell debris and, with washing, the removal of water-soluble compounds. Quite fortuitously, we also discovered that the water-soluble organic compound acetonitrile effectively precipitates the chromoplasts out of aqueous SDS without damaging the chromoplast membrane or the carotenoids inside. Thus, the use of these two solvent systems in combination potentially provides a means to extract, partially purify, and concentrate carotenoid-containing chro-

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moplasts from selected plant systems. The method, its parameters, and its limitations are discussed herein.

MATERIALS AND METHODS

Plant Materials. Watermelons and cantaloupe used for the study were from the 2001 and 2002 crops and were grown on research plots at the South Central Agricultural Research Laboratory in Lane, OK. Other fresh fruits and vegetables were purchased as fresh produce at a local supermarket, as were the processed tomato products.

Extraction into SDS of Chromoplasts from Fruit. Tissue from a ripe fruit, for example, watermelon [*Citrulles lanatus* (Thumb.) Mansf.] was homogenized (~1 min) to a smooth puree in a blender (Waring Products, New Hartford, CT). Frequently, a tissue had to be quantitatively diluted with water to effectively homogenize it. For example, 1 g of water was added to every 4 g of papaya fruit, every 2 g of mango fruit, or every 1 g of spinach. To 2 g of the tissue puree was added 6 mL of 0.4% SDS in H_2O. Fruit puree was pipetted with a large-opening pipet tip (tip was cut off to make the larger opening), but the pipetted quantity was weighed to ensure a quantitative transfer. Sodium azide (NaN_3) was included in the SDS stock solution at a level of 0.02% to prevent bacterial and mold growth if the SDS solution was to be stored at room temperature for long periods of time. Occasionally, the suspension was further ground in a 10 mL Potter−Elvenjheim homogenizer at 200 rpm to ensure complete disruption of the tissue. For acidic fruit such as grapefruit or tomato, it was usually necessary to adjust the pH of the puree to near neutrality before the SDS would effectively solubilize the chromoplasts. After standing for 1 h to allow denaturation/solubilization of cellular components, the homogenate was centrifuged at room temperature for 15 min in a 15 mL conical centrifuge tube at 1500*g* (3000 rpm) in a swinging bucket rotor of a Sorvall TC6 benchtop centrifuge (Kendro Laboratory Products, Asheville, NC). After centrifugation, the carotenoid-containing particles were suspended in the aqueous supernatant and the colorless tissue residue was pelleted at the bottom of the tube. On rare occasions, some of the residue in a sample would float rather than pellet. By resuspending the sample, briefly degassing it, and recentrifuging it, all of the solid material would pellet. Because chromoplasts have a limited solubility in SDS, it is sometimes necessary to extract the residue a second or third time with fresh solvent or to use larger volume ratios of SDS to fruit in a single extraction. The necessity for additional extractions or for using less tissue can be recognized by the presence of residual color in the residue.

Precipitation of Chromoplasts Out of SDS. A volume, nominally 2−4 mL, of chromoplasts suspended in SDS was pipetted into a 12 mL glass conical centrifuge tube fitted with a screw cap. To this was added the desired volume of acetonitrile, routinely an equal volume, and the contents were briefly mixed on a vortex mixer. Precipitation of the chromoplasts was usually immediate, although the mixture was allowed to stand for 15 min before centrifugation. Samples were centrifuged at 1000*g* for 5 min in a swinging bucket rotor of a Sorvall TC6 benchtop centrifuge (Kendro Laboratory Products).

Solubilization of Carotenoids from the Prepared Chromoplasts. The chromoplast pellet usually could not be directly solubilized into hexane easily, probably because of the water surrounding and interacting with the chromoplast membrane. Dispersion of the chromoplasts and solubilization of the carotenoids could be achieved by adding equal volumes of ethanol and acetone (0.1−5.0 mL, depending on the size of the pellet), sonicating the sample to break the pellet into fine particles, and then extracting the suspension with a volume of hexane greater than or equal to the combined volumes of ethanol and acetone.

Separation/Quantitation of Carotenoids. Lycopene in watermelon, tomatoes, red grapefruit, and papaya was routinely quantified by the low-volume hexane extraction method for lycopene determination (*6*). β-Ccarotene in cantaloupe was quantified according to the same procedure as for lycopene, but by reading the absorbance at 452 nm and with the use of an absorptivity of 13.9×10^4 M^{-1} (*7*). Total carotenoids were estimated by using the method of Lichtenthaler (*8*) as outlined for carotenoids in the presence of chlorophyll. The amount of carotenoids, as part of the chromoplasts extracted into SDS, was quantified by assaying a known aliquot of the SDS extract by using the low-volume hexane extraction method (*6*). Similarly, the amount of carotenoids precipitated out of SDS with acetonitrile was quantified by dispersion of the chromoplast pellet with ethanol/acetone and extraction with hexane as described above. The percentage carotenoid yield by SDS extraction was calculated by dividing the quantity of carotenoid extracted into SDS per unit weight of fresh material by the quantity of carotenoid directly extracted into hexane per unit weight of fresh material. The percent recovery of carotenoids from acetonitrile precipitation of chromoplasts out of SDS was determined by dividing the quantity of carotenoids per unit weight of fresh material in the chromoplast pellet by the quantity of carotenoids in the SDS extract per unit weight of fresh material.

In selected instances, the distributions of carotenoids and their geometric isomers were determined by HPLC (*9*). Thin-layer chromatography of carotenoids was carried out on commercial silica gel plates (Sigma-Aldrich, St. Louis, MO) with the use of selected solvent systems of Philip and Francis (*10*).

Quantitation of SDS. SDS was quantified according to the method of Mukerjee (*11*) as modified by Hayashi (*12*).

Table 1. Yields of Carotenoids from Selected Fruits and Vegetables by SDS Extraction Followed by Precipitation With Acetonitrile

fruit/vegetable	% of total carotenoids extracted into SDS[a]	% of carotenoids in SDS precipitated by acetonitrile[a]
red watermelon	99 ± 2[b]	95 ± 2[b]
yellow watermelon	97 ± 3	95 ± 2
cantaloupe	97 ± 3	94 ± 3
mango[c]	59 ± 1	83 ± 3
papaya	99 ± 4	99 ± 2
red grapefruit	93 ± 2	93 ± 3
frozen spinach[d]	46 ± 4	32 ± 2
fresh tomato	93 ± 5	90 ± 3
tomato catsup[e]	68 ± 4	96 ± 1
tomato sauce[e]	47 ± 2	96 ± 3
tomato juice[e]	67 ± 2	97 ± 2

[a] Determined as described under Separation/Quantitation of Carotenoids (Materials and Methods). [b] Mean and standard deviation of two to four replicates. [c] These yields are from extracting 1 g of mango flesh per 3 mL of 0.4% SDS. If the ratio of 0.4% SDS to 1 g of mango flesh was increased to 9:1, 91% of the carotenoids were extracted, but only 33% of these were recovered in the acetonitrile precipitation step. [d] These yields are from extracting 1 g of frozen spinach with 8 mL of 0.3% SDS. If the ratio of SDS to g spinach was increased to 20:1, ~100% of the carotenoids were extracted. Recovery from acetonitrile precipitation at this dilution was not attempted. [e] These yields are from extracting 1 g of processed tomato product with 16 mL of 0.3% SDS. No more than ~0.5 mL of the SDS extract was treated with 10 mL of hexane. Not all of the carotenoids were extracted into the hexane when larger quantities of the SDS extract were employed.

RESULTS

During an experiment designed to test various polar organic compounds for use with aqueous SDS to further purify watermelon fruit chromoplasts, we observed that acetonitrile quickly and effectively precipitated the chromoplasts out of solution. Upon removal of the acetonitrile, the chromoplasts readily re-extracted into aqueous SDS or, if desired, could be dissolved into a small volume of organic solvent. We elected to examine this system in greater detail as a possible means to extract and concentrate fruit chromoplasts with their carotenoids contained inside them.

Table 1 summarizes the extractability of carotenoids by SDS from a selected group of fruits, vegetables, and processed tomato products. As can be seen from this small sampling, the efficacy of the SDS in solubilizing the chromoplasts varies widely. Of the fruits and vegetables that we examined, green-leafed vegetables were one of the more recalcitrant to SDS extraction. Soft-fleshed fruits and vegetables tended to be more amenable

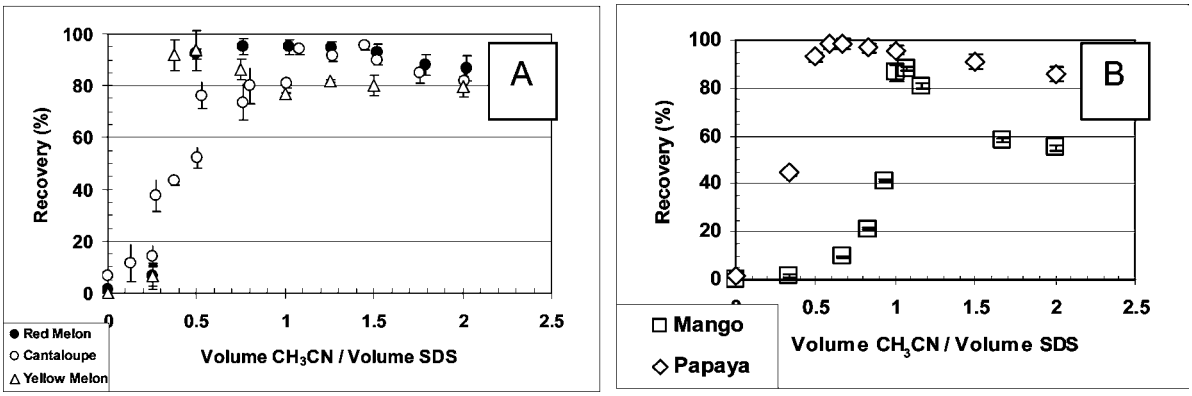

Figure 1. Recovery of carotenoids by acetonitrile precipitation as a function of the volume ratio of acetonitrile to SDS extract. Chromoplasts precipitated with acetonitrile were dissolved into a known volume of hexane, and the carotenoids were quantified spectrophotometrically. Percent recovery was determined as described under Materials and Methods. Error bars represent plus or minus one standard deviation of two or three replicates. (**A**) Watermelon and cantaloupe fruit: (●) red watermelon ('Sangria') chromoplast starting concentration in the 0.3% SDS was equivalent to 20 or 40 μg/mL of lycopene; (○) cantaloupe ('Magnum 45') chromoplast starting concentration in the SDS was equivalent to 6 or 10 μg/mL of β-carotene; (△) yellow watermelon ('Chiffon') chromoplast starting concentration in the SDS was equivalent to ~3 μg/mL total carotenoids. (**B**) Mango and papaya fruit: (□) mango chromoplast starting concentration in the SDS was equivalent to 6 μg/mL total carotenoids; (◇) papaya chromoplast starting concentration in the SDS was equivalent to 37 μg/mL total carotenoids.

to extraction in higher yields. As discussed below, the level of acetonitrile required to precipitate the chromoplasts out of SDS exhibited some variation with the source of the chromoplasts.

Fresh tomatoes and three processed tomato products offer an interesting comparison in extraction efficiencies by aqueous SDS. Three types of fresh tomatoes were tested: a common red garden tomato, a variety of Roma tomato, and grape tomatoes. All three types of fresh tomatoes were susceptible to extraction of their chromoplasts by SDS at ~90% recovery of carotenoids. The tomato products, on the other hand, were significantly more recalcitrant to chromoplast extraction. As seen in **Table 1**, 68 ± 4% of the carotenoids were extracted from tomato catsup and tomato juice with SDS, whereas only 47 ± 2% were extracted from tomato sauce. A dark red ring of precipitate was observed on the pellets from the SDS-treated tomato sauce and the tomato juice. This appearance has been interpreted to indicate the presence of insoluble carotenoid aggregates that are no longer inside a chromoplast membrane (*5*).

To examine the precipitation of chromoplasts by acetonitrile in greater detail, SDS extracts of a number of the fruits and vegetables listed in **Table 1** were treated with different ratios of acetonitrile to determine the level of precipitating agent to produce the highest yield of chromoplasts. **Figure 1** illustrates the results of this study. Between a volume ratio of acetonitrile to SDS of 0.25:1 and 0.5:1, the yield of red watermelon (**Figure 1A**) and papaya (**Figure 1B**) chromoplasts, as measured by lycopene, jumped from <10% to >90%. Cantaloupe chromoplasts, on the other hand, appeared to require a higher level of acetonitrile to fully precipitate; maximal precipitation did not occur until a volume ratio of ~1:1 of acetonitrile to SDS solvent was reached (**Figure 1A**). The yield of mango carotenoids (**Figure 1B**) peaked at a 1:1 ratio and was markedly reduced above and below that level. The yields of all precipitates began to diminish above their optimal ratio for precipitation, but all did not diminish at the same rate.

We then examined the influence of carotenoid chromoplast concentration in the SDS extract on the yield of precipitated chromoplasts at a fixed volume ratio of acetonitrile to SDS. The results are summarized in **Figure 2**. It can be seen that at a 1:1 volume ratio of acetonitrile to 0.3% SDS, cantaloupe chromoplasts and watermelon chromoplasts behaved similarly.

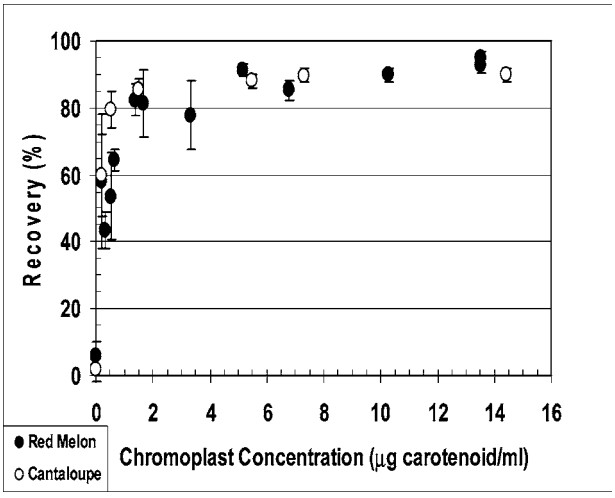

Figure 2. Recovery of chromoplasts by acetonitrile precipitation as a function of the chromoplast concentration in a 0.3% SDS extract: (●) red watermelon ('Sangria'); (○) cantaloupe ('Magnum 45'). Chromoplast concentration is a reflection of the measured carotenoid concentration. The volume ratio of acetonitrile to SDS extract was 1:1. Percent recovery was determined as described under Materials and Methods.

Maximal yield of precipitated chromoplast material was reached at or slightly below 2 μg/mL of carotenoid in the 0.3% SDS. These lower limits for watermelon and cantaloupe chromoplast concentrations should be considered only as a rough guideline for other sources of chromoplasts. For example, only 33% recovery of carotenoids was achieved with mango chromoplasts when the carotenoid level was diluted to ~2 μg/mL in 0.3% SDS (**Table 1**, footnote *c*).

Cursory experimentation was performed to see if the level of SDS used to extract the chromoplasts would affect the level of acetonitrile necessary for maximal precipitation of the chromoplasts. Watermelon chromoplasts from the same weight of watermelon were extracted into 0.3 and 0.6% SDS solutions. They were then each precipitated with 0.75 volume of acetonitrile (a volume ratio near the lowest ratio for maximal recovery), and the yields of carotenoids in the precipitates were compared. The recovery of carotenoids from 0.3% SDS was 48.4 ± 2 μg, and the recovery from 0.6% SDS was 47.6 ± 2

μg. Thus, it would appear that variations in the practical working levels of SDS will have little or no effect on the subsequent acetonitrile precipitation efficiency of the chromoplasts.

It was previously observed that in the presence of excess SDS, watermelon chromoplasts bind large quantities of SDS, presumably to the chromoplast membrane. Approximately 120 molecules of SDS were bound to the watermelon chromoplast membrane for each molecule of lycopene inside the chromoplast (*5*). We hypothesized that the precipitating effect of acetonitrile on chromoplasts in the presence of SDS might be that the polar organic solvent stripped much of the SDS off the chromoplast membrane, thereby reducing the chromoplast's solubility nearer to that of its original state. To test this, watermelon chromoplasts were prepared in 0.3% SDS. Half of the preparation was pelleted by centrifugation at 30000*g* for 1 h. It was then washed twice by suspension in a volume of water equal to that of the starting solution and pelleted by centrifugation. The other half of the preparation was precipitated with an equal volume of acetonitrile, and the precipitate was washed twice with 50% acetonitrile in water (v/v) and centrifugation. Each sample, suspended in water, was no longer soluble and was assayed for lycopene and SDS. The amount of SDS still associated with the chromoplasts washed with water amounted to 0.4 ± 0.1 mol of SDS/mol of lycopene. The amount of SDS still associated with the chromoplasts precipitated with acetonitrile was 0.07 ± 0.03 mol of SDS/mol of lycopene. Thus, extensive washing with water still left almost 6 times more SDS associated with the membrane than the acetonitrile-treated chromoplasts. Although these results do not prove our hypothesis, they are consistent with it.

The obvious application of this methodology would be to extricate carotenoids from tissue and concentrate them while they are still protected inside the chromoplast. From this point, the carotenoids could be extracted into organic solvent for subsequent qualitative or quantitative analysis. Toward this end, we examined the HPLC chromatograms of carotenoids from watermelon, cantaloupe, papaya, mango, and pink grapefruit chromoplasts that had each been prepared by SDS extraction and acetonitrile precipitation. The carotenoids from the SDS-extracted, acetonitrile-concentrated samples were extracted into *n*-hexane and subjected to HPLC analysis. No statistically significant quantitative differences could be found between these samples and samples prepared by direct hexane extraction of the fruit tissue, although relative levels of lutein in watermelon extracts frequently seemed to be slightly higher in the SDS-extracted samples than in the samples prepared by direct extraction with hexane. Additionally, chromoplasts were prepared by SDS extraction−acetonitrile precipitation from a yellow-meated watermelon, cv. 'Yellow Baby'. The TLC pattern, both qualitatively and semiquantitatively was virtually identical to that from a direct organic solvent extract/concentration of the fresh tissue. Similar observations were made for mango fruit, papaya fruit, and fresh spinach.

DISCUSSION

By interacting at the membrane level, the anionic detergent, sodium dodecyl sulfate, is moderately successful at releasing and solubilizing carotenoid-containing chromoplasts out of plant tissue cells, particularly soft tissue cells. The advantage that such a process offers is that it maintains the carotenoids packaged in more or less their natural environment (*5*, *13*) during further manipulation. We have observed that lycopene located inside intact watermelon chromoplasts that are suspended in SDS degrades at a rate much slower than it does when dissolved (monodispersed) in an organic solvent (unpublished observations). This natural protection by the chromoplast membrane thus allows the researcher additional time for the preparation or enrichment of the carotenoids.

The nature of the interaction of SDS with the chromoplast membrane appears to be quite different from that of its interaction with chloroplast thylakoid membranes of the fig (*14*). Various levels of SDS between 10^{-5} and 10^{-1}% were observed to differentially release various populations of chlorophylls and carotenoids from fig leaves. Above 0.1% SDS, the thylakoid membrane was solubilized, and all pigments were released (*14*). Our earlier work (*5*) and the results of the present study are consistent with the chromoplast membrane being resistant to disruption by SDS at the levels at which SDS completely solubilized the fig leaf chloroplast thylakoid membrane.

The unexpected ability of the polar organic solvent acetonitrile to almost quantitatively precipitate the chromoplasts out of SDS solution affords a simple concentration step. The integrity of the chromoplast membrane and its contents appear to be maintained during this precipitation process. Such an extraction/precipitation gives one a concentrated preparation with which to work. The applicability of this procedure is demonstrated by this study, and this procedure has been utilized in a recently published work (*15*).

Another interesting aspect of this methodology is that by being packaged inside the chromoplast membrane, the different chemical properties of various carotenoids will not enter into their purification yield. Only the nature of the chromoplast membrane and any surrounding cellular material will affect the membrane's interaction with SDS. Because the nature of cellular and intracellular materials is different among plants and plant tissues, the aforementioned technology will not necessarily perform adequately in all plant systems. Unless the chromoplast is more or less free of encumbering materials and remains intact, this technology is likely to be marginal at best. The poor yields of carotenoids from spinach are likely the result of interfering intracellular materials or marked chemical differences in the double membrane of the spinach chloroplast. The poor yields from processed tomato products likely result from chromoplast membrane damage or destruction during the processing of the tomatoes. The mango may owe its recalcitrance to its somewhat unique chromoplast structure and organization. A recent study demonstrated that carotenoids are deposited in the plastoglobular substructures of the mango chromoplasts (*16*). It may well be that the lipid composition of the membrane of the mango chromoplast is not as receptive to interaction with SDS as are the membranes of chromoplasts from the other fruits that we examined.

In summary, this methodology affords the researcher an approach markedly different from current methodologies for the extraction and concentration of carotenoids. The novelty of the method is that it keeps the carotenoids inside a naturally occurring organelle and in an aqueous milieu for much of the process and, thus, provides the investigator with an alternative means to effectively concentrate carotenoids from fruit tissues where yields may often be low.

ABBREVIATIONS USED

SDS, sodium dodecyl sulfate; HPLC, high-performance liquid chromatography; TLC, thin-layer chromatography.

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