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Analysis of circulating lipid-phase micronutrients in humans by HPLC: Review and overview of new developments

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Abstract

Retinol, tocopherols, coenzyme Q10, carotenoids, and vitamin D are lipophilic compounds shown to function as important health-protective agents by mitigating the damaging effects of oxidative and other injury. Scientific interest in evaluating these compounds has resurfaced in recent years, particularly in the nutritional, clinical and epidemiologic fields, and has precipitated the development of a multitude of new analytical techniques. This review considers recent developments in HPLC-based assays since 2007 for the simultaneous determination of these lipid-phase compounds utilizing exclusively serum or plasma as these matrices are mostly used in clinical and epidemiological investigations. We also provide an overview of blood measurements for selected carotenoids, tocopherols, coenzyme Q10 and retinol from the last 15 years of healthy umbilical cord blood, children, and adults.

Keywords

Plasma; Serum; Micronutrients; HPLC; Lipid-phase antioxidants

1. Introduction

Lipid-phase micronutrients (LPM), e.g. retinol, tocopherols, coenzyme Q10 (CoQ), and carotenoids, shown to function as important antioxidants, and vitamin D, involved in numerous physiological processes, assist in mitigating and/or preventing oxidative and other damage [1–7]. Such damage, if not controlled, can contribute to the development of various conditions such as cancer, of which the etiology is thought to involve oxidative or other damage [8]. Thus, several studies have investigated the role of these LPM against cancer [9–

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11], atherosclerosis, and age-related (eye) diseases [12,13]. Consequently, interest in evaluating LPM has been remarkable, resulting in the development of numerous analytical techniques for accurate and sensitive measurements of LPM.

This review considers the developments of HPLC techniques for the simultaneous determination of multiple LPM as they apply to the routine clinical and molecular epidemiology laboratory. Particular emphasis is given to methods developed since 2007 using exclusively plasma or serum. In addition, an overview of measurements of plasma and serum LPM from healthy cord blood, as well as from the circulation of children and adults is summarized.

Considerable progress has been made in the field of LPM detection and a growing number of clinical, epidemiological and other investigations continue to require LPM measurements from biological matrices. Fast and affordable analytical methods capable of resolving and quantifying multiple LPM simultaneously in a single chromatographic run from very little plasma or serum are highly desirable as they play a crucial role for investigators involved in large-scale studies. Consequently, our review will serve the needs of analysts particularly in the clinical or epidemiologic fields.

1.1. Carotenoids

Carotenoids are a group of naturally occurring lipid-phase compounds widely distributed in nature and generally found as red, yellow, and orange pigments in fruit and vegetables as well as in some animal products. Over 600 carotenoids have been acknowledged [14] and, of these, more than 50 have been identified in the human diet and biological matrices [15,16].

Carotenoids are polyunsaturated aliphatic compounds containing a polyisoprene skeleton and generally divided into two classes: carotenes and xanthophylls; the former are strictly hydrocarbons (e.g. α -carotene, β -carotene, and lycopene) and the latter are their oxygenated derivatives (e.g. lutein) [17].

Some carotenoids, e.g. α -carotene, β -carotene, and β -cryptoxanthin, display pro-vitamin A activity and are collectively called provitamin A carotenoids [18]. Carotenoids such as β -carotene, lycopene, and zeaxanthin display radical quenching abilities and, therefore, serve as antioxidants against oxidative stress. This antioxidant ability is a direct result of their molecular structure – a characteristic conjugated double bond system consisting of delocalized π -electrons susceptible to electrophilic attack [19]. Because of their antioxidant and other effects, carotenoids are thought to play a preventable role against certain diseases. Several epidemiological studies describe associations between higher intakes of dietary carotenoids and/or high serum concentrations of some carotenoids and lower incidence of cardiovascular disease and cancer [20–22]. Some studies suggest that carotenoids may be involved in regulating immune function and preventing atherosclerosis [23,24]. Conversely, other studies have found LPM to have no beneficial effect [25–27] or even be detrimental if consumed in unphysiological doses and/or by smokers [28] for disease occurrence and/or prevention.

Many of the observed favorable health effects occur only when these LPM are consumed with the habitual diet and not by supplementation. This may be due to the modes of exposure and/or the exposure to a wide variety of LPM and, thus, may help to explain the conflicting results in clinical and epidemiological studies. While supplements provide only single or few agents, the diet leads to the exposure of many micronutrients or nutritional components in combination with LPM.

A number of HPLC methods are available for carotenoid detection. Traditional and current methods include light absorbance using fixed wavelength ultraviolet/visible (UV/vis) detection or monitoring at multiple wavelengths along with the recording of the UV/vis spectrum using diode array detection (DAD) [29]. Carotenoids have also been analyzed using electrochemical detection (ED) [30] and, albeit rare, thermal lens spectrometry (TLS) [31]. Recent technological improvements have also expanded to include more sensitive techniques such as mass spectrometry (MS) [32].

1.2. Retinol

Vitamin A is a generic term referring to a group of related compounds possessing biological activity for all-trans retinol and includes retinol, retinal, retinoic acid, and retinyl esters [33,34]. Dietary vitamin A is supplied either as preformed vitamin A (i.e. retinol and retinyl esters) or provitamin A carotenoids (i.e. β -carotene, α -carotene, and β -cryptoxanthin) with the former predominately found in foods of animal origin and the latter largely supplied from plant sources [35]. Vitamin A is also available from fortified products such as margarine and sugar [36] and from supplements mainly in the form of retinyl esters [33]. These vitamin A compounds undergo intracellular conversion to the active vitamin A forms before exerting their important biological functions (for vitamin A metabolism, refer to review by [35]) such as normal cell growth, cell differentiation, vision, immunology, and hematopoiesis [37].

Structurally, all forms of vitamin A possess a β -ionone ring linked to a string of four isoprenoid units with a series of conjugated double bonds and a side chain consisting of either an alcohol (retinol), aldehyde (retinal), carboxylic acid (retinoic acid), or ester group (e.g. retinyl palmitate).

Analyses of vitamin A compounds and their metabolites have been performed using various HPLC-based techniques. The conjugated series of electron rich double bonds in the vitamin A compounds results in their ability to absorb light very strongly, particularly in the UV and often visible wavelength range depending on the resonance state [38,39]. For this reason, vitamin A detection most commonly entails UV/vis detection [40,41] or DAD [42] although ED [43] and tandem MS (MS/MS) [44] have also been used. Analyses of some retinoid compounds having native fluorescence (e.g. retinyl esters [45]) have been performed using fluorescence detection (FD) [46,47].

1.3. Vitamin E – tocopherols

Vitamin E is an umbrella term for the 8 chromanol ring homologs (four tocopherols and four tocotrienols) naturally present in foods originating from both animal and plant sources [48]. Structurally, all vitamin E homologs contain a hydroxyl group on a chromanol ring attached

to a phytyl side chain. Tocopherols have a saturated phytyl side chain and consist of four forms (α -, β -, γ -, and δ) that differ in the number and location of methyl groups on its chromanol ring. Tocotrienols are structurally identical to tocopherols except for the occurrence of three double bonds in the side chain occurring at carbons 3', 7', and 11' [17].

Vitamin E is best known for its role as a potent antioxidant and, as such, helps to maintain membrane integrity through its ability to inhibit the chain propagation of peroxidative reactions occurring within polyunsaturated lipids. This function is thought to occur through interactions between the phenolic ring hydroxy group of vitamin E and organic peroxy radicals [48,49]. γ -Tocopherol behaves exceptionally well by scavenging specifically reactive nitrogen species [50]. Independent of its antioxidant function, vitamin E has been shown to exert preventive effects against health conditions such as coronary heart disease and cancer [51–53] in addition to its involvement in inhibiting cell proliferation in vascular smooth muscle [54], modulating signal transduction, and regulating gene expression [55]. Conversely, for patients with pre-existing health conditions (e.g. diabetes and cardiac disease), high-dose vitamin E has been associated with increased risk of detrimental conditions and even death and/or higher rates of heart failure. But again, this could have been due to the exposure to high-level and/or unphysiological doses and lacking the context of exposure to a variety of phytochemicals required to achieve beneficial effects as the cases with dietary exposure (see review by [25]).

Determination of vitamin E compounds has been commonly performed using both normal phase (NP) [56] and reverse phase (RP) systems [57–60] in combination with simple detection methods such as UV [60] and DAD [59] or more sophisticated methods such as MS [57] or MSMS [58,61] instrumentation. Additional detection methods include ED [62], which uses the low oxidation potential of the compounds, or FD [47,63,64], which takes advantage of the native fluorescence of the compound and allows very selective and sensitive detection.

1.4. Vitamin D

Vitamin D belongs to a group of compounds referred to as sterols and whose backbone resembles a steroid except for a detached β -ring and a hydroxyl group on the 3-position of the A-ring. The two naturally occurring vitamers, D_3 (cholecalciferol) and D_2 (ergocalciferol), are structurally identical except for an additional double bond and methyl group in the side chain of the latter. Vitamin D_3 is produced in the human skin from 7-dehydrocholesterol when exposed to UV-B radiation in the range of 280–320 nm. Exposure to vitamin D_3 can also occur through dietary (e.g. animal), fortified, and supplemental sources [65] but is believed to account for only 10% of human exposure (except in the case of supplementation). Vitamin D_2 is produced through the UV mediated photoisomerization of ergosterol, a sterol occurring exclusively in plants and yeast [65]. Vitamin D_2 exposure also occurs from fortified products and supplements [66].

Regardless of the source, once ingested both D vitamers are hydroxylated in the liver to 25-hydroxyvitamin D (25(OH)D) – the prehormone form used to evaluate an individual's vitamin D status [65] – and are then further hydroxylated predominately in the kidneys to 1,25-dihydroxy vitamin D, the fully active hormone [67].

Vitamin D's involvement in health and disease is encyclopedic as highlighted by its control of approximately 3% of the human genome [66]. Vitamin D's basic function is to facilitate the absorption of calcium and phosphorus and, for this reason, vitamin D deficiency affects calcium and bone metabolism [66], and ultimately leads to the development of rickets in children and osteomalacia and/or osteoporosis in adults. Vitamin D deficiency is also linked to serious health conditions such as rheumatoid arthritis, infections, multiple sclerosis, type 1 and type 2 Diabetes Mellitus, autoimmune disease, cardiovascular disease, Crohn's disease, and many cancers [68–73]. The array physiologic and pathologic events in which vitamin D is involved is constantly expanding and include antioxidant potential in the central nervous system [6], in brain liposomes [2], and in astrocytes [74].

Methods for determining vitamin D include mainly enzyme-linked, and chemiluminescence immunoassay platforms. These techniques remain the most widely used platforms today because of their simplicity, speed, and accuracy despite the drawbacks of cross-reactivity, unequal detection, and recovery [75,76]. Radio-immunoassay platforms are less favored because of the need to use radioactive tracers. Liquid chromatographic methods incorporating UV [77] or ED [78] have also been reported. A comparison of immunoassay and HPLC-based methods for detection of D vitamers were provided recently [79]. The advent of HPLC–MSMS was a substantial improvement in vitamin D analysis due to its ability to facilitate separation of 25(OH)D₂ from 25(OH)D₃ [80,81] and its ability to assist in accurate and simultaneous quantification of multiple D analytes [82]. However, the drawbacks of using HPLC–MSMS include price, limitation to those laboratories with appropriate equipment [83], and its probability to epimeric interferences [84] although recent undertakings have sought to account for the latter [85,86]. An excellent discussion of vitamin D HPLC–MSMS measurements is provided in a recent review [82].

1.5. Coenzyme Q10

Coenzyme Q (CoQ) is a lipophilic molecule composed of a redox-active benzoquinone head conjugated to a side chain of repeating isoprene units [87–90]. In humans, both CoQ9 and CoQ10 (side chains of 9 or 10 five-carbon isoprene units) exist, with the latter being the dominant homologue found in plasma [91]. CoQ10 is transported in the circulation via lipoprotein particles [92], similar to that of other lipophilic compounds, and is located in the phospholipid bilayer membrane of all tissues where it functions as an electron/proton carrier during cellular respiration as well as an intracellular antioxidant [88,90,93]. CoQ is structurally similar to vitamin E [1] and can be acquired exogenously from both animal and plant sources [94,95].

CoQ10 in its chemically reduced form of ubiquinol-10 (UL10) is considered a free radical scavenger that protects low-density lipoproteins against oxidative stress in vitro [5]. As such, UL10 acts to reduce lipid peroxyl or tocopheroxyl radicals [96] which explains the rapid oxidation of UL10 in plasma [97], and also minimizes oxidative stress by dehydrogenating ubiquinone-10 (UN10), the chemically oxidized form of CoQ [98–105]. Thus, the UL10:UN10 ratio has been postulated as an in vivo marker of oxidative stress [5,106,107], whereas total Q10 (TQ10) may represent general physiological events such as cell death, as dying cells would leak Q10 into the circulation [103,108–110].

Traditionally used methods to determine total Q10 and/or UL10 have relied on UV detection [111,112] for its simplicity or ED [113] for its sensitivity. For UN10 determinations, use of ED has been the most preferred [114] while methods to simultaneously determine UL10 and UN10 have included in-line post-column reductions of UL10 to UN10 by coulometric ED [62,115,116] or online reductions using a reduction column with [117] or without [118] a semi-microcolumn switching system. UN10 has also been determined by subtraction of UL10 from total Q10 after the latter was determined by precolumn reduction with ED or sodium borohydride or by total chemical oxidation [119]. More complicated determinations have used FD after post-column derivatization [120] or MSMS with ESI in positive mode [121] or MS with APCI in negative mode [122].

2. Methods of analysis in serum/plasma

2.1. Separation using high-performance liquid chromatography (HPLC)

HPLC has become the long-standing method of choice among investigators analyzing LPM owing to its high throughput feature and ability to quantify various forms of compounds at low levels in serum and plasma. HPLC is a powerful tool that enables the separation of complex mixtures into individual components and, compared to other conventional analysis methods, e.g. open-column and thin-layer chromatography, is advantageous in terms of shorter analysis times, greater resolution, and higher sensitivity [123]. Additionally, HPLC is a highly sensitive and reproducible analytical technique. Over the years, HPLC has been combined with numerous detection methods (explained below and summarized in Table 1) and has steadily experienced increasing innovations regarding stationary phases (explained below and summarized in Table 1), which have enhanced its sensitivity and specificity.

2.1.1. Sample work-up procedures used in HPLC analyses of lipid-phase micronutrients (LPM)—Procedures developed for the analysis of multiple LPM measurements generally require 50–1000 μ L serum or plasma that is deproteinized then extracted once or several times with the appropriate solvents (see below) and preferably including butylated hydroxytoluene (BHT) and/or ascorbic acid [47,124–126] as an antioxidant stabilizer. After evaporating to dryness, samples are usually then reconstituted in the HPLC mobile phase at concentrations necessary to enable accurate detection of analyte in the lowest concentration. Some investigators have attempted to minimize sample degradation and manual handling of samples by eliminating the evaporation step thereby allowing a direct injection of the organic phase into the HPLC system for online solid-phase extraction [127].

Sample deproteinization typically uses an organic polar solvent such as ethanol, methanol, tetrahydrofurane, or acetonitrile containing an internal standard(s), which provides an accurate way to correct for any uncertainty and/or variability (i.e. analyte losses) that may have resulted during sample handling. The analytes are then extracted into non-polar solvents e.g. hexane, heptane, dichloromethane, chloroform or a combination thereof, then evaporated to dryness under a stream of nitrogen and finally reconstituted with mobile phase prior to injection into the HPLC instrument. When analytes are naturally found in low concentrations e.g. vitamin D metabolites, it is usually necessary to pre-concentrate samples prior to HPLC analysis in order to achieve sufficient sensitivity.

For multiple analyte determinations, it is important to critically evaluate the choice of extraction solvents used, as the best solvent for one compound may not be suitable for another. This may be the result of the differing structural features of the LPMs that, in turn, influence their solubility characteristics. For example, lipophilic solvents such as hexane or tetrahydrofuran commonly used to extract β -carotene from serum or plasma may be of limited use for compounds such as lutein, retinol, or tocopherol which are much more miscible in a polar solvents such as acetonitrile or different alcohols.

Carotenoids have a characteristic conjugated double bond system consisting of delocalized π -electrons that make them especially reactive and unstable. Thus, sample preparation and extraction procedures should proceed quickly, be conducted under dim or yellow light and at low temperatures in order to minimize photodegradation [124]. In addition, drying time under nitrogen flow should be carefully monitored as some investigators have obtained diverging values due to artifactual oxidation resulting from excessive drying [128]. Moreover, the temperature at which analytes are prepared at and stored post-extraction need to be taken into consideration as temperature dependent degradation and/or losses have been known to occur [128].

2.1.1.1. Detection by ultraviolet/visible absorbance (UV/vis): Perhaps the most common HPLC-based detection method used today is UV and vis absorbance detection alone or in combination due to their extended applicability with good sensitivity [129]. DAD is an advanced UV/vis detector commonly used to simultaneously and continuously acquire UV/vis data over a wide range of wavelengths-typically 190–800 nm with arrays of usually 512 photo-diodes [130]. This ability enables the measurement of compounds that display absorbance patterns in both the UV and vis spectrum and, thus, provides a significant advantage in terms of sensitivity and resolution plus potential for characterization of unknowns over use of either UV or vis detectors alone. Traditional detectors speeds are 20 Hz but this has been improved recently to 80–160 Hz, which benefits most ultra-HPLC (UHPLC) techniques.

2.1.1.2. Fluorescence detection (FD): FD is a technique based on the emission of photons from excited molecules having intrinsic fluorescence yields large enough to be detected analytically. Natively fluorescent compounds are relatively rare and include those containing at least two conjugated π bonds or specific (electron-donating) groups able to donate electrons to a π system to enhance fluorescence intensity [131]. FD has the ability to reliably achieve low detection limits (e.g. picogram range) [63,64] and is superior to UV or vis detectors with respect to sensitivity, specificity, and selectivity [130–132]. To extend the applicability of FD to compounds without natural fluorescence, chemical derivatization or fluorescence labeling techniques have been developed. These techniques, however, are cumbersome and are greatly influenced by many factors, e.g. reagent concentration, mobile phase composition, temperature, acidity, and solvent polarity [129,130] that may significantly affect fluorescence intensity and, accordingly, changes in the excitation and emission wavelengths used for quantification. Therefore, these parameters should be carefully monitored to ensure FD is not unintentionally affected.

2.1.1.3. Electrochemical detection (ED): ED takes advantage of compounds possessing an active electrophore, a substructure within the molecule that is easily reduced or oxidized when provided with a suitable electric potential [132]. ED provides higher sensitivity compared to other detection methods such as FD and UV/vis [133] as well as higher selectivity resulting from the ability to maximize detector responses by manipulating electric potentials. While the use of ED is limited to compounds possessing electroactivity, these types of compounds are generally more common than those that fluoresce or can be made to fluoresce through derivization techniques [134]. Successful application of ED necessitates not only a detailed knowledge of electrochemistry but also the optimization of operational parameters such as column type (ED best suited for RP), mobile phase pH (due to release or consumption of protons) and buffer composition to allow for an electrical current, solvent purity (to minimize background noise), and optimum electrode potential (to maximize sensitivity) [134]. This detection technique, however, is so sensitive that it is often plagued by interferences.

2.1.1.4. Detection by thermal lens spectrometry (TLS): TLS is a laser-based spectrometric technique by which the temperature of a sample is increased by means of light absorbance from an excitation laser. This irradiation results in temperature-dependent changes of the sample's physical properties that are then measured by absorbance. The indirect measure of absorbance inherent to the TLS technique provides better sensitivity compared to conventional spectrometry techniques [135]. TLS instrumentation is often divided into two groups (single and dual laser beam) based on its optical configurations. In the single beam configuration, one laser serves as both the source of excitation light and the source of the probe beam. In the dual beam configuration, commonly termed the "pump and probe" arrangement, one laser serves as the "pump beam" to excite and absorb the sample, while a second laser – usually low power – is used as a "probe beam" to detect the thermal lens of the irradiated sample [136]. The application of HPLC-TLS to LPM analyses particularly in serum or plasma has been limited, to our knowledge, to one study thus far [31] in which α -carotene, β -carotene, β -cryptoxanthin, and lycopene were evaluated in plasma. Further information on TLS theory and applications is provided by recent reviews [135,137].

2.1.1.5. Analysis by mass spectrometry (MS) and tandem mass spectrometry (MS/MS): MS is a powerful analytical technique used to identify and quantitate compounds based on their molecular mass. This technique can help to provide confirmation information of compounds that may co-elute using less specific detection methods such as UV/vis or DAD. MS is a very sensitive and specific technique that measures charged molecules; it involves sample vaporization followed by ionization, mass separation, and ultimately mass detection. Analyte ionization, the essential step in MS to generate a charged species, is performed using a variety of techniques e.g. electron, chemical, electrospray or desorption, and results in the formation of ionized species (anions or cations) that are subsequently separated in a mass analyzer (e.g. quadrupole mass filter, ion trap, or hyphenated systems) according to their mass-to-charge ratio (m/z). The presence of each product ion signal within the sample is quantitatively detected then plotted into mass spectra representing relative ion abundance as a function of the m/z ratio [138,139].

MS/MS employs the use of two or more mass analyzers and is used to obtain additional information on ions generated by ionization. MS/MS is typically a two-stage mass analysis process separated by a step involving ion fragmentation. After mass determination, specific ions (called precursor or parent ions) are selected in the first quadrupole then subjected to fragmentation by the addition of energy, usually through collisions with neutral gas molecules (collision-induced dissociation) followed by separation of the generated product ions in the second quadrupole. The resulting fragmentation patterns used with the molecular weight helps to assist in structural elucidation of the molecules of interest. While MS and MS/MS are excellent detection techniques for the unambiguous analyte identification, this characteristic does not apply to geometric isomers since such compounds have identical masses and fragmentation patterns. Additionally, MS and MS/MS operations often incur high equipment cost and highly trained personnel that often preclude its routine use in clinical evaluations [138,139].

2.1.1.5.1. HPLC stationary phases

2.1.1.5.1.1. Normal-phase (NP) and hydrophilic interaction chromatography (HILIC): In normal-phase (NP) chromatography, the mechanism of retention onto the column is regulated by hydrogen-bonding, electrostatic, and dipole-dipole interactions [130] between the compound(s) of interest and the column material. NP chromatography entails the use of a polar stationary phase and a non-polar mobile phase, generally composed of solvents such as hexane [38] so that retention of an analyte increases as its polarity increases.

NP-HPLC has been used to effectively resolve γ - from β -tocopherol [140,141], cis-trans isomers of α - from β -carotene, [142] and lutein from zeaxanthin [15]. NP has been especially useful for compounds with high lipid content [38], however, its use has decreased in popularity due to commercial unavailability, poor reproducibility [143], and its sensitivity toward small changes in mobile phase that result in long equilibrium times [38]. In contrast, hydrophilic interaction chromatography (HILIC), a type of NP chromatography, is based on the liquid-liquid partitioning between the bonded polar stationary phase ('reverse reverse-phase') consisting of bare silica or silica phases modified with various polar groups, and the mobile phase consisting usually of mixtures of water and organic modifiers such as acetonitrile. HILIC has become increasingly favored over NP chromatography due to its higher robustness [144].

2.1.1.5.2. Reverse-phase (RP): Reverse-phase (RP)-HPLC has become the most commonly used chromatography mode. In RP chromatography, the stationary phase is chemically modified (mostly by C8, C18 and recently C30 but also many other materials) to become non-polar and the mobile phase is polar. For these reasons, the retention of analytes is reverse to that of NP; that is, non-polar compounds are more strongly retained than polar compounds. RP chromatography is much more popular than NP as a result of its commercial availability, superior stability, and robustness over NP columns [38] in addition to being highly successful in separating relatively unpolar and non-polar compounds, such as carotenoids. Several different RP columns exist that vary in material, length, particle size, and internal diameter. An in-depth description of these parameters is available in a recent review [145].

Typical stationary phases employed for RP-HPLC analysis of LPM include C18 and, more recently, C30 materials. Various C18 columns continue to be employed for analysis of retinol, carotenoids, and tocopherols as they provide good separations for a wide range of analytes, especially those with relatively short chain and low molecular weight (see review by [145]). However, because many of the carotenoids are similar in structure often with subtle differences in polarity, use of the C18 column is often inadequate in resolving geometric [carotenoid] isomers. In 1994, Sander and colleagues [146] designed a RP column specifically to overcome this limitation – a silica based, non-encapped C30 material that was more hydrophobic than classical C18 columns. The thickness of this C30 column has been estimated at 25 Å, comparable in dimension to some carotenoids and, thus, has been deemed favorable for interaction with long chain compounds. This characteristic has been partly attributed as the reason for the enhanced separation of cis-trans isomers over C18 columns, which range in thickness from 17 to 21 Å, much smaller than the size of carotenoids [143]. C30 columns have been reported to display higher retention quality, enhanced geometric isomer selectivity, and superior resolution over C18 columns [143]. Since its commercial availability many investigators have applied this ‘carotenoid column’ for separation of geometric isomers in serum/plasma for carotenoids, D and E vitamers, and Q10 compounds [147–150].

2.1.2. Serial column coupling—The serial coupling of columns has been reported as a way to increase separation efficiency along with peak capacity [151–154]. In these circumstances, two [or more] columns are linked end to end with a detector attached at the end of the last column. Under optimized conditions, column coupling can provide more separation power than conventional LC. However, this comes at the expense of longer chromatographic runs as a result of the low flow rate imposed by back pressure limitations; the tradeoff of the long runs nonetheless includes savings in analysis time and sample volume. By and large, the time invested to analyze compounds on serially coupled columns is much shorter than when using individual columns. Another drawback of this technique (in addition to long run times) includes the possibility that the elution order of analytes may change compared to when single columns are used [154]. Coupling of analytical techniques, in this case, with MS provides an enhanced ability for analyte confirmation.

2.1.3. Ultra high-performance liquid chromatography (UHPLC)—UHPLC is a recent advance in high-resolution chromatography by the use of sub 2 µm particles in the stationary phase which requires specialized pumps and equipment for the high pressures up to 18,000 PSI and has been increasingly employed in routine analyses [155–157] since its introduction in 2005. UHPLC offers a number of distinct advantages over conventional HPLC including faster run times, narrower peaks, higher sensitivity, less sample and reagent requirements, better resolution and minimized matrix effects [155]. Additionally, the use of specialized columns ensures a wider pH operating range and great tolerance for resisting high back pressures (up to 100 MPa) compared to conventional HPLC (maximum pressures ca 35–40 MPa). However, the high cost of columns and the need for specialized expensive hardware are often prohibitive for its application.

2.1.4. Two-dimensional liquid chromatography (2D-LC)—Another advancement in LC separation is two-dimensional LC (also called 2D-LC or LC $\times$ LC). This technology involves the coupling of two LC techniques with similar (e.g. RP $\times$ RP) or different separation mechanisms (e.g. RP $\times$ NP) thus providing increased peak capacity. A 2D-LC system typically includes the use of one or two HPLC systems, two pumps, two columns, an injector, interface, and detector. The columns are connected by a transfer device (either an interface or modulator such as a port-switching valve) that continuously collects effluent contents from the first (slow flow) column then re-injects the contents in small, pre-defined amounts onto the second (ultra-fast flow) column [158]. The latter step ensures that no information is lost as a result of under-sampling.

The unique capability of this technology provides a substantial benefit over traditional one-dimensional LC due to its ability to provide increased resolution and increased identification power resulting from the formation of 2D chemical class patterns. Interest in the unique technology has increased over the years and its effectiveness has been consistently demonstrated by many investigators in a variety of matrices [159].

Successful application of this technology requires that many experimental parameters be highly optimized and synchronized; requirements that often makes this method operator dependent and labor intensive. In addition, this technology is prone to problems associated with mobile phase immiscibility or mobile phase incompatibility [159]. Lastly, routine use of 2D-LC is most likely limited to laboratories possessing the appropriate hardware and software capable of quantifying LC $\times$ LC separation data [160] as well as personnel able to interpret the resulting data.

3. Simultaneous measurement of multiple lipid-phase antioxidants

Several methods have been developed for the simultaneous determination of multiple LPM (Table 2).

For quantitative analysis, it is imperative that investigators are able to demonstrate the quality of their methods (e.g. by means of accuracy and precision) in order to provide confidence in the suitability and applicability of their results. The evaluation of accuracy, the degree of closeness of the experimentally derived value to the true or accepted value, can be assessed in many ways: ideally, (1) by comparing the results of a test method to that of one that has been previously validated or (2) by comparing a sample of known concentration to a reference or high-purity sample for example the Fat-Soluble Quality Assurance Program from the National Institute of Standards and Technology (NIST; Gaithersburg, MD) for carotenoids and/or the Vitamin D External Quality Assurance Scheme (DEQAS; London, UK) [157].; or alternatively, (3) by spiking a pure constituent such as a standard (i.e. standard addition method) at varying levels into a blank or sample that does not contain the constituent of interest, then assaying the result and comparing it to the expected result; and (4) by determining the percent recovery of a pure compound that has been spiked into a sample at several varying concentrations; these concentrations should cover the entire range of concentrations analytically measureable under the given sampling conditions [161].

On the other hand, precision describes the degree of repeatability (variation) of replicate analyses of the same homogenous sample. As such, precision is most often expressed in terms of standard deviation, relative standard deviation, or coefficient of variation of a series of measurements. Precision can be sometimes further divided into: intra-assay (injection repeatability), where samples have been injected multiple times into an HPLC apparatus [126] or separate analyses within the same batch or day. In contrast, inter-assay (between-assay) repeatability reflects variation of results obtained from different days, among different analysts or equipment. In general, precision should be assessed as much as possible over an extended period of time and/or a period of time typical to that encountered by unknown samples in order to account for all factors that could naturally affect the results. This assures internal consistency within studies, the most important factor for reliable results. It is also important to keep in mind that the variation of precision is distinctly related to the concentration of the analytes being measured; with the variation typically increasing as the concentration increases [161].

Khan et al. [126] evaluated three commercially available C18 RP columns (Kromasil 100, Brownlee analytical C18, and Supelcosil™ LC-18) for sensitivity, selectivity and resolution. Best separations were made in isocratic mode using a mobile phase of methanol:water (99:1, v/v) at a flow rate of 1.0 mL/min or 1.5 mL/min for the 3 µm (Supelco) or 5 µm columns (Kromasil and Brownlee), respectively and UV detection at 292 nm. Of the three test columns, the Supelco (3 µm particle size) column had the fastest retention time (ca 3 min) and lowest mobile phase consumption, however, was shown to have the shortest shelf-life, back pressure problems, and problems involving peak broadening compared to the Kromasil and Brownlee columns, whose total run time approximated 6 min. Semeraro et al. [125] also measured these compounds simultaneously in 200 µL serum using the same RP Supelcosil™ LC-C18 column but diluted their serum with 300 µL PBS before deproteinization with a butanol:ethylacetate mixture containing BHT as a stabilizer. After centrifugation, the super-natant was directly injected to the HPLC instrument. Separation was achieved with isocratic elution of methanol with a 1.0 mL/min flow rate within 15 min. Their method improved upon the 1985 method of Nierenberg and Lester [163] by reducing serum volume from 500 µL to 200 µL, FD was introduced for retinol/retinyl acetate measurements (with 240 nm used for excitation and 520 nm for emission), and α-tocopherol/α-tocopheryl acetate were detected using their maximal absorption at 292 nm rather than at 280 nm. Another study by Khan et al. [164] also simultaneously measuring retinol and α-tocopherol evaluated the effects of various chromatographic conditions and experimental parameters to using HPLC–UV/vis (set at 292 nm). In contrast to their earlier study [126], serum volume was lowered to 100 µL, the flow rate was increased to 2.2 mL/min, and the water content in the mobile phase was decreased to 4%. Separation of 2 LPM plus retinyl acetate as internal standard was achieved in less than 7 min using a Kromasil 100 RP C18 column. Additionally, optimal column temperature was found to be 35 °C; at temperatures lower than this there was too much injection-to-injection peak area variability whereas temperature exceeding 35 °C resulted in decreased sensitivity and resolution.

Online solid-phase extraction (SPE) methods have been employed as a way to minimize intervention time while maximizing unattended analysis. Ferreiro-Vera used HPLC–DAD with SPE and a Novapac C18 column (3.9 mm × 100 mm, 4 µm) to separate major

carotenoids (lutein, zeaxanthin, β -cryptoxanthin, lycopene, and β -carotene) in 200 μ L serum [127]. Serum was crashed with 400 μ L tetrahydrofuran (THF), centrifuged, and the supernatant injected into the HPLC instrument for analysis with online SPE. Recovery and analyte retention was obtained using a dual cartridge test, which negated the use of an internal standard or spiking for validation. This method maximized efficiency and cleanup while minimizing operator involvement as well as cost. Using a linear gradient dual solvent system consisting of acetonitrile:water (90:10) and THF (100%), a flow rate set at 1.5 mL/min, and column temperature optimized at 20 °C, their analytes were resolved in 18 min.

Mata-Granados [165] measured retinol, α - and δ -tocopherol, and vitamin D metabolites (25(OH)D₃, 25(OH)D₂, D₃, and D₂) from 500 μ L serum within 20 min using DAD (monitoring at 325 nm, 292 nm, and 265 nm, respectively). To improve sample load in the recovery loop and sample recovery, serum samples were diluted with an acetonitrile:water (60:40, v/v) solution containing 150 nM sodium dodecyl sulfate prior to injection into a fully automated SPE system. Separation was performed using a Intersil ODS-2 C18 column (4.6 mm $\times$ 150 mm; 5 μ m) and a mobile phase consisting of acetonitrile:methanol (85:15, v/v) with 20 mM ammonium acetate using linear gradient was used that started at 1.0 mL/min for the first 5 min followed by increase to 1.5 mL/min. Their method was rapid and cheap, and involved minimal sample handling and no internal standard owing to the closed system that maximized protection against analyte degradation.

Using the same amount of serum (200 μ L) as the Ferreiro-Vera study [127], Thibeault et al. [162] was able to resolve the same compounds plus α -, β -, γ - and δ -tocopherol and retinol by using a hyphenated DAD-FD system. Samples were deproteinized using 200 μ L ethanol containing BHT followed by extraction twice with 800 μ L hexane and subsequent drying of the hexane phases under nitrogen prior to reconstitution in mobile phase. Separation was performed on a Thermo Spherisorb ODS2 column (3 mm $\times$ 250 mm, 3 μ m) using an isocratic mobile phase of acetonitrile:dichloromethane:methanol (7:2:1, v/v/v) with 20 mM ammonium acetate at a flow rate of 0.5 mL/min. Carotenoids were detected on a DAD with wavelength set at 455 nm, while FD was used for retinol ($\lambda_{\text{excitation}}$ = 326 nm and $\lambda_{\text{emission}}$ = 460 nm) and tocopherols ($\lambda_{\text{excitation}}$ = 290 nm and $\lambda_{\text{emission}}$ = 335 nm). All analytes eluted within 13 min.

The use of FD for identifying retinol and α -, γ -, and δ -tocopherol in 200 μ L plasma was investigated by Siluk and colleagues using a single and rapid approach [47]. In their method, liquid-liquid extraction was performed using 200 μ L water, 400 μ L ethanol, and 800 μ L hexane. The resulting supernatant was evaporated to dryness under a concentrator set at 45 °C, followed by reconstitution of the residue in methanol. Using a step gradient consisting of polar organic mobile phases methanol (100%) and acetonitrile (100%) and flow rates set at 1.0–1.2 mL/min, all compounds were separated on a Zorbax Eclipse XDB-C18 column (4.6 mm $\times$ 150 mm, 5 μ m) and eluted within 15 min. Detection for retinol was optimized using excitation and emission wavelengths of 325 and 470 nm, respectively, while analysis of tocopherol isomers used wavelengths of 295 and 300 nm.

Lee and colleague [128] developed a highly sophisticated HPLC-based method to quantify 16 LPM (including tocopherols, tocotrienols, retinol, carotenoids, ubiquinol, and

ubiquinone) from 30 μ L plasma within 30 min. Their method used a multiple-detector, column switching approach with two C18 RP columns and a gradient elution with 3 mobile phases. The two columns were kept at different temperatures (25 °C and 4 °C) to enhance separation of compounds of varying polarity and especially for the common coeluting lutein/zeaxanthin pair. The mobile phases were altered to best fit the solubility and stability of the varying compounds and the reagents and samples were kept on ice prior to and throughout the assay. Tocopherols were measured using FD while ubiquinol used ED; the remaining compounds, i.e. carotenoids, retinol, and ubiquinone, were detected using DAD. This method has distinct advantages over other methods in that multiple analytes were simultaneously measured in a chromatographic run 3-fold faster than when using single analyte assays are performed separately and used a plasma volume amount 2- to 10-fold less than other published methods. However, the considerably more complicated and precise set up (e.g. sample prep, temperature control, mobile phase composition) and more operator involvement than single detector use renders this method difficult to apply in routine clinical laboratory determinations.

Recently, a simple and fast HPLC method was developed to quantify 11 carotenoids, retinol, tocopherols, and CoQ10 from several different matrices including human plasma (250 μ L) in 35 min [148]. This method, in contrast to the method from Lee and Ong [128], was much simpler and involved the use of a single detection unit (DAD) and a single YMC C30 RP column with temperature held constant at 35 °C. Their gradient elution involved three mobile phases with flow rate set a 1.0 mL/min. The use of a C30 column also allowed the detection of several cis-isomer compounds not seen by Lee and others in addition to the separation of lutein and zeaxanthin. Using this method, however, the authors measured only one tocopherol homolog (α -tocopherol) in plasma in addition to β -carotene and α -cryptoxanthin. More recently, Turner and Burri [166] described a quick and simple procedure to analyze retinol, lutein + zeaxanthin, β -cryptoxanthin, lycopene, α - and β -carotene, and α - and γ -tocopherols from plasma using a minimal amount of solvent. In their method, 200 μ L plasma was protein precipitated with 1 mL ethanol then twice extracted with 1 mL hexane. HPLC-grade water (800 μ L) was combined with a nitrogen layer during extraction to complete the organic-aqueous phase separation. Samples were detected by DAD using isocratic elution with a mobile phase consisting of acetonitrile:dichloromethane:methanol (7:2:1) and flow rate at 0.5 mL/min. Separation of compounds were achieved on a Spherisorb ODS2 column (3.0 mm $\times$ 125 mm, 3 μ m) within 8 min.

HPLC can also be combined with MS to increase detection specificity thereby serving as a powerful analytical tool. Coupling MS with HPLC systems facilitates the quick and unambiguous assignment of compounds with observed spectral interferences. Priego Capote et al. [167] used HPLC–MSMS with ESI in positive mode and a Zorbax Eclipse XDB-C18 column (150 mm 4.6 mm, 5 μ m) to identify retinol, α - and δ -tocopherol, and $\times$ vitamins K and D from serum. Gradient elution was performed using mobile phases consisting of acetonitrile:water (90:10, v/v) and methanol (100%). This method was highly selective and sensitive in detecting these compounds, however, required a large sample (1 mL) and extraction reagent volume (3 mL MeOH, 9 mL hexane) per 27 min run. More recently, Midtturn [168] used HPLC–MSMS-ESI+ to determine retinol, α -tocopherol, and vitamin D

(25-hydroxyvitamin D₂ and D₃) from 50 µL of EDTA-plasma. An automated robot was used for sample preparation, which deproteinated sample using 100 µL ethanol followed by extraction with isooctane:chloroform (3:1, v/v), drying under nitrogen flow and reconstitution in 50 µL methanol. Separation was performed using a C18 column (4.6 mm × 50 mm; 2.7 µm) and gradient mobile phase consisting of methanol and water with flow rate set at 1.1 mL/min. All analytes eluted within 5 min.

The coupling of two [or more] columns in series, i.e. serial coupling, was first presented by Lyan [169], who combined two C18 columns (Nucleosil C18 to a Vydac C18) together and was able to resolve 13 carotenoids and their geometric isomers in plasma. Use of either column alone resulted in many co-eluting compounds including cis-trans isomers of lycopenes and β-carotene. Coupling of the two columns not only increased sensitivity but also resolved lutein and zeaxanthin, a commonly co-eluting pair. However, total retention time was moderately long (50 min) and required a fairly large amount (600 µL) of plasma. Since then, column coupling between C18 [154,170] as well as C30 [154] columns has gained attention. Karppi et al. [170] coupled two Synergy Hydro-RP 80A C18 columns to an HPLC–DAD system for detection of retinol, α-tocopherol, and 6 carotenoids from 200 µL plasma. In their method, extraction and sampling time was shortened by the addition of water during the extraction step. The addition of water (500 µL) during the extraction followed by freezing for 30 min (at –70 °C) enabled the investigators to decant, rather than pipette the upper organic (now frozen) aqueous layer. This decanting step eliminated the laborious pipetting and also prevented phase mixing which allowed for a cleaner sample thus improving precision and repeatability. Application of an isocratic elution using a tertiary mobile phase of acetonitrile:methanol:chloroform (60:25:15) and flow rate set at 1.0 mL/min allowed all compounds to elute within 35 min. In the past, only two columns of the same type were combined until we started analyzing LPM using a combination of C18 and C30 columns in series that included for the first time in a single chromatographic run the simultaneous analysis of circulating 25-hydroxy-vitamin D₃, 25-hydroxy-vitamin D₂, retinol, all major tocopherols and carotenoids including isomers, and oxidized and reduced coenzyme Q10 by HPLC with DAD [171].

Several investigators have detected LPM using UHPLC, which utilizes stationary columns packed with smaller diameter particles and requires higher pressures than conventional HPLC instrumentation resulting in better chromatographic efficacy and foremost higher speed. Granado-Lorencio et al. [157] assessed the suitability of using UHPLC with UV/vis detection for retinol, retinyl esters, tocopherols (α- and γ-), vitamin D, and the major carotenoids (lutein, zeaxanthin, α- and β-carotene, β-cryptoxanthin, and lycopene) from 300 to 500 µL serum. Samples were protein precipitated using 300–500 µL ethanol then twice extracted with 500 µL dichloromethane; gradient elution was performed using acetonitrile:methanol (85:15, v/v) and isopropanol (100%) with flow rate set at 0.5 mL/min. Separation was achieved on a HSS T3 column (2.1 mm × 100 mm, 1.8 µm) with the total run time of 4.5 min. In this method, vitamin D (measured as total 25(OH)D) analysis was less sensitive compared to previous HPLC–MS reports but comparable with regards to precision. Paliakov et al. [156] also used UHPLC to obtain an even faster run time of 2 min for the simultaneous analysis of retinol, γ + β-, and α- tocopherol, β-carotene, and CoQ10 using DAD. In their method, 500 µL serum was protein precipitated with 1 mL THF with

BHT and 500 μ L ethanol followed by addition of 3 mL (4%) NaCl and extraction with 4 mL hexane. Gradient elution was performed with mobile phases A (acetonitrile:water, 90:10, v/v) and B (methanol:isopropanol, 70:30, v/v) with flow rate set at 1.25 mL/min. Although all UHPLC methods are fast, they often lack selectivity.

4. Lipid-phase plasma/serum antioxidant levels in healthy populations

Umbilical cord and peripheral blood concentrations of selected carotenoids and retinol from children to adults are listed in Tables 3a–3c. Blood concentrations from pregnant and postpartum females are listed separated from adults in Table 3d. For cord blood levels, cord plasma concentrations relative to maternal peripheral plasma levels are provided (expressed as a percentage) when information was available. For all tables, a weighted mean and medians for each listed analyte is provided to account for number of samples used to provide the given value (n).

4.1. Umbilical cord

Median concentrations of all analytes varied across studies (Table 3a). Cord blood concentrations among studies were, on average, 8% (β -carotene and lycopene) to 67% (retinol) of maternal levels. Mixed results were found among studies separating data according to maternal smoking habit; for retinol, one study found higher concentrations in non-smokers vs. smokers while another study found the opposite; however, neither study had statistically significant results. For α -tocopherol and β -carotene, two studies observed higher concentrations in samples derived from non-smokers compared to smokers while a third study found no difference in β -carotene levels between the two different groups. Mixed results were also observed when mode of delivery was taken into account: mean cord α -tocopherol levels were significantly higher in mothers who underwent vaginal vs. cesarean delivery. A similar finding was found for β -carotene, however, this difference was not significant. For retinol levels, there were conflicting findings: one study found significantly higher levels from samples of vaginal delivery while another study did not.

4.2. Children and adolescents

Peripheral blood concentrations of selected carotenoids and retinol from healthy children and adolescents (ages 4 days–18 years) are listed in Table 3b. Median concentrations of all analytes varied across studies with the largest variation in lycopene levels (range 0.02–0.54 μ M). Consistent and similar concentrations among studies were found for lutein + zeaxanthin, γ -tocopherol, retinol, and β -cryptoxanthin, with concentrations varying 1.2–2.8-fold across studies. In general, mean concentrations of all analytes appeared to increase with age and did not seem to differ with gender in those studies separating males from females.

4.3. Adults

Peripheral blood concentrations of all analytes in healthy adults (ages > 18 years) were higher than those from cord and child/adolescent levels (Table 3c). Mean and median concentrations in adults varied much less overall and between genders among studies compared to younger aged individuals and cord blood. Aside from α -carotene, concentrations among other listed analytes varied only 1.3–6.4-fold among studies.

Blood concentrations (and weighted means) of analytes in pregnant and postpartum women overall were similar to or slightly higher than those in non-pregnant, non-lactating women (Table 3d). Among studies separating data according to smoking habit, no differences in retinol concentrations were observed between smokers and non-smokers; however, for β -carotene and α -tocopherol, smoking mothers had lower concentrations compared to non-smoking mothers. Among studies stratifying data by mode of delivery, similar values for retinol and β -carotene were found between mothers who underwent vaginal vs. cesarean delivery. For α -tocopherol, however, individuals who underwent cesarean delivery had higher concentrations compared to those who gave birth naturally.

Discrepancies in the reported LPM values shown in Tables 3a–3d can be due to a wide variety of factors. These include: (1) differences in the target population, whom may be exposed to a diversity of LPM in diet depending on age and health status, especially relevant for umbilical cord values, which are representative of those of the mother; (2) analytical procedures that do not follow cautionary laboratory practices such as including [relevant] internal standard(s), protecting samples from degradation by minimizing exposure to light and heat during handling and preparation, avoiding multiple freeze-thawing cycles, not using analyte stabilizers (e.g. BHT, ascorbic acid) and not choosing suitable extraction solvents (refer to Section 2.1.1); (3) the systems/methods not being validated (refer to Section 3). The medians reported in Tables 3a–3d, however, reflect a good estimate of ‘normal’ or accurate levels as it takes a better mid-point of measure than the mean, which is affected by extreme values that may not have been excluded during analysis.

5. Conclusion

This review provided an overview on new and improved HPLC-based detection techniques for the simultaneous analysis of circulating LPM within the last 5 years. Considerable progress has been made in this field over the years with regards to the amount and combination of analytes analyzed. Previous studies have focused on analyzing only a few or combinations of a few analytes together. However, the currently available detection techniques, which range in complexity from simple (UV/vis) to inherently challenging (2-D LC), labor demanding (MS/MS), time-consuming (serial-column coupling), very involved (multiple pump and detector systems), and miniaturized and expensive systems (UHPLC) in addition to novel reagent combinations, sample preparation and extraction procedures, varying hyphenated systems, and advanced columns, have not only enabled researchers to simultaneously analyze varying combinations of multiple LPM but also their geometric isomers all in less time, with increased sensitivity and selectivity, at lower costs, and with decreased serum/plasma volume requirements than before. These factors are crucial for large population studies that seek to understand disease causes and relationships with circulating LPM.

Abbreviations

CoQ10	coenzyme Q10
TQ10	total coenzyme Q10

UN10	ubiquinone-10
UL10	ubiquinol-10
LPM	lipid-phase micronutrients
FD	fluorescence detection
ECD	electrochemical detection
TLS	thermal lens spectrometry
MS	mass spectrometry
MSMS	tandem mass spectrometry
HILIC	hydrophilic interaction chromatography
2D-LC	two-dimensional liquid chromatography

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Table 1

HPLC phases and detectors.

Detection method/phases	Characteristics	Advantages	Disadvantages
UV, vis	Measures the light absorbance at a fixed wavelength in the ultraviolet or visible region, typically 190–800 nm. Multiple wavelengths can be monitored simultaneously with certain instruments	Enables detection of a wide variety of compounds containing a chromophore non-destructively. Able to monitor co-eluting compounds at variable wavelengths. Eluate can be used for other measurements or for preparative purposes Simple and inexpensive	Sensitivity dependent on chromophore in molecule Not highly specific for some molecules particularly those absorbing at low wavelengths Dependent on solution conditions Compound identification based exclusively on retention time and absorbance wavelength
DAD	Simultaneously and continuously measures absorbed light during the entire HPLC run throughout the UV/vis region, typically 190–800 nm at up to 200 Hz or higher in certain instruments	As UV/vis but can acquire all UV/vis absorbance data in a given run allowing UV/vis scans for all peaks detected and 3D images (time vs. intensity vs. wavelength). Characterization of peaks by UV/vis scans possible Simple and more expensive	As above but prone to larger noise than UV/vis detection Compound identification based exclusively on retention time and UV/vis absorbance pattern
FD	Monitors the emission of light, typically at 200–700 nm at up to 200 Hz, after 2D or 3D excitation of light at higher wavelength	Very sensitive Very specific (only compounds that fluoresce are detected) Minimum interference. Simple and inexpensive	Few compounds show native fluorescence Natively non-fluorescent compounds require pre-column or post-column derivitization techniques which are additional steps, often not applicable to certain analytes, and can contribute to quantitation errors
ED	Measures compounds that can be oxidized or reduced	Very sensitive	Requires electrolytes in the mobile phase and special equipment and detailed knowledge of electrochemistry. Measuring cells need to be cleaned often or replaced at relatively high cost. Software often not ideal Often plagued by inconsistent responses and interferences
MS	Measures molecules based on their mass and/or mass fragments when present charged in the gas phase	Very selective. Accurate quantitation, ideal internal standards by using stable isotopes of the analyte Provides structural information when exact masses can be determined (high resolution instruments) and by identifying distinctive fragmentation patterns. Ideal for multiple analytes unless isobaric compounds coelute	High cost of instrument acquisition, operation, and maintenance/repair Time-consuming maintenance and troubleshooting

Detection method/phases	Characteristics	Advantages	Disadvantages
MS/MS	Employs the use of two or more mass analyzers in a single instrument; used to provide structural information on ions produced by single-stage MS by fragmenting specific ions then identifying the fragments	Sample purity not important	Often unable to distinguish between isomers Requires experienced technical knowledge
		Allows more powerful structural information than single-stage MS	Same as MS
		Very selective Very accurate Sample purity not important	
TLS	Measures the rise in temperature in a sample that has been illuminated by an excitation laser	Very high sensitivity	Instruments often not commercially available or at very high cost
NP	Mode of chromatography entailing use of polar stationary phase and non-polar mobile phase	Effectively resolves common eluting pairs (e.g. γ - from β -tocopherol and lutein from zeaxanthin) and geometric isomers	Time-consuming maintenance and trouble-shooting Columns commercially unavailable
	Compounds interact with polar groups on column through hydrogen-bonding, electrostatic, and dipole-dipole interactions	Useful for very hydrophobic compounds (e.g. compounds with high lipid content)	Poor reproducibility
	Most hydrophilic compounds elute first		Sensitive to small changes in mobile phase composition Not ideal for gradient elution Entails use of solvents not miscible with water
HILIC	Mode of chromatography based on the liquid-liquid partitioning between the bonded polar stationary phase and mobile phase	Negates need for drying phase resulting from use of organic components thus shorter preparation time	pH sensitive
	Variation of NP chromatography, also called "reverse reverse-phase"	Higher robustness than NP	Relatively small variety of phases commercially available
	Stationary phase is polar and mobile phase composed of highly organic and some polar solvents	Modifiable to electrostatic repulsion HILIC (ERLIC)	
RP	Mode of chromatography entailing use of non-polar stationary phase and polar mobile phase	Many columns of various sizes and chemistries are commercially available and stable	Not suitable for very polar analytes
	Most commonly used chromatography mode	Highly reproducible	Eluate contain mostly water and are difficult to dry
	Most hydrophobic compounds elute first	Provides high resolution for a wide range of molecules	
	Stationary phase most commonly chemically modified with C8, C18, and C30 materials	Robust	

UV, ultraviolet; vis, visible; DAD, photo diode array detection; FD, fluorescence detection; ED, electrochemical detection; MS, mass spectrometry; MS/MS, tandem mass spectrometry; TLS, thermal lens spectrometry; NP, normal phase; HILIC, hydrophilic interaction chromatography; RP, reverse phase.

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Table 2

HPLC analysis of human circulating lipid-soluble antioxidants.

Matrix (amt per assay)	Analytes measured	Internal Standard(s)	Deproteinization solvent	Extraction solvent	Matrix concentration before LC injection	Injection volume [equivalent injected matrix amt]	Technique/detection	Stationary phase(s)
Serum (1 mL)	Retinol, K ₁ , K ₃ , 25(OH)D ₂ , 25(OH)D ₃ , 1,25(OH)D ₃ , D ₂ , D ₃ , αδ-tocopherol	Not used	MeOH	Hexane	3.3	100 µl [333 µL]	(+)ESI-MS/MS	C18
Plasma (0.2 mL)	Retinol, α/γ/δ-tocopherol	Retinol acetate	EtOH w/BHT	Hexane w/BHT	1.0	20 µL [20 µL]	HPLC/FD	C18
Plasma (0.2 mL)	Retinol, α-tocopherol, lutein, zeaxanthin, β-cryptoxanthin, lycopene, α-/β-carotene	α-Tocopherol acetate, β-apo-8'-carotenal	EtOH w/BHT	Hexane w/BHT	1.0	50 µL [50 µL]	HPLC/DAD	2-C18
Serum/Plasma (0.1 mL)	Retinol, retinyl palmitate, α-tocopherol, γ-tocopherol	Retinyl acetate	EtOH w/ascorbic acid	Hexane	2.0	15 µL [30 µL]	UHPLC/UV	C18
Plasma (0.03 mL)	Retinol, lutein, zeaxanthin, canthaxanthin, β-cryptoxanthin, lycopene, α-/β-carotene, UN10, UL10, δ/γ-tocopherol, β/γ/α-tocotrienol	Retinol acetate, tocopherol acetate, echinenone	EtOH:tert-BuOH w/BHT	Hexane	0.5	30 µL [15 µL]	HPLC/DAD-FD-ED	2-C18
Serum (0.5 mL)	Retinol, D ₂ , D ₃ , 25(OH)D ₂ , 25(OH)D ₃ , α/δ-tocopherol	Not used	MeCN:H ₂ O w/SDS	n/a, SPE	0.5	600 µL [300 µL]	HPLC/DAD	C18
Serum (0.5 mL)	Retinol, γ-tocopherol, β-carotene, CoQ10	Vitamin K ₁	THF w/BHT	Hexane	3.3	2 µL [6.67 µL]	UHPLC/DAD	C18
Serum (0.2 mL)	Retinol, α-tocopherol	Retinyl acetate, α-tocopheryl acetate	BuOH-Ethylacetate w/BHT	K ₂ HPO ₄	0.8	20 µL [16 µL]	HPLC/UV-vis-FD	C18
Serum (0.2 mL)	Lutein, zeaxanthin, β-cryptoxanthin, lycopene, α-/β-carotene, retinol, α/β/γ/δ-tocopherol	Echinenone, tocol	EtOH w/BHT	Hexane	2.0	20 µL [40 µL]	HPLC/DAD/FD	C18
Plasma (0.2 mL)	25-OHD ₃ , 25-OHD ₂ , 24,25-(OH) ₂ D ₃ , 1,25(OH) ₂ D ₃ , 1,25-(OH) ₂ D ₂	D6-1,25(OH) ₂ D ₃ , D6-1,25 (OH) ₂ D ₂ , D6-25-OH D ₃ , D6-25 OHD ₂	MeCN	n/a, SPE	0.25	10 µL [2.5 µL]	UHPLC/(+)ESI-MS/MS	C18
Serum (0.3-0.5 mL)	Retinol, retinyl palmitate, α/γ-tocopherol, total 25(OH)D, lutein + zeaxanthin, α-/β-carotene, α-/β-cryptoxanthin, lycopene	Not used	EtOH	Hexane:DCM	5.0	1 µL [5 µL]	UHPLC/UV-vis	C18
Serum (0.25 mL)	Retinol, α-tocopherol	Retinyl acetate	EtOH:MeOH	Hexane:DCM w/BHT	1.0	20 µL [20 µL]	HPLC/UV-vis	C18
Serum (0.1 mL)	Retinol, α-tocopherol	Retinyl acetate	EtOH w/BHT	Hexane:DCM	0.2	20 µL [4 µL]	HPLC/UV-vis	C18
Serum (0.2 mL)	Lutein, zeaxanthin, cryptoxanthin, lycopene, carotene	Not used	THF	n/a, SPE	0.3	500 µL [167 µL]	HPLC/DAD	C18
Plasma (1.5 mL)	D ₂ , D ₃ , 25(OH)D ₂ , 25(OH)D ₃	1α-(OH)D ₃	MeOH w/ASC	Heptane	7.5	50 µL [375 µL]	HPLC/UV	C30
Plasma (0.05 mL)	Retinol, 25(OH)D ₂ , 25(OH)D ₃ , α-tocopherol	² H ₆ -all trans retinol, ² H ₉ -α-tocopherol, ² H ₆ -25 hydroxyvitamin D ₃	EtOH w/BHT	Isooctane:CF	1.0	20 µL [20 µL]	HPLC/(+)-ESI-MS/MS	C18
Plasma (0.25 mL)	Retinol, α-tocopherol, lutein, zeaxanthin, β-cryptoxanthin, UL10, UN10, β-carotene, 9-cis-β-carotene, all <i>trans</i> -lycopene, 13-cis-lycopene, 9-cis-lycopene, 5-cis-lycopene	Echinenone	EtOH	Hexane	2.5	50 µL [125 µL]	HPLC/DAD	C30
Plasma (0.2 mL)	Retinol, lutein + zeaxanthin, β-cryptoxanthin, lycopene, α-/β-carotene, α-/γ-tocopherol	Tocol	EtOH w/BHT	Hexane	2.0	40 µL [80 µL]	HPLC/DAD	C18

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Matrix (amt per assay)	Analytes measured	Internal Standard(s)	Deproteinization solvent	Extraction solvent	Matrix concentration before LC injection	Injection volume [equivalent injected matrix amt]	Technique/detection	Stationary phase(s)
Plasma (0.2 mL)	Retinol, $\alpha\beta\delta\gamma$ -tocopherol, UN10, UL10, 25(OH)D ₂ , 25(OH)D ₃ , lutein, zeaxanthin, $\alpha\beta$ -cryptoxanthin, $\alpha\beta$ -carotene, lycopene, lycopene isomers, many non-identified carotenoids	1 α (OH)D ₃ , tocol, retinyl laurate, echinenone, Bu/Et-UL10, Bu/Et-UN10	ETOH w/BHT	Hexane	1.0–4.0	20 μ L (20–80 μ L)	HPLC/DAD	C18, C30
Mobile phase components (v/v ratio)								
			Flow rate (mL/min)	Total run time (min)	Precision	Accuracy	Notes	Novelty
Zorbax Eclipse XDB-C18 (4.6mm $\times$ 150mm; 5 μ m)	A: MeCN:H ₂ O (90:10) w/5 mM AmF; B: MeOH (100)w/5 mM AmF		1	27	Y	Y, E	Large sample volume required but vitamin K ₁ and K ₃ included	High selectivity and sensitivity [167]
Zorbax Eclipse XDB-C18 (4.6mm $\times$ 150mm; 5 μ m)	A: MeOH (100); B: MeCN (100)		1–1.2	15	Y	Y, E	Only 4 analytes, β - and γ -tocopherol not separated	Rapid (ca 10') method analyzing retinol + tocopherol isomers using step gradient and polar organic mobile phase [47]
Synergy Hydro-RP 80 A (4.6mm $\times$ 150mm; 4 μ m)	MeCN:MeOH:CF(60:25:15) w/0.01% BHT		1	35	Y	Y, A	No resolution of cis-isomers	Inclusion of ultrapure water and freezing step in sample prep to facilitate phase separation. Decanting vs. pipetting aq phase decreases labor and time [170]
Agilent Zorbax Rapid Resolution HT Eclipse Plus (4.6 $\times$ 50 mm, 1.8 μ m)	A: MeOH:H ₂ O (90:10); B: Ethyl acetate:IPA (90:10)		2	ca 2	Y	Y, A	Only 4 analytes but fast	Extremely Fast! (<2 min). Modification of existing method led to decreased run time, mobile phase consumption and sample injection volume [172]
Agilent Zorbax SB-C18 (3.9mm $\times$ 150mm; 4 μ m), Whaman Partisphere-5C18 (4.7mm $\times$ 110mm; 5 μ m)	A: MeCN (100); B: MeOH (100); C: ETOH:tert-BuOH (80:20)		ns	ca 30	Y	Y, E	UL10 = on-line pre-column reduction, β -tocopherol and β -tocotrienol not detected	Comprehensive chromatographic conditions with multi-channel chromatography and column switching. Run time 3 $\times$ faster and sample size 5 $\times$ smaller compared to separately run assays [128]
Intersil ODS-2 (4.6mm $\times$ 150mm; 5 μ m)	MeCN:MeOH (85:15)		1	20	Y	Y, E	Vitamin D and their 25-hydroxy metabolites included; only 7 analytes	Rapid (ca 13'), cheap, minimal sample handling, automatic valve switching and cartridge exchange [165]
Acquity Shield RP18 BEH (2.1 mm $\times$ 50mm; 1.7 μ m)	A:MeCN:H ₂ O (90:10);B: MeOH:IPA (70:30)		1.25	2	Y	Y, E	Only 4 analytes but fast	Extremely fast (2') method using UHPLC. No interferences. [156]
Supelcosil/TM LC-18 (4.6mm $\times$ 250mm; 5 μ m)	MeOH (100)		1	14.5	Y	Y, B	Only 2 analytes	Method fully validated and evaluation of measurement uncertainty [125]
Thermo Spherisorb ODS2 (3.0mm $\times$ 250mm; 3 μ m)	MeCN:DCM:MeOH(7:2:1) w/20 mM AmAct		0.5	13	Y	Y, B	No cis-isomers resolved. Lutein & zeaxanthin not separated	Use of titane frit packed column, low front pressure (<80 bars), low flow rate, dual internal standard (echinone + tocol) permitted simultaneous use of DAD and fluorescent detectors, relatively fast(13') [162]
Waters Acquity BEH C18 (2.1 mm $\times$ 50mm, 1.7 μ m)	A: H ₂ O w/0.1% FA + 5mM methylamine (100); B: MeOH w/0.1% FA (100)		0.3	<4	Y	Y, E	PTAD derivatization required after SPE followed by addition of methylamine, satisfactory recovery (55–85%)	Resolved all hydroxy vitamin D metabolites, low limit of quantification [173]
Acquity HSS T3 (2.1 mm $\times$ 100mm; 1.8 μ m)	A: MeCN:MeOH (85:15); B: IPA (100)		0.5	4.5	Y	Y, B, C	No internal standards!; no resolution of geometric isomers or 25(OH)D ₃ from 25(OH)D ₂	Fast (2.5') but low resolution [157]

Mobile phase components (v/v ratio)	Flow rate (mL/min)	Total run time (min)	Precision	Accuracy	Notes	Novelty	Reference
Kromasil 100 C18 (4.6mm × 150mm, 5 μm), Brownlee analytical (Perkin Elmer) C18 (4.6mm × 150mm, 5 μm), and Supelco™ LC-18 (3.0mm × 150mm, 3 μm)	1 for 3 μm, 1.5 for 5 μm	<12	Y	Y, E	3 different particulate types of C18 columns tested	Comparison of various reverse-phase particulate columns under various chromatographic conditions and experimental parameters	[126]
Kromasil 100 RP18 (4.6mm × 150mm; 5 μm)	2.2	<7	Y	Y, E	Only 2 analytes but fast	Optimization simultaneously determining all-trans retinol and α-tocopherol in serum, brain slices, and cosmetic products.	[164]
Novapak C18 (3.9mm × 100mm; 4 μm)	1.5	18	Y	Y, E	Not sensitive	Automated sample preparation (online SPE with automatic valve switching and cartridge exchange), closed system for continuous analysis	[127]
YMC Europe GmbH (4.6 mm × 250 mm; 5 μm)	1	ca 25	Y	Y, B	Vitamin D and their 25-hydroxy metabolites included; large sample volume required (1.5 mL)	Clean separation of vitamin D metabolites. Use of C30 column, MeCN-free MP	[150]
Ascentis Express (4.6 mm × 50 mm; 2.7 μm)	1.1	5	Y	Y, C	Only 4 analytes but fast	Fast (5'), low plasma volume requirement, simple and automated (robotic) sample preparation	[168]
YMC (4.6 mm × 250 mm; 5 μm)	1	35	Y	Y, C	Typical G30 run but UL10 and UN10 included	Included UL10 and UN10 determination	[148]
Waters Spherisorb ODS2 (3.0 mm × 125 mm; 3 μm)	0.5	8	Y	Y, B	Lutein and zeaxanthin not separated, cis-isomers not resolved	Short analysis time HPLC (8 min), low solvent usage (ca 5 mL per sample)	[166]
Kinetex C18, ProntoSIL C30 (3.0mm × 150 mm; 2.6 μm 2.0 mm × 150 mm; 3 μm)	0.4	15–42	Y	Y, B, C	All analytes included in one run; simple system or sophisticated with valve but always one HPLC system	Combination analysis of all analytes or parts of analytes with shorter run times; C18 + C30 columns alone or in series; custom design to analytical needs; little sample relative to number of analytes	[171]

Precision; Y = done, N = not done/not stated; Accuracy; Y = done, N = not done/not stated; (A) another proficient lab/another established method; (B) NIST; (C) DEQAS; (D) other Round Robins; (E) standard addition method/recovery; equiv. amt sample inject = amt starting mat/reconstituted amt × injection volume; conc. factor = equiv. amt sample inject/injection volume; Bu/Et-UL10, Bu/Et-UN10 = butylated/ethylated ubiquinol-10, butylated/ethylated ubiquinone-10; *a* + *b* = isomers are in one peak; *a/b/c* = geometr. Isomers are determined separately.

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Table 3a

Healthy umbilical cord blood values.^a

Gender	Gestational age, weeks	Maternal smoking habit	Delivery mode	n	ret	α-car	β-car	β-crx	lyc	lut + zea	α-toc	δ-toc	HPLC method ^{b,c}	Matrix	Reference
M/F	Mean 39	S/NS		375	0.55								DAD ^b	na	[175]
M/F	Mean 39.5	S/NS		107	0.59								DAD ^b	na	[175]
M/F	Mean 39.6	S/NS		112	0.71								DAD ^b	na	[175]
na	Mean 40	NS	vd/cs	17							6.70		ECD ^b	EDTA-plasma	[176]
				<i>d</i>							20%				
M	26–36	na	vd/cs	123	0.54						7.00		na	Serum	[177]
F	26–36	na	vd/cs	119	0.56						8.00		na	Serum	[177]
M	37-<42	na	vd/cs	129	0.70						6.00		na	Serum	[177]
F	37-<42	na	vd/cs	119	0.76						6.00		na	Serum	[177]
na	Mean 39.8	S/NS	na	175	1.10								UV ^b	Serum	[178]
na	Mean 39.6	S/NS	na	138	1.27								UV ^b	Serum	[178]
na	Mean 39	na	na	222	1.29								Spectrophotometric dosage	Serum	[179]
				<i>d</i>	76%										
na	Term	na	na	40	1.01	0.01	0.04	0.04	0.04	0.13	7.21		^b na	Heparin plasma	[180]
				<i>d</i>	54%	20%	17%	29%	10%	28%	35%				
na	36–42	S	vd/cs	296	0.88		0.01				4.21		FD ^c	Heparin plasma	[181]
				<i>d</i>	66%		2%				14%				
na	36–42	NS	vd/cs	405	0.92		0.02				4.28		FD ^c	Heparin plasma	[181]
				<i>d</i>	71%		4%				13%				
na	36–42	S/NS	vd	634	0.91		0.02				4.30		FD ^c	Heparin plasma	[181]
				<i>d</i>	68%		3%				14%				
na	36–42	S/NS	cs	64	0.83		0.01				3.76		FD ^c	Heparin plasma	[181]

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Gender	Gestational age, weeks	Maternal smoking habit	Delivery mode	<i>n</i>	ret	α-car	β-car	β-crx	lyc	lut + zea	α-toc	δ-toc	HPLC method ^{b,c}	Matrix	Reference
M/F	37–42	S	na	62	75%	2%	0.03				14%		UV ^b	Serum	[182]
M/F	37–42	NS	na	153		0.05	10%						UV ^b	Serum	[182]
M/F	Mean 39.5	na	na	143	0.69						15.90		UV ^b	Serum	[183]
M/F	34–42	na	vd	56	61%						62%		DAD ^b	Arterial EDTA-plasma	[184]
M/F	34–42	na	vd	56	62%						18%		DAD ^b	Venous EDTA-plasma	[184]
M/F	34–42	na	cs	56	0.99						5.49		DAD ^b	Arterial EDTA-plasma	[184]
M/F	34–42	na	cs	56	69%						15%		DAD ^b	Venous EDTA-plasma	[184]
M/F	Mean 39.7	S/NS	na	10	0.79	0.01	0.03		0.01	0.06	3.63		DAD ^b , FD ^b	EDTA-plasma	[185]
M/F	na	na	na	27	47%	5%	4%		3%	15%	11%		UV ^b / _{vis} ^b , FD ^b	EDTA-plasma	[186]
M/F	Mean 39.6	na	vd/cs	251	0.67	0.01	0.03		0.04		7.74	0.05	DAD ^b	EDTA-plasma	[187]
M/F	31–42 (median 40)	NS	vd/cs	33	51%	17%	11%		8%		20%		UV ^b	EDTA-plasma	[188]
				<i>d</i>	55%	9%	7%	13%	7%	18%	19%				
				<i>d</i>	0.67	0.09	18%				6.52				
				<i>d</i>	69%						19%				

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Gender	Gestational age, weeks	Maternal smoking habit	Delivery mode	<i>n</i>	ret	α-car	β-car	β-crx	lyc	lut + zea	α-toc	δ-toc	HPLC method ^{<i>b c</i>}	Matrix	Reference
M/F	30–41 (median 38)	S	vd/cs	33	0.81	0.09	0.09				4.70		UV ^{<i>b</i>}	EDTA-plasma	[188]
				<i>d</i>	80%	27%					16%				
M/F	Term	na	vd/cs	100	0.73	0.004	0.02	0.02	0.02	0.06	4.97		DAD ^{<i>b</i>}	Plasma	[174]
			Weighed mean	0.84	0.03	0.03	0.04	0.02	0.08	5.88	1.07				
			Medians	0.83	0.01	0.03	0.04	0.02	0.08	6.00	0.43				
				Relative to mother ^{<i>d</i>}	67%	13%	8%	21%	8%	18%	17%				

S, smoker during pregnancy; NS, non-smoker during pregnancy; vd, vaginal delivery; cs, cesarean delivery; na, not available; α-car, α-carotene; β-car, β-carotene; β-crx, β-cryptoxanthin; lyc, lycopene; lut + zea, lutein + zeaxanthin; α-toc, α-tocopherol; β + γ-toc, β + γ-tocopherol; δ-toc, δ-tocopherol.

^{*a*}Mean values expressed as μmol/L.

^{*b*}Reverse phase.

^{*c*}Normal phase.

^{*d*}Percent of maternal supply.

Table 3b

Peripheral blood values in healthy children and adolescents.^a

Age, years	n	ret	α -car	β -car	β -crx	lyc	lut + zeo	α -toc	γ -toc	δ -toc	RP-HPLC method	Reference
<i>Males</i>												
0.4–6.25	35	1.09	0.04	0.20		0.15		15.70			DAD	[189]
6–9	36	0.80									UV/vis	[190]
9–12	55	0.94									UV/vis	[190]
12–14	45	1.05									UV/vis	[190]
12–17	153	1.57	0.05	0.22	0.15	0.54	0.22	16.90			DAD	[191]
14–16	41	1.12									UV/vis	[190]
16–18	60	1.22									UV/vis	[190]
<i>Females</i>												
0.75–6.7	35	1.10	0.06	0.17		0.16		17.80			DAD	[189]
6–9	33	0.77									UV/vis	[190]
9–12	57	0.84									UV/vis	[190]
10–19	118	0.92									UV	[192]
12–14	44	0.91									UV/vis	[190]
12–17	132	1.50	0.06	0.24	0.14	0.50	0.23	17.30			DAD	[191]
14–16	37	1.05									UV/vis	[190]
16–18	57	1.08									UV/vis	[190]
<i>Males/females</i>												
na	49	0.99	0.03	0.12	0.09	0.02	0.09				HPLC	[193]
na	32	0.90	0.01	0.09	0.06	0.02	0.11				HPLC	[193]
na	41	0.94	0.01	0.07	0.06	0.02	0.10				HPLC	[193]
4 days ^b	18	0.72		0.11				20.88			UV	[188]
4 days ^c	15	0.65		0.09				21.10			UV	[188]
0–4	187	1.12									UV	[194]
1–6	676	1.02	0.06	0.16	0.15	0.08	0.46				UV/vis	[195]
Mean 6	56	2.44						21.95			UV	[196]
2–11	50	1.10	0.04	0.31	0.12	0.07		17.71			UV/vis	[197]
2–3	15							7.82	2.15		UV	[198]

Age, years	n	ret	α -car	β -car	β -crx	lyc	lut + zea	α -toc	γ -toc	δ -toc	RP-HPLC method	Reference
3–4	100	0.98									HPLC	[199]
4–5	7							5.34	1.87		UV	[198]
6–7	839		0.07	0.34	0.21		0.35				DAD	[200]
10,13	156		0.08	0.34	0.17	0.20		17.86	1.44		HPLC	[201]
Mean 16	16	1.87	0.04	0.40				17.10		0.15	UV, FD	[202]
Weighted mean	1.12	0.06	0.25	0.17	0.18	0.35	17.53	1.52				
Median values	0.94	0.05	0.12	0.08	0.12	0.10	17.45	1.87				

a-car = α -carotene; β -car = β -carotene; β -crx = β -cryptoxanthin; lyc = lycopene; lut + zea = lutein + zeaxanthin; α -toc = α -tocopherol; γ -toc = γ -tocopherol; δ -toc = δ -tocopherol.

^aMean or median plasma or serum values expressed as $\mu\text{mol/L}$.

^bBorn to exclusively non-smoking mothers.

^cBorn to exclusively smoking mothers.

Table 3c

Peripheral blood values in healthy adults^a.

Age, years	n	ret	α -car	β -car	β -crx	lyc	lut + zeax	α -toc	γ -toc	δ -toc	RP-HPLC method	Reference
<i>Males</i>												
18–45	5193 ^b		0.05	0.20	0.17	0.48	0.25				DAD	[203] ^f
18–45	1585 ^c		0.06	0.22	0.17	0.45	0.23				DAD	[203] ^f
18–45	604 ^d		0.04	0.19	0.16	0.40	0.22				DAD	[203] ^f
18–45	414 ^d		0.05	0.15	0.12	0.35	0.17				DAD	[203] ^f
18–63	159	1.77		0.29		0.71		21.30			UV/vis	[204]
19–62	45	2.20	0.06	0.46		0.35		30.00			UV/vis	[205]
20–29	218	1.93	0.19	0.65	0.3	1.02		28.47	3.75	0.24	DAD, FD	[173]
20–45	87			0.51		0.47		28.97			UV/vis	[206]
24–44	40	2.24						26.2	1.87		DAD	[207]
24–45	33	2.18						23.9	2.3		DAD	[207]
24–45	38	2.24						26.9	1.28		DAD	[207]
25–45	32	2.01						27.4	1.14		DAD	[207]
26–46	32	2.05						26.00	1.64		DAD	[207]
46–75	89			0.46		0.46		28.24			UV/vis	[206]
46–90	5193 ^b		0.09	0.36	0.11	0.56	0.41				DAD	[203] ^f
46–90	1585 ^c		0.10	0.36	0.12	0.49	0.42				DAD	[203] ^f
46–90	604 ^d		0.09	0.37	0.11	0.56	0.41				DAD	[203] ^f
46–90	414 ^d		0.08	0.324198 ^f	0.11	0.55	0.41				DAD	[203] ^f
65+	252		0.06	0.20	0.11	0.47	0.14				DAD	[208]
Mean 40	284		0.08	0.24	0.17	0.62	0.32				DAD	[209]
Mean 51	92		0.08	0.39	0.31	1.29	0.66				DAD	[210]
Mean 52	1487			0.33				27.5			DAD	[211]
Mean 52	1307			0.18				31.80			na	[212]
Mean 54	97		0.12	0.39	0.26	1.01	0.62				DAD	[210]
Mean 54	99		0.06	0.25	0.35	0.66	0.41				DAD	[210]

Age, years	n	ref	α -car	β -car	β -crx	lyc	lut + zeax	α -toc	γ -toc	δ -toc	RP-HPLC method	Reference
Mean 54	97		0.08	0.21	0.30	0.53	0.41				DAD	[210]
Mean 54	98		0.11	0.37	0.17	0.60	0.34				DAD	[210]
Mean 54	99		0.17	0.48	0.16	0.62	0.38				DAD	[210]
Mean 54	99		0.11	0.30	0.13	0.46	0.34				DAD	[210]
Mean 55	95		0.08	0.40	0.33	0.90	0.55				DAD	[210]
Mean 55	97		0.07	0.24	0.42	0.69	0.44				DAD	[210]
Mean 55	99		0.25	0.53	0.19	0.98	0.47				DAD	[210]
Mean 55	98		0.16	0.41	0.14	0.72	0.30				DAD	[210]
Mean 55	97		0.08	0.29	0.17	0.54	0.35				DAD	[210]
Mean 55	99		0.15	0.37	0.14	0.56	0.32				DAD	[210]
Mean 56	121	2.13	0.11	0.47		0.82		27.10	1.44		Vis-FD	[213]
Mean 57	99		0.15	0.31	0.11	0.58	0.33				DAD	[210]
Mean 61	777	2.05		0.32	0.17	0.89	0.26	31.99			DAD	[214]
Mean 65	180		0.06	0.21	0.12	0.43	0.25				na	[215]
Mean 70	264			0.18							UV/vis	[216]
Mean 73	252		0.06	0.20	0.11	0.47	0.14				DAD	[208]
Mean 54	99		0.15	0.42	0.32	1.03	0.73				DAD	[210]
<i>Females</i>												
18–45	4643 ^b		0.02	0.15	0.15	0.44	0.29				DAD	[203] ^f
18–45	1554 ^c		0.03	0.19	0.16	0.43	0.30				DAD	[203] ^f
18–45	1200 ^d		0.00	0.10	0.15	0.45	0.27				DAD	[203] ^f
18–45	1414 ^d		0.02	0.19	0.14	0.41	0.28				DAD	[203] ^f
18–63	101	1.74		0.88		1.31		17.30			UV/vis	[204]
19–45	33	1.97						27.6	1.55		DAD	[207]
19–62	66	1.90	0.07	0.58		0.41		30.40			UV/vis	[205]
20–29	491	1.96	0.12	0.39	0.23	1.09		27.61	4.33	0.30	DAD, FD	[173]
20–45	81			0.58		0.73		24.45			UV/vis	[206]
24–43	39	2.1						25.5	2.32		DAD	[207]
24–45	33	1.83						26.5	1.74		DAD	[207]
25–45	37	1.87						26.5	1.05		DAD	[207]

Age, years	n	ref	α -car	β -car	β -crx	lyc	lut + zea	α -toc	γ -toc	δ -toc	RP-HPLC method	Reference
25–45	32	1.78						28.3	0.88		DAD	[207]
46–75	37			0.63		0.77		22.13			UV/vis	[206]
46–90	4643 ^b		0.12	0.45	0.12	0.52	0.43				DAD	[203] ^f
46–90	1554 ^c		0.13	0.54	0.10	0.50	0.43				DAD	[203] ^f
46–90	1200 ^d		0.12	0.47	0.11	0.52	0.41				DAD	[203] ^f
46–90	1414 ^d		0.12	0.44	0.12	0.53	0.42				DAD	[203] ^f
65+	293		0.09	0.30	0.14	0.7	0.15				DAD	[208]
Mean 28	13	1.69						23.07			DAD	[184]
Mean 40	287		0.12	0.30	0.23	0.66	0.39				DAD	[209]
Mean 47	2034			0.44				28.2			DAD	[211]
Mean 47	1821			0.28				30.90			na	[212]
Mean 52	100		0.19	0.67	0.53	1.32	0.81				DAD	[210]
Mean 53	186	1.91	0.12	0.67		0.76		26.20	1.44		Vis-FD	[213]
Mean 54	99		0.23	0.62	0.48	0.90	0.81				DAD	[210]
Mean 54	99		0.16	0.55	0.35	0.90	0.77				DAD	[210]
Mean 54	100		0.11	0.36	0.48	0.69	0.43				DAD	[210]
Mean 54	99		0.32	0.68	0.24	0.89	0.53				DAD	[210]
Mean 54	99		0.24	0.53	0.21	0.77	0.37				DAD	[210]
Mean 54	99		0.23	0.64	0.27	0.69	0.44				DAD	[210]
Mean 54	97		0.20	0.54	0.20	0.52	0.34				DAD	[210]
Mean 55	99		0.13	0.53	0.44	0.87	0.63				DAD	[210]
Mean 55	98		0.11	0.38	0.52	0.74	0.47				DAD	[210]
Mean 55	100		0.10	0.28	0.47	0.43	0.39				DAD	[210]
Mean 55	99		0.22	0.65	0.25	0.54	0.44				DAD	[210]
Mean 55	99		0.12	0.37	0.27	0.47	0.40				DAD	[210]
Mean 55	99		0.29	0.66	0.24	0.44	0.37				DAD	[210]
Mean 57	98		0.22	0.47	0.23	0.53	0.41				DAD	[210]
Mean 59	839	1.91		0.40	0.19	0.89	0.27	34.50			DAD	[214]
Mean 65	220		0.08	0.32	0.17	0.43	0.27				na	[215]
Mean 70	204			0.28							UV/vis	[216]

Age, years	n	ref	α-car	β-car	β-crx	lyc	lut + zea	α-toc	γ-toc	δ-toc	RP-HPLC method	Reference
Mean 74	293		0.09	0.30	0.14	0.7	0.15				DAD	[208]
<i>Males/females</i>												
20–29	740							20.92			DAD	[217]
20–70+	4087							27.39	4.79		DAD	[217]
30–39	712							24.17			DAD	[217]
40–49	666							28.54			DAD	[217]
42–65	20	1.82						29.40	5.10		FD	[218]
50–59	515							32.50			DAD	[217]
60–69	684							35.14			DAD	[217]
70+	770							35.01			DAD	[217]
Mean 32	25		0.10	0.28	0.42	0.41	0.23				DAD	[171]
Mean 55	291	1.95	0.02	0.17	0.01	0.35	0.14	20.63	2.54		na	[219]
Weighted mean	1.96	0.08	0.32	0.15	0.54	0.34	28.82	4.23				
Median all values	1.91	0.08	0.30	0.14	0.14	0.48	0.28	29.40	1.94			
Mean males	2.12	0.07	0.29	0.14	0.52	0.30	28.03	3.75				
Median males	2.12	0.06	0.22	0.12	0.49	0.26	28.55	3.75				
Mean females	1.84	0.07	0.35	0.14	0.51	0.33	27.06	1.93				
Median females	1.90	0.08	0.31	0.14	0.14	0.48	0.29	28.30	1.50			

Number of individuals per age category per gender for each marital status is unknown. na, information not available; α-car, α-carotene; β-car, β-carotene; β-crx, β-cryptoxanthin; lyc, lycopene; lut + zea, lutein + zeaxanthin; α-toc, α-tocopherol; β + γ-toc, β + γ-tocopherol; δ-toc, δ-tocopherol.

^a mean plasma or serum values expressed as μmol/L.

^b Married.

^c Never married.

^d Divorced.

^e Widowed.

^f _n represents number of individuals within gender and marital status category of ages 18–90 years.

Table 3d

Peripheral blood values in healthy pregnant and postpartum individuals.^a

Age, years	Gestation stage at blood draw	Smoking habit	Delivery mode	n	ret	α -car	β -car	β -crx	lyc	lut +zea	α -toc	γ -toc	δ -toc	HPLC method ^{b,c}	Reference
19–43	14 weeks	na	na	35	1.37	0.06	0.30		0.62		27.26		0.17	UV/vis ^b , FD ^b	[186]
16–40	10–20 weeks	na	na	66	1.88	0.05	0.23	0.14	0.4	0.46	20.65	2.17	0.35	RP-HPLC	[180]
15–44	20 weeks	S	vd/cs	492	1.39		0.44				22.98			FD ^c	[181]
15–44	36–42 weeks	S	vd/cs	486	1.33		0.56				30.95			FD ^c	[181]
15–44	20 weeks	NS	vd/cs	599	1.41		0.50				23.6			FD ^c	[181]
15–44	36–42 weeks	NS	vd/cs	582	1.29		0.63				32.00			FD ^c	[181]
15–44	20 weeks	S/NS	vd	1003	1.4		0.47				23.31			FD ^c	[181]
15–44	36–42 weeks	S/NS	vd	983	1.33		0.6				31.85			FD ^c	[181]
15–44	20 weeks	S/NS	cs	88	1.4		0.5				23.47			FD ^c	[181]
19–43	22 weeks	na	na	35	1.33	0.06	0.27		0.55		33.25		0.20	UV/vis ^b , FD ^b	[186]
19–43	32 weeks	na	na	35	1.11	0.05	0.24		0.57		38.90		0.24	UV/vis ^b , FD ^b	[186]
15–44	36–42 weeks	S/NS	cs	85	1.11		0.55				27.71			FD ^c	[181]
16–34+	37–42 weeks	S	na	62			0.27							UV ^b	[182]
16–34+	37–42 weeks	NS	na	153			0.45							UV ^b	[182]
Mean 25	Mean 39.5 weeks	na	na	143	1.13						25.7			UV ^b	[183]
Mean 28	Postpartum	na	vd	35	1.44						37.48			DAD ^b	[184]
Mean 28	Postpartum	na	cs	21	1.47						28.29			DAD ^b	[184]
Mean 29	Postpartum	S/NS	na	10	1.67	0.19	0.69		0.32	0.41	32.18	3.73		DAD ^b , FD ^b	[185]
19–43	Postpartum	na	na	32	1.32	0.06	0.28		0.48		38.59		0.17	UV/vis ^b , FD ^b	[186]
18–35	Postpartum	NS	vd/cs	251	1.33	0.33	0.87	0.32	0.30	0.50	38.73			DAD ^b	[187]
16–36	Postpartum	NS	vd/cs	18	0.97		0.49				34.47			UV ^b	[188]

Age, years	Gestation stage at blood draw	Smoking habit	Delivery mode	n	ret	α-car	β-car	β-crx	lyc	lut +zea	α-toc	γ-toc	δ-toc	HPLC method ^{b,c}	Reference
16–43	Postpartum	S	vd/cs	14	1.01	0.33					30.20			UV ^b	Lai and Franke [188]
22–36	Postpartum	NS	vd/cs	19							33.4	1.9		ECD ^b	[176]
Mean 26	Postpartum	na	na	222	1.7									Spectrophotometric dosage	[179]
		Weighted mean	1.37	0.21	0.53	0.28	0.39	0.49	28.14	2.28	0.25				
		Median all values	1.33	0.06	0.47	0.23	0.48	0.46	30.95	2.17	0.20				
		Median smoking			1.33		0.39				30.20				
		Median non-smoking			1.31	0.33	0.57	0.32	0.30	0.50	33.24				
		Median vaginal delivery	1.40		0.54				31.85						
		Median cesarean section	1.40		0.53				27.71						

NS, non-smoker, S, smoker; na, information not available; vd, vaginal delivery, cs, cesarean section; α-car, α-carotene; β-car, β-carotene; β-crx, β-cryptoxanthin; lyc, lycopene; lut + zea, lutein + zeaxanthin; α-toc, α-tocopherol; β + γ-toc, β + γ-tocopherol; δ-toc, δ-tocopherol.

^aMean plasma or serum values expressed as μmol/L.

^bReverse phase.

^cNormal phase.