analytical chemistry

Article

pubs.acs.org/ac

Combined Measurement of 6 Fat-Soluble Vitamins and 26 Water-Soluble Functional Vitamin Markers and Amino Acids in 50 μL of Serum or Plasma by High-Throughput Mass Spectrometry

Øivind Midttun,[*,†] Adrian McCann,[†] Ove Aarseth,[†] Marit Krokeide,[†] Gry Kvalheim,[†,‡] Klaus Meyer,[†] and Per M. Ueland[‡,§]

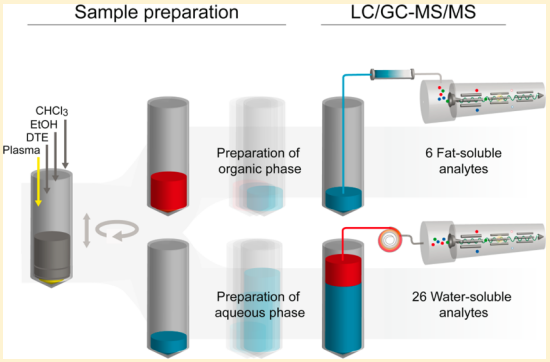
[†]Bevital AS, Laboratory Building, Ninth Floor, Jonas Lies veg 87, 5021 Bergen, Norway

[‡]Department of Clinical Science, University of Bergen, 5021 Bergen, Norway

[§]Laboratory of Clinical Biochemistry, Haukeland University Hospital, 5021 Bergen, Norway

Supporting Information

ABSTRACT: Targeted metabolic profiling characterized by complementary platforms, multiplexing and low volume consumption are increasingly used for studies using biobank material. Using liquid−liquid extraction, we developed a sample workup suitable for quantification of 6 fat- and 26 water-soluble biomarkers. 50 μL of serum/plasma was mixed with dithioerythritol, ethanol, and isooctane/chloroform. The organic layer was used for analysis of the fat-soluble vitamins all-trans retinol (A), 25-hydroxyvitamin D2, 25-hydroxyvitamin D3, α-tocopherol (E), γ-tocopherol (E), and phylloquinone (K1) by LC-MS/MS. The remaining aqueous fraction was mixed with ethanol, water, pyridine, and methylchloroformate (in toluene) to derivatize the water-soluble biomarkers. The resulting toluene layer was used for GC-MS/MS analysis of alanine, α-ketoglutarate, asparagine, aspartic acid, cystathionine, total cysteine, glutamic acid, glutamine, glycine, histidine, total homocysteine, isoleucine, kynurenine, leucine, lysine, methionine, methylmalonic acid, ornithine, phenylalanine, proline, sarcosine, serine, threonine, tryptophan, tyrosine, and valine. Isotope-labeled internal standards were used for all analytes. Chromatographic run times for the LC-MS/MS and GC-MS/MS were 4.5 and 11 min, respectively. The limits of detection (LOD) for the low-concentration analytes (25-hydroxyvitamin D2, 25-hydroxyvitamin D3, and phylloquinone) were 25, 17, and 0.33 nM, respectively, while all other analytes demonstrated sensitivity significantly lower than endogenous concentrations. Recoveries ranged from 85.5−109.9% and within- and between-day coefficients of variance (CVs) were 0.7−9.4% and 1.1−17.5%, respectively. This low-volume, high-throughput multianalyte assay is currently in use in our laboratory for quantification of 32 serum/plasma biomarkers in epidemiological studies.



Interest in circulating levels of nutrients and metabolites, such as vitamins and amino acids, has grown significantly in recent years, and there is a growing need for high performance assays for quantification of nutritional biomarkers. Such assays are important for investigating the multifaceted mechanisms and biological systems underlying certain health and disease processes. Circulating concentrations of fat-soluble vitamins, amino acids, and metabolically related compounds are influenced by factors such as intake[1,2] and disease status.[3−8] An increased understanding of the complex metabolic interactions can be achieved by analyzing large panels of metabolically related biomarkers.[9]

In epidemiological and clinical studies, only limited amounts of precious biobank material are often available, and assays that are capable of combined measurement of several analytes simultaneously using a small sample volume are therefore desirable. However, development of such multianalyte assays represents a methodological challenge, and the combinations of analytes that can be included in an assay are restricted by several factors. These issues include the large spread in analyte concentrations, chemical diversity (fat- and water-soluble), as well as protein binding of analytes. Consequently, each analytical assay may quantify only a restricted number or a chemical class of analytes. Therefore, the sample volume consumption generally increases with the number of biomarkers included in a study.

Specifically, some methods for the simultaneous quantification of several fat-soluble vitamins in serum/plasma have been published.[10−17] Most of these methods are constrained by relatively long retention times (15−35 min)[10−13,16] and/or large sample volume requirements.[10,12,14−16] Only one of these methods[12] is capable of quantifying phylloquinone at its low endogenous concentrations. The downside of this method is the consumption of a very large sample volume of 1 mL.

Received: June 16, 2016
Accepted: October 7, 2016
Published: October 7, 2016

DOI: 10.1021/acs.analchem.6b02325
Anal. Chem. 2016, 88, 10427−10436

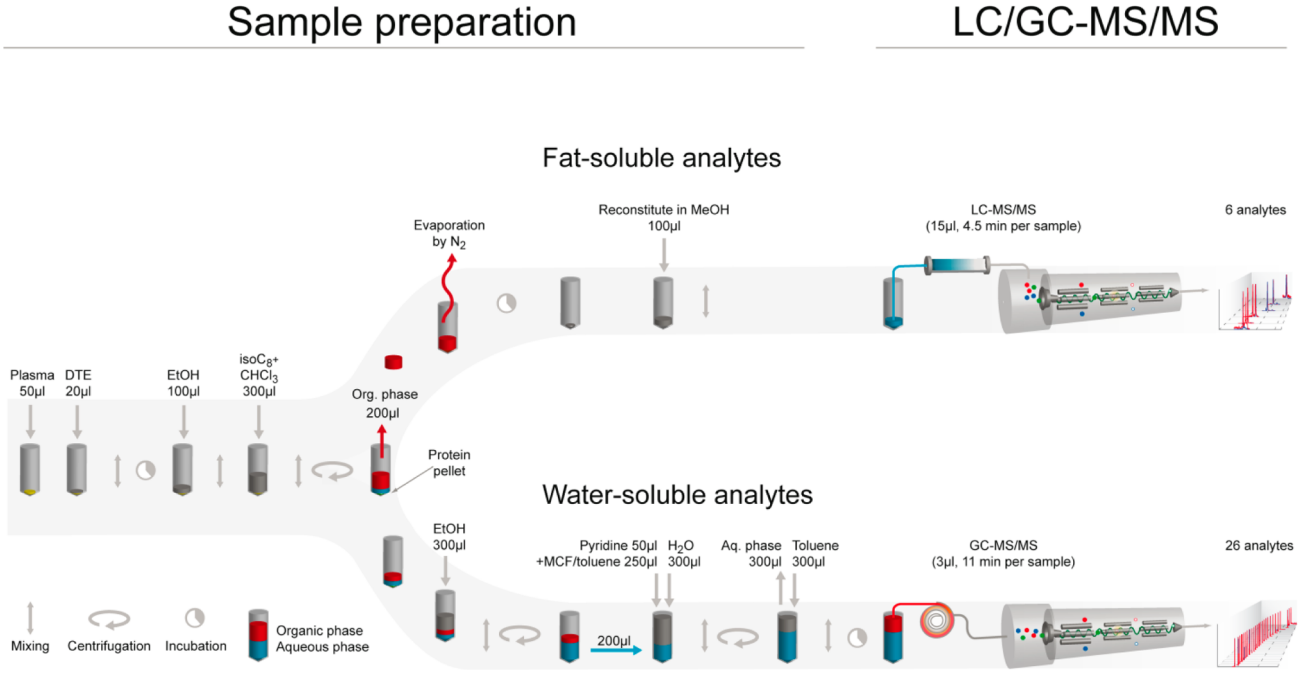


Figure 1. Work flow for combined sample preparation. Based on liquid−liquid extraction (LLE) principles, 50 μL of serum/plasma was mixed with dithioerythritol (DTE), ethanol (EtOH), and isooctane/chloroform (isoC8+CHCl3), and then centrifuged. The resulting organic layer containing the fat-soluble vitamins was transferred to a new plate, evaporated under nitrogen (N2), and then reconstituted in methanol (MeOH) containing butylated hydroxytoluene (BHT), before analysis by LC-MS/MS. The aqueous fraction was mixed with ethanol, water, pyridine, and methylchloroformate (MCF) in toluene to derivatize the water-soluble biomarkers (making them collect in the organic toluene phase) for analysis by GC-MS/MS. Isotope-labeled internal standards were used for all analytes. Chromatographic run times for the LC-MS/MS and GC-MS/MS were 4.5 and 11 min, respectively.

Similarly, methods capable of quantification of several water-soluble amino and organic acid compounds have also been published,[18−23] but are often restricted by factors such as few analytes[23] or lack of internal standards,[18] or again, long retention times.[19,20,22]

Targeted approaches to metabolic profiling investigates the analytes within defined pathways, in contrast to untargeted metabolomics, which often captures only abundant analytes, retaining the inherent weakness of possible assay interference. Normally, targeted analytical techniques are restricted to measuring analytes with similar chemical properties and concentration ranges. When the target analytes are chemically diverse and span a wide range of concentrations, multiple methods and multiple techniques are often required. Thus, few methods allowing quantification of both fat- and water-soluble analytes in single assays have been published. These methods utilize solid phase extraction (SPE) followed by independent chromatographic separation,[24] time-consuming LC separations,[25−27] or a combined LC/supercritical fluid chromatography method.[28]

In liquid−liquid extraction (LLE) the extraction solvent is chosen to optimize the solubility of the analytes, and LLE is used to extract analytes from different liquid sample types,[29,30] including serum. For analysis of fat-soluble analytes, sample preparation often consist of a protein precipitation step followed by LLE, which is performed by addition of an organic solvent to the mixture. The fat-soluble analytes accumulate in the organic phase, which is then used for analysis. Conveniently, the water-soluble analytes are retained in the aqueous phase, which can also be carried on to subsequent analytical steps. Although the physical-chemical principle of separating water- and fat-soluble biomarkers by LLE is simple, due to a number of inherent challenges including those referred to above, published methods describing combined sample preparation and subsequent quantitative analysis of both fat and water-soluble compounds are comparatively rare.[31,32]

Here, we describe the use of LLE to increase both the number of target biomarkers, as well as the chemical diversity of analytes, which can be analyzed using only 50 μL of serum/plasma. Despite low sample volume consumption and a wide range in analyte concentrations, this high-throughput assay enables targeted quantification of 6 fat-soluble and 26 water-soluble biomarkers. The assay is particularly attractive for use in biobank studies where available sample volume is limited.

EXPERIMENTAL SECTION

Materials. Details of materials used are included in Supporting Table 1. Standard Reference Material (SRM) 968e, 972a, 1950 were obtained from National Institutes of Standards and Technology (NIST, Gaithersburg, MD). Individual stock solutions of fat-soluble compounds were prepared on ice in methanol with butylated hydroxytoluene (BHT; 1 g/L), while water-soluble compounds were prepared in phosphate-buffered saline (PBS). The stock solutions of all-trans retinol, α-tocopherol, γ-tocopherol, $^2H_6$-all-trans retinol, $^2H_9$-α-tocopherol, and $^2H_4$-γ-tocopherol were prepared in red light under nitrogen atmosphere. $^2H_6$-all-trans-retinol contained 4% all-trans-retinol, $^2H_4$-γ-tocopherol contained 5% γ-tocopherol; no other isotope labeled internal standards contained unlabeled compounds. All stock solutions were stored at −80 °C until use.

Sample Collection and Preparation. Current Norwegian regulations categorize method development within the Quality Control Category, which is exempt from review by the

DOI: 10.1021/acs.analchem.6b02325
Anal. Chem. 2016, 88, 10427−10436

Table 1a. LC-MS/MS Retention Times and Instrument Settings[a]

analyte	T_r (min)	transition ions (m/z) precursor	product	DP (V)	EP (V)	CE (V)	CXP (V)
$^2H_6$-25-hydroxyvitamin D3	1.28	407.2	371.4	50	12	20	25
25-hydroxyvitamin D3	1.28	401.4	365.4	50	12	20	25
$^2H_6$-all-trans retinol	1.29	276.5	97.2	30	12	30	10
all-trans retinol	1.30	270.1	94.2	30	12	30	10
$^2H_6$-25-hydroxyvitamin D2	1.30	419.2	337.2	40	12	15	10
25-hydroxyvitamin D2	1.30	413.5	355.5	40	12	15	10
$^2H_4$-γ-tocopherol	1.89	421.5	155.0	30	14	30	15
γ-tocopherol	1.90	417.5	151.0	30	14	30	15
$^2H_9$-α-tocopherol	2.02	440.4	146.4	30	12	60	15
α-tocopherol	2.04	431.4	137.4	40	12	60	15
$^2H_4$-phylloquinone	2.75	455.2	191.2	40	12	30	15
phylloquinone	2.78	451.2	187.2	40	12	30	15

[a]Abbreviations: T_r, retention time; DP, declustering potential; EP entrance potential; CE, collision energy; CXP, collision cell exit potential

Institutional Review Board. Serum was obtained by collecting blood into Vacutainer Tubes with no additive. Blood was allowed to clot at room temperature for 30 min before isolation of the serum fraction. All samples were stored at −80 °C until use. Pooled EDTA plasma from healthy individuals was purchased from Innovative Research, Inc. (www.innov-research.com). The endogenous analyte concentrations of this plasma were estimated by the standard addition method using the current assay. For routine analyses a spiked version of the pooled plasma was used as calibrator, and the analyte concentrations of this spiked plasma are given in Supporting Table 2.

Sample processing (illustrated in Figure 1) was performed by a robotic workstation (MicrolabAT Plus, Reno, NV, USA) fitted with Hamilton disposable tips with liquid detection. The samples were protected from unnecessary light at all stages. In 1 mL Costar polypropylene plates, 50 μL of serum/plasma was mixed with 20 μL of D,L-dithioerythritol (DTE; 37.5 mM) containing the internal standards $^2H_4$-homocystine and $^2H_2$-cysteine (30 and 800 μM, respectively) and incubated at room temperature for 5 min to reduce the disulfide bonds of homocystine and cystine symmetric and mixed disulfides. Deproteinization was then obtained by adding 100 μL of ethanol containing the remaining internal standards (concentrations included in Supporting Table 2). This was followed by rigorous mixing for 20 s. Three hundred microliters of isooctane/chloroform (3:1, v/v) was then added, followed by mixing and 5 min of centrifugation (4000 g/4 °C).

Processing of the organic phase was carried out as follows: 200 μL of the organic layer (containing the fat-soluble analytes) was transferred to a new plate, and the solvent in this plate was evaporated at 35 °C under a flow of nitrogen gas, using a TurboVap96 (Caliper Lifescience, Hopkinton, MA, USA). The resulting material was then reconstituted in methanol (100 μL) containing BHT (1 g/L), and vigorously mixed for 20 s. The plate was then sealed and placed in the cooled autosampler compartment of the LC-MS/MS system.

Processing of the aqueous phase was performed using the following steps: To the remaining sample volume, 300 μL of ethanol was added, the plate was then vigorously mixed for 20 s followed by 5 min of centrifugation (6000 g/4 °C). 200 μL of the aqueous phase was then transferred to a new plate, to which 300 μL of water, 50 μL of pyridine, and 250 μL of 200 mL/L methylchloroformate (MCF) in toluene (to derivatize the water-soluble analytes) were added. Mixing was achieved by repeated pipetting. Following incubation at room temperature for 6 min to obtain phase separation, 300 μL of the aqueous phase was then removed and replaced with 300 μL of toluene, and the samples were mixed again before the plates were sealed and transferred to the cooled autoinjector sample tray on the GC-MS/MS system.

Instruments: LC-MS/MS for Quantification of Fat-Soluble Analytes. Fat-soluble analytes were analyzed using an Agilent 1290 Infinity LC system (binary pump, degasser, autosampler with thermostat, column compartment), and an API 5500 QTRAP tandem mass spectrometer from AB Sciex fitted with an atmospheric pressure chemical ionization (APCI) ion source. The instruments were controlled by Analyst software version 1.6.3. Prepared samples were kept at 4 °C in the autosampler compartment under subdued light. Fifteen microliters of each sample was injected into a Poroshell 120 (50 × 4.6 mm, particle size 2.7 μm) stable-bond C18 column, positioned in a thermostated compartment at 20 °C. The two-component mobile phase was delivered at a flow rate of 1600 μL/min according to the following time table: 0−0.1 min, 80% methanol/20% water; 1.0−3.30 min, 2.5 mM ammonium formate in methanol; 3.35−4.5 min, 80% methanol/20% water. Samples were injected every 5.33 min, and delivered to the mass spectrometer via splitless injection. Mass spectrometric parameters were optimized by infusing 100−200 μM solutions of each analyte at a rate of 5.0 μL/min. These solutions were mixed with 2.5 mM ammonium formate in methanol delivered at a flow rate of 1000 μL/min, using the T-junction located at the ion-source of the mass spectrometer. For sample runs we used scheduled positive mode multiple reaction monitoring (MRM) with a detection window of 50 s, and a target scan time of 0.4 s at unit resolution for both Q1 and Q3. The following parameters were identical for all analytes and internal standards: collision-activated dissociation gas (medium), ion source temperature (450 °C), curtain gas (10 psig), ion source gas 1 (50 psig) and 2 (70 psig) and needle current (4 μA). The acquisition parameters including retention times, protonated precursor and product ions, as well as ion-pair specific parameters for the analytes and internal standards, are summarized in Table 1a.

Instruments: GC-MS/MS for Quantification of Water-Soluble Analytes. An Agilent 7890B GC system coupled to an Agilent 7010 GC-MS triple quad mass spectrometer was

DOI: 10.1021/acs.analchem.6b02325
Anal. Chem. 2016, 88, 10427−10436

used in the electron ionization mode for the analysis of the water-soluble analytes. Mass spectrometric parameters were optimized by injecting 500 μM solutions of each analyte at a rate of 1.2 mL/min. These prepared solutions were repeatedly injected enabling identification of suitable precursor and product ions, and optimal dwell time and collision energies. Prepared samples were kept at 4 °C in the autoinjector sample tray and a 3.0 μL aliquot of the toluene layer from each sample containing the derivatized water-soluble analytes was injected every 12.5 min and delivered via splitless injection. The analytes were separated on a CP Sil 24-CB low-bleed/MS capillary column from Varian (15 m, 0.25 mm [i.d.]; film thickness 0.25 μm). The initial oven temperature of 75 °C was increased at a rate of 45 °C/min to 85 °C, and maintained for 1 min. The oven temperature was then increased at a rate of 45 °C/min to 125 °C followed by a further increase at 60 °C/min to 260 °C. This temperature was maintained for 2.5 min, before a final increase at 60 °C/min to 310 °C, which was maintained for 1.5 min. Helium was used as carrier gas and delivered at a constant flow rate of 1.2 mL/min, while pressure increased linearly from 25.7 to 94.6 kPa during the run. The interface temperature was 290 °C, the source temperature was 250 °C, and the electron energy was 70 eV. Scheduled MRM was used, and the acquisition parameters, including retention times, protonated precursor and product ions, dwell time, and collision energy, for each analyte and corresponding internal standard are summarized in Table 1b.

Quantification. Serum/plasma concentrations were calculated by dividing the analyte peak area by the area of the corresponding deuterated internal standard and comparing these ratios with the area ratios obtained from calibrator plasma with known analyte concentrations.

Matrix Effects. We investigated matrix effects for the LC-MS/MS system by postcolumn infusion[33] of a solution containing 150 μmol/L all-trans retinol, 16.9 μmol/L 25-hydroxyvitamin D2 and 25-hydroxyvitamin D3, 843.6 μmol/L α-tocopherol, 422.5 μmol/L γ-tocopherol and 844 nmol/L phylloquinone. Signal intensities during the infusion were recorded from injections of mobile phase, treated blank (water) and 12 different plasmas.

Limit of Detection, Lower Limit of Quantification, and Linearity. Limit of detection (LOD), lower limit of quantification (LLQ) and linearity were determined by preparing 24 PBS/methanol solutions containing all the analytes. Signal-to-noise (S/N) ratios were calculated for each analyte measured by LC-MS/MS using the S/N-Script (Analyst software version 1.6.3), or as the peak height of the analyte divided by the standard deviation of the baseline noise using the Agilent MassHunter Workstation software (version B.07.00) for analytes measured by GC-MS/MS. LODs were determined as the lowest concentrations that gave S/N values higher than three, and LLQs as two times LOD. Serum concentrations of all-trans retinol, α-tocopherol, γ-tocopherol, and all analytes quantified by GC-MS/MS (see Supporting Table 3) were very high compared to LOD and LLQ, so we did not perform a detailed study of these parameters for these analytes. Linearity parameters were assessed by linear regression of the peak area ratios of each analyte to its corresponding internal standard using concentrations above LLQ and up to indications of detector saturation for each analyte.

Precision and Recovery. Serum from three presumed healthy persons was pooled, and because of low endogenous

Table 1b. GC-MS/MS Retention Times and Instrument Settings[a]

analyte	T_r (min)	transition ions (m/z) precursor	transition ions (m/z) product	DT (ms)	CE (V)
$^2H_3$-methylmalonic acid	2.57	177.0	103.0	15	2
methylmalonic acid	2.58	174.0	100.0	15	2
$^2H_3$-alanine	2.96	178.0	105.0	8	6
alanine	2.97	175.0	102.0	8	6
$^2H_2$-glycine	3.05	163.0	90.0	8	6
glycine	3.06	161.0	88.0	8	6
$^2H_3$-sarcosine	3.08	178.0	105.0	8	6
sarcosine	3.09	175.0	102.0	8	6
$^2H_2$-valine	3.25	146.0	100.0	6	10
valine	3.26	144.0	98.0	6	10
$^2H_{10}$-leucine	3.29	154.0	90.0	6	8
leucine	3.31	144.0	88.0	6	8
$^2H_{10}$-isoleucine	3.33	154.0	90.0	6	8
isoleucine	3.35	144.0	88.0	6	8
$^2H_6$-α-ketoglutarate	3.52	133.0	105.0	8	8
α-ketoglutarate	3.53	129.0	101.0	8	8
$^2H_2$-threonine	3.60	134.0	58.0	15	8
threonine	3.61	132.0	56.0	15	8
$^2H_3$-proline	3.72	204.0	131.0	8	8
proline	3.73	201.0	128.0	8	8
$^2H_3$-$^{15}N_2$-asparagine	3.74	132.0	60.0	8	8
asparagine	3.75	127.0	56.0	8	8
$^2H_3$-aspartic acid	3.94	145.0	98.0	15	6
aspartic acid	3.95	142.0	96.0	15	6
$^2H_5$-glutamine	3.99	146.0	114.0	10	8
glutamine	4.00	141.0	109.0	10	8
$^2H_3$-serine	4.01	179.0	103.0	10	5
serine	4.01	176.0	100.0	10	5
$^2H_4$-methionine	4.12	239.0	162.0	8	6
methionine	4.13	235.0	161.0	8	6
$^2H_5$-glutamic acid	4.16	193.0	119.0	8	6
glutamic acid	4.17	188.0	114.0	8	6
$^2H_5$-phenyl-$^2H_3$-alanine	4.29	186.0	154.0	8	6
phenylalanine	4.29	178.0	146.0	8	6
$^2H_2$-cysteine	4.36	208.0	152.0	8	6
total cysteine	4.37	206.0	150.0	8	6
$^2H_4$-homocysteine	4.58	237.0	178.0	8	5
total homocysteine	4.59	233.0	174.0	8	5
$^2H_3$-ornitine	4.80	237.0	148.0	8	6
ornitine	4.81	230.0	142.0	8	6
$^2H_9$-lysine	5.04	268.0	151.0	10	3
lysine	5.05	259.0	142.0	10	3
$^2H_3$-histidine	5.38	229.0	153.0	10	3
histidine	5.39	224.0	152.0	10	3
$^2H_2$-tyrosine	5.68	296.0	222.0	15	6
tyrosine	5.69	294.0	220.0	15	6
$^2H_5$-tryptophan	7.30	295.0	220.0	8	5
tryptophan	7.31	290.0	215.0	8	5
$^2H_4$-cystathionine	7.39	164.0	116.0	8	4
cystathionine	7.40	160.0	114.0	8	4
$^2H_3$-kynurenine	7.54	208.0	164.0	10	4
kynurenine	7.55	204.0	160.0	10	4

[a]Abbreviations: T_r, retention time; DT, dwell time; CE, collision energy.

DOI: 10.1021/acs.analchem.6b02325
Anal. Chem. 2016, 88, 10427−10436

Table 2. Analytical Recovery and Precision of the Assay[a]

	concentration				within-day CV (N = 19), (%)			between-day CV (N = 20), (%)		
	endogenous diluted 1:1	endogenous	endogenous + added	recovery (%)						
analyte	low	medium	high	high	low	medium	high	low	medium	high
25-hydroxyvitamin D3 (nM)	37.8	75.5	83.5	82.5	9.4	8.3	8.6	9.2	6.6	4.8
all-trans retinol (μM)	0.92	1.84	5.18	103.7	5.4	4.5	3.2	3.6	4.2	4.0
25-hydroxyvitamin D2 (nM)	<LLQ	57.4	140.8	101.7		8.5	8.3		14.2	10.4
γ-tocopherol (μM)	1.17	2.33	7.39	94.3	2.9	3.1	2.9	4.4	4.3	3.5
α-tocopherol (μM)	16.8	33.6	74.1	98.2	4.1	4.1	3.8	5.7	5.8	9.1
phylloquinone (nM)	1.12	2.24	3.60	91.3	6.3	8.1	5.7	17.5	14.3	8.1
methylmalonic acid (μM)	0.07	0.13	0.25	102.4	4.5	3.7	3.2	2.8	2.9	2.6
alanine (μM)	208	417	625	109.9	1.4	2.1	1.2	2.0	1.3	1.8
glycine (μM)	148	296	358	99.2	1.7	2.2	1.3	1.5	1.5	2.9
sarcosine (μM)	0.72	1.39	3.21	101.8	1.4	2.3	1.3	1.8	1.7	1.3
valine (μM)	133	262	332	98.4	3.0	2.8	2.9	1.7	1.4	3.9
leucine (μM)	67.7	135	250	99.8	2.4	2.3	1.8	1.5	1.3	4.0
isoleucine (μM)	32.3	63.9	204	101.1	2.0	2.3	1.5	1.4	1.5	3.5
α-ketogluturate (μM)	3.89	7.61	14.0	95.4	4.5	3.7	2.2	3.0	3.7	3.2
threonine (μM)	71.0	138	250	98.8	3.2	3.9	0.7	2.1	1.8	4.0
proline (μM)	115	224	322	103.6	2.3	2.0	2.1	1.6	1.7	4.1
asparagine (μM)	25.7	49.0	111	98.4	4.1	2.5	3.1	3.1	2.5	2.7
aspartic acid (μM)	11.9	24.8	47.3	95.6	1.5	2.0	1.8	2.9	4.4	4.6
glutamine (μM)	299	599	804	92.0	1.8	2.6	1.1	2.6	2.0	2.7
serine (μM)	65.6	130	250	101.2	1.7	2.1	1.4	1.6	1.5	3.9
methionine (μM)	12.1	23.9	46.8	97.7	1.1	2.1	1.6	1.7	1.8	1.2
glutamic acid (μM)	27.2	54.6	69.9	100.3	1.7	2.6	1.5	4.3	4.2	3.1
phenylalanine (μM)	34.5	69.8	202	98.4	1.3	1.5	1.4	2.2	2.4	3.6
total cysteine (μM)	100	179	264	107.1	0.8	2.4	1.5	1.2	1.5	2.6
total homocysteine (μM)	4.88	9.50	13.6	106.7	1.1	2.6	1.0	1.1	1.1	1.2
ornithine (μM)	40.6	81.0	121	102.4	3.0	2.5	1.3	1.8	1.6	3.9
lysine (μM)	87.8	176	285	102.3	1.5	2.5	1.3	1.8	2.0	4.0
histidine (μM)	46.9	92.3	225	102.3	1.6	2.3	1.2	1.4	1.5	4.1
tyrosine (μM)	36.0	63.7	195	97.2	2.5	2.2	1.1	3.1	1.9	4.7
tryptophan (μM)	29.3	57.5	116	98.4	1.4	2.2	1.5	1.7	1.8	2.9
cystathionine (μM)	0.12	0.22	0.49	107.8	1.8	1.8	0.7	2.1	2.5	3.6
kynurenine (μM)	0.89	1.70	2.81	101.9	1.6	1.9	3.2	3.0	2.6	2.8

[a]Recoveries and within-day CVs were calculated from the same experiments. 25-Hydroxyvitamin D2 concentrations were below the LLQ for the low concentration between- and within-day experiments.

concentrations spiked with 40 nmol/L 25-hydroxyvitamin D2. The pooled serum was then divided into three portions. One portion was diluted 1:1 using PBS, creating a solution that contained a "low" concentration of the analytes. A second, undiluted, portion containing endogenous levels (except for 25-hydroxyvitamin D2) represented a "medium" concentration. The third portion of pooled serum assigned "high" concentration, was spiked with all analytes, to concentrations given in Table 2. Within-day precision (CV) was investigated by analyzing 19 replicates of these low, medium and high sera on the same day. The results from the high and medium concentrations were used to calculate the recovery (%) as

$$\text{recovery} = \frac{\text{measured concentration} - \text{endogenous concentration}}{\text{added concentration}} \times 100\%$$

Between-day precision was estimated by analyzing the sera containing low, medium and high analyte concentrations on 20 different days over a period of 27 days.

Accuracy: Serum Concentrations in Presumed Healthy Individuals. We investigated analyte concentrations in serum collected from 163 anonymous, presumed healthy Norwegians (39.9% female) with mean age 44.4 (range: = 21−68) years. Accuracy of quantification was verified by participation in the Vitamin D External Quality Assessment Scheme (DEQAS, www.deqas.org) for 25-hydroxyvitamin D3, Vitamin K External Quality Assurance Scheme (KEQAS, www.keqas.com) for phylloquinone, and the Danish Institute for External Quality Assurance for Hospital Laboratories (DEKS, www.deksonline.dk) for methylmalonic acid (MMA) and total homocysteine (tHcy). Results from the presented assay were compared with the target value of each external quality assessment scheme of 18−20 samples (from the period 2013−2015) by Passing-Bablok regression, using the Excel tool from Acomed Statistics (www.acomed-statistics.com).

Trueness. We analyzed the NIST SRM materials 968e (fat-soluble vitamins), 972a (vitamin D metabolites), and 1950 (amino acids and fat-soluble vitamins) in duplicate on 5 different days to investigate the trueness for available analytes included in the assay. The trueness was expressed as percent recovery of the NIST-certified and NIST-reference values.

DOI: 10.1021/acs.analchem.6b02325
Anal. Chem. 2016, 88, 10427−10436

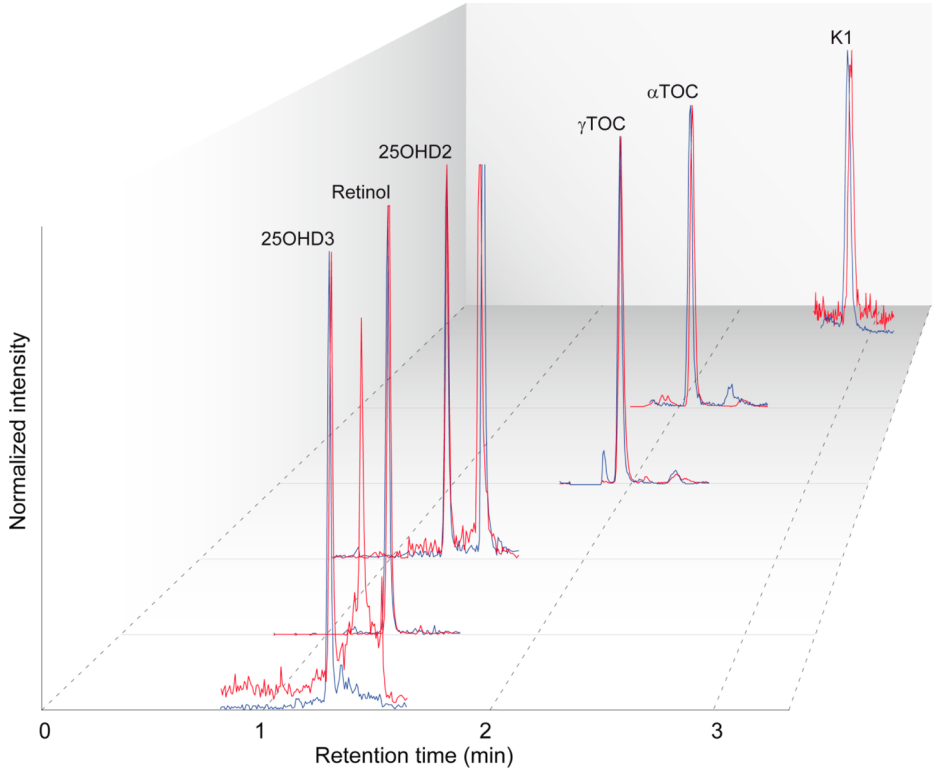


Figure 2. Normalized LC-MS/MS Chromatograms of Fat-soluble Vitamins in Calibrator Plasma. Concentrations are given in Supporting Table 2. Abbreviations: 25OHD3, 25-hydroxyvitamin D3; Retinol, All-trans retinol; 25OHD2, 25-hydroxyvitamin D2; γ-TOC, γ-tocopherol; α-TOC, α-tocopherol; K1, Phylloquinone.

RESULTS AND DISCUSSION

The present work utilized both the organic and the aqueous phases from liquid−liquid extraction (LLE) to quantify 6 fat- and 26 water-soluble biomarkers in only 50 μL of serum/plasma. All sample handling was performed by a robotic workstation, chromatographic run times for the LC-MS/MS and GC-MS/MS were 4.5 and 11 min respectively, and sample throughput was 86 samples/24 h.

Sample Treatment, Stability of Analytes, and Internal Standards. Sample storage and treatment protocol were guided by published data on analyte stability. Samples and analyte stock solutions were stored at −80 °C to avoid degradation of analytes and internal standards. In order to avoid degradation of all-trans retinol[34] and α-tocopherol,[35] which are susceptible to oxidation, we used butylated hydroxytoluene (BHT) as an antioxidant when reconstituting the fat-soluble vitamins. We observed no degradation of analytes or internal standards during storage of prepared samples at 4 °C under subdued light in the autosampler compartments.

DTE, which was used as reductant to liberate homocysteine and cysteine from their symmetric and mixed disulfides, may consume chloroformate and cause assay interference.[35] However, quantification of the analytes were similar in the absence and presence of DTE (data not shown). Further, we observed that DTE reduced the signal intensity of phylloquinone and $^{2}H_{4}$-phylloquinone, so the amount of this reagent was kept at a minimum (final centration of 57 mM), ensuring only a small difference in intensity when comparing samples with and without DTE (data not shown).

MCF was used to derivatize the water-soluble analytes, which were analyzed as N(S)-methoxycarbonyl ethyl esters extracted into toluene, by GC-MS/MS.[36,37] For protein precipitation we also avoided using acid, which may consume methylchloroformate (MCF) and form pyridine salts that negatively influence GC column lifetime.[38] Furthermore, the use of deuterated internal standards in this method corrects for variable derivatization, which may occur if the amount of MCF becomes limiting.

After centrifugation of the mixture containing serum/plasma, DTE in water, ethanol and the isooctane/chloroform solvent, three distinct layers were formed: a protein pellet at the bottom of the well, an intermediate aqueous layer, and an organic layer at the top (see Figure 1). The use of ethanol as protein-precipitating agent made it possible to use the organic and the aqueous phase for analysis of fat- and water-soluble analytes, respectively. The layering formed by combining chloroform with isooctane also made transfer of organic and aqueous phases to new plates easy in comparison to methods using only chloroform, which can result in a protein precipitate located between a lower organic phase and an upper aqueous phase.[39] Another advantage of LLE is that lipids will collect in the organic phase, thus preventing possible adverse effects of such material on the GC column.[38]

For routine runs, each 96-well plate contained 86 serum/plasma samples, 6 calibrator samples, 3 control samples, and one blank (deionized water). Potential cross-contamination between wells in the microtiter plate was prevented by using appropriate well- and liquid volumes,[30] as well as establishing suitable mixing and pipetting procedures.

Analytical Platform: LC-MS/MS. The chromatographic run time was 4.5 min, with samples injected every 5.33 min, giving a total run-time for each plate of 8.5 h.

DOI: 10.1021/acs.analchem.6b02325
Anal. Chem. 2016, 88, 10427−10436

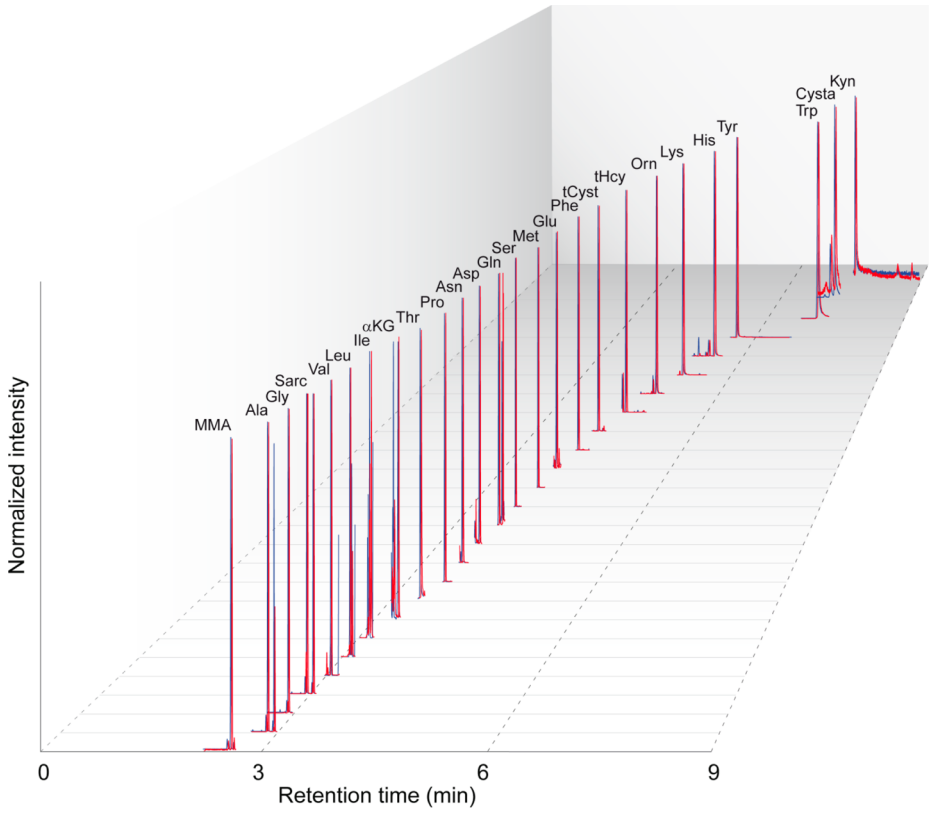


Figure 3. Normalized GC-MS/MS chromatograms of water-soluble functional vitamin markers and Amino Acids in Calibrator Plasma. Concentrations are given in Supplemental Table 2. Abbreviations: MMA, methylmalonic acid; Ala, alanine; Gly, glycine; Sarc, sarcosine; Val, valine; Leu, leucine; Ile, isoleucine; α-KG, α-ketogluturate; Thr, threonine; Pro, proline; Asn, asparagine; Asp, aspartic acid; Gln, glutamine; Ser, serine; Met, methionine; Glu, glutamic acid; Phe, phenylalanine; tCyst, total cysteine; tHcy, total homocysteine; Orn, ornithine; Lys, lysine; His, histidine; Tyr, tyrosine; Trp, tryptophan; Cysta, cystathionine; Kyn, kynurenine.

The chromatogram of the fat-soluble analytes in the calibrator is shown in Figure 2. 25-Hydroxyvitamin D3, all-trans retinol, and 25-hydroxyvitamin D2 eluted at 1.28−1.30 min (See Table 1a and Figure 2). Retention times for vitamin E forms γ-tocopherol and α-tocopherol were 1.90 and 2.04 min respectively, while phylloquinone eluted at 2.78 min. There was essentially no difference (0.01−0.02 min) in retention times for the analytes and their isotope-labeled internal standards, demonstrating that isotope effects were negligible with regard to chromatography.

While giving higher sensitivity for 25-hydroxyvitamin D2 and 25-hydroxyvitamin D3, electrospray ionization did not provide sufficient sensitivity to detect phylloquinone at endogenous concentrations; we therefore used an APCI ion source. Based on infusion of solvent solutions, we selected the ion-pairs with the highest S/N for each analyte, providing endogenous concentrations would not lead to detector saturation. The second most abundant product ion was used for detection of all-trans retinol and the third most abundant was used for α-tocopherol in order to avoid detector saturation.[17] For all isotope-labeled internal standards we selected the product ions with the highest S/N yield, with the exception of $^{6}H_{2}$-all-trans retinol, where we selected the second most abundant isotope.

The presented assay did not separate 25-hydroxyvitamin D3 from 3-epi-25-hydroxyvitamin D3, which exists in significant amount in infants. Interference from 3-epi-25-hydroxyvitamin D3 may result in elevated 25-hydroxyvitamin D3 concentrations being obtained for some samples.[40] Existing assays[41,42] are typically unable to separate and differentiate between β- and γ-tocopherol as the two vitamin E forms have the same mass and very similar structure.[43] It is plausible β- and γ-tocopherol eluted at the exact same retention time in the present assay. Since serum/plasma concentrations of β-tocopherol have been reported to be much lower than those of γ-tocopherol[44−47] we did not investigate this further. We have therefore used the term γ-tocopherol for all results originating from the chromatographic peak at the retention time of γ-tocopherol (identified in standard solutions) originating from the 417.5/151.0 ion-pair.

Analytical Platform: GC-MS/MS. The order of analyte elution, retention times and acquisition parameters for the water-soluble analytes and their corresponding internal standards are provided in Table 1b. A chromatogram of the calibrator is shown in Figure 3. Retention times for the water-soluble analytes ranged from 2.57−7.55 min, chromatographic run-time for each sample was 11 min, and the total run-time for each plate was 19.6 h. The temperature in the GC compartment was increased from 260 to 310 °C in order to accommodate the late eluting analyte, kynurenine, and its internal standard.

We used scheduled multiple reaction monitoring (MRM), and selection of ion-pairs was based on the same criteria used for LC-MS/MS discussed above. To obtain maximum sensitivity on the GC-MS/MS, we divided the data acquisition into different retention windows (with adjustable, analyte-specific dwell time and collision energy).

Matrix Effects on LC-MS/MS Analytes. Matrix effects can have a negative impact on the performance of LC-MS/MS assays by signal reduction or enhancement, thereby reducing

DOI: 10.1021/acs.analchem.6b02325
Anal. Chem. 2016, 88, 10427−10436

precision and accuracy.[48] Since matrix effects may vary between different plasma specimens, 12 different plasma samples were tested. Typical signals from the infusion experiments with injected mobile phase, processed blank and a plasma sample are shown in Supporting Figure 1. The signal traces for injected plasmas showed no differences from traces for injected mobile phase or treated blank at the retention times of any of the analytes. These results are in accordance with the report that LLE and APCI are both less liable to matrix effects.[33]

Performance of the Method. Results for limit of detection (LOD), lower limit of quantification (LLQ) and linearity are shown in Table 3. LOD and LLQ were far below published concentrations in human serum/plasma for all-trans retinol,[17,49] α-tocopherol,[17,49] γ-tocopherol,[49] and water-soluble analytes[20,23,50] and concentrations in a healthy population obtained by us using the current method (Supporting Table 3).

For 25-hydroxyvitamin D2 and 25-hydroxyvitamin D3 LLQ was 50 and 34 nM, respectively, and for phylloquinone LLQ was 0.66 nM. Our LLQs for 25-hydroxyvitamin D and phylloquinone are higher than those observed for assays specifically designed for the measurement of 25-hydroxyvitamin D[51−56] or phylloquinone.[57−62] However, the present assay allows quantitation of serum/plasma 25-hydroxyvitamin D3 concentrations well below the cutoff for deficiency, often defined as 50 nM.[63] The reported lower end of plasma/serum reference ranges for phylloquinone vary significantly between populations, with a range of 0.17−0.93 nM.[57,58,64] It is thus possible that the presented assay, which enables quantification of phylloquinone in 80−95% of healthy subjects investigated here (Supporting Tables 3 and 4) and by others,[57,64] may not be able to quantify phylloquinone in samples from deficient subjects. Using a larger sample volume increased the noise in the signal from the ion-pair used for phylloquinone, and thus did not increase the sensitivity for this analyte. Importantly, sample volume requirements are large for methods measuring only phylloquinone (100−500 μL),[57−62] and also for reference measurement procedures for vitamin D forms (250−2000 μL)[54−56] while our assay is capable of high-throughput quantification of 32 chemically diverse biomarkers over a wide concentration range (0.66 nM−1 mM) using only 50 μL of sample.

All regression parameters for the relative signal intensity versus concentration are shown in Table 2 and demonstrate excellent linearity for all the analytes, with r^2 values ranging from 0.994 to 0.999. Linear ranges spanned from LLQ to well above the normal concentration ranges for all analytes (Supporting Table 3). Analytical recoveries were between 82.5−109.9% (Table 2), within-day CVs ranged from 0.7 to 9.4%, and between-day CVs ranged from 1.1 to 17.5% (Table 2). The CVs that were above 10% were observed in the between-day experiments for 25-hydroxyvitamin D2 or phylloquinone, analytes that are present in nanomolar concentrations.

For 163 presumed healthy individuals the serum analyte concentrations quantified by our assay, presented in Supporting Table 3, were in accordance with those published for the respective fat-[17,49,57,64] and water-soluble[20,23,50] analytes. Comparing results from our assay with target values from External Quality Assessment Schemes (EQAS) for phylloquinone and MMA by Passing-Bablok regression gave slopes that did not differ from one and intercepts that did not differ from zero. For 25-hydroxyvitamin D3 the slope (1.182, 95% CI 1.038, 1.442) was slightly different from one and the intercept (−10.4, 95% CI −27.1, −0.06) was slightly different from zero. These small deviations from ideal results (slope = 1, intercept = 0) may be related to the inability of our assay to separate the 3-epi-25-hydroxyvitamin D3 from 25-hydroxyvitamin D3. For tHcy, the slope differed slightly from one (0.945, 95% CI 0.923−0.985) and the intercept did not differ from zero.

Table 3. Limit of Detection and Linearity[a]

analyte	linear range	LOD[b]	regression parameters		
			slope	intercept	r^2
25-hydroxyvitamin D3 (nM)	17−1000	17	0.0043	−0.069	0.996
all-trans retinol (μM)	0.03−10	n.d.[c]	3.39	−0.476	0.997
25-hydroxyvitamin D2 (nM)	25−1000	25	0.0086	−0.060	0.994
γ-tocopherol (μM)	0.17−100	n.d.[c]	0.174	0.013	0.997
α-tocopherol (μM)	0.1−100	n.d.[c]	0.334	0.161	0.996
phylloquinone (nM)	0.33−33	0.33	0.0227	0.0039	0.994
methylmalonic acid (μM)	0.025−50	0.025	0.554	−0.0815	0.999
alanine (μM)	1−1000	1	0.003	−0.0166	0.998
glycine (μM)	1−1000	1	0.005	−0.0183	0.998
sarcosine (μM)	0.035−70	0.035	0.324	−0.080	0.998
valine (μM)	0.5−1000	0.5	0.0076	0.0193	0.999
leucine (μM)	0.5−1000	0.5	0.0101	−0.0333	0.999
isoleucine (μM)	0.5−1000	0.5	0.0074	−0.0328	0.998
α-ketogluturate (μM)	0.375−150	0.375	0.0535	0.0047	0.998
threonine (μM)	1.25−1000	1.25	0.0061	0.0047	0.999
proline (μM)	0.5−1000	0.5	0.014	0.069	0.999
asparagine (μM)	0.625−500	0.625	0.0266	0.172	0.999
aspartic acid (μM)	0.5−1000	0.5	0.0695	0.297	0.999
glutamine (μM)	10−1000	10	0.0036	−0.025	0.999
serine (μM)	0.5−1000	0.5	0.016	−0.0488	0.998
methionine (μM)	0.5−667	0.5	0.0292	0.109	0.999
glutamic acid (μM)	0.5−1000	0.5	0.0897	−0.289	0.998
phenylalanine (μM)	0.5−1000	0.5	0.0234	−0.0595	0.998
total cysteine (μM)	80−800	80	0.0055	1.108	0.996
total homocysteine (μM)	0.1−200	0.1	0.147	−0.0324	0.999
ornithine (μM)	1−400	1	0.0372	−0.0746	0.999
lysine (μM)	1.25−1000	1.25	0.0123	−0.0616	0.999
histidine (μM)	1−1000	1	0.0452	−0.0091	0.999
tyrosine (μM)	0.5−1000	0.5	0.0062	0.0182	0.999
tryptophan (μM)	0.625−500	0.625	0.0124	−0.0154	0.999
cystathionine (μM)	0.05−100	0.05	0.374	−0.148	0.999
kynurenine (μM)	0.125−50	0.125	0.2705	−0.0611	0.999

[a]Performed in PBS. [b]LOD, limit of detection, defined as $S/N > 3$. [c]Not determined due to high plasma concentrations.

DOI: 10.1021/acs.analchem.6b02325
Anal. Chem. 2016, 88, 10427−10436

Results from participation in EQAS thus demonstrated that our assay showed good agreement with EQAS target values for 25-hydroxyvitamin D3, phylloquinone, MMA, and tHcy (Supporting Table 4).

Trueness for 25-hydroxyvitamin D3 ranged from 113.1 to 117.1% for the NIST SRM samples (except for SMR 972a level 4, which contained a very high concentration of epi-25-hydroxyvitamin D3), Supporting Table 5. Only one of the NIST SRM samples (972a level 3, 32.3 nM) contained 25-hydroxyvitamin D2 at a concentration above the LOD, but below the LLQ (50 nM), of our assay, and the low concentration may explain the high trueness of 180.9% obtained. For the other analytes with certified/reference values, trueness ranged from 92.8 to 126.3%.

In summary, the overall performance of the present method is comparable to published assays measuring fat-soluble[10−17,57,60] or water-soluble analytes.[18−21,23,36]

Robustness. During routine analysis the quality of the results are followed by monitoring the chromatography for all peaks, as well as the precision and accuracy of all analytes. The sensitivity, measured as the intensity of the internal standard peaks, drops slowly over time on the GC-MS/MS system and the ion source is therefore cleaned after 3−4000 sample runs. On the LC-MS/MS system we have not observed significant changes in sensitivity over several thousand sample runs. On the GC-MS/MS system, 20 cm of the column is cut and the liners are replaced after approximately 500 runs. The GC column is replaced after approximately 1500−2000 sample runs. On the LC-MS/MS system we normally replace the analytical column after approximately 1000 sample runs. Because of these maintenance routines, the CVs do not vary significantly over time on any of the instruments.

CONCLUSION

Targeted metabolic profiling and quantitative analysis of biomarkers has been constrained by methodological challenges related to wide-ranging analyte concentrations, chemical diversity of biomarkers and large sample volume requirements. We have developed and validated a fully automated assay that combines low sample volume requirements (50 μL) with high-throughput for the combined quantification of 6 fat- and 26 water-soluble serum/plasma biomarkers related to nutritional status, lifestyle and disease. Combining LLE with LC- (for fat-soluble analytes) or GC- (for water-soluble analytes) and isotope dilution tandem mass spectrometry, ensured accurate and precise quantification of analytes ranging from low nanomolar to high micromolar concentrations. The method is presently used in our laboratory to analyze biobank samples for epidemiological studies investigating complex metabolic networks.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.analchem.6b02325.

Chemicals, concentrations of analytes in calibrator plasma and internal standards in precipitating agent, analyte concentrations in serum from 163 nonfasting, presumed healthy individuals, results from participation in external quality assessment schemes, trueness data from analysis of NIST SRM 968e, 972a, and 1950, and matrix effects for fat-soluble vitamins (PDF)

AUTHOR INFORMATION

Corresponding Author

*E-mail nkjbm@uib.no.

Author Contributions

O.M. and A.M. contributed equally. The manuscript was written through contributions of all authors, who have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

The Norwegian Cancer Society and the Foundation to Support Research in Functional Vitamin B12 Deficiency supported the work undertaken by the authors.

REFERENCES

(1) Booth, S. L.; Tucker, K. L.; McKeown, N. M.; Davidson, K. W.; Dallal, G. E.; Sadowski, J. A. *J. Nutr.* **1997**, *127*, 587−592.

(2) Schmidt, J. A.; Rinaldi, S.; Scalbert, A.; Ferrari, P.; Achaintre, D.; Gunter, M. J.; Appleby, P. N.; Key, T. J.; Travis, R. C. *Eur. J. Clin. Nutr.* **2016**, *70*, 306−312.

(3) Wang, T. J.; Pencina, M. J.; Booth, S. L.; Jacques, P. F.; Ingelsson, E.; Lanier, K.; Benjamin, E. J.; D'Agostino, R. B.; Wolf, M.; Vasan, R. S. *Circulation* **2008**, *117*, 503−511.

(4) Maillard, V.; Kuriki, K.; Lefebvre, B.; Boutron-Ruault, M. C.; Lenoir, G. M.; Joulin, V.; Clavel-Chapelon, F.; Chajes, V. *Int. J. Cancer* **2010**, *127*, 1188−1196.

(5) Kimura, T.; Noguchi, Y.; Shikata, N.; Takahashi, M. *Curr. Opin. Clin. Nutr. Metab. Care* **2009**, *12*, 49−53.

(6) Nygard, O.; Vollset, S. E.; Refsum, H.; Brattstrom, L.; Ueland, P. M. *J. Intern. Med.* **1999**, *246*, 425−454.

(7) Johansson, M.; Relton, C.; Ueland, P. M.; Vollset, S. E.; Midttun, O.; Nygard, O.; Slimani, N.; Boffetta, P.; Jenab, M.; Clavel-Chapelon, F.; Boutron-Ruault, M. C.; Fagherazzi, G.; Kaaks, R.; Rohrmann, S.; Boeing, H.; Weikert, C.; Bueno-de-Mesquita, H. B.; Ros, M. M.; van Gils, C. H.; Peeters, P. H.; Agudo, A.; Barricarte, A.; Navarro, C.; Rodriguez, L.; Sanchez, M. J.; Larranaga, N.; Khaw, K. T.; Wareham, N.; Allen, N. E.; Crowe, F.; Gallo, V.; Norat, T.; Krogh, V.; Masala, G.; Panico, S.; Sacerdote, C.; Tumino, R.; Trichopoulou, A.; Lagiou, P.; Trichopoulos, D.; Rasmuson, T.; Hallmans, G.; Riboli, E.; Vineis, P.; Brennan, P. *JAMA* **2010**, *303*, 2377−2385.

(8) Suzuki, Y.; Suda, T.; Furuhashi, K.; Suzuki, M.; Fujie, M.; Hahimoto, D.; Nakamura, Y.; Inui, N.; Nakamura, H.; Chida, K. *Lung Cancer* **2010**, *67*, 361−365.

(9) Hustad, S.; Midttun, O.; Schneede, J.; Vollset, S. E.; Grotmol, T.; Ueland, P. M. *Am. J. Hum. Genet.* **2007**, *80*, 846−855.

(10) Quesada, J. M.; Mata-Granados, J. M.; Luque De Castro, M. D. *J. Steroid Biochem. Mol. Biol.* **2004**, *89−90*, 473−477.

(11) Chatzimichalakis, P. F.; Samanidou, V. F.; Papadoyannis, I. N. *J. Chromatogr. B: Anal. Technol. Biomed. Life Sci.* **2004**, *805*, 289−296.

(12) Priego Capote, F.; Jimenez, J. R.; Granados, J. M.; de Castro, M. D. *Rapid Commun. Mass Spectrom.* **2007**, *21*, 1745−1754.

(13) Alvarez, J. C.; De Mazancourt, P. *J. Chromatogr., Biomed. Appl.* **2001**, *755*, 129−135.

(14) Granado-Lorencio, F.; Herrero-Barbudo, C.; Blanco-Navarro, I.; Perez-Sacristan, B. *Anal. Bioanal. Chem.* **2010**, *397*, 1389−1393.

(15) Mata-Granados, J. M.; Quesada Gomez, J. M.; Luque de Castro, M. D. *Clin. Chim. Acta* **2009**, *403*, 126−130.

(16) Mata-Granados, J. M.; Luque De Castro, M. D.; Quesada, J. M. *J. Pharm. Biomed. Anal.* **2004**, *35*, 575−582.

(17) Midttun, O.; Ueland, P. M. *Rapid Commun. Mass Spectrom.* **2011**, *25*, 1942−1948.

DOI: 10.1021/acs.analchem.6b02325
Anal. Chem. 2016, 88, 10427−10436

(18) Casetta, B.; Tagliacozzi, D.; Shushan, B.; Federici, G. *Clin. Chem. Lab. Med.* **2000**, *38*, 391−401.
(19) Armstrong, M.; Jonscher, K.; Reisdorph, N. A. *Rapid Commun. Mass Spectrom.* **2007**, *21*, 2717−2726.
(20) Kaspar, H.; Dettmer, K.; Gronwald, W.; Oefner, P. J. *J. Chromatogr. B: Anal. Technol. Biomed. Life Sci.* **2008**, *870*, 222−232.
(21) Harder, U.; Koletzko, B.; Peissner, W. *J. Chromatogr. B: Anal. Technol. Biomed. Life Sci.* **2011**, *879*, 495−504.
(22) Kvitvang, H. F.; Andreassen, T.; Adam, T.; Villas-Boas, S. G.; Bruheim, P. *Anal. Chem.* **2011**, *83*, 2705−2711.
(23) Yang, R.; Dong, J.; Guo, H.; Li, H.; Wang, S.; Zhao, H.; Zhou, W.; Yu, S.; Wang, M.; Chen, W. *PLoS One* **2013**, *8*, e81144.
(24) Papadoyannis, I. N.; Tsioni, G. K.; Samanidou, V. F. *J. Liq. Chromatogr. Relat. Technol.* **1997**, *20*, 3203−3231.
(25) Li, H. B.; Chen, F. *Chromatographia* **2001**, *54*, 270−273.
(26) Dabre, R.; Azad, N.; Schwammle, A.; Lammerhofer, M.; Lindner, W. *J. Sep Sci.* **2011**, *34*, 761−772.
(27) Buszewski, B.; Zbanyszek, W. *J. Liq. Chromatogr. Relat. Technol.* **2002**, *25*, 1229−1241.
(28) Taguchi, K.; Fukusaki, E.; Bamba, T. *J. Chromatogr A* **2014**, *1362*, 270−277.
(29) Ramos, L. *J. Chromatogr A* **2012**, *1221*, 84−98.
(30) Chang, M. S.; Ji, Q.; Zhang, J.; El-Shourbagy, T. A. *Drug Dev. Res.* **2007**, *68*, 107−133.
(31) Santos, J.; Mendiola, J. A.; Oliveira, M. B.; Ibanez, E.; Herrero, M. *J. Chromatogr A* **2012**, *1261*, 179−188.
(32) Koc, H.; Mar, M. H.; Ranasinghe, A.; Swenberg, J. A.; Zeisel, S. H. *Anal. Chem.* **2002**, *74*, 4734−4740.
(33) Souverain, S.; Rudaz, S.; Veuthey, J. L. *J. Chromatogr A* **2004**, *1058*, 61−66.
(34) Gundersen, T. E.; Blomhoff, R. *J. Chromatogr A* **2001**, *935*, 13−43.
(35) Mottier, P.; Gremaud, E.; Guy, P. A.; Turesky, R. *Anal. Biochem.* **2002**, *301*, 128−135.
(36) Windelberg, A.; Arseth, O.; Kvalheim, G.; Ueland, P. M. *Clin. Chem.* **2005**, *51*, 2103−2109.
(37) Wang, J.; Huang, Z. H.; Gage, D. A.; Watson, J. T. *J. Chromatogr A* **1994**, *663*, 71−78.
(38) Husek, P. *J. Chromatogr., Biomed. Appl.* **2000**, *740*, 289−290.
(39) Whiley, L.; Godzien, J.; Ruperez, F. J.; Legido-Quigley, C.; Barbas, C. *Anal. Chem.* **2012**, *84*, 5992−5999.
(40) van den Ouweland, J. M.; Beijers, A. M.; van Daal, H. *Clin. Chem.* **2011**, *57*, 1618−1619.
(41) Thibeault, D.; Su, H.; MacNamara, E.; Schipper, H. M. *J. Chromatogr. B: Anal. Technol. Biomed. Life Sci.* **2009**, *877*, 1077−1083.
(42) Oostenbrug, G. S.; Mensink, R. P.; Al, M. D.; van Houwelingen, A. C.; Hornstra, G. *Br. J. Nutr.* **1998**, *80*, 67−73.
(43) Nagy, K.; Courtet-Compondu, M. C.; Holst, B.; Kussmann, M. *Anal. Chem.* **2007**, *79*, 7087−7096.
(44) Chow, C. K. *Am. J. Clin. Nutr.* **1975**, *28*, 756−760.
(45) Mino, M.; Kijima, Y.; Nishida, Y.; Nakagawa, S. *J. Nutr. Sci. Vitaminol.* **1980**, *26*, 103−112.
(46) Jansson, L.; Nilsson, B.; Lindgren, R. *J. Chromatogr., Biomed. Appl.* **1980**, *181*, 242−247.
(47) Jambazian, P. R.; Haddad, E.; Rajaram, S.; Tanzman, J.; Sabate, J. *J. Am. Diet. Assoc.* **2005**, *105*, 449−454.
(48) Gosetti, F.; Mazzucco, E.; Zampieri, D.; Gennaro, M. C. *J. Chromatogr A* **2010**, *1217*, 3929−3937.
(49) Jenab, M.; Riboli, E.; Ferrari, P.; Friesen, M.; Sabate, J.; Norat, T.; Slimani, N.; Tjonneland, A.; Olsen, A.; Overvad, K.; Boutron-Ruault, M. C.; Clavel-Chapelon, F.; Boeing, H.; Schulz, M.; Linseisen, J.; Nagel, G.; Trichopoulou, A.; Naska, A.; Oikonomou, E.; Berrino, F.; Panico, S.; Palli, D.; Sacerdote, C.; Tumino, R.; Peeters, P. H.; Numans, M. E.; Bueno-de-Mesquita, H. B.; Buchner, F. L.; Lund, E.; Pera, G.; Chirlaque, M. D.; Sanchez, M. J.; Arriola, L.; Barricarte, A.; Quiros, J. R.; Johansson, I.; Johansson, A.; Berglund, G.; Bingham, S.; Khaw, K. T.; Allen, N.; Key, T.; Carneiro, F.; Save, V.; Del Giudice, G.; Plebani, M.; Kaaks, R.; Gonzalez, C. A. *Br. J. Cancer* **2006**, *95*, 406−415.
(50) Davids, M.; Peters, J. H.; de Jong, S.; Teerlink, T. *Clin. Chim. Acta* **2013**, *421*, 164−167.
(51) Zerwekh, J. E. *Am. J. Clin. Nutr.* **2008**, *87*, 1087S−1091S.
(52) Tsugawa, N.; Suhara, Y.; Kamao, M.; Okano, T. *Anal. Chem.* **2005**, *77*, 3001−3007.
(53) Maunsell, Z.; Wright, D. J.; Rainbow, S. J. *Clin. Chem.* **2005**, *51*, 1683−1690.
(54) Mineva, E. M.; Schleicher, R. L.; Chaudhary-Webb, M.; Maw, K. L.; Botelho, J. C.; Vesper, H. W.; Pfeiffer, C. M. *Anal. Bioanal. Chem.* **2015**, *407*, 5615−5624.
(55) Stepman, H. C.; Vanderroost, A.; Van Uytfanghe, K.; Thienpont, L. M. *Clin. Chem.* **2011**, *57*, 441−448.
(56) Tai, S. S.; Nelson, M. A. *Anal. Chem.* **2015**, *87*, 7964−7970.
(57) Paroni, R.; Faioni, E. M.; Razzari, C.; Fontana, G.; Cattaneo, M. *J. Chromatogr. B: Anal. Technol. Biomed. Life Sci.* **2009**, *877*, 351−354.
(58) Wang, L. Y.; Bates, C. J.; Yan, L.; Harrington, D. J.; Shearer, M. J.; Prentice, A. *Clin. Chim. Acta* **2004**, *347*, 199−207.
(59) Davidson, K. W.; Sadowski, J. A. *Methods Enzymol.* **1997**, *282*, 408−421.
(60) Gentili, A.; Cafolla, A.; Gasperi, T.; Bellante, S.; Caretti, F.; Curini, R.; Fernandez, V. P. *J. Chromatogr A* **2014**, *1338*, 102−110.
(61) Fu, X.; Peterson, J. W.; Hdeib, M.; Booth, S. L.; Grusak, M. A.; Lichtenstein, A. H.; Dolnikowski, G. G. *Anal. Chem.* **2009**, *81*, 5421−5425.
(62) Jones, K. S.; Bluck, L. J.; Coward, W. A. *Rapid Commun. Mass Spectrom.* **2006**, *20*, 1894−1898.
(63) Holick, M. F. *N. Engl. J. Med.* **2007**, *357*, 266−281.
(64) Sadowski, J. A.; Hood, S. J.; Dallal, G. E.; Garry, P. J. *Am. J. Clin. Nutr.* **1989**, *50*, 100−108.

DOI: 10.1021/acs.analchem.6b02325
Anal. Chem. 2016, 88, 10427−10436

Supporting Information:

Combined Measurement of 6 Fat-soluble Vitamins and 26 Water Soluble Functional Vitamin Markers and Amino Acids in 50 µL of Serum or Plasma by High-throughput Mass Spectrometry

Øivind Midttun[1], Adrian McCann[1], Ove Aarseth[1], Marit Krokeide[1], Gry Kvalheim[1,2], Klaus Meyer[1] and Per M Ueland[2,3]

[1]Bevital AS, Laboratory Building, 9th Floor, Jonas Lies veg 87, 5021 Bergen, Norway. [2]Department of Clinical Science, University of Bergen, 5021 Bergen, Norway. [3]Laboratory of Clinical Biochemistry, Haukeland University Hospital, 5021 Bergen, Norway.

This document contains tables with information on chemicals used (Table S-1), concentrations of analytes and internal standards (Table S-2), serum biomarker concentrations in healthy adults (Table S-3), results from participation in external quality assessment schemes for 25-OH vitamin D3, phylloquinone, total homocysteine and MMA (Table S-4), data on the trueness of the method (Table S-5). Matrix effects are shown in Figure S-1.

15

16

17 **Table S-1. Chemicals**

Analyte/Internal Standard	Purchased from
$^{2}H_{6}$-25-hydroxyvitamin D3 (95%)	Synthetica[a]
25-hydroxyvitamin D3 (98%)	Sigma-Aldrich[b]
$^{2}H_{6}$-all-trans retinol (99.2%)	Buchem BV[c]
All-trans retinol (95%)	Sigma-Aldrich[b]
$^{2}H_{6}$-25-hydroxyvitamin D2 (99%)	Medical Isotopes[d]
25-hydroxyvitamin D2 (>98%)	Sigma-Aldrich[b]
$^{2}H_{4}$-γ-tocopherol (94%)	Toronto Research Chemical[e]
γ-tocopherol (99%)	Sigma-Aldrich[b]
$^{2}H_{9}$-α-tocopherol (99%)	Chemaphor[f]
α-tocopherol (99.9%)	Sigma-Aldrich[b]
$^{2}H_{4}$-phylloquinone (98%)	Buchem BV[c]
Phylloquinone (99.9%)	Sigma-Aldrich[b]
$^{2}H_{3}$-methylmalonic acid (98%)	Cambridge Isotopes Laboratories[g]
Methylmalonic acid (99%)	Sigma-Aldrich[b]
$^{2}H_{3}$-alanine (99%)	C/D/N Isotopes[h]
Alanine (>98%)	Sigma-Aldrich[b]
$^{2}H_{2}$-glycine (98%)	Medical Isotopes[d]
Glycine (99.7%)	Sigma-Aldrich[b]
$^{2}H_{3}$-sarcosine (99%)	C/D/N Isotopes[h]
Sarcosine (99%)	Sigma-Aldrich[b]
$^{2}H_{2}$-valine (98%)	C/D/N Isotopes[h]
Valine	Sigma-Aldrich[b]
$^{2}H_{10}$-leucine (98%)	C/D/N Isotopes[h]
Leucine	Sigma-Aldrich[b]
$^{2}H_{10}$-isoleucine (98%)	Cambridge Isotope Laboratories[g]
Isoleucine	Sigma-Aldrich[b]
$^{2}H_{6}$-α–ketoglutaric acid (98%)	Sigma-Aldrich[b]
α–Ketoglutaric acid (>99%)	Sigma-Aldrich[b]
$^{2}H_{2}$-threonine (>98%)	C/D/N Isotopes[h]
Threonine	Sigma-Aldrich[b]
$^{2}H_{3}$-proline (99%)	C/D/N Isotopes[h]
Proline	Sigma-Aldrich[b]
$^{2}H_{3}$-$^{15}N_{2}$-asparagine (98%)	Cambridge Isotope Laboratories[g]
Asparagine	Sigma-Aldrich[b]
$^{2}H_{3}$-aspartic acid (98%)	C/D/N Isotopes[h]
Aspartic acid	Sigma-Aldrich[b]
$^{2}H_{5}$-glutamine (>98%)	C/D/N Isotopes[h]
Glutamine (98%)	Sigma-Aldrich[b]
$^{2}H_{3}$-serine (98%)	Medical Isotopes[d]
Serine (99%)	Sigma-Aldrich[b]

$^{2}H_4$-methionine (>99%)	C/D/N Isotopes[h]
Methionine (>99%)	Sigma-Aldrich[b]
$^{2}H_5$-glutamic acid (98%)	C/D/N Isotopes[h]
Glutamic acid	Sigma-Aldrich[b]
$^{2}H_5$-phenyl-$^{2}H_3$-alanine	C/D/N Isotopes[h]
Phenylalanine	Sigma-Aldrich[b]
$^{2}H_2$-cysteine (98%)	Cambridge Isotope Laboratories[g]
Cysteine (99.5%)	Sigma-Aldrich[b]
$^{2}H_4$-homocysteine (99%)	C/D/N Isotopes[h]
Homocysteine (98%)	Sigma-Aldrich[b]
$^{2}H_7$-ornithine (98%)	C/D/N Isotopes[h]
Ornithine	Sigma-Aldrich[b]
$^{2}H_9$-lysine (99%)	C/D/N Isotopes[h]
Lysine	Sigma-Aldrich[b]
$^{2}H_3$-histidine (98%)	C/D/N Isotopes[h]
Histidine	Sigma-Aldrich[b]
$^{2}H_2$-tyrosine (99%)	C/D/N Isotopes[h]
Tyrosine	Sigma-Aldrich[b]
$^{2}H_5$-tryptophan (>98%)	C/D/N Isotopes[h]
Tryptophan (98%)	Sigma-Aldrich[b]
$^{2}H_4$-cystathionine (>95%)	C/D/N Isotopes[h]
Cystathionine (>95%)	Sigma-Aldrich[b]
$^{2}H_3$-kynurenine (>98%)	Buchem BV[c]
Kynurenine (99%)	Sigma-Aldrich[b]
Ammonium formate (99 %)	Sigma-Aldrich[b]
Butylated hydroxutoluene (BHT, 99 %)	Sigma-Aldrich[b]
Isooctane (>99.5 %),	Sigma-Aldrich[b]
Chloroform (>99 %)	Merck[i]
Methanol (99.99%)	Fisher Scientific[j]
Ethanol (96 %)	Arcus[k]

Key: [a]www.synthetica.no, [b]www.sigmaaldrich.com, [c]www.buchem.com, [d]www.medicalisotopes.com, [e]www.trc-canada.com, [f]www.chemaphor.com, [g]www.isotope.com [h]www.cdnisotopes.com, [i]www.merck.com, [j]www.fisher.co.uk, [k]www.arcus.no

Table S-2. Concentrations of Analytes in Calibrator Plasma and Internal Standards in Precipitating Agent

Analyte		Internal standard	
Name	**Concentration in calibrator plasma**	**Name**	**Concentration in precipitating agent**
25-hydroxyvitamin D3 (nM)	112	$^{2}H_6$-25-OH D3 (nM)	100
all-trans retinol (μM)	1.85	$^{2}H_6$-all-trans retinol (μM)	1
25-hydroxyvitamin D2 (nM)	147	$^{2}H_6$-25-OH D2 (nM)	100
γ-tocopherol (μM)	4.50	$^{2}H_4$-γ-tocopherol (μM)	5
α-tocopherol (μM)	21.0	$^{2}H_9$-α-tocopherol (μM)	1
Phylloquinone (nM)	3.60	$^{2}H_4$-phylloquinone (nM)	100
Methylmalonic acid (μM)	0.21	$^{2}H_3$-methylmalonic acid (μM)	1.2
Alanine (μM)	444	$^{2}H_3$-alanine (μM)	182
Glycine (μM)	217	$^{2}H_2$-glycine (μM)	90
Sarcosine (μM)	1.7	$^{2}H_3$-sarcosine (μM)	2.7
Valine (μM)	242	$^{2}H_2$-valine (μM)	95
Leucine (μM)	135	$^{2}H_{10}$-leucine (μM)	50
Isoleucine (μM)	90	$^{2}H_{10}$-isoleucine (μM)	61
α-ketoglutaric acid(μM)	27.6	$^{2}H_6$-α–ketoglutaric acid (μM)	64
Threonine (μM)	113	$^{2}H_2$-threonine (μM)	87
Proline (μM)	236	$^{2}H_3$-proline (μM)	48
Asparagine (μM)	26.5	$^{2}H_3$-$^{15}N_2$-asparagine (μM)	47
Aspartic acid (μM)	8.7	$^{2}H_3$-aspartic acid (μM)	9
Glutamine (μM)	116	$^{2}H_5$-glutamine (μM)	239
Serine (μM)	402	$^{2}H_3$-serine (μM)	68
Methionine (μM)	30.1	$^{2}H_4$-methionine (μM)	23
Glutamic acid (μM)	295	$^{2}H_5$-glutamic acid (μM)	32
Phenylalanine (μM)	58	$^{2}H_5$-phenyl-$^{2}H_3$-alanine (μM)	31
Total cysteine (μM)	209	$^{2}H_2$-cysteine (μM)	1
Total homocysteine (μM)	12.3	$^{2}H_4$-homocysteine (μM)	30
Ornithine (μM)	72	$^{2}H_7$-ornithine (μM)	42
Lysine (μM)	182	$^{2}H_9$-lysine (μM)	40
Histidine (μM)	65	$^{2}H_3$-histidine (μM)	40
Tyrosine (μM)	52	$^{2}H_2$-tyrosine (μM)	230
Tryptophan (μM)	65	$^{2}H_5$-tryptophan (μM)	36
Cystathionine (μM)	0.33	$^{2}H_4$-cystathionine (μM)	2.3
Kynurenine (μM)	1.8	$^{2}H_3$-kynurenine (μM)	1.8

Table S-3. Analyte Concentrations in Serum from 163 Nonfasting, Presumed Healthy Individuals

Analyte name	Median (5th, 95th percentile)
25-hydroxyvitamin D3 (nM)	54.7 (<LLQ, 93.5)
all-trans retinol (μM)	2.2 (1.5, 3.0)
25-hydroxyvitamin D2 (nM)	<LLQ (<LLQ, <LLQ)
γ-tocopherol (μM)	2.9 (1.4, 5.3)
α-tocopherol (μM)	34.0 (23.2, 50.2)
Phylloquinone (nM)	1.6 (<LLQ, 5.4)
Methylmalonic acid (μM)	0.18 (0.13, 0.27)
Alanine (μM)	484 (349, 643)
Glycine (μM)	315 (234, 457)
Sarcosine (μM)	1.5 (0.9, 2.3)
Valine (μM)	305 (217, 398)
Leucine (μM)	172 (111, 238)
Isoleucine (μM)	88 (55, 137)
α-ketoglutaric acid(μM)	7.1 (3.4, 15.0)
Threonine (μM)	151 (112, 211)
Proline (μM)	296 (193, 447)
Asparagine (μM)	66 (51, 87)
Aspartic acid (μM)	33.3 (25.2, 47.6)
Glutamine (μM)	575 (453, 675)
Serine (μM)	171 (135, 220)
Methionine (μM)	33.0 (23.0, 47.5)
Glutamic acid (μM)	102 (68, 168)
Phenylalanine (μM)	99 (76, 130)
Total cysteine (μM)	273 (238, 315)
Total homocysteine (μM)	10.2 (6.4, 14.7)
Ornithine (μM)	94 (61, 130)
Lysine (μM)	210 (149, 280)
Histidine (μM)	99 (80, 120)
Tyrosine (μM)	78 (59, 107)
Tryptophan (μM)	82 (67, 107)
Cystathionine (μM)	0.28 (0.14, 0.69)
Kynurenine (μM)	1.6 (1.3, 2.1)

Table S-4. Results from External Quality Assessment Schemes

Analyte	EQAS	n	Concentration		Passing-Bablok regression coefficients[a]	
			Min.	Max.	Slope (95%CI)	Intercept (95% CI)
25-hydroxyvitamin D3 (nM)	DEQAS	20	32.3	118	1.182 (1.038,1.442)	-10.4 (-27.1,-0.06)
Phylloquinone (nM)	KEQAS	18[b]	0. 49	8.4	1.000 (0.937,1.102)	-0.133 (-0.236,0.03)
Methylmalonic acid (µM)	DEKS	18	0.17	0.64	0.987 (0.923,1.082)	-0.005 (-0.030,0.017)
Total homocysteine (µM)	DEKS	18	6.7	51.4	0.945 (0.923,0.985)	-0.222 (-0.730,0.088)

[a]Passing-Bablok performed using EQAS Target Values (for methylmalonic acid, total Homocysteine and 25-hydroxyvitamin D3) or All Laboratories Trimmed Mean (ALTM, for phylloquinone) as x and results with the current assay as y.

[b]Two samples were excluded because KEQAS ALTM were below 0.66 nM (LLQ of the current assay).

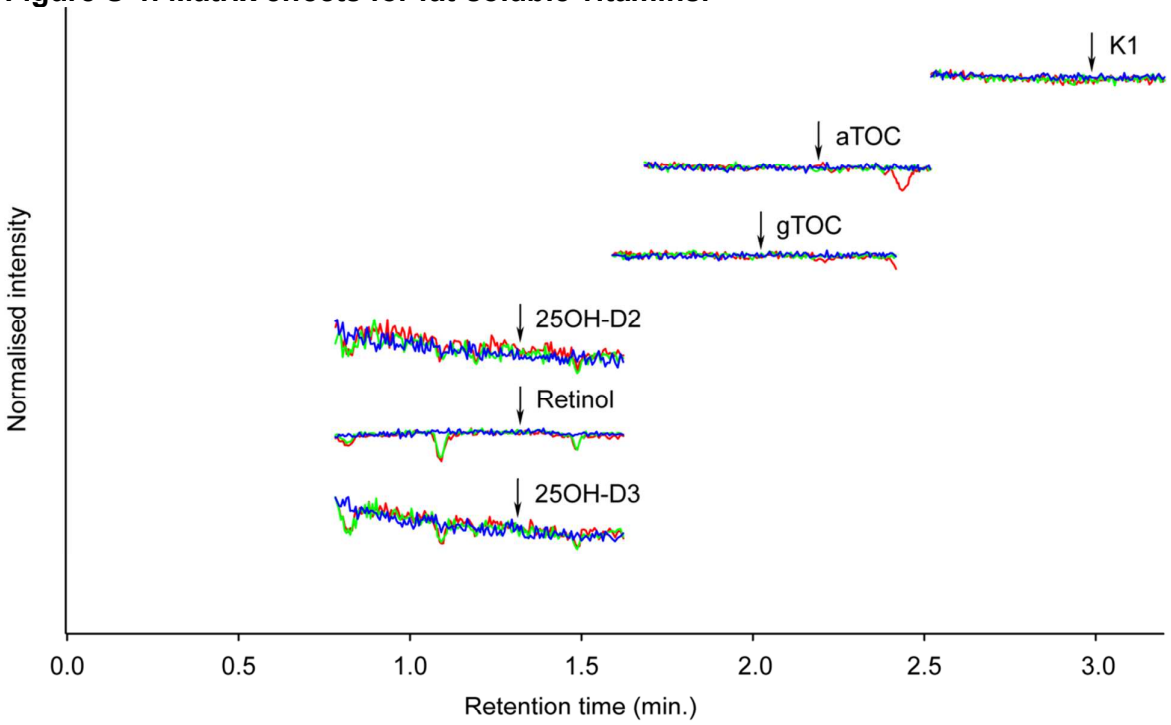
Table S-5. Trueness data from analysis of NIST SRM 968e, 972a and 1950[a]

		SRM 968e			SRM 972a				SRM 1959
Analyte name		**Level 1**	**Level 2**	**Level 3**	**Level 1**	**Level 2**	**Level 3**	**Level 4**	
25-hydroxyvitamin D3 (nM)	Target±U,[b] (nM)	17.7 ± 0.4	32.2 ± 0.7	49.6 ± 1.0	71.8 ± 2.7	45.1 ± 1.0	49.5 ± 1.1	73.4 ± 2.3	61.9 ± 1.9
	Mean[c] (95 % CI, nM)	<LLQ	37.7 (36.2-39.2)	56.9 (54.1-59.7)	82.7 (79.9-85.4)	52.8 (50.9-54.6)	56.0 (53.8-58.2)	163 (156-169)	71.9 (68.5-75.2)
	Trueness[d] (95 % CI), %		117.1 (112.3-121.8)	114.7 (109.1-120.4)	115.1 (111.2-119.0)	117.0 (112.9-121.0)	113.1 (108.6-117.5)	221.4 (212.4-230.4)	116.2 (110.8-121.5)
all-trans retinol (μM)	Target±U,[b] (μM)	1.19 ± 0.06	1.68 ± 0.10	2.26 ± 0.073					
	Mean[c] (95 % CI, μM)	1.28 (1.24-1.31)	1.78 (1.75-1.81)	2.33 (2.29-2.37)					
	Trueness[d] (95 % CI), %	107.3 (104.2-110.4)	106.0 (104.3-107.6)	103.2 (101.5-104.9)					
25-hydroxyvitamin D2 (nM)	Target±U,[b] (nM)					1.3 ± 0.2	2.0 ± 0.2	32.3 ± 0.8	1.3 ± 0.2
	Mean[c] (95 % CI, nM)					<LOD	<LOD	58.4 (56.6-60.2)	<LOD
	Trueness[d] (95 % CI), %							180.9 (175.3-186.5)	
γ-tocopherol (μM)	Target±U,[b] (μM)	4.47 ± 0.38	3.44 ± 0.19	5.45 ± 0.41					
	Mean[c] (95 % CI, μM)	4.73 (4.65-4.80)	3.66 (3.60-3.73)	5.86 (5.76-5.95)					
	Trueness[d] (95 % CI), %	105.7 (104.1-107.3)	106.5 (104.7-108.3)	107.4 (105.7-109.2)					
α-tocopherol (μM)	Target±U,[b] (μM)	15.2 ± 2.0	23.98 ± 0.34	45.0 ± 1.5					
	Mean[c] (95 % CI, μM)	19.2 (18.9-19.5)	28.3 (27.5-29.1)	50.8 (49.6-51.9)					
	Trueness[d] (95 % CI), %	126.3 (124.2-128.3)	117.9 (114.5-121.3)	112.8 (110.3-115.4)					
Alanine (μM)	Target±U,[b] (μM)								300 ± 26
	Mean[c] (95 % CI, μM)								307 (300-313)
	Trueness[d] (95 % CI), %								102.2 (100.1-104.3)
Glycine (μM)	Target±U,[b] (μM)								245 ± 16
	Mean[c] (95 % CI, μM)								270 (266-274)
	Trueness[d] (95 % CI), %								110.2 (108.6-111.9)
Valine (μM)	Target±U,[b] (μM)								182.2 ± 10.4
	Mean[c] (95 % CI, μM)								195 (191-199)
	Trueness[d] (95 % CI), %								107.0 (104.8-109.2)
Leucine (μM)	Target±U,[b] (μM)								100.4 ± 6.3
	Mean[c] (95 % CI, μM)								105 (103-106)
	Trueness[d] (95 % CI), %								104.3 (102.6-106.0)
Isoleucine (μM)	Target±U,[b] (μM)								55.5 ± 3.4
	Mean[c] (95 % CI, μM)								59.9 (58.7-61.0)
	Trueness[d] (95 % CI), %								107.9 (105.8-109.9)
Threonine (μM)	Target±U,[b] (μM)								119.5 ± 6.1
	Mean[c] (95 % CI, μM)								126 (123-128)

	Trueness[d] (95 % CI), %	105.3 (103.2-107.3)
Proline (µM)	Target±U,[b] (µM)	177 ± 9
	Mean[c] (95 % CI, µM)	208 (204-213)
	Trueness[d] (95 % CI), %	117.7 (115.2-120.1)
Serine (µM)	Target±U,[b] (µM)	95.9 ± 4.3
	Mean[c] (95 % CI, µM)	99.3 (97.5-101.1)
	Trueness[d] (95 % CI), %	103.5 (101.6-105.4)
Methionine (µM)	Target±U,[b] (µM)	22.3 ± 1.8
	Mean[c] (95 % CI, µM)	24.6 (24.1-25.1)
	Trueness[d] (95 % CI), %	110.4 (108.1-112.7)
Phenylalanine (µM)	Target±U,[b] (µM)	51 ± 7
	Mean[c] (95 % CI, µM)	53.5 (52.1-54.9)
	Trueness[d] (95 % CI), %	104.8 (102.1-107.6)
Total homocysteine (µM)	Target±U,[b] (µM)	8.50 ± 0.20
	Mean[c] (95 % CI, µM)	7.89 (7.71-8.07)
	Trueness[d] (95 % CI), %	92.8 (90.7-94.9)
Lysine (µM)	Target±U,[b] (µM)	140 ± 14
	Mean[c] (95 % CI, µM)	142 (139-146)
	Trueness[d] (95 % CI), %	101.8 (99.5-104.1)
Histidine (µM)	Target±U,[b] (µM)	72.6 ± 3.6
	Mean[c] (95 % CI, µM)	69.4 (67.9-71.0)
	Trueness[d] (95 % CI), %	95.6 (93.5-97.8)
Tyrosine (µM)	Target±U,[b] (µM)	57.3 ± 3.0
	Mean[c] (95 % CI, µM)	56.4 (55.2-57.6)
	Trueness[d] (95 % CI), %	98.4 (96.3-100.5)

[a]Each SRM sample was prepared and analysed 10 times.
[b]The target value and the uncertainty (U) listed in the NIST certificates of analysis.
[c]Results from analysis of SRM samples.
[d]Difference between concentrations obtained by the present assay and target concentration, as a percentage of target concentration.

Figure S-1. Matrix effects for fat-soluble vitamins.



Supplemental Figure 1. Post-column infusion chromatograms from the matrix effect experiments. Chromatogram from injection of mobile phase is shown in blue, treated blank in green and plasma in red. Arrows indicate the retention times for each analyte.