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Simple Saponification Method for the Quantitative Determination of Carotenoids in Green Vegetables

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A simple, reliable, and gentle saponification method for the quantitative determination of carotenoids in green vegetables was developed. The method involves an extraction procedure with acetone and the selective removal of the chlorophylls and esterified fatty acids from the organic phase using a strongly basic resin (Ambersep 900 OH). Extracts from common green vegetables (beans, broccoli, green bell pepper, chive, lettuce, parsley, peas, and spinach) were analyzed by high-performance liquid chromatography (HPLC) for their content of major carotenoids before and after action of Ambersep 900 OH. The mean recovery percentages for most carotenoids [(*all-E*)-violaxanthin, (*all-E*)-lutein epoxide, (*all-E*)-lutein, neolutein A, and (*all-E*)-β-carotene] after saponification of the vegetable extracts with Ambersep 900 OH were close to 100% (99−104%), while the mean recovery percentages of (9′*Z*)-neoxanthin increased to 119% and that of (*all-E*)-neoxanthin and neolutein B decreased to 90% and 72%, respectively.

KEYWORDS: Carotenoids; xanthophylls; chlorophylls; saponification; Ambersep 900 OH; solid-phase extraction

INTRODUCTION

The carotenoids are one of the most important groups of natural pigments because of their wide distribution in the plant and animal kingdoms, their structural diversity, and their numerous functions (*1*−*3*). Carotenoids, several of which have vitamin A activity, occur invariably in photosynthetic tissues and are together with anthocyanins responsible for the yellow, orange, and red colors of fruits and vegetables. Humans and animals cannot synthesize carotenoids but are able to deposit dietary carotenoids as absorbed or with slight modification of their structure. The intestinal cleavage of these to form vitamin A-active retinoids represents a major contribution to nutrition. Apart from their provitamin A capacity, animal studies and epidemiological investigations have shown that carotenoids are important constituents as antioxidants and as preventing agents against cardiovascular diseases, age-related muscular degeneration, anticarcinogenic activity, and cataracts (*4*−*6*). Owing to the great interest of the role of carotenoids in relation to human health, the carotenoid composition of foods has been analyzed extensively (*7*).

Several methods for extraction, isolation, and quantification of carotenoids have been described in the literature. Basic saponification has long been an integrated part of both vitamin A (retinol) and carotenoid analyses (*7*−*16*). For vitamin A analyses, basic saponification serves to free retinol from the food matrix, convert the retinol esters to retinol, and remove bulk components such as triglycerides (*7*, *16*). In carotenoid analyses, saponification is carried out with the dual aim of eliminating chlorophylls that interfere in the spectrophotometric assay of carotenes and xanthophylls and of hydrolyzing carotenol esters and removing unwanted lipids (*9*, *10*, *17*). This step in the analysis may be carried out directly in the homogenized matrix but is also frequently performed after extraction with an organic solvent. For more fibrous vegetables, in which pigments are not directly accessible, saponification is often done after extraction to reduce, for example, the saponification time and hence the possibility for degradation of carotenoids. Saponification is usually performed with methanolic sodium or potassium hydroxide (5−30%), which is added to the pigment extract, and the mixture is stirred either in an open container under an atmosphere of nitrogen or under reflux for various time periods (*9*−*11*, *15*, *17*−*19*). A decrease in the carotenoid content is observed during saponification varying from a few percent to 100% depending on the concentration and structure of the carotenoids and on the saponification procedure (*9*−*11*, *15*).

The saponification treatment may be superfluous for plant species such as carrots, tomatoes, pumpkin, and sweet potatoes that contain carotenes as a major pigment and do not contain chlorophylls and major esterifiable groups. In addition, these plants are relatively poor in lipids, and thus saponification does not change the carotene content nor does it cause the appearance of additional α- and β-carotene isomers.

In light of this, many have chosen to avoid saponification, because even when justified, this very time-consuming step of the carotenoid analysis also may lead to degradation of the pigments being studied. A more simple and reliable saponification method therefore seems to be necessary.

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Table 1. Concentration of Major Carotenoids and Total Chlorophylls in Green Vegetables Used in Saponification Experiments[a]

vegetables	total chlorophylls[c]	total carotenoids[d]	carotenoids and total chlorophylls (μg/100 g vegetable sample)[b]							
			1	**2**	**3**	**4**	**5**	**6**	**7**	**8**
beans	8400	2090	52	135	226	152	883	84	50	503
broccoli	32 800	8230	90	1070	1220	424	3300	207	172	1750
chive	63 400	12 200	453	869	1895	292	5670	546	287	2170
green bell pepper	8700	2180	25	183	530	88	911	65	44	335
lettuce (curly)	37 700	6150	135	594	185	110	3000	90	82	1950
lettuce (feld)	56 200	11 500	94	1460	1950	86	4710	267	206	2750
lettuce (roquette)	57 500	11 500	138	1360	2070	139	4780	402	245	2340
parsley	106 000	23 200	280	824	3790	443	9990	920	564	6410
peas	11 300	3160	21	138	365	192	1700	130	79	538
spinach	52 100	15 600	75	2160	2720	205	6380	268	340	3410

[a] Mean of quadruplicates. Coefficient of variation (CV) < 5% between four replications for the carotenoids **1**–**5** and **8** and CV between 8 and 14% for the xanthophylls **6** and **7**. [b] (*all-E*)-Neoxanthin (**1**), (9′*Z*)-neoxanthin (**2**), (*all-E*)-violaxanthin (**3**), (*all-E*)-lutein epoxide (**4**), (*all-E*)-lutein (**5**), neolutein B (**6**), neolutein A (**7**), and (*all-E*)-β-carotene (**8**). [c] Sum of chlorophylls *a*, *a-1*, *a′*, *b*, *b-1*, and *b′* and pheophytins *a* and *b*. [d] Sum of the carotenoids **1**–**8**.

In the present study, we describe a very simple, gentle, and reliable saponification method of extracts from green vegetables for the quantification of carotenoids. The saponification procedure is based on hydrolysis of carotenoid esters and chlorophylls and other compounds containing esterifiable groups such as lipids using a strong basic quaternary amine styrene-divinylbenzene anion resin (Ambersep 900 OH).

MATERIALS AND METHODS

Plant Materials. Samples of fresh [green bell pepper, broccoli, lettuce (roquette, feld, and curly)] and frozen (peas, beans, parsley, chive, and spinach) vegetables were obtained from a local grocery store. Fresh vegetables were extracted on the day they were obtained whereas frozen vegetables were stored at −20 °C until use.

Chemicals. Ambersep 900 OH was obtained from Aldrich-Chemie, Steinheim, Germany. Ambersep 900 OH is a strong basic resin, which is composed of approximately 35−55% quaternary amine styrene-divinylbenzene copolymer of the OH form and 45−65% water. Acetone, ethyl acetate (EtOAc), methanol (MeOH), ethanol (EtOH), hexane, and tetrahydrofuran (THF) were of Ratborn high-performance liquid chromatography (HPLC) grade (99.8% or 99.9% HPLC grade) obtained from Aldrich-Chemie. The water used for activating Ambersep 900 OH and HPLC analysis was ultrapure generated by an Elgastat Maxima Analytica Water Purification System (Elga Ltd., United Kingdom). All eluents for HPLC were filtered through a 0.45-μm Minisart SRP 25 filter (Bie & Berntsen, Rødovre, Denmark) and were degassed with ultrasound for 20 min before use. Reference samples of (*all-E*)-lutein, (*all-E*)-β-carotene, and chlorophylls *a* and *b* were purchased from Sigma (Diesenhofen, Germany) and were used without further purification.

Extraction of Pigments. Chlorophylls and carotenoids were extracted from the plant material at room temperature under dim laboratory light. Fresh or frozen vegetable samples (2 g) were homogenized for 60 s with 18 mL cold 100% acetone with an Ultra-Turrax T25 in a centrifuge tube (30 mL) with stopper and were left for 20 min. The mixture was centrifuged for 4 min using a Sorvall SA-600 head (G_{max} 20,845; Buch & Holm, Herlev, Denmark), and the resulting supernatant was filtered through a 0.45-μm Minisart SRP 25 filter directly into the reaction vial for saponification (10 mL) and into a 4-mL brown vial (Merck Kebo Lab, Albertslund, Denmark) for HPLC analysis. Extractions were performed in quadruplicates.

Saponification. Ambersep 900 OH was washed with water using a glass filter funnel and was dried on a filter paper prior to use. The acetone extract (10 mL) was added to Ambersep 900 OH (1.0 g) and a stirring magnet in a centrifuge tube (30 mL) with stopper. After stirring for 30 min, the solution was filtered through a 0.45-μm Minisart SRP 25 filter into a 4-mL brown vial for HPLC analysis. Saponifications were performed in quadruplicates.

HPLC Analysis of Pigments. Analytical HPLC was carried out on a SUMMIT/Dionex HPLC system (Dionex Denmark A/S, Denmark) equipped with a diode array detector (DAD) operating between 300 and 700 nm. The DAD was employed at 440 nm and absorption spectra of carotenoids and chlorophylls were recorded between 300 and 700 nm. Separations were performed on a LiChrospher 100 reversed-phase (RP)-18 column (5 μm; 244 × 4 mm i.d., Merck Kebo Lab, Albertslund, Denmark) protected with a LiChrosorb RP-18 guard cartridge (5 μm; 15 × 4 mm i.d., Merck Kebo Lab, Albertslund, Denmark). The column temperature was maintained at 30 °C and the mobile phases consisted of solvent A (80% MeOH−20% H_2O) and solvent B (100% EtOAc). Separations were performed by the following solvent gradient: 0 min 20% B, 2.5 min 22.5% B, 20−22.5 min 50% B, 24−26 min 80% B, 31−34 min 100% B, 42−47 min 20% B. All increases of solvent B were linear programmed. The flow rate was 1 mL/min and the injection volume was 25 μL. Usually, the samples are analyzed within 12 h on HPLC while they are kept refrigerated (5 °C) on the HPLC-apparatus. Otherwise, the samples were kept at −20 °C until analysis within a few days. The HPLC method was validated with regard to linearity, precission, and accuracy, and no degradation of carotenoids were observed from preparation of extracts to HPLC analysis. Cis-trans isomerization of β-carotene was checked by analyzing the samples on a C-30 column (Carotenoid S-5 μm, 250 × 4.6 mm i.d., YMC/Waters, Microlab, Aarhus, Denmark) by gradient elution with solvent A (MeOH−THF (v/v), 95:5) and solvent B (H_2O−EtOH−THF (v/v/v), 90:5:5). The gradient was raised linear from 0 to 60 min with solvent B: 0 → 100% and solvent A: 100 → 0%. The flow rate was 1.5 mL/min and the injection volume was 20 μL.

Identification and Quantification of Pigments. Identification was based on chromatographic behavior on reversed-phase HPLC and visible absorption spectra (*20*). The various carotenoids and chlorophylls in the plant extracts were quantified using an external calibration method for (*all-E*)-lutein, (*all-E*)-β-carotene, and chlorophylls *a* and *b*. (*all-E*)-β-Carotene was dissolved in $CHCl_3$ (1 mg in 3 mL). The other pigments were dissolved in $CHCl_3$ (1 mg in 100 μL) to a final volume of 2 mL with 80% acetone. Several standard dilutions in 80% acetone were made from these stock solutions and the concentrations of (*all-E*)-lutein, (*all-E*)-β-carotene, and chlorophylls *a* and *b* were determined spectrophotometrically. (*all-E*)-Lutein was determined with an absorption maximum at 453 nm in dioxane (ϵ 152,000) and (*all-E*)-β-carotene with absorption maximum at 450 nm in $CHCl_3$ (ϵ 139,057) (*21*). The chlorophyll *a* and *b* concentrations in the nonsaponified extracts were calculated according to the method of Lichtenthaler (*22*), which takes into account the specific absorption coefficients of chlorophyll *a* and *b* in different solvent systems including 80% acetone. The concentration calculated from the absorbance reading was corrected for pigment purity determined by analytical HPLC. All xanthophylls were calculated relative to (*all-E*)-lutein, whereas chlorophylls *a-1*, *a′*, and pheophytin *a* were calculated relative to chlorophyll *a* and chlorophylls *b-1*, *b′*, and pheophytin *b* were calculated relative to chlorophyll *b*. The total content of chlorophylls and the total content of carotenoids as well as the content of individual carotenoids in different vegetable samples are shown in **Table 1**.

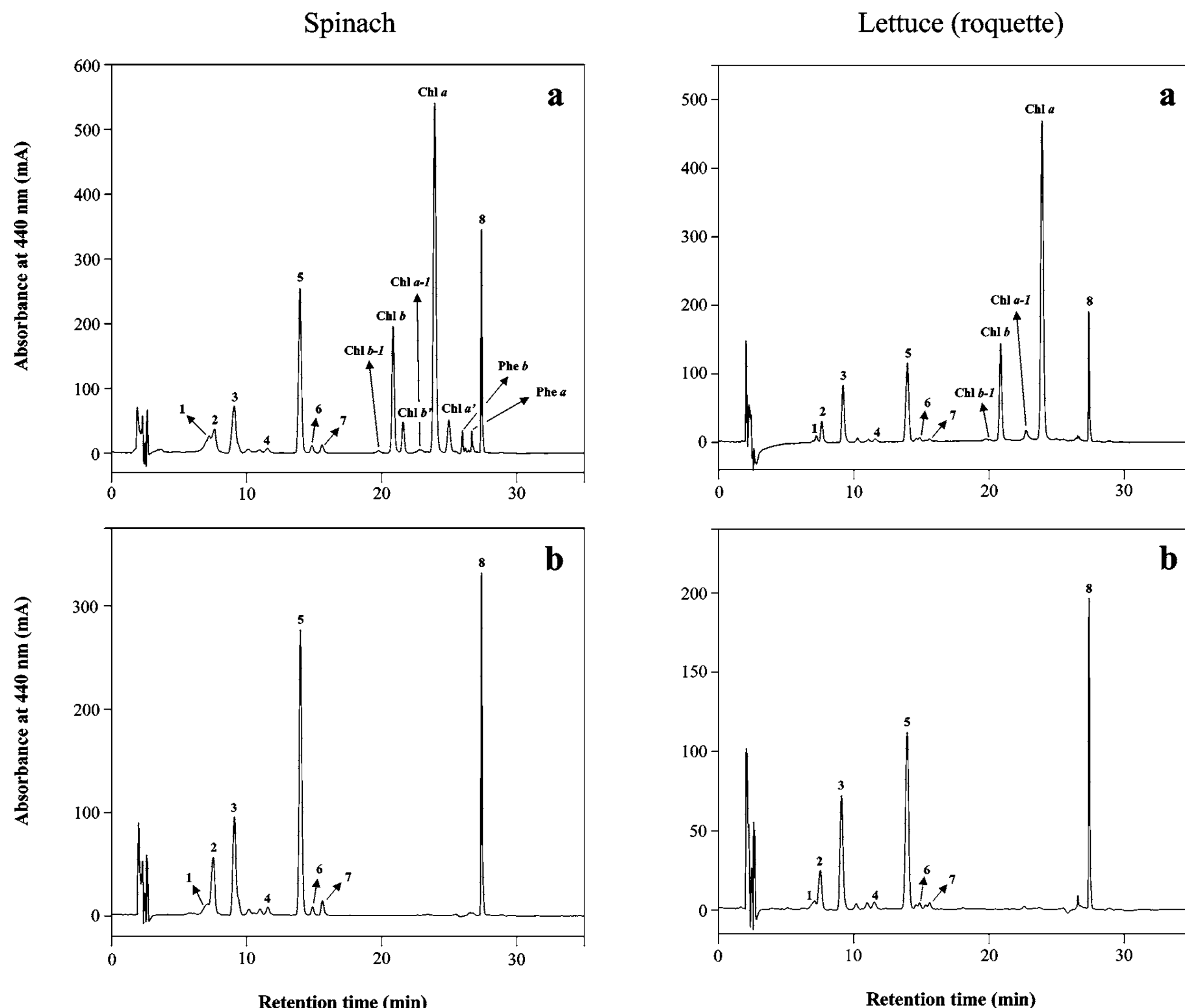

Figure 1. HPLC chromatograms of acetone extracts of spinach and lettuce (roquette), respectively, before (**a**) and after (**b**) saponification with Ambersep 900 OH. Peak identification: (*all-E*)-neoxanthin (**1**), (9′Z)-neoxanthin (**2**), (*all-E*)-violaxanthin (**3**), (*all-E*)-lutein epoxide (**4**), (*all-E*)-lutein (**5**), neolutein B (**6**), neolutein A (**7**), and (*all-E*)-β-carotene (**8**). *Chl* = chlorophyll, *Phe* = pheophytin. Chromatographic conditions described in Materials and Methods.

RESULTS AND DISCUSSION

The saponification of extracts of green vegetables usually involves adding methanolic potassium or sodium hydroxide and either refluxing the solution or leaving it under nitrogen for several hours. Afterward, the mixture needs to be extracted with water to remove the alkali salts (*9*−*11*, *15*, *17*−*19*). It is obvious that these operations are time and labor intensive. Depending on the conditions used (hydrolysis time, temperature, hydroxide concentration, number, and volume for partition and washing), carotenoids may produce artifacts, isomerize, and degrade (*9*, *10*, *15*), and losses may occur because of incomplete recovery during the various partition steps. Despite these complications, a saponification of plant extracts may still be justified to remove unwanted chlorophylls, lipids, and other interfering substances to improve resolution and to reduce HPLC analysis time.

To reduce time and costs and to improve stability of carotenoids during the saponification step, we have developed a new saponification method that is more simple and gentle compared to standard saponification procedures for carotenoid analysis of green vegetables. The extraction of the plant material was done with a single extraction of 2 g of homogenized material in 18 mL 100% acetone, as multiple extractions gave no significant improvement (greater than 5%) of the yield of carotenoids and chlorophylls (data not shown). This is in good agreement with the findings of Dunn et al. (*23*) who compared different solvent extraction methods and found that extraction with 100% acetone on leaf tissues gave no significant improvement of the yield of carotenoids and chlorophylls using either a single or a triple extraction. Further, we have used twice the amount of acetone per gram of plant tissue compared to that used in multiple extraction methods (*23*). In addition, a multiple extraction procedure is rather time-consuming with respect to mixing, centrifugation, and combining as well as to providing uncertainty to the accuracy of the analysis.

Adding a strongly basic resin (Ambersep 900 OH) to these acetone extracts was very efficient in removing chlorophylls. The extracts (before and after action of Ambersep 900 OH) were analyzed by HPLC-DAD, and the chromatograms showed complete removal of the chlorophylls (**Figure 1**), while the concentration of the carotenoids were mostly unchanged as illustrated by the relative high mean recoveries for most carotenoids (**Table 2**). During stirring, the solution changes color from green to yellow and the polymeric beads of the Ambersep 900 OH change color to light green. To avoid long reaction times, stirring (or some kind of mechanical action like shaking using an incubator shaker) is necessary, and usually it takes about 30 min to completely remove the chlorophylls from the solution (see Materials and Methods). Ultrasonic agitation was

Table 2. Recovery Percentages of Some of the Major Carotenoids in Green Vegetable Extracts after Saponification with Ambersep 900 OH Treatment[a]

vegetables	carotenoids (recoveries in %)[b]							
	1	2	3	4	5	6	7	8
beans	90 ± 5	121 ± 6	101 ± 4	108 ± 11	98 ± 4	75 ± 5	105 ± 6	97 ± 3
broccoli	95 ± 6	118 ± 6	103 ± 5	100 ± 5	100 ± 2	70 ± 9	104 ± 6	99 ± 2
chive	95 ± 9	128 ± 11	103 ± 7	105 ± 7	101 ± 2	69 ± 7	103 ± 4	99 ± 1
green bell pepper	91 ± 9	116 ± 8	102 ± 5	103 ± 7	100 ± 3	77 ± 8	102 ± 5	98 ± 2
lettuce (curly)	94 ± 8	120 ± 5	102 ± 4	106 ± 5	101 ± 3	76 ± 5	108 ± 7	102 ± 4
lettuce (feld)	92 ± 6	116 ± 6	101 ± 4	104 ± 7	100 ± 2	75 ± 6	102 ± 4	100 ± 2
lettuce (roquette)	88 ± 8	106 ± 6	102 ± 4	105 ± 8	102 ± 3	70 ± 6	101 ± 6	101 ± 1
parsley	74 ± 12	128 ± 12	108 ± 9	104 ± 4	103 ± 4	68 ± 5	108 ± 5	97 ± 3
peas	88 ± 10	118 ± 11	103 ± 5	99 ± 7	101 ± 2	68 ± 9	96 ± 7	100 ± 2
spinach	92 ± 9	116 ± 8	104 ± 4	103 ± 4	100 ± 2	72 ± 5	109 ± 7	99 ± 2
mean recovery (%)	~90	~119	~103	~104	~101	~72	~104	~99

[a] Analysis performed in quadruplicate (mean ± standard deviation). [b] (*all-E*)-Neoxanthin (**1**), (9′*Z*)-neoxanthin (**2**), (*all-E*)-violaxanthin (**3**), (*all-E*)-lutein epoxide (**4**), (*all-E*)-lutein (**5**), neolutein B (**6**), neolutein A (**7**), and (*all-E*)-*β*-carotene (**8**).

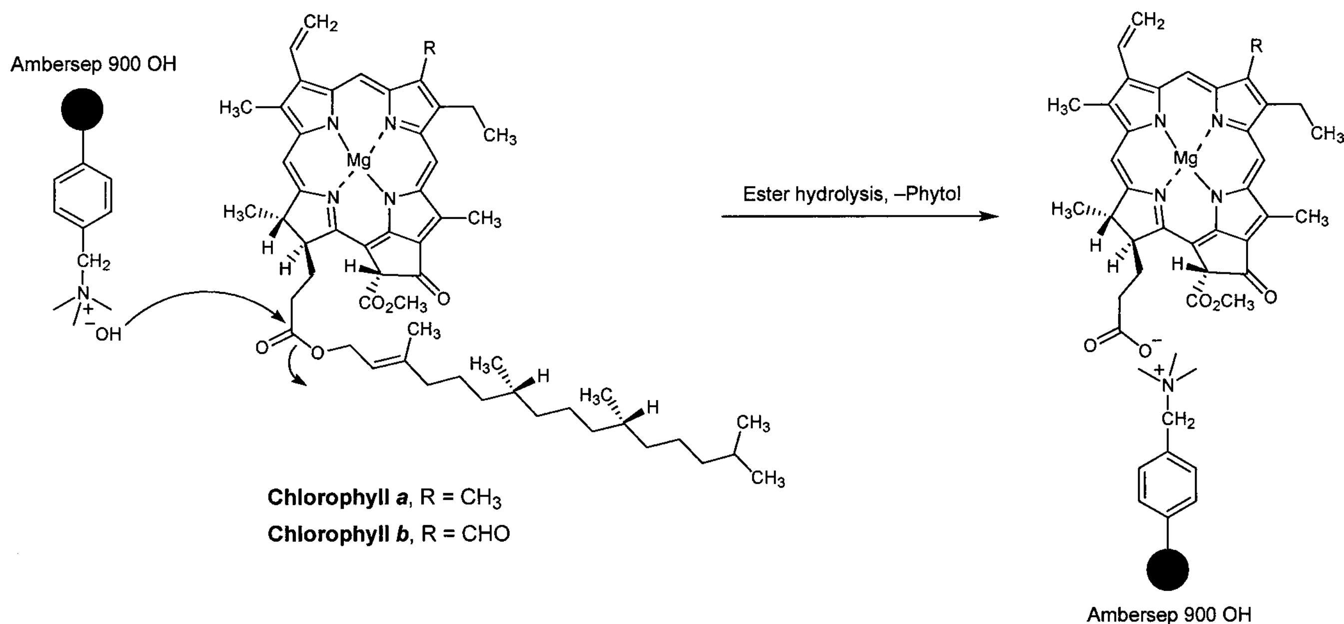

Figure 2. The most likely reaction between chlorophylls and the strong basic resin Ambersep 900 OH, which is composed of a quaternary amine styrene-divinylbenzene copolymer of the OH form.

not able to substitute this stirring/shaking step. Other extraction solvents such as tetrahydrofuran, *tert*-butyl methyl ether, or a mixture of acetone−hexane gave no reaction at all probably because of the lack of contact between the basic moieties on the resin and the chlorophylls in the solution (data not shown). A likely action of Ambersep 900 OH on the chlorophylls is shown in **Figure 2**. The hydroxide ion coordinated to the resin reacts with the carboxylic ester part of the chlorophyll molecule in a nucleophilic substitution reaction (ester hydrolysis) thereby immobilizing the chlorophyll as a salt to the resin and releasing phytol. A similar reaction should happen with any other carboxylic esters such as triglycerides of fatty acids and carotenoid esters. The extracts used in this study did not contain any carotenoid esters, as it is necessary to use a mixture of hexane−acetone to completely extract these esters while this solvent composition is not compatible with our saponification procedure. Therefore, the present saponification method is only suitable for total quantification of carotenoids in vegetables that do not contain carotenoid esters such as green vegetables.

The green vegetables we have analyzed by this method are different varieties of lettuce, peas, spinach, parsley, green bell pepper, broccoli, beans, and chive. The concentration of the major carotenoids in these vegetables before Ambersep 900 OH treatment is shown in **Table 1**. The total concentration of the chlorophylls (chlorophylls *a*, *a-1*, *a*′, *b*, *b-1*, and *b*′ and pheophytins *a* and *b*) varied from approximately 10 to 100 mg/100 g vegetable sample (**Table 1**). After Ambersep 900 OH treatment, the chlorophylls were completely removed (**Figure 1**) while leaving the major carotenoids mostly unchanged as can be seen from the recovery percentages in **Table 2**. The mean recovery percentages for most carotenoids [(*all-E*)-violaxanthin, (*all-E*)-lutein epoxide, (*all-E*)-lutein, neolutein A, and (*all-E*)-*β*-carotene] after saponification with Ambersep 900 OH were close to 100% (99−104%), while the mean recovery percentages of (9′*Z*)-neoxanthin increased to 119% and that of (*all-E*)-neoxanthin and neolutein B decreased to 90% and 72%, respectively (**Table 2**). Although the recovery percentages of most major carotenoids were close to 100%, minor conversions of some of these pigments may have occurred during saponification. It is well-known that (*all-E*)-lutein isomerizes easily to the two *Z*-isomers of lutein, neolutein A and B, and vice versa (*11*, *20*). From the present investigation, it appears that the net isomerization of these lutein isomers was in favor of (*all-E*)-lutein and neolutein A. Further, it cannot be excluded that (*all-E*)-lutein can be oxidized to (*all-E*)-lutein epoxide during saponification; however, this reaction was absent or at

least insignificant during saponification with Ambersep 900 OH. In addition, no isomerization of (*all-E*)-β-carotene to α-carotene or other *Z*-isomers of β-carotene was observed as shown by HPLC analysis on a C-30 column (see Materials and Methods). However, the relative high mean recovery for (9′*Z*)-neoxanthin of 119% indicates that some (*all-E*)-neoxanthin and minor quantities of (*all-E*)-violaxanthin and perhaps some of the minor unidentified carotenoids in the extracts are converted to (9′*Z*)-neoxanthin during saponification (**Table 2**). The observed minor transformations of single carotenoids during saponification with Ambersep 900 OH are, however, considerable lower compared to those obtained by standard saponification methods (*9*, *11*, *12*, *15*, *19*). Using a standard treatment with methanolic potassium hydroxide (*11*, *12*, *19*), the recovery percentages were comparable with those found by Khachik et al. (*11*) (data not shown) being significantly lower for all carotenoids except for β-carotene, compared to saponification with Ambersep 900 OH, because of a longer contact time with hydroxide ions resulting in oxidation and isomerization and because of many laboratory operations involving multiple extractions and evaporations.

The method described here for the selective removal of chlorophylls from extracts of green vegetables is very simple and reliable compared to the standard methods used for basic hydrolysis. This should allow more laboratories to introduce a chlorophyll removal step to shorten the time for carotenoid analysis of green vegetables on HPLC. Finally, the method may also find use for saponification of complex vegetable food matrixes for the removal of both chlorophylls and unwanted lipids.

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