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Method for beta-carotene extraction from processed baby foods as a model for plant-based fatty food products.

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Key words: Carotenoid analysis, Baby foods, Plant-based fatty products, Antioxidant

Highlights

- A one-pot β-carotene extraction method was developed for plant-based baby foods
- Improved β-carotene recovery by enzyme-aided extraction
- Phenolic antioxidants added to calibration solutions avoid systematic analytical errors
- The apparent recovery of β-carotene in fatty matrices after saponification was 80%
- β-Carotene in 50 vegetable- and 22 fruit-based processed baby foods was determined.

Abstract

Reliable quantitative determination of carotenoids in complex food matrices such as processed baby food products is challenging because of their incorporation in rigid cellular structures, their sensitivity to oxidation and their lipophilic character. A one-pot liquid-liquid β-carotene extraction procedure is described for solid baby foods, in the presence of enzymes (Clara-Diastase and Rapidase) facilitating matrix disintegration.

The combined extraction and enzymatic dissolution not only protected β-carotene from oxidation compared to the sequential approach, but also reduced the use of solvents and amount of filtrations steps, favouring a higher recovery.

The addition of phenolic antioxidants (BHT, TBHQ and BHA) to calibration solutions and during the procedure at 25 mg/mL resulted in an up to 2.5-fold higher absorbance of β-carotene solutions which was not observed for trans-β-apo-8'-carotenal (used as internal standard) solutions.

When applying the full procedure on β-carotene spiked sunflower oil, an apparent recovery of 80% for β-carotene was obtained. Finally, this protocol was applied to 50 vegetable-based and 22 fruit-based processed baby foods (range 0 to 1179 and 504 µg/100g, respectively), and it was concluded that this extraction procedure may be used for similar processed foods products. The procedure proved to be sensitive (LOD = 0.12 µg/mL) and reproducible (CV for baby foods: 4-10%).

Introduction

Carotenoids are plant pigments and their consumption is associated with beneficial health effects. Some carotenoids, such as β-carotene, have pro-vitamin A activity, and may therefore contribute to epithelial cell development, growth, fertility and eye functioning (Wiseman, Bar-El Dadon, & Reifen, 2017). Carotenoids have also been associated with a reduction of metabolic syndrome (Goncalves & Amiot, 2017), improved bone health (Xu, Song, Song, Zhang, & Li, 2017) and reduced cancer development (Shareck, Rousseau, Koushik, Siemiatycki, & Parent, 2017), although some controversy still exists (Goralczyk, 2009). Therefore, their adequate determination in foods and the estimation of their intake and bioavailability is of outmost importance for advisory bodies giving dietary guidelines to the general population.

Many carotenoid extraction protocols have been described in literature, (R. K. Saini & Y.-S. Keum, 2018), but few of them are generally applicable for all types of foods, because in contrast with blood or human/animal tissues, food matrices can be of a very diverse nature. In plants, carotenoids are located within liquid-crystalline or solid-crystalline structures present in cellular chromoplasts (Schweiggert, Mezger, Schimpf, Steingass, & Carle, 2012), where they appear in free or esterified forms together with other lipophilic components (Mercadante, Rodrigues, Petry, & Mariutti, 2017). Within these subcellular structures, carotenoids are quite well protected from oxidation and the impact of the digestive system with its mechanical stress, pH extremes and enzymatic activities. Food processing, such as heating, may result in a (partial) release of carotenoids from the cellular environment, improving their bioavailability. A further improvement can be achieved by the presence of lipids in the food matrix (Brown et al., 2004), while the presence of dietary fibre, pectin, and cell wall components will result in a decreased release (Cervantes-Paz et al., 2017; Palafox-Carlos, Ayala-Zavala, & Gonzalez-Aguilar, 2011).

The analytical recovery of carotenoids from plants is affected by the matrix. The presence of lipids typically requires a saponification step to remove the bulk triacylglycerols, but as the carotenoids are then liberated from their matrix, they are particularly prone to degradation. In addition, saponification may lead towards further degradation of the carotenoids, depending on their structure (Kimura, Rodriguez-Amaya, & Godoy, 1990). The presence of cell wall components can in addition affect the extraction efficiency of the carotenoids (R. K. Saini & Y. S. Keum, 2018). These aspects make carotenoid analysis in vegetable matrices containing lipids particularly challenging. In this context, the addition of enzymes, was successfully applied for both analytical and commercial purposes (Kumar, Sharma, Ratrey, & Datta, 2016; R. K. Saini & Y. S. Keum, 2018; Schierle, Pietsch, Ceresa, Fizet, & Waysek, 2004).

In this paper, a step-wise description of a new procedure to extract and analyze β-carotene in vegetable-based matrices containing lipids is presented, with particular focus on its release from a food matrix using an enzymatic pre-treatment and removal of interfering lipids using saponification while retaining stability using different types of anti-oxidants. After validation, the method was applied to quantify β-carotene in processed baby foods, including vegetable and fruit-based meals.

Materials and methods

Chemicals and enzymes

Trans-β-apo-8'-carotenal, β-carotene, potassium hydroxide, triethylamine, sodium chloride, anhydrous sodium sulphate, ammonium acetate, magnesium carbonate and Clara-Diastase were purchased from Sigma (Bornem, Belgium) and Rapidase solution from DSM (Delft, the Netherlands). Hexane, methanol and ethanol were from Fisher-Scientific (Geel, Belgium), and pentane and acetone were from Chemlab (Zedelgem, Belgium). Ethyl acetate,

acetonitrile, and methanol were purchased from Biosolve (BV, Valkenswaard, The Netherlands) and were of HPLC grade quality. Denaturol (ethanol with 2% isopropanol and 2% methyl ethyl ketone) was from Tailer Made Chemicals. Butylhydroxytoluene (BHT), tert-butylhydroquinone (TBHQ), butylhydroxyanysole (BHA) were purchased from Sigma. All reagents were of analytical grade.

Preparation of the standards

All extractions steps were performed in brown or aluminium-foil wrapped glassware and under dimmed-light conditions to avoid photo-oxidation of the carotenoids. The use of plastics was completely avoided. Stock solutions of β-carotene and trans-β-apo-8'-carotenal (internal standard) were prepared in dichloromethane containing 1 g/L BHT, at concentrations of 250 mg/L and 175 mg/L, respectively, and aliquots were stored at -20°C. Spectrophotometric measurements showed no significant degradation of these stock solutions during the course of the experiments which lasted at least two years. Working solutions were prepared by dilution of the stock solutions in dichloromethane (1:9 *v/v*), and were stored at -20°C. Calibration solutions were prepared daily. The concentration of β-carotene and trans-β-apo-8'-carotenal were determined by measuring the absorbance of a 1:49 *v/v* dilution of the working solutions in methanol at λ = 450 nm for β-carotene, and λ = 470 nm for trans-β-apo-8'-carotenal. Their actual concentrations were calculated based on the Lambert-Beer law according to the following formula:

$C(mg/L) = ((A_{sample}-A_{blank})*10000)/(E^{1\%}_{1cm})$

with A_{sample} the absorbance of the diluted carotenoid working solution, A_{blank} = absorbance of methanol, $E^{1\%}_{1cm}$ = 2592 for β-carotene, and $E^{1\%}_{1cm}$ = 2640 for trans-β-apo-8'-carotenal (Vandekinderen et al., 2008).

To prepare the calibration solutions, HPLC vials were first filled with 1mL denaturol, or 1 mL of either a BHT, TBHQ or BHA solution (2.5% *w/v* in denaturol) and dried under a gentle nitrogen gas flow. Next, 0, 60, 120, 180 and 250 µL of β-carotene working solution was added to the vials, as well as 150 µL trans-β-apo-8'-carotenal working solution. Again, the vial was dried and the residue was redissolved in 1mL HPLC-grade methanol prior to injection. A methanol blank (either with or without BHT, TBHQ or BHA) was included.

Extraction and enzymatic pretreatment

The extraction process is visualized in Figure 1. Three g sample was weighed in a 100 mL glass centrifugation tube. Conditions with and without enzymatic pretreatment were compared, as well as the use of different solvents for extraction. Next to the tested food products, a control sample consisting of freeze-dried mango powder (±19 µg β-carotene per g dry weight), stored in light-protected and vacuum aliquots at -80°C, was extracted and analysed daily to monitor and control inter-day variability.

For the enzymatic pretreatment, a saturated aqueous NaCl solution (5 mL), TBHQ (2.5% *w/v* stock solution in denaturol, 1 mL), Clara-Diastase (powder ≥35U/mg, 10% *w/v* stock solution in water, 2 mL), Rapidase (1 mL undiluted solution), and an acetone/pentane mixture (4:6 *v/v*, 5 mL) were added and incubated for 30 min at 30°C in a warm water bath. Next, an additional 10 mL acetone/pentane solution (4:6 *v/v*) was added to the tube, the content was manually mixed during 1 min, and centrifuged for 10 min at 4000 *g* using a Hettich bucket-swing centrifuge (Voor 't Labo, Eeklo, Belgium). The top solvent layer was transferred to a 20 mL glass tube using a pasteur pipet, and the acetone/pentane extraction of the water phase was repeated twice using each time 10 mL of the solvent mixture. Depending on the expected carotenoid concentration, either the full extract was used for low concentrations, or the volume was adjusted with pentane to 50 mL. In that case, 10 mL was taken for further

analysis. Due to the high volatility of the solvents, no rota-evaporation was necessary, and therefore, for both cases, the solvent was evaporated at room temperature using a gentle nitrogen flow. Apart from the acetone/pentane (4:6) mixture, also pentane and ethanol/hexane (4:3) were evaluated.

Saponification

To the test tube containing the dried extracted residue, 1 mL magnesium carbonate solution (10 mg/mL stock solution in denaturol), 13 mL denaturol and 4 mL potassium hydroxide solution (50% *w/v* stock solution in water) were added and vortexed. Three saponification conditions were tested. For the first two conditions, the sample was present in a capped test tube, and incubated either overnight at 30°C, or 90 min at 50°C, using a rotating device placed in an incubator. For the third condition, the sample was transferred to an erlenmeyer, and heated to 100°C for 30 min under reflux.

Carotenoid extraction after saponification

The saponified mixture was transferred in a separatory funnel, and the glass recipient was washed with 20 mL of an aqueous NaCl (10% *w/v*) solution which was added to the mixture. Next, 10 mL hexane and 150 µL internal standard (trans-β-apo-8'-carotenal working solution) were added, the funnel was capped, and the content was vigorously shaken (3 to 5 times) and the phases were allowed to separate. The aqueous layer was removed from the bottom and retained in a glass recipient. The hexane top layer was collected from the top of the funnel in another glass recipient. The aqueous phase was added to the separatory funnel, the glass recipient was rinsed with 10 mL hexane which was then used to extract the aqueous phase again. The whole procedure was repeated one more time.

The combined organic fractions were subsequently transferred to the same separatory funnel, the glass recipient was rinsed with an aqueous 10% NaCl solution which was then used to wash the organic fraction. The aqueous phase was discarded and the washing was repeated twice. Next, the same procedure (i.e., the three washing steps as described above) was performed with 10 mL distilled water.

Finally, the hexane fraction was transferred to a folded paper filter (130 mm, Novolab) with anhydrous Na_2SO_4 to remove the remaining water, and the filter was washed with hexane until all colour was removed (up to 50 mL for high carotenoid containing samples). Next, most of the solvent was removed using a rotary evaporator at 30°C, and the remaining extract (approximately 1 mL) was dried under a gentle nitrogen flow. The dried residue was redissolved in 1 mL methanol, filtered (0.45 µm pore diameter, Millipore) and injected into the HPLC. AOACal formation was not observed.

HPLC analysis of β-carotene

Carotenoids were analysed by reversed-phase high-performance liquid chromatography (HPLC) with DAD detection (Ultimate 3000, Dionex, Thermo, Landsmeer, The Netherlands). Samples were placed in an autosampler with light-cover and without extra cooling to avoid precipitation of the extract in the vials. A reversed phased YMC-pack C30 column (250 mm x 4.6 mm i.d., S-5µm, Schermbeck, Germany) was used with methanol:acetonitrile (9:1 *v/v*) as mobile phase A and ethyl acetate with 0.25% triethylamine as mobile phase B. The flow rate was 1 mL/min and the column temperature was 30°C. The gradient profile was at 0 min 100% A, linear gradient to 40% A and 60% B over 25 min, isocratic for 5 min, linear gradient to 100% A over 5 min, isocratic for 5 min. β-Carotene and trans-β-apo-8'-carotenal were analysed at λ= 450 and 470 nm, respectively.

Five-points calibration lines were constructed by linear interpolation of the ratio of the experimentally obtained area of β-carotene/trans-β-apo-8'-carotenal in function of the ratio of the added concentrations of β-carotene/ trans-β-apo-8'-carotenal. Recoveries of β-carotene and trans-β-apo-8'-carotenal of the extracts are expressed as percentage to the expected concentration.

Electrospray ionisation-time-of-flight/mass spectrometer (ESI-TOF/MS) infusion experiments

A β-carotene solution (5.6 mg β-carotene/mL) alone or with 25 mg/mL TBHQ was directly infused into the microTOF II mass spectrometer (Bruker Daltonics, Germany). The electrospray ionization source was operated in positive mode; the nebulizer flow (nitrogen gas) was set at 4 L/min and its pressure was 0.4 bar and the dry temperature at 190°C. The capillary voltage was maintained at 4500V, the capillary exit voltage at 90V, the skimmer potential was 30V and the hexapole RF was set at 150V. The MS-data was recorded using a scan range of *m/z* 50 to 3000.

Statistics

Data were analyzed using a Kruskal-Wallis test in SPSS.

Results and discussion

The current protocol was based on earlier published methods, including the AOAC method for determination of β-carotene in supplements and raw materials by HPLC. The methodology is thus strongly adapted from the procedures as published earlier (Bunea et al., 2008; Taungbodhitham, Jones, Wahlqvist, & Briggs, 1998; Vandekinderen et al., 2008) (Schierle et

al., 2004). Briefly, these protocols describe liquid extraction of carotenoids from the food matrix using different mixtures of solvents such as hexane, ethanol, petroleum ether and diethyl ether, after mechanical and/or enzymatic destruction of the food matrix, overnight saponification using methanolic KOH, and HPLC-DAD analysis. When applying these methods to an industrially processed baby food, which is a model matrix for (mainly) plant-based food products to which extra lipids are added, three major drawbacks were identified. These included (i) carotenoid losses because of the extensive and time-consuming procedures (i.e., lengthy filtration step of the extracted sample), (ii) a remarkable impact of added anti-oxidants (BHT, TBHQ and BHA) on the absorbance intensity of β-carotene (but not of trans-β-apo-8'-carotenal) and (iii) a strong degradation of the carotenoids during saponification.

Development of a one-pot extraction method for the extraction of the food sample

A first modification of the published protocols was to introduce the concept of a one-pot extraction procedure in the first extraction steps prior to saponification. Previously, carotenoid release from the matrix was performed by intensive ultraturrax mixing (thereby introducing air into the slurry), and then filtering the mixture and washing the filter with hexane. This step is tedious due to prolonged filtration, requiring moreover relatively high amounts of solvents to ensure that the filtration residue is colorless (typically >50 mL) and all carotenoids are extracted. Due to the lengthy exposure to air however, carotenoids are susceptible to losses as a result of oxidation. In the presented protocol, the ultra turrax mixing and filtration step were omitted. Rota-evaporation was avoided as well because of following elements. It would have required an additional manipulation of the extract by transferring to another recipient which was considered as an extra potential source of analytical error. In addition upon evaporation using a rota-vapor it cannot be excluded that samples run dry and leave for a particular time unattended. As during this time β-carotene, spread over the whole glass surface, is exposed to

a high temperature, an additional risk of degradation is introduced. Instead, solvent evaporation under a gentle nitrogen flow was introduced prior to saponification. In order to reduce the time needed to evaporate the solvent (10-30 mL), the use of acetone and pentane as more volatile alternatives for ethanol and hexane was proposed. Then, evaluate the extraction efficiency of this alternative solvent mixture, 120 µL working solution (containing 3 µg β-carotene) was spiked to 7.5 mL water, containing 25 mg BHT and 10 mg magnesium carbonate, and extracted using three types of solvents: acetone/pentane (4:6), pentane, and ethanol/hexane (4:3). β-Carotene recoveries were 262 ± 2%, 261 ± 6%, 232 ± 16% for acetone/pentane, pentane and ethanol/hexane, respectively, and of trans-β-apo-8'-carotenal 111 ± 1%, 109 ± 2%, 104 ± 6%, respectively (n=3 for each condition). A first conclusion is that the solvents gave similar recoveries according to a Kruskal-Wallis test (SPSS), and therefore, the acetone/pentane mixture was chosen because it combines a strictly hydrophobic solvent with a more polar one, which is known to favour the extraction of all lipid species. However, a second conclusion is that the apparent recoveries of β-carotene were more than double of the expected recoveries. This was due to the observed substantial increase in absorbance of β-carotene, and not of trans-β-apo-8'-carotenal, during HPLC-DAD analysis, which has, as far as we know, never been described before. Therefore, this phenomenon was further investigated.

Increase of the absorbance of β-carotene calibration solutions in the presence phenolic antioxidants

To investigate the unexpected increase in absorbance of β-carotene, respectively (i) 5 mL of acetone/pentane (6:4 *v:v*) mixture, (ii) 1 mL denaturol, (iii) 10 mg magnesium carbonate in 1 mL denaturol or (iv) 25 mg BHT in 1 mL of denaturol were spiked with β-carotene (3 and 4.5

µg), dried and redissolved in methanol. Recoveries ranged between 85 and 95%, (i to iii) except in the presence of BHT (iv), for which recoveries of 172% were obtained (detailed results not shown). This suggested that the increased absorbance of β-carotene was caused due to the presence of the phenolic antioxidant.

In a second experiment, to check whether this effect was antioxidant specific, three different phenolic antioxidants, more specifically BHT, TBHQ or BHA were added to the calibration solutions at a concentration of 25 mg/mL. Control calibration solutions without phenolic antioxidant were considered as well. As such four sets were obtained, which all contained appr. 2.8 µg/mL trans-β-apo-8'-carotenal, and appr. 0.0, 1.4, 2.7, 4.0 and 5.5 µg/mL β-carotene (Supplementary Table 1). Each set was injected consecutively and this procedure was carried out 5 times to take into account oxidation phenomena in the solutions over time. The time between the injection of the same calibration solution was 1000 min. For each set, five calibration lines were constructed, and the last calibration line was obtained for calibration solutions which were 'aged' for about 4000 min (about 66h). Figure 2 presents the calibration curves of the first and fifth injection sequence whereas Table 1 describes their characteristics. The raw data including absorbance and retention times of β-carotene and trans-β-apo-8'-carotenal, and apparent recoveries are presented in Supplementary Table 1 and 2, respectively.

A first observation was that all antioxidants gave a substantial significant ($p<0.05$) increase in absorbance of β-carotene compared to antioxidant-free calibration solutions (appr. 50 to 100% increase in apparent recovery depending on concentration and antioxidant type, see Supplementary Table 2), whereas this was not observed for trans-β-apo-8'-carotenal. This points to a β-carotene-specific effect that is generally visible for all tested antioxidants. Note that no additional peaks were visible in the chromatogram, and therefore it was assumed that

this effect was not the result of photodegradation reactions, such as the ones described by Pesek et al. (Pesek & Warthesen, 1990).

When considering the calibration curve characteristics presented in Figure 2, Table 1 and Supplementary Table 1, it is observed that the calibration lines obtained by injection the β-carotene solutions without any phenolic antioxidant showed a good linearity for the freshly prepared solutions and the solutions 'aged' for about 1000 min (so up to the second injection sequence indicated with 2 or inject.2). Similarly, the slope of the calibration lines remained constant. Upon further aging however a loss in absorbance of the highest calibration solutions (containing 5.6 µg β-carotene/mL) was observed resulting in a loss of linearity of the calibration lines. Overall, after 4000 min of 'aging' a loss in absorbance of 24% was observed for this particular calibration solution. Omitting the areas obtained from the injection of these solutions, restored the linearity and the slopes proved to be rather similar, even after aging the solutions up to 4000min. It is hypothesized that the solution containing the highest β-carotene concentration was prone to oxidation, which can be explained by the pro-oxidative character of this carotenoid at higher concentrations as reported before (Palozza, Serini, Di Nicuolo, Piccioni, & Calviello, 2003). The fact that β-carotene was prone to oxidation in the calibration solution was confirmed via qualitative ESI-TOF-MS infusion measurements. By infusing the pure β-carotene solutions without antioxidants (containing 5.6 µg β-carotene/mL), the mass spectrum clearly indicated the presence of ions with an *m/z* value which could be attributed to oxidized colorless β-carotenes species as reported before (Zeb, 2012), such as β-ionylidene, 12'-apo-β-carotenal and β-carotene di-epoxides, having $[M+H]^+$ at *m/z* 219.0317, 5351.0177 and 569.9132 respectively.By adding BHT to the β-carotene solutions, a significant increase compared to antioxidant-free conditions ($p<0.05$) of the slopes of the linear calibration lines obtained by injecting the (freshly) prepared calibration solutions was observed again, showing the reproducible character of the earlier described phenomenon. A 70% increase in the slope

of the calibration lines compared to those obtained for β-carotene solutions void of phenolic antioxidants was observed, corresponding to the earlier observed 'apparent recovery' of 172%. Upon 'aging' the BHT-containing calibration solutions, a limited decrease of the slope and R^2 of the calibration lines was observed. Especially the calibration solution containing the highest β-carotene concentration (5.3 µg/mL) showed a gradual decrease in absorbance (area loss of 13% over 4000 min). Omitting this experimental point from the calibration line, slopes and linearity of the calibration lines was stable over the total time of the experiment. As mentioned before, this was not the case for the calibration lines obtained with β-carotene solutions not containing BHT. As the deviation of linearity of the calibration lines of pure β-carotene solutions was attributed to pro-oxidative effects of this carotenoid, it seemed that BHT could temper somewhat the observed pro-oxidative effect of β-carotene. No bathochromic or hypsochromic shifts in the UV-VIS absorbance spectrum of β-carotene were observed due to the presence of BHT (Supplementary Figure 2).

For TBHQ, linearity of the freshly prepared calibration solutions was already suboptimal, again due to a lower than expected absorbance of the calibration solution with the highest concentration. Omitting this solution improved linearity, but better results were obtained by restricting the calibration range from 0 to 3 µg β-carotene/mL. The slopes of the calibration solutions were even higher compared to those obtained from the solutions containing BHT: the slopes more than doubled compared to the calibration lines obtained by injecting β-carotene solutions void of phenolic antioxidants. Similar as before, a time dependent decrease was observed in the absorbance of the most concentrated β-carotene calibration solutions. A 40% drop in absorbance of the 5.3 µg β-carotene solution was observed in the most 'aged' solution compared to the freshly prepared solution. So although higher slopes were obtained for calibration solutions containing TBHQ, indicating that the presence of TBHQ protected β-carotene from oxidation, the solution containing the highest β-carotene concentration seemed

more sensitive to degradation in the presence of this phenolic antioxidant compared the pure β-carotene solution. So it seems that at high β-carotene concentrations, TBHQ also exacerbates the pro-oxidative character of the carotenoid which can be related to the pro-oxidative character of some phenolic antioxidants, on the contrary this synergistic effect was not observed for BHT. Similarly to the pure β-carotene calibration solutions, by infusing the TBHQ-containing β-carotene solutions on the TOF/MS instrument oxidized β-carotene-species could be detected. So despite the fact TBHQ seemed to prevent to a considerably extent β-carotene oxidation, a full protection from degradation could not be realized. In an additional experiment in which the impact of TBHQ concentration on the increased absorbance of a single β-carotene solution (2.7 µg/mL) was evaluated, it was shown that also at the lowest TBHQ concentration evaluated (8.9 mg/mL) an increase in absorbance was observed that was independent upon the TBHQ concentration in the tested range (up to 42 mg TBHQ/mL) (Supplementary Figure 1A). In another additional experiment, TBHQ was pre-exposed to air for 2 and 24 h before addition to a β-carotene calibration solution (Supplementary Figure 1B), and it was observed that the impact on the absorbance of β-carotene of the ’24 h aged’ TBHQ was much lower than the freshly prepared TBHQ, thereby confirming the hypothesis of a potential oxidation-mediated mechanism leading to β-carotene degradation. In all these experiments, no bathochromic or hypsochromic shifts in the UV-VIS absorbance spectrum of β-carotene could be observed due to the presence of TBHQ (Supplementary Figure 2).

For freshly prepared calibration solutions containing 25 mg BHA/mL, a good linearity was observed and again the slope was considerably higher compared to those of the calibration lines obtained by injecting pure β-carotene solutions. However, chromatograms showed many additional peaks which eluted earlier compared to β-carotene, suggesting polar (oxidation) compound formation (Figure 3). Remarkably, these peaks were not observed in the other sets

of calibration solutions. When comparing the DAD spectra to those of β-carotene (absorbance maxima at 452/478 nm, %III/II = 18), we found that peak 2 (max. 425/450 nm, %III/II = 53), peak 3 (max. 445/469 nm, %III/II = 4, $\%Ab/A_{II}$ = 35) and peak 4 (max 447/473 nm, %III/II = 28, $\%Ab/A_{II}$ = 3) showed strong similarities with the DAD-spectra of 5,8-epoxy-β-carotene, 15-*cis*-β-carotene and 9-*cis*-β-carotene (Rodriguez-Amaya, Institute, & OMNI, 2001). It is therefore speculated that BHA has a stronger pro-oxidant character compared to TBHQ.

To our knowledge this is the first time that the observed chemical interactions between β-carotene and phenolic antioxidants are reported. The observations suggest that a complex balance between the pro- and antioxidant nature of both β-carotene, its oxidation products and the respective phenolic antioxidants is in play. On the basis of these observations it is hypothesized that in pure β-carotene solutions, a substantial part of β-carotene is quickly oxidized, a process which can be reduced by the phenolic antioxidants. Remarkably, a further progressive degradation of β-carotene in the time course considered in this study was not observed at least when the β-carotene remained lower then about 5 µg/mL: the slope of the calibration lines obtained by injecting pure β-carotene solutions did not decrease over the time course of these experiments. This allows to speculate that some of the oxidized β-carotene species also exhibit anti-oxidative effects. Although care should be taken to generalize the polar paradox theory of antioxidants (Shahidi & Zhong, 2011) these observations could be a currently unknown practical illustration of this theory. In conclusion it seems pivotal to supplement the β-carotene calibration solutions with an appropriate phenolic antioxidant and also to ensure that the concentration of the β-carotene solutions remains low enough to avoid pro-oxidative effects caused by this carotenoid.

As addition of an antioxidant to the samples prior to the extensive carotenoid extraction procedure is absolutely warranted, it is considered necessary in view of the reported observations that the same amount of anti-oxidant should be added to the vials during the preparation of the calibration solutions. In a next experiment, extra TBHQ (25 mg/mL) was added to the calibration solutions, and the first part of the extraction procedure (enzymatic pretreatment and solvent extraction) was performed on 60, 120, 180 and 240 µL (corresponding with 1.5, 3, 4.5 and 6.25 µg, respectively) of the β-carotene working solutions. The average recovery was 100 ± 3 % for β-carotene and 102 ± 1 % for trans-β-apo-8'-carotenal, so TBHQ could indeed be accounted for protecting β-carotene in the calibration solutions. Retrospectively, the use of BHT could probably be preferred in view of the synergistic pro-oxidative effect of TBHQ observed at the highest β-carotene concentrations. Nevertheless, a case-per-case evaluation of β-carotene analysis in the presence of TBHQ for improved sensitivity, or BHT for a larger linear range, is recommended. It should be stressed however that especially with respect to the analysis of the real food samples, the actually injected extracts contained never more than 3 µg β-carotene/mL.

Effect of enzymatic pretreatment

In this study, two enzymatic preparations (Clara-Diastase and Rapidase) were applied to improve the solubilization of processed baby food products and hence the extraction efficiency of β-carotene. According to the manufacturers data sheets, Clara-Diastase is a mixture of α-amylase, cellulase, invertase, peptidase, phosphatase and sulfatase, whereas Rapidase is a liquid form of pectinase from *Aspergillus niger*. This broad spectrum of added enzymes therefore facilitates the degradation of cell wall polysaccharides and proteins, which strongly impact carotenoid (bio-)accessibility. In a first set of experiments, Clara-Diastase (10%) was added to baby food products which were expected to be either low and high in

carotenoids (A: beans, potato and beef, and B: beef, carrots and tomatoes, both brand B1), and incubated at room temperature (approx. 21°C), 30°C and 40°C. It was visibly observed that sufficient solubilisation was achieved after 30 min of incubation at all tested temperatures In a next experiment, Clara-Diastase concentrations of 10, 5, 1, 0.5 and 0% alone, or in the presence of 1 mL Rapidase were added to the same baby foods, incubated at 30°C for 30 min. A complete solubilization was achieved with 5 and 10% of Clara-Diastase in presence of Rapidase.

In order to minimize β-carotene degradation, the simultaneous addition of apolar solvents and enzymes to the food matrix to facilitate *in situ* transport of β-carotene to the organic phase was evaluated. Thus, the exposure to aqueous soluble pro-oxidants such as metal ions (Choe & Min, 2009) could be minimized . To this end, the extraction solvents, i.e. acetone/pentane (4:6) were added to a β-carotene working solution (120 µL, 3 µg β-carotene) either during or after enzymatic incubation. Recoveries of β-carotene were 94 ± 2% and 49 ± 13%, respectively. These results show indeed an improved recovery when solvents were added simultaneously with the enzymes, and therefore, this approach was incorporated in the final protocol. Based on the visual observations of the solubilized sample slurries, it was also concluded that the presence of organic solvents did not impact enzymatic activity, thereby suggesting that the presence of solvents was not visibly inducing protein denaturation.

Enzymatic pretreatment of plant products prior to carotenoid extraction has already been proposed earlier (Barzana et al., 2002), where enzymes such as Viscozyme, Pectinex, Neutrase, Corolase and HT-Proteolytic were used to release carotenoids from organelles of flower cells. Also Kha et al. used enzymatic pretreatment to release carotenoids from Gac oil (Kha, Phan-Tai, & Nguyen, 2014). In milk, carotenoid release was enhanced by the use of proteases and lipases (Liu, Xu, & Canfield, 1998), and the AOAC method (Schierle et al., 2004) recommends the use of proteases (Protex 6L) for water-dispersible formulations. This

research demonstrates that also ClaraDiastase and Rapidase may be added to the list of appropriate enzymes to facilitate carotenoid extraction, and that solubilization performed in solvent gave a higher β-carotene yield.

Effect of saponification

To remove residual fats and chlorophylls and to saponify esterified carotenoids, AOAC protocols recommend to conduct saponification with methanolic KOH at room temperature for 16 h or at 56°C for 20 min, or a simultaneous extraction and saponification using hexane-ethanol-acetone-toluene (10:6:7:7 *v/v*) and methanolic KOH (40% *w/v*) (Delgado-Vargas & Paredes-Lopez, 1997). In this study, the recoveries of β-carotene (120 µL working solution, 3µg) using the three adopted different saponification steps, were 73 ± 3% for overnight saponification at 30°C, 76 ± 4% for 90 min at 50°C, and 47 ± 4% for 30 min at 100°C. However, in all cases, trans-β-apo-8'-carotenal was extensively degraded (Figure 4A). It was shown that even a mild saponification gave strong degradation of trans-β-apo-8'-carotenal, whereas the β-carotene recovery was relatively acceptable at overnight saponification at 30°C. This is in line with literature (Kimura et al., 1990), in which it was shown that during saponification according to the AOAC protocol, trans-β-apo-8'-carotenal is converted to citranaxanthin when traces of acetone are present, by aldolcondensation with acetone in an alkaline environment. The same authors also showed that β-carotene is more stable than trans-β-apo-8'-carotenal.

Carotenoid recoveries after each step of the fully optimised extraction procedure

The newly developed procedure was tested and the recoveries of each of the steps were determined. The average recoveries of β-carotene and trans-β-apo-8'-carotenal after performing only the first extraction procedure on working solutions containing 1.5, 3, 4.5 and

6.25 µg β-carotene, and 3 µg trans-β-apo-8'-carotenal, were 100 ± 3% for β-carotene, and 102 ± 1% for trans-β-apo-8'-carotenal (Supplementary Table 3). It can be concluded that the carotenoid loss was negligible in this step.

Next, the working solutions were spiked to 100 mg sunflower oil (Vandemoortele, Gent, Belgium), as this is the maximal amount of fat in 3 g of baby food samples according to the specification on the package. After the first extraction step, a mild saponification (overnight, 30°C, rotating tube) was performed. This resulted in an extensive degradation of trans-β-apo-8'-carotenal. Therefore, in the following experiments, trans-β-apo-8'-carotenal was added as an internal standard after the saponification step as losses during the first extraction step were negligible. Recoveries of β-carotene are presented in Table 2. The recovery of the internal standard (trans-β-apo-8'-carotenal), when added after saponification, amounted 93%, indicating that about 10% was lost during the extraction process as such. Similarly it is assumed that ±10% of β-carotene is lost as a result of extraction. As the overall recovery of β-carotene amounted 72%, this implies that 20% of β-carotene is lost during saponification, which is in line with previous literature (Kimura et al., 1990). The apparent recovery of β-carotene, on the basis of the ratio of the β-carotene/IS peak amounted on average 79%.

Small-scale validation of the protocol

The limit of detection (LOD) and limit of quantification (LOQ) were determined by analysis of the calibration solutions of β-carotene containing 0, 1.46, 2.93, 4.39, 5.86 µg per mL, and trans-β-apo-8'-carotenal (3 µg/mL), prepared on seven different days to account for inter-day variability. The calibration curves were constructed using the internal standard method, and the average slope was 0.26 µg/mL, the average intercept was 0.06 µg/mL and the standard deviation on the intercept was 0.009 µg/mL. The LOD was calculated as 3.3*(standard

deviation intercept/average slope) and was 0.12 µg/mL. The LOQ was calculated as 10*(standard deviation intercept/average slope) and was 0.36 µg/mL.

The intra-day (n=3) and inter-day (n=5) variability (coefficient of variation, CV) of the optimized analytical method was investigated using a lyophilised mango powder (1.35 ± 0.34mg β-carotene/100mg, n=9), stored at -80°C in vacuum packages, and was 18% and 25%, respectively. Moreover, the CV on the β-carotene content was measured by extraction of lyophylized carrot powder and a baby food product containing beef, carrot and tomato (n=3 extractions). The average β-carotene concentrations of carrot powder and baby food was 24.95 ± 1.05 and 1.54 ± 0.06 mg/100g, with a CV of 4.2 and 3.8%, respectively. Finally 4 baby food samples were analysed in duplicate, on which basis an overall CV of 5.3% was obtained whereas the concentrations of the samples ranged between 0.1 and 1.6 mg of β-carotene per 100 g of sample.

Application to processed baby foods

The developed protocol was applied to 50 different vegetable-based and 22 different fruit-based processed baby food products (Table 3 and 4), bought from local Belgian supermarkets in 2015-2016. For the calculation of the β-carotene content, the apparent recovery was taken into consideration. Standard solutions (containing appr. 0.0, 1.4, 2.7, 4.0 and 5.5 µg/mL β-carotene µg/mL) were prepared in presence of TBHQ – the antioxidant yielding calibration lines with the highest β-carotene absorbances for all tested concentrations and the linearity (R^2=0.98±0.01) of the standard curves was ensured as they were freshly prepared immediately before HPLC analysis. The amount of samples analyzed during one run was maximum 9, including the mango powder extract as internal control, to avoid β-carotene degradation. The data have been used to investigate the intake of fat-soluble vitamins in Belgian infants and toddlers, and additional information about the content of other fat-soluble vitamins (A, D, E,

K) of the baby foods used in this study has been gathered in the study of Moyersoen et al. (Moyersoen et al., 2018).

Conclusions

It is concluded that (i) the one-pot approach using acetone/pentane and enzymatic pretreatment with Clara-Diastase and Rapidase followed by centrifugation helped to reduce losses during extraction, (ii) addition of equal amounts of anti-oxidants prevented oxidation of β-carotene in calibration solutions and thus overestimation of the actual β-carotene content of the analysed sample, and (iii) saponification accounted for 20% of β-carotene losses. The high recovery from the complex matrix was obtained by some unique approaches compared to conventional methods, amongst which (i) the use of centrifugation to separate the solvents instead of filtration during the first extraction, (ii) the addition of organic solvents to the enzyme cocktail, (iii) and the careful consideration of β-carotene absorbance interferences in the presence of different antioxidants. The developed procedure is useful for extraction of processed baby food products, and shows potential to be used for other types of plant-based fat-containing food matrices.

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conceptualization, funding acquisition, MVS and AVDM: analysis, data analysis: CG: writing, funding acquisition.

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Highlights

- A one-pot β-carotene extraction method was developed for plant-based baby foods
- Improved β-carotene recovery by enzyme-aided extraction
- Phenolic antioxidants added to calibration solutions avoid systematic analytical errors
- The apparent recovery of β-carotene in fatty matrices after saponification was 80%
- β-Carotene in 50 vegetable- and 22 fruit-based processed baby foods was determined.

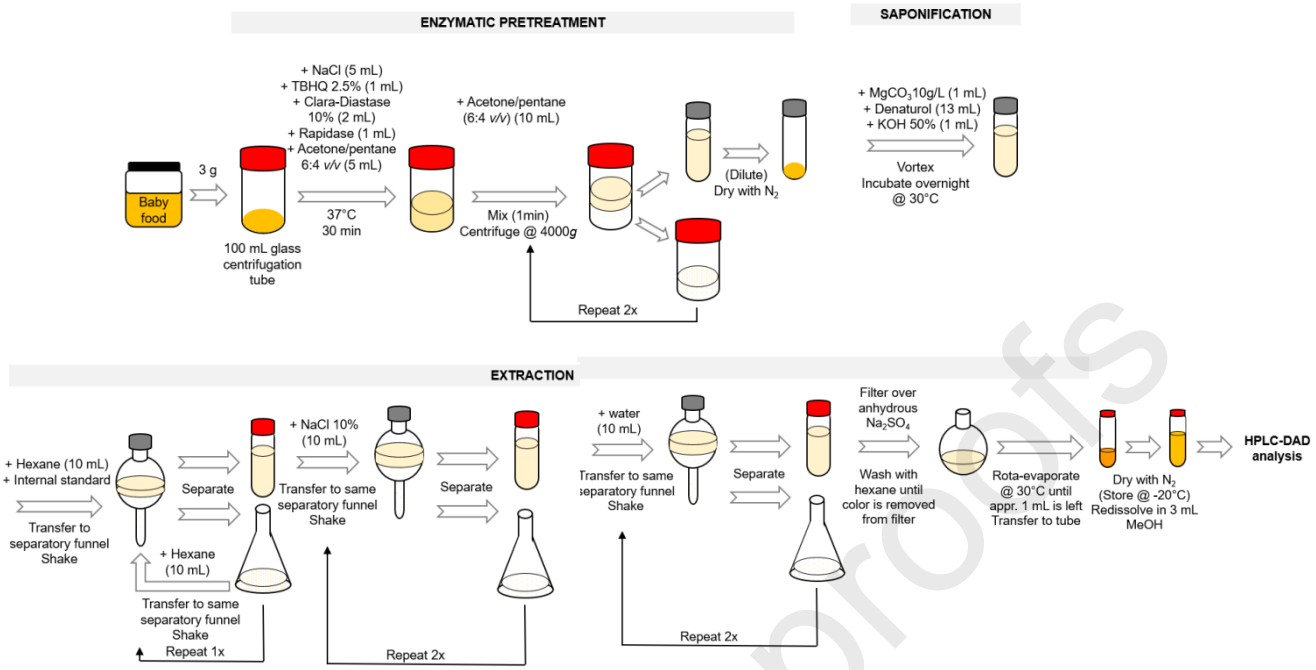

Figure 1: Overview of the extraction process.

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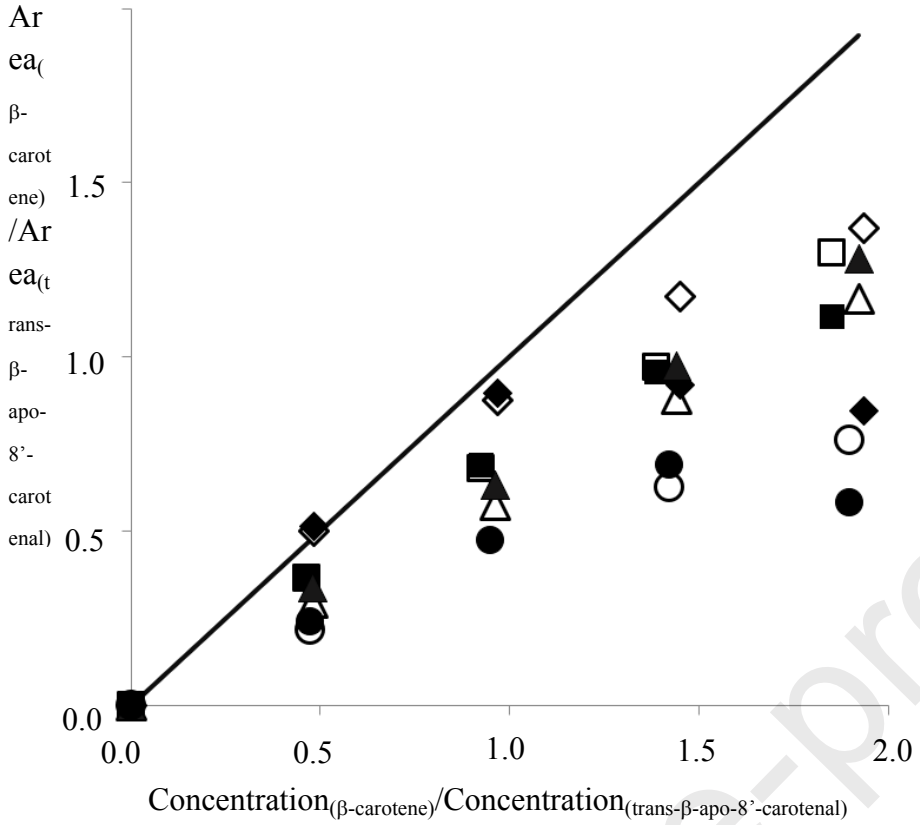

Figure 2. Effect of different phenolic antioxidants on calibration curve characteristics. Calibration curves of β-carotene without and in presence of antioxidants (○ no antioxidant, 1st injection, ● no antioxidant 5th injection, ◊ TBHQ 1st injection, ♦ TBHQ 5th injection, □ BHT 1st injection, ■ BHT 5th injection, Δ BHA 1st injection, ▲ BHA 5th injection., concentrations 25 mg/mL) from calibration solutions containing approximately 2.8µg/mL trans-β-apo-8'-carotenal, and approximately 0.0, 1.4, 2.7, 4.0 and 5.5µg/mL β-carotene based on HPLC-DAD analysis (see Supplementary Table 1 for more detailed values). Each of the calibration solutions was injected 5 times to follow up possible degradation. The time between each injection was 979 min, and the fifth injection was performed after 3916 min. The theoretical curve (black line) indicates the values when 100% β-carotene recovery is assumed.

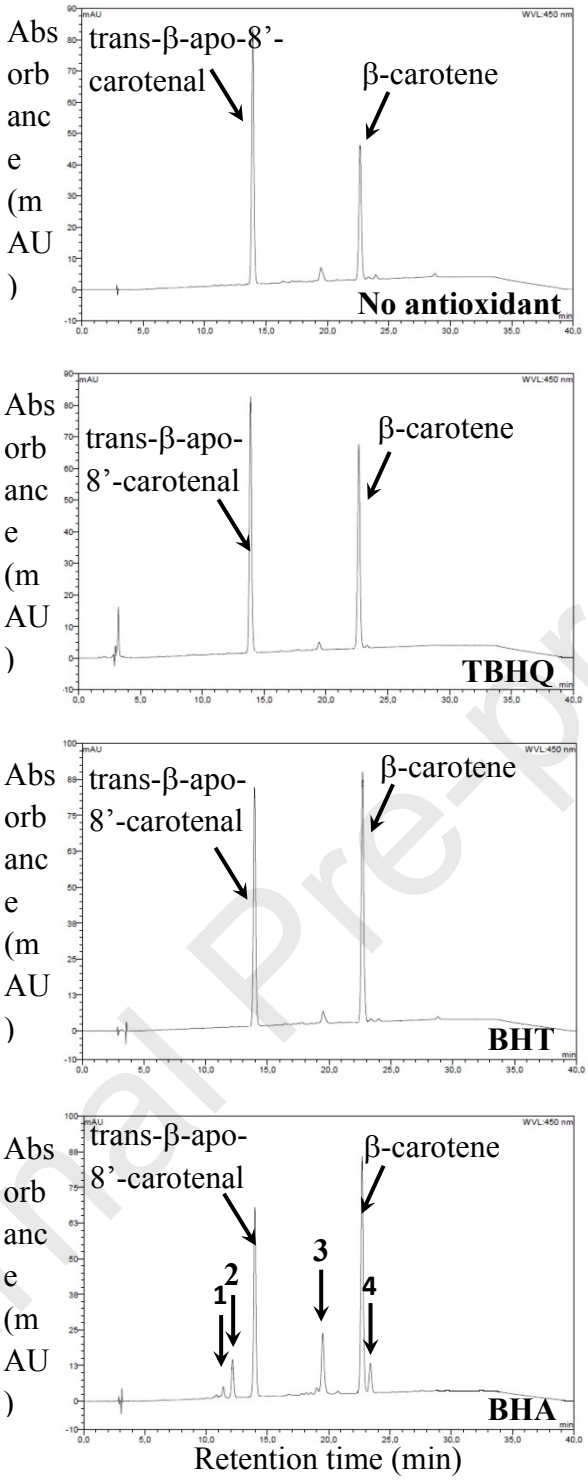

Figure 3. Representative chromatograms of β-carotene (5.5 µg/mL) and trans-β-apo-8'-carotenal, from calibrations solutions without antioxidants, or in presence of TBHQ, BHT and BHA, after injection 5. Dotted arrows indicate degradation products of the tested carotenoids. 1. Unknown, 2, 3 and 4.: presumably 5,8-epoxy-β-carotene, 15-cis-β-carotene isomer, and 9-cis-β-carotene, respectively, based on DAD-spectra of the peaks. A reversed

phased YMC-pack C30 column (250 mm x 4.6 mm i.d., S-5µm) was used with methanol:acetonitrile (9:1 *v/v*) as mobile phase A and ethyl acetate with 0.25% triethylamine as mobile phase B.

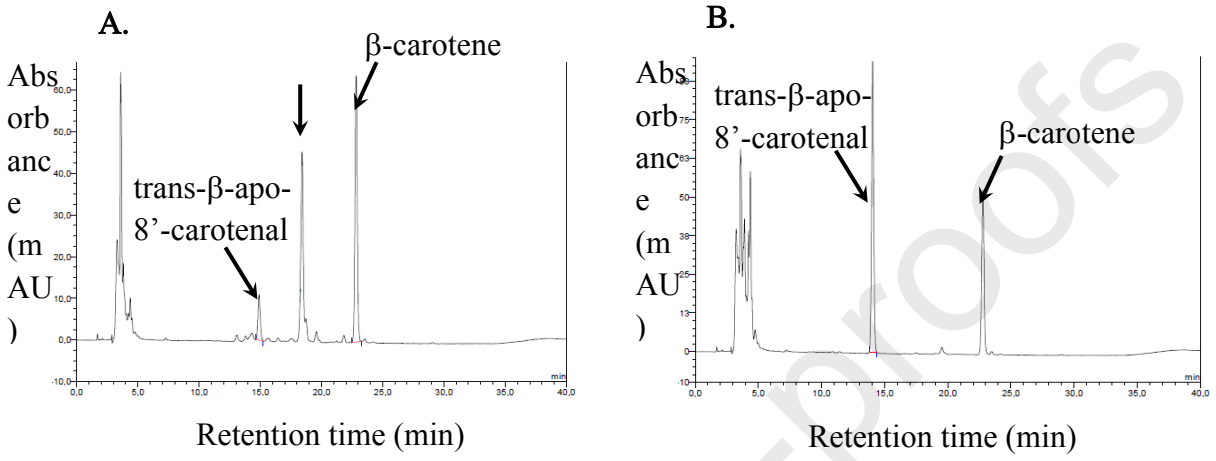

Figure 4. Effect of saponification on stability of trans-β-apo-8'-carotenal. Chromatogram of trans-β-apo-8'-carotenal (150µL, 2.6µg) when added either before (A) or after (B) the mildest saponification procedure (18h, 30°C). Dotted arrows indicate degradation products of the tested carotenoids. A reversed phased YMC-pack C30 column (250 mm x 4.6 mm i.d., S-5µm) was used with methanol:acetonitrile (9:1 *v/v*) as mobile phase A and ethyl acetate with 0.25% triethylamine as mobile phase B.

Table 1. Slopes and correlation coefficients of linear calibration lines fitting the areas and concentration ratios of β-carotene and trans-β-apo-8'-carotenal, respectively obtained after injecting β-carotene calibration solutions (0-5.25 mg/L), each in the presence of respectively 25 mg/ml BHT, TBHQ and BHA as antioxidant or in the absence of an antioxidant. The time gap between each injection series was about 1000 min.

Antioxidant	# points	1st injection		2nd injection		3rd injection		4th injection		5th injection	
		Slope	R^2	Slope	R^2	Slope	R^2	Slope	R^2	Slope	R^2
Absent	5[a]	0.41	0.99	0.41	0.99	0.38	0.95	0.35	0.90	0.34	0.84
Absent	4[a,b]	0.44	1.00	0.47	1.00	0.47	1.00	0.48	1.00	0.49	1.00
BHT	5	0.69	1.00	0.68	1.00	0.68	1.00	0.66	1.00	0.61	0.98

BHT	4[b]	0.70	1.00	0.69	1.00	0.69	1.00	0.69	1.00	0.69	0.99
TBHQ	5	0.70	0.98	0.58	0.89	0.53	0.83	0.48	0.78	0.43	0.72
TBHQ	4[b]	0.80	0.98	0.75	0.97	0.73	0.95	0.69	0.92	0.65	0.89
TBHQ	3[c]	0.90	0.99	0.91	0.99	0.92	0.99	0.93	0.99	0.93	0.99
BHA	5	0.60	1.00	0.62	0.99	0.65	0.99	0.67	0.99	0.67	0.99

[a] for the first injection series, the calibration solution containing 2.79mg β-carotene/ml was not injected due to a technical issue with the injector

[b] calibration solution containing 5.25mg β-carotene/L was not included

[c] calibration solutions containing 4.27 and 5.25mg β-carotene/L were not included

Table 2. Recovery of β-carotene (βC, 2.93 µg) spiked to 100 mg sunflower oil after extraction of the unsaponifiable fraction and the recovery of trans-β-apo-8'-carotenal (IS, 17.88µg) spiked after saponification of the oil.

Repeat	**Area βC (mAU)**	**Area IS (mAU)**	**Ratio[a]**	**Amount βC[b] (µg)**	**Amount βC[c] (µg)**	**Recovery βC[b] (%)**	**Recovery βC[c] (%)**	**Recovery IS (%)**
1	14.03	16.61	0.84	2.22	2.44	76	83	93
2	13.01	16.67	0.78	2.05	2.25	70	77	93
3	13.32	16.58	0.80	2.10	2.31	72	79	93
Aver.	13.45±	16.62	0.81	2.12 ±	2.33 ±	**72±3**	**79±3**	93±0
STD	0.52	± 0.05	± 0.03	0.09	0.10			

[a] Ratio = (Area βC)/(Area IS)

[b] Calculated without considering IS

[c] Calculated considering IS

Tabel 3. β-Carotene content of vegetable-based processed baby food products.

Vegetable based processed baby foods			**β-Carotene**
Producer	**Brand**	**Content**	**(µg/100g)**
P1	B1	Beans, chicken and sweet potato	218
P1	B1	Vegetable, rice and chicken	154
P1	B1	Chicken with mixed vegetables	302

P1	B1	Chicken, carrot and rice	925
P1	B1	Stew with chicken, carrot and tomato	618
P1	B1	Turkey, peas and pumpkin	194
P1	B1	Tunafish, carrot, tomato and rice	494
P1	B1	Tunafish, risotto, red pepper, zucchini	120
P1	B1	Ham with cauliflower	<LOD
P1	B1	Endive stew with tomato and ham	120
P1	B1	Brown beans with ham and potato	<LOQ
P1	B1	Macaroni dish	30
P1	B1	Spinach with potato	123
P1	B1	Carrot with potato	1050
P1	B1	Cauliflower	21
P1	B1	Peas	41
P1	B1	Vegetables with rice	428
P1	B1	Stew with beef, carrot and tomato	1058
P1	B1	Vegetables from the garden with beef	165
P1	B1	Beef with sweet potato	148
P1	B1	Spinach with beef and potato	114
P1	B1	Beans, potato, beef	<LOQ
P1	B1	Provencal vegetables with beef and potato	46
P1	B1	Spaghetti bolognaise with carrot and peas	253
P2	B2	Mashed potatoes and ham	25
P3	B3	Vegetables, chicken and rice	59
P3	B3	Peas and veal	<LOQ
P3	B3	Salmon and vegetables	170

P3	B3	Pasta with carrots and peas	18
P3	B3	Southern vegetables and turkey	32
P3	B3	Provencal vegetables and fish	27
P3	B3	Carrot, zucchini and coalfish	52
P3	B3	Mini pasta with vegetables	22
P4	B4	Vegetables, pasta and chicken	308
P4	B4	Vegetables, rice and turkey	296
P4	B4	Tagliatelles, spinach and cheese	105
P4	B4	Vegetable lasagne	192
P5	B5	Zucchini, rice and turkey	177
P5	B5	Spaghetti, tomato and beef	177
P5	B5	Potato, tomato and beef	831
P6	B6	Rice dish with chicken and cauliflower	223
P6	B6	Stew with spinach and chicken	120
P6	B6	Pasta with fish and vegetables in tomato sauce	189
P6	B6	Pasta with fish and spinach	202
P6	B6	Pasta with broccoli and carrots	612
P6	B6	Parsnip	<LOD
P6	B6	Quinoa with spinach and carrot	1179
P6	B6	Potato with beef and carrot	637
P7	B7	kippilav with rice	25
P7	B7	Chili con carne	51

Table 4. β-Carotene content of fruit-based processed baby food products.

Fruit based processed baby foods			**β-Carotene**
Producer	**Brand**	**Content**	**(µg/100g)**
P1	B1	Apple-Pear	<LOQ
P1	B1	Banana, apple and cookies	<LOQ
P1	B1	Apple, banana, mango	504
P1	B1	Apple, orange, banana	46
P1	B1	Plum, apricot, pear and grapes	67
P1	B1	Banana, strawberry, blackberry	87
P1	B1	Apple, orange, grape and kiwi	<LOD
P8	B8	The red one	<LOD
P5	B5	Apple-raspberry	<LOD
P5	B9	Fruit from the garden	61
P5	B5	Summerfruits	44
P3	B2	Apple-banana-orange with cookies	<LOQ
P3	B2	Apple-banana-apricot	36
P3	B2	Apple-pineapple	<LOD
P9	B10	Apple-plum	<LOQ
P6	B11	Mango-pear	105
P6	B12	Mango, orange and apple	147
P7	B13	Banana	<LOQ
P7	B13	Mixed fruit	37
P7	B13	Apple, banana, mango and grains	49
P7	B13	Banana, mandarin, pear	104

Credit author statement

Charlotte Grootaert was the principle author of the manuscript and co-coordinated the research

Margot Vansteenland was the labtechnician who was mainly involved in the laboratory work.

Angelique Vandemoortele was involved in the TOF-MS experiments

John Van Camp co-coordinated the research and was the promotor of the research project in which the work was carried out

Bruno De Meulenaer was the main coordinator of the research work presented

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: