

## Incorporation of carotenoids from paprika oleoresin into human chylomicrons

Antonio Pérez-Gálvez<sup>1</sup>, Hans D. Martin<sup>2</sup>, Helmut Sies<sup>1</sup> and Wilhelm Stahl<sup>1\*</sup>

<sup>1</sup>*Institute for Physiological Chemistry I and*

<sup>2</sup>*Institute for Organic Chemistry I, Heinrich-Heine-University Düsseldorf, Düsseldorf, Germany*

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The intake of a carotenoid-rich diet is epidemiologically related to a lower risk for different chronic disorders like cardiovascular disease, some types of cancer or age-related macular degeneration. Red pepper (*Capsicum annuum* L.) and its dietary products contain a variety of carotenoids, which may contribute to the carotenoid pattern of human blood and tissues. The objective of the present study was to assess the availability of carotenoids from paprika oleoresin, including zeaxanthin,  $\beta$ -cryptoxanthin,  $\beta$ -carotene and the paprika-specific oxocarotenoids capsanthin and capsorubin. After overnight fasting, the volunteers ( $n$  9) ingested a single dose of the paprika oleoresin containing 6.4 mg zeaxanthin, 4.2 mg  $\beta$ -cryptoxanthin, 6.2 mg  $\beta$ -carotene, 35.0 mg capsanthin and 2.0 mg capsorubin. At different time points the carotenoid pattern in the chylomicron fraction was analysed to evaluate carotenoid absorption. From the major carotenoids present in the paprika oleoresin only zeaxanthin,  $\beta$ -cryptoxanthin and  $\beta$ -carotene were detectable in considerable amounts. Although the xanthophylls in paprika oleoresin were mainly present as mono- or di-esters, only free zeaxanthin and  $\beta$ -cryptoxanthin were found in human samples. The bioavailability of the pepper-specific carotenoids capsanthin and capsorubin from paprika oleoresin is very low. However, oleoresin is a suitable source for the provitamin A carotenoids  $\beta$ -carotene and  $\beta$ -cryptoxanthin and the macular pigment zeaxanthin.

### Bioavailability: Paprika carotenoids: Xanthophyll esters

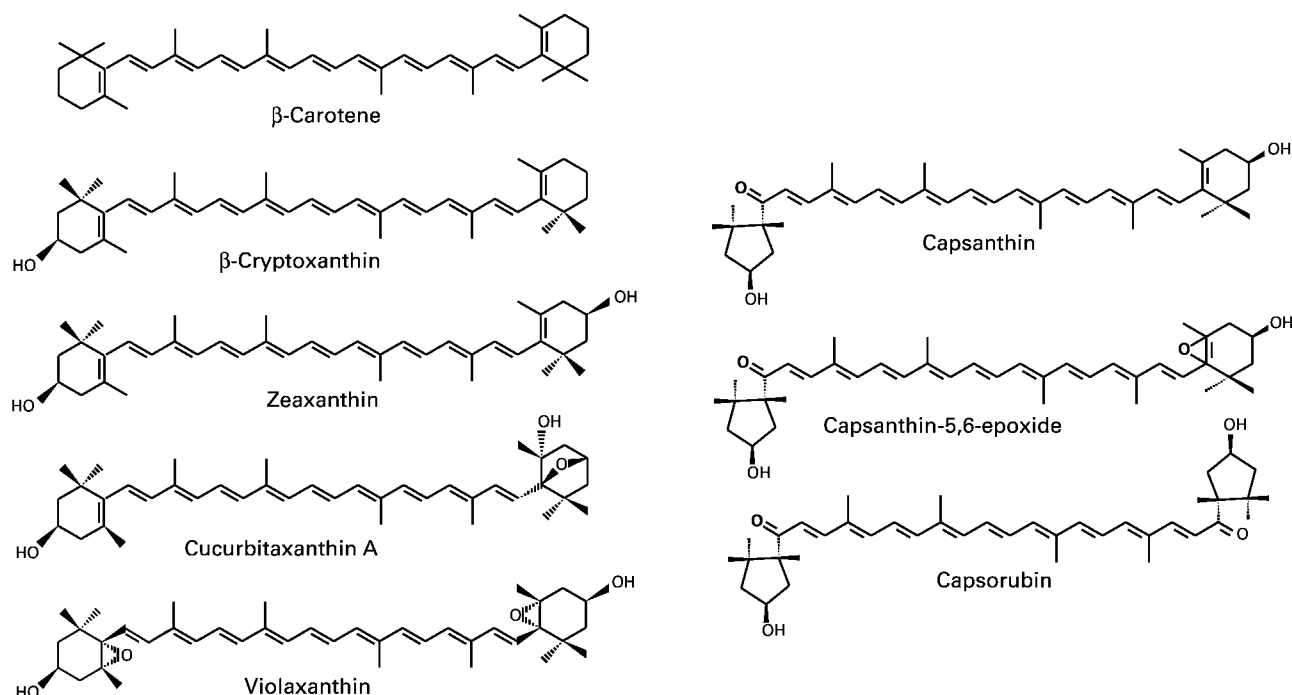
Paprika oleoresin is a product derived from red pepper (*Capsicum annuum* L.) (Mínguez-Mosquera & Pérez-Gálvez, 1998a). It is an oil with high amounts of carotenoids, commonly used in the food industry as a colourant for sauces, soups, or meat-based meals. It is also used in cosmetic products such as liquid emulsions and creams (Govindarajan, 1986). The colouration capacity of paprika oleoresin is due to its high content of carotenoids, which represent the pigment pattern of red pepper. This includes seven carotenoids, of which capsanthin, epoxy-capsanthin and capsorubin are exclusively synthesised in red pepper (Curl, 1962; Davies *et al.* 1970). In addition,  $\beta$ -carotene and  $\beta$ -cryptoxanthin, both provitamin A carotenoids, and the dihydroxylated xanthophylls zeaxanthin, cucurbitaxanthin A and violaxanthin are present. Fig. 1 shows the structure of the carotenoids present in the fruit and its products paprika and paprika oleoresin. The xanthophylls are almost completely esterified with

fatty acids, saturated fatty acids in the case of capsanthin and capsorubin; mainly unsaturated fatty acids are conjugated to the other xanthophylls (Philip *et al.* 1971; Camara & Moneger, 1978; Mínguez-Mosquera & Hornero-Méndez, 1994).

Carotenoids present in paprika oleoresin may be absorbed from the diet. In this case, not only carotenoids with provitamin A activity are available, but also carotenoids with particular structural features that might exhibit biological properties, including antioxidant and non-antioxidant activities (Britton, 1995; Stahl *et al.* 2002a). The oxocarotenoids capsanthin and capsorubin exhibit greater antioxidant capacity than other xanthophylls (Pérez-Gálvez & Mínguez-Mosquera, 2002). This was attributed to structure-related properties, particularly the presence of the keto groups, which, similarly to other oxocarotenoids, improve antioxidant activity by lowering the rate of autoxidation (Terao, 1989; Martin *et al.* 1999;

**Abbreviation:** AUC, area under the time–response curve.

\* **Corresponding author:** Professor Wilhelm Stahl, fax +49 211 811 3029, email wilhelm.stahl@uni-duesseldorf.de



**Fig. 1.** Structures of the carotenoids present in red pepper (*Capsicum annuum* L.) and in its processed products, paprika and paprika oleoresins.

Beutner *et al.* 2001). Paprika oleoresin also contains considerable amounts of zeaxanthin, a carotenoid that together with lutein forms the human macular pigment. The macular carotenoids are suggested to play a role in the protection against macular degeneration, a common eye disease in the elderly (Mares-Perlman *et al.* 2002).

The processes involved in carotenoid uptake and transport in the human organism are complex and are only partially understood. Absorption is influenced by various factors, including structural features, matrix effects, fibre consumption, availability of additional dietary fat components, the vitamin A status of the organism or food processing (Parker, 1997; van Het Hof *et al.* 2000; Stahl *et al.* 2002b). After ingestion, carotenoids are incorporated into micelles formed from lipids and bile acids. The carotenoid-loaded micelles are transferred into intestinal mucosa cells, where part of the carotenoids and retinyl esters are assembled into chylomicrons and transported to the blood via the lymphatic system. The analysis of the carotenoid pattern in the chylomicron fraction of blood plasma at different time points allows investigation of absorption processes.

## Subjects and methods

### Materials

Paprika oleoresin was kindly provided by EVESA (La Línea de la Concepción, Cádiz, Spain). All other chemicals were obtained from Merck (Darmstadt, Germany) with analytical or HPLC grade purity. The raw material is an oil rich in carotenoids with the xanthophylls almost completely esterified with fatty acids (Mínguez-Mosquera & Hornero-Méndez, 1994). The analysis of the carotenoid

concentration was done according to Mínguez-Mosquera & Hornero-Méndez (1993);  $\beta$ -apo-8'-carotenol was used as internal standard. The method for ester cleavage was published previously by Mínguez-Mosquera & Pérez-Gálvez (1998b). The procedure does not affect the stability of the carotenoids: recovery was about 90%. For ester cleavage, a sample of 25 mg was dissolved in 50 ml diethyl ether and 25 ml KOH (100 g/l methanol) was added. An appropriate amount of the internal standard was added for subsequent quantification. After complete de-esterification (1 h at room temperature) 200 ml NaCl (100 g/l water) was added and the aqueous and organic phases were allowed to separate. The aqueous phase was discarded, and the organic phase was washed several times with 200 ml distilled water until neutral pH. The organic phase, containing the carotenoids, was filtered through a solid bed of anhydrous sodium sulfate, and the filtrate was evaporated to dryness under vacuum. The residue was dissolved in 10 ml acetone (HPLC grade) and stored at  $-20^{\circ}\text{C}$  until analysis by HPLC.

### Study design

Nine healthy volunteers (four female and five male, 23–31 years old, non-smokers) took part in the study. They fasted overnight before the start of the study; during the study they consumed a controlled diet with negligible amounts of carotenoids and fat. Oleoresin (1 g) was ingested together with 5 g oil and white bread. The dose of individual carotenoids ingested is given in detail in Table 1. The study was in accordance with the Helsinki Declaration, updated in Tokyo, Japan in 1975. All subjects gave their informed consent for all procedures.

**Table 1.** Carotenoid pattern of paprika oleoresin used in the study\*  
(Mean values with their standard errors for four analyses)

Carotenoid	mg/g of sample†		%	
	Mean	SEM	Mean	SEM
Capsorubin	2.04	0.04	3.27	0.04
Violaxanthin	2.38	0.08	3.82	0.07
Capsanthin-5,6-epoxide	1.83	0.11	2.93	0.11
Capsanthin	24.6	0.44	39.4	0.41
9- <i>cis</i> -Capsanthin	10.4	0.29	16.7	0.26
Curcubitaxanthin A	4.25	0.03	6.81	0.03
Zeaxanthin	5.29	0.03	8.48	0.03
9- <i>cis</i> -Zeaxanthin	1.11	0.02	1.78	0.02
$\beta$ -Cryptoxanthin	4.24	0.06	6.80	0.06
$\beta$ -Carotene	6.23	0.08	9.99	0.07
Total carotenoids	62.4	0.06	100	

\* Paprika oleoresin was provided by EVESA (La Línea de la Concepción, Cádiz, Spain).

† Since 1 g paprika oleoresin was ingested as a single dose, these values also represent the doses of individual carotenoids consumed in a single ingestion by each volunteer.

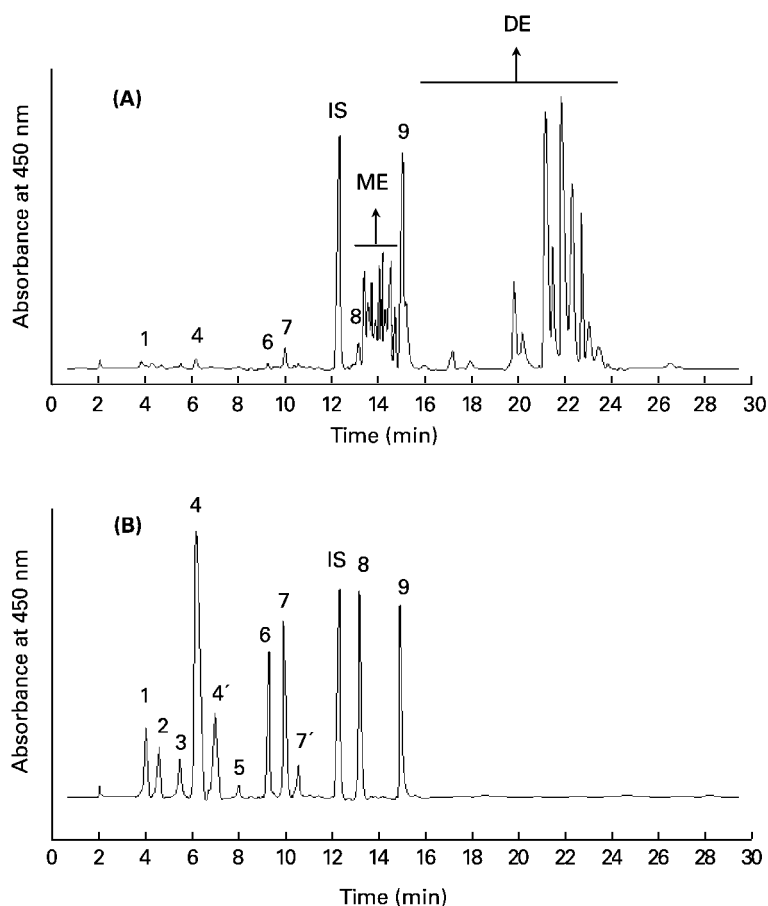
#### Sample preparation for chylomicrons

Blood (10 ml) was taken just before (0 h) and 2, 4, 5, 6, 7, 9 and 12 h after intake of the paprika oleoresin. Blood

samples were centrifuged after clotting (30 min in the dark at room temperature) for 10 min at 2000 *g* at 4°C to obtain serum. Chylomicrons were prepared according to the method described by Terpstra (1985). Serum (2 ml) was mixed with KBr (770 mg), sucrose (50 mg) and ethylene glycol (200  $\mu$ l). The mixture was overlaid with 2 ml KBr solution ( $\delta$  1.225 kg/l), 4 ml KBr ( $\delta$  1.100 kg/l) and 4 ml distilled water. Chylomicrons were isolated after ultracentrifugation (100 000 *g* at 20°C for 40 min) and stored at -70°C until analysis.

#### Sample preparation for HPLC

All the following steps were performed under diminished light. An appropriate amount of the sample (500  $\mu$ l chylomicron preparation) was mixed with 1 ml buffer (2 mM-KH<sub>2</sub>PO<sub>4</sub>-K<sub>2</sub>HPO<sub>4</sub> and 0.7 mM-EDTA, pH 7.2), 1 ml ethanol and  $\beta$ -apo-8'-carotenol as internal standard. The carotenoids were extracted with 6 ml hexane-dichloromethane (5:1, v/v). This mixture was vortexed for 1 min, sonicated for 5 min and vortexed again for 1 min and finally centrifuged for 10 min at 2000 *g* at 20°C. A portion of the upper phase (5 ml) was withdrawn and the solvent evaporated under N<sub>2</sub>. The extract was dissolved in 100  $\mu$ l



**Fig. 2.** HPLC chromatograms of paprika oleoresin before (A) and after (B) esters hydrolysis. Peak identification: 1, capsorubin; 2, violaxanthin; 3, capsanthin-5,6-epoxide; 4, capsanthin; 4', 9-*cis*-capsanthin; 5, mutatoxanthin; 6, cucurbitaxanthin A; 7, zeaxanthin; 7', 9-*cis*-zeaxanthin; 8,  $\beta$ -cryptoxanthin; 9,  $\beta$ -carotene. IS, internal standard; ME, mono-esterified xanthophylls; DE, di-esterified xanthophylls. For details of procedures, see p. 788.

acetone and stored at  $-20^{\circ}\text{C}$  until analysis by HPLC; HPLC analysis was performed within 1 week.

#### Carotenoid quantification by HPLC

The chromatographic separation was performed on a reversed-phase column (Spherisorb  $\text{C}_{18}$  ODS2, particle size  $5\ \mu\text{m}$ ,  $250\ \text{mm} \times 4\ \text{mm}$ ; Merck KGaA, Darmstadt, Germany). The eluent comprises a binary gradient at a constant flow of  $1.5\ \text{ml/min}$  and detection at  $450\ \text{nm}$ . The initial composition of the eluent (acetone– $\text{H}_2\text{O}$  (75:25, v/v)) is held for 5 min. A linear gradient is then applied for 5 min to yield a final composition of acetone– $\text{H}_2\text{O}$  of 95:5 (v/v). This composition is held for 7 min. Finally the column is washed for 3 min with acetone. Carotenoids present in the chylomicron fraction were identified by their spectral and chromatographic characteristics. For comparison, purified standards were used, isolated from red pepper (*Capsicum annuum* L.). For quantification the internal standard method was applied, using  $\beta$ -apo-8'-carotenol. Standards isolation, chromatographic separation and carotenoid quantification are described in detail by Mínguez-Mosquera & Hornero-Méndez (1993).

#### Statistical analysis

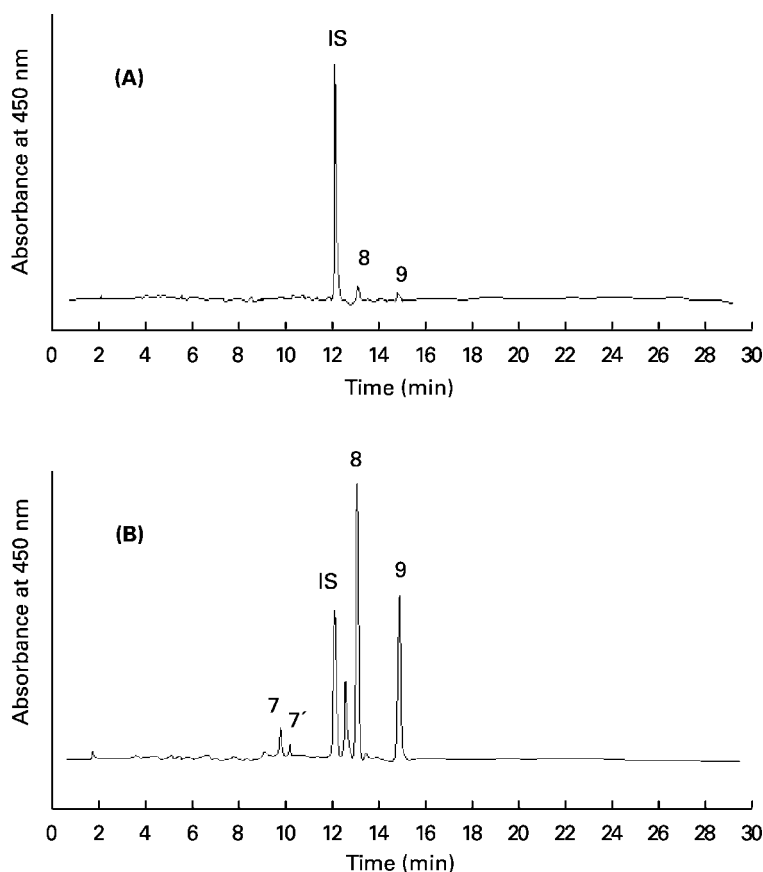
The area under the time–response curve ( $\text{AUC}_{0-12\text{h}}$ ) for each carotenoid was calculated using trapezoidal

approximation. Values in the text are means with their standard errors. Data were normally distributed (Kolmogorov–Smirnov test) and analysed parametrically. For each carotenoid detected in the chylomicron extracts, mean values at each time point were compared to test for significant differences (Student's *t* test). Significance was set at  $P < 0.01$ . The statistical analysis was performed with a statistical software package (STATISTICA for Windows, 5.5, 1999; Statsoft, Inc., Tulsa, OK, USA).

#### Results

The carotenoid pattern of the paprika oleoresin used in the present study and the amounts of single carotenoids applied are summarized in Table 1. The major carotenoid is capsanthin with about 56 % total carotenoid content in the sample.  $\beta$ -Carotene and zeaxanthin contribute about 10 % to the total amount, followed by  $\beta$ -cryptoxanthin and cucurbitaxanthin A with about 7 %. Capsorubin is also present but in minor amounts (3 % total carotenoids) as others like violaxanthin or capsanthin-5,6-epoxide. Fig. 2 shows the HPLC traces of the carotenoid pattern in paprika oleoresin before (Fig. 2(A)) and after hydrolysis (Fig. 2(B)). Only small amounts of free (unesterified) xanthophylls are present (retention time 2–10 min, Fig. 2(A)). About 30 % xanthophylls are present as monoesters and about 60 % as diesters/g.

Fig. 3 shows the HPLC chromatograms of carotenoids in chylomicrons obtained from one individual before (*t* 0)



**Fig. 3.** Chromatograms of chylomicron samples from one subject before (A) and 6 h after (B) ingestion of paprika oleoresin. Peak identification: 7, zeaxanthin; 7', 9-*cis*-zeaxanthin; 8,  $\beta$ -cryptoxanthin; 9,  $\beta$ -carotene. IS, internal standard. For details of procedures, see p. 788.

(Fig. 3(A)) and at  $t$  6 h (Fig. 3(B)) after ingestion of the paprika oleoresin. From the carotenoids present in the paprika oleoresin (see Fig. 2) only  $\beta$ -carotene,  $\beta$ -cryptoxanthin and zeaxanthin were detectable in chylomicrons; the xanthophylls present were unesterified. No significant amounts of other free xanthophylls or their esters were detected in chylomicrons. Very low concentrations of capsanthin below the limit of quantification ( $<0.5$  nmol/l) were found in two subjects.

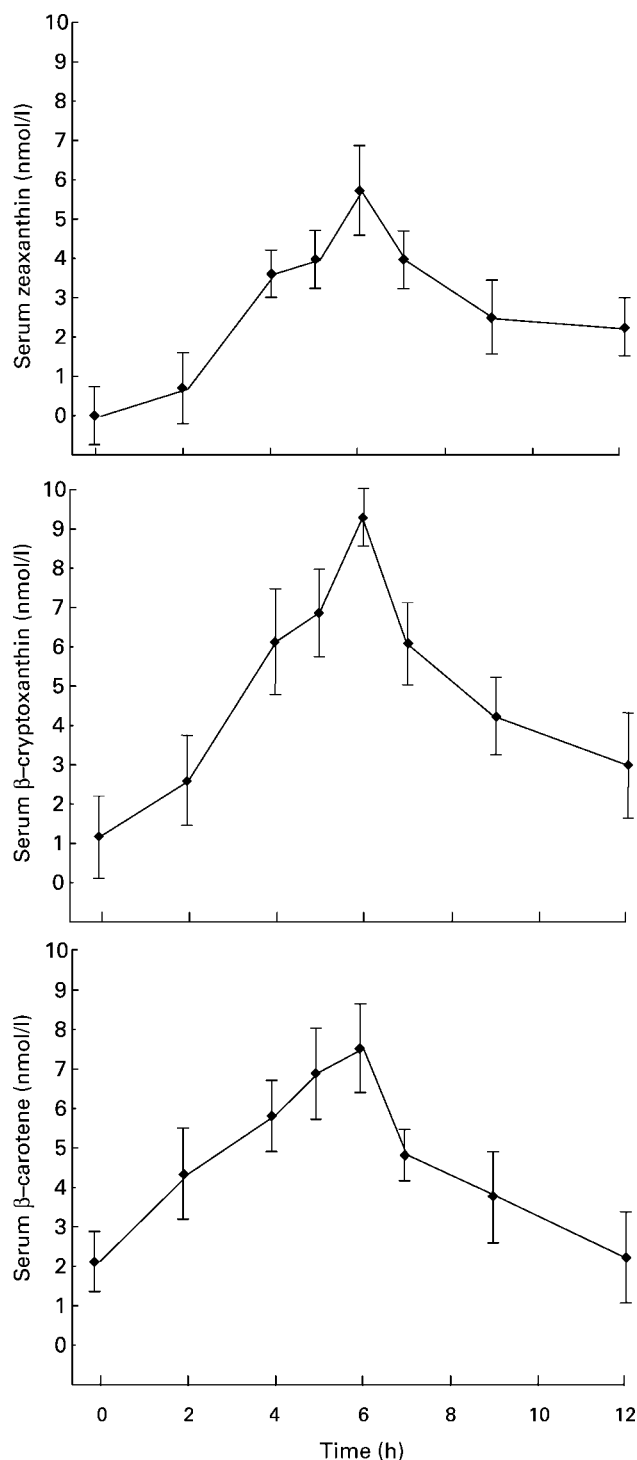
The time courses of the mean chylomicron carotenoid levels (nmol/l serum) after ingestion of 1 g paprika oleoresin are shown in Fig. 4. The concentration of the three carotenoids incorporated into the chylomicrons reached a maximum at about 6 h after intake: mean serum maximal levels were (nmol/l): zeaxanthin 5.73 (SEM 1.14);  $\beta$ -cryptoxanthin 9.27 (SEM 0.73);  $\beta$ -carotene 7.53 (SEM 1.12). The  $AUC_{0-12h}$  were (nmol·h/l): zeaxanthin 32.3 (SEM 2.3);  $\beta$ -cryptoxanthin 56.0 (SEM 5.5);  $\beta$ -carotene 54.5 (SEM 5.8). Zeaxanthin exhibited the lowest  $AUC_{0-12h}$  value among the carotenoids detected. The value was significantly lower compared with  $\beta$ -cryptoxanthin and  $\beta$ -carotene ( $P < 0.01$ ).

### Discussion

Paprika oleoresin contains the provitamin A carotenoids  $\beta$ -carotene and  $\beta$ -cryptoxanthin as well as several other xanthophylls, which are mainly present as mono- or diesters of fatty acids. Some of the xanthophylls, capsanthin and capsorubin, are only found in pepper. Although a relative high dose of capsanthin was ingested, the carotenoid was not detectable in human chylomicrons. In addition, no response was observed for capsorubin, violaxanthin, capsanthin epoxide, and cucurbitaxanthin A.

However, the concentration of  $\beta$ -carotene,  $\beta$ -cryptoxanthin and zeaxanthin increased significantly ( $P < 0.01$ ) in the chylomicron fraction. Maximum levels of the individual carotenoids in chylomicrons were reached at about 6 h, followed by a decrease close to the starting value within 12 h. Such time courses of carotenoids in the chylomicron fraction are typical after ingestion of a supplement or a carotenoid-rich meal (Gärtner *et al.* 1996; van den Berg H & van Vliet T, 1998). Only free  $\beta$ -cryptoxanthin and zeaxanthin were detected in the lipoprotein fraction, although those xanthophylls were mainly esterified in the oleoresin. Apparently, most of the esters are cleaved in the gut or before incorporation into the chylomicrons, an observation that has been described previously (Wingerath *et al.* 1995). The levels of carotenyl esters in human plasma and tissues are very low even after ingestion of high amounts in the diet (Granado *et al.* 1998).

There are several factors which influence the uptake of carotenoids from the gut, but it is yet unknown why some of the carotenoids from the paprika oleoresin do not appear in the chylomicron fraction. Although epoxides of  $\beta$ -carotene have been described in human plasma (Barua, 1999), the epoxide-containing compounds violaxanthin and capsanthin-5,6-epoxide were not present in the chylomicron extracts. They might be chemically degraded under acidic conditions in the stomach or



**Fig. 4.** Time course of the carotenoids present in chylomicrons after ingestion of paprika oleoresin. For details of subjects and procedures, see p. 788. Values are means with their standard errors shown by vertical bars for nine subjects.

enzymatically modified in the gut mucosa cell (Eugster, 1995). However, we did not detect any signals in the HPLC at retention times earlier than capsorubin, as was expected from polar metabolites. King *et al.* (1997) identified twenty-two structurally different carotenoids (not including *cis-trans* isomers) in human plasma and

breast-milk. Among them, there were no epoxide-containing carotenoids like violaxanthin, which occurs in several dietary products but is known to be degraded under acidic conditions. Thus, chemical instability is a reasonable explanation for the lack of carotenoid epoxides in the chylomicrons.

However, we found that cucurbitaxanthin A, capsorubin and capsanthin are relatively stable compounds even at low pH (A Pérez-Gálvez and W Stahl, unpublished results). Therefore, reasons other than chemical instability during the absorption process must be responsible for the poor availability of these carotenoids. It might be that they are not incorporated into mixed micelles or are rapidly metabolized by specific enzymes in the mucosa cell. As xanthophylls were ingested in their esterified form, it is possible that lipases do not cleave esters of oxocarotenoids like capsanthin; this has been recently suggested based on *in vitro* experiments (Breithaupt *et al.* 2002). However, an increase of capsanthin in human plasma after ingestion of paprika juice was observed in a previous study (Oshima *et al.* 1997). The inconsistent results might be due to matrix effects. In agreement with our present results, the authors did not detect the epoxide-containing carotenoids or capsorubin and cucurbitaxanthin A before or after supplementation.

It has been shown that there are differences in the availability of carotenoids depending on their polarity. Xanthophylls lutein and zeaxanthin are preferentially incorporated into chylomicrons as compared with  $\beta$ -carotene (Gärtner *et al.* 1996). From the present study it can be concluded that paprika oleoresin, and probably red pepper, are suitable sources for the provitamin A carotenoids  $\beta$ -carotene and  $\beta$ -cryptoxanthin, and the macular carotenoid zeaxanthin.

### Acknowledgements

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