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Separation and quantification of 15 carotenoids by reversed phase high performance liquid chromatography coupled to diode array detection with isosbestic wavelength approach

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ABSTRACT

The manuscript presents the development of a new reverse phase high performance liquid chromatography (RP-HPLC) photo diode array detection method allowing the separation and quantification of 15 carotenoids (adonirubin, adonixanthin, astaxanthin, astaxanthin dimethyl disuccinate, asteroidenone, beta-apo-8′-carotenal, beta-apo-8′-carotenoic acid ethyl ester, beta-carotene, canthaxanthin, capsanthin, citranaxanthin, echinenone, lutein, lycopene, and zeaxanthin), 10 of which are feed additives authorised within the European Union. The developed method allows for the reliable determination of the total carotenoid content in one run using the corresponding *E*-isomer as calibration standard while taking into account the *E*/*Z*-isomers composition. This is a key criterion for the application of the method, since for most of the analytes included in this study analytical standards are only available for the *E*-isomers. This goal was achieved by applying the isosbestic concept, in order to identify specific wavelengths, at which the absorption coefficients are identical for all stereoisomers concerned. The second target referred to the optimisation of the LC conditions. By means of an experimental design, an optimised RP-HPLC method was developed allowing for a sufficient chromatographic separation of all carotenoids. The selected method uses a Suplex pKb-100 HPLC column and applying a gradient with a mixture of acetonitrile, tert-butyl-methyl ether and water as mobile phases. The limits of detection and limits of quantification ranged from 0.06 mg L^{-1} to 0.14 mg L^{-1} and from 0.20 mg L^{-1} to 0.48 mg L^{-1}, respectively.

1. Introduction

Carotenoids are a class of hydrocarbons (carotenes) and their oxygenated derivatives (xanthophylls) consisting of eight isoprene building blocks. Because of the numerous conjugated double bonds and the cyclic end groups, carotenoids present a variety of stereoisomers with different chemical and physical properties. The most important forms commonly found among carotenoids are stereoisomers abbreviated as *E*- or *Z*-isomers. Stereoisomers of this type are interconvertible in solution and exert a marked influence on the physical properties. *E*- and *Z*-isomers do not only differ in their melting points, solubility and stability, but also in respect to absorption affinity, colour and colour intensity [1]. Also the ultraviolet/visible (UV/Vis) spectra of the *E*/*Z*-isomers show significant differences, for instance the appearance of a new absorbance band in the spectra of the Z-isomers compared to the corresponding *E*-isomers [2].

Apart from the nutritional importance in human and animal health as metabolic precursors of vitamin A and antioxidants, carotenoids are used for the direct colouring of foodstuff as well as for pigmentation of animal products via their addition to complete feedingstuffs. In this study we included 15 carotenoids (Fig. 1), namely adonirubin, adonixanthin, asteroidenone, echinenone, lycopene and the feed additives astaxanthin, astaxanthin dimethyl disuccinate, beta-apo-8′-carotenal, beta-apo-8′-carotenoic acid ethyl ester, beta-carotene, canthaxanthin, capsanthin, citranaxanthin, lutein, and zeaxanthin. The feed additives are authorised within the European Union under Regulation (EC) No 1831/2003 classified in the category "sensory additives" and functional group "colourants: substances which, when fed to animals, add colours to food of animal origin" [3]. For instance, astaxanthin and canthaxanthin are added to salmon and trout feed

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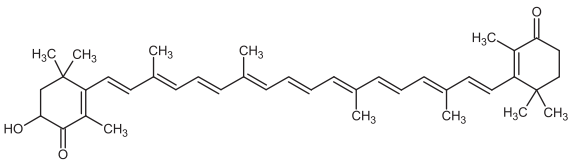
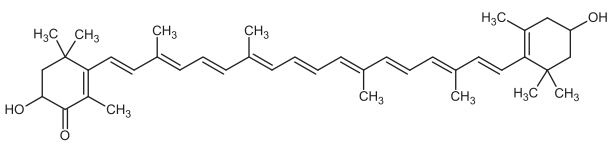
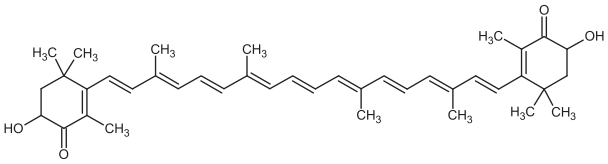


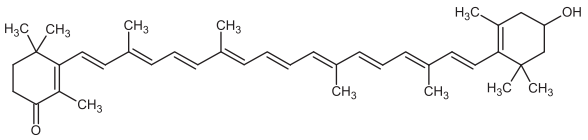
Adonirubin (ADR) $C_{40}H_{52}O_3$



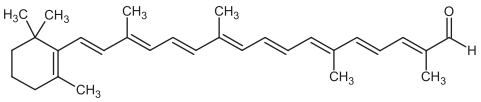
Adonixanthin (ADXN) $C_{40}H_{54}O_3$



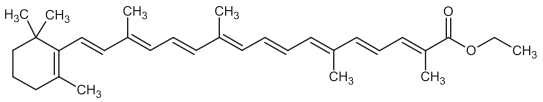
Astaxanthin (AXN) $C_{40}H_{52}O_4$



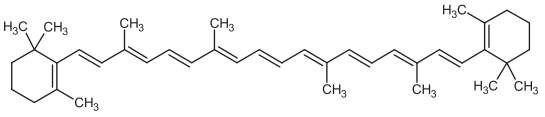
Asteroidenone (AST) $C_{40}H_{54}O_2$



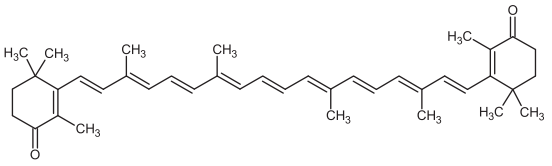
Beta-apo-8'-carotenal (BACAR) $C_{30}H_{40}O$



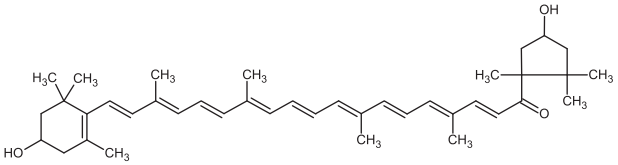
Beta-apo-8'-carotenoic acid ethyl ester (BACARE) $C_{32}H_{44}O_2$



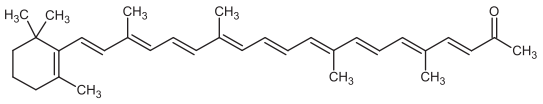
Beta-carotene (BCAR) $C_{40}H_{56}$



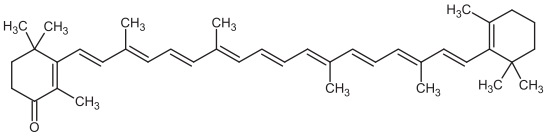
Canthaxanthin (CXN) $C_{40}H_{52}O_2$



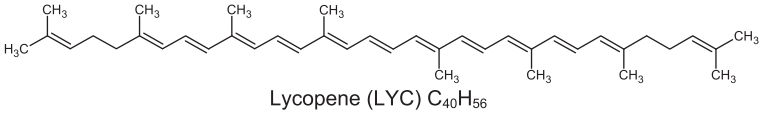
Capsanthin (CSN) $C_{40}H_{56}O_3$



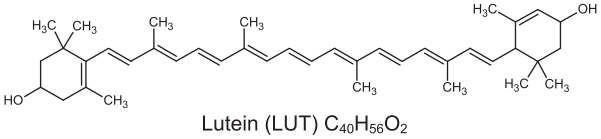
Citranaxanthin (CIXN) $C_{33}H_{44}O$



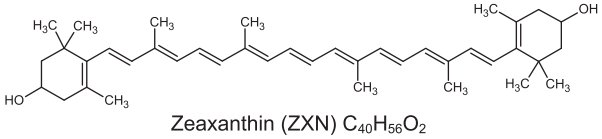
Echinenone (ECH) $C_{40}H_{54}O$



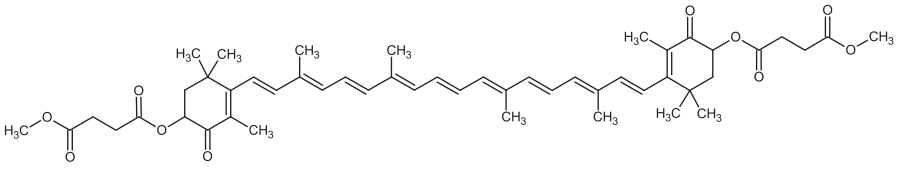
Lycopene (LYC) $C_{40}H_{56}$



Lutein (LUT) $C_{40}H_{56}O_2$



Zeaxanthin (ZXN) $C_{40}H_{56}O_2$



Astaxanthin dimethyl disuccinate (AXN-DMDS) $C_{50}H_{64}O_{10}$

Fig. 1. Chemical structures of the target all-*E* carotenoids.

for flesh colouration, whereas lutein is widely used in poultry farming for egg yolk coloration [4]. In addition, authorisation of these substances includes target concentration limits in complete feedingstuffs as specified in respective Commission Regulations. For instance, astaxanthin dimethyl disuccinate is authorised by Commission Regulation (EC) No 393/2008 for salmon and trout [5], with a maximum level of this compound in feed at 138 mg kg^{-1}.

In order to carry out official controls to check compliance with feed and food law, animal health and animal welfare rules (Regulation (EC) No 882/2004), improved methods of quantitative analysis of carotenoids are required [6]. In particular there is a strong need for multianalyte methods allowing for the simultaneous determination of the different carotenoids. Since legal limits of carotenoids when utilised as feed additives are expressed in terms of the *sum* of the *Z*-and all-*E*-forms, analytical methods that are fit for the intended purpose need to address the presence of the various *E*/*Z*-isomers of the target carotenoid.

Many procedures have been described for the quantitative analysis of carotenoids in a wide range of carotenoids mixtures and extracts [7–23]. They are mainly based on the spectrophotometric [7–20] or mass spectrometry analysis [21] combined with high performance liquid chromatography. The different methods are generally designed to meet different objectives. Some provide a rapid single-analyte method [7–9], whereas others are more complex procedures which give a complete profile of the carotenoids present [11–16,21–23], including separation of stereoisomers with [19,20] or without optical properties [8,10,17–19].

Quantitative analysis depends on the availability of carotenoid standards including the various isomers. However, not all isomers are available, which especially applies to the *Z*-isomer. Moreover, *Z*- and all-*E*-forms do not have the same absorption coefficient and these absorption coefficients are often not tabulated, thus rendering the measurement of carotenoids extremely difficult. Furthermore, calibration of different isomers against the stable and often available all-*E*-isomers leads in general to an underestimation of the total content of the different isomers of a specific carotenoid. In order to cope with the lack of all required standard substances, we applied the isosbestic concept, which has been previously used by Lessin for quantification of *E*- and *Z*-isomers of three carotenoids (alpha-carotene, beta-carotene and beta-cryptoxanthin) [10] and by Schierle for spectrophotometric determination of beta-carotene [7]. This concept is based on the principle that a specific wavelength is selected for the measurement, where the absorption coefficients are identical for all stereoisomers, thus allowing the determination of the total carotenoid content independently of the *E*/*Z*-isomers composition against the corresponding all-*E*-form. The only drawback of this method is its lower sensitivity, since the wavelength with the highest absorption coefficient is often different from the isosbestic wavelength. However, this is not a major problem, since the legal limits of carotenoids in target matrices in complete feedingstuffs are most often at relatively high level (above 50 mg kg^{-1}).

The research presented in this paper had two goals, namely (1) the development of a reverse phase liquid chromatography with diode array detection (RP-HPLC-DAD) allowing for the separation of all 15 carotenoids included in this study and (2) establishing the isosbestic wavelengths for the individual carotenoids, thus facilitating their quantification. To our knowledge, to date, there are no published methods enabling separation and quantification of as many as 15 different carotenoids belonging to both carotenes and xanthophylls in one run.

Since the isosbestic concept when applied to the determination of feed additives focuses on the quantification of the *sum* of *E*/*Z*-isomers, the optimisation of LC conditions allowing for a chromatographic separation of these isomers was not a target of this study.

2. Experimental

2.1. Reagents and solvents

All chemicals and solvents used were of analytical grade and suitable for HPLC. The selected carotenoids are shown in Fig. 1 and were all-*E*-isomers. Adonirubin (ADR) 95.0%, adonixanthin (ADXN) 98.0%, asteroidenone (AST) 98.0%, echinenone (ECH) 98.0% were purchased at CaroteNature GmbH (Lupsingen, Switzerland). Astaxanthin (AXN) ≥ 98.5%, canthaxanthin (CXN) 94.0%, beta-apo-8′-carotenal (BACAR) > 96.0%, beta-carotene (BCAR) 95.0%, lycopene (LYC) ≥ 97.0% were obtained from Sigma–Aldrich (St. Louis, MO, USA). Lutein (LUT), zeaxanthin (ZXN) were provided by Applichem Lifescience (Darmstadt, Germany). Astaxanthin dimethyl disuccinate (AXN-DMDS), beta-apo-8′-carotenoic acid ethyl ester (BACARE), capsanthin (CSN), and citranaxanthin (CIXN) were kindly provided by DSM Nutritional Products Ltd (Kaiseraugst, Switzerland).

Acetonitrile (ACN), ethanol (EtOH), methanol (MeOH), and tert-butyl-methyl ether (tBME) 99% were of LC gradient grade (VWR, Lutterworth, England) whereas tetrahydrofuran 99% stabilised with 250–350 ppm butylated hydroxytoluene (THF with BHT) was obtained from Alfa Aesar GmbH & Co KG (Karlsruhe, Germany). Pure water (H_2O) 18.2 MΩ cm^{-1} quality was obtained from a Milli-Q Integral 5 System (Millipore, Molsheim, France).

2.2. Standard solutions

Stock solutions for ADR, ADXN, AXN, AXN-DMDS, AST, BACAR, BACARE, BCAR, CXN, CSN, CIXN, ECH, LUT, LYC and ZXN at approximately 1 mg mL^{-1} were prepared in THF with BHT.

Using the respective absorption coefficients A (1%, 1 cm) the exact concentrations of the individual stock solutions were determined spectrophotometrically after dilution in suitable solvents. Next the individual working solutions of each carotenoid prepared in ACN at the concentration of approx. 10 mg L^{-1} were injected in the HPLC system for purity check (purity based on the peak area of the individual carotenoid to the sum of all chromatogram peak areas ratio) and the absorbance of the solutions was corrected for the impurities.

A first mixture working solution consisted of a dilution of each individual stock solution at 50 mg L^{-1} in ACN. The second mixture working solution was obtained from the first solution by dilution in ACN at the concentration of 5 mg L^{-1}. All these working solutions were prepared in amber glass and kept at −30 °C when not in use.

2.3. HPLC conditions for the separation of 15 all-E-carotenoids

All chromatographic measurements were performed using a HPLC Shimadzu system CLASS-VP (Shimadzu, Duisburg, Germany) equipped with a quaternary pump, a degasser, an autosampler and a column heater, and coupled to a diode array detector. The HPLC solution version 1.23 SP1 software controlled the HPLC-DAD system and processed the data. The best HPLC separation of the all-*E*-carotenoids after optimisation as specified in Section 3.1.2 was achieved on a Suplex pKb-100 column, 5 μm, 250 mm × 4.6 mm in gradient mode. The mobile phase A containing ACN, tBME and H_2O (696:200:104; v:v:v) and the mobile phase B containing ACN and tBME (700:300; v:v) were used to employ the following gradient elution: 100% of the mobile phase A was held for 10 min, decreased to 40% at 14 min and held for 14 min. From 28 to 45 min the system was re-equilibrated with the initial composition of the mobile phase A (100%). The flow rate of the mobile phase was 0.5 mL min^{-1}. The column temperature was 20 °C and the sample temperature was kept at 4 °C by means of the thermostated HPLC autosampler. The injection volume in the HPLC system was 5 μL for all the

samples injected. The UV/Vis spectra of all peaks were recorded through the diode array detector in the range from 190 to 700 nm.

3. Results and discussion

Since the absorption coefficients of the target analytes depend on the medium in which they are solved, the study consisted first of the optimisation of the chromatographic separation of 15 all-*E*-carotenoids in a standard solution. Secondly the concept of the isosbestic approach to allow reliable quantification of all 15 carotenoids was developed.

3.1. Determination of appropriate HPLC conditions

3.1.1. Initial optimisation of HPLC conditions

For the selection of the HPLC column and also for the mobile phase, as a starting point systems from literature have been chosen [7–23]. Several reverse phase columns (Lichrospher RP select B, Zorbax RX-C8, Ascentis RP-Amide, YMC Carotenoid C 30, Suplex pKb-100) were tested through a traditional and sequential approach with different combinations of solvents, namely acetonitrile, methanol, tert-butyl-methyl ether and water in order to find optimal HPLC conditions for the separation of the carotenoids. Compared to the other phases examined in this work the Suplex pKb-100 with a mixture of ACN, tBME and H_2O (700:200:100; v:v:v) provided the best separation, and was thus chosen for this study. The Suplex pKb-100 is a column commonly used for carotenoid analyses and is known to exhibit enhanced shape selectivity useful for separation of compounds with rigid, well-defined molecular shape such as carotenoids [8,18]. The maximum injected volume was set at 10 μL, since larger volumes made the peak shape wider and asymmetrical. Then three Suplex pKb-100 chromatographic columns with different internal diameters (IDs) were tested: 4.6 mm, 3.0 mm and 2.1 mm. It was found that the chromatographic columns having smaller IDs than 4.6 mm (3.0 mm and 2.1 mm) turned out to be unsuitable to separate 15 all-*E*-carotenoids. Moreover chromatographic conditions were not easily transferred from the column of 4.6 mm ID to the one of 3.0 mm ID or 2.1 mm ID. The influence of column temperature and flow rate was also examined. Applying a lower flow rate and performing the analysis at lower temperature resulted in better chromatographic separation.

Besides the selection of the specific stationary phase and the other chromatographic conditions, a HPLC gradient elution

Table 1
Ruggedness test 1: The experimental design and the results of analysis expressed in terms of the chromatographic response function (CRF) as defined in the text. The experimental design was based on the initial optimised conditions of phase A: ACN, tBME and H_2O (700:200:100; v:v:v). High values of the CRF stand for improved chromatographic conditions.

Trial number	Replication	H_2O (mL)	tBME (mL)	ACN (mL)	CRF
1	1	96	196	708	36.17
1	2	96	196	708	37.30
2	1	96	204	692	41.12
2	2	96	204	692	40.56
3	1	96	196	692	40.72
3	2	96	196	692	40.40
4	1	96	204	708	40.08
4	2	96	204	708	40.01
5	1	104	196	708	40.98
5	2	104	196	708	41.02
6	1	104	204	692	40.77
6	2	104	204	692	41.27
7	1	104	196	692	41.27
7	2	104	196	692	41.16
8	1	104	204	708	41.43
8	2	104	204	708	41.29

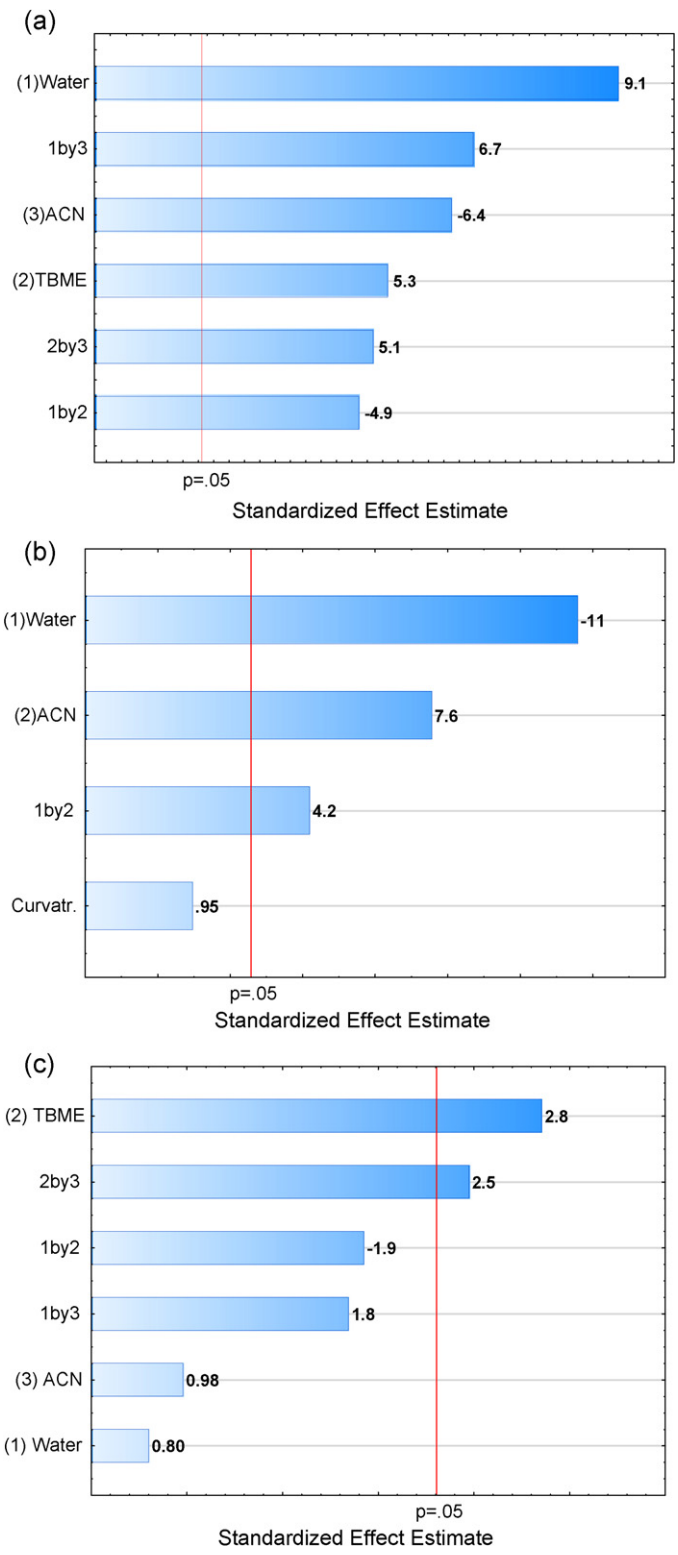


Fig. 2. Standardised effects of factors and interactions on the chromatographic performance including the significance level (vertical line) at $\alpha = 0.05$ for (a) ruggedness test 1, (b) ruggedness test 2 and (c) ruggedness test 3. Interactions of specific factors are indicated by the corresponding numbers. For instance "2by3" means that there is an interaction between TBME and water on the chromatographic performance. Only factors that exceed the line have a significant influence on the chromatography. The LC conditions of ruggedness 3 were considered more robust compared to the conditions of the other ruggedness tests, since the number of factors exceeding the vertical line was much less.

Table 2
Ruggedness test 2. The experimental design was based on the results from ruggedness test 1 indicating that at the initial optimised conditions small variation had a strong influence on CRF. Description as in caption of Table 1.

Trial number	Replication	H_2O (mL)	ACN (mL)	CRF
1	1	104	668	40.30
1	2	104	668	40.63
2	1	104	692	40.99
2	2	104	692	41.10
3	1	120	668	37.49
3	2	120	668	38.04
4	1	120	692	39.90
4	2	120	692	39.65
5	1	112	680	39.79
5	2	112	680	40.10

(described in Section 2.3) was chosen increasing the concentration of the organic solvent in order to minimise the retention time of the most retained carotenoids (LYC and BCAR) within the shortest total analysis time. Thus the total analysis time was shortened from 60 min in isocratic mode to 45 min in gradient mode. When establishing best HPLC conditions we also applied the simplex optimisation procedure [24], starting with the parameter combinations described here. However, even after 14 trials the starting conditions still turned out to be the best one. Therefore we stopped here and proceeded with the ruggedness test of mobile phase A (mixture of ACN, tBME and H_2O (700:200:100; v:v:v)).

3.1.2. Ruggedness tests of the HPLC separation

In order to establish the robustness of the chromatographic separation of the target all-*E*-15 carotenoids against small variation of the composition of the mobile phase, we conducted three ruggedness tests. The tests are based on the principle that small variations of the composition of mobile phase A are introduced on purpose and the effect of these variations on the chromatographic response function (CRF) [25] which was calculated according the following equation:

$$\mathrm{CRF} = \sum_{i=1}^{p-1} R_i + aP + b(t_M - t_L)$$

where R_i is the resolution between adjacent peak pairs, P is the number of peaks detected, t_M is the target retention time (in this case 30 min), t_L is the retention time for the last peak, and "a" and "b" are two arbitrary weighting factors. In order to adjust the value of CRF to the visual inspection of the chromatograms thus to make the difference between CRF values more significant the values for "a" and "b" were set a 1 and 0.3, respectively. Better chromatographic conditions are indicated by higher values for CRF indicating

Table 3
Ruggedness test 3. The experimental design was based on the following conditions that have been slightly modified based on the results from ruggedness test 2.: ACN:tBME:H_2O; 696:200:104; v:v:v. Description as in caption of Table 2.

Trial number	Replication	H_2O (mL)	tBME (mL)	ACN (mL)	CRF
1	1	102	196	686	40.81
1	2	102	196	686	40.60
2	1	102	204	706	41.31
2	2	102	204	706	40.92
3	1	102	196	706	41.14
3	2	102	196	706	41.05
4	1	102	204	686	41.45
4	2	102	204	686	41.23
5	1	106	196	686	41.23
5	2	106	196	686	41.00
6	1	106	204	706	40.97
6	2	106	204	706	40.94
7	1	106	196	706	41.08
7	2	106	196	706	40.91
8	1	106	204	686	41.39
8	2	106	204	686	41.23

(1) sufficient separation of adjacent peaks and (2) an acceptable retention time for the last eluting peak. The small variations are implemented by adjusting the variables at two different levels – specified as high and low – and performing HPLC measurements accordingly. In order to measure the effect of the small variation of a specific variable on CRF, the average of the values for CRF obtained from the experiments at the high and low level is calculated respectively. The difference of both average values is then the effect of this specific variable on CRF. The set up of the experimental design and the statistical assessment was done with the software package Statistica®, Version 10 (StatSoft, Tulsa, OK, USA).

In ruggedness test 1 we selected three variables defining the composition of the mobile phase, which were the volumes of ACN, tBME and H_2O and introduced variations of each variable at two levels into the preparation of the mobile phase. For instance, for the variable ACN one set of experiments was conducted using 692 mL for the preparation of the mobile phase, whereas the second set of experiments was done with 708 mL of ACN. Eight trials of the various combinations of the variable variations were conducted in duplicates (Table 1), in order to estimate the analytical error, against which the significance of the calculated effects and interactions was checked. The results of the statistical assessment as shown in Fig. 2a indicated that CRF was significantly influenced by water, followed by ACN, whereas tBME turned out to be less important. We also observed significant interactions between the factors. For instance, the influence of the water content on the CRL function was much higher, when the concentration of tBME was at the high level. The sign of the factors (Fig. 2a)

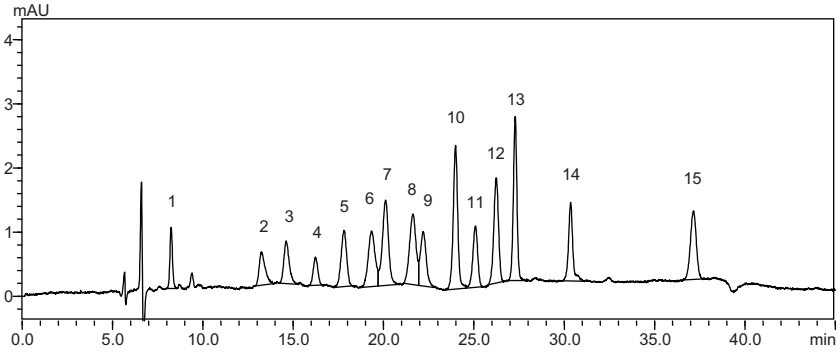


Fig. 3. HPLC separation of 15 all-*E* carotenoids (0.5 mg L^{-1} in ACN); column: Suplex pKb-100, 5 μm, 250 mm × 4.6 mm; mobile phase A: ACN:tBME:H_2O (696:200:104; v:v:v); mobile phase B: ACN:tBME (700:300; v:v); the isocratic conditions: 0–10 min: 100% A; the gradient: 10–14 min gradient 100–40% A, 14–28 min 40% A, 28–45 min 100% A; flow rate: 0.5 mL min^{-1}; column temperature: 20 °C; λ: 410 nm; identification: 1 – AXN-DMDS, 2 – AXN, 3 – ADR, 4 – CXN, 5 – CSN, 6 – ADXN, 7 – BACAR, 8 – AST, 9 – CIXN, 10 – BACARE, 11 – LUT, 12 – ZXN, 13 – ECH, 14 – LYC, and 15 – BCAR.

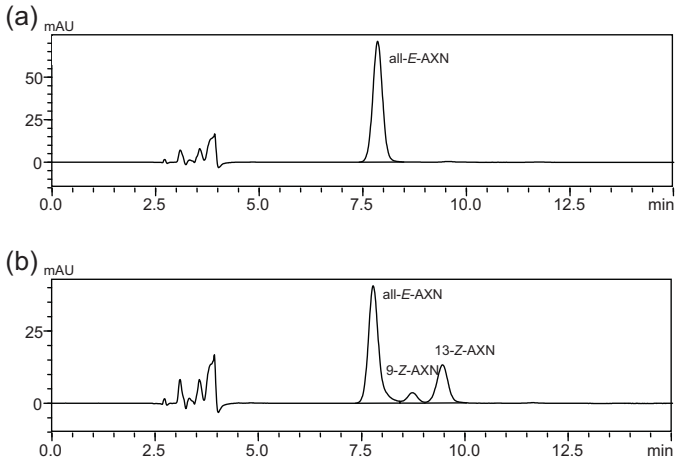


Fig. 4. HPLC separation of AXN isomers (a) before incubation – only all-*E*-AXN isomer and (b) after incubation – a mixture of all-*E* and *Z* isomers; column: Ascentis RP Amide, 5 μm, 250 mm × 2.1 mm; mobile phase: ACN:tBME:H_2O, 600:200:200; v:v:v; flow rate: 0.2 mL min^{-1}; column temperature: 30 °C.

revealed that better chromatographic conditions were observed, when the water and ACN content was high and the tBME was low. The result of the statistical assessment is also reflected in the visual presentation of the chromatograms. For example, in the chromatogram corresponding to combination 1 (ACN:tBME:H_2O (708:196:96; v:v:v), Table 1) delivering the worst mean value for CRF (36.17) we could see the co-elution of ADXN with BACAR and AST with CIXN. The HPLC separation of 15 carotenoids obtained applying the composition of the mobile phase A in combination 8 (ACN:tBME:H_2O (708:204:104; v:v:v), Table 1) gave the best optimised HPLC separation of all compounds (CRF 41.43). Nevertheless, the large variation of the chromatographic separation obtained in the experiments strongly indicated that the method was not robust enough, thus indicating that we had not found yet optimal chromatographic conditions. Therefore we conducted additional experiments to establish better HPLC conditions at which the separation power was still high and the effect of small variations of the composition of the mobile phase on the separation of the target analysed was minimised. This optimisation was pursued by a second factorial design, making use of the results from ruggedness test 1.

The experimental design only included two factors namely (1) water adjusted at higher concentrations and (2) ACN adjusted at lower concentrations. The content of tBME was kept constant, since ruggedness test 1 indicated that the effect of this factor was minor. Furthermore, the remaining two factors were tested at three levels in order to check whether the optimal conditions were already within the variation realised by the experiments of this test. The experimental design and the results are shown in Table 2. The statistical evaluation (Fig. 2b) revealed that water had the most significant impact on the separation of the target analyte, but – in contrast to ruggedness test 1 – the best result was observed when the water content was at the low level (104 mL) and the worst result was observed when the water content was at the high level (120 mL). Furthermore, no significant curve linear effect could be observed in this range. However, the pooled data from ruggedness tests 1 and 2 indicated that the CRF value passes a maximum at a water content of about 104 mL, whereas a lower CRF values were obtained at 112 and 96 mL, respectively. Likewise for ACN, optimal conditions were observed at 692 mL and lower CRF values were obtained for 668 and 708 mL, respectively. Based on these results the initial conditions as established in Section 3.1.1 were slightly modified and the following optimal composition of mobile phase A was established: ACN:tBME:H_2O; 696:200:104; v:v:v.

The objective of the third ruggedness test was to check the impact of small variations of the optimal conditions established in ruggedness test 2 on the CRF value. All three components were included in the study, each of them varied at two levels, resulting in 8 experiments performed in duplicates. The experimental design and the results are shown in Table 3. The results of the statistical assessment as presented in Fig. 2c indicate that most of the factors did not have a significant influence on the CRF value, whereas a significant but very small effect was observed for tBME. Therefore, the selected conditions (ACN:tBME:H_2O; 696:200:104; v:v:v) are considered as robust enough for the intended purpose. This is also confirmed by visual inspection of the 16 chromatograms obtained in this exercise, since in all cases a sufficient separation of the target analytes was obtained.

The best conditions in terms of retention times, peak shapes, repeatability of the signal and highest possible signal to noise ratio are detailed in Section 2.3. The separation of the target 15 all-*E*-carotenoids obtained at the selected conditions is presented in Fig. 3.

3.2. Optimisation of the DAD quantification

The identification of isosbestic wavelengths included two steps, namely (1) the formation of *Z*-isomers from the corresponding all-*E*-isomers by conducting experiments under defined conditions (incubation) and (2) measuring the absorption characteristics of

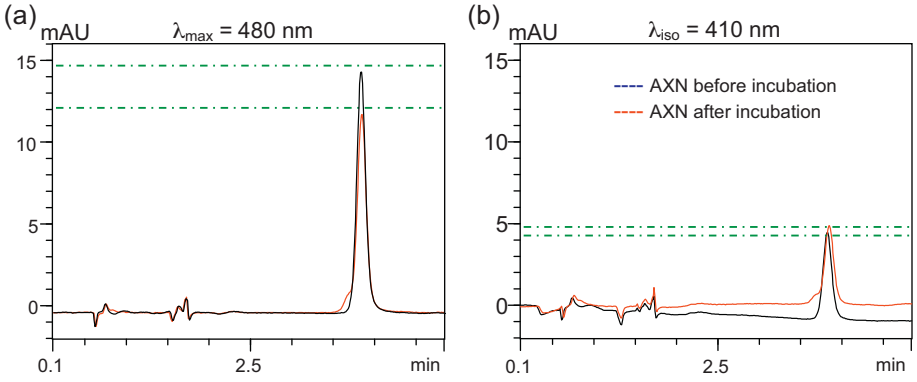


Fig. 5. Chromatograms of the three stereoisomers of AXA that were coeluting at the LC conditions selected, before (black line) and after the incubation (red line). The chromatograms in (a) refer to measurements at λ_{max}: 480 nm and (b) λ_{iso}: 410 nm; column: Suplex pKb-100, 5 μm, 250 mm × 4.6 mm; mobile phase: ACN:tBME:H_2O; 600:200:200; v:v:v; flow rate: 2 mL min^{-1}; column temperature: 20 °C. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

Table 4
The measured wavelengths for the different carotenoids at maximum absorbance (λ_{max}), at isosbestic point (λ_{iso}) and at chosen common wavelength for all carotenoids (λ_{com}); (a) the difference of the absorbances before and after incubation at specific λ and (b) the corresponding sensitivities of the measurement conducted at specific λ expressed as relative absorbance compared to the absorbance at λ_{max}.

Carotenoid			Difference[a] at specific λ (%)	Sensitivity[b] at specific λ (%)
AXN-DMDS	λ_{max}	480 nm	18	100
	λ_{iso}	410 nm	0	29
	λ_{com}	410 nm	0	29
AXN	λ_{max}	480 nm	16	100
	λ_{iso}	410 nm	2	31
	λ_{com}	410 nm	2	31
ADR	λ_{max}	480 nm	25	100
	λ_{iso}	405 nm	1	25
	λ_{com}	410 nm	5	30
CXN	λ_{max}	475 nm	20	100
	λ_{iso}	410 nm	2	31
	λ_{com}	410 nm	2	31
CSN	λ_{max}	475 nm	23	100
	λ_{iso}	385 nm	1	14
	λ_{com}	410 nm	9	32
ADXN	λ_{max}	465 nm	19	100
	λ_{iso}	400 nm	2	26
	λ_{com}	410 nm	4	36
BACAR	λ_{max}	460 nm	10	100
	λ_{iso}	385 nm	1	20
	λ_{com}	410 nm	5	46
AST	λ_{max}	460 nm	19	100
	λ_{iso}	390 nm	1	19
	λ_{com}	410 nm	8	38
CIXN	λ_{max}	470 nm	25	100
	λ_{iso}	385 nm	1	14
	λ_{com}	410 nm	13	33
BACARE	λ_{max}	445 nm	13	100
	λ_{iso}	360 nm	0	9
	λ_{com}	410 nm	9	58
LUT	λ_{max}	445 nm	18	100
	λ_{iso}	380 nm	1	15
	λ_{com}	410 nm	9	48
ZXN	λ_{max}	455 nm	16	100
	λ_{iso}	375 nm	0	12
	λ_{com}	410 nm	5	44
ECH	λ_{max}	460 nm	18	100
	λ_{iso}	400 nm	1	27
	λ_{com}	410 nm	5	37
LYC	λ_{max}	475 nm	35	100
	λ_{iso}	395 nm	0	14
	λ_{com}	410 nm	7	25
BCAR	λ_{max}	455 nm	22	100
	λ_{iso}	375 nm	0	12
	λ_{com}	410 nm	8	41

the solution before and after the incubation against various wavelengths.

3.2.1. Formation of Z-isomers

In order to conduct experiments to establish the isosbestic wavelength we need to look in more details at the physico-chemical characteristics of the *E*/*Z*-isomers and the conditions of their formation. *Z*-isomers of carotenoids may be naturally formed in certain organisms. However, as an example, all-*E*-AXN is the biologically favoured form but can be isomerised to its *Z*-isomer, especially the 9-*Z*- and 13-*Z*-AXN, by environment factors such as heat, light and oxygen [17]. Theoretically, a higher number of many different cis-*Z*-isomers may be formed, the actual number of *Z*-isomers however is quite reduced [1]. Furthermore, high temperature markedly promotes the isomerisation of all-*E*-AXN, thereby leading to the formation of the corresponding *Z*-isomers. We made use of this reaction in order to produce *Z*-isomers from the corresponding all-*E*-isomers. For instance, in the case of AXN the formation of the above mentioned *Z*-isomers was checked by subjecting the solution *after* incubation to LC analysis. Three different chromatographic columns were tested regarding their capability of separating the target analytes, namely Suplex pKb-100, Zorbax RX C8, Ascentis RP Amide. The best separation of all-*E*- and *Z*-isomers of AXN was achieved with the Ascentis RP Amide column

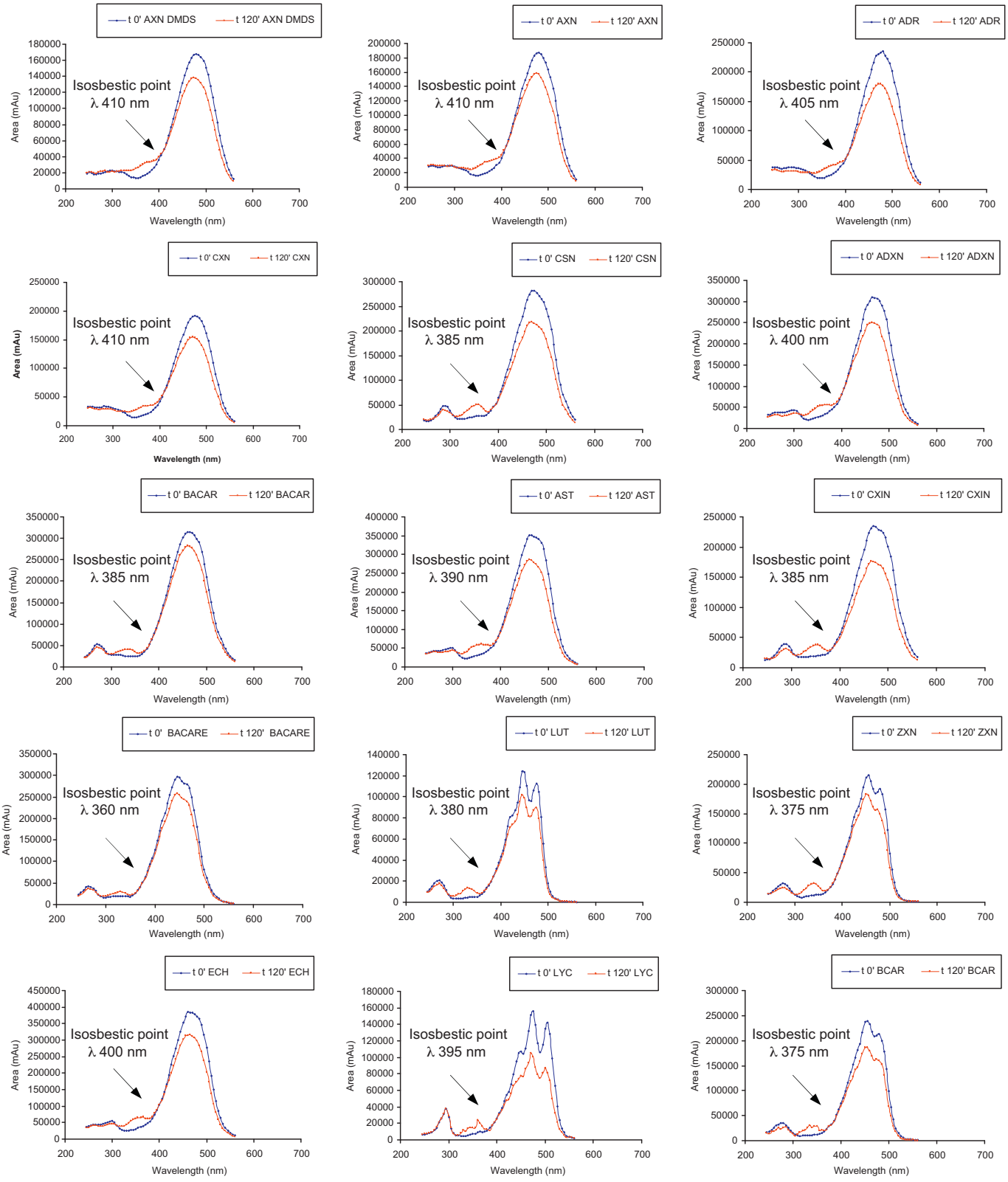


Fig. 6. UV/Vis spectra of standard solutions before (*t* 0′) and after (*t* 120′) incubation; the isosbestic point corresponds to the wavelength at which these spectra cross each other. The abbreviations of the analytes are defined in Fig. 1 and in Section 2.1.

as shown in Fig. 4. The chromatogram obtained from the analysis of the solution *after* incubation showed two additional peaks close to the peak of the all-*E*-isomer that were identified as 9-*Z*- and 13-*Z*-AXN [1].

For the incubation experiments, standard solutions containing individually the all-*E*-isomer of each carotenoid (approx. 10 mg L^{-1} in ACN) were treated at 80 °C in a heating block for 2 h. The incubation conditions were identical for all carotenoids [7]. In addition,

we performed corresponding incubation experiments at 50 °C, in order to check for an impact of a different incubation temperature on the determination of the isosbestic wavelengths.

3.2.2. Determination of isosbestic wavelengths

In the next step the standard solutions were subjected to LC analysis, measuring at different wavelengths ranging from 245 to 560 nm in steps of 5 nm. This analysis was done on both standard solutions, before and after the incubation. For this experiment it was required that specific HPLC conditions were selected in which the all-*E*- and *Z*-isomers of the same carotenoid coeluted. For that purpose we applied the HPLC conditions already optimised in Section 3.1 with some modifications: a decrease in the amount of water in the mobile phase and an increase in the flow rate of the mobile phase up to 2 mL min^{-1}. The test is based on the principle that at the isosbestic wavelength the peaks of the carotenoid before and after the heat treatment have the same area. Fig. 5 shows the result for AXN obtained at two wavelengths. At 480 nm the absorption coefficients for the all-*E*- and *Z*-isomers are different, thus leading to a different peak area for the solution before and after the incubation. In contrast, at 410 nm the difference of the area of both peaks is much smaller, indicating that this wavelength is very close to the ideal isosbestic conditions. The results from the incubation experiments of all carotenoids are shown in Fig. 6, in which the measured peak areas of the standard solutions before and after the incubation were plotted against the adjusted wavelengths, thus showing the spectra of both cases. The isosbestic point corresponded to a wavelength at which these spectra cross each other. The results of experiments are summarised in Table 4, showing individually for each carotenoid the wavelengths of maximum absorbance (λ_{max}) and those of isosbestic conditions (λ_{iso}). The positive effect on the accuracy of the measurement by measuring at λ_{iso} is indicated by the differences of the absorbances obtained before and after the incubation when selecting λ_{max} for the measurement and varied from 10% for BACAR to 35% for LYC. In addition, Table 4 shows the sensitivity obtained at λ_{iso} compared to λ_{max}, which ranged between 9% for BACARE and 31% for CXN.

The results from the experiments at 50 °C demonstrated in most cases a lower formation of *Z*-isomers compared to the corresponding experiments performed at 80 °C. However, the impact of the different incubation conditions on the isosbestic wavelength was in all cases negligible, thus confirming the robustness of the selected isosbestic wavelengths presented in this paper (figures not shown).

Using this isosbestic wavelength the total carotenoid content can be quantified exclusively against the all-*E*-isomer without the risk of underestimation of the sum of all isomers but with a loss of sensitivity of around 3 times.

3.2.3. Applying a common isosbestic wavelength for quantification of 15 carotenoids

Because isosbestic points individually determined for all 15 carotenoids were relatively close to each other covering a range from 360 to 410 nm, we investigated the option of selecting 410 nm as a common wavelength which applied to all analytes and the corresponding isomers. The same wavelength has already been used by Lessin for recording absorbance and quantification of *E*- and *Z*-isomers of alpha-carotene, beta-carotene and beta-cryptoxanthin [10]. In our study measuring all analytes at this common wavelength revealed that the difference in absorbance of a standard solution before and after incubation was in all cases except one below or equal 9%. For CIXN the difference was 13%. The absorbance measured at the chosen common isosbestic wavelength in comparison to the one measured at the maximum wavelength specifically established for each analyte ranged from 25% to 58% for all tested carotenoids. In contrast, the absorbance measured accurately at the isosbestic wavelength in comparison to the one measured at maximum wavelength ranged from 9% to 31% for all tested carotenoids, showing much less sensitivity of the measurement (Table 4).

Applying one common isosbestic wavelength for the quantification of 15 carotenoids is a compromise between quantifying all isomers of carotenoids exclusively against all-*E*-isomers at a maximum wavelength (the risk of underestimation) and quantifying at an isosbestic point (the risk of too low sensitivity). The separation of the target 15 carotenoids quantified at a common isosbestic wavelength is presented in Fig. 3.

In addition, it has been observed that while injecting incubated single standard solutions of individual carotenoids using a final optimised method for the separation of 15 carotenoids some isomers of one carotenoid may slightly coelute with isomers of another adjacent carotenoid. It means that *Z*-isomers of some carotenoids may show up under two or more peaks under optimised HPLC conditions. It should be taken into account that in a real sample there could be an unlimited number of *Z*-isomers and other carotenoids which may coelute and interfere with the carotenoids we have included in our method.

3.3. Calibration curves, limits of detection and qualification

As a part of single-laboratory validation, linearity, correlation coefficient, limit of detection (LOD) and limit of quantification (LOQ) of the method for the determination of carotenoids at 410 nm were assessed.

The calibration curves were linear in the range from 5 mg L^{-1} to 25 mg L^{-1} while correlation coefficient for all 15 carotenoids was above 0.9987.

Table 5
Calibration curves, LODs and LOQs for 15 carotenoids (n = 3).

Carotenoid	Slope	Intercept	Correlation coefficient	LOD (mg L^{-1})	LOQ (mg L^{-1})
AXN-DMDS	17,830	−1330	0.9998	0.09	0.29
AXN	30,040	117	0.9997	0.12	0.40
ADR	35,043	−10,451	0.9996	0.13	0.43
CXN	20,405	3122	0.9994	0.12	0.41
CSN	33,768	−3640	0.9998	0.08	0.26
ADXN	39,407	−12,754	0.9987	0.07	0.23
BACAR	68,307	−16,660	0.9994	0.10	0.33
AST	57,324	−8356	0.9995	0.10	0.32
CIXN	32,432	−6824	0.9993	0.14	0.48
BACARE	75,816	−26,378	0.9997	0.06	0.20
LUT	32,041	−6056	0.9998	0.09	0.30
ZXN	53,930	−13,790	0.9997	0.09	0.32
ECH	64,133	−17,916	0.9997	0.08	0.27
LYC	38,648	−7903	0.9997	0.12	0.39
BCAR	44,191	−11,717	0.9997	0.08	0.26

LODs calculated as three times the signal-to-noise ratio and LOQs calculated as ten times the signal-to-noise ratio were determined using calibration curves established at appropriate low concentration levels of the target analytes and ranged from 0.06 mg L^{-1} to 0.14 mg L^{-1} and from 0.20 mg L^{-1} to 0.48 mg L^{-1}, respectively. Additionally the values calculated were confirmed experimentally by injecting the standard solutions of carotenoids at the concentration level corresponding to the obtained LODs and LOQs (Table 5).

4. Conclusion

A new RP-HPLC-DAD method for the separation and the quantification of adonirubin, adonixanthin, astaxanthin, astaxanthin dimethyl disuccinate, asteroidenone, beta-apo-8′-carotenal, beta-apo-8′-carotenoic acid ethyl ester, beta-carotene, canthaxanthin, capsanthin, citranaxanthin, echinenone, lutein, lycopene, zeaxanthin in standard solution has been developed.

The isosbestic point approach has been applied to calculate the concentration of 15 carotenoids. Isosbestic points for all 15 carotenoids have been determined and the chromatogram of a standard solution mixture of 15 carotenoids at one common isosbestic wavelength has been presented.

This analytical method is designed for the determination of the content of carotenoids against all-*E*-standards. It has been demonstrated that while measuring at an isosbestic wavelength the result for the content of carotenoids does not depend on the ratio of the *E*/*Z*-isomers present in the product forms.

Going further calculating the concentration of all 15 carotenoids at one common isosbestic wavelength (410 nm) reduces the risk of underestimation by quantifying *Z*-isomers exclusively against all-*E*-isomers and ensures high sensitivity. Another advantage of this approach is the possibility of using single-wavelength UV/Vis detectors instead of multi-wavelength acquisition with a DAD in one run. Alternatively analyte specific wavelengths can be adjusted, when using a HPLC system equipped with a DAD.

It can be finally concluded that the developed multi-analyte method based on multi-lambda acquisition with a DAD in one run could be an appropriate tool for the detection and determination of carotenoids in a wide range of carotenoids mixtures and extracts.

The next step will be to complete the optimisation of the sample preparation of feed samples and to apply the complete methodology to the determination of the target analytes in complete feedingstuffs and to develop a single-laboratory validated method which meets the criteria of relevant European legislation [6].

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