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Evaluation of a method based on liquid chromatography–diode array detector–tandem mass spectrometry for a rapid and comprehensive characterization of the fat-soluble vitamin and carotenoid profile of selected plant foods

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ABSTRACT

The feasibility of using reversed-phase liquid chromatography/diode array/tandem mass spectrometry (LC–DAD–MS/MS) for a rapid and comprehensive profiling of fat soluble vitamins and pigments in some foods of plant origin (maize flour, green and golden kiwi) was evaluated. The instrumental approach was planned for obtaining two main outcomes within the same chromatographic run: (i) the quantitative analysis of ten target analytes, whose standards are commercially available; (ii) the screening of pigments occurring in the selected matrices. The quantitative analysis was performed simultaneously for four carotenoids (lutein, zeaxanthin, β-cryptoxanthin, and β-carotene) and six compounds with fat-soluble activity (α-tocopherol, δ-tocopherol, γ-tocopherol, ergocalciferol, phylloquinone and menaquinone-4), separated on a C30 reversed-phase column and detected by atmospheric pressure chemical ionization (APCI) tandem mass spectrometry, operating in Selected Reaction Monitoring (SRM) mode. Extraction procedure was based on matrix solid-phase dispersion with recoveries of all compounds under study exceeding 78 and 60% from maize flour and kiwi, respectively. The method intra-day precision ranged between 3 and 7%, while the inter-day one was below 12%. The mild isolation conditions precluded artefacts creation, such as *cis*-isomerization phenomena for carotenoids. During the quantitative LC-SRM determination of the ten target analytes, the identification power of the diode array detector joined to that of the triple quadrupole (QqQ) allowed the tentatively identification of several pigments (chlorophylls and carotenoids), without the aid of standards, on the basis of: (i) the UV–vis spectra recorded in the range of 200–700 nm; (ii) the expected retention time; (iii) the two SRM transitions, chosen for the target carotenoids but also common to many of isomeric carotenoids occurring in the selected foods.

1. Introduction

Fat-soluble vitamins are classified into four groups of compounds involved in important biological functions, such as vision (vitamin A), calcium adsorption (vitamin D), antioxidative protection of cell membranes (vitamin E) and blood coagulation (vitamin K) [1], just to mention a few.

Vitamin A-active compounds occur in foods of animal origin as retinoids while in those of plant origin as carotenoids (provitamins A) [1]. Nevertheless, only carotenoids with one unsubstituted β-ionone ring and with an 11-carbon polyene chain at least possess vitamin A activity: β-carotene, with two β-rings and polyene chains, is the most important provitamin A, followed by α-carotene and β-cryptoxanthin. Xanthophylls such as lutein and zeaxanthin are not provitamins A, but they are important antioxidants, effective in preventing age-related macular degeneration. All of these compounds are very sensitive to light and oxidants, so special care has to be taken during sampling, extraction procedure and the final chromatographic separation in order to avoid isomerization and oxidation phenomena [2,3]. Ascorbic acid, hydroquinone or pyrogallol are some of antioxidants utilized to prevent their degradation. Sample pretreatment may consist either of (i) saponification followed by solvent extraction for quantifying the total free form (for example, xanthophylls may occur in fruits and vegetables as free or esterified forms), or of (ii) direct extraction for determining esterified forms [2,3].

Foods of plant origin may contain vitamin D2 (ergocalciferol), while only fish liver is an abundant source of vitamin D3 (cholecalciferol) [1]. Sensitivity to light and oxygen, and the presence of fats and proteins makes their analysis complicated [2–4]. As for

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vitamin A, two procedures can be applied for sample preparation: the former one includes hot saponification (to release bound forms, to hydrolyze triglycerides and to dissolve any gelatine) and extraction of the unsaponificable fraction; the second one is based on a direct extraction with organic solvents. Saponification is omitted or carried out at room temperature overnight for reducing thermal isomerization, while the addition of an antioxidant is useful to prevent oxidation [2–4].

Predominant forms of vitamin E in nature are α- and γ-tocopherol, while δ-form is the least widespread one [1,2]. During its extraction, vitamin E must be protected from light and oxygen in order to quantify its actual content. It can be directly analyzed in oils after sample dilution with hexane, but hot saponification is always applied for its determination in other foods; nevertheless, alkaline conditions must be carefully balanced for avoiding losses [2,3,5].

Vitamin K is a collective term which includes forms characterized by a common 2-methyl-1,4-naphtoquinone nucleus (menadione moiety) and by a different isoprenoid side chain at the 3-position [1]. Phylloquinone or vitamin K1 has the same phytyl side chain as chlorophyll and it occurs in green plants, while vitamin K2 is represented by a family of bacterially synthesized menaquinones with multiprenyl side chains (MK-*n*, with *n* number of prenyl units ranging from 4 to 13) [1]. This group of compounds is relatively stable to heat and oxygen exposure, but it is destroyed by light and alkali [2]. Therefore, hot saponification cannot be used and it was substituted with enzymatic hydrolysis (lipase) followed by liquid–liquid extraction [3,6] or direct solvent extraction [6,7].

Currently, the conventional methods are addressed to the determination of one or few forms belonging to a specific fat-soluble vitamin group in food products, mainly by HPLC with UV or fluorescence detections [8–12] after having applied a quantitative and non-destructive extraction procedure [13,14]. In the literature, few analytical methods have been proposed for the simultaneous determination of fat-soluble vitamins in foods by chromatographic techniques [15,16]. The reason is due to some fundamental difficulties in developing multi-analyte methods: (i) chemical heterogeneity which requires specific detection parameters; (ii) occurrence of vitamers and geometrical isomers, hard to separate and to identify due to unavailability of standards; (iii) different stability and endogenous levels of analytes (vitamin D content is sometimes 100-fold less than the one of vitamin E or vitamin A), presence of bound forms to macromolecular food components (polysaccharides, proteins, and lipids), and complexity of matrix, all factors that make extraction a critical step.

Hot saponification is the most used extraction procedure, especially for foods with a high fat content; moreover, it simplifies vitamin analysis which is only performed on the free forms. However, some troubles can occur: first of all, K vitamers rapidly decompose in alkaline media and at high temperature, vitamin D and carotenoids might suffer thermal isomerization, while the free forms of vitamins and carotenoids are more sensitive to light and oxygen [1–3].

Among the published multi-vitamin methods, only two are based on liquid chromatography tandem mass spectrometry (LC–MS/MS): one determines retinol, α-tocopherol, and cholecalciferol in fortified infant formulae [17]; the other one is addressed to quantify several fat-soluble vitamins (retinol, D2, D3, 25OH-D2, 25OH-D3, β-carotene, K1, MK-4 and MK-7) in human breast milk, adopting a separated extraction procedure for K vitamers [18]. For carotenoids analysis in fruit, the literature describes several LC–DAD–MS^n methods [19–22]: some characterize via MS the analytes detected by DAD, carrying out more chromatographic runs for finding the *m*/*z* values of pseudomolecular ions [19], prior, and those of fragment ions, then [20,21]; only one [22] uses theoretical ion currents for a provisional identification of the interested compounds.

The main objective of this work was to propose an effective analytical approach for quickly obtaining the profile of fat-soluble vitamers and of pigments (chlorophylls, carotenes, xanthophylls and their derivates) in some selected foods of plant origin (maize flour, green and golden kiwi), using the LC–DAD–APCI-QqQ hyphenation. MSPD was used as a relatively mild technique for the sample extraction/clean-up, with little modifications depending on the matrix. Saponification was omitted for preserving vitamin K and for avoiding isomerization of carotenoids. Trans-β-apo-8′-carotenal, α-tocopherol acetate, 1α-hydroxyvitamin D3, and phylloquinone-d_7 were used as internal standards (volumetric method) for the quantitative analysis of carotenoids (lutein, zeaxanthin, β-cryptoxanthin, β-carotene), vitamers E (α-tocopherol, δ-tocopherol, γ-tocopherol), vitamin D2 (ergocalciferol), vitamers K (phylloquinone, menaquinone-4), respectively. Since many carotenes and xanthophylls are structural isomers, the two SRM transitions selected for the target analytes resulted common to many other carotenoids, detected by DAD ($\lambda = 450$ nm) in the extracts from various foods. The agreement between UV–vis spectra (200–700 nm), the SRM transitions and the expected retention time allowed the tentative identification of many pigments without the use of standards, expensive and/or difficult to find, during the same chromatographic run used for the quantitative analysis. Besides the extensive characterization, some interesting results deserve to be cited such as the finding of menaquinone-4 in foods of plant origin, and the detection of geometric isomers which were not surely artefacts of the applied extraction procedure.

2. Materials and methods

2.1. Chemicals and materials

The following carotenoids and fat-soluble vitamin standards were purchased from Aldrich-Fluka-Sigma S.r.l. (Milano, Italia): lutein, zeaxanthin, β-cryptoxanthin, β-carotene, α-tocopherol, δ-tocopherol, γ-tocopherol, ergocalciferol, phylloquinone, and menaquinone-4. Trans-β-apo-8′-carotenal, (D,L) α-tocopherol acetate, 1α-hydroxyvitamin D3, and phylloquinone-d_7 (5,6,7,8-d_4, 2-methyl-d_3), bought from Aldrich-Fluka-Sigma S.r.l. (Milano, Italia), were chosen as internal standards (IS) for carotenoids, vitamers E, ergocalciferol, and vitamers K, respectively. All chemicals had a purity grade greater than 90% and were used without further purification.

Butylated hydroxytoluene (BHT), provided by Aldrich-Fluka-Sigma S.r.l. (Milano, Italia), was used as antioxidant both in standard solutions and during the steps of extraction.

Acetonitrile and methanol were RS-Plus grade (special grade reagents); isopropyl alcohol, hexane and chloroform were RS grade; absolute ethanol was RPE grade (elevated purity grade or analytical grade). All these solvents were bought from Carlo Erba (Milano, Italy).

The following materials were employed for extraction studies: as adsorbents, SPE Bulk Sorbent C-18 (6%)-bonded silica with particles of 35–70 μm diameter and porosity of 60 Å, supplied by Alltech Associates Inc. (Deerfield, IL, USA), and diatomaceous earth SPE-ED™ MATRIX 38, supplied by Applied Separations (Allentown, PA-USA); glass cartridges (i.d. 1 cm, capacity 6 mL) with frits made of Teflon, purchased from Supelco Inc. (Bellafonte, PA, USA); polypropylene tubes (i.d. 2.5 cm, capacity 20 mL) with polyethylene frits, purchased from Alltech Associates Inc. (Deerfield, IL, USA).

2.2. Standard solutions

On the basis of results achieved by a stability study, carried out over a period of forty days, the individual stock solutions of analytes and internal standards were prepared monthly at 1 μg μL^{-1} concentration for: ergocalciferol, phylloquinone, menaquinone-4, δ-tocopherol, γ-tocopherol, α-tocopheryl acetate, 1α-hydroxyvitamin D3, and phylloquinone-d_7 in acetonitrile containing 0.1% (w/v) BHT; α-tocopherol in methanol containing 0.1% (w/v) BHT; trans-β-apo-8′-carotenal in chloroform containing 0.1% BHT. Owing to their lower stability and/or solubility, the individual solutions of the other carotenoids were prepared fortnightly in chloroform containing 0.1% (w/v) BHT at the following concentrations: β-carotene (1 μg μL^{-1}); lutein, zeaxanthin, and β-cryptoxanthin (500 ng μL^{-1}).

Both working multianalyte solution and the composite one of internal standards were prepared weekly, at different concentrations depending on the purpose, from the individual solutions diluting in methanol with 0.1% (w/v) BHT, in the first case, and in isopropanol:hexane (50:50, v/v) with 0.1% (w/v) BHT, in the second one. All solutions were degassed with nitrogen and stored in dark glass flasks at −18 °C.

The precise concentration and stability of every standard was periodically checked using the corresponding extinction coefficient and verifying LC–MS area constancy, respectively.

2.3. Sample treatment

The extraction procedures used for the different food matrices were rapid, simple and relatively mild in order to minimize the exposure of vitamins to light, heat and air and to avoid undesired losses. All operations were performed in subdued light.

Maize flour was ready to be directly subjected to the extraction procedure, while kiwi had to be homogenized. For this purpose, one kiwifruit was peeled off and the edible part was cut in small pieces and homogenized by a rotating blade (∼100 rpm for 30 s).

The MSPD technique [23,24] was applied to extract analytes from the different foods, using C18 sorbent as dispersing medium in the case of maize flour and diatomaceous earth for kiwi due to its high water content.

Before dispersion, C_{18} sorbent and diatomaceous earth were submitted to a cleaning procedure: 10 g of sorbent were filled into a polypropylene tube (i.d. 2.5 cm, capacity 20 mL), washed with 30 mL of methanol and dried with a gentle nitrogen flow.

2.3.1. Maize flour

Maize flour was obtained from a miller of centre Italy (Abruzzo Region). Two grams of sample, 15 mg of BHT and 2 g of C18 sorbent were weighted and transferred in a glass mortar. Subsequently, the MSPD technique was applied: the food matrix and the solid support were blended with a glass pestle until the mixture was of an uniformed colour and consistency. Once the dispersion was completed, an empty glass cartridge (i.d. 1 cm, capacity 6 mL) was before filled with the blend, retained by two Teflon frits, and then it was connected to a vacuum flask. Analytes were extracted from matrix by forcing 4 mL of methanol, 4 mL of isopropanol and 4 mL of hexane to pass through the cartridge, in sequence. The whole extract was collected into a glass tube with conical bottom (i.d. 1.4 cm) and spiked with the composite solution of the internal standards. Afterwards, it was evaporated up to 500 μL in a thermostated bath at 30 °C, under a kind nitrogen flow, and finally diluted at a volume of 1 mL with an isopropanol:hexane (50:50, v/v) solution. Subsequently, it was transferred into an Eppendorf tube and centrifuged at 4000 rpm for 5 min. The final concentration of the internal standards was as follows: 0.75 ng μL^{-1} of trans-β-apo-8′-carotenal, 25 ng μL^{-1} of α-tocopheryl acetate, 0.01 ng μL^{-1} of 1α-hydroxyvitamin D3, and 0.025 ng μL^{-1} of phylloquinone-d_7. Twenty μL of the final extract were injected into the LC–DAD–MS/MS system.

2.3.2. Kiwifruits

The kiwi species studied were the two commercial fruits *Actinidia deliciosa* cultivar Hayward (green kiwi) and a yellow-fleshed genotype *Actinidia chinensis* cultivar Hort16A (known commercially as ZESPRI Gold kiwifruit or golden kiwi). The sample (2 g), BHT (15 mg), diatomaceous earth (3 g) and Na_2SO_4 (1 g) were weighed, put in a glass mortar and dispersed, as described for maize flour sample. The extraction/purification cartridge was prepared by introducing first 0.5 g of the C18 sorbent in a 6 mL syringe-like glass tube and then the food sample, treated as above illustrated. Teflon frits were located above and below the sorbent/food matrix bed. The cartridge was connected to a side-arm flask, and the extractants (5 mL of methanol, 10 mL of isopropanol and 5 mL of hexane) were forced to pass through it by a water pump vacuum.

The whole extract was collected into a 50 mL Falcon tube and centrifuged at 6000 rpm for 5 min. The supernatant was transferred in a glass tube with conical bottom (i.d. 1.4 cm), and spiked with the composite solution of the internal standards. After evaporation up to 500 μL (as maize flour), its volume was corrected at final value of 1 mL with an isopropanol:hexane (50:50, v/v) solution. The extract was then centrifuged at 4000 rpm for 5 min into an Eppendorf tube. The final concentration of the internal standards was as follows: 1.25 ng μL^{-1} of trans-β-apo-8′-carotenal, 250 ng μL^{-1} of α-tocopheryl acetate, 0.75 ng μL^{-1} of 1α-hydroxyvitamin D3 and of phylloquinone-d_7. Twenty μL of the extract were injected into the LC–DAD–MS/MS system.

2.4. Instrumentation

2.4.1. Liquid chromatography

Chromatographic analysis was performed using a micro-HPLC/autosampler/vacuum degasser system PE Series 200 (Perkin Elmer, Norwalk, CT).

The analytes were separated on a ProntoSIL C30 column (250 × 4.6 mm i.d., particle size 3 μm) protected by a guard column of the same type (4.0 × 10 mm, 5 μm particle size), from Bischoff Chromatography (Leonberg Germany), using methanol as phase A and an isopropanol/hexane (50:50, v/v) solution as phase B; the latter was also used as washing solution for the autosampler injection system. Elution was effected working for the first minute in isocratic mode (100% A), then linearly increasing phase B from 0% to 75% in 14 min and then from 75% to 99.5% in 0.1 min; finally, phase B was kept at 99.5% for 15 min (the 0.5% percentage of methanol was maintained in this final part of the chromatographic run to support atmospheric pressure chemical ionization of analytes with higher capacity factors).

Flow rate of the mobile phase was 1 mL min^{-1} and it was entirely introduced into the DAD–tandem MS detection system.

2.4.2. Diode array detector

A Series 200 model (Perkin Elmer, Norwalk, CT) of diode array detector, equipped with a flow Z cell of 12 μL volume and optical path of 10 mm, was coupled on-line between the chromatographic column and the mass spectrometer.

The LC–DAD chromatograms were acquired selecting the 450 nm wavelength (all-*trans* form λ_{max} of β-carotene, zeaxanthin and β-cryptoxanthin) and a bandwidth of 10 nm; afterwards, the UV–vis spectra, recorded in the range of 200–700 nm, were extracted and used for the tentative identification of carotenoids and their geometrical isomers, in correspondence of their expected retention times.

Table 1
LC–MS/MS parameters for fat-soluble vitamins, carotenoids and internal standards, selected in this study.

Analyte	Retention time (min)	Qualifier and quantifier SRM transitions[a]	Declustering potential (V)	Collision potential (V)	Relative abundance[b] Mean ± SD
δ-Tocopherol	7.52	402/177	40	34	0.89 ± 0.02
		402/137		47	
γ-Tocopherol	8.09	416/151	57	40	0.66 ± 0.01
		417/151	35	35	
Ergocalciferol	8.16	397/159	36	30	0.85 ± 0.05
		397/125		22	
α-Tocopherol	8.75	431/111	20	20	0.03 ± 0.01
		431/165		25	
Lutein	8.83	551/175	45	25	0.39 ± 0.02
		551/459		17	
Menaquinone-4	8.95	445/149	45	30	0.45 ± 0.02
		445/187		35	
Zeaxanthin	9.23	569/551	40	20	0.46 ± 0.05
		569/135		30	
Phylloquinone	10.80	451/197	45	35	0.18 ± 0.04
		451/187		38	
β-Cryptoxanthin	11.91	553/135	40	20	0.91 ± 0.04
		553/119		30	
β-Carotene	15.80	537/137	47	25	0.64 ± 0.02
		537/177		28	
Internal standards					
α-Tocopherol acetate	10.39	472/430	25	30	–
1α-HydroxyvitaminD3	6.82	383/365	35	25	–
Phylloquinone-d_7	10.80	458/194	45	38	–
trans-β-Apo-8′-carotenal	11.63	417/177	47	20	–

[a] The first line reports the least intense SRM transition (qualifier) and the second line the most intense one (quantifier).
[b] The relative abundance is calculated as ratio of qualifier intensity/quantifier intensity; the results are reported as arithmetic average of six replicates plus the corresponding standard deviation (SD).

The softwares used for running the instrument and acquiring spectral data were Totalchrom Navigator and Iris.

2.4.3. Mass spectrometry

Target analytes were identified and quantified by a Applied Biosystem API 3000 triple-quadrupole mass spectrometer (Applied Biosystem Sciex, Ontario, Canada), set to acquire in Selected Reaction Monitoring (SRM) scan mode.

APCI source operated in positive ionization, with a needle current (NC) of 3 μA and a probe temperature of 450 °C. High-purity nitrogen was used as curtain (2 L min^{-1}) and collision (4 mTorr) gas, while air as nebulizer (1.5 L min^{-1}) and make-up (2 L min^{-1}) gas.

A preliminary mass axis calibration of each quadrupole mass-analyzer, Q_1 and Q_3, was carried out by the infusion of a polypropylene glycol solution at 10 μL min^{-1}. Unit mass resolution was established and kept in the mass-resolving quadrupoles by maintaining a full width at half maximum (FWHM) of approximately 0.7 ± 0.1 Da.

For the quantitative analysis of the ten analytes, two SRM transitions were selected for each of them, after having observed their product ion scan spectra, by flow injection analysis (10 ng injected; 1 mL min^{-1} of flow rate). Afterwards, these ion currents were optimized tuning the instrumental parameters of the APCI source and the triple quadrupole. Table 1 resumes the most important LC–APCI(+)–MS/MS parameters useful for the analysis of the ten target compounds in the chosen food matrices. Taking into account the criteria expressed in the Commission Decision 2002/657/EC [25] for confirming contaminant residues in animal food products, each analyte was identified on the basis of its retention time, two selected SRM transitions and their relative abundance. Figs. 1 and 2 show typical APCI(+)-SRM chromatograms of a working standard solution of an extract from golden kiwi, respectively.

The inseries coupling of DAD and MS was indispensable for the screening of pigments; also in this case, mass spectrometer operated in SRM scan, enforcing the DAD detection of those carotenoids which are structural and geometric isomers of four target carotenoids.

2.5. Validation method

Since blank samples were not available, quantitative analysis was performed by means of standard-addition method; in this way, besides estimating the unknown amount of the analytes occurring in the different foods, it was possible to evaluate sensitivity and linear dynamic range in the various matrices. Recoveries, precision, limits of detection (LODs) and limits of quantitation (LOQs) were calculated after having determined the natural levels of each form in the specific food.

Regarding the two SRM transitions, the most intense one (quantifier transition) was used for quantitative analysis, while the least one (qualifier transition) for identification purposes and for defining the method limits (LODs and LOQs).

The software employed for acquiring and elaborating LC–MS/MS data was Analyst 1.5.1.

3. Results and discussion

3.1. Fragmentation study and optimization of MS/MS conditions

Structural information to confirm analytes identity were obtained by means of a preliminary fragmentation study.

Q1 scan spectra of almost all of fat-soluble vitamins and carotenoids showed the protonated molecule $[M+H]^+$ as base peak, but some exceptions were noticed. Each tocopherol showed three ion species: $[M+H]^+$ (base peak for α- and γ-tocopherol), $[M]^{+\bullet}$ (base peak for δ-tocopherol) and $[M-H]^+$. Vitamin D2 generated a $[MH-H_2O]^+$ ion, but it was not more intense than $[M+H]^+$. Differently from what reported van Breemen [26], we did not observe the radical ion $[M]^{+\bullet}$ for β-carotene, but only $[M+H]^+$ at m/z 537 as the base peak and $[M-H]^+$ at m/z 535; this latter ion species, being

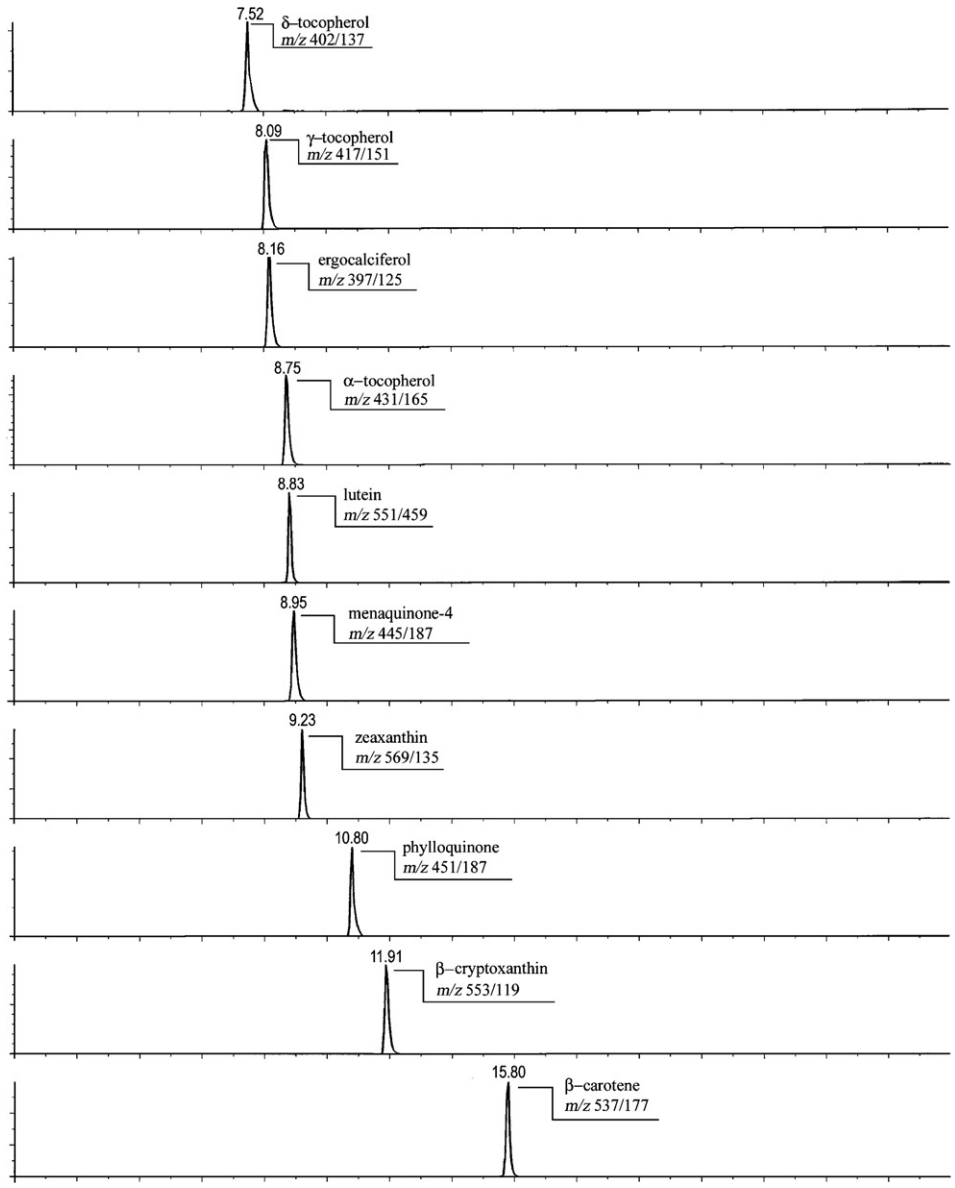

Fig. 1. LC-SRM chromatogram, acquired by an APCI source operating in positive ionization mode, of the ten target analytes (25 ng injected); for each analyte the most intense SRM transition is shown.

very few intense, was confirmed by chromatographic analysis. Q1 full-scan spectra of xanthophylls presented two very abundant ions: $[M+H]^+$ (base peak for zeaxanthin and β-cryptoxanthin) and $[MH-H_2O]^+$ (base peak for lutein); while, other less intense species, i.e. $[M]^{+\bullet}$ and $[MH-H_2O-92]^+$ (92 Da corresponding to the toluene loss), were observed for lutein and β-cryptoxanthin only.

Product ion scan spectra and the hypothesized fragmentation schemes of the studied analytes are reported in Figs. 3–5.

Upon collision-induced decomposition (CID), vitamin D2 (Fig. 3A) lost one molecule of water generating the ion at *m/z* 379.6, while the fragment at *m/z* 271 was due to the cleavage of its side chain (−126 Da).

Protonated molecular ions of both K vitamers lost their phytyl chain, giving an intense product ion at *m/z* 187 (Fig. 3B and C). Fragmentation of menaquinone-4 was more pronounced and resulted in several fragments of appreciable intensity.

Fragmentation of tocopherols (Fig. 4A–C) caused the cleavage of the chromanol ring, giving abundant product ions at *m/z* 165 (from $[M+H]^+$, $[M]^{+\bullet}$ and $[M-H]^+$ of α-tocopherol), at *m/z* 151 and at *m/z* 137 (from $[M+H]^+$, $[M]^{+\bullet}$ and $[M-H]^+$ of γ- and δ-tocopherol, respectively). The isoprenoid chain rupture generated an intense ion at *m/z* 177 for δ-tocopherol only.

Product ion scan spectrum of β-carotene highlighted two series of fragment ions (Fig. 5A). The first was probably originated from breaking the C7–C8, C9–C10, C11–C12, C13–C14, and C15–C15′ double bonds; the most intense ion at *m/z* 177 might be stabilized by the formation of a ring structure. The second series was due to the cleavage of single bonds between C6–C7 (fragments at *m/z* 123 or 413), C10–C11, C12–C13, and C14–C15.

At low collision energy, $[MH-H_2O]^+$ of lutein lost a second water molecule (fragment at *m/z* 533), toluene (fragment at *m/z* 459) and the terminal ring (fragment at *m/z* 429). Product ions at *m/z* 551, 477 and 429 were generated from $[M+H]^+$ of zeaxanthin for loss of water, toluene and water plus the terminal ring, respectively, while β-cryptoxanthin lost water giving the $[MH-H_2O]^+$ species at *m/z* 535.5. At higher collision energies, all these xanthophylls showed a fragmentation pattern analogous to that described for β-carotene (Fig. 5B–D).

On the basis of this fragmentation study, the two most intense SRM transitions of each analyte (listed in Table 1) were chosen for

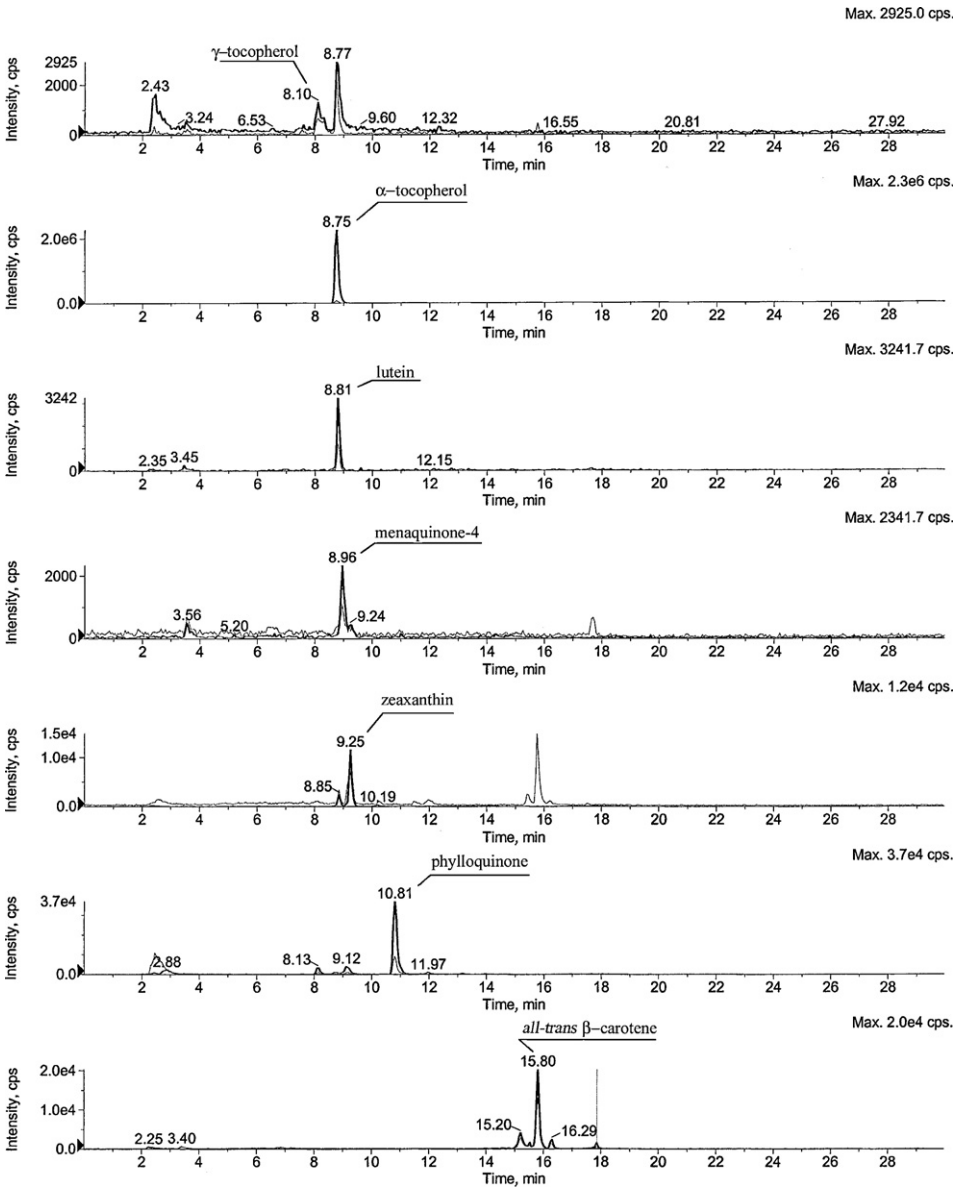

Fig. 2. LC-SRM chromatogram of a golden kiwi extract. For each detected analyte, the two overlapped extracted ion currents are shown.

performing quantitative and confirmative analysis on the selected food matrices. In summary:

- $[M+H]^+$ was selected as precursor ion for α-tocopherol, γ-tocopherol (for the most intense SRM transition), ergocalciferol, phylloquinone, menaquinone-4, β-carotene, zeaxanthin, and β-cryptoxanthin;
- $[MH-H_2O]^+$ for lutein;
- $[M]^{+\bullet}$ for δ-tocopherol and γ-tocopherol (for the least intense SRM transition).

3.2. Recovery studies

Matrix solid phase dispersion (MSPD) is a relatively new extraction technique [23,24], mainly employed for extracting target analytes from solid or semisolid samples. It involves the use of abrasive dispersing medium, blended with sample by mortar and pestle. By adopting an adequate solid sorbent, this technique was also applied to liquid foodstuffs such as milk and fruit juice. The shearing forces, developed during the dispersion process, disrupt the sample matrix and disperse its components on the solid support, thus generating a finely divided material suitable for a chromatographic-like extraction. In fact, the solid blend, achieved at the end of the MSPD process, is transferred into a column and retained between two frits; thereafter, a suitable solvent has to be chosen to extract analytes from the dispersed sample. Due to its mildness and rapidity, MSPD was used for extracting carotenoids stereoisomers from spinach samples without inducing analyte isomerization and/or oxidation [27].

For maize flour, a C18 sorbent proved to be the best dispersion medium. After having packed the cartridge as described in Section 2, different eluents were tested. Using methanol (12 mL) as extractant, recoveries ranged between 70 and 90% for all analytes. With 12 mL of a methanol:isopropanol (50:50, v/v) solution recoveries exceeded 74%, but the best results were achieved eluting sequentially with methanol (4 mL), isopropanol (4 mL) and hexane (4 mL). In this way, lutein was recovered with a percentage close to 80%.

C18 sorbent could not be utilized for the dispersion of kiwifruits due to their high content of water and to the formation of fractures and preferential flow paths of the solid blend, during the elution

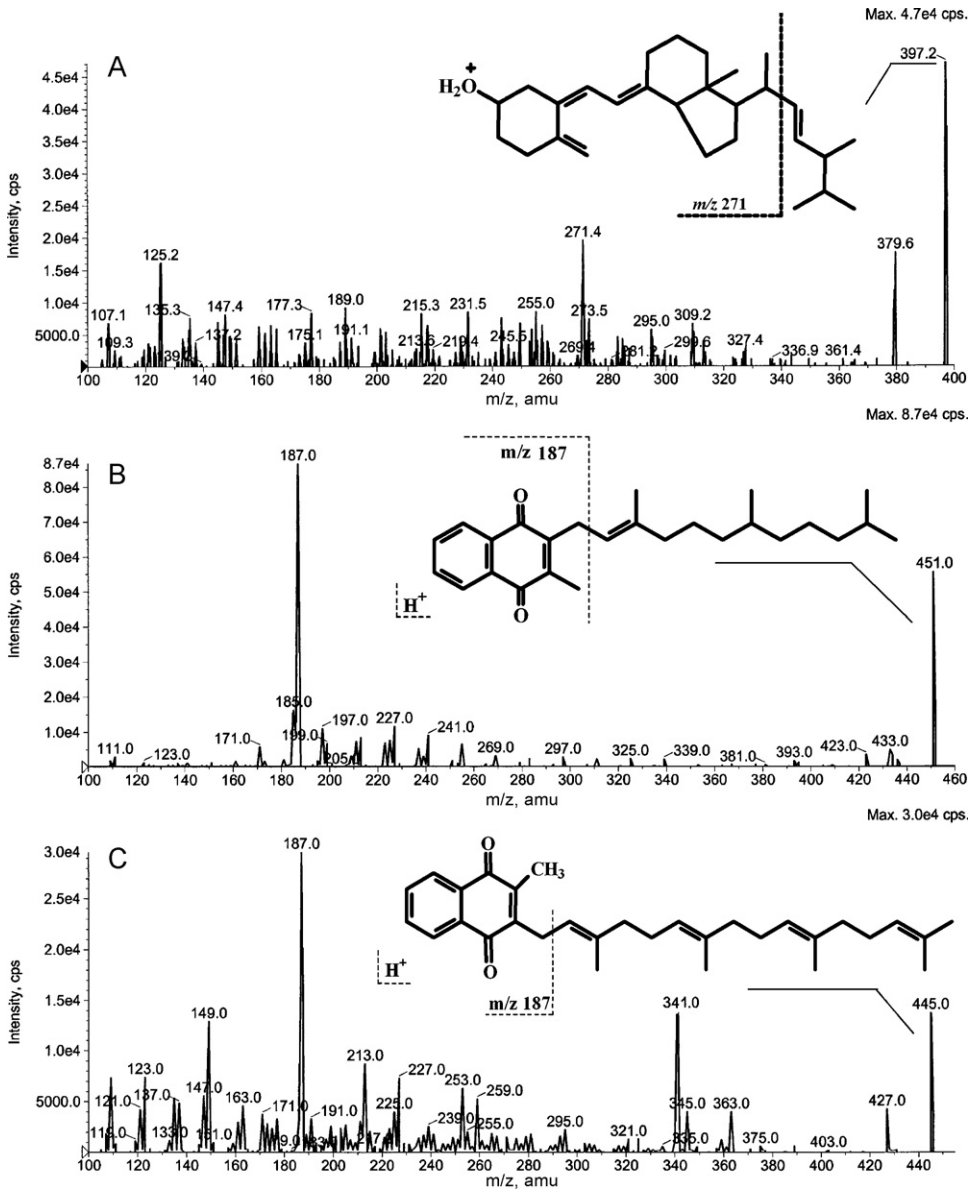

Fig. 3. Product ion scan mass spectra and corresponding fragmentation schemes for ergocalciferol (A), phylloquinone (B), and menaquinone-4 (C).

step with the same solvents employed for maize flour. For these reasons, C18 was substituted by diatomaceous earth and by a drying agent such as anhydrous Na_2SO_4, in order to obtain a quite dry solid blend suitable for the subsequent extraction. This solution permitted recoveries ranging between 60 and 85% from the two species of kiwi.

3.3. Validation results

3.3.1. Recovery and precision

After a preliminary determination of the natural vitamin and carotenoid content in each food (Table 2 reports the definitive values of the estimated quantities by the standard addition method), analyte recovery was assessed by weighing 4 g of sample and then dividing it in two aliquots. One was spiked with known amounts of the analytes and, after 30 min of equilibration time, submitted to the extraction process (spiked sample). The second aliquot was directly extracted and the fortification was applied on the final extract with the same nominal amounts of analytes (control sample). It has to be pointed out that the spiked level was chosen in order to increase the original content of the vitamins and carotenoids in the specific food by a factor of 2–3. Recovery of each analyte was calculated as follows

$$R(\%) = \frac{(\text{Analyte Area/Internal Standard Area})_{\text{Spiked Sample}}}{(\text{Analyte Area/Internal Standard Area})_{\text{Control Sample}}} \times 100$$

$$= \frac{\text{total amount (endogenous + added) of analyte recovered from the spiked sample}}{\text{endogenous amount of analyte extracted from the control sample + analyte amount added to the final extract of the control sample}} \times 100$$

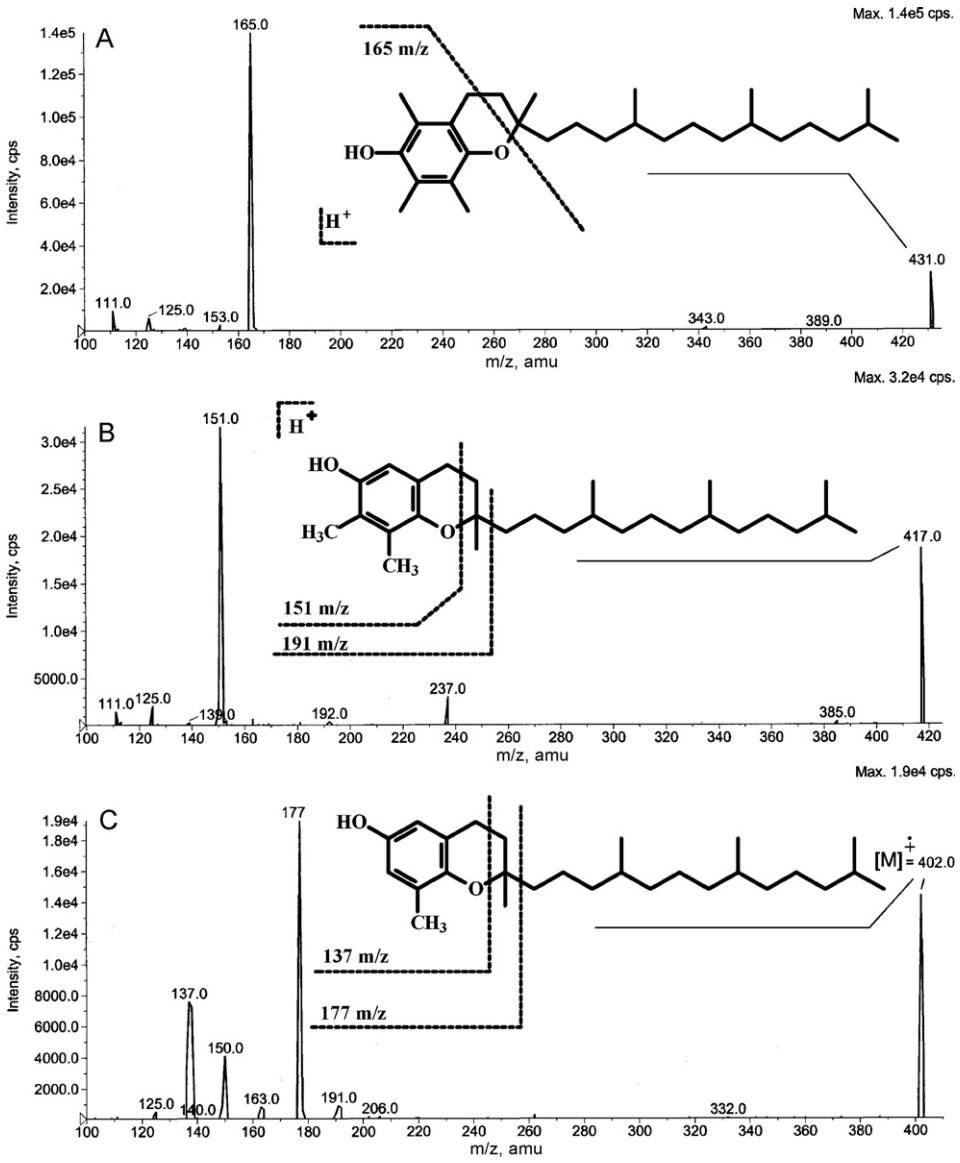

Fig. 4. Product ion scan mass spectra and corresponding fragmentation schemes for α-tocopherol (A), γ-tocopherol (B), and δ-tocopherol (C).

Mean recovery was calculated for each vitamin by averaging six replicates at the applied spike level, while the corresponding relative standard deviation was representative for intra-day precision.

Recoveries ranged between 60 and 100%, and resulted independent by the applied fortification level. Table 2 shows recovery and precision data, relative to the lowest spiked concentration level.

Inter-day precision was estimated as the relative standard deviation of six replicates performed within two weeks and the respective CV was less than 12% for all analytes.

3.3.2. Standard addition method and memory effect

Analyte quantitation was performed by the standard addition methods (five points). For this purpose, the extraction/purification cartridge was prepared by using 5 g of food sample and maintaining the same ratios between *g sample:g sorbent* and *mL extractant:g solid blend* as described in Section 2.3. Eluate from cartridge was subdivided into five aliquots which were spiked with the same volume of the internal standards composite solution; four of these were then spiked with appropriate volumes of working standard solution. Afterwards, each aliquot was evaporated to 250 μL, and its final volume was adjusted to 500 μL with an isopropanol:hexane (50:50, v/v) solution. The final concentrations of the internal standards were the following: 0.5 ng μL^{-1} of trans-β-apo-8′-carotenal, 25 ng μL^{-1} of α-tocopheryl acetate, 0.25 ng μL^{-1} of 1α-hydroxyvitamin D3 and of phylloquinone-d_7. Twenty μL of every aliquot was injected into LC–DAD–MS/MS system. Signal *vs* injected amount curves were then constructed by relating the peak area resulting from the most intense SRM transition of each analyte to that of the corresponding internal standard.

Linear dynamic range was investigated up to 50 ng injected for all analytes, except for: (i) α-tocopherol in kiwi (up to 1000 ng injected); (ii) lutein in kiwi and maize flour, and zeaxanthin in maize flour (up to 200 ng injected).

Linear regression parameters are reported in detail in Table 3. A good linearity was verified over two or three orders with all correlation coefficients exceeding 0.9960. Slopes, which are representative of the method sensitivity, resulted very similar in the different matrices; this suggests that the applied extraction procedure cleaned up the extracts from the various food samples analogously, producing a comparable matrix effect which the use of the standard addition method avoided to have to value.

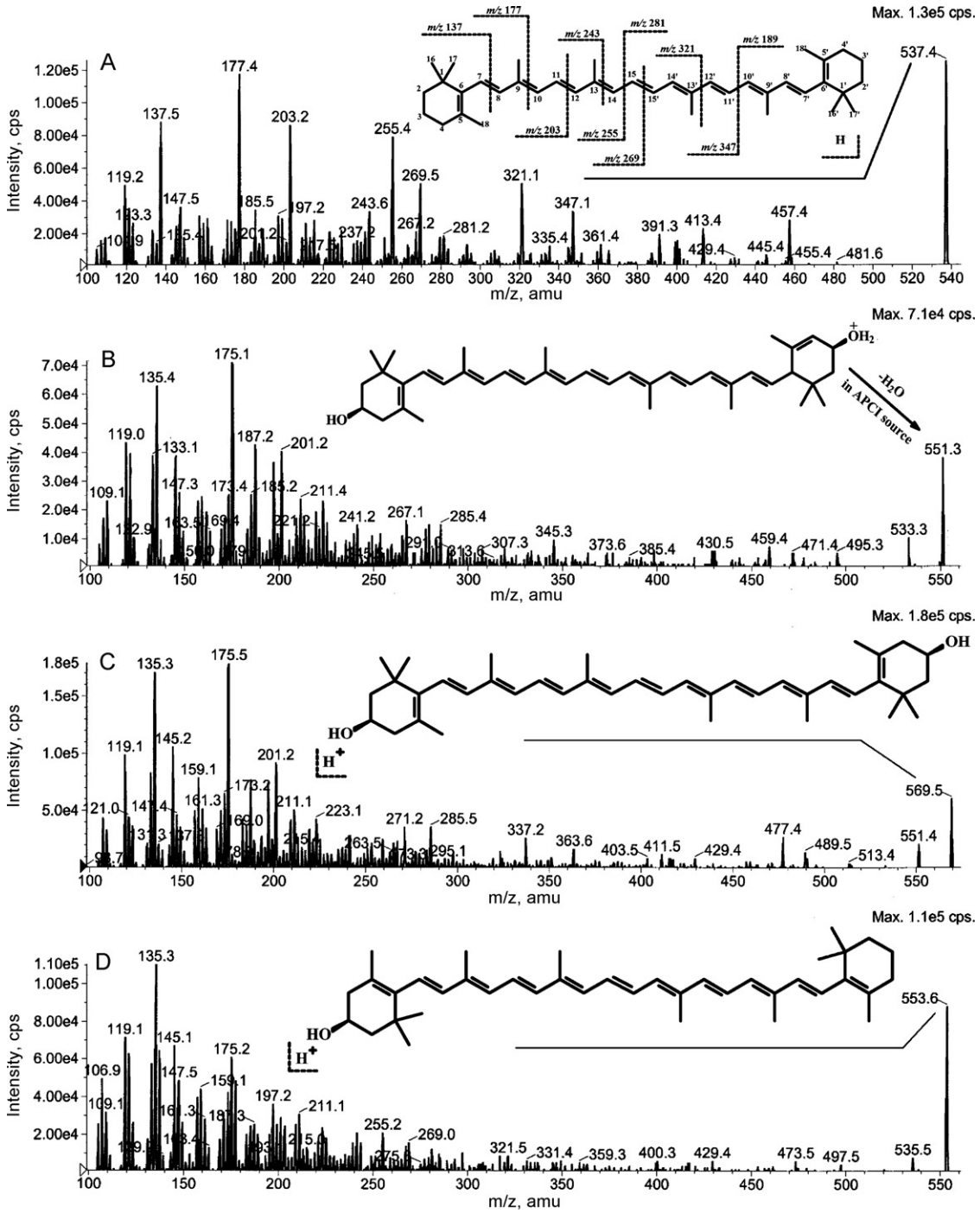

Fig. 5. Product ion scan mass spectra and corresponding fragmentation schemes for β-carotene (A), lutein (B), zeaxanthin (C) and β-cryptoxanthin (D).

Carry-over in the LC–MS system during sequential analyses of samples can adversely affect the accuracy of subsequent measurements. So, in this study, the memory effect was evaluated by running a blank LC–MS experiment (solvent injection) following the analysis of the sample spiked at the highest level of the calibration curve, obtained by means of the standard addition method. No memory effect was observed during the blank runs.

3.3.3. Limits of detection and limits of quantitations

Method limits were calculated using the least intense transition for confirming identity of each analyte also at low concentrations: in this way, in fact, both SRM ion currents could be observed with the characteristic *qualifier intensity*/*quantifier intensity* ratio.

The limit of detection (LOD) was calculated as the quantity of analyte able to produce a chromatographic peak three times higher than the noise of the baseline in a chromatogram (S/N = 3) of a non-fortified sample, after having estimated the endogenous amount. The limit of quantitation (LOQ) was set at three times the LOD. The noise level depended on the matrix and, therefore, the same analyte was characterized by different LODs in the several foods. LODs and LOQs are reported in Table 3 as mean of six replicates.

3.4. Application of the method for the analysis of food products

One of the goals of this work has been that of demonstrating the feasibility of the developed analytical method for the direct and

Table 2
Estimated quantities and recoveries (relative standard deviations) of fat-soluble vitamins and carotenoids from selected food matrices.

Analyte	Maize flour			Green kiwi			Golden kiwi		
	Estimated quantity	Spiked level[a]	Recovery (RSD)	Estimated quantity	Spiked level[a]	Recovery (RSD)	Estimated quantity	Spiked level[a]	Recovery (RSD)
	$\mu g\,g^{-1}$		%	$\mu g\,g^{-1}$		%	$\mu g\,g^{-1}$		%
δ-Tocopherol	n.d.[b]	–	–	n.d.	–	–	n.d.	–	–
γ-Tocopherol	0.12	0.3	100 (5)	0.23[c]	0.5	63 (6)	0.12[c]	0.3	63 (6)
Ergocalciferol	n.d.	–	–	n.d.	–	–	n.d.	–	–
α-Tocopherol	0.32	1.0	100 (3)	15.9	30	85 (4)	48.4	100	82 (7)
Lutein	3.16	10	78 (7)	3.04	10	73 (6)	2.1	5	70 (5)
Menaquinone-4	<LOQ	0.03	100 (5)	0.10	0.3	75 (4)	0.16	0.3	75 (5)
Zeaxanthin	4.87	10	84 (6)	<LOQ	0.5	65 (5)	0.56	1.0	63 (6)
Phylloquinone	0.005	0.01	100 (5)	0.86	2.5	62 (7)	0.53	1.5	60 (4)
β-Cryptoxanthin	1.22	2.5	83 (6)	n.d.	–	–	n.d.	–	–
β-Carotene	0.08	0.01	80 (7)	0.51	1.5	60 (5)	0.75	1.5	60 (5)

[a] The lowest spiked level was applied for doubling or tripling the endogenous concentration of each compound.
[b] n.d.: not detected by using the least intense SRM transition.
[c] For kiwifruits, the estimated amount should be ascribed to the sum of β- and γ-tocopherol.

Table 3
Method validation parameters: slopes (m), correlation coefficients (R^2) and limits (LOD and LOQ).

Analyte	Maize flour				Green kiwi				Golden kiwi			
	m ($\times 10^3$)	R^2	LOD	LOQ	m ($\times 10^3$)	R^2	LOD	LOQ	m ($\times 10^3$)	R^2	LOD	LOQ
			$\mu g\,g^{-1}$				$\mu g\,g^{-1}$				$\mu g\,g^{-1}$	
γ-Tocopherol	16.5	1.0000	0.018	0.054	14.6	0.9991	0.020	0.060	16.5	0.9997	0.021	0.063
α-Tocopherol	15.6	1.0000	0.070	0.210	16.0	0.9995	0.180	0.540	15.3	0.9964	0.150	0.450
Lutein	0.20	0.9983	0.075	0.226	0.20	0.9978	0.221	0.663	0.20	0.9993	0.162	0.486
Menaquinone-4	38.6	0.9998	0.005	0.015	41.0	0.9995	0.001	0.003	46.3	0.9983	0.003	0.009
Zeaxanthin	9.60	0.9996	0.049	0.147	9.10	0.9996	0.083	0.249	9.70	0.9988	0.076	0.228
Phylloquinone	37.6	0.9995	0.0004	0.001	36.7	0.9992	0.018	0.054	44.1	0.9971	0.013	0.039
β-Cryptoxanthin	9.70	0.9989	0.036	0.108	–	–	–	–	–	–	–	–
β-Carotene	10.7	0.9960	0.001	0.003	11.6	0.9994	0.030	0.090	13.8	0.9992	0.023	0.069

rapid definition of the fat-soluble vitamin and carotenoid profile in the selected foods. The results concerning the quantitative determination of the target analytes are summarized in Table 2, while those related to the screening of pigments (xanthophylls, carotenes, chlorophylls and their derivates) are schemed in Table 4.

Among the target analytes, ergocalciferol and δ-tocopherol were never detected.

Alfa- and γ-tocopherol were found in all analyzed matrices. The major homologue always resulted the α-form, determined in golden kiwi at a level three times higher than in the green one. Extracting the ion currents of γ-tocopherol from the LC–MS chromatograms, obtained by the analysis of foods under study, its chromatographic peak (t_r = 8.09 min) showed a shoulder which might to be attributed to β-tocopherol (5,8-dimethyltocol), isomer of the γ-form (7,8-dimethyltocol). Their coelution was only observed for kiwifruits, so the estimated amount, reported in Table 2, should be ascribed to the sum of the two tocopherols.

Most food composition tables do not include values for vitamers K and information is still very limited for menaquinones particularly. The literature describes vitamers K2 as microbial origin compounds which occur in animal food products and in the intestine [1]. Their contribution to vitamin K status is considered to be minor, but MK-4 may constitute an exception since it seems to be the most widespread among menaquinones. Nevertheless, recent reports [28] suggest that phylloquinone can be converted in animal tissues to menaquinone-4, indicating that the bacterial action is not necessary for its synthesis. Unexpectedly, besides phylloquinone, we found MK-4 in the analyzed samples of maize flour and kiwifruits. Explaining its occurrence in plant origin foods is beyond the scope of this work, but this finding deserves to be deepened and understood by means of a specific study.

Diode array detector is for its own account a very effectual technique in identifying carotenoids and other pigments. The UV–vis spectra of most carotenoids [14,29] show a three-peak spectrum; the chromophore identification is based both on the position of absorption maxima and on the shape (fine structure) of the spectrum. The *Fieser–Kuhn* rules [30] (valid for conjugated polyenes containing more than 4 double bonds) were applied to estimate wavelength of the central peak (λ_{II} or λ_{max}) of carotenoids selected and found during this study (see Table 4). The λ_{max} shifts to longer wavelengths, as the number of conjugated double bonds increases, while the introduction of hydroxy substituents into the carotenoid molecule does not affect the chromophore absorption. The fine structure of the spectrum was numerically expressed as the percentage ratio between peak heights, measured from minimum between the peaks II and III, i.e. % (III/II).

It is known that solvent [14,29] influences both λ_{max} (a bathochromic shift is observed, as the solvent refractive index increases) and spectral fine structure, which is greater in solvent with low polarity such as hexane. For practical purposes, the λ_{max} values for any carotenoid are similar in petroleum ether, hexane, diethyl ether, methanol, ethanol, and acetonitrile. In our study, the carotenoids UV–vis spectra were extracted from a HPLC–DAD chromatogram, achieved working in gradient elution; since the used solvents were methanol, isopropanol, and hexane, the observed λ_{max} values resulted very close to those calculated by the Fieser–Kuhn rules (Table 4). The differences with the values reported in the literature for the same carotenoid can be due both to the type of utilized solvent and to a reading error (±1–2 nm).

The provisional identification of *cis* isomers [14,29] was accomplished comparing the fine structure of their spectra with the all-*trans* isomer one, evaluating the following differences: (i) a small hypsochromic shift in λ_{max} (usually 2–6 nm for mono-*cis*, 10 nm for di-*cis* and 50 nm for poli-*cis* isomers); (ii) an ipochromic effect; (iii) a reduction in fine structure; (iv) the appearance of a *cis*-peak (λ_{cis} in the near-UV between 330 and 350 nm), at a characteristic position under 142 nm below λ_{III} in the spectrum of the all-*trans* compound. Intensity of the *cis*-peak is greater as the *cis* double bond is nearer to the centre of the chromophore; in this case, a further indicator of the spectrum fine structure is the percentage ratio between the heights (measured from the baseline of spectrum) of II and B peaks, i.e. % (II/B) which is known as *Q*-ratio [31] (some other authors prefer to calculate *Q*-ratio as % (B/II) [14]).

3.4.1. Maize

It's well known that maize is the richest natural source of zeaxanthin with 5.28 μg g^{-1} [32]. The samples from Abruzzo Region, analyzed in our laboratories, contained analogous amounts of this pigment, while lutein resulted to be the second carotenoid in term of abundance (see Table 2). Their significant occurrence makes maize a food with important nutraceutical implications; in fact, these two isomeric xanthophylls are not metabolized to vitamin A, and they accumulate in the macular region of the human retina, where they prevent damage by absorbing high-energy blue light through their antioxidant properties [32]. With one unsubstituted β-ring and polyene chain, the main source of vitamin A in maize is resulted to be β-cryptoxanthin (Table 2), followed by α-cryptoxanthin (whose occurrence was supposed on the basis of the experimental data reported in Table 4), and β-carotene.

Presence of derivates and geometric isomers of all these carotenoids was hypothesized combining information from DAD–MS detection (Table 4). Chromatographic peak 1 was attributed to 13- or 13′-*cis* isomer of 5,6-epoxy-lutein. In the literature [31,33] is reported that the all-*trans* form of this monoepoxide (known as taraxanthin or isolutein) has lower λ_{max} of 6 nm in comparison to the lutein, while a further hypsochromic shift is ascribable to the *cis* isomery. The probable identification as 13-*cis* or 13′-*cis* (contrary to β-carotene, lutein is not centrosymmetric) is justified by the *Q*-ratio value. Peaks 4, 5, and 6 were observed both on LC–DAD–MS and on LC-SRM chromatogram in correspondence of the extracted currents of lutein and zeaxanthin; their presumed identification as lutein and zeaxanthin *cis*-isomers was based on the λ_{max} and *Q*-ratio values. The same considerations were made for assigning peaks 10, 11 and 12 to *cis*-isomers of α- and β-cryptoxanthin. Other carotenes, deduced only on the basis of their characteristic UV–vis spectra, were 15-*cis*-phytofluene, all-*trans*-phytofluene, β-zeacarotene, phytoene and ζ-carotene. The UV–vis spectra extracted from peaks 9 and 17 resulted similar; nevertheless, the tentative identification as β-zeacarotene was assigned to peak 17 according to the expected retention time.

3.4.2. Kiwifruits

Within the genus *Actinidia*, fruits show a range of colours, including green, red, purple, yellow, and orange [34]. Nevertheless, almost all kiwifruits in commerce belong to one single species, *A. deliciosa* cultivar Hayward, whose colour is green. In 2000, a yellow-fleshed kiwifruit of the species *Actinidia chinensis* cultivar Hort16A, was launched on the worldwide market under the trade name Zespri Gold [34].

Kiwifruit of *A. deliciosa*, analyzed in our laboratories, showed a content of lutein slightly higher than the golden kiwi one which, nevertheless, resulted richest of α-tocopherol and zeaxanthin, while their β-carotene amounts were comparable (Table 2). The green colour of *A. deliciosa* fruit is due to the presence of chlorophyll *a*, chlorophyll *b*, pheophytin *a* and pheophytin *b*, which are retained during ripening, masking the colour of the occurring carotenoids [34]. Kiwifruit of *A. chinensis* contains pheophytin-*a* only, which is not able to cover the yellow colour of its flesh [34]. According to the literature, our LC–DAD analyses screened all these pigments and allowed us to attribute the colour of golden kiwi to α-tocopherol, lutein, zeaxanthin, and β-carotene, mainly.

Table 4
LC–UV–vis–MS data for the tentative identification of carotenoids in the selected foods.

Peak no.	Retention time[a] (min)	Compound	SRM transitions (*m*/*z*)	Calculated λ_{max}[b] (nm)	Observed λ (nm)	Reported λ (nm)	Found		Reported	
							%(III/II)	*Q*-ratio	%(III/II)	*Q*-ratio
Maize flour										
1	8.31	13 or 13′-*cis*-5,6-Epoxy-lutein		436	336, 411, 432, 466	334, 412, 436, 466 [31] 328, 410, 432, 460 [33]	7	3.1	–	2.5 [31] 2.2[c] [33]
2	8.58	All-*trans*-lutein	551/175 551/459 569/135	448	426, 448, 475	426, 448, 472 [29]	60	–	60 [29]	–
3	8.99	All-*trans*-zeaxanthin		453	425, 454, 480	428, 450, 478 [29]	25	–	25 [29]	–
4	9.43	13-*cis*-Lutein		442	334, 420, 443, 471	334, 419, 442, 466 [31]	31	3.7	22 [36]	3.0 [31]
5	9.78	9-*cis*-Lutein		446	330, 421, 444, 471	334, 419, 442, 466 [31]	31	3.7	–	5.2 [31]
6	10.02	13-*cis*-Zeaxanthin		447	340, 427, 449, 476	340, 419, 448, 472 [31]	20	3.1	–	2.5 [31]
7	11.09	All-*trans*-α-cryptoxanthin	553/135 553/119	448	427, 449, 477	423, 446, 473 [29]	60	–	60 [29]	–
8	11.65	All-*trans*-β-cryptoxanthin		453	425, 453, 478	425, 454, 478 [31]	23	–	25 [29]	–
9	11.99	Unknown 1		–	411, 431, 457	–	67	–	–	–
10	12.23	13-*cis*-β-Cryptoxanthin	553/135 553/119	447	333, 428, 451, 474	334, 419, 448, 472 [31]	12	2.4	–	4.3 [31]
11	12.99	9-*cis*-α-Cryptoxanthin		446	331, 427, 448, 472	–	55	4.5	–	–
12	13.29	9-*cis*-β-Cryptoxanthin		451	330, 431, 452, 479	–	18	3.0	–	–
13	13.55	Phytoene		–[d]	280, 290, 303	277, 287, 298 [37]	–	–	10 [37]	–
14	13.77	15-*cis*-Phytofluene		–[d]	336, 353, 371	331, 347, 367 [29]	50	–	–	–
15	14.21	All-*trans*-phytofluene		337	336, 353, 371	331, 347, 367 [29]	78	–	89 [37]	–
16	15.54	All-*trans*-β-carotene	537/137 537/177	453	425, 454, 480	450, 478 [29]	25	–	25 [29]	–
17	15.73	β-Zeacarotene		438	408, 430, 455	402, 428, 453 [29]	65	–	62 [38]	–
18	15.89	ζ-Carotene		397	383, 405, 430	378, 400, 425 [29]	100	–	96 [29]	–
19	15.97	*cis*-ζ-Carotene		–	383, 405, 430	–	100	–	–	–
20	16.63	Unknown 2		–	357, 376, 397	–	65	–	–	–
Green kiwi										
1	6.48	*cis*-Neochrome		–	401, 425, 452	400, 422, 450 [39]	80	–	93 [39]	–
2	6.85	All-*trans*-neochrome		423[e]	403, 426, 452	400, 422, 450 [36,39]	80	–	92 [39]	
3	7.07	Violaxanthin		442[e]	416, 439, 468	419, 440, 470 [29]	75	–	95 [29]	–
4	7.76	All-*trans*-auroxanthin		401[e]	385, 405, 428	382, 405, 428 [35]	91	–	95 [29]	
5	8.07	Flavoxanthin		423[e]	402, 424, 451	400, 423, 448 [35,36]	81	–	85 [29]	–
6	8.26	Cryptochrome		401[e]	385, 405, 428	380, 402, 428 [29]	89	–	–	–
7	8.58	All-*trans*-lutein	551/175 551/459	448	426, 448, 475	426, 448, 472 [29]	57	–	60 [29]	–
8	8.67	Chlorophyll *b*			474, 607, 654	463, 600, 647 [40]				
9	9.30	13-*cis*-Lutein	551/175 551/459	442	334, 420, 443, 471	333, 420, 442, 468 [39]	31	3.5	22 [39]	3.0 [31]
10	10.06	Chlorophyll *a*			436, 623, 670	431, 616, 663 [40]				
11	12.83	Pheophytin *b*			441, 532, 604, 662	436, 528, 600, 654 [39]				
12	11.12	All-*trans*-α-cryptoxanthin	553/135 553/119	448	427, 449, 477	423, 446, 473 [29]	58	–	60 [29]	–
13	13.75	Pheophytin *a*			412, 511, 542, 613, 670	410, 506, 536, 666 [39]				
14	15.55	All-*trans*-β-carotene	537/137	453	425, 454, 480	450, 478 [29]	23	–	25 [29]	–
15	17.36	γ-Carotene	537/177	465	Not detected by DAD	437, 462, 494 [29]	–	–	40 [28]	–
Golden kiwi										
1	6.48	*cis*-Neochrome		–	402, 426, 452	400, 422, 450 [39]	100	–	93 [39]	–
2	6.85	All-*trans*-neochrome		423[e]	402, 426, 452	400, 422, 450 [36,39]	92	–	92 [39]	
3	7.33	Antheraxanthin		445[e]	423, 449, 477	421, 444, 474 [40]	40	–	52 [40]	–
4	8.07	Flavoxanthin		423[e]	402, 425, 452	400, 423, 448 [35]	81	–	85 [29]	–
5	8.57	All-*trans*-lutein	551/175 551/459 569/135	448	425, 449, 477	426, 448, 472 [29]	57	–	60 [29]	–

Table 4 (*Continued*)

Peak no.	Retention time[a] (min)	Compound	SRM transitions (*m*/*z*)	Calculated λ_{max}[b] (nm)	Observed λ (nm)	Reported λ (nm)	Found		Reported	
							%(III/II)	Q-ratio	%(III/II)	Q-ratio
6	8.99	All-*trans*-zeaxanthin		453	431, 453, 480	428, 450, 478 [29]	24	–	25 [29]	–
7	11.02	All-*trans*-α-cryptoxanthin	553/135 553/119	448	427, 449, 477	423, 446, 473 [29]	59	–	60 [29]	–
8	12.58	Unknown 1			426, 449, 478		57	–	–	–
9	12.85	Unknown 2			427, 448, 473		20	–	–	–
10	13.25	Unknown 3			426, 450, 472		–	–	–	–
11	13.68	Unknown 4			408, 427, 451		90	–	–	–
12	13.73	Pheophytin *a*			414, 511, 542, 613, 671	410, 506, 536, 666 [39]				
13	14.95	13-*cis*-β-Carotene	537/137 537/177	447	345, 428, 448, 472	338, 449, 478 [36]	5	3.0	7 [36]	2.3[c] [19]
14	15.05	9-*cis*-β-Carotene		451–447	347, 428, 449, 478	352, 448, 472 [31]	23	5.7	20 [38]	6.5 [31]
15	15.55	All-*trans*-β-carotene		453	431, 455, 482	450, 478 [29]	26	–	25 [29]	–
16	16.05	*cis*-β-Carotene		451–447	347, 426, 448, 477		–	7.0	–	–
17	17.36	γ-Carotene		465	Not detected by DAD	437, 462, 494 [29]	–	–	40 [29]	–

[a] Analyte retention times, recorded on the LC–DAD chromatogram, were early of about 0.25 s compared to the corresponding ones, observed on the LC–MS chromatogram and listed in Table 2 for the target analytes.

[b] The Fieser–Kuhn rule [23] was applied for calculating λ_{max} (λ_{II}) of all-*trans* forms of carotenoids found in the several food matrices; $\lambda_{max} = 114 + 5M + n(48 - 1.7n) - 16.5R_{endo} - 10R_{exo}$, where n = number of double conjugated bonds, M = number of alkyl or alkyl-like substituents on the conjugated system, R_{endo} = number of rings with endocyclic double bonds in the conjugated system, R_{exo} = number of rings with exocyclic bonds. The presence of hydroxy groups on the molecule does not influence the λ_{max} value which is instead lowered by an epoxide substituent of about 6 nm. For *cis*-isomers, their λ_{max} value was estimated subtracting to the theoretical one, calculated as explained for the all-*trans* form, 2–6 nm for a mono-*cis* isomer (major decrement when *cis*-double-bond becoming more-central), 10 nm for di-*cis* isomer and 50 nm for poli-*cis* isomer.

[c] The $1/Q_{ratio}$ value was reported, since the authors calculated Q_{ratio} as (B/II)%.

[d] The Fieser–Kuhn rule was not applied since phytoene has three double bonds only.

[e] For carotenoid epoxides [23], the Fieser–Kuhn rule that has to be applied is as follows: $\lambda_{max} = 118 + 5M + n(48 - 1.7n) - 23R_j$, where R_j = number of rings with an internal double bond.

Other carotenoids common to the two species were supposed to be α-cryptoxanthin, neochrome, violaxanthin, flavoxanthin, and γ-carotene, while β-cryptoxanthin was not detected. Moreover, auroxanthin, cryptochrome, and 13-*cis*-lutein were provisionally identified in the sample of *A. deliciosa*, while antheraxanthin, and a series of geometrical isomers of β-carotene in that of *A. chinensis*. Always in golden kiwi, a series of poor intensity chromatographic peaks, indicated as unknowns 1–4 in Table 4, was attributed to xanthophylls esterified with fatty acids; the disappearance of these peaks was noticed after having applied overnight cold saponification, chosen instead of hot saponification for avoiding degradation of free xanthophylls. Some of these results agree with those of other works [34,35] addressed to the definition of pigment compositions for both species of kiwifruits.

4. Conclusions

Chemical heterogeneity, linkage with the matrix macromolecular components and the lack of standards are the main difficulties in developing a method for the simultaneous analysis of fat-soluble vitamins and carotenoids in foods. For these reasons, the number of publications in this research area is still limited; moreover, the two classes of micronutrients are treated separately, even if it is possible to find methods which comprise analysis of β-carotene and fat-soluble vitamins.

To the best of our knowledge, this is the first work addressed to the extensive characterization both the fat-soluble vitamin and carotenoid fraction of a food, performing in a single chromatographic run the quantitative analysis of several target analytes and the screening of many pigments. Matrix solid phase dispersion proved to be a technique suitable for a concurrent extraction of these micronutrients with good recovery values, even if not quantitative from kiwifruits. The mechanic disruption of the food matrix and the dispersion of its components permitted to the extractant of recovering free and esterified forms, avoiding their degradation or hydrolysis due to the mild applied conditions. Surely, the proposed procedure was simple, fast, and it avoided artefacts such as carotenoid isomerization; in this way, it was possible to deduce the presence of several β-carotene *cis*-isomers as endogenous compounds of golden kiwi.

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