TECHNICAL NOTE 21997

Rapid quantitative analysis of vitamins K_1 and K_2 with chromatographic resolution from matrix interferences for clinical research

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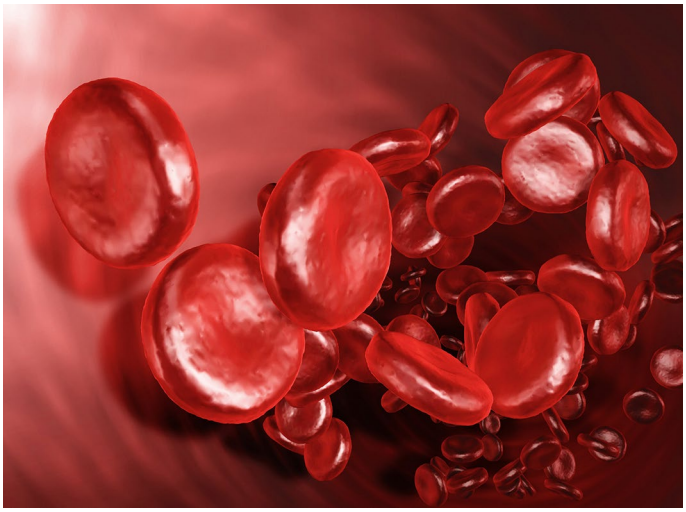

Goal

- Develop a simple sample preparation, a protein precipitation extraction procedure, when coupled with chromatographic separation
- Elute the target analytes away from known problematic matrix components using liquid chromatography
- Provide a robust assay for the analysis of vitamin K_1 and vitamin K_2 (MK-4 and MK-7)

Introduction

Vitamin K_1 (phylloquinone) and vitamin K_2 (group of compounds; menaquinones) are crucial for their roles in controlling blood clotting and regulation of bone metabolism. These fat-soluble vitamins are problematic for typical reversed-phase liquid chromatography as they are very lipophilic and often suffer from matrix interferences from co-eluting phospholipids. Typically, complex, multi-step sample preparation is employed meaning a lengthy analysis time. Here, a simplified sample preparation approach was explored and shown to be suitable for clinical research when coupled with a Thermo Scientific™ Accucore™ Biphenyl analytical column providing excellent separation from endogenous matrix components. High performance liquid chromatography was achieved on the Thermo Scientific™ Vanquish™ Horizon UHPLC system with excellent retention time stability. Detection was provided by the Thermo Scientific™ TSQ Endura™ triple-stage quadrupole mass spectrometer.

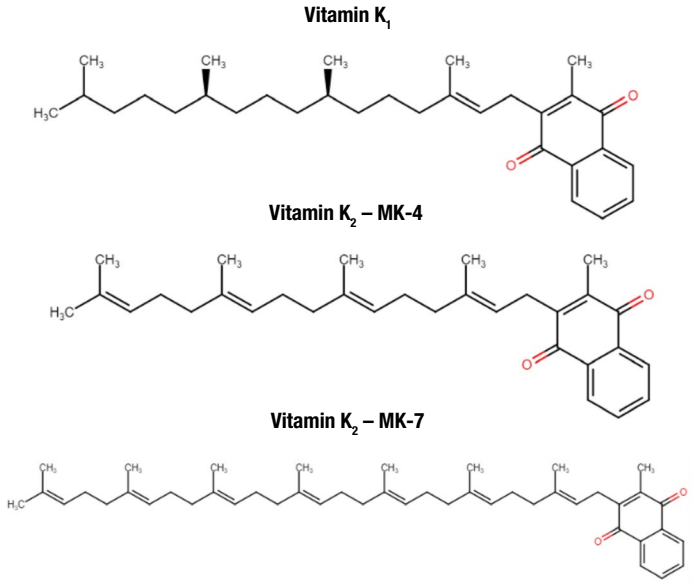

Figure 1. Target compounds and structures

Experimental

Chromatography consumables

- Thermo Scientific™ Accucore™ Biphenyl column 2.6 µm, 50 × 2.1 (P/N 17826-052130)
- Thermo Scientific™ WebSeal™ 96-well plate, square well (pack of 5) (P/N 60180-P212)
- Thermo Scientific™ WebSeal™ 96-well plate, square well (pack of 50) (P/N 60180-P202)
- Thermo Scientific™ WebSeal™ mat, blue silicone (pack of 5) (P/N 60180-M122)

Reagents

- Fisher Scientific™ Optima™ UHPLC-MS grade water (P/N 10154604)
- Fisher Scientific™ Optima™ UHPLC-MS grade methanol (P/N A458-1)
- Fisher Scientific™ Optima™ UHPLC-MS grade acetonitrile (P/N A956-1)
- Fisher Scientific™ analytical grade formic acid (P/N F/1900/PB08)
- Fisher Scientific™ Optima™ LC/MS grade ammonium formate (P/N A11550)

Instrumentation

- Vanquish Horizon UHPLC system consisting of the following:
  - System base Vanquish Horizon (P/N VH-S01-A)
  - Binary pump H (P/N VH-P10-A)
  - Split sampler HT (P/N VH-A10-A)
  - Column compartment H (P/N VH-C10-A)
  - Active pre-heater (P/N 6732.0110)
- TSQ Endura triple-stage quadrupole mass spectrometer (P/N IQLAAEGAAXFAPJMBFU)

UHPLC-MS/MS conditions	
Mobile phase A	Water with 5 mM ammonium acetate and 0.1% formic acid
Mobile phase B	Methanol with 0.1% formic acid
Flow rate	0.6 mL/min
Run time	3.5 min
Column temperature	40 °C, with active pre-heating and still air mode
Injection volume	10 µL
Auxiliary gas	5 AU

MS/MS conditions	
Source	Thermo Scientific™ Ion Max ion source with HESI-II probe
Polarity	Positive
Spray voltage	4500 V
Vaporizer temperature	420 °C
Sheath gas pressure	52 Arb
Aux gas pressure	16 Arb
Ion transfer tube temperature	356 °C
CID pressure	2 mTorr

Table 1. Compound transition details

Compound	Polarity	MS quantitation peak (*m/z*)	MS confirmation peak (*m/z*)	Collision energy (V)
Vitamin K_1	+	451.4 / 187.1	451.4 / 197.2	25
MK-4	+	445.3 / 187.1	445.3 / 81.4	27
MK-7	+	649.5 / 187.1	649.5 / 81.4	26

Sample preparation

Charcoal stripped human plasma was used as a surrogate matrix. A calibration range of 0.1–10 ng/mL was assessed along with three QC levels at 0.1, 5, and 10 ng/mL (n=6). Deuterated (d_7) vitamin K_1 was used as an internal standard. An aliquot of 500 µL of each sample was fortified with vitamin K1, MK-4 and MK-7 and extracted with acetonitrile at a ratio of 1:3 before evaporation and reconstitution in 50 µL of initial mobile phase conditions. The WebSeal 96-well glass-coated plate was used to facilitate high-throughput processing. The plate is glass coated to ensure sample cleanliness and sealed to prevent sample loss.

Data processing

The Thermo Scientific™ Chromeleon™ Chromatography Data System (CDS) software, version 7.2.9, was used for data acquisition and analysis.

Results and discussion

Using a simple approach to sample preparation, a lower limit of quantitation (LLOQ) of 0.1 ng/mL and a linear range to 10 ng/mL was successfully achieved using charcoal stripped plasma as a surrogate matrix. Excellent accuracy and precision were demonstrated, and good selectivity was observed for each analyte (Figures 2–4).

Electrospray ionization (ESI) was used for its excellent ionization efficiency of the target analytes; however, the technique is known to suffer from ion suppression effects caused by co-eluting matrix components from biological samples, predominantly phospholipids.[1,2] As the sample preparation was simple, but with limited selectivity, phospholipids were also monitored during analysis for co-elution by neutral loss of fragment *m/z* 184. The compounds of interest were chromatographically resolved from these matrix interferences, reducing the risk of ion suppression effects on the target analytes and improving the robustness of the analysis (Figure 5).

Linear calibration curves for all three compounds were achieved (Figure 6 and Table 2), along with excellent accuracy and precision data as shown in Table 3.

Cis/trans isomerism of vitamin K_1

Further separation of cis/trans isomers can be achieved by using the Thermo Scientific™ Accucore™ C30 analytical column, offering high shape selectivity for hydrophobic, long chain, structurally related isomers.[3]

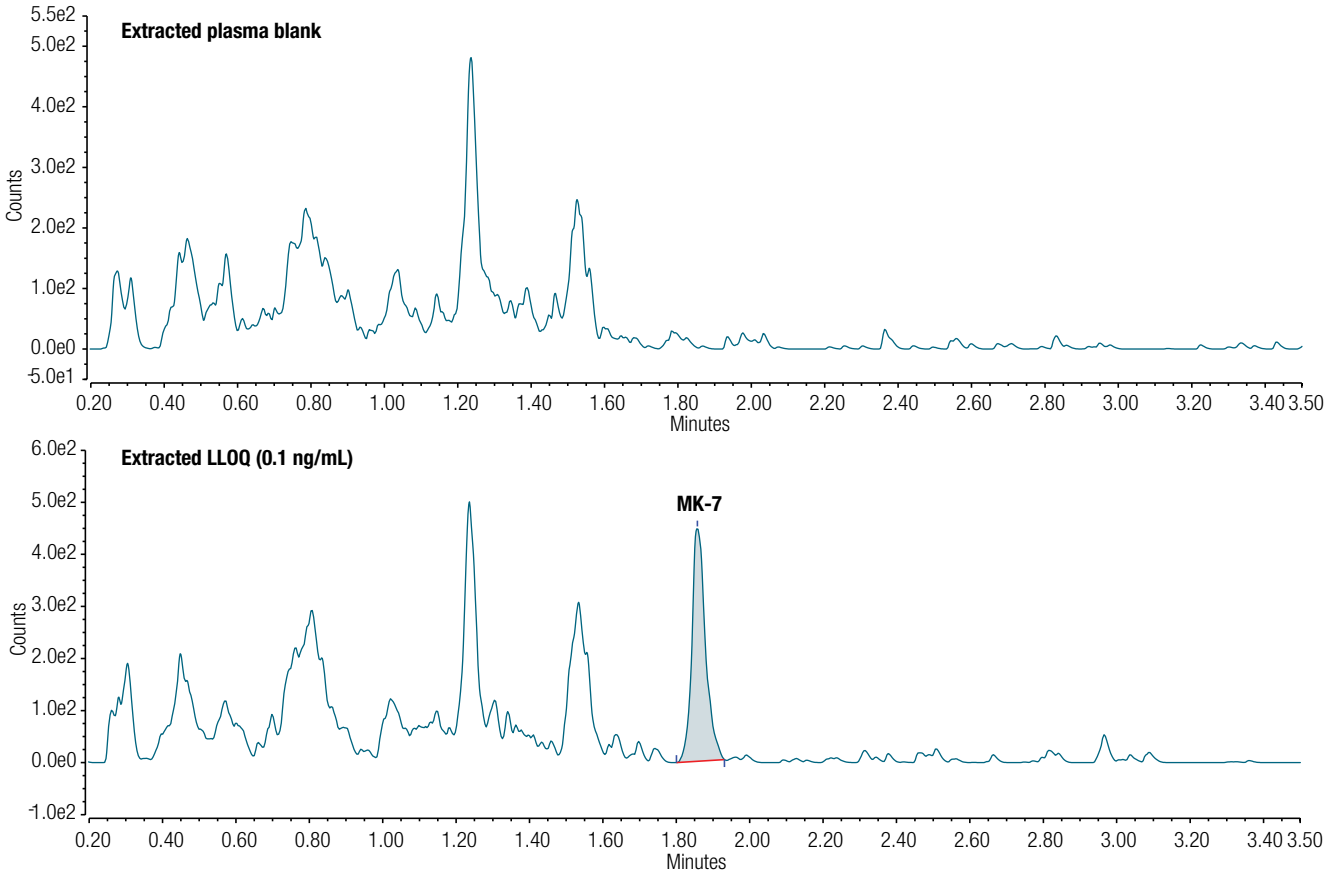

Figure 2. Example chromatogram for MK-7 showing selectivity against a blank extract

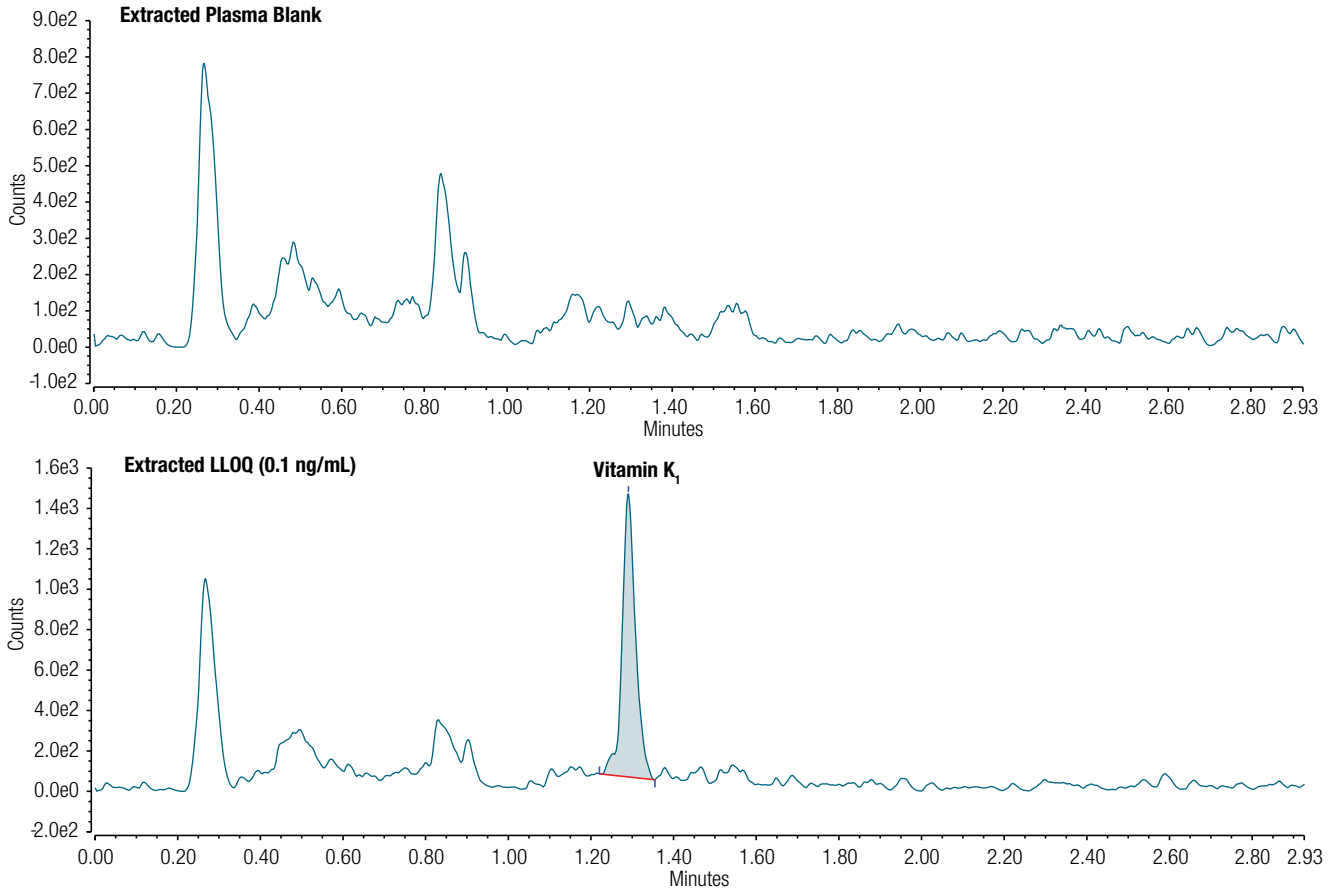

Figure 3. Example chromatogram for vitamin K_1 showing selectivity against a blank extract

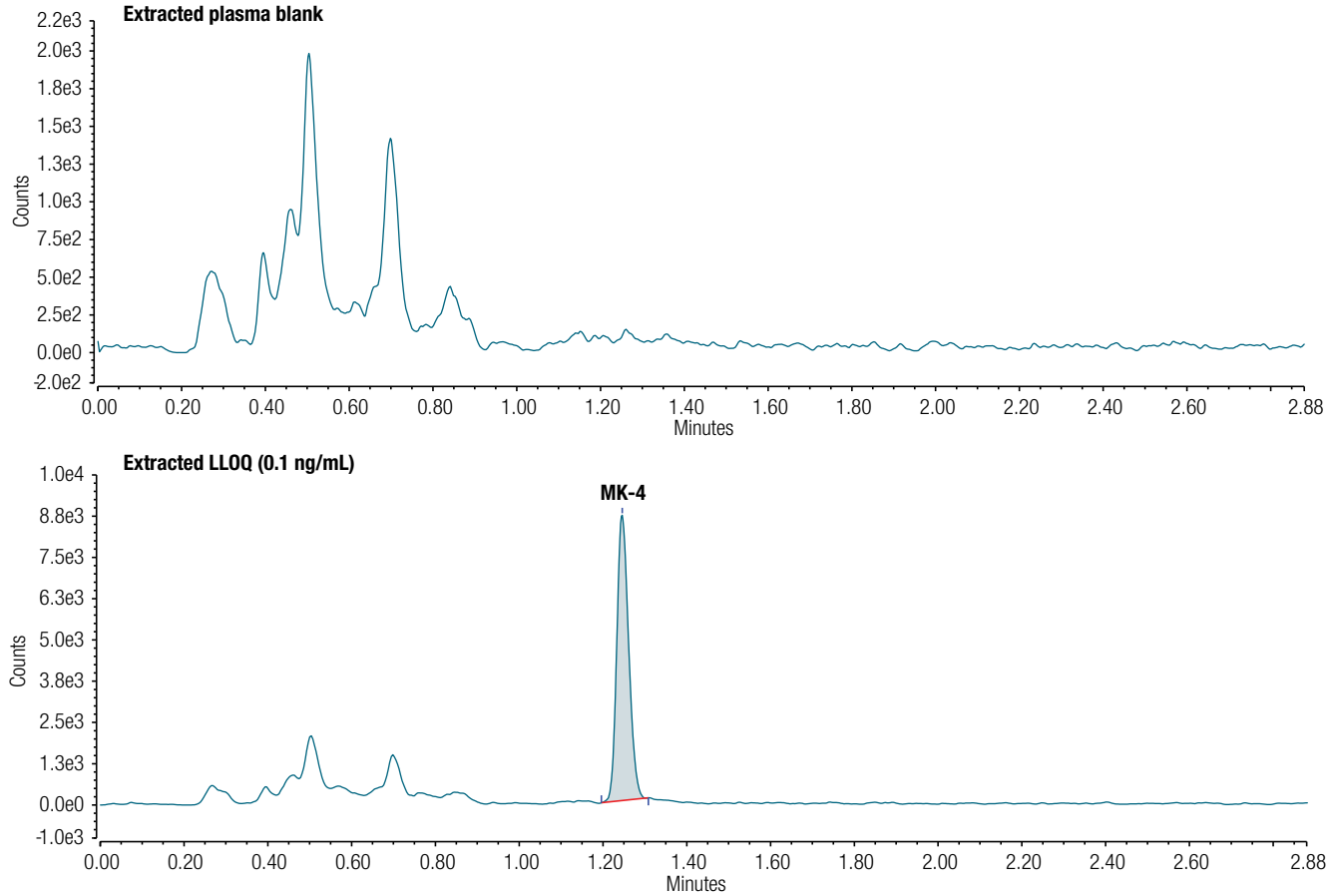

Figure 4. Example chromatogram for MK-4 showing selectivity against a blank extract

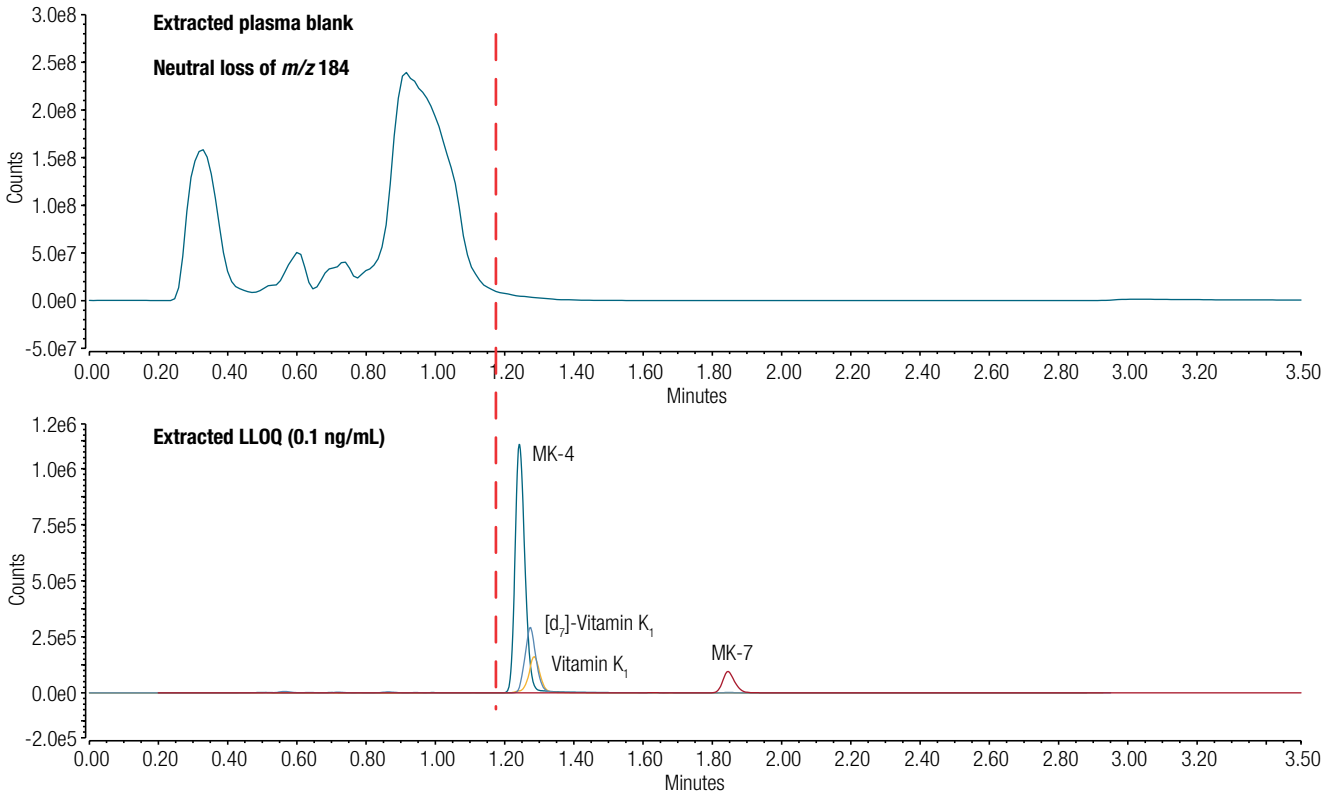

Figure 5. Retention time comparison between neutral loss scan of *m/z* 184 (top) and target analytes (bottom)

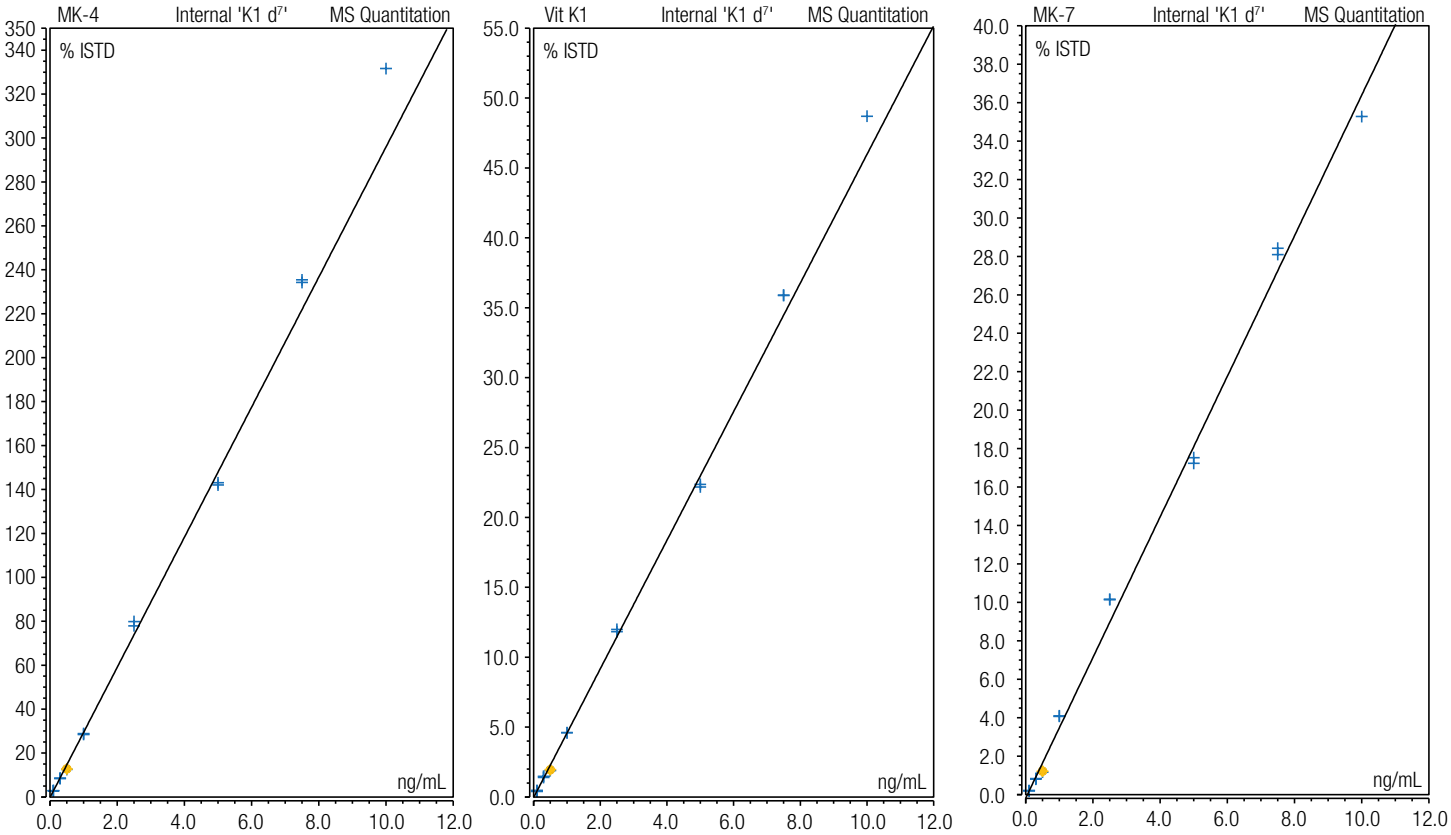

Figure 6. Calibration lines for all three components from 0.1 to 10 ng/mL

Table 2. Average bias and correlation data for the calibration lines

	Range 0.1–10 ng/mL)	
	Average % bias	Coeff. of det.
MK-4	-0.063	0.99213
Vit K_1	3.406	0.99171
MK-7	2.4	0.99537

Table 3. Average bias and precision data for calibration line and QC samples, n=6

n=6	Lower limit of quantitation (0.1 ng/mL)		Mid range QC sample (5 ng/mL)		Upper limit of quantitation (10 ng/mL)	
	X bias (%)	%CV	X bias (%)	%CV	X bias	%CV
MK-4	-0.660	3.16	-4.77	1.72	3.72	1.13
Vit K_1	1.70	0.343	-2.90	0.327	3.47	0.402
MK-7	5.71	0.420	-3.31	0.127	3.91	0.765

Conclusion

Simple sample preparation is desirable but can often lead to non-selective assays that are prone to effects on the target analyte by matrix components. The separation afforded by the Accucore Biphenyl analytical column allowed a simple protein precipitation extraction to be utilized without affecting the accuracy of the analytical method for clinical research.

References

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2. Ismaiel, O. et al. Monitoring phospholipids for assessment of ion enhancement and ion suppression in ESI and APCI LC/MS/MS for chlorpheniramine in human plasma and the importance of multiple source matrix effect evaluations. *Journal of Chromatography B* **2008,** *875*(2), 333–43.
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