JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

pubs.acs.org/JAFC

Comprehensive Profiling of Carotenoids and Fat-Soluble Vitamins in Milk from Different Animal Species by LC-DAD-MS/MS Hyphenation

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Supporting Information

ABSTRACT: This paper describes a novel and efficient analytical method to define the profile of fat-soluble micronutrients in milk from different animal species. Overnight cold saponification was optimized as a simultaneous extraction procedure. Analytes were separated by nonaqueous reversed-phase (NARP) chromatography: carotenoids on a C_{30} column and fat-soluble vitamins on a tandem C_{18} column system. Besides 12 target analytes for which standards are available (*all-trans*-lutein, *all-trans*-zeaxanthin, *all-trans*-β-cryptoxanthin, *all-trans*-β-carotene, *all-trans*-retinol, α-tocopherol, γ-tocopherol, δ-tocopherol, ergocalciferol, cholecalciferol, phylloquinone, and menaquinone-4), the DAD-MS combined detection allowed the provisional identification of other carotenoids on the basis of the expected retention times, the absorbance spectra, and the mass spectrometric data. Retinol and α-tocopherol were the most abundant fat-soluble micronutrients and the only ones found in donkey's milk along with γ-tocopherol. Ewe's milk also proved to be a good source of vitamin K vitamers. Bovine milk showed a large variety of carotenoids that were absent in milk samples from other species with the only exception of *all-trans*-lutein and *all-trans*-zeaxanthin.

KEYWORDS: *fat-soluble vitamins, carotenoids, HPLC-APCI-DAD-MS/MS, milk*

INTRODUCTION

Vitamin and carotenoid determination is a complex and challenging task, especially when addressed to the analysis of food and biological samples.[1] Milk is a unique matrix because it is both food and biological fluid with the characteristics of three chemical phases: emulsion, colloidal suspension, and solution.[2] Moreover, it is an almost complete single food containing significant amounts of essential nutrients more than any other; its whey is a good source of water-soluble vitamins, whereas its lipid fraction is an important delivery medium of fat-soluble vitamins.[3]

In general, numerous studies have focused on bovine milk, although milk from other animal species is essential for the human diet in various parts of the world. For example, India and Pakistan are the largest producers of buffalo's milk (the second most produced in the world), whereas the dairy sheep industry is concentrated in Europe and the countries of the Mediterranean area. On the other hand, donkey's and goat's milks are viable alternatives for children who suffer from allergies to cow's milk. It has not been possible yet to perform a comparative valuation on the nutritional properties of these types of milk because there is no detailed information about the composition of their fat-soluble micronutrients in the scientific literature.

Vitamin A-active compounds occur in human and animal milks mainly as retinoids[4,5] and, to a lesser degree, as carotenoids, which, present in all green tissues of plants in association with chlorophyll, are the only natural source of vitamin A for herbivores.[6]

Vitamin D is the name given to a series of compounds with antirachitic activity, produced by solar irradiation in plants, fungi, and yeasts (ergocalciferol or vitamin D_2) or in human and animal skin (cholecalciferol or vitamin D_3).[4] Up to now, traces of D vitamers were found in bovine,[7,8] caprine,[9] and human milk.[10]

Vitamin E counts eight biologically active forms, represented by four tocopherols (saturated isoprenoid side chain) and four tocotrienols (unsaturated isoprenoid side chain), designated α-, β-, γ-, and δ- according to the number and position of methyl groups on the chromanol ring.[4] α-Tocopherol is the main source of vitamin E in bovine milk, whereas a smaller contribution derives from γ-tocopherol.[11,12]

Vitamin K vitamers are a family of 2-methyl-1,4-naphthoquinones differing for the side chain attached at C_3.[4] Phylloquinone (vitamin K_1) has a phytyl side chain and is found in green plants. Vitamin K_2 includes a group of compounds synthesized by bacteria and characterized by a polyisoprene side chain; they are designated menaquinones-*n* (MK-*n*, with *n* from 4 to 13) according to their number of isoprenyl units. Besides phylloquinone, the other K homologues found in bovine milk were menaquinones from MK-4 to MK-9.[13,14]

At the present time, the conventional methods for analysis of fat-soluble vitamins in food products determine one or only a few forms of each vitamin by HPLC with UV or fluorescence

Special Issue: IX Italian Congress of Food Chemistry

Received: June 29, 2012
Revised: September 13, 2012
Accepted: September 14, 2012
Published: September 27, 2012

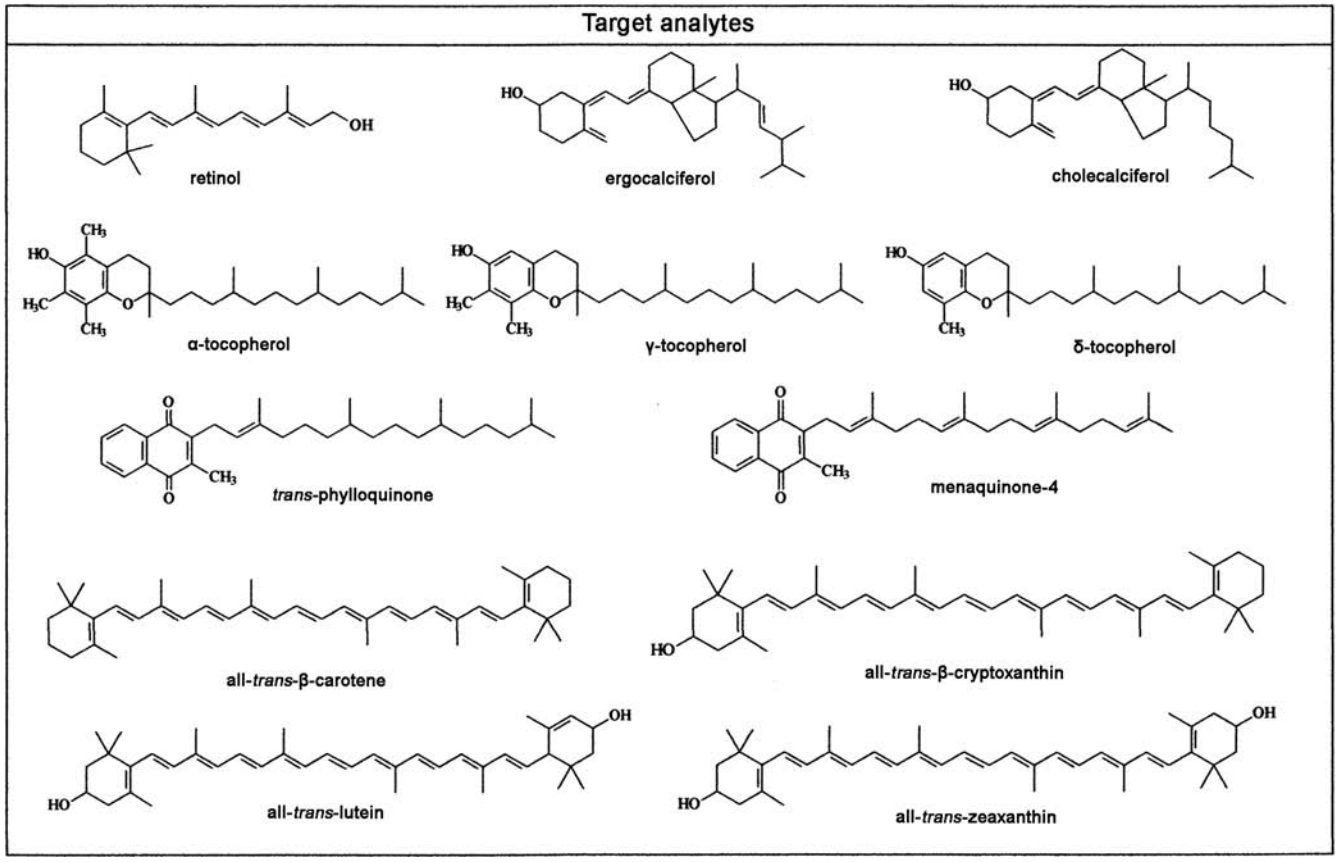

Figure 1. Names and structures of the 12 target analytes.

detection.[15−20] On the other hand, the literature has described several analytical methods for the simultaneous determination of fat-soluble vitamins in real matrices by chromatographic techniques,[10,11,21−29] some of which are based on liquid chromatography−tandem mass spectrometry (LC-MS/MS).[10,25,27−29] Nevertheless, very few methods have dealt with the determination of a considerable number of endogenous forms,[10,28,29] which can be ascribed to a combination of different analytical difficulties. A first problem concerns the commercial unavailability of authentic standards of some vitamin forms, carotenoids and their geometrical isomers. The subtle structural difference between homologues belonging to the same group hampers their chromatographic separation, whereas the chemical heterogeneity among vitamin groups makes it difficult to find common conditions of extraction and detection. The food matrix complexity, the dissimilar stabilities, and the different endogenous levels of analytes contribute to make the extraction procedure a critical step. For animal food products, another problem is due to the occurrence of fat-soluble vitamins and carotenoids within the lipid fraction, both as free forms and as esterified with fatty acids. Glycerides, sterols, and phospholipids,[2,3] exhibiting an analogous solubility, constitute a potential source of interference;[4] moreover, the high fat content could affect chromatographic efficiency, shorten column life, and decrease the analyte recovery if the applied extraction protocol cannot remove it efficiently.

Hot saponification is the most common procedure to remove neutral lipids, hydrolyzing their ester bonds, and to free endogenous vitamins and xanthophylls from any combined form.[1,4,15] It is considered to be essential for a quantitative extraction from fatty foods, and it is used for the individual and simultaneous determination of vitamins A, E, and D.[10,16−19,21,25,27] The reaction conditions are drastic and can cause thermal isomerization of vitamin D to provitamin D,[4,30] isomerization of carotenoids,[4,31] and degradation of vitamin K[4] and xanthophylls.[4,31] Moreover, saponification simplifies the analysis of vitamin A and xanthophylls because the chromatographic separation, identification, and quantification are performed on the free forms, for example, retinol instead of retinyl esters; nevertheless, due to the more pronounced sensitivity to light and oxygen of free forms, it is essential to use an adequate antioxidant[1,4,11]

Hence, the purposes of this work were (1) to overcome the described problems to develop a cost/time-effective approach for the characterization of the fat-soluble micronutrients in foods of animal origin (milk in the specific case) and (2) to obtain detailed information on the vitamin and carotenoid composition of milk from different animal species lacking in the literature. To this end, the milk samples were saponified under mild temperature conditions and then analyzed to quantify 12 target analytes (names and structures are shown in Figure 1) and to screen other pigments on the basis of the data collected by the HPLC-DAD-MS/MS hyphenation. The overall analytical strategy was applied to the analysis of cow's, buffalo's, goat's, ewe's and donkey's milk samples to define a comprehensive profile of the occurring vitamins and carotenoids.

All data on the composition of the different kinds of milk will be presented and discussed along with the results related to the development of the whole approach and validation of the quantitative method. This study has not examined the effect of

stage of lactation, season of year, and other variability parameters on the concentrations of the micronutrients.

MATERIALS AND METHODS

Chemicals and Materials. The following standards of carotenoids and fat-soluble vitamins were purchased from Aldrich-Fluka-Sigma Chemical (Milan, Italy): *all-trans*-lutein, *all-trans*-zeaxanthin, *all-trans*-β-cryptoxanthin, *all-trans*-β-carotene, *all-trans*-retinol, α-tocopherol, γ-tocopherol, δ-tocopherol, ergocalciferol, cholecalciferol, phylloquinone, and menaquinone-4. α-Tocopherol-d_6 [α-tocopherol-(*ring*-5,7-dimethyl-d_6)], cholecalciferol-d_3 [cholecalciferol (6,19,19-d_3)], phylloquinone-d_7 (5,6,7,8-d_4, 2-methyl-d_3), and *trans*-β-apo-8′-carotenal, bought from Aldrich-Fluka-Sigma Chemica, were chosen as internal standards (ISs) for vitamin E vitamers, vitamin D vitamers, vitamin K vitamers, and carotenoids, respectively. Retinol-d_5 acetate [retinol-(*ring*-3,3-d_2, 2-methyl-d_3) acetate], obtained from Chemical Research 2000 (Rome, Italy), was hydrolyzed to retinol-d_5 and used as internal standard for retinol. All chemicals had a purity grade of >90%.

Butylated hydroxytoluene (BHT), provided by Aldrich-Fluka-Sigma Chemical, was used as antioxidant both in standard solutions and during the several steps of the extraction procedure.

Acetonitrile and methanol were of RS-Plus grade (special grade reagents); isopropyl alcohol, hexane, and chloroform were of RS grade (elevated purity grade); absolute ethanol was of RPE grade (analytical grade). All of these solvents and potassium hydroxide (KOH) were purchased from Carlo Erba (Milan, Italy). Distilled water, used in the extraction procedure based on the cold saponification, was further purified by passing it through a Milli-Q Plus apparatus (Millipore, Bedford, MA USA).

Standard Solutions. The individual stock solutions of analytes and internal standards were prepared with a regularity established on the basis of results achieved by a stability study, carried out over a period of 40 days (Table S1 in the Supporting Information summarizes solvents, concentrations, and preparation frequency of the individual stock standard solutions).

A working multistandard solution as well as the composite solution of the internal standards was prepared every two weeks from the individual solutions by dilution in methanol with 0.1% (w/v) BHT. In all cases, different final concentrations were reached depending on the purpose.

The precise concentration of every standard was periodically checked by UV−vis spectrophotometry using the corresponding absorption coefficient.

All solutions were degassed with nitrogen and stored in dark glass flasks at −18 °C.

Milk Samples. Raw milk samples from different animal species were obtained from direct producers of central Italy (Latium and Campania) using pasture-based feeding systems. Cow's, buffalo's, and donkey's milk samples were sourced from an incoming cooled reception silo, whereas goat's and sheep's milks were taken from a single donor. Milk to develop the method was sampled on September 2011, whereas milks were submitted to the final characterization in April 2012. All samples were stored at 4 °C for 1 day and subsampled, and the subsamples were stored at −18 °C prior to analysis. The thawed milk samples were stirred for 15 min before assaying.

The fat amounts of the samples were declared by the producers: about 1.0% (w/v) for donkey's milk, 3.5% (w/v) for cow's milk, 5.0% (w/v) for buffalo's and goat's milk, and 5.5% (w/v) for ewe's milk.

Sample Treatment. A procedure based on overnight cold saponification was developed for the simultaneous extraction of fat-soluble vitamins and carotenoids. All operations were performed in subdued light, using BHT as antioxidant.

Into a 50 mL polypropylene centrifuge tube, equipped with a screw cap, was poured a 6 mL aliquot of milk, which was then spiked with known amounts of the internal standards; a 30 min period was allowed for equilibration at room temperature. Thereafter, 18 mL of absolute ethanol containing 0.1% (w/v) BHT, and x mL of 50% (w/v) aqueous KOH were also added (x = 1 mL for donkey's and cow's milk; x = 3 mL for buffalo's, goat's, and ewe's milk). The saponification tube was flushed with oxygen-free nitrogen, promptly closed, and thermostated at 25 °C overnight (approximately 15 h in the dark) in a water bath under continuous magnetic stirring to ensure an efficient lipid saponification.

At the end of incubation, the digest was diluted with 8.5 mL of Milli-Q water and the analytes were extracted with 12 mL of hexane with 0.1% (w/v) BHT. The extraction was repeated twice for cow's and donkey's milk, three times for buffalo's milk, and four times for ewe's and goat's milk, depending on different endogenous amounts of the analytes. Following the addition of each hexane aliquot, the tube was capped and the mixture was stirred for 5 min and then vortex-mixed for a further 5 min; finally, after centrifugation at 0 °C for 10 min at 6000 rpm, a definite separation between protein precipitate, aqueous phase, and organic phase was obtained. The combined hexane layers were then washed with 12 mL portions of Milli-Q water; two washings were necessary to remove alkalis completely (no color was observed on phenolphthalein addition).

The whole extract was collected into a glass tube with a conical bottom (i.d. = 2 cm), evaporated to 100 μL in a thermostated bath at 30 °C, under a slow nitrogen flow, and diluted to a final volume of 200 μL with an isopropanol/hexane (75:25, v/v) solution containing 0.1% (w/v) BHT. Eventually, 20 μL was injected into the HPLC-DAD-MS/MS system; owing to their high concentrations in the extracts, retinol and α-tocopherol were quantified by injecting 5 μL.

Instrumentation. *Liquid Chromatography.* Liquid chromatography was performed by means of a micro HPLC/autosampler/vacuum degasser system PE series 200 (Perkin-Elmer, Norwalk, CT, USA); solvent B of mobile phase was used as washing solution of the autosampler injection system.

Two C18 columns, a Supelcosil C_{18} (4.6 mm × 50 mm, 5 μm particle size) from Supelco-Sigma-Aldrich (Bellefonte, PA, USA) and an Alltima C_{18} (4.6 mm × 250 mm, 5 μm particle size) from Grace-Alltech (Deerfield, IL, USA), were connected in series to increase efficiency in separating fat-soluble vitamins from interfering compounds. Simultaneous separation of carotenoids was carried out on a ProntoSIL C_{30} column (4.6 × 250 mm, 3 μm; Bischoff Chromatography, Leonberg, Germany) thermostated at 19 °C and equipped with a guard column of the same type (4.0 mm × 10 mm, 5 μm particle size). Nonaqueous mobile phase, consisting of methanol (phase A) and an isopropanol/hexane (50:50, v/v) solution (phase B), was the same for both chromatographic separations and was entirely introduced into the DAD-MS/MS detection system at a flow rate of 1 mL min^{-1}. The linear gradient profiles were as follows (t in min): t_0, B = 0%; t_1, B = 0%; t_{15}, B = 75%; $t_{15.1}$, B = 99.5%; t_{22}, A = 99.5% to separate fat-soluble vitamins on the tandem C_{18} column system; t_0, B = 0%; t_1, B = 0%; t_{15}, B = 75%; $t_{15.1}$, B = 99.5%; t_{30}, A = 99.5% to separate carotenoids on the C_{30} column.

The 0.5% percentage of methanol was maintained in the final part of the chromatographic runs to support the atmospheric pressure chemical ionization of analytes with higher capacity factors.

Diode Array Detection. Carotenoids were identified by a series 200 model (Perkin-Elmer) diode array detector, equipped with a flow Z cell (12 μL volume; optical path of 10 mm). It was coupled online between the chromatographic column and the mass spectrometer.

The LC-DAD chromatograms were acquired by selecting the 450 nm wavelength (*all-trans* form λ_{max} of β-carotene, zeaxanthin, and β-cryptoxanthin) and a bandwidth of 10 nm; the UV−vis spectra were recorded in the range of 200−700 nm.

The softwares used for running the instrument and acquiring spectral data were Totalchrom Navigator 6.3.1 and Iris (Perkin-Elmer).

Mass Spectrometry. Analytes were identified and quantified by a 4000 Qtrap (AB Sciex, Foster City, CA, USA) mass spectrometer. Only conventional scan modes of triple quadrupole were used for this work. Detection was performed in positive ionization, placing the APCI probe in the Turbo V source and setting a needle current (NC) of 3 μA and a probe temperature of 450 °C. High-purity nitrogen was used as a curtain gas (5 L min^{-1}) and collision gas (4 mTorr), whereas air was used as nebulizer gas (2 L min^{-1}) and makeup gas (30 psi).

Table 1. LC-SRM Parameters of the Fat-Soluble Micronutrients and Their Internal Standards, Selected in This Study

analyte	retention time[a] (min), mean ± SD	qualifier and quantifier SRM transitions (m/z)	declustering potential (V)	collision potential (V)	ion ratio,[c] mean ± RSD (%)
vitamins					
retinol	6.14 ± 0.02	269.1/107.1	40	40	33 (6)
		269.1/93.1		30	
δ-tocopherol	9.13 ± 0.02	402.4/137.0	40	40	75 (6)
		402.4/177.2		35	
ergocalciferol	9.46 ± 0.01	397.3/107.1	50	42	94 (5)
		397.3/379.3		25	
γ-tocopherol	9.62 ± 0.02	416.3/191.2	60	40	23 (5)
		416.3/151.1			
cholecalciferol	9.64 ± 0.01	385.3/259.2	30	40	69 (5)
		385.3/367.3	30	25	
α-tocopherol	10.02 ± 0.02	430.2/205.1	65	40	9 (6)
		430.2/165.1		45	
menaquinone-4	10.41 ± 0.01	445.3/81.3	80	60	41 (10)
		445.3/187.1		40	
phylloquinone	12.16 ± 0.01	451.5/199.2	65	41	16 (7)
		451.5/187.1		35	
internal standards					
retinol-d_5	6.10 ± 0.02	275.2/95.1	40	40	
cholecalciferol-d_3	9.63 ± 0.01	388.3/370.2	40	30	
α-tocopherol-d_6	9.98 ± 0.01	436.4/171.2	40	45	
phylloquinone-d_7	12.07 ± 0.01	458.4/194.1	73	40	
carotenoids					
lutein	9.27 ± 0.02	551.4/135.2	20	50	85 (9)
		551.4/175.0		40	
zeaxanthin	9.70 ± 0.02	569.4/477.2	30	23	32 (8)
		551.4/135.2	20	40	
β-cryptoxanthin	12.41 ± 0.01	553.5/135.1	60	45	54 (9)
		553.5/119.1		55	
β-carotene	16.39 ± 0.02	537.5/119.1	30	60	78 (9)
		537.5/177.2		40	
internal standard					
trans-β-apo-8′-carotenal	11.41 ± 0.02	417.3/177.1	80	20	

[a]The retention times are reported as the arithmetic average of 10 replicates plus the corresponding standard deviation (SD). They are referred to the tandem C_{18} column system for the fat-soluble vitamins and to the C_{30} column for the carotenoids. [b]The first line reports the least intense SRM transition (qualifier) and the second line the most intense one (quantifier). [c]The ion ratio (relative abundance) between the two SRM transitions is calculated as the percentage ratio of qualifier intensity/quantifier intensity; the results are reported as the arithmetic average of 10 replicates plus the corresponding relative standard deviation (RSD).

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 each mass-resolving quadrupole by maintaining a full width at half-maximum (fwhm) of approximately 0.7 ± 0.1 u.

The quantitative analysis of the 12 target analytes was carried out in selected reaction monitoring (SRM) mode, after their corresponding fragmentation spectra had been studied; the instrumental parameters of the APCI source and the analyzers were optimized for every compound by working in flow injection analysis (1−10 ng injected; 1 mL min^{-1} flow rate). Table 1 summarizes the quantitation channels chosen for the analytes and their internal standards and the parameters used for their identification in the milk samples (retention time, two SRM transitions selected for each analyte, and their ion ratio).

Representative LC-APCI(+)-SRM chromatograms of fat-soluble vitamins and carotenoids extracted by means of cold overnight saponification are shown in Figures 2 and 3, respectively.

Method Validation. The bovine milk was used for developing the analytical method, which was then optimized and validated for each type of milk. Because blank samples were not available, the LC-SRM quantitative analysis of fat-soluble vitamins and carotenoids was

dx.doi.org/10.1021/jf302811a | J. Agric. Food Chem. 2013, 61, 1628−1639

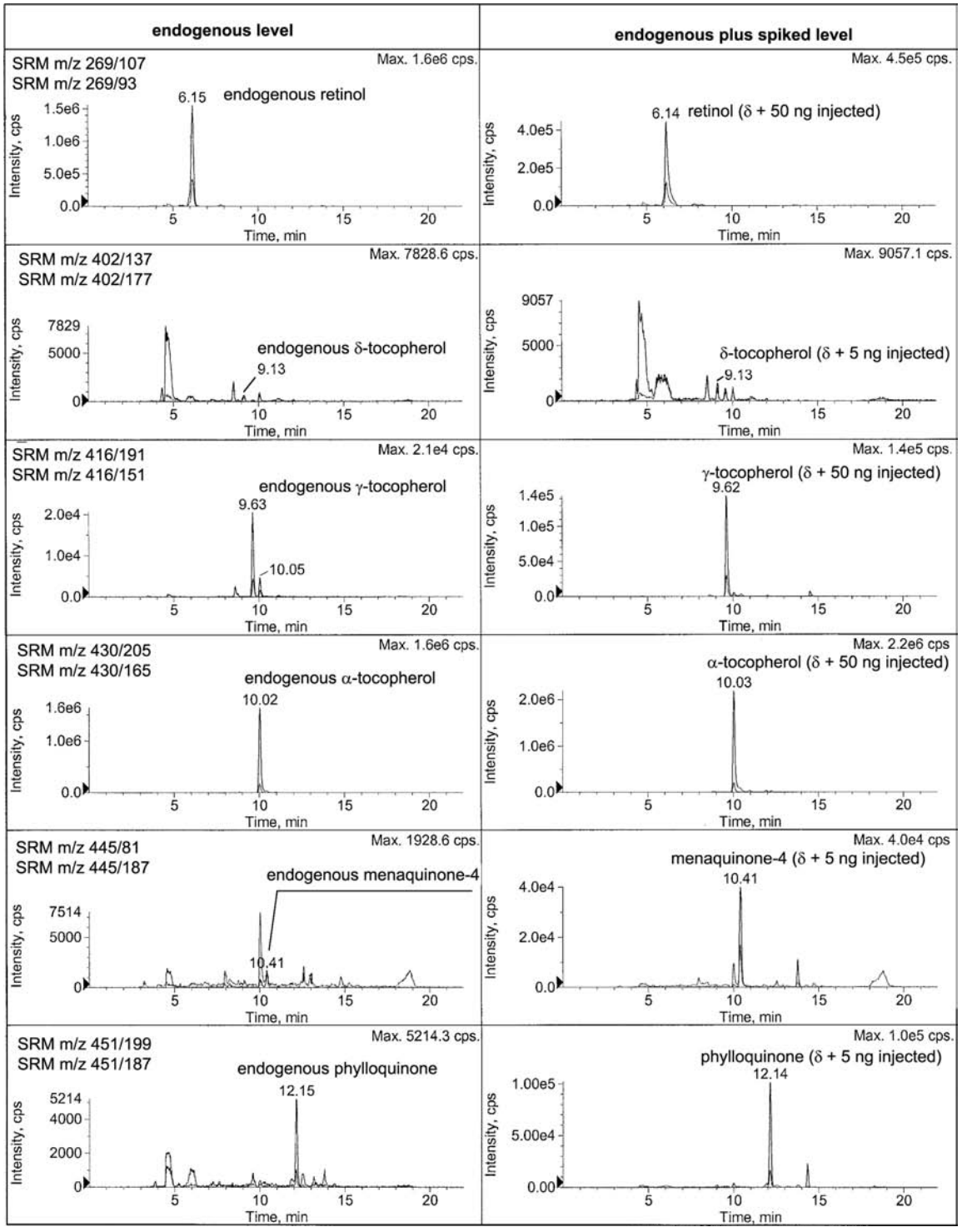

Figure 2. LC-SRM chromatogram of the fat-soluble vitamins found in a ewe's milk extract and separated on the tandem C_{18} column system. Each form has been identified unequivocally on the basis of its retention time, the two selected SRM transitions, and their relative abundance (ion ratio).

performed by means of a standard addition method; in this way, besides estimating the unknown quantity of the analytes occurring in the several samples, it was possible to evaluate sensitivity and linear dynamic range. Recoveries, precision, and method limits (LODs and LOQs) were calculated after determination of the endogenous concentrations of each compound.

The most intense SRM transition (quantifier transition) was selected for quantitation purposes and the least intense one (qualifier transition) for identification purposes.

The software used for acquiring and elaborating LC-MS/MS data was Analyst 1.5.1 (AB Sciex).

Recovery and Precision. After a preliminary determination of the natural vitamin and carotenoid contents in the selected milk samples, their recovery was assessed by spiking a 6 mL aliquot of milk with known amounts of analytes and internal standards. Spike levels were chosen to increase the original content of the vitamins and carotenoids by a factor of 2−3 (i.e., as close as possible to the endogenous value); an arbitrary level at about 2 times the LOQ was chosen for those analytes that were not detected. The samples were placed under

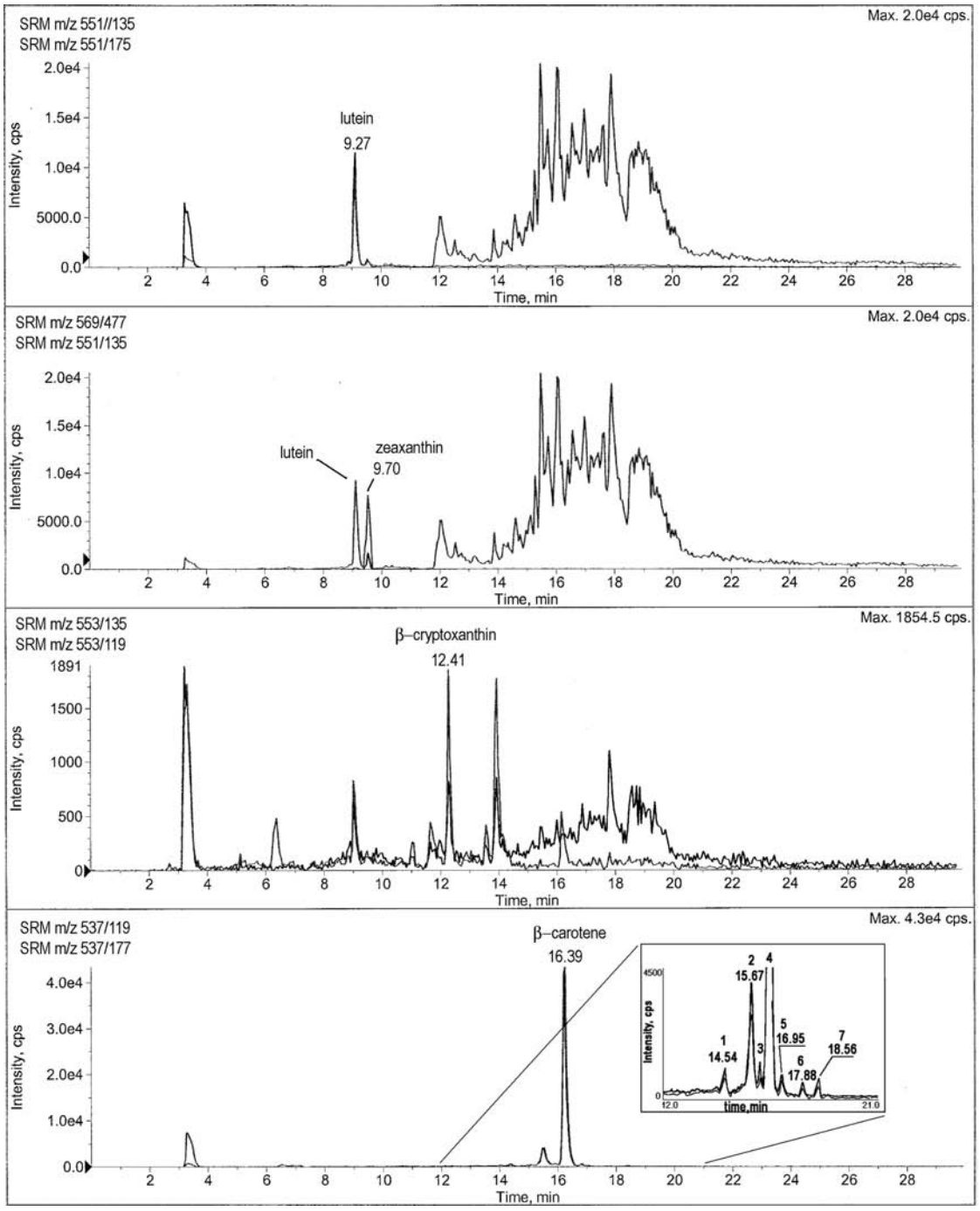

Figure 3. LC-SRM chromatogram of the carotenoids found in a bovine milk extract and separated on the C_{30} column. By zooming the ion current profiles of *β*-carotene (*m*/*z* 537/177 and 537/119 SRM transitions) and combining these MS data with those obtained by diode array detector (see Table 4), the numbered peaks in the enlargement were identified as follows: 1, *cis*-*β*-carotene; 2, 13-*cis*-*β*-carotene; 3, *all*-*trans*-*α*-carotene; 4, *all*-*trans*-*β*-carotene; 4, *β*-zeacarotene; 5, *γ*-carotene.

continuous agitation (30 min) for equilibration at room temperature and then submitted to the extraction process.

Recovery was calculated as the mean of six replicates, whereas the corresponding relative standard deviation (RSD) was representative of intraday precision. Interday precision was estimated as the RSD of 12 replicates performed within 2 weeks.

Standard Addition Method and Memory Effect. Linear dynamic range, sensitivity, and goodness of the linear model were evaluated by applying the standard addition method (six points). For this purpose, six 6 mL aliquots were spiked with 250 μL of a solution containing the internal standards at the following concentrations: 80 ng μL^{-1} retinol-d_5 and α-tocopherol-d_6; 2 ng μL^{-1} phylloquinone-d_7, cholecalciferol-d_3, and *trans*-*β*-apo-8′-carotenal. Five of these aliquots were spiked with increasing concentrations of the standards (for the applied fortification levels see Table S2 in the Supporting Information); a 30 min period was allowed for equilibration at room temperature under continuous stirring. The extraction procedure was performed according to that described under Sample Treatment. Twenty microliters was injected into the HPLC-DAD-MS/MS system, using the C_{30} column for the carotenoid analysis and the tandem C_{18} column system for the fat-soluble vitamin analysis; due to the high contents of retinol and *α*-tocopherol, they were reanalyzed by injecting 5 μL.

The washing of injection system with phase B avoided a possible memory effect due to the use of the autosampler and the high

dx.doi.org/10.1021/jf302811a | J. Agric. Food Chem. 2013, 61, 1628−1639

Table 2. Validation Parameters Related to the Confirmatory Method of the Target Analytes in Bovine Milk

		precision (RSD) (%)		method limits ($\mu g\ L^{-1}$)		linear regression parameters	
analyte	recovery (%)	intraday	interday	LOD	LOQ	slope ($\times 10^4$)	correlation coefficient
vitamins							
retinol	100	7.0	13	15.6	46.8	3.36	0.9982
α-tocopherol	100	5.0	10	3.70	11.1	1.68	0.9974
δ-tocopherol	80	10	15	3.13	9.40	0.09	0.9949
γ-tocopherol	88	5.0	12	3.24	9.72	0.66	0.9983
ergocalciferol	97	5.0	12	0.90	2.70	0.57	0.9990
cholecalciferol	99	6.0	10	1.12	3.36	1.32	0.9950
phylloquinone	67	10	18	1.70	5.10	7.51	0.9986
menaquinone-4	54	10	20	2.21	6.63	2.78	0.9989
carotenoids							
lutein	100	9.0	12	1.20	3.60	1.06	0.9983
zeaxanthin	80	9.0	15	2.32	6.96	0.34	0.9986
β-cryptoxanthin	87	8.0	15	1.85	5.55	0.93	0.9962
β-carotene	100	10	18	7.41	22.2	0.27	0.9965

sensitivity of the LC-MS instrumentation. The absence of carry-over was checked by injecting methanol (e.g., solvent instead of a blank sample which is unavailable) after the analysis of the sample spiked at the highest point of the standard addition curve.

Method Detection Limits. The least intense transition was used for the estimation of the method detection limits. In this way, both SRM transitions could be observed with the characteristic ion ratio (reported in Table 1) respecting the identification criteria of the target analytes also at limit of detection (LOD) level.

The LOD was calculated as the amount of analyte able to produce a chromatographic peak 3 times higher than the noise of the baseline in a LC-SRM-chromatogram (S/N = 3) of an unspiked sample, after having estimated its naturally occurring concentration. The limit of quantitation (LOQ) was set at 3 times the LOD.

LODs and LOQs were estimated as the mean of six replicates.

RESULTS AND DISCUSSION

Recovery Studies. The literature describes a number of procedures to isolate every vitamin from a food or a biological fluid singly.[1,4,31,32] Among these, the hot alkaline hydrolysis is the most used to achieve a high recovery of retinol, tocopherols, and cholecalciferol; the loss of vitamins E and D is low, whereas that of xanthophylls and K vitamers may be very severe. An alternative procedure to remove triglycerides without causing degradation of vitamin K is the enzymatic hydrolysis with lipase,[13,33] which was also applied for the simultaneous recovery of vitamins A, D, E, and K from fortified milk.[34]

In the early stages of this work, the efficiency of the direct extraction with solvent was tested to isolate the analytes most susceptible to alkalis (K vitamers and xanthophylls). Notwithstanding the several optimization trials, this procedure could recover a limited number of vitamins: retinol, α- and γ-tocopherol, and menaquinone-4. In particular, phylloquinone and carotenoids remained sequestered in the milk fat, also after the attempt to disrupt lipoprotein globules and to promote the analyte release by means of sample sonication in the presence of ethanol and hexane.

At this point, overnight cold saponification was assessed as a simultaneous extraction procedure of the 12 analytes targeted in this study. The first step of optimization was to establish the lowest number of moles of potassium hydroxide (KOH) needed for an effective removal of glycerides in the different kinds of milk (see Milk Samples for the fat content of the milk varieties); it was calculated considering that 3 mol of KOH hydrolyzes 1 mol of triolein, taken as a representative molecule of milk triglycerides (oleic acid is the most abundant among fatty acids). The volume (moles) of 50% (w/v) aqueous KOH was established as the best compromise between the amount of the released analytes and extent of the degradation of K vitamers and xanthophylls. The other optimized factors were the sample volume, the ratio between volumes of sample and ethanol, essential for a quantitative protein precipitation, and the hexane volume fundamental for obtaining the best extractive efficiency. The volume of Milli-Q water, added after overnight digestion and before extraction with hexane, was important to improve the phase separation and to lower the ethanol percentage under 40% to avoid losses of more polar analytes such as retinol and tocopherols;[4] adding a volume of 8.5 mL, the percentage of ethanol resulted to be <37% (v/v).

Validation Results. Validation results have been very similar for the different types of milk because of an efficient extraction procedure and an adequate internal standardization. Table 2 summarizes the validation data for cow's milk and Table S3 in the Supporting Information for the other kinds of milk.

Relative recoveries were >80% for all analytes, with the exception of phylloquinone (67%) and menaquinone-4 (54%). The method precision was good, and the higher relative standard deviations were obtained for carotenoids and K vitamers, independent from the type of milk. The linear dynamic range was investigated up to 200 ng injected for all analytes, except α-tocopherol, retinol, and γ-tocopherol (2000 ng injected). Squared linear regression coefficients (r^2) were >0.99 for every analyte in the different milk samples. Vitamin D vitamers, phylloquinone, and lutein were the analytes characterized by lower LODs and LOQs.

A selectivity study was performed for each kind of milk. At the beginning of this work, the 12 analytes were all chromatographed on the C_{30} column, indispensable for separating lutein and zeaxanthin, structural isomers. To verify the absence of interferences from matrix, the characteristic SRM transitions of each compound were extracted from LC-SRM chromatograms. The identification criteria (matching of retention times and ion ratios with those of authentic standards in solvent) were verified for all analytes except menaquinone-4 and cholecalciferol, the ion ratios of which were different from those reported in Table 2.

Table 3. Results of the Quantitative Analysis[a] of Raw Milk from Different Animal Species

analyte		μg L^{-1}				
		cow	buffalo	sheep	goat	donkey
vitamin A	retinol	2100	2870	4323	4317	586
provitamins A	β-carotene	243	nd[b]	nd	nd	nd
	β-cryptoxanthin	7.01	nd	nd	nd	nd
non-provitamin A carotenoids	lutein	10.0	nd	9.87	LOD	nd
	zeaxanthin	<LOQ	nd	<LOQ	nd	nd
vitamin E	α-tocopherol	3780	1210	3048	6545	807
	γ-tocopherol	38.1	188	208	384	260
	δ-tocopherol	nd	nd	LOD	LOD	nd
vitamin D	ergocalciferol	nd	nd	nd	nd	nd
	cholecalciferol	nd	LOD	nd	nd	nd
vitamin K	phylloquinone	8.82	5.49	97.9	LOD	nd
	menaquinone-4	8.60	nd	17.4	nd	nd

[a]All data are the means of duplicate or triplicate values. [b]nd, least intense SRM transition or both SRM transitions were not detected.

To check the coelution of isobaric interfering compounds responsible for this alteration, a tandem C_{18} column system (Supelcosil C_{18} plus Alltima C_{18}; as described under Instrumentation) was employed to improve the chromatographic efficiency. In this way, it was possible to separate the interfering peaks from those of analytes and to obtain proper ion ratios (see Figure S1 in the Supporting Information).

Identification of Fat-Soluble Vitamins and Carotenoids by HPLC-DAD-MS/MS. As just seen, the correct identification of each target analyte in milk was assessed by criteria that avoided false identifications and quantitations.

The provisional identification of other carotenoids, for which authentic standards were unavailable, was supported by the expected retention time and the UV−vis absorption spectrum; moreover, a further element corroborating the identification of the structural or geometric isomers of the target analytes was the sharing of their SRM transitions.

In formulating a hypothesis for the identification of a pigment, the UV−vis spectrum is basic. Most carotenoids show a characteristic three-peak spectrum:[31,35] its shape (fine structure) and absorption maxima allow chromophore identification. The Fieser rules,[36] valid for conjugated polyenes containing more than four double bonds, were applied to estimate the wavelength of the central peak (λ_{II} or λ_{max}). The degree of spectral fine structure was expressed as the percentage ratio between the heights of peaks II and III, measured from minimum (i.e., %III/II). The identification of a *cis* isomer[31,35] was based on the comparison between its spectrum and that of the corresponding *all-trans* isomer and on the evaluation of some differences: (i) a decrease of %III/II; (ii) a hypochromic effect accompanied by (iii) a hypsochromic shift of about 2−6 nm for mono-*cis*, 10 nm for di-*cis*, and 50 nm for poly-*cis* isomers; and (iv) the appearance of a *cis* peak (peak B) in the ultraviolet region. Because a stereoisomer with a *cis* double bond at (or near) the center of the conjugated system shows a very high *cis* peak, a parameter used to establish the position of the *cis* double bond is the Q ratio,[31] that is, the percentage ratio between the heights of peaks II and B, measured from the baseline of spectrum; this is sometimes calculated as (B/II)%.[31]

A representative LC-DAD chromatogram of carotenoids extracted from a cow's milk sample is shown in Figure S2 in the Supporting Information.

The APCI(+) full scan mass spectra of fat-soluble vitamins and carotenoids show a series of ionic species: $[M + H]^+$, $[M]^{\bullet+}$, $[M - H]^+$, $[MH - H_2O]^+$. This behavior was verified by studying the ionization of the target analytes. The base peak for the majority of them was $[M + H]^+$, but the dehydrated pseudomolecular ion was the most intense peak for retinol and lutein. $[M]^{\bullet+}$ was the base peak for δ-tocopherol. On the basis of the fragmentation study, already described in previous papers,[28,29,37] two SRM transitions were selected on the basis of the best S/N chromatographic ratio and used to identify each target analyte as well as to support the identification of isomeric unknowns.

Characterization of the Fat-Soluble Vitamin and Carotenoid Fraction of Milk Samples from Different Animal Species. The developed analytical approach was applied to characterize raw cow's, buffalo's, sheep's, goat's, and donkey's milks. Raw milk samples were obtained from pasture-fed animals to avoid the occurrence of vitamins due to the intake of fortified feedstuffs or losses due to the processing.[38] The quantitative results, summarized in Table 3, are related to the analysis of a limited number of samples; this means they have to be interpreted as a profile, because variability factors[6,39] such as season, stage of lactation, status of health, and individual variability were not taken into consideration in this study.

It is known that milk is a good source of vitamins A and E, but the milk samples analyzed in this work were found to be particularly rich in these micronutrients, probably because they were obtained from pasture-grazed animals and analyzed as soon as possible after the sampling. In fact, pasteurized bovine milk samples, bought in supermarkets of Rome and analyzed by means of this method, had concentrations of retinol ranging between 400 and 1900 μg L^{-1} and of α-tocopherol between 600 and 1280 μg L^{-1} (see Table S4 in the Supporting Information). These latter values were more consistent with those from the literature.[2,4,12,24,39,40]

Table 4. LC-UV-Vis-MS/MS Data for the Tentative Identification of Carotenoids in Bovine Milk

	retention time[a] (min)							found		reported	
peak	LC-DAD	LC-MS	compound	SRM transitions (m/z)	calcd λ_{max}[b] (nm)	obsd λ[c] (nm)	reported λ (nm)	%(III/II)	Q ratio	%(III/II)	Q ratio
1	7.71		unknown 1			404, 424					
2	9.01	9.27	*all-trans*-lutein	551.4/135.2 551.4/175.0	448	424, 448, 478	422, 445, 474[35]	60		60[35]	
3	9.44	9.70	*all-trans*- zeaxanthin	569.4/477.2 551.4/135.2	453	431, 455, 482	428, 450, 478[35]	25		25[35]	
4	10.88	11.14	*all-trans*-zeinoxanthin	553.5/119.1	448	422, 448, 479	420, 444, 472[35]	58	55	60[35]	
5	11.31	11.56	*all-trans*-α-cryptoxanthin	553.5/135.1 553.5/119.1	448	421, 447, 478	423, 446, 473[35]	60		60[35]	
6	11.54	11.80	*cis*-α-cryptoxanthin	553.5/135.1 553.5/119.1	446−442	422, 448, 476		55			
7	11.95		unknown 2			433, 454, 480		23			
8	12.15	12.41	*all-trans*-β-cryptoxanthin	553.5/135.1 553.5/119.1	453	430, 454, 480	425, 454, 478[44]	23		25[35]	
9	13.09		unknown 3			393, 415, 441		86			
10	13.77	14.04	*all-trans*-3-hydroxy-β-zeacarotene	*[d]	432	410, 431, 457	405, 428, 453[35]	33			
11	14.28	14.54	*cis*-β-carotene	537.5/119.1 537.5/177.2		346, 430, 451, 480		21			
12	15.40	15.67	13-*cis*-β-carotene	537.5/119.1 537.5/177.2	451−447	345, 426, 448, 471	338, 449, 478[45]	5	2.6	7[45]	2.3[46e]
13	15.75	16.01	*all-trans*-α-carotene	537.5/119.1 537.5/177.2	447	427, 448, 474	422, 445, 473[35]	60		60[35]	
14	16.13	16.39	*all-trans*-β-carotene	537.5/119.1 537.5/177.2	453	433, 455, 482	450, 478[35]	23		25[35]	
15	16.69	16.95	*all-trans*-β-zeacarotene	*[d]	432	410, 430, 449	402, 428, 453[47]	60		62[47]	
16	17.63	17.88	unknown 4	537.5/119.1 537.5/177.2		low intensity					
17	18.30	18.56	*all-trans*-γ-carotene	537.5/119.1 537.5/177.2	465	439, 465, 494	437, 462, 494[35]	38		40[35]	

[a]Retention times recorded on the LC-DAD chromatogram were early by about 0.26 min compared to the corresponding ones observed on the LC-MS/MS chromatogram. [b]The Fieser rule[36] was applied for calculating λ_{max} (λ_{II}) of the *all-trans* form of carotenoids found in cow's milk; $\lambda_{max} = 114 + 5M + n\ (48 - 1.7n) - 16.5R_{endo} - 10R_{exo}$, 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. For *cis* isomers, their λ_{max} value was estimated by subtracting the theoretical one of the *all-trans* form: 2-6 nm for a mono-*cis* isomer (major decrement when the *cis* double bond became more central), 10 nm for di-*cis* isomer, and 50 nm for poly-*cis* isomer. [c]It is known that solvent[35,36] influences both λ_{max} and spectral fine structure, but the λ_{max} values for any carotenoid are similar in petroleum ether, hexane, diethyl ether, methanol, ethanol, and acetonitrile. In our study, the UV−vis spectra of carotenoids were extracted from HPLC-DAD chromatograms, achieved working in gradient elution; because the used solvents were methanol, isopropanol, and hexane, the observed λ_{max} values were very close to those calculated by the Fieser rule. [d]Notwithstanding the slight mass difference, both 3-hydroxy-β-zeacarotene and β-zeacarotene responded to the SRM transitions of β-cryptoxanthin and β-carotene, respectively. That might be possible considering triple quadrupole works in unit mass resolution mode (0.7 ± 0.1 u) and carotenoids can generate an ion species such as $[M - H]^+$, besides $[M]^{\bullet+}$ and $[M + H]^+$. [e]The $1/Q_{ratio}$ value was reported because the authors calculated Q_{ratio} as (B/II)%.

Milk from small ruminants was very rich in vitamin A, but bovine milk was the only one to show the occurrence of provitamins A in a significant amount (see Table 3) and a variety of carotenoids (see Table 4; Figure 4; and Figure S2 in the Supporting Information) missing in the other types of milk, with the exception of lutein and zeaxanthin. In this case, the concentration of *all-trans*-β-carotene resulted close to the data from the literature.[2,22,39,40]

The results of the LC-DAD-SRM analysis, applied to screen pigments occurring in bovine milk, are summarized in Table 4. The characteristic absorption in the Soret band of unknown 1 has supported its identification as a chlorophyll *a* degradation product, generated in animal rumen. Peak 4 has been assigned to zeinoxanthin, a carotenol occurring in the petals of some flowers,[35] in corn grain, and in citrus fruits along with *all-trans*-α- and β-cryptoxanthin (peaks 5 and 8). The MS/MS and UV−vis data set have allowed attributing peak 6 to *cis*-α-cryptoxanthin, whereas peak 7 (unknown 2) could not be identified because of the low signals of both detectors. Notwithstanding the characteristic UV−vis spectrum extracted from peak 9 (unknown 3), no identification has been established due to the lack of MS data. Peaks 10 and 15 have been assigned to 3-hydroxy-β-zeacarotene and β-zeacarotene, respectively, on the basis of matching UV−vis spectra (the hydroxyl group does not affect the chromophore absorption), retention times consistent with the expected ones, and sharing of the ion currents of the target isomeric carotenoids. Extraction of the ion currents of *all-trans*-β-carotene (16.39 min) from the LC-SRM chromatogram of Figure 3 permitted the observation of the other six peaks at 14.54, 15.67, 16.01,

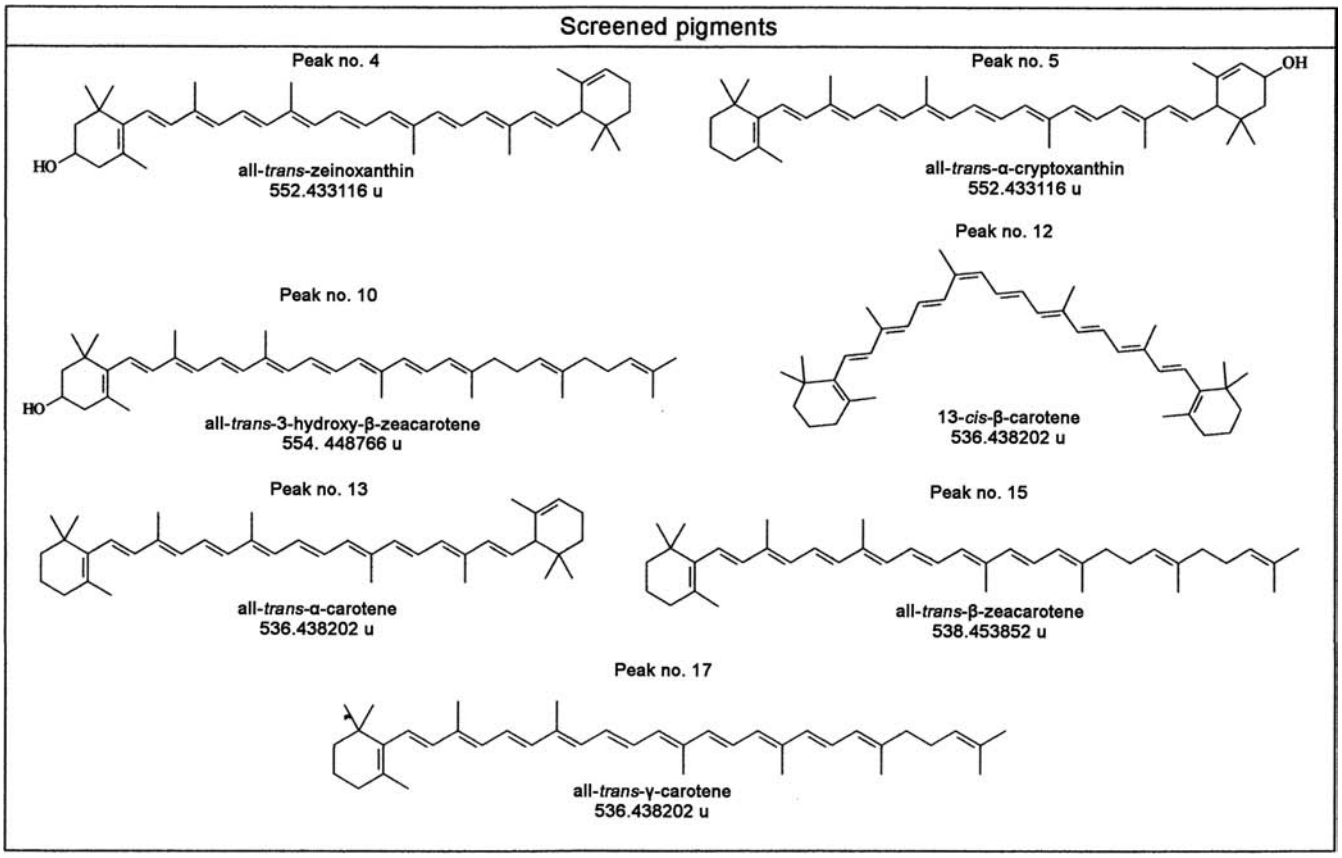

Figure 4. Names, structures, and monoisotopic masses of the screened pigments.

16.95, 17.88, and 18.56 min. The related UV−vis spectra have allowed their tentative identification as reported in Table 4.

It is known that carotenoids modify the nutritional and sensory properties of bovine milk,[6] contributing to its yellowish color and oxidative stability along with other antioxidants such as lactoferrin, vitamin C, and vitamin E. As a matter of fact, the bovine milk analyzed in this work showed a green-yellow color, whereas the other kinds of milk were white, being almost devoid of these pigments. The literature[6] explains these differences among species as due to a higher efficiency of conversion of β-carotene into retinal in ovine enterocytes than in bovine ones. However, their concentration in milk also depends on nature and the amount of the consumed forage with variations related to season (for instance, sunlight exposure decreases their grass concentration because they are UV-sensitive). Our samples were taken in April 2012, and the concentrations of carotenoids in bovine milk have been consistent with those measured by Chauveau-Duriot et al.[24] in forages, where lutein (230 μg g^{-1}) is the most abundant form, followed by *all-trans*-β-carotene (60 μg g^{-1}), zeaxanthin (19 μg g^{-1}), neoxanthin (18 μg g^{-1}), 9-*cis*-β-carotene (10 μg g^{-1}), 13-*cis*-β-carotene (8 μg g^{-1}), and violaxanthin (6 μg g^{-1}); α-carotene and β-cryptoxanthin[2] were also detected but not quantified.[43]

With regard to vitamin E, α-tocopherol (5,7,8-trimethyltocol) has been the only form detected in grass-based forages so far;[3] it has also been the most abundant vitamin form (more than vitamin A) found in all kinds of milk analyzed in this work, with the exception of the buffalo one. γ-Tocopherol occurs in lower amount, whereas δ-homologue is absent in cow's, buffalo's, and donkey's milk. It is important to point out that when the ion currents of γ-tocopherol (7,8-dimethyltocol) were extracted from the LC-SRM chromatogram (Figure 2), two peaks were noticeable: the first one at 9.63 min (γ form) and the second one at 10.05 min; the latter might be improperly attributed to β-tocopherol (5,8-dimethyltocol), an isomer of γ-form. It is actually ascribable to α-tocopherol, which, losing a $-CH_3$ group in the APCI source, generates a fragment ion with the same m/z value of the γ-tocopherol pseudomolecular ion; moreover, correspondence with the retention time of the α-form is perfect. This ion current was observed with an appreciable intensity by analyzing the extracts of bovine and caprine milk due to the very high concentration of α-tocopherol.

There is not much information in the literature concerning the content of vitamin K vitamers in foods[13,14,33,41,42] and, when available, they are often referred to phylloquinone. Indyk extracted phylloquinone and menaquinone-4 from pasteurized bovine milk[13] and processed foods[33] by enzymatic digestion with lipase, finding values in the range of 1−10 μg L^{-1} for both homologues. Our analyses have provided analogous results; moreover, they have shown that ewe's milk is a good source of vitamin K vitamers.

The concentration of cholecalciferol in milk depends on the animal's exposure to sunlight, whereas the presence of ergocalciferol is influenced by the feeding with sun-dried green forage (hay). The literature[2,7,8,30] gives very different indications about the concentration of cholecalciferol in bovine milk (from 0.001 to 2 μg L^{-1}). Although the LODs of our LC-SRM method are very low (1.12 μg L^{-1} for D_3 and 0.90 μg L^{-1} for D_2), we have never detected vitamin D vitamers in bovine milk. Evidently, their endogenous concentrations are below the

LOD and a higher enrichment factor should have been needed for their detection; as a matter of fact, some authors could quantify vitamin D_3 only after preconcentration of the alkaline digest on a semipreparative column.[7,8] However, our analyses have revealed the occurrence of vitamin D_3 at the LOD level in buffalo's milk, whereas traces of both vitamin D vitamers in buffalo's, goat's, and donkey's milk could not be confirmed because only their quantifier transitions were observed on the LC-SRM chromatograms.

In conclusion, the feasibility of this novel analytical approach, making use of cold overnight saponification and LC-DAD-APCI-MS/MS hyphenation, was tested by analyzing milk from different animal species. A comprehensive characterization of every type of milk has been obtained by performing the quantitative analysis of 12 target micronutrients and the screening of unknown pigments without the support of authentic standards. The high method sensitivity and selectivity have allowed a detailed composition of cow's, buffalo, goat's, ewe's, and donkey's milk to be obtained, providing information lacking in the literature. Nevertheless, it must be stressed that the variability parameters affecting these profiles were not considered in this study.

■ ASSOCIATED CONTENT

Supporting Information

Solvents, concentrations, and preparation frequency of the individual stock standard solutions (Table S1); fortification levels applied for performing standard addition method (Table S2); validation parameters related to the confirmatory method of the target analytes in buffalo's, ewe's, goat's, and donkey's milk (Table S3); results of the quantitative analysis of several Italian bovine milk samples (Table S4); results of selectivity study for menaquinone-4 extracted from a ewe's milk sample (Figure S1); LC-DAD chromatogram of carotenoids extracted from a bovine milk sample (Figure S2). This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We acknowledge gratefully Antonio Antinozzi, owner of a biological farm in Cisterna di Latina (Latium, Italy), and Mario Ventura, veterinarian at the Health Service of Sora (Frosinone, Latium, Italy), for their help in providing raw milk samples from pasture-fed animals.

■ ABBREVIATIONS USED

NARP, nonaqueous reversed-phase chromatography; MK-*n*, menaquinones-*n*; MK-4, menaquinone-4; MK-9, menaquinone-9; BHT, butylated hydroxytoluene; RS-Plus, special grade reagents; RS, elevated purity grade; RPE, analytical grade; KOH, potassium hydroxide; APCI, atmospheric pressure chemical ionization; fwhm, full width at half-maximum; SRM, selected reaction monitoring; UV−vis, ultraviolet−visible; HPLC, high-performance liquid chromatography; DAD, diode array detector; S/N, signal-to-noise ratio; Q, quadrupole mass analyzer; CE, collision energy; DP, declustering potential; FP, focusing potential; FS, full scan; IS, internal standard; LOD, limit of detection; LOQ, limit of quantification; RSD, relative standard deviation; LC-MS/MS, tandem mass spectrometry coupled to liquid chromatography.

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