Determination of Eight Estrogens in Milk by UHPLC and the Agilent 6495 Triple Quadrupole Mass Spectrometer

Application Note

Authors

Dan-Hui Dorothy Yang,
Agilent Technologies Inc.,
Santa Clara, California,
USA

Jian-Zhong Li,
Agilent Technologies, Inc.,
Beijing, P.R. China

Bernhard Wuest,
Agilent Technologies R&D and
Marketing GmbH & Co. KG,
Waldbronn,
Germany

Abstract

This Application Note demonstrates a complete method to rapidly and precisely determine estrogens in milk with the Agilent 6495 Triple Quadrupole Mass Spectrometer coupled with the Agilent 1290 Infinity UHPLC. Using dynamic multiple reaction monitoring (DMRM) in negative ion mode, quantitation of estrogens was achieved with a simple QuEChERS procedure without any derivatization. The lower limits of quantitation (LLOQ) for all estrogens in milk were less than 0.1 µg/kg, far below the regulatory requirement, which is set at 1 µg/kg[4].

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Introduction

Human exposure to estrogens through the consumption of cow milk and other dairy products is an area of increasing concern due to the considerable amounts of female sex hormones they contain. On modern dairy farms, cows are milked approximately 300 days a year, and are pregnant for the majority of that time. The milk produced by a pregnant cow is higher in estrogens. This leads to an increased exposure of these hormones when consumed by the public.

The presence of estrogens in milk impacts the development of infants and children, causing early maturation in girls and delayed maturation in boys[1]. Regular exposure to high levels of estrogens has been linked to hormone-sensitive cancers, such as breast cancer and prostate cancer[2,3]. Routine determination of estrogens in milk or milk powder is required by many governmental agencies[4,5].

Generally accepted methods for analyzing estrogen in milk employ a traditional solvent extraction followed by solid phase extraction (SPE)[1]. Although they have been widely accepted, these traditional methods are labor-intensive, use a large amount of solvent, and generate a high volume of waste. The use of highly-sensitive instrumentation avoids the need of enrichment of a large sample size or analyte derivatization. We have developed a method using an Agilent QuEChERS kit[6] in combination with the highly sensitive Agilent 6495 Triple Quadrupole Mass Spectrometer to quantify estrogens in milk. Several modifications to the triple quadrupole mass spectrometer have resulted in better analytical performance. These modifications include a new mass filter one optics for increased precursor ion transmission, an improved curved and tapered collision cell for enhanced MS/MS spectral fidelity, a new ion detector operating at dynode accelerating voltages of up to 20 kV, and an optimized autotune for speed and sensitivity. This method provides significant advantages over the traditional methods, including minimal labor, high recovery, high precision, and limited solvent usage. We have achieved LLOQs far below regulatory requirements for the following eight estrogens: estriol, estradiol (two isomers: 17-α-estradiol and 17-β-estradiol), estrone, dienestrol, ethynyl estradiol, diethylstilbestrol, and hexestrol. Figure 1 shows the structure of eight estrogens detected with the method.

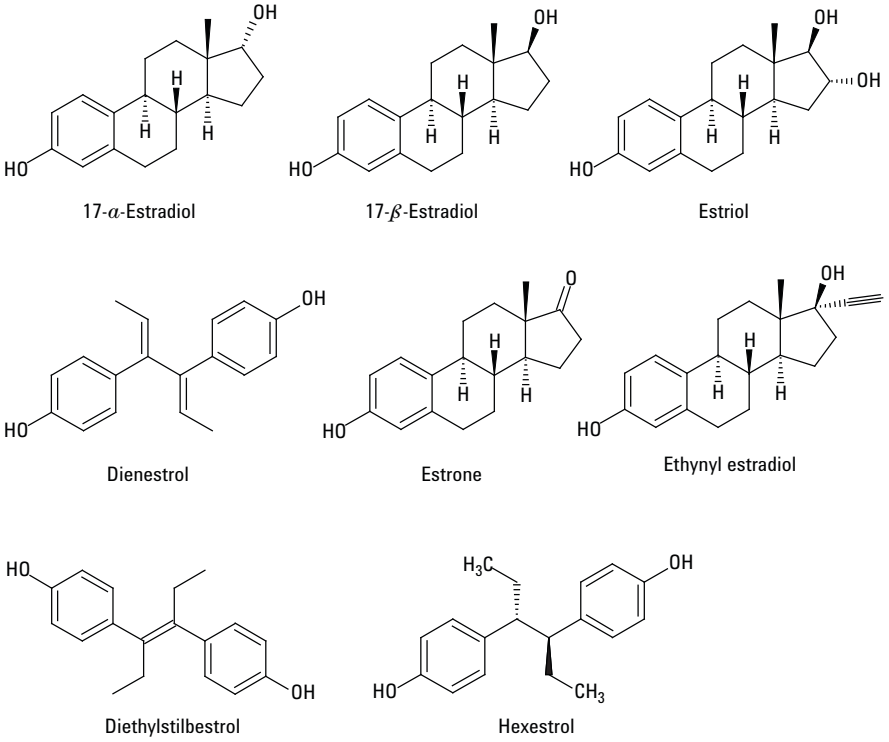

Figure 1. Structures of estrogens detected with the LC/MS/MS method.

Experimental

Reagents and chemicals

All reagents and solvents were HPLC or analytical grade. Methanol and acetonitrile were purchased from Honeywell (Catalog number 230-4 and 015-4 respectively). Ultrapure water was obtained from a Milli-Q Integral system equipped with LC-Pak Polisher and a 0.22-µm membrane point-of-use cartridge (Millipak). Ammonium fluoride was purchased from Fluka (338869-25 g). A 5 M stock solution was made by dissolving ammonium fluoride in Milli-Q water. Estrogen standards were obtained from the Chinese Academy of Inspection and Quarantine at concentrations of 10 ppm, 1 ppm, and 100 ppb in acetonitrile.

Instrumentation and conditions

- Agilent 1290 Infinity Binary Pump (G4220A)
- Agilent 1290 Infinity Standard Autosampler (G4226A) and sample cooler (G1330B)
- Agilent 1290 Infinity Thermostatted Column Compartment (G1316C)

UHPLC conditions are listed in Table 1.

MS detection

- Agilent 6495 Triple Quadrupole Mass Spectrometer with Agilent JetStream electrospray ionization source

Software

- Agilent MassHunter data acquisition for triple quadruple mass spectrometer, Version B.07.00
- Agilent MassHunter Qualitative Software, Version B.06.0.633.10 SP1
- Agilent MassHunter Quantitative Software, Version B.06.00.388.00

MS conditions are listed in Table 1. Table 2 lists the precursor ions/product ions and their corresponding optimized collision energy values for eight estrogens.

Caliper LifeSciences Turbo Vap LV
Eppendorf Centrifuge 5451 R
Heraeus Labofuge 400 R

Table 1. Chromatographic conditions.

UHPLC conditions		
Column	Agilent Poroshell 120 Phenyl-Hexyl 2.1 × 100 mm, 2.7 µm (p/n 695775-912)	
Column temperature	35 °C	
Injection volume	5 µL	
Speed	Draw 100 µL/min; Eject 200 µL/min	
Autosampler temperature	6 °C	
Needle wash	20 seconds (80 % MEOH/20 % water)	
Mobile phase	A) Water with 0.4 mM ammonium fluoride	
	B) Methanol:acetonitrile (1:1 v/v)	
Flow rate	0.4 mL/min	
Gradient program	Time	B %
	0	15
	0.5	15
	2.5	40
	6.0	65
	7.0	95
	9.0	95
	9.1	15
	Stop time	10 minutes
	Post time	2 minutes
MS conditions		
Ion mode	Negative	
Drying gas temperature	200	
Drying gas flow	16	
Sheath gas temperature	390	
Sheath gas flow	12	
Nebulizer pressure	35	
Capillary voltage	3,000 (neg)	
Nozzle voltage	300 (neg)	
Delta EMV	400 (neg)	
LPF RF	60 (neg)	
HPF RF	100 (neg)	
MS1 and MS2 resolution	Unit	

Sample preparation

The sample preparation procedure includes extraction/partitioning and dispersive SPE cleanup. Two percent organic milk was purchased from a local grocery store. Ten-gram milk samples were fortified with the appropriate amount of estrogens. After a brief vortex, 10 mL of acetonitrile was added and the tube was vortexed for 30 seconds. One pouch of Agilent Bond Elute extraction/partitioning reagent (p/n 5982-5755) was then added to each tube, followed by vigorous shaking for 1 minute. The tubes were then centrifuged for 5 minutes at 3,500 rpm.

A 6 mL amount of supernatant acetonitrile was transferred to an Agilent Bond Elut QuEChERS dispersive SPE 15-mL tube for animal tissue (p/n 6982-4956). The tubes were vortexed for 1 minute and centrifuged at 3,500 rpm for 5 minutes. Then, 4 mL of liquid layer was transferred to a glass test tube and dried at 40 °C under constant nitrogen flow. The samples were reconstituted with 2 mL of 50 % methanol water. After reconstitution, the samples were transferred to a 2-mL Eppendorf tube and centrifuged at 14,000 rpm for 7 minutes at 4 °C. The supernatant clear solution was then transferred to an HPLC vial for injection. Figure 2 shows the flow chart of the QuEChERS procedure for the determination of estrogens in milk.

Table 2. Optimum precursor/product ions and their corresponding collision energy (CE) values. Dwell time depends on the number of MRMs in a dynamic MRM method. Min/max dwell time of 31.14 ms and 224.11 ms were defined with cycle time 450 ms.

Compound name	Precursor ion	Product ion	RT (min)	Delta RT	Fragmentor	CE	CAV	Polarity
17-α-estradiol	271.1	145	5.2	1	380	45	3	Negative
17-α-estradiol	271.1	183	5.2	1	380	45	3	Negative
17-β-estradiol	271.15	183.1	5.4	1	380	45	3	Negative
17-β-estradiol	271.15	145.1	5.4	1	380	45	3	Negative
Dienestrol	265.1	249	6.17	1	380	25	3	Negative
Dienestrol	265.1	93	6.17	1	380	30	3	Negative
Diethylstilbestrol	267.1	251	5.94	1	380	27	3	Negative
Diethylstibestrol	267.1	237	5.94	1	380	30	3	Negative
Estriol	287.1	171	3.53	1	380	43	3	Negative
Estriol	287.1	145	3.53	1	380	50	3	Negative
Estrone	269.1	159	5.81	1	380	42	3	Negative
Estrone	269.1	145	5.81	1	380	45	3	Negative
Ethynyl estradiol	295.2	269	5.55	1	380	35	3	Negative
Ethynyl estradiol	295.2	145	5.55	1	380	45	3	Negative
Hexestrol	269.1	133	6.12	1	380	15	3	Negative
Hexestrol	269.1	119	6.12	1	380	43	3	Negative

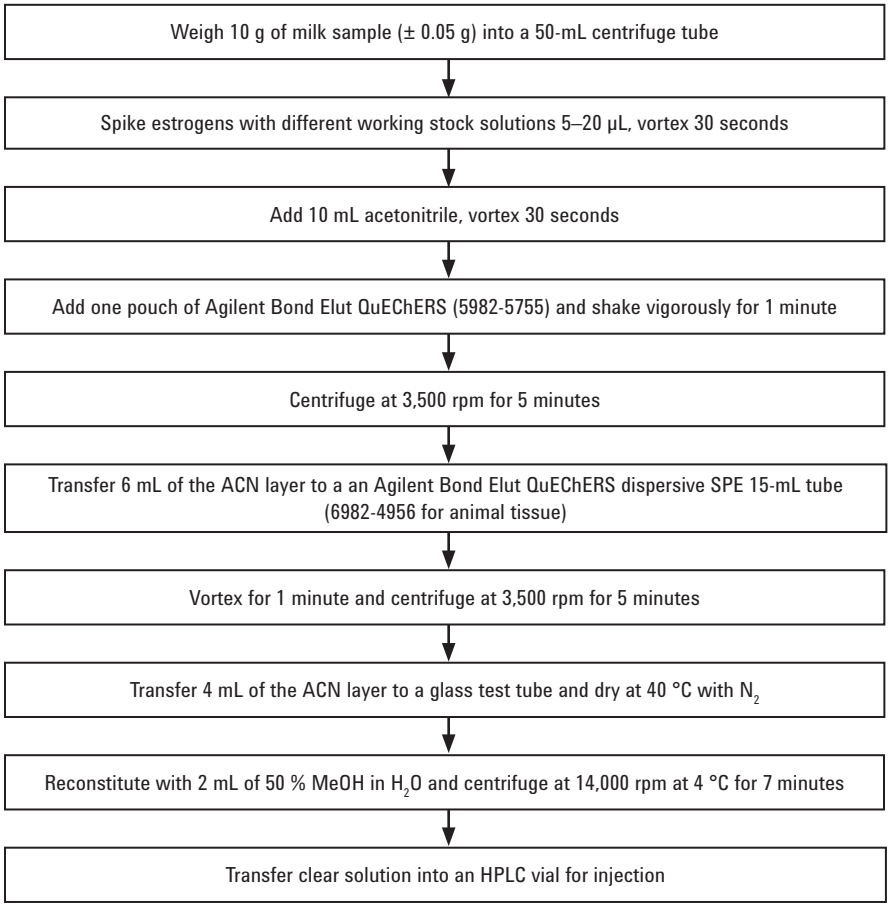

Figure 2. Flow chart of QuEChERS procedure for the determination of estrogens in milk.

Results and Discussion

Instrument detection limit (IDL) of estrogens in solvent

To test the sensitivity of the instrument, and the feasibility of estrogen detection, eight estrogens were diluted in 50 % methanol water from 0.2 ppt to 100 ppb from a working solution of 1 ppm at 12 levels. IDL refers to the minimum amount of analyte required to produce a signal that is statistically distinguishable from background noise with a given confidence level. This approach helps avoid ambiguity related to the variation in the chemical noise and the different ways in which signal-to-noise ratios (S/N) are determined[7]. The LLOQs for these compounds range from 5 to 10 ppt, and the IDL ranges roughly from 1.2 to 3.7 ppt as listed in Table 3. The linear dynamic range covers 10 ppt to 100 ppb with a correlation coefficient of $R^2 > 0.994$ for all analytes. These results demonstrate the high sensitivity of the 6495 Triple Quadrupole Mass Spectrometer for estrogen detection.

Linearity and limit of quantitation (LOQ) of estrogens in milk

The linear calibration range for all estrogens in milk was from 0.1 µg/kg to 10 µg/kg. Appropriate amounts of estrogens were spiked into 10 g of milk. The spike levels were 0.1, 0.2, 0.5, 1, 2, 5, and 10 µg/kg from working stock solutions of 100 ppb to 10 ppm (5–20 µL). The sample preparation procedure is outlined in Figure 2. The calibration curves were generated by plotting the relative response (peak area) versus the concentration of estrogens. The LOQ should be less than the lowest spike concentration tested at 0.1 µg/kg, which is far below the regulatory requirement of 1 µg/kg. Figure 3 shows the normalized chromatogram of 0.1 µg/kg spiked estrogens in milk and their associated retention time. Figure 4 shows the overlay of a blank milk sample and the 0.1 µg/kg spiked milk sample.

Table 3. IDL and LLOQ for eight estrogens in solvent, demonstrating highly sensitive detection in negative ion mode.

Analytes	IDL (ppt)	LLOQ (ppt)
Estriol	1.78	5.0
17-α-Estradiol	2.65	10.0
17-β-Estradiol	3.60	10.0
Ethynyl estradiol	3.69	10.0
Estrone	1.50	5.0
Diethylstilbestrol	2.02	10.0
Hexestrol	1.74	5.0
Dienestrol	1.20	5.0

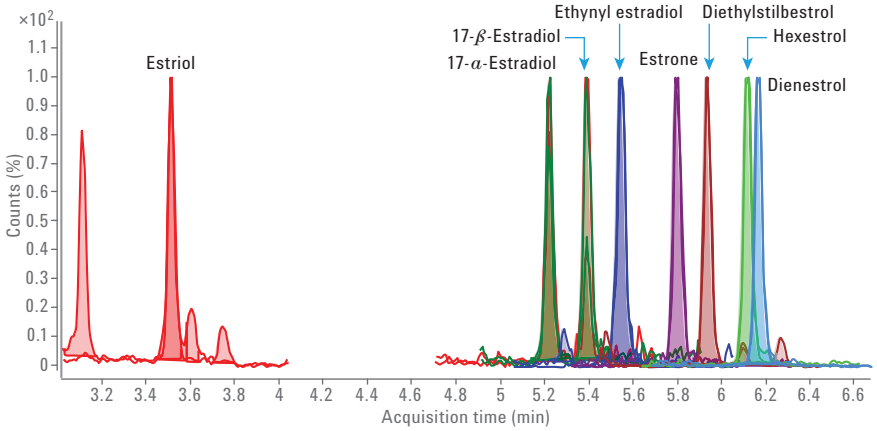

Figure 3. Normalized chromatogram of the 0.1 µg/kg spiked estrogens in milk.

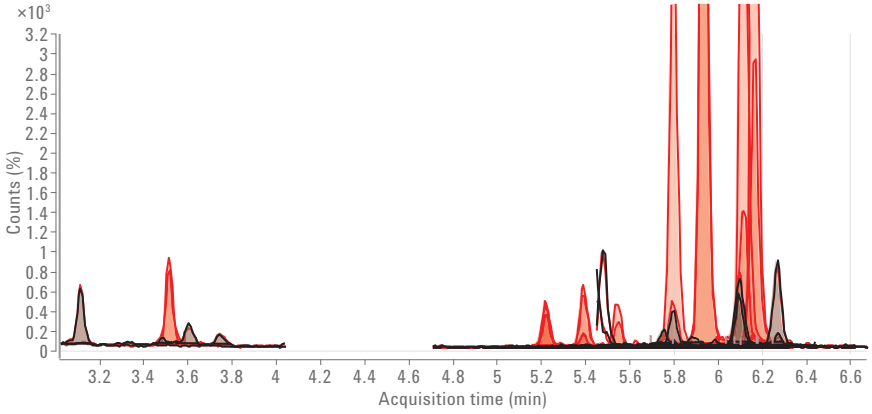

Figure 4. Overlay of 0.1 µg/kg estrogens spiked in milk (red) versus milk blank (black).

The milk used for the blank and spiked injections may contain a very small amount of estrone, but its quantity is far less than the 0.1 µg/kg spiked concentration. Thus, this should not be a concern. Table 4 shows the regression equation and correlation coefficient (R^2) for eight estrogens from 0.1 µg/kg to 10 µg/kg at seven calibration levels. Linear regression fit of 1/x was applied. Results indicated excellent linearity for all analytes.

Table 4. Linearity of estrogens in milk.

Analytes	Regression equation	R^2
Estriol	Y = 23308.11X - 235.73	0.9988
17-α-Estradiol	Y = 11519.06X - 244.56	0.9973
17-β-Estradiol	Y = 16435.07X - 324.27	0.9985
Ethynyl estradiol	Y = 13872.40X - 281.85	0.9984
Estrone	Y = 113277.15X - 1685.45	0.9987
Diethylstilbestrol	Y = 212726.81X + 572.60	0.9985
Hexestrol	Y = 223738X - 8467.75	0.9981
Dienestrol	Y = 169692X - 1949.39	0.9995

Recovery and reproducibility

The recovery and reproducibility were evaluated by fortifying estrogen standards in milk at levels of 0.2 µg/kg, 1 µg/kg, and 5 µg/kg. The analysis included six spiked replicates at each concentration. The QuEChERS procedure recovery was calculated as the ratio of the area for the pre-extraction spike (average of six replicates) and post-extraction spike at the same final concentration. Table 5 shows the recovery and reproducibility (listed as RSD % for six replicates). Results indicate that all eight estrogens gave good recoveries, especially for more polar compounds. Precision values were excellent for all analytes at three levels, primarily < 10 %.

Table 5. Recovery and reproducibility of estrogens spiked in milk.

	0.2 µg/kg (n = 6)		1 µg/kg (n = 6)		5 µg/kg (n = 6)	
Analytes	Recovery	RSD	Recovery	RSD	Recovery	RSD
Estriol	92.9	5.2	102.8	4.9	85.8	4.8
17-α-Estradiol	92.2	7.8	99.4	8.5	93.4	8.5
17-β-Estradiol	92.9	3.3	106.0	5.8	96.9	8.8
Ethynyl estradiol	90.2	4.8	104.9	4.7	94.0	8.4
Estrone	94.0	2.9	101.7	5.6	96.3	6.8
Diethylstilbestrol	83.5	3.7	98.6	3.7	91.0	14.6
Hexestrol	86.1	7.0	98.4	3.1	91.8	13.0
Dienestrol	82.9	3.6	92.8	2.0	85.3	9.5

Conclusion

The highly sensitive Agilent 6495 Triple Quadrupole Mass Spectrometer is a potential tool for estrogen measurement in food matrixes where, by regulation, MRLs are set low. The advanced instrumentation also allows the use of simpler sample preparation procedures, as significant enrichment or derivatization is unnecessary. The Agilent Bond Elut Buffered QuEChERS method is easier to implement and saves solvent when compared to other methods such as typical LLE and SPE. The recovery and precision of the method, based on the matrix-spiked standards, were excellent for multi-residue estrogen determination in milk. The impurities and observed matrix effect were minimal for the milk blank, which did not interfere with the quantitation of the analytes at 10x less than the MRL. This method can be applied to the measurement of estrogens in other similar matrixes, such as milk powder.

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