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Automated Liquid-Liquid Extraction of Vitamins in Infant Formula using a Bench-top Workstation.

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Abstract
Liquid-liquid extractions are used to extract and concentrate analytes from aqueous matrices. This extraction technique is widely accepted as shown by its inclusion within many official methods. Analytical laboratories are looking to automation to help reduce solvent usage and increase sample throughput while ensuring the high quality of the resulting data.

The GERSTEL MultiPurpose Sampler (MPS), commonly used for sample introduction in GC or HPLC can be used to perform a wide variety of sample preparation techniques using a single instrument and controlling software. The sampler can be configured as part of a GC or LC system or can be configured as a bench-top workstation.

In this study, the automation of the liquid-liquid extraction of vitamins in infant formula using the MPS is discussed. Two key GERSTEL options make the automated extraction possible: (1) The quickMIX option that allows samples to be rapidly and effectively mixed using agitation speeds of up to 3000 rpm, (2) The CF200 centrifuge option that enables the centrifugation of extracts. Automation of the extraction of infant formula samples and subsequent HPLC determination of the vitamins listed in the official AOAC® 2012.09 [1] and 2015.09 Official Methods℠ [2] are examined and resulting precision and accuracy data are provided.

Introduction
Due to its extreme importance to the health and development of human infants, analysis of infant formula and its constituents is highly regulated. Manufacturers and the laboratories in charge of testing their products are required to follow regulated methods to ensure that reproducible and accurate results are obtained and reported. Because of the extreme demand for infant formula, there is a great need to increase the turnaround time from sample receipt to reported results. Automation of manual extractions listed within the official methods not only increases throughput, but also enhances data reproducibility and accuracy.

The AOAC® Official Method℠ 2012.09 involves the quantification of vitamin A palmitate, vitamin A acetate, vitamin E acetate, and α-tocopherol in infant formula and adult/pediatric nutritional formula samples. The AOAC® Official MethodSM 2015.09 involves the quantification of vitamin K1 in infant formula and adult/pediatric nutritional formula samples. Briefly, the samples are initially mixed with methanol to precipitate proteins and free lipids for extraction and then extracted using isooctane. After centrifugation to separate the isooctane layer from the alcohol water layer, an aliquot is injected into the HPLC system for analysis. In this study, we show that the liquid-liquid extraction of vitamins in infant formula can be automated using the GERSTEL MPS while achieving both accurate and precise data.

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Experimental

Materials

An infant formula sample (SRM 1869 from NIST) was provided by Eurofins. Vitamin A palmitate, vitamin A acetate, vitamin E acetate, vitamin K1, and α-tocopherol reference standards were obtained from USP®. All other reagents and solvents used were reagent grade or better.

Stock solutions of vitamin A palmitate (1000 µg/mL), vitamin A acetate (200 µg/mL), vitamin E acetate (1 mg/mL), α-tocopherol (1 mg/mL), and vitamin K1 (220 µg/mL) were prepared in isooctane. Calibration curves were prepared using dilutions of the vitamin stock solutions in isooctane at the concentrations listed in Table 1.

Reconstituted infant formula sample was prepared by combining 10 grams of infant formula with 100 grams of LC-MS grade water, mixing well, and protecting from light. Powdered infant formula sample was used as received. For Vitamins A and E, 0.0875 grams of the powdered infant formula and 0.700 grams of the reconstituted infant formula sample were used. For Vitamin K, 0.0438 grams of the powdered infant formula and 0.350 grams of the reconstituted infant formula sample were used. During the preparation, extraction and analysis of samples, care was taken to protect samples from light by using amber glassware throughout. Six replicates of both the powdered and reconstituted infant formula samples were extracted over three separate days.

Table 1: Calibration curve concentrations for vitamins.

	Vitamin A Acetate [mg/ml]	Vitamin A Palmitate [mg/ml]	Vitamin E Acetate [mg/ml]	Vitamin E [mg/ml]	Vitamin K1 [mg/mL]
1	1.70	2.40	0.0200	0.0200	0.0800
2	0.700	1.00	0.00800	0.00120	0.0400
3	0.300	0.500	0.00400	0.00400	0.0240
4	0.0800	1.100	0.00100	0.00100	0.0120
5					0.00400

Automated Sample Preparation

Vitamins A and E

1. The MPS adds 0.700 mL of water to the powdered sample. No water is added to the reconstituted sample.
2. The powdered sample is vortexed at 1500 rpm for 30 seconds using the GERSTEL quickMIX.
3. The MPS adds a total of 4.375 mL of methanol to the sample mixing for 30 seconds at 1500 rpm using the GERSTEL quickMIX after each methanol aliquot addition.
4. The MPS allows the sample to settle for 10 minutes.
5. The MPS adds 1.750 mL of isooctane to the sample.
6. The sample is vortexed at 1500 rpm for 45 seconds using the GERSTEL quickMIX.
7. The MPS adds 0.875 mL of water to the sample.
8. The sample is vortexed at 1500 rpm for 20 seconds.
9. The sample is centrifuged at 3000 rpm for 10 minutes.
10. The MPS transfers 0.500 mL of the isooctane supernatant into a clean, amber 2 mL autosampler vial.

Vitamin K1

1. The MPS adds 0.700 mL of water to the powdered sample and 0.350 mL of water to the reconstituted sample.
2. The sample is vortexed at 1500 rpm for 30 seconds using the GERSTEL quickMIX.
3. The MPS adds a total of 4.375 mL of methanol to the sample mixing for 30 seconds at 1500 rpm using the GERSTEL quickMIX after each methanol aliquot addition.
4. The MPS allows the sample to settle for 10 minutes.
5. The MPS adds 1.750 mL of isooctane to the sample.
6. The sample is vortexed at 1500 rpm for 45 seconds using the GERSTEL quickMIX.

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7. The MPS adds 0.875 mL of water to the sample.
8. The sample is vortexed at 1500 rpm for 20 seconds.
9. The sample is centrifuged at 3000 rpm for 10 minutes.
10. The MPS transfers 0.500 mL of the isooctane supernatant into a clean, amber 2 mL autosampler vial.

Instrumentation

All automated liquid-liquid extractions were performed using an MPS robotic^{PRO} sampler like the one shown in Figure 1. The determination of vitamins A and E were performed using an Agilent 1200 series HPLC consisting of a 6 port, 2 position valve, two isocratic pumps, a UV detector, a Fluorescence detector, ES Industries Chromegasphere Si-60 guard column, (3.0 x 30 mm, 3 µm) and a Thermo Fisher Betasil 100 Si-60 analytical column (3.0 x 200 mm, 3 µm).

Figure 1: GERSTEL MPS robotic^{PRO} sampler with Agilent LC System. The MPS is configured with Filtration Option, which was not used in this work.

LC Method Parameters

Compounds	vitamins A and E
Mobile phase	98.94% isooctane: 1% dichloromethane: 0.06% isopropanol

LC pump 1	Time (min)	Flow (mL/min)
	0	0.4
	11	0.4
	11.1	0.8
	29	0.8
	29.1	0.4
	30	0.4

LC pump 2	isocratic; 0.6 mL/min
Run time	30 minutes
Injection volume	20.0 µL
Column temperature	25 °C
UV detector	325 nm and 285 nm
Fluorescence detector	excitation – 295 nm; emission – 330 nm

The determination of vitamin K1 was performed using an Agilent 1200 series HPLC consisting of two isocratic pumps, a Fluorescence detector, a Thermo Hypersil Gold Silica analytical column (3.0 x 150 mm, 5 µm), a high-pressure mixing tee, and a Zinc reactor column, (4.0 x 20 mm).

LC Method Parameters for Vitamin K1

Mobile phase	99.6% isooctane: 0.4% isopropanol-
Post-column electrolyte solution	3.67 mM zinc chloride/2.44 mM sodium acetate solution with 0.015% acetic acid
LC pump 1	isocratic; 0.4 mL/min
LC pump 2	isocratic; 0.4 mL/min
Run time:	20 minutes
Injection volume:	20.0 µL
Column temperature:	ambient
Fluorescence detector:	excitation – 245 nm; emission – 440 nm

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Results & Discussion

Figure 2 A-B show representative LC-UV chromatograms for vitamin A palmitate, vitamin A acetate, and vitamin E acetate from extracted powdered infant formula samples. Figure 3 A-B show representative LC-fluorescence chromatograms for vitamin E and vitamin K1 from extracted powdered infant formula samples. These data show that vitamins extracted from infant formula samples using the automated extraction procedures result in good chromatography.

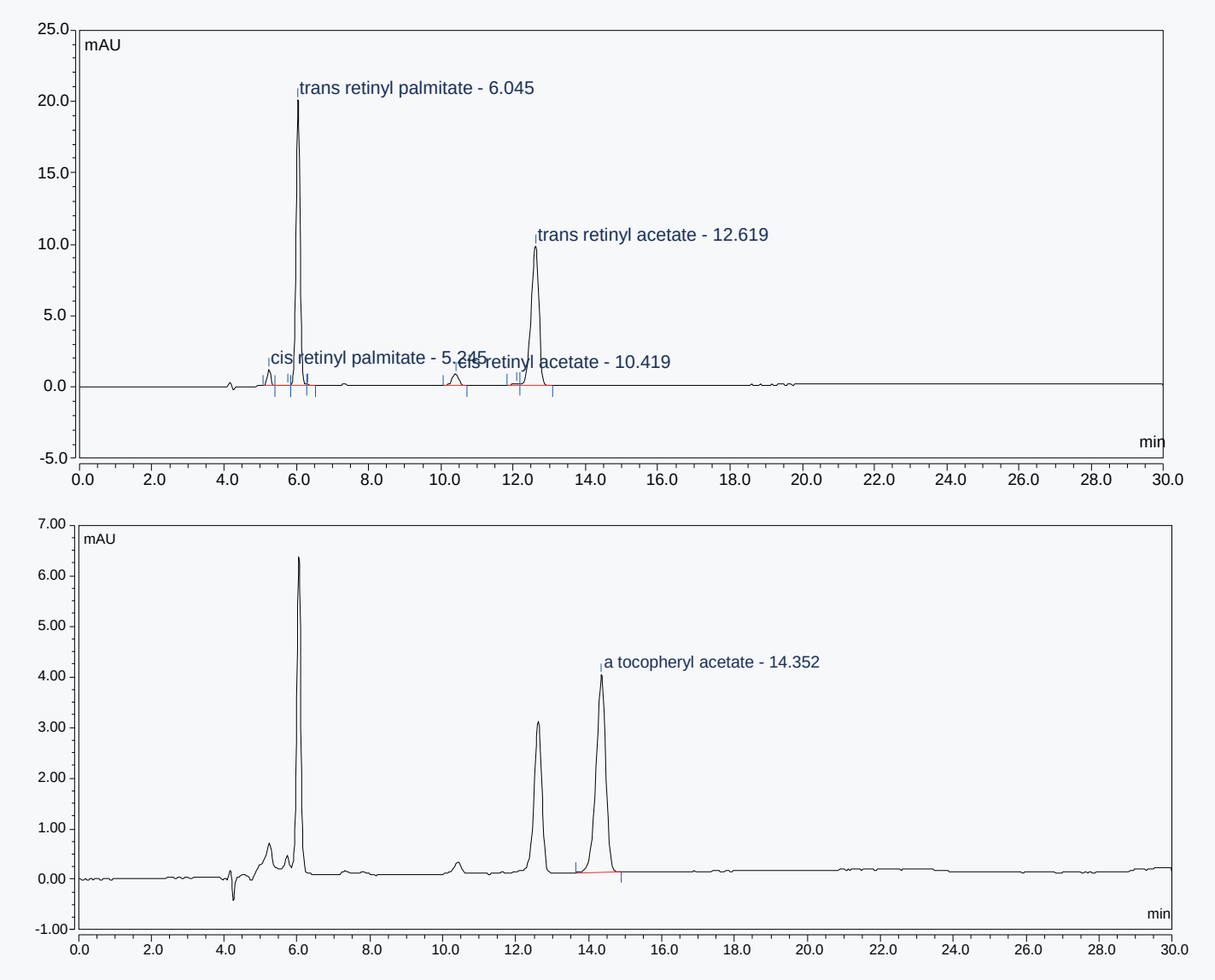

Figure 2 A-B: Representative LC-UV chromatograms of vitamin A palmitate (A), vitamin A acetate (A), and vitamin E acetate (B) from extracted powdered infant formula.

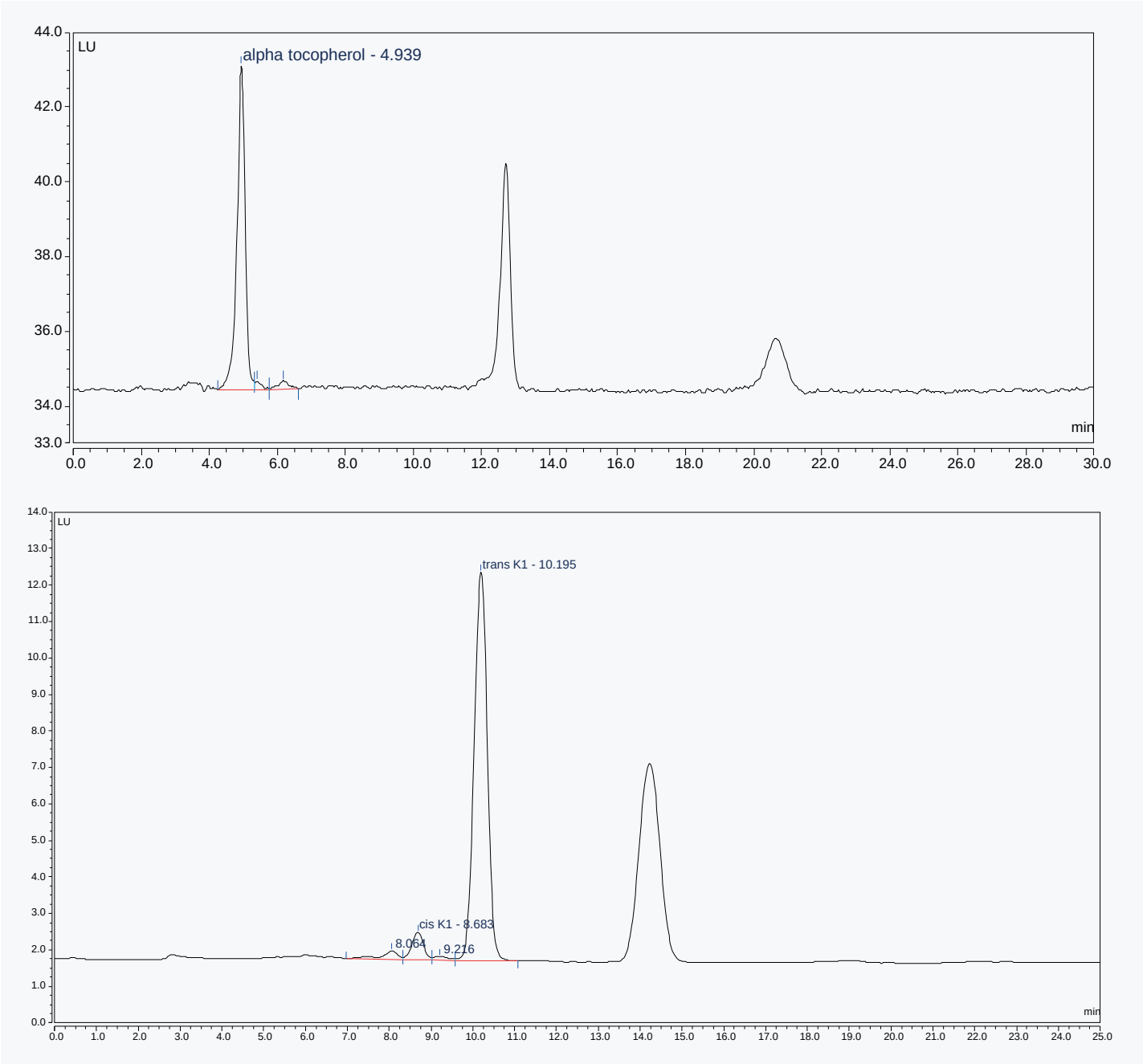

Figure 3 A-B: Representative LC-fluorescence chromatograms of vitamin E (A) and vitamin K1 (B) from extracted powdered infant formula.

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The lower limits of quantitation for the vitamins determined using the two methods in either liquid or powder infant formula are shown in table 2. Representative calibration curves for vitamin A acetate, vitamin E acetate, and vitamin K1 are shown in Figures 4 A-C. Regression analysis for all vitamins analyzed within this method resulted in R^2 values of 0.99 or greater.

Table 2: Lower limits of quantitation for vitamins in liquid and powder infant formula.

Analyte	LLOQ for Liquid Infant Formula [µg/g]	LLOQ for Powder Infant Formula [µg/g]
Vitamin K1	0.0100	0.0800
Vitamin A Acetate	0.210	1.68
Vitamin A Palmitate	0.160	1.28
Vitamin E Acetate	2.50	20.0
Vitamin E	2.50	20.0

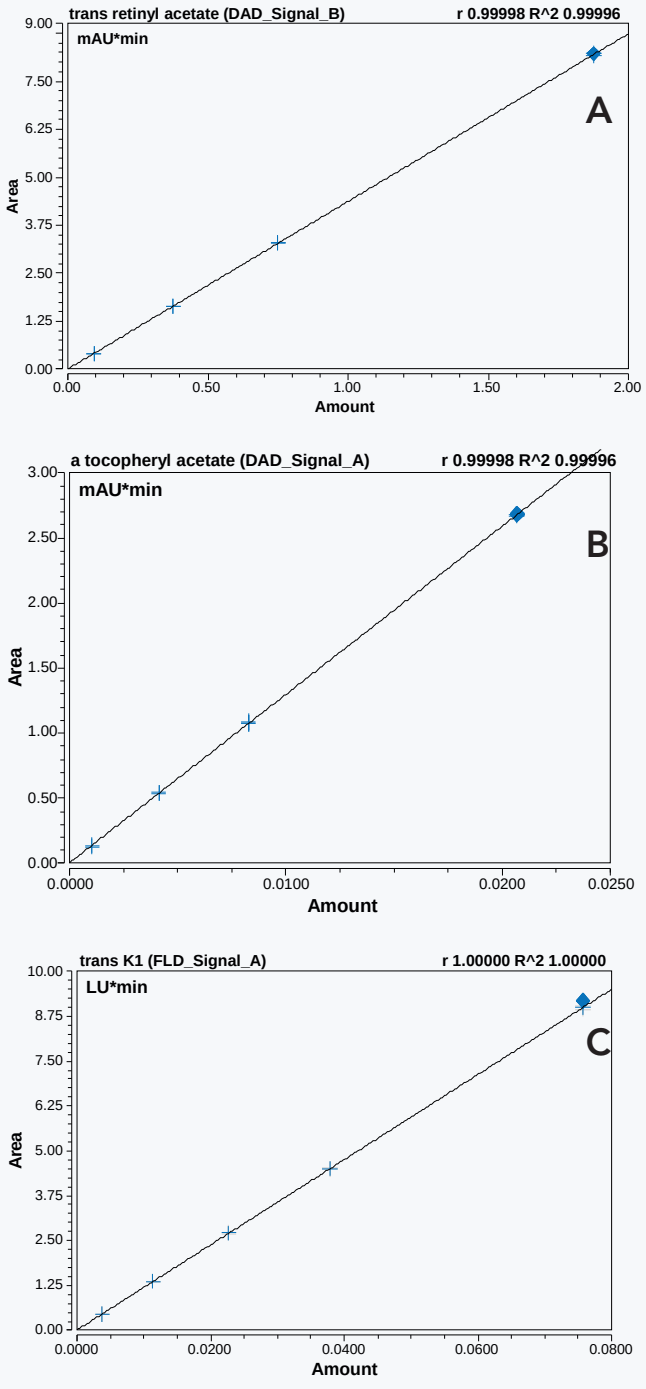

Figure 4 A-C: Representative calibration curves for vitamin A acetate (A), vitamin E acetate (B), and vitamin K1 (C).

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Table 3 shows the resulting accuracy and precision data for all vitamins determined based on automated liquid-liquid extraction of six replicates of both the powdered and reconstituted infant formula samples over three separate days. Accuracy data averaged 99.3% (range: 96.2% - 101%) and precision data averaged 1.24% RSD (range: 0.800% - 2.10%) for all vitamins in powdered infant formula. Accuracy data averaged 99.2% (range: 97.5% - 101%) and precision data averaged 1.14% RSD (range: 0.700% - 2.10%) for all vitamins in reconstituted infant formula. These data show that the liquid-liquid extraction of vitamins in infant formula could be automated using the MPS sampler and that the quickMIX was able to provide the thorough mixing required to ensure proteins are sufficiently precipitated allowing the accurate and reproducible extraction of vitamins from infant formula.

Table 3: Accuracy and precision results for all vitamins analyzed from extracted powdered and reconstituted infant formula samples.

Powder					
Analyte	Vitamin K	Vitamin A Palmitate	Vitamin A Acetate	Vitamin E Acetate	Vitamin E
Target Value	1.28	31.7	32.8	180	55.9
avg Day 1 value	1.28	30.4	32.7	182	58.0
avg Day 2 Value	1.27	30.5	32.3	181	55.9
avg Day 3 Value	1.25	30.7	32.4	180	56.7
Avg Day1+2+3 Value	1.27	30.5	32.5	181	56.7
% of target	99.2	96.2	99.1	101	101
Target RSD	2.70	1.40	0.900	1.70	1.40
Day 1 RSD	0.400	1.10	0.800	1.10	1.00
Day 2 RSD	1.40	0.800	0.600	0.600	1.20
Day 3 RSD	1.10	0.400	0.400	1.30	0.400
Day 1+2+3 RSD	1.30	0.900	0.800	1.10	2.10

Liquid Infant Formula					
Analyte	Vitamin K (mcg/g)	Vitamin A Palmitate (IU/g)	Vitamin A Acetate (IU/g)	Vitamin E Acetate (mcg/g)	Vitamin E (mcg/g)
Target Value	1.28	31.7	32.8	180	55.9
avg Day 1 value	1.30	30.8	32.3	181	56.0
avg Day 2 Value	1.25	31.0	32.4	180	56.4
avg Day 3 Value	1.27	30.9	32.3	180	56.6
Avg Day1+2+3 Value	1.27	30.9	32.3	180	56.3
% of target	99.2	97.5	98.5	100	101.
Target RSD	2.70	1.40	0.900	1,70	1.40
Day 1 RSD	1.20	0.500	0.500	0.700	1.20
Day 2 RSD	1.30	1.20	0.900	1.30	1.40
Day 3 RSD	2.20	0700	0.600	1.20	0.500
Day 1+2+3 RSD	2.10	0.800	0.700	1.00	1.10

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Conclusions

As a result of this study, we were able to show:

- Liquid-liquid extractions, such as those found in AOAC® Official Methods SM 2012.09 and 2015.09, are readily automated using the GERSTEL MPS robotic^{PRO} sampler.
- Vitamins K1, A, and E extractions from infant formula using the automated method proved to be accurate and precise. Accuracy data averaged 99.3% (range: 96.2% - 101%) and precision data averaged 1.24% RSD (range: 0.800% - 2.10%) for all vitamins in powdered infant formula. Accuracy data averaged 99.2% (range: 97.5% - 101%) and precision data averaged 1.14% RSD (range: 0.700% - 2.10%) for all vitamins in reconstituted infant formula.
- The GERSTEL MPS robotic sampler can efficiently extract infant formula samples using the ^{quick}MIX and CF-200 centrifuge options and, if desired, can be used to inject the resulting extracts into the HPLC system for complete automation of the entire infant formula analysis.

References

[1] Official Methods of Analysis of AOAC INTERNATIONAL (2012) 19th Ed., AOAC INTERNATIONAL, Gaithersburg, MD, USA, Official Method 2012.09.

[2] Official Methods of Analysis of AOAC INTERNATIONAL (2019) 21st Ed., AOAC INTERNATIONAL, Gaithersburg, MD, USA, Official Method 2015.09.