Elsevier Editorial System(tm) for Journal of Chromatography A

Manuscript Draft

Manuscript Number: JCA-18-1662R1

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Article Type: Full length article

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Abstract: An analytical method was validated for the quantitative determination of isoprenoids compounds in faecal samples, based on liquid-liquid extraction from a small aliquot (0.3-0.5 g of sample) and subsequent analysis by Rapid Resolution Liquid Chromatography (RRLC) on a C30 column. An excellent linear response was observed over the range specified for all dietary isoprenoids, as confirmed by the correlation coefficient, which ranged from 0.9977 to 0.9999. LODs ranged from 0.002 µg to 0.036 µg for lutein and α-tocopherol, respectively. Depending on the compound, LOQs ranged from 0.001 µg (lutein) to 0.120 µg (α-tocopherol). For accuracy testing, spiking of faeces samples with trans-□-apo-8'-carotenal, α-tocopherol and chlorophyll a were performed (three concentration levels). Excellent recoveries were obtained in the all levels (>90%). The intra-day RSD % ranged from 0.86 to 9.78%. The inter-day RSD% was not higher than 10%, except to α-tocopherol (11.34%).
In order to assess the applicability of the method faecal samples from a baby fed with different purees formulated from various vegetables were analysed during a six month period. α-carotene, β-carotene, capsanthin, lycopene, lutein, phytoene, phytofluene, violaxanthin, zeaxanthin and ζ-carotene), and their isomers were identified and quantified using this method. Besides, 2 tocopherols and 9 chlorophylls and derivatives were identified and quantified in the faecal samples analysed. This method is suitable to determine dietary isoprenoids from complex matrices such as human faeces within 28 min.

Simultaneous determination of dietary isoprenoids (carotenoids, chlorophylls and tocopherols) in human faeces by Rapid Resolution Liquid Chromatography

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Abstract

An analytical method was validated for the quantitative determination of isoprenoids compounds in faecal samples, based on liquid-liquid extraction from a small aliquot (0.3-0.5 g of sample) and subsequent analysis by Rapid Resolution Liquid Chromatography (RRLC) on a C30 column. An excellent linear response was observed over the range specified for all dietary isoprenoids, as confirmed by the correlation coefficient, which ranged from 0.9977 to 0.9999. LODs ranged from 0.002 μg to 0.036 μg for lutein and α-tocopherol, respectively. Depending on the compound, LOQs ranged from 0.001 μg (lutein) to 0.120 μg (α-tocopherol). For accuracy testing, spiking of faeces samples with trans-β-apo-8'-carotenal, α-tocopherol and chlorophyll a were performed (three concentration levels). Excellent recoveries were obtained in the all levels (>90%). The intra-day RSD % ranged from 0.86 to 9.78%. The inter-day RSD% was not higher than 10%, except to α-tocopherol (11.34%).

In order to assess the applicability of the method faecal samples from a baby fed with different purees formulated from various vegetables were analysed during a six month

period. α-carotene, β-carotene, capsanthin, lycopene, lutein, phytoene, phytofluene, violaxanthin, zeaxanthin and ζ-carotene), and their isomers were identified and quantified using this method. Besides, 2 tocopherols and 9 chlorophylls and derivatives were identified and quantified in the faecal samples analysed. This method is suitable to determine dietary isoprenoids from complex matrices such as human faeces within 28 min.

Keywords: faecal samples, geometrical (*cis/trans*, *E/Z*) isomers, isoprenoids, phytoene, phytofluene, RRLC

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1. Introduction

Isoprenoids is a class of organic compounds composed of two or more units of hydrocarbons with each unit consisting of five carbon atoms arranged in a specific pattern. They comprise relevant compounds like carotenoids (CARS), chlorophylls (CHLS), and tocopherols (TOCS), that have been widely studied because of their beneficial properties on health and/or their influence on the organoleptic characteristic of food [1,2]. The biological significance of isoprenoids has been sometimes attributed to their antioxidant properties [3–5], although there may be other mechanisms involved. Thus, some carotenoids have pro-vitamin A activity and also, for some others, a prooxidant activity which modulate gene expression or membrane properties has been described [6,7]. TOCS have vitamin E activity and have long been considered potent lipophilic antioxidants, although it appears that they could also act by regulating gene expression [8].

The principal sources of isoprenoids are fruits and vegetables. Their health benefits in the human diet are well-known and they play an important role in nutrition that is strongly supported by scientific evidence [9–12].

Their bioavailability from the natural sources is the main handicap to take advantage of their health benefits, which in turn, relies on the individual biotransformation systems, the chemical structure and properties of each compound, and the complexity of the food matrix [9,13–15]. Bioavailability refers to the fraction of the food nutrient ingested that is available for use in physiologic functions or to be stored in body [16]. Assessing bioavailability is a difficult task which has been tackled using different approaches, from humans or animal models studies [17,18], which are tedious and time consuming, to "*in vitro* models" which resemble the digestion process and evaluate what is called bioaccessibility or the fraction available for absorption [19,20]. The complex and time-consuming *in vitro* digestion procedure, followed by extraction and analysis of unstable

components like CARS, is not straightforward [21]. Moreover the predictive value of *in vitro* digestion models should be assessed with *in vivo* human studies [22], that allow a wider evaluation of digestive changes (i.e. ester hydrolysis, isomerization) occurring *in vivo*. In this sense, faeces is a valuable matrix to study *in vivo* digestion changes and bioavailability of dietary components [19,23]. However, few studies on isoprenoids identification and quantification have been conducted in human faeces. Briviba et al. [24] and Schnäbele et al. [25] determined the content of lycopene, α-carotene and β-carotene in faeces after vegetables juices supplementation. Other studies reported the content of lutein, zeaxanthin, β-cryptoxanthin and *trans*- and *Z*-isomers of lycopene, β-carotene, α- and γ-tocopherol in faeces [19,23]. None of them reported the analytical validation of the procedure. Only recently Eriksen et al. [26] developed and validated a method for carotenoid quantification in several matrices such as spinach, serum, chylomicrons, and faeces. However, to the best of our knowledge, there are no studies in the literature describing the simultaneous determination of isoprenoids and their isomers in faeces, including the validation parameters of the optimized method.

Several methods have been proposed to determine dietary isoprenoids (CARS, CHLS and TOCS) from different matrices [27] either separately [28–30] or simultaneously [31–33], typically by reverse phase HPLC-DAD. Rapid-resolution (RRLC) and ultra-high performance liquid chromatography (UHPLC) have greatly improved traditional HPLC methods, with higher throughput of samples and without compromising resolution and with a remarkable solvents reduction. RRLC also offers higher resolution and sensitivity, and shorter retention times than HPLC [27,34] with the advantage of achieving a good resolution of isomeric forms due to the usage of C30 column.

The aim of this work was to set up and validate a rapid and effective RRLC method for measuring dietary isoprenoids in human faeces as a non-invasive approach to gain insight into their bioavailability in nutritional studies. To achieve this objective liquid-

97 liquid extraction and RRLC analysis has been applied to stool samples from one baby
98 fed with different vegetables formulated purees.
99

2. Materials and Methods

2.1. Chemicals and standards

Extraction solvents (Hexane and diethyl ether) were of analytical grade (VWR, Seattle, WA, USA). HPLC solvents, i.e. methanol (MeOH) and methyl tert-butyl ether (MTBE) were of HPLC grade and were acquired from Merck (Darmstadt, Germany). All analyses were performed with purified water (NANOpure® DIamond™, Barnsted Inc. Dubuque, IO). α-carotene, β-carotene, β-cryptoxantin, lutein, lycopene, zeaxanthin, chlorophylls (A and B) and trans-β-apo-8'-carotenal were from Sigma-Aldrich (Steinheim, Germany), whereas violaxanthin, phytoene and phytofluene were isolated from appropriate sources in accordance to standard procedures [35].

The tocopherol standards (α-tocopherol, β-tocopherol, δ-tocopherol and γ-tocopherol) were purchased from Calbiochem (Merck, Darmstadt, Germany).

Pheophytins a and b were obtained from their respective chlorophylls by adding diluted HCl (0.1 N) [36,37].

2.2 Samples and experimental design

For validating the extraction procedure and the precision and accuracy of the proposed method, stool samples corresponding to a 5-month-old baby´s depositions were used (S0). The reason for selecting such sample is that during this stage of life, babies have a very controlled diet and they start the complementary feeding (CF). The CF is defined by the World Health Organization (WHO) as ''the process starting when breast milk alone is no longer sufficient to meet the nutritional requirements of infants'' so that ''other foods and liquids are needed, along with breast milk''. Vegetables and fruits are gradually incorporated to the diet from 6 to 12 months, and the most allergenic foods (eggs, fish) are introduced after nine month [39].

The sample used for validating the method corresponded to the first stage of the CF (S0) when the diet consisted in a vegetable puree with the simplest isoprenoid profile (pumpkin, carrot, potato and olive oil).

For the applicability of the method, ten samples of faeces (S1-S10) were selected during the following period of life (from 6 to 12 month) each corresponding to the introduction of a new food with a characteristic isoprenoid profile. All vegetables were purchased in retail shops and were included in the diet of the baby as homemade purees always made with the same proceeding: olive oil aprox. 10g + a selection of vegetables (including one or several of the following: potato, tomato, broccoli, spinach, zucchini, red pepper, green-peas, corn or onion) to make 200g of puree. The samples were stored at -20ºC until analysis.

The study protocol was approved by the Comité Coordinador de Ética de la Investigación Biomédica de Andalucía, Junta de Andalucía (Reg. No.1440-N-16).

2.3. Extraction procedure

The ability of three solvents to extract the isoprenoids compounds from the one faecal samples were tested, namely solvent A: Hexane, solvent B hexane: ethyl ether (1:1) and, solvent C: ethyl ether.

Approximately, 0.3-0.5 g of faeces samples (S0) were homogenized with 10.0 mL of cool Phosphate Buffered Saline (PBS) solution (pH = 7.4) and 2 mL of ethanol. Subsequently, 10 mL of extraction solvent were added and then the mixtures were vortexed and finally centrifuged at 4000 rpm for 10 min at 4ºC using Eppendorf Centrifuge5810R (Brinkman Instruments Inc., Westbury, NY). After recovering the coloured fraction the residue was extracted again with another aliquot of 10.0 mL extraction solvent. This procedure was repeated until the colour disappeared (4-5 extractions) and the supernatants were combined. The organic coloured fractions were

pooled and evaporated to dryness in a rotary evaporator at a temperature below 30°C and stored under N_2 at -20°C until analysis. The residue was dissolved in 1 mL of HPLC-grade ethyl acetate and centrifuged at 18000 g for 5 min. at 4°C. Finally, the supernatants were filtered through a 0.22 µm a nylon membrane filter and transferred to a vial prior to their injection in the RRLC system. The analyses were carried out in triplicate.

2.4. Rapid resolution liquid chromatography conditions (RRLC)

Isoprenoids analyses were performed by RRLC on a Agilent 1260 system (Agilent, Waldbronn, Germany) equipped with UV/VIS diode array detector, which was set at 285 nm for phytoene and TOCS, 350 nm for phytofluene, 410 nm for ζ-carotene and pheophytin A, 430 nm for chlorophyll A and pheophytin B, 472 nm for lycopene and 450 nm for the rest of the CARS (α-carotene, β-carotene, β-cryptoxanthin, capsanthin, lutein, violaxanthin and zeaxanthin) and chlorophyll B. Separation was accomplished on a C30 column (150 × 4.6 mm I.D. 3 µm particle size; YMC Europe, Dinslaken, Germany) kept at 28 ºC with a guard precolumn (10 x × 4.0 mm I.D. 3 µm particle size; YMC Europe, Dinslaken, Germany).

Methanol (solvent A), methyl-*tert*-butyl ether (solvent B) and water (solvent C) were used in the mobile phase. Separation was achieved using the following gradient: 0 min 90% A + 5% B + 5% C, 0−5 min, 95% A+ 5% B ; 5−10 min, 89% A + 11% B, 10-16, min,75% A + 25% B; 16- 20 min, 40% A + 60% B; 22.5-25 min, 15% A + 85% B, 25-28 min, 90% A+ 5% B + 5% C. A 2 min re-equilibration time back to the initial mobile phase composition was used after each analysis.

The mobile phase was pumped at 1 mL/min, and the chromatograms were monitored at different wavelengths, using open lab ChemStation software. The injection volume was set at 1 µL.

177 The identification of isoprenoids compounds was carried out by comparing the retention
178 times, UV/vis spectroscopic characteristics with those of standards.
179 External calibration was used for quantification.
180 The identification of *Z* isomers (*cis* isomers) in the faecal samples was carried out by
181 comparing their chromatographic and spectroscopic features with the data reported by
182 other authors [33,40–44]. All the isomers were quantified with the calibration curve
183 made with the corresponding all-trans standard.
184

185 **2.5. Method validation**

186 The method was validated in terms of linearity, precision (repeatability, reproducibility
187 and instrumental precision), accuracy, and sensitivity (limit of detection (LOD) and
188 limit of quantification (LOQ)) according to internationally recognized guidelines [45].
189 Linearity of the method was evaluated by considering the detector response (area units)
190 to different amounts (μg) of isoprenoids by means of linear regression.
191 Homoscedasticity and linearity were performed by F-test and the residual plot (95%
192 significance level) [46].
193 To quantify the concentration of the standards a spectrophometer UV-visible Agilent
194 8453 (Agilent Technologies) was used and the corresponding molar absorptivity were
195 considered [47–49]. The standard curves were obtained by plotting the response of the
196 different dilutions of the quantified standards against the concentration injected The
197 LOD and LOQ were calculated from the calibration curves as the three and ten times
198 relative standard deviation of the analytical blank, respectively.
199 The accuracy of the method was determined by recovery studies using the standard
200 addition method, for that, trans-β-apo-8'-carotenal (APO), chlorophyll a (CHLA) and α-
201 tocopherol (ATOC) were selected as standards. The study of recovery was performed
202 by spiking one sample of faeces (S0) in quadruplicate with standard solutions at three

concentration levels (low, medium and high) and then were extracted by the methodology described above (section 2.3). The spiking levels were 5.49, 120.79 and 244.05 μg of APO; 8.69, 245.49 and 437.02 μg of ATOC, and 10.58, 52.88 and 169.20 μg of CHLA.

Finally, the spiked samples were analyzed using the instrumental conditions of the proposed RRLC method. The recovery was calculated comparing the values obtained for each compound ‘‘spiked” in relation to the initial value contained in the sample.

Instrumental precision, repeatability (intra-day) and reproducibility (inter-day) were determined to stablish the precision of the method. It was ascertained by analyzing in triplicate the isoprenoids content in three replicates of the same samples (S0), under the same analytical conditions. For the reproducibility assessment, the same sample was extracted and analyzed at 2-day intervals during 3 days. Instrumental precision was evaluated by six replicate injections of a standards mixture, containing all dietary isoprenoids. from the same vial and in the same day with the same chromatographic conditions. Precision was expressed as relative standard deviation (RSD %).

3. Results and discussion

3.1. Optimization of chromatographic separation conditions

Different chromatographic conditions were tested in order to obtain a simultaneous and optimal separation of CARS, TOCS, CHLS and derivatives, both using standards and fecal extracts. **Fig. 1 A, B** and **C** show the chromatographic profiles corresponding to standard mixtures of CARS, TOCS and CHLS, respectively, using the optimized conditions, detailed in section 2.4.

The developed method allows the separation in a 28-min run of up to 20 isoprenoid compounds belonging to three different groups: twelve CARS (violaxanthin, lutein, capsanthin, zeaxanthin, zeinoxanthin, β-cryptoxathin, α-carotene, β-carotene, lycopene,

phytoene, phytofluene, ζ-carotene), four TOCS (α, β, δ and γ-tocopherols) and four CHLS and derivatives (pheophythins).

3.2. Optimization of solvent extraction procedure

Faeces are a complex matrix for the analysis of isoprenoids. Like for all matrices, the first stage of the analysis is the extraction of the analytes with an optimized and efficient extraction procedure. The most commonly used solvents reported in the literature for this type of sample are hexane and diethyl ether [19,23–25]. Since hexane is regarded a good extracting solvent for carotenes and diethyl ether for both carotenes and xanthophylls [50], they were tested separately and in a 1:1 mixture as described in more detail in Materials and Methods. To assess the efficiency of extraction of the different solvents tested, the average contents corresponding to total (calculated as the sum of the content of individual compounds) and individual isoprenoids were selected as responses of interest.

The results of total isoprenoids content obtained for the different extraction procedures are shown in **Fig. 2.** Using diethyl ether as extracting solvent increased by 26% and 66% the amount obtained with hexane and the mixture, respectively. With respect to the individual isoprenoids contents, significant differences ($p<0.05$) in the extraction were found depending on the solvent (**Table 1**). The highest extraction efficiency was achieved with diethyl ether for all compounds, followed by the mixture hexane: diethyl ether. The major differences were found for lutein and ζ-carotene, the mean contents obtained with ethyl ether were ca 2.5-fold higher to those obtained with hexane, and ca 1.7-fold higher than the one obtained with the mixture, respectively. These results were expected since hexane is more apolar than diethyl ether, so it is less efficient dissolving xanthophylls (lutein and β-cryptoxanthin) [50].

In view of these results, diethyl ether was the solvent chosen for the best extraction of dietary isoprenoids from faecal samples, since the isoprenoids content extracted from the matrix was always higher ($p < 0.05$) for this solvent.

3.3. Method validation

Linearity and Limits of Detection and Quantification

In **Table 2** the validation parameters: slope, intercept, coefficients of determination, LOD and LOQ are shown. The homoscedasticity of the linear calibration range was tested to confirm if the linear least-squares method (constant variance) was applicable.

The linearity was excellent ($R^2 > 0.999$) for most of the carotenoids, except β-carotene and capsanthin ($R^2 = 0.998$). Also, for all the tocopherols and chlorophylls the linearity was excellent ($R^2 > 0.999$) within the selected range of concentrations.

LODs and LOQs were calculated as those corresponding to signal to noise ratios of 3:1 and 10:1, respectively. LODs for all the carotenoids were in the range from 0.003 μg for phytofluene and zeaxanthin to 0.013 μg for β-cryptoxanthin (**Table 2**). Concerning the TOCS, LODs ranged between 0.021 μg for δ-tocopherol and 0.036 μg for α-tocopherol, while those of CHL ranged from 0.002 μg (for pheophytin b) to 0.021 μg (for chlorophyll a).

LOQ ranged from 0.001 μg to 0.120 μg (for lutein and α-tocopherol, respectively). The results obtained for the quantitation limits indicate that the proposed RRLC method is suitable for a rapid and sensitive detection of the dietary isoprenoids compounds in feaces.

Accuracy

The accuracy of the analytical method was evaluated by calculating the recoveries for the spiked samples. The average percentage of recovery and standard deviations of the three standards selected (APO; ATOC and CHLA) are shown in **Table 3**.

The spiked samples were extracted using the developed procedures and analysed by RRLC, in quadruplicate. For APO the recoveries were 102.7, 101.1 and 100.7% for the three spiked levels (low, medium and high level, respectively). Similarly, for ATOC the recoveries were 95.6, 90.9 and 99.3 %. For CHLA the recovery obtained varied from 90.6% for the medium level, 94.9% for the low level and 103.3 % for the high level of spiked sample. According to the good recoveries obtained the proposed extraction method can be recommended to analyse carotenoids in feaces for its accurancy.

Recent studies related to carotenoid recoveries in faecal samples reported lower results. Eriksen et al (2017) reported recoveries in faeces with levels of approximately 60−70% for zeaxanthin, lutein, β-cryptoxanthin, α-carotene, and >85% for β-carotene. Results on lycopene were even lower, specifically around 50%.

Precision

The instrumental precision expressed as relative standard deviation (RSD %) ranged from 0.89% to 3.20% for CARS, from 0.91 % to 1.35% for TOCS and, between 2.99-3.71 % for CHLS (**Table 2**).

Concerning repeatability, the RSD values of the method for faecal samples were under 10% for all the isoprenoids analysed (**Table 4)**. The highest values corresponded to α-tocopherol (9.38%) and the lowest ones to β-cryptoxanthin (1.06%). The highest RSD observed in the reproducibility corresponded to α-tocopherol (11.34%) and the lowest ones to α-carotene (2.36%). All results were below the established limits, according to the variation accepted (RSD% ≤12%) indicating a low variability between the values obtained, and a satisfactory precision of the analytical method. These results are in

303 agreement with those of Eriksen et al. [26] that reported RSD% values of a faecal
304 sample under 10% for intra- and interday analysis, except for lycopene (11.4%).
305
306 **3.2 Analysis of isoprenoids in faecal samples by the RRLC method**
307 In order to demonstrate the applicability of the new optimized method, it was applied to
308 faecal samples corresponding to the intake of formulated vegetable-based purees (S1-
309 S10) with different isoprenoids profiles. The ingredients used to prepare them were
310 carefully selected from vegetables with widely known carotenoid profiles (broccoli,
311 carrot, green peas, onion, potato, pumpkin, red pepper, spinach, zucchini and tomato),
312 egg, and olive oil in order to obtain different isoprenoid profiles in faeces**.**
313 A total of 25 carotenoids and isomers, 2 tocopherols and 9 chlorophylls and derivatives
314 were identified and quantified in the different faecal samples. **Table 5** summarizes the
315 mean values of the isoprenoids determined. **Fig. 3** and **4** show the chromatograms of
316 faecal samples 5 and 10 using the proposed method. As far as the macular carotenoids
317 lutein and zeaxanthin were concerned, the former was present in all samples, whereas
318 zeaxanthin was only found in high levels in stool samples obtained after the intake of
319 purees elaborated with egg. The highest lutein contents were found in the samples
320 obtained after the intake of a vegetable pure made with spinach (12.95-16.89 μg/g).
321 Moreover, a lutein isomer tentatively identified as (13*Z* + 13'*Z*) lutein was found in
322 some faecal samples. To the best of our knowledge, this is the first time that this
323 geometrical isomer of this carotenoid is reported in this kind of sample.
324 Concerning the provitamin carotenoids, β-carotene and a Z isomer of β-carotene (9-*Z*)
325 were found in all the samples, which was to be expected since it was present in the
326 ingredients used to formulate the purees. α-Carotene was found in all samples, except
327 the faecal samples 4, 5 and 9, which were obtained after the intake of vegetable purees
328 with ingredients that were poor in this compound like spinach, broccoli and tomato. β-

329 cryptoxanthin was not found in the samples. Other carotenoids like capsanthin and their
330 derivative were found, mainly, in the samples containing red pepper as ingredient.
331 Another interesting point of this methodology was the separation of geometrical isomers
332 of different carotenoids. In samples 5, 8, 9 and 10 (**Fig. 4)** four *Z* lycopene isomers, in
333 addition to (all-*E*)-lycopene, were well resolved with this method. The spectroscopic
334 and chromatographic characteristics used for identification are shown in the
335 **supplementary table.** The geometrical isomers tentatively identified were (5*Z*)-
336 lycopene, (9*Z*)-lycopene, (5*Z*, 9′*Z*)-lycopene and (13*Z*)-lycopene. All these samples
337 came from the intake of vegetables purees containing tomato as an ingredient. Similarly,
338 five phytofluene isomers (sample 10, **Fig. 4**) and four ζ-carotene isomers (samples 1, 6
339 and 7) were detected. A phytoene isomer identified as (15*Z*)-phytoene was found in all
340 samples, except in the samples 2, 4 and 9.
341 The presence of isomers in faeces can be due to their original presence in the foods
342 ingested or their formation during the diverse processes that take place during digestion.
343 As an example, there is wide evidence that lycopene undergoes extensive isomerization
344 once tomatoes are ingested [41]. Although, the differentiation of geometrical isomers of
345 carotenoids in faeces has not been common, this appears of great importance to get
346 further insight into the isomerization of carotenoids during digestive processes. In this
347 sense, it is important to note that discerning different geometrical isomers of carotenoids
348 is of great interest as they can sometimes exhibit different properties (shape, stability,
349 solubility) that can result in clear differences in bioavailability or functionality [41,51].
350 In relation to tocopherols, α-tocopherol was found in all samples, ranging from 10.74 to
351 72.43 μg/g. These results were expected, since all the samples were obtained after the
352 intake of purees made with olive oil and vegetables rich in this compound, like carrot
353 and tomato [52,53].

γ-tocopherol was identified in the sample 5 (**Fig. 3**), which was obtained after the intake of a pure containing green peas as main ingredient (Boschin & Arnoldi, 2011; Padhi et al., 2017). Finally, chlorophylls (a and b), pheophytins (a and b) and derivatives were identified and quantified in all faecal samples obtained after the intake of purees formulated with green vegetables (broccoli, spinach, green peas, and zucchini).

4. Conclusions

A chromatographic method for the rapid determination of dietary isoprenoids in human faeces is proposed. Its applicability has been demonstrated by validation criteria considering the linearity, repeatability, reproducibility and accuracy. A total of 36 isoprenoids compounds (carotenoids, chlorophylls and tocopherols) can be separated and identified in different faecal samples in 28 min by RRLC. Moreover, five different geometrical *Z* isomers of lycopene, five of phytofluene isomers, four of ζ-carotene and one of lutein, phytoene, α-carotene and β-carotene were tentatively identified and quantified using this method. The procedure was successfully applied to analyse a set of 10 samples of faeces corresponding to different isoprenoid profiles in the diet. The main route of isoprenoids excretion after digestion is faeces and its analysis in this matrix can be a valuable noninvasive biomarker of its bioavailability.

Acknowledgements

This work was partially supported by the project P11-AGR-7783 (Consejería de Innovación Ciencia y Empresa, Junta de Andalucía). CMS holds a postdoctoral research grant from the Junta de Andalucía. AJMM acknowledges funding from the Andalusian Council of Economy, Innovation, Science and Employment (project ref. CAROTINCO-P12-AGR-1287). Quality assistance from the technical staff of the Service of Biology (SGI, Universidad de Sevilla) is also acknowledged.

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Abbreviations:

α-tocopherol (ATOC), Carotenoids (CARS), Chlorophylls (CHLS), Chlorophyll a (CHLA), Complementary feeding (CF), Limit of detection (LOD), Limit of quantification (LOQ), Rapid-resolution liquid chromatography (RRLC), Relative standard deviation (RSD %), Tocopherols (TOCS), *Trans*-β-apo-8'-carotenal (APO), Ultra-high performance liquid chromatography (UHPLC).

Figure captions

Fig. 1A.

Chromatograms corresponding to the mixture of carotenoids standards in the optimized chromatography conditions at the corresponding wavelengths of maximum absorption. The maximum absorption spectra of each peak is also shown in separate figures. Peaks: 1 violaxanthin, 2 lutein, 3 capsanthin, 4 zeaxanthin, 5 phytoene, 6 zeinoxanthin, 7 β-cryptoxanthin, 8 phytofluene, 9 α-carotene, 10 β-carotene, 11 ζ-carotene, 12 lycopene.

Fig. 1B.

Chromatograms and spectra corresponding to the mixture of tocopherols standards at 285 nm in the optimized chromatography conditions. Peaks: 1 δ-tocopherol, 2 γ-tocopherol, 3 β-tocopherol, 4 α-tocopherol

Fig. 1C.

Chromatograms and spectra corresponding to the mixture of chlorophylls standards in the optimized chromatography conditions. Peaks: 1 and 2 chlorophyll b (450nm), 3 chlorophyll a (430nm), 4 and 5 pheophytin b (430nm), 6 pheophytin a (410 nm).

Fig. 2. Average recoveries corresponding to the total isoprenoid content after extraction of faecal samples using three different extraction solvents.

Fig. 3.

RRLC of the sample 5 in the optimized chromatography conditions. Peaks: 1 γ-tocopherol (285 nm), 2 α-tocopherol (285 nm), 3 (13*Z*+ 13'*Z*)-lutein (450 nm), 4 chlorophyll b (450nm), 5 chlorophyll b derivative (450nm), 6 lutein (450 nm), 7 capsanthin (450 nm), 8 zeaxanthin (450 nm), 9 chlorophyll a (410nm), 10 chlorophyll a derivative (410nm), 11 pheophytin b (450nm), 11 pheophytin b (450nm), 12

pheophytin b (450 nm), 13 pheophytin a (410 nm), 14 pheophytin a (410 nm), 15 β-carotene, 16 pheophytin b (450 nm), 17 pheophytin a (410 nm), 18 (15*Z*)-phytoene.

Fig. 4.

RRLC of the sample 10 in the optimized chromatography conditions. Peaks: 1 α-tocopherol (285nm), 2 (13*Z* + 13'*Z*)-lutein (450nm), 3 lutein (450nm), 4 capsanthin (450 nm), 5 capsanthin derivative (450nm), 6 (15*Z*)-phytoene (285nm), 7 phytofluene isomers (350 nm), 8 α-carotene (450nm), 9 (9*Z*)-α-carotene (450nm), 10 β-carotene (450 nm), 11 (9*Z*)-β-carotene isomer(450nm), 12 ζ-carotene (410nm), 13 capsanthin derivative (450nm), 14 (13*Z*)- lycopene (450nm), 15 (5*Z*, 9´*Z*)- lycopene (450nm), 16 (9*Z*)-lycopene, 17 (all-*E*)-lycopene, 18 (5*Z*)-lycopene

Figure

Fig 1 A

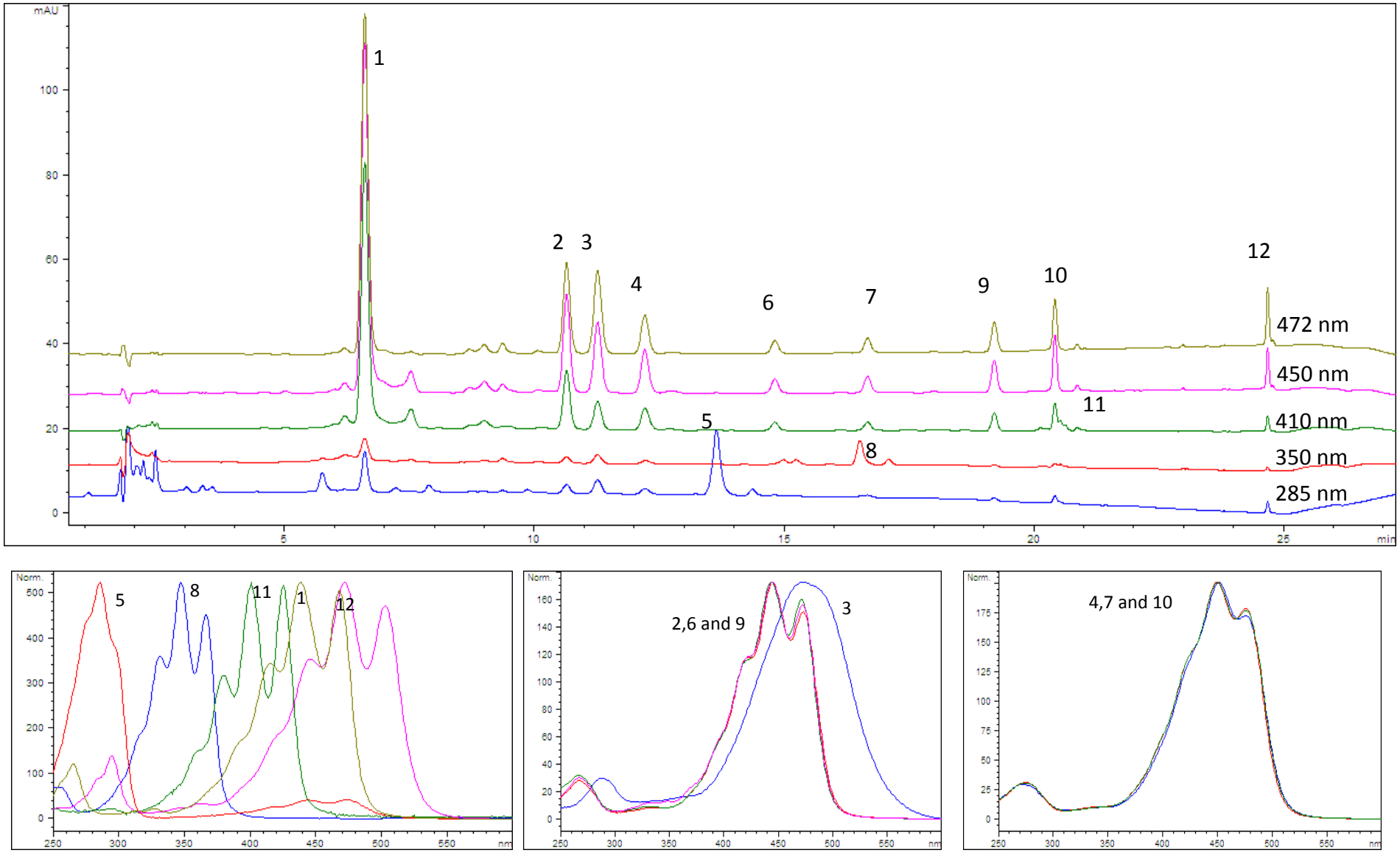

Fig 1 B

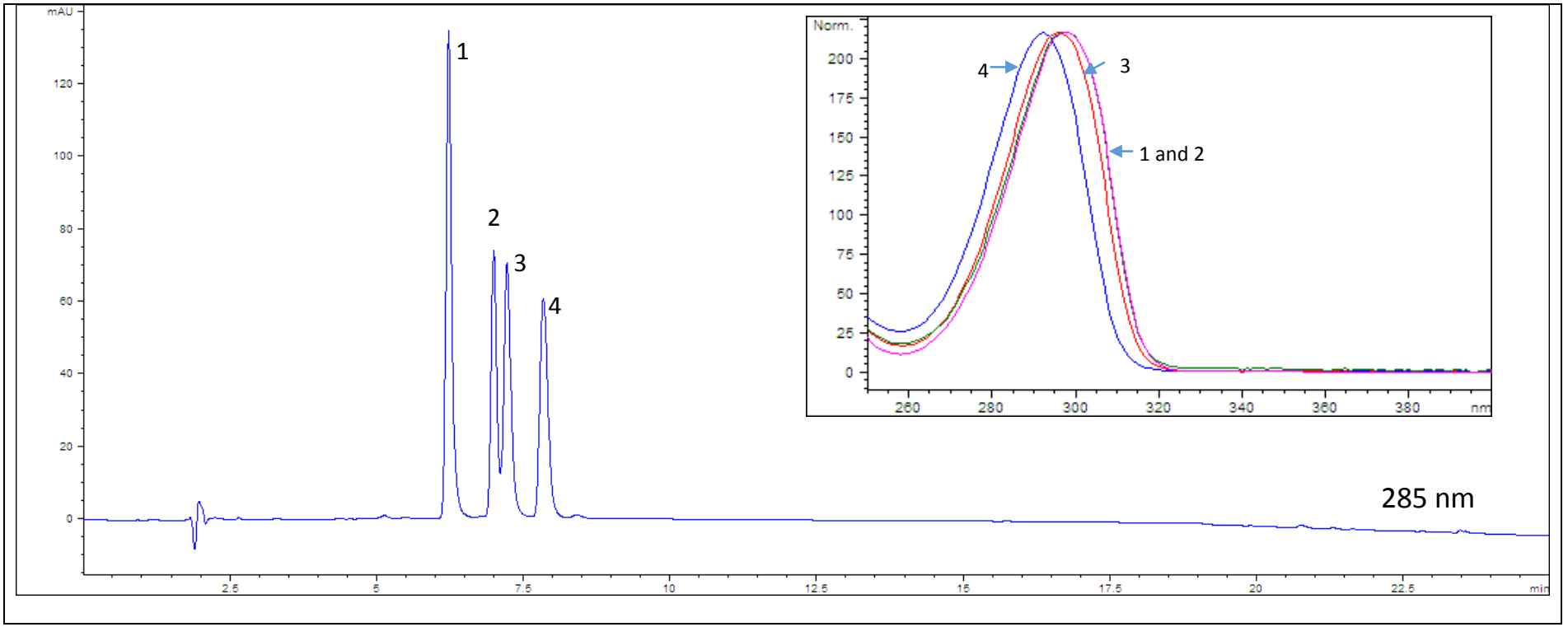
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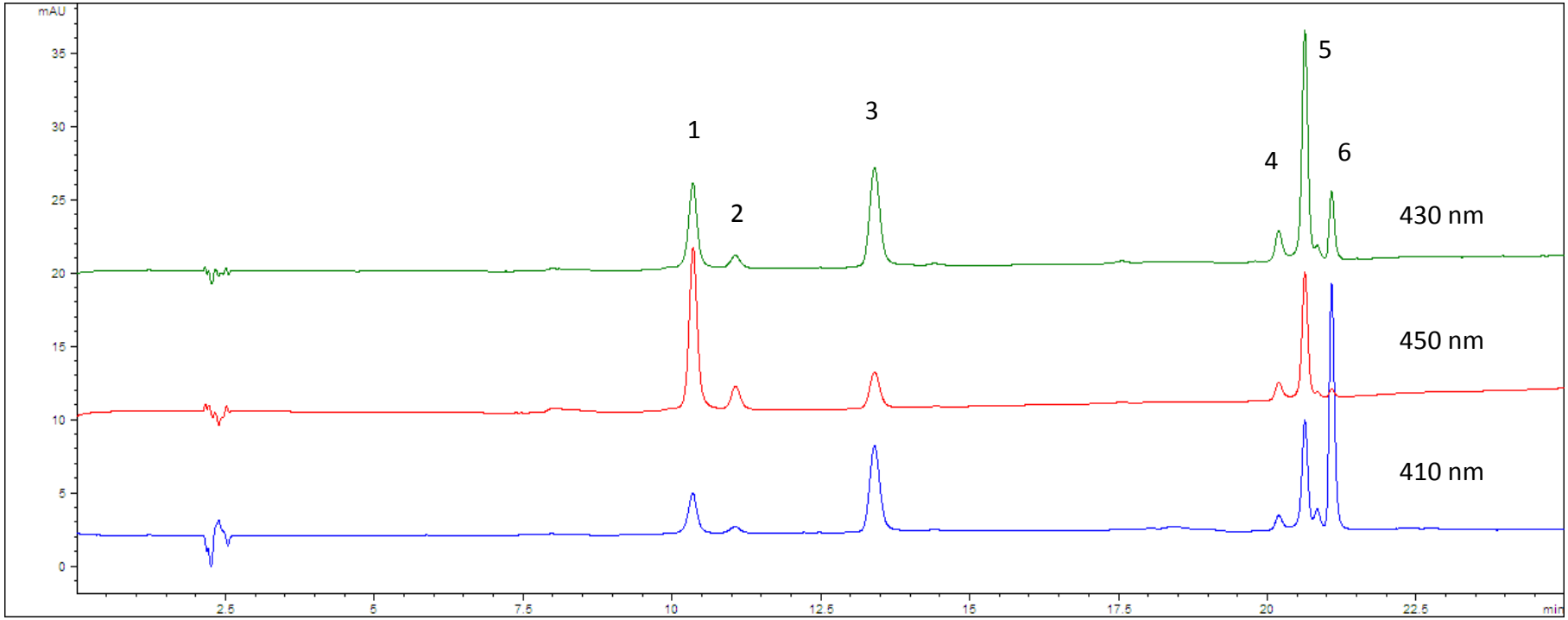
Fig 1 C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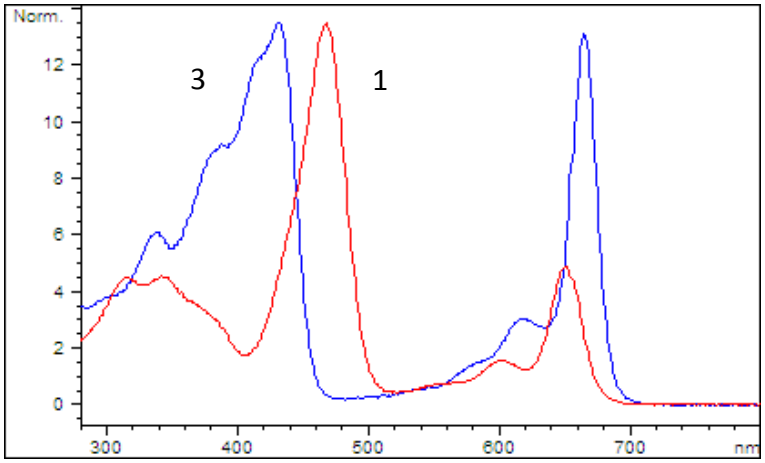
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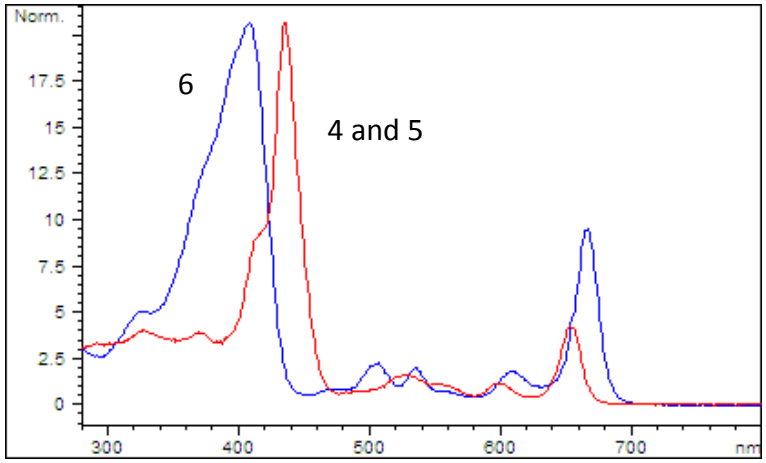
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Fig 2.

Different letters in the same by-product indicate significant differences by ANOVA test ($p<0.05$)

Fig 3.

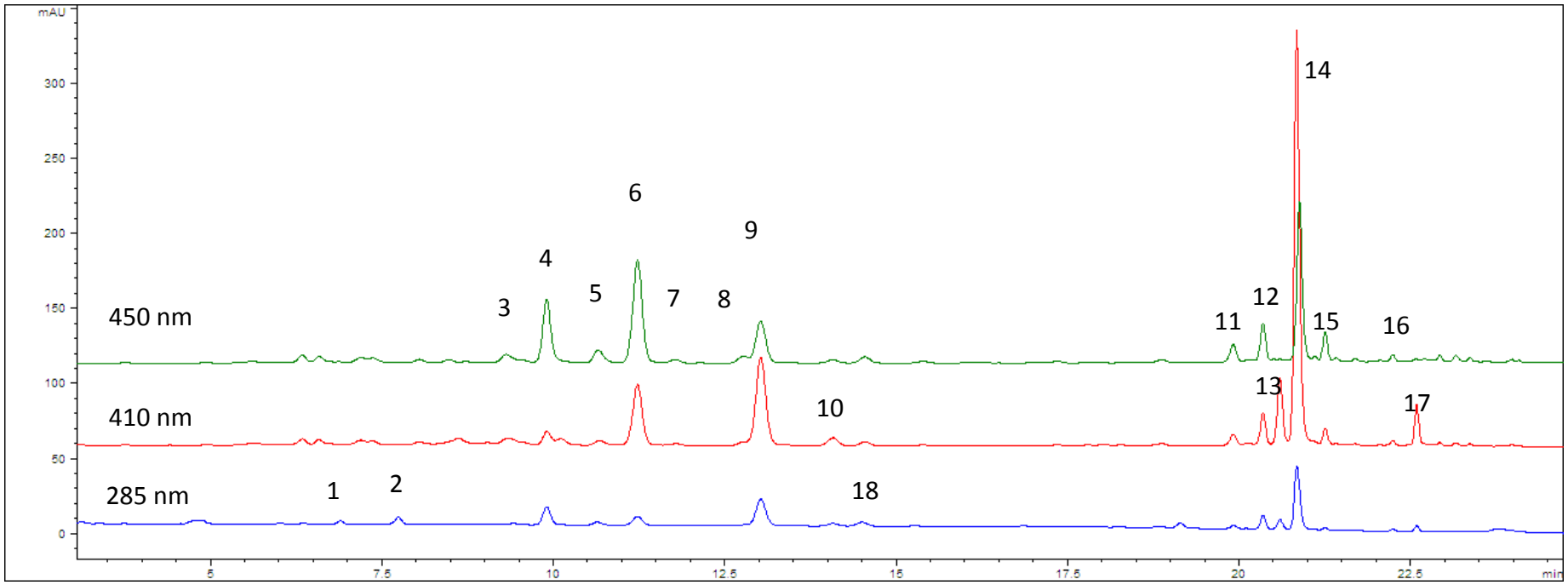
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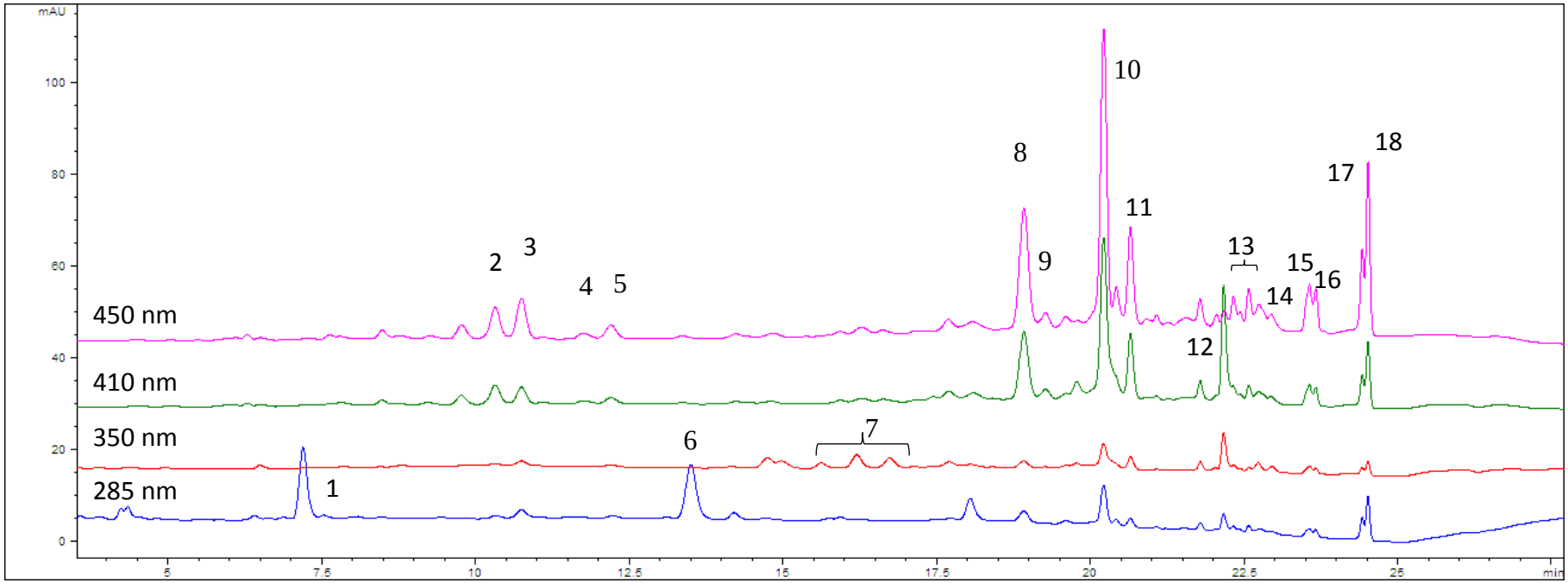
Fig 4.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Tables

Table 1. Mean content of isoprenoids after extraction of faecal samples using three different solvents.

Isoprenoids	Wavelength (nm)	hexane	hexane-diethyl ether	diethyl ether
Lutein	450	1.38±0.09[a]	1.92±0.02[a]	3.33±0.32[b]
β-cryptoxanthin	450	2.31±0.28[a]	2.72±0.19[a]	3.37±0.23[b]
α-carotene	450	29.06±1.33[a]	31.19±1.63[a]	42.76±3.77[b]
α-carotene isomer	450	4.50±0.03[a]	4.29±0.05[a]	5.57±0.14[b]
β-carotene	450	21.68±0.52[a]	26.34±0.95[a]	37.98±4.03[b]
β-carotene isomer	450	2.90±0.17[a]	3.50±0.21[a]	4.89±0.38[b]
ζ-carotene	410	0.23±0.05[a]	0.35±0.02[b]	0.59±0.05[c]
phytofluene isomer 1	350	1.68±0.08[a]	2.29±0.17[b]	3.51±0.25[c]
phytofluene isomer 2	350	1.95±0.18[a]	1.77±0.19[a]	2.42±0.14[b]
phytoene	285	2.54±0.22[a]	3.85±0.35[ab]	5.31±0.81[b]
α-tocopherol	285	33.93±3.93[a]	52.97±1.24[b]	60.52±4.54[b]

Different letters in the same row indicate significant differences by ANOVA test ($p<0.05$)

Table2. Summary of data about linearity, LOD, LOQ, linear range and instrumental precision of the method

	Analyte	Wavelength (nm)	Lineal range (µg)	Intercept ± SD	Slope ± SD	Coefficient of determination (R^2)	LOD (µg) [a]	LOQ (µg) [b]	Instrumental precision [c]
Carotenoids									
	α-carotene	450	0.008-0.457	-22.58±11.04	3831.12±56.34	0.9989	0.009	0.029	1.41
	β-carotene	450	0.002-0.405	-4.70±26.62	8653.36±130.37	0.9977	0.009	0.031	1.76
	β-cryptoxanthin	450	0.009-0.571	-11.46±18.00	4035.03±63.61	0.9985	0.013	0.045	2.76
	capsanthin	450	0.007-0.160	-12.55±8.37	6245.24±109.59	0.9985	0.004	0.013	3.01
	lutein	450	0.003-0.256	1.88±1.09	8759.00±8.716	1.0000	0.001	0.001	1.37
	lycopene	472	0.003-0.291	3.33±7.84	6566.74±58.38	0.9997	0.004	0.012	1.94
	phytoene	285	0.001-0.290	14.60±6.64	4016.67±44.56	0.9989	0.005	0.017	1.76
	phytofluene	350	0.004-0.225	-1.04±3.35	2892.53±30.40	0.9992	0.003	0.012	1.41
	violaxanthin	450	0.005-0.549	-10.16±13.87	7871.37±60.02	0.9996	0.005	0.018	2.01
	zeaxanthin	450	0.005-0.506	-16.80±6.43	6291.73±28.70	0.9999	0.003	0.010	0.89
	ζ-carotene	410	0.005-0.411	5.02±6.04	2432.75±30.98	0.9990	0.007	0.025	3.20
Tocopherols									
	α-tocopherol	285	0.073-10.341	3.94±3.66	304.17±0.79	0.9999	0.036	0.120	1.35
	β-tocopherol	285	0.0095-6.097	2.80±3.82	326.09±1.54	0.9999	0.035	0.117	1.20
	δ-tocopherol	285	0.104-9.637	-6.67±2.19	306.59±0.56	1.0000	0.021	0.072	0.91
	γ-tocopherol	285	0.063-5.560	13.13±3.47	297.83±1.44	0.9999	0.035	0.117	1.03
Chlorophylls									
	chlorophyll a	430	0.012 - 1.294	-13.06±13.02	1867.27±19.42	0.9995	0.021	0.070	3.71
	chlorophyll b	450	0.006 - 0.647	-7.99±6.57	1650.14±13.03	0.9997	0.004	0.014	3.01
	pheophytin a	410	0.001 - 0.346	2.61±7.57	5602.69±45.69	0.9997	0.004	0.014	3.67
	pheophytin b	430	0.003 - 0.288	-9.54±5.31	6933.87±38.75	0.9998	0.002	0.008	2.99

Values are expressed as means ± standard deviation; LOD a: limit of detection; LOQ b: limit of quantification; instrumental precision c : n=6

Table 3. Summary data from the recovery studies.

Compound	Concentration	Quantity added (μg)	Recorded amount (μg)	Recovery (%)[a]	SD (%)[a]
trans-β-apo-8'-carotenal	low	5.49	5.64	102.67	3.78
	medium	120.79	122.15	101.12	0.64
	high	244.05	245.70	100.68	3.04
α-tocopherol	low	8.69	8.31	95.61	5.01
	medium	245.49	223.33	90.97	0.82
	high	437.02	434.11	99.33	3.60
chlorophyll a	low	10.58	9.52	94.93	2.73
	medium	52.88	45.40	90.56	5.04
	high	169.20	165.71	103.30	8.20

[a] n=4

Table 4. Summary of results about repeatability (intra-day assay) and reproducibility (inter-day assay) for the proposed RRLC method.

Isoprenoid compunds		Concentration Mean ± SD (µg/g FW)	Repeatability RSD% (n=3)	Reproducibility RSD% (n=9)
Carotenoids	*α-carotene*	12.54±0.30	0.86	2.36
	β-cryptoxanthin	5.62±0.20	1.06	3.56
	β-carotene	16.56±0.46	2.49	2.80
	lycopene	5.51±0.25	4.44	4.47
	lutein	7.94±0.59	6.00	7.49
	phytoene	6.63±0.42	4.58	6.29
	phytofluene	0.97±0.07	6.94	7.59
	ζ-carotene	5.65±0.56	6.62	9.92
Tocopherols	*α-tocopherol*	36.09±4.09	9.78	11.34
Chlorophylls	*pheophytin a*	7.31±0.33	3.85	4.55

Table 5. Summary of carotenoid, tocopherol and chlorophyll contents of the faecal samples analyzed (μg/g FW).

Compounds	Food consumed	Pumpkin, carrot, potato, olive oil	Potato,eggs, red pepper, olive oil	Potato,carrot, corn, olive oil	Spinach, brocoli, olive oil	Green peas, brocoli, egg, olive oil	Pumpkin, carrot, potato,red peper, eggs olive oil	Pumpkin, carrot, potato, red pepper, olive oil	Zucchini, spinach, potato tomato, olive oil	Spinach, potato, tomato, olive oil	Tomato, carrot, onion, red pepper, olive oil
	Faecal Samples	1	2	3	4	5	6	7	8	9	10
Carotenoids	*violaxanthin*	nd	nd	nd	nd	0.82 ± 0.01	nd	nd	0.86 ± 0.11	nd	nd
	(*13Z* + 13'*Z*)-*lutein*	nd	nd	nd	0.73 ± 0.03	0.95 ± 0.04	0.71 ± 0.02	0.50 ± 0.09	1.21 ± 0.10	nd	0.30 ± 0.02
	lutein	5.42 ± 0.37	2.03 ± 0.04	3.20 ± 0.32	12.95 ± 0.17	9.56 ± 0.12	11.11 ± 0.70	4.77 ± 1.23	15.35 ± 2.17	16.89 ± 0.75	1.24 ± 0.04
	capsanthin	nd	2.28 ± 0.12	nd	1.29 ± 0.02	0.82 ± 0.05	1.69 ± 0.19	1.72 ± 0.08	1.12 ± 0.28	nd	3.91 ± 0.72
	zeaxanthin	nd	1.52 ± 0.06	1.25 ± 0.05	1.07 ± 0.16	1.61 ± 0.41	2.55 ± 0.20	1.28 ± 0.20	1.47 ± 0.15	nd	nd
	α-carotene	96.33 ± 4.54	1.81 ± 0.28	36.76 ± 1.12	nd	nd	116.82 ± 9.32	92.08 ± 6.61	20.62 ± 2.85	nd	12.96 ± 0.69
	(9*Z*)-α-*carotene*	5.35 ± 0.01	nd	2.49 ± 0.06	nd	nd	9.70 ± 0.48	4.83 ± 0.92	nd	nd	3.07 ± 0.31
	β-*carotene*	83.21 ± 4.50	1.09 ± 0.19	34.21 ± 1.19	9.76 ± 0.01	8.73 ± 0.21	86.93 ± 7.34	79.43 ± 9.19	25.18 ± 1.06	13.18 ± 1.18	12.90 ± 1.04
	(9*Z*)-β-*carotene*	6.25 ± 0.10	0.23 ± 0.01	2.89 ± 0.29	1.47 ± 0.06	1.46 ± 0.01	11.97 ± 0.71	6.83 ± 1.62	3.32 ± 0.17	1.73 ± 0.07	4.13 ± 0.61
	capsanthin derivatives	nd	0.76 ± 0.06	nd	nd	nd	nd	nd	nd	nd	4.83 ± 0.51
	(13*Z*)- *lycopene*	nd	nd	nd	nd	nd	nd	nd	0.49 ± 0.03	0.44 ± 0.08	7.39 ± 0.49
	(5*Z*, 9´*Z*)- *lycopene*	nd	nd	nd	nd	nd	nd	nd	0.46 ± 0.09	0.34 ± 0.08	6.34 ± 0.57
	(9*Z*)-*lycopene*	nd	nd	nd	nd	nd	nd	nd	0.13 ± 0.03	0.10 ± 0.04	4.39 ± 0.32
	(all-*E*)-*lycopene*	nd	nd	nd	nd	nd	nd	nd	3.08 ± 0.78	4.26 ± 1.25	26.88 ± 2.14
	(5*Z*)- *lycopene*	nd	nd	nd	nd	nd	nd	nd	2.24 ± 0.39	1.08 ± 0.42	26.27 ± 1.54
	(15*Z*)-*phytoene*	15.14 ± 0.62	nd	5.82 ± 0.28	nd	0.47 ± 0.04	15.94 ± 0.99	16.08 ± 0.41	3.37 ± 0.53	nd	5.17 ± 0.28
	phytofluene isomer 1	9.76 ± 0.30	nd	4.35 ± 0.09	nd	nd	4.71 ± 0.34	10.35 ± 1.18	1.66 ± 0.50	0.44 ± 0.08	1.30 ± 0.10
	phytofluene isomer 2	2.60 ± 0.01	nd	1.16 ± 0.03	nd	nd	3.47 ± 0.18	2.53 ± 0.16	nd	nd	1.50 ± 0.05
	phytofluene isomer 3	nd	nd	nd	nd	nd	1.38 ± 0.20	0.55 ± 0.03	nd	nd	1.11 ± 0.09
	phytofluene isomer 4	5.77 ± 0.34	nd	2.20 ± 0.12	nd	nd	nd	6.37 ± 0.48	1.72 ± 0.32	0.63 ± 0.02	2.39 ± 0.20
	phytofluene isomer 5	nd	nd	0.53 ± 0.03	nd	nd	nd	nd	nd	nd	1.87 ± 0.09
	ζ-carotene isomer	1.82 ± 0.02	nd	nd	nd	nd	1.78 ± 1.01	1.79 ± 0.06	4.02 ± 1.00	7.32 ± 0.04	nd
	(15*Z*)- *ζ-carotene*	4.82 ± 0.26	nd	2.75 ± 0.18	nd	nd	2.77 ± 0.68	4.86 ± 0.65	nd	nd	nd
	(*9Z*)-*ζ-carotene*	17.12 ± 1.00	nd	6.45 ± 0.65	nd	nd	13.05 ± 1.20	16.03 ± 1.61	nd	nd	0.97 ± 0.02
	(all-*E*)- *ζ-carotene*	11.60 ± 0.43	nd	3.85 ± 0.14	nd	nd	17.45 ± 1.37	10.77 ± 0.78	3.07 ± 0.29	nd	2.30 ± 0.09
	Total carotenoids	**265.20 ± 12.48**	**9.73 ± 0.45**	**107.93 ± 3.89**	**27.29 ± 0.45**	**24.43 ± 0.22**	**302.02 ± 21.56**	**260.78 ± 16.51**	**89.30 ± 1.16**	**46.40 ± 4.02**	**131.24 ± 9.87**
Tocopherols	*α-tocopherol*	29.41 ± 0.05	44.47 ± 6.13	72.43 ± 11.60	16.75 ± 1.46	10.74 ± 1.10	30.64 ± 0.94	16.20 ± 4.76	42.11 ± 1.14	13.59 ± 0.10	20.27 ± 5.89
	γ-tocopherol	nd	nd	nd	nd	0.46 ± 0.18	nd	nd	nd	nd	nd
	Total tocopherols	**29.41 ± 0.05**	**44.47 ± 6.13**	**72.43 ± 11.60**	**16.75 ± 1.46**	**11.19 ± 0.92**	**30.64 ± 0.94**	**16.20 ± 4.76**	**42.11 ± 1.14**	**13.59 ± 0.10**	**20.27 ± 5.89**
Chlorophylls	*chlorophyll b*	nd	nd	nd	16.50 ± 1.58	26.25 ± 2.47	nd	nd	42.41 ± 8.91	5.53 ± 0.13	nd
	chlorophyll b derivative	nd	nd	nd	6.53 ± 0.08	7.11 ± 0.17	nd	nd	11.70 ± 1.02	11.33 ± 0.95	nd
	chlorophyll a	nd	nd	nd	28.02 ± 1.96	49.01 ± 9.58	nd	nd	61.77 ± 23.01	31.98 ± 8.15	nd
	chlorophyll a derivative	nd	nd	nd	nd	5.99 ± 1.12	nd	nd	7.06 ± 1.30	3.68 ± 0.30	nd
	pheophytin b	nd	nd	nd	17.63 ± 0.71	4.65 ± 0.17	nd	nd	12.75 ± 3.20	28.73 ± 5.18	nd
	pheophytin b derivative 1	nd	nd	nd	1.11 ± 0.21	0.91 ± 0.01	nd	nd	0.69 ± 0.02	0.72 ± 0.20	nd
	pheophytin b derivative 2	nd	nd	nd	1.11 ± 0.21	0.91 ± 0.01	nd	nd	0.69 ± 0.02	0.72 ± 0.20	nd
	pheophytin a	nd	nd	nd	12.80 ± 0.02	5.44 ± 0.03	nd	nd	14.03 ± 3.36	19.19 ± 2.40	nd
	pheophytin a derivative 1	nd	nd	nd	3.40 ± 0.33	2.58 ± 0.04	nd	nd	1.10 ± 0.09	1.27 ± 0.01	nd
	Total chlorophylls	**nd**	**nd**	**nd**	**87.10 ± 3.68**	**96.85 ± 12.31**	**nd**	**nd**	**145.14 ± 39.54**	**99.47 ± 0.90**	**nd**

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