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Analysis of carotenoids with emphasis on 9-*cis* β-carotene in vegetables and fruits commonly consumed in Israel

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Recent epidemiological studies have directed the attention from the synthetic all-*trans* β-carotene to natural carotenoids predominant in fruits and vegetables as possible active ingredients for prevention of cancer and cardiovascular diseases. Seventeen fruits and 17 vegetables commonly consumed in Israel and the β-carotene-rich alga, *Dunaliella bardawil*, were analysed for their content of carotenoids with emphasis on 9-*cis* β-carotene by reversed-phase, 3D photodiode array HPLC. Fourteen carotenoids were eluted in order of decreasing polarity, from polar oxycarotenoids to lipophilic hydrocarbons, and quantified in μg carotenoid per gram freeze-dried plant sample. The richest sources of total carotenoids (>100 μg/g dry weight) in Israeli fruits were pittango, mango and papaya while, in vegetables, the predominant types were carrot, dill, parsley, tomato, lettuce, sweet potato and red pepper. Red fruits and vegetables contained mainly lycopene. Yellow and orange fruits and vegetables had high contents of hydrocarbon carotenes with substantial levels of cryptoxanthins and xanthophylls. The green vegetables had high contents of both xanthophylls and hydrocarbon carotenes. Relatively high ratios (9-*cis* to all-*trans* β-carotene) of above 0.2 g/g were noted in sweet potato, papaya, parsley, lettuce, dill, apricot, pepper, prune and pumpkin, compared to the high ratio of 9-*cis* to all-*trans* β-carotene in the alga *Dunaliella* (~1.0 g/g). The high content of 9-*cis* β-carotene in certain fruits and vegetables and the wide variety of carotenoids and stereoisomers of carotenoids in all plants should shift nutritional and medical attention from the synthetic all-*trans* β-carotene toward natural carotenoids as potential candidates for chemoprevention.

INTRODUCTION

β-Carotene has been used for many years as a food colouring agent, as pro-vitamin A in food and animal feed, as an additive to cosmetics, multivitamin preparations, and in the last decade as a health food product under the claim 'anti-oxidant'. Many epidemiological and oncological studies suggest that humans fed on a diet high in carotenoid-rich vegetables and fruits, who maintain higher than average levels of serum carotenoids, have a lower incidence of several types of cancer and cardiovascular disease. Recently, the Alpha-Tocopherol, β-Carotene Cancer Prevention Study Group (ATBC), the Physicians Health Study (PHS), and the β-Carotene and Retinol Efficacy Trial (CARET) clearly showed that not only did β-carotene fail to reduce the incidence of cancer and cardiovascular disease, but in fact it increased these diseases in smokers and in workers exposed to asbestos (Albanes *et al.*, 1996; Hennekens *et al.*, 1996; Omenn *et al.*, 1996*a*,*b*; The ATBC Study, 1994). Despite the fact that the most convincing reports support a direct connection between high intake of fruits and vegetables and low incidence of cancer and cardiovascular disease, the two USA National Cancer Institutes supported studies, the ATBC Study in Finland and many earlier trials which dealt with the question of the protective role of β-carotene against chronic diseases, used only synthetic all-*trans* β-carotene and none checked the effect of natural carotenoids.

Carotenoids are a diverse group of over 600 structurally related isoprenoids biosynthesized by plants, fungi and bacteria and are divided into two main groups: the hydrocarbon carotenes and the xanthophylls or oxycarotenoids.

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Isoprenoid biosynthesis, common in nature, yields many different geometric stereoisomers of carotenoids as well as carotenoid esters produced by subsequent carotenogenesis esterification. β-Carotene is present in most higher plants in small amounts of ~0.2% of the dry weight with variable content of 9-*cis* β-carotene (Goodwin, 1988; Hart and Scott, 1995; Mueller, 1997). The alga *Dunaliella* represents an extreme case of accumulating large amounts of β-carotene to more than 10% of the algal dry weight, of which about 50% is 9-*cis* β-carotene (Ben-Amotz and Avron, 1990). The unique stereoisomeric β-carotene content in *Dunaliella* gave impetus to studies on the metabolism, storage and function of 9-*cis* β-carotene in animals and humans with emphasis on the possible role of the 9-*cis* β-carotene in scavenging reactive oxygen species (Ben-Amotz *et al.*, 1989; Ben-Amotz and Levy, 1996; Challem, 1997; Gaziano *et al.*, 1995; Johnson *et al.*, 1996; Parker, 1996; Stahl *et al.*, 1993; Suzuki *et al.*, 1996). Recently, such studies were expanded to the stereoisomeric configuration of lycopene in relation to human prostate (Clinton *et al.*, 1996), but the present available nutritional and medical studies on carotenoid geometric isomers are scarce in comparison to the related studies on all-*trans* β-carotene. Similarly, the analytical literature lacks a modern database of carotenoid stereoisomers in human-consumed vegetables and fruits, and only recently has a new database of individual carotenoids been established by the development of the more sensitive and accurate method of HPLC (Ben-Amotz, 1995; Hart and Scott, 1995; Mangles, 1993; Riso and Porrini, 1996; Wills and Rangga, 1996; Yang *et al.*, 1996). The present paper expands the analysis by HPLC diode array to carotenoids, and carotenoid stereoisomers in commonly consumed Israeli fruits and vegetables. Special emphasis is given to the detection and quantification of 9-*cis* β-carotene in the selected plants.

MATERIALS AND METHODS

Sampling and preparation of food items

Three samples of common Israeli fruits and vegetables cultivated in open fields were purchased from local markets in the Haifa area during the year 1996. Fresh samples were used by selection of about 5 g from each sample. After cutting, the samples were weighed fresh, freeze-dried for 24 h, and weighed again for determination of dry weight percentage (Table 1). Immediately after freeze-drying the samples were ground in a mortar and pestle to a plant powder with small amount of liquid nitrogen. Duplicate (from 0.5 to 1 g) aliquots of the freeze-dried ground sample were extracted by 5 ml of tetrahydrofuran (TFH) and methanol (1:1 v/v, THF:MeOH) with internal standard, β-apo-8′-carotenal or echinenone. Carotenoids were extracted easily from the ground, freeze-dried samples but, to ensure complete extraction, the process was repeated twice. Plant debris was removed by centrifugation and the two supernatants were combined. Hexane, 10 ml, was added to the combined THF/MeOH and mixed with 2 ml 10% sodium chloride in a separating funnel. After phase separation the upper hexane phase was transferred and evaporated under a stream of nitrogen at 35°C. The dried carotenoid extract was dissolved and diluted in 200 μl methylene chloride and 10 μl were injected into the 5 μl HPLC loop.

Table 1. Percentage of dry weight of Israeli fruits and vegetables as determined by freeze-drying

Fruits	% Dry weight	Vegetables	% Dry weight
Apricot	12.6	Carrot	11.3
Banana	28.3	Cucumber	4.6
Carob	85.0	Dill	15.4
Cherry	12.9	Eggplant	44.4
Dates	30.0	Kale	8.6
Grape	12.5	Lettuce	4.8
Guava	22.2	Onion, green	11.6
Mango	17.1	Parsley	10.4
Nectarine	13.4	Pepper red	9.5
Opuntia	44.0	Pepper red, hot	10.2
Papaya	18.3	Pepper yellow	7.2
Peach	12.0	Potato	20.0
Pear	16.1	Pumpkin	6.2
Persimmon	23.0	Sweet potato	15.7
Pittango	23.2	Sweet corn	32.1
Prune	13.0	Tomato	9.0
Prune (yellow)	13.1	Zucchini	42.3

HPLC system

The HPLC was based on a Waters HPLC system, Millipore, Marlborough, MA. The system included pumps 501 and 510 and a Waters 996 photodiode array detector attached to Waters Millennium 2010, Chromatography Manager, Version 2.10, run on an IBM-compatible computer connected to HP Deskjet 1200 plotter, Hewlett Packard, Avondale, PA.

The column was a Vydac 201 TP54 stainless steel column of 25 cm×4.6 mm (internal diameter) packed with C18 reversed-phase material with particle size of 5 μm and a pore size of 30 nm, The Separation Group, Hysperia, CA. The column was maintained at 30±0.2°C in HPLC Column 7955 Heater/Chiller, JonesChromatography, Glamorgan, UK. The column was protected by a 5-cm C18 ODS guard column, Shimadzu, Kyoto, Japan and with a small preguard column, a Guard-Pak, inserted with a C18 μBondapak cartridge, Waters Chromatography, Milford, PA.

Elution was performed at 30±0.2°C with an isocratic solvent, HPLC grade methanol: acetonitrile (9:1 by vol), at a constant flow of 1.0 ml/min, which is a well-documented system for distinguishing the different carotenoids and their isomers (Ben-Amotz *et al.*, 1989; Shaish *et al.*, 1990; Riso and Porrini, 1996). The mobile phase was

flushed with nitrogen to avoid air-gassing in the solvents. Samples were injected via a 7725i syringe loading sample injector fitted with a 5 μl loop, Rheodyne Inc., Cotati, CA.

Peak responses were measured and assessed at maximum using the photodiode array and detected by the Millennium 3-D "Max" absorption, as described previously (Ben-Amotz, 1995). Peak responses of carotenoids were measured at selected channel wavelength of 450 nm. Quantification of the HPLC data was done by Excel (Microsoft, USA).

Standards

Synthetic lutein, zeaxanthin, canthaxanthin, β-apo-8′-carotenal, β-cryptoxanthin, echinenone, all-*trans*-β-carotene, 15-*cis* β-carotene, all-*trans* γ-carotene and all-*trans* ξ-carotene were provided by Hoffmann-La Roche, Basle, Switzerland. Lutein from alfalfa, lycopene from tomato, α-carotene from carrot, 9-*cis* β-carotene, neoxanthin, violaxanthin and zeaxanthin from *Dunaliella bardawil* (Ben-Amotz and Levy, 1996). All standards were kept at −70°C under nitrogen, dried by a stream of nitrogen before being analysed and injected into the HPLC system in methylene chloride. The concentration of the standards was determined by spectral measurement and calculated using the appropriate extinction coefficients in ethanol (Bauernfeind, 1981).

β-Apo-8′ carotenal or echinenone were used as internal standards in all runs, depending on the carotenoid profile of the specific plant. A 2.5 μg aliquot of standard was added to the freeze-dried plant sample with THF:MeOH and then the internal standard was extracted similarly as described above for injection into the HPLC at 125 ng/5 μl. No loss in the quantity of the internal standard was noted along the extraction process; nevertheless, linearity was ensured by using three concentrations of the standard.

RESULTS AND DISCUSSION

One example of the 3D chromatogram obtained from separation of the different selected vegetables and fruits by reversed phase HPLC is illustrated for apricot in Fig. 1. The 3D chromatogram detected and memorized

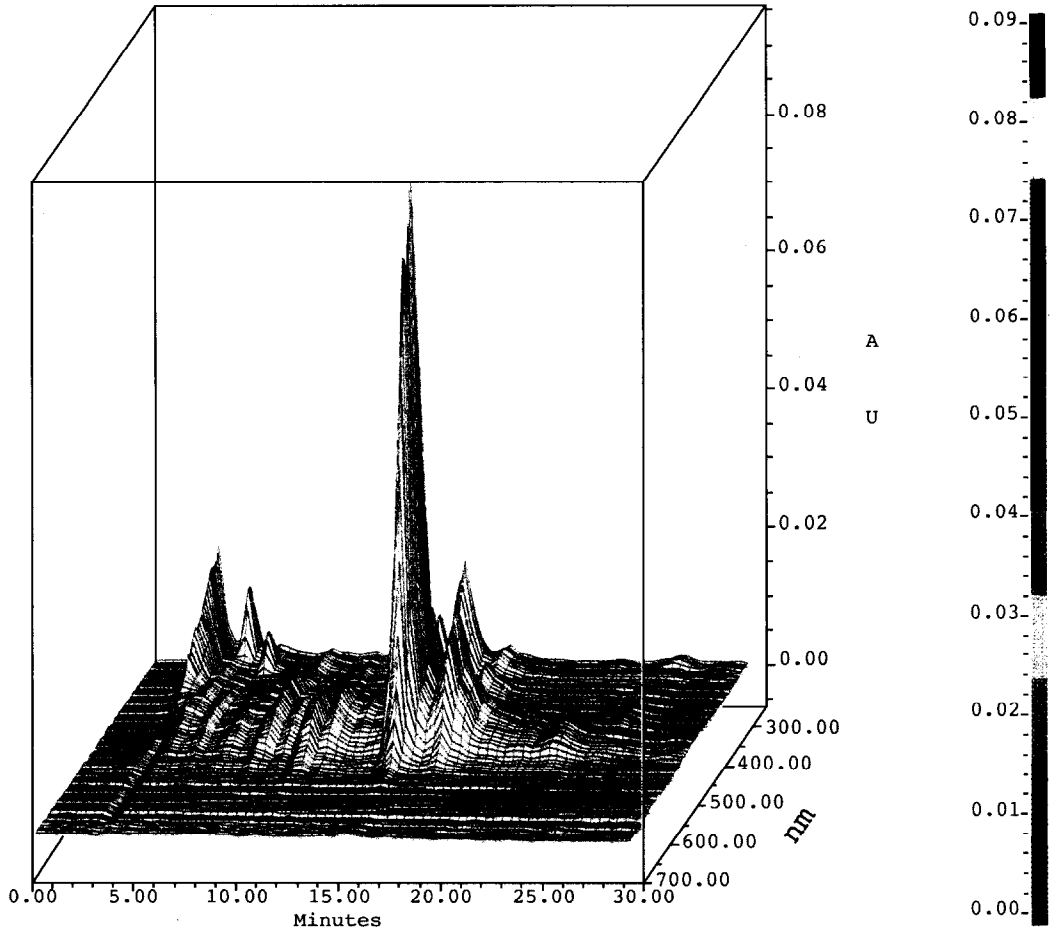

Fig. 1. Three-dimensional HPLC profile of an extract of freeze-dried apricot. Elution was performed at 30 ± 0.2°C with an isocratic solvent, HPLC grade methanol: acetonitrile (9:1 by vol), at a constant flow of 1.0 ml/min. Absorbance was monitored from 250 to 700 nm at a resolution of 1.2 nm.

all components absorbing over a wide range of spectra from 250 to 700 nm, by the Chromatogram Manager for post-run analysis of each eluted peak at its absorption maxima. A total of 14 different prominent peaks were noted in the selected plants at 450 nm, a channel closed to the absorption maximum of all carotenoids. Identification of these peaks, as described before (Ben-Amotz, 1995), was based on comparison of unknown peaks to authentic standards in terms of elution time and absorption profile. The absorption maxima of all available standards were initially memorized in the HPLC database and were compared by overlay to each unknown peak. Thus, the peak presenting absorption maxima, λmax = 274.6, 417.0, 450.8, 475.1 nm, and the following peak of λmax = 268.4, 341.5, 436.4, 446.0, 472.7 nm were identified as all-*trans* β-carotene and 9-*cis* β-carotene, respectively. Our identification was confirmed by the elution time of each peak on the reversed phase column. The components were eluted in order of decreasing polarity from polar oxycarotenoids to lipophilic hydrocarbons. Lutein, β-cryptoxanthin, β-carotene and lycopene, the prominent carotenoids, were detected under the employed running conditions at 5.0, 6.8, 14.5 and 17 min, respectively. Lutein, the widely distributed xanthophyll, exhibited a clear λmax = 269.9, 331.9, 446.1, 475.2 nm. The other prominent xanthophylls were neoxanthin, violaxanthin, and zeaxanthin, eluted around 5 min with detected *cis* stereoisomers to violaxanthin and zeaxanthin. The semi-polar carotenoids were eluted at around 7 min and were identified as α-cryptoxanthin, β-cryptoxanthin (λmax = 274.7, 426.8, 450.8, 480.1 nm), *cis* β-cryptoxanthin and β-zeacarotene, while the last non-polar hydrocarbons eluting above 13 min were α-carotene, all-*trans* β-carotene, 9-*cis* β-carotene and lycopene (λmax = 293.7, 446.1, *470.3*, 504.4 nm).

Quantification of the data in general followed the procedure reported recently by Hart and Scott (1995) using, for internal standards, either echinenone or β-apo-8′-carotenal, depending on the tested sample. Response factors relative to internal standard for individual carotenoids were established from the response equivalent to 1 μg/ml, for individual carotenoids, to the response equivalent to 1 μg/ml of internal standard. The peak area of the internal standard was monitored for freshness of the solution and changed on observed variability. The reproducibility and extraction efficiency were routinely checked by the standard quantity during the extraction and analysis, from the freeze-dried plant to the HPLC injection.

Table 2 shows the carotenoid contents of fruits commonly consumed in Israel. Rich sources of carotenoids (of over 40 μg/g dry weight) were pittango, mango, papaya, guava, persimmon and prune. Pittango, an uncommon edible fruit, and red guava had the highest content of lycopene, while mango and persimmon were highest for β-carotene. High contents of the xanthophylls, neoxanthin, violaxanthin, lutein and zeaxanthin were detected in mango, papaya, pear and prune, while the cryptoxanthins were high in mango, papaya and pear. 9-*cis* β-Carotene was prominent in papaya, prune and persimmon, less in apricot and pittango and below detection in other fruits. The ratio of 9-*cis* to all-*trans* β-carotene in papaya and prune was high, 0.66 and 0.22, respectively.

Table 3 shows the carotenoid contents of vegetables commonly consumed in Israel with reference to the β-carotene-rich alga, *Dunaliella*. Plants with the highest content of total carotenoids in decreasing order were *Dunaliella*, carrot, dill, parsley, tomato, lettuce, sweet potato and red pepper. β-Carotene content generally followed this order and represented the major carotenoid in carrot, dill, parsley and sweet potato. In contrast to fruits, where the content of 9-*cis* β-carotene was low, all the β-carotene-containing vegetables presented high ratios of 9-*cis* β-carotene to all-*trans*, ranging from 0.81 in sweet potato, 0.48 in parsley, 0.33 in lettuce, down to 0.21 in red pepper. Obviously many vegetables do have a high stereoisomeric ratio of β-carotene with varying amounts of 9-*cis* β-carotene as the predominant component. The lack of literature information on the content of 9-*cis* β-carotene in plants may be related to previous use of non-appropriate analytical conditions for identification of 9-*cis* β-carotene and other stereoisomers of carotenoids.

Good sources of xanthophylls were carrot, dill, lettuce, red pepper and tomato; and good origins of cryptoxanthins were dill, lettuce, parsley, red pepper, sweet potato, tomato and zucchini, in agreement with previously published data (Mangles, 1993; Hart and Scott, 1995; Mueller, 1997). Our 3D HPLC analysis detected, in all fruits and vegetables, varying contents of phytoene, phytofluene, their stereoisomers and other intermediates of carotenogenesis, to be reported elsewhere. *Dunaliella* contained, in addition to the extreme content of β-carotene, high amounts of xanthophylls but not cryptoxanthins.

Experimental nutritional medical studies with natural carotenoids, isomers of carotenes and carotenoids originating from different plant sources, have been limited, and such research is only now stimulated by the negative results of the recent CARET, ATBC and PHS studies (Hennekens *et al.*, 1996; Omenn, 1997; Challem, 1997). The present study, and related previously published studies on natural carotenoids in plants, show that, even within the scope of β-carotene itself, more attention should be paid to the stereoisomeric configuration. The possible metabolic conversion of 9-*cis* β-carotene to 9-*cis* retinoic acid was recently reported (Wang *et al.*, 1994) and it is now considered as a potential medicine in certain types of tumours (Mertz *et al.*, 1997). Similar attention should be given to carotenoid esters, which have different physicochemical features from the saponified carotenes and which have not yet been studied nutritionally or clinically.

Table 2. The carotenoid contents of Israeli fruits (μg/g dry weight)[a]

Fruit	Neox	Viol	*cis*-Viol	Lutein	Zeax	*cis*-Zeax	α-Cryp	β-Cryp	*cis*-β-Cryp	β-Zea carot	α-Carot	β-Carot	9-*cis* β-Carot	Lycop	TOTAL
Apricot	0.1	0.1	0.2	0.1	0.1	0.1	0.2	—	0.5	0.7	—	16.0	4.4	—	22.5
Banana	0.3	0.4	0.5	0.4	1.8	0.8	0.7	0.9	0.5	11.1	5.7	—	0.3	—	23.3
Carob	0.1	0.3	0.1	0.2	0.3	0.2	—	—	—	0.1	—	0.7	—	—	2.0
Cherry	0.1	0.1	0.1	0.1	0.2	0.1	0.1	—	0.1	0.1	0.2	0.6	—	0.3	1.9
Date	—	—	0.1	—	1.1	—	—	—	—	0.3	0.1	0.6	—	—	2.2
Grape	—	0.3	0.1	0.1	0.5	0.3	—	—	0.1	0.1	0.2	6.0	—	0.3	8.0
Guava	—	—	—	2.7	—	—	0.8	4.9	1.0	4.9	—	49.6	—	9.0	73.0
Mango	5.9	10.4	9.2	25.8	22.5	—	—	26.4	—	—	6.7	49.3	—	—	156
Nectarine	0.1	0.4	0.2	1.5	—	—	0.4	—	0.6	0.1	1.2	8.4	—	2.0	15.0
Opuntia	0.1	0.1	0.5	4.6	—	0.5	1.3	—	—	0.1	—	0.1	—	—	7.2
Papaya	2.0	3.9	2.2	7.1	4.6	2.9	16.5	24.3	8.3	7.5	5.6	10.6	7.0	—	103
Peach	0.1	0.1	0.1	0.1	0.2	—	—	0.2	—	0.2	—	0.1	—	—	1.1
Pear	0.8	1.6	1.7	1.2	10.1	0.8	8.6	—	—	0.1	—	5.1	—	—	29.9
Persimmon	—	—	1.7	—	—	—	—	—	—	—	5.1	52.8	3.2	—	62.8
Pittango	0.5	2.3	0.3	0.9	4.5	0.9	6.2	—	—	—	—	8.0	0.7	532.1	556
Prune	2.1	2.2	2.6	2.2	4.1	2.9	2.0	2.8	—	0.1	0.8	17.4	3.8	—	42.9
Prune (yellow)	2.3	2.3	1.9	2.3	0.1	—	—	—	1.4	0.2	2.1	4.7	—	1.1	18.5

[a]Neox, neoxanthin; Viol, violaxanthin; Zeax, zeaxanthin; Cryp, cryptoxanthin; β-Zea carot, β-zeacarotene; α-Carot, α-carotene; β-Carot, β-carotene; Lycop, lycopene.

Table 3. The carotenoids contents of Israeli vegetables and *Dunaliella* (μg/g dry weight)[a]

Vegetable	Neox	Viol	*cis*-Viol	Lutein	Zeax	*cis*-Zeax	α-Cryp	β-Cryp	*cis*-β-Cryp	β-Zea carot	α-Carot	β-Carot	9-*cis* β-Carot	Lycop	TOTAL
Cabbage	—	0.1	0.2	0.5	—	—	—	1.3	0.3	—	—	0.4	—	—	2.8
Carrot	0.5	0.1	9.0	7.8	57.4	7.8	0.6	1.4	—	10.1	425	1030	57.1	—	1608
Cucumber	0.4	0.6	3.8	0.5	3.9	—	—	—	2.3	0.4	16.8	2.9	—	—	31.6
Dill	38.5	1.7	10.4	341	40.0	—	159	37.5	23.7	5.1	12.2	99.8	27.7	—	811
Dunaliella	65.9	77.9	293	970	599	205	—	—	—	820	3092	38 500	37 800	—	82 622
Eggplant	2.0	—	—	9.6	1.3	—	4.8	2.0	1.0	2.0	2.3	—	—	27.4	52.3
Lettuce	1.1	2.2	1.7	13.1	1.1	—	20.0	—	11.5	—	0.3	104	41.0	—	198
Onion, green	0.2	0.7	0.2	3.0	—	0.4	1.0	—	0.3	—	0.3	0.1	—	—	6.4
Parsley	0.5	1.6	2.0	1.5	8.3	2.7	33.8	—	13.6	23.7	27.4	229	111.2	—	455
Pepper red	3.6	—	27.1	3.2	15.8	—	—	12.4	9.0	14.4	21.3	26.5	6.0	5.5	145
Pepper red, hot	0.2	0.3	1.3	0.2	0.6	—	—	1.7	0.6	—	0.2	45.1	9.6	4.1	59.8
Potato	—	—	0.5	0.5	0.2	0.1	—	2.1	0.2	—	—	0.3	—	—	3.9
Pumpkin	0.1	0.5	—	1.4	—	0.2	0.8	0.6	0.7	2.3	5.4	9.7	2.7	0.6	25.2
Sweet potato	0.3	0.6	1.0	0.2	0.2	0.2	3.8	6.0	21.8	8.1	—	79.8	65.1	—	187
Sweet corn	0.4	1.1	1.6	4.3	6.3	1.6	—	—	0.3	—	0.1	0.2	—	—	15.8
Tomato	7.6	9.0	15.3	14.3	—	—	5.3	5.0	—	—	—	14.5	—	243.1	314
Zucchini	0.9	0.6	—	33.2	—	4.5	10.4	—	5.4	—	0.1	1.3	—	0.3	56.6

[a]Abbreviations, see Table 2.

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