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Simultaneous HPLC determination of carotenoids used as food coloring additives: applicability of accelerated solvent extraction

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Abstract

A sensitive HPLC multimethod was developed for the determination of the carotenoid food additives (CFA) norbixin, bixin, capsanthin, lutein, canthaxanthin, β-apo-8′-carotenal, β-apo-8′-carotenoic acid ethyl ester, β-carotene, and lycopene in processed food using an RP C30 column. For unequivocal identification, the mass spectra of all analytes were recorded using LC–(APcI)MS. For extraction, a manual process as well as accelerated solvent extraction (ASE) was applied. Important ASE parameters were optimized. ASE was used for the first time to extract CFA from various food matrices. Average recoveries for all analytes ranged from 88.7–103.3% (manual extraction) and 91.0–99.6% (ASE), with exception of norbixin using ASE (67.4%). Limits of quantitation (LOQ) ranged from 0.53–0.79 mg/L. The presented ASE method can be used to monitor both, forbidden application of CFA or the compliance of food with legal limits.

Keywords: Carotenoid food additives; LC–(APcI)MS; Accelerated solvent extraction

1. Introduction

Natural as well as synthetic food colorants are widely used by food manufacturers to gain attention of consumers. A current trend towards natural food colors is strengthened since a possible correlation between consumption of certain carotenoids (e.g., lycopene and prostate cancer (Kucuk et al., 2002; Giovannucci, 2002) or lutein and macula degeneration (Landrum & Bone, 2001; Olmedilla, Granado, Blanco, Vaquero, & Cajigal, 2001) was reported. Up-to-dateness of the topic is demonstrated by new products placed on the market over the past years, e.g., a semi-fat margarine containing lycopene and lutein as supplements. With respect to EU laws, this paper focuses on the analyses of natural carotenoids as well as of synthetic carotenoids, summarized as "carotenoid food additives (CFA)". Coloring plant extracts, which are not regarded as food additives under legal aspects, were not considered. To the best of our knowledge, this is the first method detecting simultaneously all CFA allowed in the EU.

CFA can be produced starting from various materials (Richtlinie, 95/45/EG). β-Carotene (E 160 a (i)) is obtained by solvent extraction of edible plants (e.g., lucerne) or from algae (*Dunaliella salina*), and can be naturally accompanied by minor carotenoids (e.g., α-carotene). Nearly pure β-carotene (E 160 a (ii)) is available from the fungus *Blaskeslea trispora*. Annatto (E 160 b) is derived from the pericarp of the seeds of *Bixa orellana*. It is supplied as an oil-soluble extract which contains mainly bixin (a mono methyl ester) or as a water-soluble product, which is produced by extraction with aqueous alkali and therefore contains mainly norbixin, the free acid. The coloring principle of red pepper extracts (E 160 c) is capsanthin, accompanied by capsorubin and various minor carotenoids. Lycopene (E 160 d) is found in several fruits (e.g., papaya) and represents the main carotenoid of red tomatoes. Lutein (E 161 b) is found in several plants (e.g., alfalfa) or flowers (e.g., marigold; *Tagetes erecta* L.). In marigold lutein is acylated with various fatty acids. Thus, as is the case for

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red pepper oleoresins, food may contain esterified or free lutein, depending on the application form. β-Apo-8′-carotenal (E 160 e), β-apo-8′-carotenoic acid ethyl ester (E 160 f), and canthaxanthin (E 161 g), introduced by Roche in the 1960s (Gordon & Bauernfeind, 1982), are synthesized as bulk products and are used worldwide in various formulations.

Methods for quantitative analyses of CFA in foodstuffs are rather scarce. The German 'Official Collection of Methods According to paragraph 35 LMBG' (2002) lists only one high performance liquid chromatograph (HPLC) method (L 00.00-63/2) for detection of α-carotene, β-carotene, and lycopene in extracts obtained after saponification. Method L 26.11.03-13 allows for the spectrophotometric quantification of lycopene in extracts of tomato puree. Additional spectrophotometric applications for determination of carotenoids in fresh and dried plant materials, macaroni, flour, and egg yolk were available in the American collection of 'Official Methods of Analysis of AOAC International' (2000). Only few further methods for the simultaneous detection of CFA have been described in literature. Mínguez-Mosquera, Hornero-Méndez, & Garrido-Fernández (1995) presented a method for detection of bixin, lycopene, canthaxanthin, and β-apo-8′-carotenal using saponification in products derived from red pepper. Although they stated that the method may be used to detect these carotenoids in food matrices, no experimental data were presented. Norbixin, β-apo-8′-carotenoic acid ethyl ester as well as carotenes were not covered by this study. Amakawa, Ogiwara, Ohnishi, Kano, & Yamagishi (1992) detected β-apo-8′-carotenal and β-apo-8′-carotenoic acid ethyl ester by HPTLC and HPLC in oil-soluble pigments and Italian pasta. Sapers (1994) analyzed cocktail cherries spiked with carotenoids and compared them to commercial cherries by spectrocolorimetry. Tantillo, Storelli, Aprile, & Matrella (2000) described a method for determination of canthaxanthin and astaxanthin in salmon fillets. Recently, a method for analysis of β-carotene stereoisomers in carrot juice and vitamin supplemented drinks has been published (Marx, Schieber, & Carle, 2000). None of these methods covers all CFA nor uses LC–MS using an atmospheric pressure chemical ionization interface [LC–(APcI)MS] for their unequivocal identification. Thus, a reliable RP-HPLC method for determination of CFA in different foodstuffs was developed.

Additionally, the applicability of accelerated solvent extraction (ASE), which has become an important extraction technique in residue analysis, was examined. ASE currently is attracting interest as it features short extraction times, low solvent use, high extraction yields, and provides a high level of automation (Hofler, 2002). Giergielewicz-Mozajska, Dabrowski, & Namiesnik (2001) extracted environmental pollutants from solid samples and Wennrich, Popp, & Moeder (2000) used ASE for the determination of chlorophenols in soil. Benzo[a]pyrene was extracted from mussel tissue by Michalski & Germuska (2002). Furthermore, ASE was successfully applied prior to mass spectrometrical analysis of octyl- and nonylphenols and bisphenol A in fish liver (Tavazzi, Benfenati, & Barcelo, 2002) and to monitor dioxin in flue gas (Hashimoto, Suga, Yamada, Takada, Waki, & Sakaira, 2001). To the best of our knowledge, ASE has not been applied for extraction of CFA from food so far.

2. Materials and methods

2.1. Materials

β-Apo-8′-carotenal (>96%), β-carotene (>97%), canthaxanthin (>98%), β-apo-8′-carotenoic acid ethyl ester (>80%), potassium hydroxide, sodium carbonate (anhydrous), sodium chloride and triethyl amine were obtained from Fluka (Steinheim, Germany). Oil-soluble (4% bixin) and water-soluble (2.5% norbixin) annatto was kindly provided by Dr. Marcus GmbH (Geesthacht, Germany), paprika (*Capsicum annuum* L.) and marigold oleoresins (*Tagetes erecta* L.) were gifts from Euram Trade GmbH (Stuttgart, Germany), and echinenone was kindly provided by Roche Vitamins Ltd. (Basel, Switzerland). All organic solvents (methanol, diethyl ether, ethanol, ethyl acetate, methyl-*tert*-butyl ether (MTBE), light petroleum (40–60 °C), acetic acid (glacial), and tetra-n-butylammonium hydrogen sulfate (>99%, TBAH) were obtained from Merck (Darmstadt, Germany); all solvents were distilled before use. For ASE, hydromatrix (Bulk Isolute® Sorbent) from Separtis GmbH (Grenzach-Wyhlen, Germany) was used. For HPLC analysis, ultrapure water from a Milli-Q185 plus apparatus (Millipore, Eschborn, Germany) was employed. Carrots, parsley, red and orange pepper, tomato paste, and all samples (beverages, pudding mixes, breakfast cereals, cookies) and sausages (Saucisses de Strasbourg) were obtained from German and French supermarkets.

2.2. Instrumentation

2.2.1. High performance liquid chromatograph and LC–MS using an atmospheric pressure chemical ionization interface

The HPLC consists of a modular system HP1050 (Hewlett-Packard GmbH, Waldbronn, Germany) with diode array detector (DAD). Chromatograms were monitored at 450 nm (4 nm bandwidth; reference wavelength at 550 nm with 50 nm bandwidth). A YMC (Schermbeck, Germany) C30 analytical column (5.0 μm, 250 × 4.6 mm I.D.) including a C30 guard column (5.0 μm, 10 × 4.0 mm I.D.) was used and kept at 35 °C. For data processing a HP3D ChemStation software (A.04.02) was used. For gradient elution two mobile phases were employed: (A) methanol, water, triethyl

amine (90:10:0.1 v/v/v); (B) MTBE, methanol, water, triethyl amine (90:6:4:0.1 v/v/v/v). The following gradient was used (min/%A): 0/93.5; 34/0; 38/93.5; 43/93.5 (flow rate: 1 mL/min, injection volume: 20 μL). Prior to HPLC analysis, all samples were filtered using Chromafil-PET-45/25 filters (Macherey-Nagel, Düren, Germany) with 0.45 μm pore size. LC–(APcI)MS was run on an HP1100 HPLC system, coupled to a Micromass (Manchester, UK) VG platform II quadrupole mass spectrometer. Eluents A and B were used without addition of triethyl amine. Further MS parameters have been detailed by Breithaupt, Wirt, & Bamedi (2002).

2.2.2. Accelerated solvent extraction equipment

The following equipment was used: ASE 200 (Dionex, Germany). The optimized settings were as follows: pressure, 70 bar; temperature, 40 °C; preheat, 0 min; heat, 5 min; static, 2 min; flush, 100%; purge, 30 s; cycles, 3; cell volume, 11 mL.

2.3. Extraction processes

2.3.1. Manual extraction

Liquid samples: Sparkling beverages were degassed (1 min) using an ultrasonic device. An aliquot (1–20 mL), depending on the carotenoid content, was extracted thrice with a ternary solvent mixture (methanol/ethyl acetate/light petroleum 1:1:1 (v/v/v); 15 mL each) using a separating funnel. Before extraction, the internal standard echinenon (1 mL; $c = 25$ mg/L in MTBE/methanol 1:1, v/v) and saturated sodium chloride solution (2 mL) were added to assist phase separation. The upper layers were separated, combined, and evaporated in vacuum at 30 °C using a rotary evaporator. To remove traces of water, 2 mL ethanol were added and the extract was evaporated to dryness. The residue was dissolved in MTBE/methanol (1:1:v/v; 4 mL), membrane filtered (45 μm), and subjected to HPLC or LC–MS analyses immediately. All work was done under dim light using amber glass equipment.

Solid samples: An aliquot (0.5–2.0 g) was suspended in water (10 mL) and the internal standard solution (1 mL, see above) and saturated sodium chloride solution (2 mL) were added. If the sample contained citric acid, the mixture was blended with solid sodium carbonate (100 mg). To enhance recovery of bixin and norbixin, these samples were extracted with 0.05% (v/v) acetic acid (10 mL) (Mínguez-Mosquera et al. (1995)). Further extraction was carried out as described for liquid samples. If paprika oleoresin was labelled on the product, the extract was saponified in diethyl ether (50 mL) by addition of methanolic KOH (30%, w/v; 2.5 mL) according to a procedure described by Breithaupt, 2000.

2.3.2. Accelerated solvent extraction

Various parameters were tested to optimize extraction conditions (temperature range: 25–80 °C; pressure range: 70–140 bar). A ternary solvent system (see Section 2.3.1) was applied for manual extraction. Additionally, mixtures of MTBE/methanol (1:1, v/v) and of ethanol/n-hexane (4:3, v/v; Taungbodhitham, Jones, Wahlqvist, & Briggs, 1998) were used (40 °C, 70 bar).

Liquid samples: An aliquot (1–5 mL) was pipetted on a bed of hydromatrix in the extraction cell, covered with hydromatrix, and extracted immediately. After addition of saturated sodium chloride solution (2 mL), the organic layer was separated and treated as described above (final volume: 2 mL).

Solid samples: An aliquot (1.0–2.0 g) was suspended in water (1–2 mL) and mixed with hydromatrix to get a free-flowing powder. If the sample contained citric acid, the mixture was blended with solid sodium carbonate (100 mg). For extraction of norbixin, samples were suspended in 0.05% (v/v) acetic acid. The extracts were treated as described above (final volume: 2 mL).

2.4. CFA standard mix for method development

Carrot (α-/β-carotene), parsley (lutein), red (capsanthin) and orange pepper (zeaxanthin) (20 g each) were cut into cubes, minced together with tomato paste (lycopene) (5 g) by an Ultra Turrax (Kunkel, Germany), and extracted four times with a ternary solvent mixture (see Section 2.3.1; 50 mL each). Saponification of the extract was carried out as described earlier (Breithaupt, 2000). The residue was dissolved in 50 mL light petroleum. An aliquot (2 mL) was spiked with solutions of bixin, norbixin, canthaxanthin, β-apo-8′-carotenal, β-apo-8′-carotenoic acid ethyl ester, and echinenone, corresponding to ~30 μg of each CFA. Thus, a standard mix containing all relevant CFA was obtained. The solvent was distilled off and the residue was dissolved in 2 mL MTBE / methanol 1:1 (v/v). This solution was subjected to HPLC and LC–(APcI)MS analyses.

2.5. Calibration

Lutein and capsanthin were isolated from the respective oleoresins after saponification in our laboratory (Breithaupt, 2000), lycopene was extracted from tomato paste. Pure carotenoids were isolated using semipreparative HPLC on a C30 column with the following eluents (MTBE/methanol, v/v): lutein and capsanthin: 30/70, lycopene: 70/30. Extracts of bixin and norbixin (obtained from annatto formulations) were used without further clean-up. Small amounts (1 mg) of all other CFA were dissolved in an appropriate solvent in volumetric flasks. The concentrations of the resulting solutions (lp = light petroleum) were calculated spectrophotometrically on the basis of E (1%, 1 cm): bixin: 4200, lp, 456 nm; norbixin: 4355, lp, 456 nm; capsanthin: 2069, toluol, 483 nm; lutein: 2551, ethanol, 445 nm; canthaxanthin: 1900, lp, 463 nm; β-apo-8′-carotenal:

2640, lp, 457 nm; β-apo-8′-carotenoic acid ethyl ester: 2362, lp, 448 nm; echinenone: 2158, lp, 458 nm; β-carotene: 2500, lp, 451 nm; lycopene: 2939, lp, 474 nm (calculated from Köst, 1988). For preparing calibration curves (calibration range: 0.5–25.0 mg/L each), appropriate volumes of the respective stock solutions were further diluted with MTBE/methanol 1:1 (v/v) and subjected to HPLC analysis. The resulting peak areas were plotted against the concentrations.

2.6. Recovery studies

For recovery experiments, aliquots of all CFA solutions were combined to give concentrations of about 3 mg/L each. For determination of the recovery using the manual extraction process, the standard mix (2 mL) was suspended in 0.05% (v/v) acetic acid (10 mL) and extracted as described (final volume: 2 mL). Based on the use of 1.0 g solid sample (see Section 2.3), this corresponds to a typical CFA concentration of about 6 mg/kg of a real sample. For determination of the recovery using ASE, 2 mL were pipetted on a bed of hydromatrix in the extraction cell, covered with hydromatrix, and immediately analyzed as described (final volume: 2 mL). In each case, samples were protected from direct light and processed immediately. Recoveries ($n = 3$) were calculated on the basis of AOAC methods, 2000 as follows: %Recovery $= [(c_F - c_U)/c_S] \times 100$, where $c_F =$ concentration of CFA measured in the fortified sample, $c_U =$ concentration of CFA measured in the unfortified sample (set to zero), and $c_S =$ concentration of CFA added to the fortified sample. Additionally, the concentration of echinenone (ISTD) was monitored to determine possible losses during the extraction procedure.

2.7. Limit of quantitation and determination

Limits of quantitation and determination were calculated from the respective calibration graphs (HPLC) according to the recommendations of the Deutsche Forschungsgemeinschaft, 1991.

3. Results and discussion

3.1. Development of a HPLC method

For development of a reliable HPLC method, a plant extract obtained from a mixture of carrot (α-carotene, β-carotene), parsley (lutein), red pepper (capsanthin), orange pepper (zeaxanthin), and tomato paste (lycopene) was used. This extract was spiked with solutions of bixin, norbixin, β-apo-8′-carotenal, β-apo-8′-carotenoic acid ethyl ester, and canthaxanthin. Thus, a mixture containing all CFA was obtained to evaluate the applicability of a HPLC method.

Special attention was addressed to the determination of bixin and norbixin because of their acidic character, which complicates the analysis in RP-HPLC systems. To extend their retention times, tetrabutyl ammoium hydrogen phosphate (TBAH), a typical ion pair reagent, was added to eluent A (0.1% (w/v)). Thus the retention

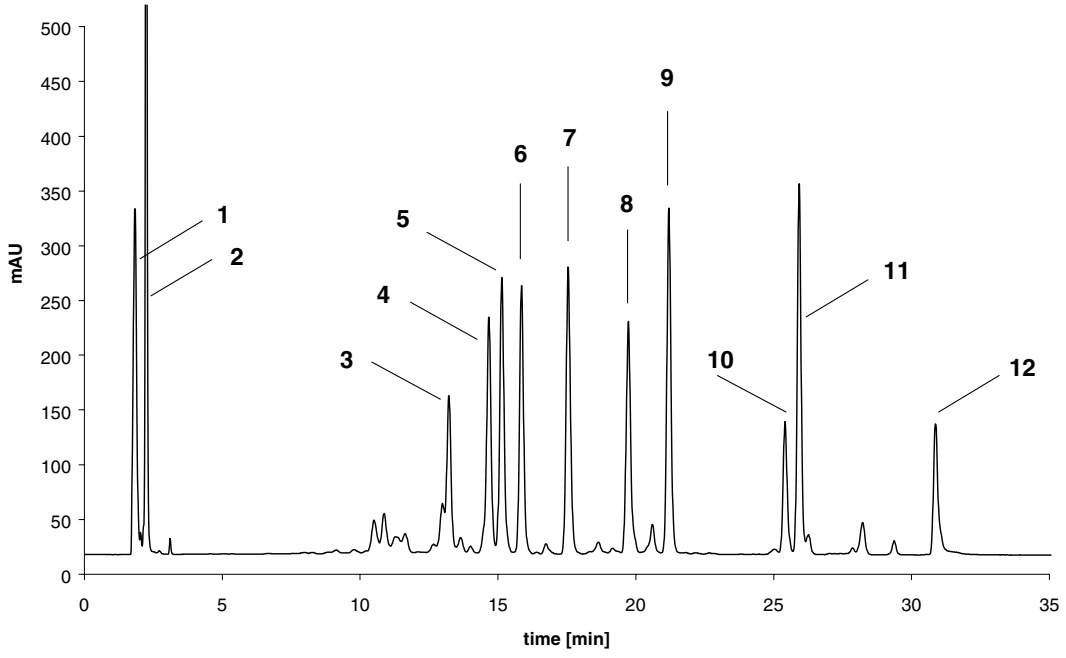

Fig. 1. HPLC chromatogram (DAD, 450 nm) of a CFA standard mixture (spiked plant extract). The operating conditions are reported in the HPLC section. For peak assignment see Table 1.

Table 1
LC–(APcI)MS (positive mode) and UV/VIS data of CFA (peak assignment according to Fig. 1)

Peak	Carotenoid	Typical ions (*m/z*)	UV/VIS data (nm)
1	Norbixin	381 $[M+H]^+$ (100%)	432/456/486
2	Bixin	395 $[M+H]^+$ (100%) 363 $[M+H–CH_3OH]^+$ (23%)	435/460/488
3	Capsanthin	585 $[M+H]^+$ (100%) 567 $[M+H–H_2O]^+$ (10%)	470
4	Lutein	569 $[M+H]^+$ (9%) 551 $[M+H–H_2O]^+$ (100%)	424/446/474
5	Zeaxanthin	569 $[M+H]^+$ (100%) 551 $[M+H–H_2O]^+$ (12%)	430/452/478
6	Canthaxanthin	565 $[M+H]^+$ (100%)	478
7	β-Apo-8′-carotenal	417 $[M+H]^+$ (100%)	462
8	β-Apo-8′-carotenoic acid ethyl ester	461 $[M+H]^+$ (100%) 415 $[M+H–C_2H_5OH]^+$ (3%)	420/446/465
9	Echinenon (ISTD)	551 $[M+H]^+$ (100%)	464
10	α-Carotene	537 $[M+H]^+$ (100%)	424/446/474
11	β-Carotene	537 $[M+H]^+$ (100%)	430/452/480
12	Lycopene	537 $[M+H]^+$ (100%)	448/474/504

The signal intensities are given in parentheses. UV/VIS data (DAD) are reported in the HPLC eluents during LC–MS analyses.

time of norbixin was shifted from 2.5 to 5.5 min and a Gaussian peak was obtained. Unfortunately, the addition of TBAH resulted in significantly reduced recovery rates of CFA, especially for β-carotene and lycopene. Hence, this additive was excluded from further analyses, accepting short retention times for bixin and norbixin. Nevertheless, both peaks were baseline separated. A typical HPLC chromatogram (total runtime: 43 min) is depicted in Fig. 1.

3.2. LC–(APcI)MS analyses

For unequivocal CFA identification, LC–(APcI)MS (positive mode) was used. The data set for identification of the respective peaks is presented in Table 1. Since bixin and norbixin bear at least one carboxyl group, (APcI)MS measurements in the negative mode were tested additionally. However, with respect to both carotenoids, no remarkable enhancement of sensitivity was observed. In fact, the sensitivity of all other xanthophylls was 5–50 times higher using the positive mode, with β-apo-8′-carotenal being the carotenoid which can be detected most sensitive.

3.3. Method performance

Various carotenoids (astaxanthin, citranaxanthin, echinenon) were tested for their suitability as internal standard. Only echinenon did not co-elute with other CFA. Thus, echinenon was used as ISTD to monitor losses during the work-up procedure. The recovery from various samples accounted for >96%. Identity of CFA was confirmed in routine analyses by comparing retention times and UV/VIS spectra of the respective peaks with those obtained from reference material. The calibration curves for CFA were found to be linear from 0.5 to 25.0 mg/L, based on peak area determinations (coefficient of correlation: $r = 0.99986 - 0.99997$), permitting quantification of all CFA under investigation. Limits of detection (LOD) and quantitation (LOQ) for all CFA, calculated from the calibration graphs according to the recommendations of the Deutsche Forschungsgemeinschaft (DFG, 1991), ranged from 0.36–0.53 mg/L (LOD)

Table 2
Recoveries of CFA ($c = 3$ mg/L) using two different extraction methods

CFA	Recovery (%)	
	ASE	Manual procedure
Norbixin	67.4 ± 1.0	88.7 ± 6.2
Bixin	91.0 ± 2.7	98.0 ± 1.7
Capsanthin	97.8 ± 0.7	100.2 ± 0.4
Lutein	97.6 ± 0.7	99.8 ± 0.4
Canthaxanthin	99.1 ± 0.1	103.3 ± 2.7
β-Apo-8′-carotenal	99.0 ± 0.2	97.9 ± 0.2
β-Apo-8′-carotenoic acid ethyl ester	99.6 ± 0.3	98.5 ± 1.3
β-Carotene	99.1 ± 0.5	101.5 ± 1.7
Lycopene	97.1 ± 1.2	89.9 ± 3.8

Recoveries are given as means ± standard deviations ($n = 3$).

Table 3
Concentration of CFA [liquid samples (mg/L); solid samples (mg/kg)] determined in beverages, pudding mixes, breakfast cereals, cookies, and sausages

Product	Declaration	Extraction	CFA	
Beverage			β-*Carotene*[a]	β-*Apo*-8′-*carotenal*
			E 160 a	E 160 e
1	E 160 a/e	Manual	1.70 ± 0.07	0.62 ± 0.01
		ASE	1.61 ± 0.05 (95%)	0.58 ± 0.01 (94%)
2	E 160 a/e	Manual	1.60 ± 0.08	0.55 ± 0.03
		ASE	1.58 ± 0.01 (99%)	0.57 ± 0.01 (104%)
3	E 160 a/e	Manual	1.06 ± 0.01	0.41 ± 0.02
		ASE	1.07 ± 0.02 (101%)	0.40 ± 0.01 (98%)
4	E 160 a	Manual	1.62 ± 0.14	–
		ASE	1.67 ± 0.06 (103%)	–
5	E 160 e	Manual	–	5.52 ± 0.15
		ASE	–	5.48 ± 0.05 (99%)
6	E 160 a	Manual	7.16 ± 0.33	–
		ASE	7.33 ± 0.19 (102%)	–
7[b]	With dye	Manual	27.78 ± 0.17	–
		ASE	27.80 ± 1.15 (100%)	–
Pudding mix			β-*Carotene*[a]	*Norbixin*
			E 160 a	E 160 b
1	E 160 a	Manual	19.67 ± 0.12	–
		ASE	20.41 ± 0.63 (104%)	–
2	E 160 a	Manual	39.34 ± 0.98	–
		ASE	37.72 ± 0.39 (96%)	–
3	E 160 a	Manual	31.23 ± 2.17	–
		ASE	29.51 ± 0.74 (94%)	–
4	E 160 a	Manual	34.63 ± 0.55	–
		ASE	33.08 ± 1.11 (96%)	–
5[c]	E 160 a	Manual	30.41 ± 0.14	–
		ASE	31.70 ± 0.87 (104%)	–
6[d]	E 160 b	Manual	–	4.83 ± 0.20
7[d]	E 160 b	Manual	–	16.43 ± 1.06
Cereals			β-*Carotene*[a]	*Capsanthin*[e]
			E 160 a	E 160 c
1	E 160 a	Manual	17.71 ± 1.15	–
		ASE	18.17 ± 1.55 (103%)	–
2	E 160 c	Manual	–	1.63 ± 0.18
		ASE	–	1.65 ± 0.14 (101%)
3	E 160 a/c	Manual	17.65 ± 0.04	5.62 ± 0.47
		ASE	18.46 ± 0.06 (105%)	5.26 ± 0.10 (94%)
				Norbixin
				E 160 b
4	E 160 b	Manual	–	1.22 ± 0.12
Cookies			*Lutein (total)*[e]	
			E 161 b	
1	161 b	Manual	2.54 ± 0.21	–
		ASE	3.03 ± 0.02 (119%)	–
2	161 b	Manual	4.16 ± 0.21	–
		ASE	4.49 ± 0.05 (108%)	–
Sausages			*Canthaxanthin*	
			E 161 g	
1	161 g	Manual	6.68 ± 0.24	–
		ASE	6.41 ± 0.19 (96%)	–

Values represent means ± standard deviations of at least three replications. If ASE extraction was applied, the concentration is given in % in relation to that determined after manual extraction.

[a] *Cis* and *trans* isomers were calculated together as *all-trans*-β-carotene.

[b] The sample contained 4% alcohol.

[c] Since the sample contained citric acid, 100 mg Na_2CO_3 were added prior to extraction.

[d] Since the sample contained only norbixin, extraction by ASE was not performed.

[e] Determined after saponification with methanolic KOH (30%, w/v) over night.

and 0.53–0.79 mg/L (LOQ), respectively. Recoveries of CFA, determined by the manual procedure, were compared to those after ASE (manual: 88.7–103.3%; ASE: 91.0–99.6%; with exception of norbixin; Table 2). Lower recoveries were obtained for the extraction of norbixin with ASE (67.4%). The adsorption of this comparatively polar carotenoid to the hydromatrix could explain this finding. To simulate starch containing samples (e.g., cereals), both methods were performed after adding wheat starch (1 g) to selected samples. In case of ASE, recoveries were 3–4% lower than those specified in Table 2, whereas after applying manual extraction no effect was noticed (data not shown).

3.4. Optimization of ASE parameters

The parameters temperature and pressure influence the extraction yields significantly. Thus, both parameters were optimized using a β-carotene containing pudding mix. The following settings were tested: 25–80 °C and 70–140 bar. A ternary solvent system (see Section 2.3.1) was used. Best results were obtained with 40 °C and 70 bar. Above 40 °C, starch started to agglutinate, forestalling complete extraction. Using pressure above 70 bar resulted in decreased β-carotene concentrations in the final solutions. Likewise, the solvent mixtures MTBE/methanol and ethanol/*n*-hexane resulted in decreased extraction yields.

3.5. Analysis of commercial samples

Commercial beverages, pudding mixes, breakfast cereals, cookies, and sausages were analyzed by the optimized method (Table 3). The levels of CFA determined for instance in beverages, ranged from 1.1 to 27.8 mg/L β-carotene and 0.4–5.5 mg/L β-apo-8′-carotenal, independant from the extraction method applied. These results clearly demonstrate applicability of ASE for extraction of CFA from beverages. With the exception of norbixin, which was not extracted quantitatively by ASE, the same applies for CFA analyses of pudding mixes. Generally, the extracts obtained by ASE did not contain food compounds, forestalling CFA analyses by RP-HPLC.

To evaluate applicability of ASE, CFA concentrations determined after applying ASE were compared to those obtained after manual extraction (set to 100%). With exception of two samples, all concentrations determined by ASE ranged from 94% to 105%. In two cookie samples the concentrations of total lutein determined by ASE were notably higher (119% and 108%) than the concentration obtained after manual extraction. Possibly, CFA are enclosed in polymers of high molecular weight during baking, inhibiting extraction without drastic conditions as provided by ASE. Thus, ASE is especially suitable for extremely complex food matrices. Regarding labeling of CFA, with exception of one sample (beverage 7) in all cases declaration was accurate. Beverage 7 was colored with β-carotene which was itemized "with dye", without designation of the customary name or the corresponding E number. This is not in accordance with EU laws.

All extracts obtained from β-carotene containing samples showed two additional peaks corresponding to *cis*-isomers of β-carotene (Strohschein, Pursch, Händel, & Albert, 1997). An example of a chromatogram

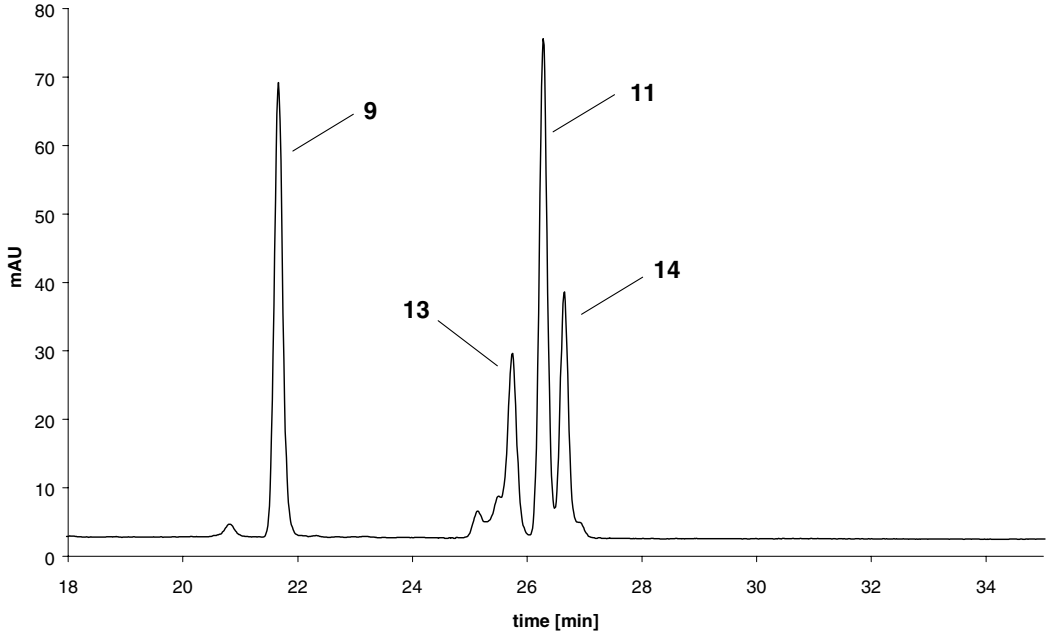

Fig. 2. HPLC chromatogram (DAD, 450 nm; extended section) of a pudding mix extract (sample 3, Table 3). Peak assignment: **9**: echinenone (internal standard); **11**: *all-trans*-β-carotene; **13**: 13-*cis*-β-carotene; **14**: 9-*cis*β-carotene.

showing the typical pattern is presented in Fig. 2. Remarkably, the degree of isomerization was found to be not dependent on the method used for extraction. Since *cis*-β-carotene isomers were not on-hand as standard materials, the areas of the respective peaks were summed and calculated as *all-trans*-β-carotene.

Interestingly, extracts obtained from cookies showed in addition to lutein and zeaxanthin five intense peaks in the apolar zone of the chromatograms. Comparison with chromatograms obtained from authentic marigold oleoresin (Breithaupt et al., 2002) clearly proved utilization of marigold oleoresin. Since conventional marigold oleoresin contains only a minute amount of free lutein and the additional peaks mimic completely the lutein ester pattern of marigold, the chromatographic procedure provides the opportunity to calculate "added" lutein separately. According to cereals which contained paprika oleoresin, "total" lutein was quantified in both cookie samples after saponification with methanolic KOH.

This study proves the suitability of ASE for extraction of CFA from various kinds of food matrices in routine analysis. All CFA may be analyzed by HPLC simultaneously in one run.

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