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Application Note : 2107A

Analysis of Vitamin A and E in Serum as a Biomarker of Clinical Disorders:

Vitamin A and E Quantification in Serum at 8 Seconds per Sample Using LDTD-MS/MS

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Keywords: High-Throughput, Vitamin, LDTD-MS/MS

Introduction

Vitamin A (Retinol) and E (Tocopherol) have important roles in physiological functions to maintain good health. Scientific publications report their potential implication in different pathogenesis caused by the oxidative stress. Their role as biomarkers is evaluated. For large epidemiologic studies, high-throughput and accurate analytical techniques are needed.

Our goal for this application note is to use an automated sample preparation method for the quantification of Vitamin A and E in serum using a single operation in LUXON-MS/MS.

LUXON-MS/MS offers specificity combined with an ultra-fast analysis for an unrivaled quantification method. To develop this application, we focused on performing a quick and simple sample preparation. Vitamin A and E are analyzed, and results are obtained in less than 8 seconds per sample.

Luxon Ionization Source

The Luxon Ion Source® (**Figure 1**) is the second-generation sample introduction and ionization source based on the LDTD® technology for mass spectrometry. Luxon Ion Source® uses Fiber-Coupled Laser Diode (**Figure 2**) to obtain unmatchable thermal uniformity providing more precision, accuracy, and speed. The process begins with dry samples which are rapidly evaporated using indirect heat. The thermally desorbed neutral molecules are carried into a corona discharge region. High efficiency protonation and strong resistance to ionic saturation characterize this type of ionization and is the result of the absence of solvent and mobile phase. This thermal desorption process yields high-intensity molecular ion signal in less than 1 second sample-to-sample and allows working with very small volumes.

Figure 1 - Luxon Ion Source®

Figure 2 - Schematic of the Luxon Ionization Source

Sample Preparation Method

Automated Sample Extraction

Due to the instability of Vitamin A and E, stock solutions were prepared in ethanol (0.1% BHT). Pooled serum was exposed to UV light for at least 24h to photodegrade the endogenous vitamin. The pooled exposed serum was then spiked to generate a calibration curve and QCs.

Serum samples were transferred into barcoded tubes, readable by the Azeo extraction system. Each barcoded vial was scanned by the Azeo Liquid Handler and an automatic batch file was created. The Azeo extraction system (Figure 3) is used to extract the samples using the following conditions:

- 5 µL of Internal standard (Retinol-d_5 and Tocopherol-d_6 at 10 µg/mL in EtOH (0.1%BHT)) were added to each sample
- 25 µL of serum sample were transferred from the vials to a deep-well plate placed in the Lumo Vortexer
  - Mix (30 seconds at 1000 rpm)
- 25 µL of extraction buffer (0.25 mM NaOH in IPA:Water / 3.5:7.5) were added into a deep-well plate
  - Mix (30 seconds at 1000 rpm)
- 300 µL Hexane were added into a deep-well plate
  - Mix for 5 minutes at 1000 rpm
  - Pause of 5 minutes for phase separation
- Spot 6 µL upper layer phase onto a LazWell™ 96 plate
  - Dry 1 minute at room temperature with air flow.
- Spot 6 µL Butylated Hydroxytoluene (BHT, 100 µg/mL in hexane) onto a LazWell™ 96 plate
  - Dry 1 minute at room temperature with air flow.

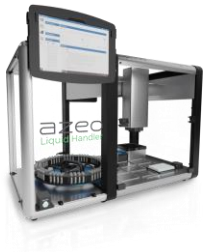

Figure 3 - Automated extraction system

LDTD®-MS/MS Parameters

LDTD
Model: Luxon S-960, Phytronix
Carrier gas: 9 L/min (air)
Laser pattern:

- 6-second ramp to 65% power
- Hold 2 seconds

MS/MS
MS model: Q-Trap System® 5500, Sciex
Scan Time: 40 msec
Curtain: 30
Total run time: 8 seconds per sample
Ionization: APCI
Analysis Method: Positive MRM mode

Table 1 - MRM transitions for LDTD-MS/MS

	Transition	CE
Retinol	269.2 → 93.1	32
Retinol-d_5	274.2 → 93.1	32
Tocopherol	431.2 → 165.1	25
Tocopherol-d_6	437.2 → 171.1	25

Results and Discussion

Validation Test

Calibration curves ranging from 191 to 2191 ng/mL for Retinol and from 3.1 to 43.1 µg/mL for Tocopherol were prepared in a serum exposed to UV light. Three human serum samples were used as QC (endogenic concentration values were evaluated with a reference method and used as a nominal value). Replicate extractions were deposited onto a LazWell™ plate and dried before analysis. The peak area against the internal standard (IS) ratio was used to normalize the signal.

Linearity

The calibration curves were plotted using the peak area ratio and the nominal concentration of standards. For the linearity test, the following acceptance criteria was used:

- Linear regression (r) must be ≥ 0.995

Table 2 shows the inter-day correlation coefficients for Tocopherol. Values greater than 0.995 are obtained. **Figure 4** shows a typical calibration curve result for Tocopherol. Similar results are obtained for Retinol.

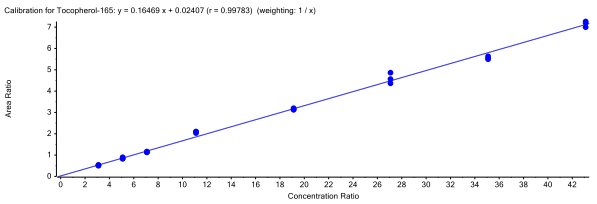

Figure 4 – Tocopherol calibration curve

Table 2 – Inter-day calibration curve correlation coefficients

	Tocopherol
Curve 1	0.99783
Curve 2	0.99576
Curve 3	0.99624
Curve 4	0.99665
Curve 5	0.99598

Precision and Accuracy

For the accuracy and precision evaluation, the following acceptance criteria were used:

- Each concentration must not exceed 15% CV
- Each concentration must be within 100 ± 15% of the nominal concentration

For the inter-run precision and accuracy experiment, each standard was analyzed in triplicate, on five different days. **Table 3** and **4** shows the inter-run precision and accuracy results for Retinol and Tocopherol, respectively. The obtained %CV was below 15% and the accuracy was within 15% of the nominal value.

Table 3 - Inter-Run Precision and Accuracy of Retinol

Retinol	QC-L	QC-M	QC-H
Conc (ng/ml)	391	991	1791
N	15	15	15
Mean (ng/ml)	405.0	942.2	1755.5
SD	23.1	63.4	118.8
%CV	5.7	6.7	6.8
%Nom	103.6	95.1	98.0

Table 4 - Inter-Run Precision and Accuracy of Tocopherol

Tocopherol	QC-L	QC-M	QC-H
Conc (µg/ml)	7.1	19.1	35.1
N	15	15	15
Mean (µg/ml)	7.7	19.6	33.3
SD	0.8	1.0	0.7
%CV	10.4	5.1	2.3
%Nom	108.4	102.5	94.9

Wet Stability of Sample Extracts

Following the extraction, sample extracts are kept at -20°C in closed containers. After 1 day, sample extracts are spotted on a LazWell™ plate, dried and analyzed. Precision and accuracy of QC samples are reported in **Table 5**. All the results are within the acceptable criteria range for 1 day at -20°C.

Dry Stability of Samples Spotted in LazWell™

Extracted samples are spotted onto a LazWell™ plate, dried and kept at room temperature for 1 hour before analysis. Tocopherol and Retinol must be stabilized with BHT to avoid drug degradation on the LazWell™ plate. The precision and accuracy results of QC samples are reported in **Table 5**. All the results are within the acceptable criteria range for 1 hour at room temperature. An important signal lost is observed after 1 hour. Analysis within 30 minutes after sample evaporation is strongly recommended.

Table 5 - Wet and Dry Stability of Tocopherol

Parameters	Dry stability (1 hour / RT)			Wet stability (1 day / 4°C)		
QC	QCL	QCM	QCH	QCL	QCM	QCH
Conc (µg/ml)	7.1	19.1	35.1	7.1	19.1	35.1
N	3	3	3	3	3	3
Mean (µg/ml)	8.1	20.4	33.1	8.2	20.1	33.8
%CV	2.9	3.9	3.1	0.8	2.1	1.3
%Nom	114.5	106.7	94.4	115.0	105.0	96.3

Cross validation study

Real patients' serum samples (N=6) have been tested with this method to correlate with results obtained by traditional LC-MS/MS. The percentage of difference between the values are evaluated. A difference below 10% is obtained. Results are reported in **Table 6**.

Table 6 – Cross validation result

	Vitamin A (retinol)			Vitamin E (tocopherol)		
	LC (ng/mL)	Luxon (ng/mL)	%Diff (%)	LC (µg/mL)	Luxon (µg/mL)	%Diff (%)
M1	479	449	-3.3	13.7	15.6	6.5
M2	411	423	1.4	10.0	11.1	5.3
M3	551	554	0.3	9.9	11.1	5.8
M4	581	538	-3.8	15.2	17.7	7.5
M5	820	701	-7.8	13.9	16.1	7.2
M6	553	609	4.7	10.8	12.2	6.0

Conclusion

The Luxon Ion Source® combined with Sciex Q-Trap 5500 mass spectrometer system allows ultra-fast (**8 seconds per sample**) analysis of Vitamin A and E in serum.

Phytronix Breakaway Technologies

Shimadzu

Clinical

Application Note : 2107B

Analysis Vitamin A and E in Serum as a Biomarker of Clinical Disorders:

Vitamin A and E Quantification in Serum at 8 Seconds per Sample Using LDTD-MS/MS

Pierre Picard, Jean Lacoursière and Serge Auger
Phytronix Technologies, Québec, Canada
Keywords: High-Throughput, Vitamin, Serum, LDTD-MS/MS

Introduction

Vitamin A (Retinol) and E (Tocopherol) have important roles in physiological functions to maintain good health. Scientific publications report their potential implication in different pathogenesis caused by the oxidative stress. Their role as biomarkers is evaluated. For large epidemiologic studies, high-throughput and accurate analytical techniques are needed.

Our goal for this application note is to use an automated sample preparation method for the quantification of Vitamin A and E in serum using a single operation in LUXON-MS/MS.

LUXON-MS/MS offers specificity combined with an ultra-fast analysis for an unrivaled quantification method. To develop this application, we focused on performing a quick and simple sample preparation. Vitamin A and E are analyzed, and results are obtained in less than 8 seconds per sample.

Luxon Ionization Source

The Luxon Ion Source® (**Figure 1**) is the second-generation sample introduction and ionization source based on the LDTD® technology for mass spectrometry. Luxon Ion Source® uses Fiber-Coupled Laser Diode (**Figure 2**) to obtain unmatchable thermal uniformity providing more precision, accuracy, and speed. The process begins with dry samples which are rapidly evaporated using indirect heat. The thermally desorbed neutral molecules are carried into a corona discharge region. High efficiency protonation and strong resistance to ionic saturation characterize this type of ionization and is the result of the absence of solvent and mobile phase. This thermal desorption process yields high-intensity molecular ion signal in less than 1 second sample-to-sample and allows working with very small volumes.

Figure 1 - Luxon Ion Source®

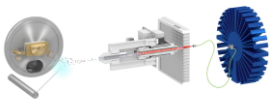

Figure 2 - Schematic of the Luxon Ionization Source

Sample Preparation Method

Automated Sample Extraction

Due to the instability of Vitamin A and E, stock solutions were prepared in ethanol (0.1% BHT). Pooled serum was exposed to UV light for at least 24h to photodegrade the endogenous vitamin. The pooled exposed serum was then spiked to generate a calibration curve and QCs.

Serum samples were transferred into barcoded tubes, readable by the Azeo extraction system. Each barcoded vial was scanned by the Azeo Liquid Handler and an automatic batch file was created. The Azeo extraction system (Figure 3) is used to extract the samples using the following conditions:

- 5 µL of Internal standard (Retinol-d_5 and Tocopherol-d_6 at 10 µg/mL in EtOH (0.1%BHT)) were added to each sample
- 25 µL of serum sample were transferred from the vials to a deep-well plate placed in the Lumo Vortexer
  - Mix (30 seconds at 1000 rpm)
- 25 µL of extraction buffer (0.25 mM NaOH in IPA:Water / 3.5:7.5) were added into a deep-well plate
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- 300 µL Hexane were added into a deep-well plate
  - Mix for 5 minutes at 1000 rpm
  - Pause of 5 minutes for phase separation
- Spot 6 µL upper layer phase onto a LazWell™ 96 plate
  - Dry 1 minute at room temperature with air flow.
- Spot 6 µL Butylated Hydroxytoluene (BHT, 100 µg/mL in hexane) onto a LazWell™ 96 plate
  - Dry 1 minute at room temperature with air flow.

Figure 3 - Automated extraction system

LDTD®-MS/MS Parameters

LDTD
Model: Luxon SH-960, Phytronix
Carrier gas: 9 L/min (air)
Laser pattern:

- 6-second ramp to 65% power
- Hold 2 seconds

MS/MS
MS model: LCMS-8060, Shimadzu
Scan Time: 50 msec
Total run time: 8 seconds per sample
Ionization: APCI
Analysis Method: Positive MRM mode

Table 1 - MRM transitions for LDTD-MS/MS

	Transition	CE
Retinol	269.2 → 93.1	25
Retinol-d_5	274.2 → 93.1	25
Tocopherol	431.2 → 165.1	20
Tocopherol-d_6	437.2 → 171.1	20

Results and Discussion

Data preparation process

Mass spectrometers are data acquisition systems that were not designed to deal with few second signal per sample. The synchronization sequence adds 6 to more than 15 seconds between each sample. To bypass this, all samples are acquired in a single file (**Figure 4**). To allow the analysis of such data, Cascade software is designed to detect, split and integrate every sample peak acquired in a single file.

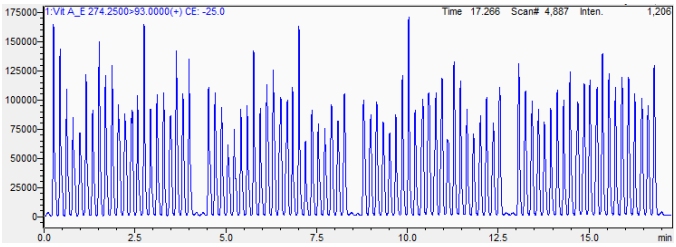

Figure 4 – Single file mass spectrometer data for 96 samples. Retinol-d_5 transition.

Validation Test

Calibration curves ranging from 191 to 2191 ng/mL for Retinol and from 3.1 to 43.1 µg/mL for Tocopherol were prepared in a serum exposed to UV light. Three human serum samples were used as QC (endogenic concentration values were evaluated with a reference method and used as a nominal value). Replicate extractions were deposited onto a LazWell™ plate and dried before analysis. The peak area against the internal standard (IS) ratio was used to normalize the signal.

Linearity

The calibration curves were plotted using the peak area ratio and the nominal concentration of standards. For the linearity test, the following acceptance criteria was used:

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Figure 5 shows a typical calibration curve result for Tocopherol. Similar results are obtained for Retinol.

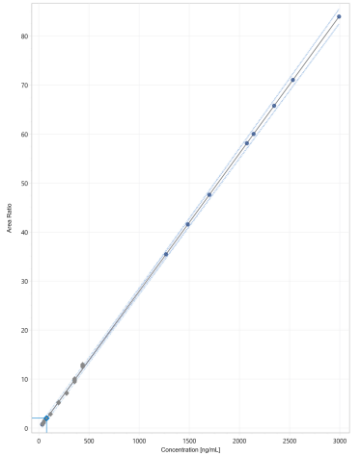

Figure 5 – Tocopherol calibration curve

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Mean (µg/ml)	7.7	19.6	33.3
SD	0.8	1.0	0.7
%CV	10.4	5.1	2.3
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Cross validation study

Real patients' serum samples (N=6) have been tested with this method to correlate with results obtained by traditional LC-MS/MS. The percentage of difference between the values are evaluated. A difference below 10% is obtained. Results are reported in **Table 4**.

Table 4 – Cross validation result

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Conclusion

The Luxon Ion Source® combined with Shimadzu LCMS-8060 mass spectrometer system allows ultra-fast (**8 seconds per sample**) analysis of Vitamin A and E in serum.

Phytronix Breakaway Technologies

Thermo

Clinical

Application Note : 2107C

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Figure 1 - Luxon Ion Source®

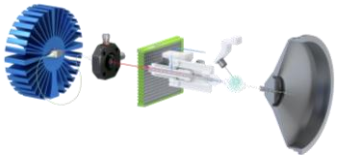

Figure 2 - Schematic of the Luxon Ionization Source

Sample Preparation Method

Automated Sample Extraction

Due to the instability of Vitamin A and E, stock solutions were prepared in ethanol (0.1% BHT). Bovine serum albumin solution (40 mg/mL) was used as negative matrix. The BSA matrix was then spiked to generate a calibration curve and QCs.

Samples were transferred into barcoded tubes, readable by the Azeo extraction system. Each barcoded vial was scanned by the Azeo Liquid Handler and an automatic batch file was created. The Azeo extraction system (Figure 3) is used to extract the samples using the following conditions:

- 5 µL of Internal standard (Retinol-d_5 and Tocopherol-d_6 at 10 µg/mL in EtOH (0.1%BHT)) were added to each sample
- 25 µL of serum sample were transferred from the vials to a deep-well plate placed in the Lumo Vortexer
  - Mix (30 seconds at 1000 rpm)
- 25 µL of extraction buffer (0.25 mM NaOH in IPA:Water / 3.5:7.5) were added into a deep-well plate
  - Mix (30 seconds at 1000 rpm)
- 300 µL Hexane were added into a deep-well plate
  - Mix for 5 minutes at 1000 rpm
  - Pause of 5 minutes for phase separation
- Spot 6 µL upper layer phase onto a LazWell™ 96 plate
  - Dry 1 minute at room temperature with air flow.
- Spot 6 µL Butylated Hydroxytoluene (BHT, 100 µg/mL in hexane) onto a LazWell™ 96 plate
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Figure 3 - Automated extraction system

LDTD®-MS/MS Parameters

LDTD
Model: Luxon T-960, Phytronix
Carrier gas: 9 L/min (air)
Laser pattern:

- 6-second ramp to 65% power

MS/MS
MS model: Thermo Vantage, Thermo Fisher Scientific
Scan Time: 50 msec
Total run time: 8 seconds per sample
Ionization: APCI
Analysis Method: Positive MRM mode

Table 1 - MRM transitions for LDTD-MS/MS

	Transition	CE
Retinol	269.2 → 93.1	25
Retinol-d_5	274.2 → 93.1	25
Tocopherol	431.2 → 165.1	20
Tocopherol-d_6	437.2 → 171.1	20

Results and Discussion

Validation Test

Calibration curves ranging from 200 to 4000 ng/mL for Retinol and from 1.5 to 30 µg/mL for Tocopherol were prepared in BSA (40 mg/mL). Three QCs were prepared in same negative matrix. Replicate extractions were deposited onto a LazWell™ plate and dried before analysis. The peak area against the internal standard (IS) ratio was used to normalize the signal.

Linearity

The calibration curves were plotted using the peak area ratio and the nominal concentration of standards. For the linearity test, the following acceptance criteria was used:

- Linear regression (r^2) must be ≥ 0.99

Table 2 shows the inter-day correlation coefficients for Retinol and Tocopherol. Values greater than 0.995 are obtained. **Figure 4** shows a typical calibration curve result for Tocopherol. Similar results are obtained for Retinol.

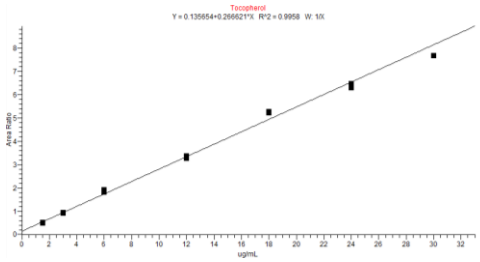

Figure 4 – Tocopherol calibration curve

Table 2 – Inter-day calibration curve correlation coefficients

	Retinol	Tocopherol
Curve 1	0.9974	0.9958
Curve 2	0.9976	0.9972
Curve 3	0.9981	0.9954
Curve 4	0.9977	0.9953
Curve 5	0.9981	0.9950

Precision and Accuracy

For the accuracy and precision evaluation, the following acceptance criteria were used:

- Each concentration must not exceed 15% CV
- Each concentration must be within 100 ± 15% of the nominal concentration

For the inter-run precision and accuracy experiment, each QC was analyzed in triplicate, on five different days. **Table 3** and **4** shows the inter-run precision and accuracy results for Retinol and Tocopherol, respectively. The obtained %CV was below 15% and the accuracy was within 15% of the nominal value.

Table 3 - Inter-Run Precision and Accuracy of Retinol

Retinol	QC-L	QC-M	QC-H
Conc (ng/mL)	600	2000	3000
N	15	15	15
Mean (ng/mL)	658.6	1940.0	3018.8
SD	32.1	193.0	253.0
%CV	4.9	9.9	8.4
%Nom	109.8	97.0	100.6

Table 4 - Inter-Run Precision and Accuracy of Tocopherol

Tocopherol	QC-L	QC-M	QC-H
Conc (µg/mL)	4.5	15	22.5
N	15	15	15
Mean (µg/mL)	4.7	15.1	21.6
SD	0.1	1.1	1.9
%CV	2.8	7.4	8.6
%Nom	103.8	100.6	96.0

Wet Stability of Sample Extracts

Following the extraction, sample extracts are kept at -20°C in closed containers. After 1 day, sample extracts are spotted on a LazWell™ plate, dried and analyzed. Precision and accuracy of Tocopherol QC samples are reported in **Table 5**. Similar results are obtained for Retinol. All the results are within the acceptable criteria range for 1 day at -20°C.

Dry Stability of Samples Spotted in LazWell™

Extracted samples are spotted onto a LazWell™ plate, dried and kept at room temperature for 1 hour before analysis. Tocopherol and Retinol must be stabilized with BHT to avoid drug degradation on the LazWell™ plate. The precision and accuracy results of Tocopherol QC samples are reported in **Table 5**. All the results are within the acceptable criteria range for 1 hour at room temperature. Similar results are obtained for Retinol.

An important signal lost is observed after 1 hour. Analysis within 30 minutes after sample evaporation is strongly recommended.

Table 5 - Wet and Dry Stability of Tocopherol

Parameters	Dry stability (1 hour / RT)			Wet stability (1 day / -20°C)		
QC	QCL	QCM	QCH	QCL	QCM	QCH
Conc (µg/mL)	4.5	15	22.5	4.5	15	22.5
N	3	3	3	3	3	3
Mean (µg/mL)	4.6	15.4	21.9	4.7	15.2	22.5
%CV	2.0	9.6	8.3	3.2	6.6	10.4
%Nom	102.9	102.8	97.1	105.3	101.3	100.0

Cross validation study

Real patients' serum samples (N=6) have been tested with this method to correlate with results obtained by traditional LC-MS/MS. The percentage of difference between the values are evaluated. A difference below 15% is obtained. Results are reported in **Table 6**.

Table 6 – Cross validation result

	Vitamin A (retinol)			Vitamin E (tocopherol)		
	LC (ng/mL)	Luxon (ng/mL)	%Diff (%)	LC (µg/mL)	Luxon (µg/mL)	%Diff (%)
M1	405,4	375,8	7,6	7,8	7,4	6,2
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M3	539,7	521,5	3,4	9,0	7,9	13,2
M4	600,1	596,5	0,6	13,9	12,6	10,2
M5	759,1	787,7	-3,7	10,7	10,1	5,7
M6	496,2	553,6	-10,9	9,8	9,1	6,8

Conclusion

The Luxon Ion Source® combined with Thermo Vantage mass spectrometer system allows ultra-fast (**8 seconds per sample**) analysis of Vitamin A and E in serum.