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An analytical method for the quantitation (20-8,000 ppb) of Ergot Alkaloids in Wheat grain.

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We use this protocol and it's working

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Abstract

Ten ergot alkaloids are quantified in wheat and rye grains at concentrations ranging 20-8,000 ppb using HPLC-MS/MS. Briefly, 5 grams of ground sample is mixed with 40 mL of an extraction solution (ACN-water; 84/16, v/v + 200 mg/L Ammonium Carbonate, pH 8.5) was added to each tube, agitated for 30 min on a horizontal shaker at 150 cycles per min, let to sit for 1h to ensure adequate extraction of analytes and filtered through a Whatman 54 filter paper into a new tube. An aliquot of 4mL was pushed through Mycosep 150 Ergot column. An aliquot of 500 mL was mixed with 500 mL of dihydroergocristine at 100 ng/mL in the extraction buffer (internal standard) and centrifuged at 10 000 RPM for 5 min to remove any possible particulate matter. An aliquot of 100 mL was transferred into autosampler vial and injected (2 mL) into Agilent 6460C UHPLC–MS/MS equipped with guarded Agilent Zorbax Eclipse Plus C18 column (2.1 50mm, 1.8 micron).

Validation data (in-house and via collaborative studies such as Blinded Method Test) are available in the following publication: **https://pubmed.ncbi.nlm.nih.gov/33394021/**

Materials

APARATUS:

- Mettler top load balance (or equivalent)
- Ohaus Analytical balance (or equivalent)
- Agilent 6460C LC-MS QQQ
- Agilent HPLC – 1260 Infinity
- Agilent MassHunter Software
- Horizontal Shaker
- Micro-Centrifuge
- Bottle Top Dispenser
- Pipettes
- Agilent Polypropylene vials (5190-2242) or equivalent
- Nitrogen Evaporator

REAGENTS:

Extracting Solution: ACN-water; 84/16, v/v + 200 mg/L Ammonium Carbonate:

- Place 3360mL of HPLC grade acetonitrile (ACN), 640 ml of 200mg/L Ammonium carbonate in nanopure water in a 4L reagent bottle and mix well.Use 100mL bottle top dispenser to dispense appropriate amount of extracting solvent.

200 mg/L Ammonium Carbonate in Water

- Weigh out 200 mg of Ammonium Carbonate
- Dissolve in 1 L Nanopure Water

1.5 M Ammonium Carbonate

- Weigh out 3.60 gms of Ammonium Carbonate and dissolve in 25 mls Nanopure Water

HPLC Solvent A- 3mM Ammonium Carbonate in Nanopure Water

- Add 1 ml of 1.5M Ammonium Carbonate to 499 ml of Nanopure Water

HPLC Solvent B – 90/10 Acetonitrile:Water

- Mix 50 mls of Nanopure water with 450 mls of Acetonitrile (LC-MS grade)

ISTD 100 PPB Dihydroergocristine in Extracting Solut

- Make up desired volume of 100 PPB Dihydroergocristine using stock standard and dilute in 84/16 Extracting Solution.

Ergot Reference standards

- Commercially prepared reference standards are purchased from Biopure (Romer Labs)
- Ines standards purchased as thin film, dried down standard at 0.5 mg to be reconstituted in 5 mls for 100 ug/mL.
- Inines standards purchased as thin film, dried down standard at 0.5 mg to be reconstituted in 5 mls for 25 ug/mL.
- After reconstitution and aliquot removal, reference standards are dried down under Nitrogen and stored in Freezer (-20C)

Troubleshooting

Safety warnings

- Mycotoxins are toxic. Wear a dust mask, laboratory coat and gloves when preparing stock standards.
- Acetonitrile is toxic. Avoid skin contact and breathing fumes. Prepare extraction solutions in fume hood. Waste ACN/water should be placed in marked waste containers and collected for disposal.

Standard Curve:

1 **Prepare serial dilution**

1.1 Make up a 500 PPB solution of 500 PPB each of Ergocornine, Ergocorninine, Ergocristine, Ergocristinine, Ergocryptine, Ergocryptinine, Ergosine, Ergosinine, Ergotamine, and Ergotaminine from reference standards.

1.2 Transfer 50 µL of [M] 500 Parts per Billion (PPB) standard to poly vial for Standard 10

2 **500 ppb stock standards in individual aliquots for one serial dilution**

2.1 Make up from reference standards 500 ppb solution of 500 ppb of each ergot alkaloids.

2.2 Transfer 150 ul of 500ppb solution to amber vials and dry down under nitrogen.

2.3 Store these vials in freezer (-20C)

2.4 To use take out one vial and let warm to room temperature.

2.5 From your ISTD 100 PPB Dihyroergocristine in extracting solution make up 800 ul of 50 PPB Dihydroergocristine in extracting solution. (1:1 dilution)

2.6 Bring up vial in 150 ul of 50 PPB Dihydroergocistine in extracting solution. Mix well.

2.7 Use this to perform serial dilution of 10 standards. Use the same 50 PPB Dihydroergocristine in extracting solution to dilute with during serial dilution.

2.8

	A	B	C
	Standard	PPB	16x PPB
	10	500	8000
	9	250	4000
	8	125	2000
	7	62.5	1000
	6	31.2	500
	5	15.6	250
	4	7.8	125
	3	3.9	62
	2	2.0	32
	1	1.0	16

The standard curve has the following ten Calibrators

PROCEDURE for sample preparation

3 Weigh out 5 grams of dried and ground sample into Centrifuge tube:
- Wheat ground on Perten Mill or Glen Mill particle size within 100-355 um.
- Wheat sample moisture is typical of wheat in long-term storage conditions or < 14 to 15% moisture. If upon physical evaluation it appears/feels wet samples are dried overnight in 60C oven.

4 Add 40 mls of Extracting Solution. Cap and invert to mix.

5 Shake for 30 minutes on Horizontal Shaker at 150 +/- 10 Cycles per minute.

6 Let extract sit for at least 1 hour.

7 Filter entire extract thru Whatman 54 filter paper into a new labeled centrifuge tubes.

8 Transfer 4 mls of filtered extract to the glass tube provided with Mycosep kit.

9 Push the 4 mls thru Mycosep 150 Ergot Column.

10 Transfer 500ul of extract to a microcentrifuge tube.

11 Dilute with 500 ul of 100 PPB Dihydroergocristine in extracting solution

12 Final concentration of Dihydroergocristine is 50 PPB.

13 Centrifuge at 10,000 RPM for 5 mins

14 Transfer 100ul to Polypropylene vial for injection on QQQ.

15 Finale extract is a 16x dilution of sample.

16 Prepare 10-point Standard curve by serial dilution and run standard curve with samples daily.

LC-MS QQQ Method

17 Analysis is carried out on an Agilent 6460C Triple Quad LC/MS.It is carried out with positive electrospray ionization in Dynamic MRM mode using three major transitions per target compound.

18 **Samples are run with the LC by a method with the following parameters.**

18.1 UHPLC Column**:** Agilent Zorbax Eclipse Plus C18 2.1 × 50 mm, 1.8 micron (P.N. 959757-902)

18.2 Guard Column: Zorbax Eclipse Plus C18, 2.1 × 5 mm, 1.8 micron, (P.N. 821725-901)

18.3 Mobile Phase: A: 3 mM ammonium carbonate in Water B: premixed Acetonitrile (90%) – Water (10%)

18.4

Auto Injector Parameters

	A	B
	Injection Volume	2 µl
	Needle Wash	5 seconds with Acetonitrile
	Column Temp	30.00 °C

18.5 **Binary Pump:**

Flow: 0.200 mL/min
High Pressure Limit: 550 bar
Stop Time: 12.00 min
Post Time: 1.0 min

Gradient Program:

	A	B	C
	Time	**A%**	**B%**
	0.70 min	95%	5%
	1.3 min	50%	50%
	8.0 min	10%	90%
	10.0 min	10%	90%
	12.0 min	95%	5%

19 **LC-MS QQQ**

	A	B
	Agilent 6460C Triple Quadrupole Parameters	
	Ionization mode	Positive ESI with Agilent Jet Stream
	Scan type	Dynamic MRM
	Gas temperature	200 °C
	Gas Flow	8 L/min
	Nebulizer pressure	45 psi
	Sheath gas temperature	400 °C
	Sheath gas flow	12 L/min
	Capillary voltage	3000 V
	Nozzle voltage	500 V
	Delta EMV	500
	Cycle Time	500 ms

20 **MRM transitions**

	A	B	C	D	E	F	G	H	I	J
	Compound Name	**RT**	**Prec Ion**	**Prod Ion**	**Frag (V)**	**CE (V)**	**Cell Acc (V)**	**Ret window**	**Polarity**	**ION**

	A	B	C	D	E	F	G	H	I	J
	1. Ergosine	5.4	548.0	530.2	150	15	4	1	Positive	Qual
	1. Ergosine	5.4	548.0	223.1	150	35	6	1	Positive	Quant
	1. Ergosine	5.4	548.0	208.0	150	50	4	1	Positive	Qual
	2. Ergotamine	5.7	582.1	277.1	140	25	6	1	Positive	Qual
	2. Ergotamine	5.7	582.1	223.1	140	35	6	1	Positive	Quant
	2. Ergotamine	5.7	582.1	208.0	140	45	4	1	Positive	Qual
	3. Ergocornine	6.4	562.1	305.1	140	25	6	1	Positive	Qual
	3. Ergocornine	6.4	562.1	277.1	140	30	4	1	Positive	Qual
	3. Ergocornine	6.4	562.1	223.1	140	35	6	1	Positive	Quant
	4. Ergocryptine	6.9	576.0	305.1	120	25	6	1	Positive	Qual
	4. Ergocryptine	6.9	576.0	291.1	120	25	4	1	Positive	Qual
	4. Ergocrypti	6.9	576.0	223.1	120	35	6	1	Positiv	Quant

	A	B	C	D	E	F	G	H	I	J
	ne								e	
	5. Ergocristine	7.1	610.0	305.0	140	25	4	1	Positive	Qual
	5. Ergocristine	7.1	610.0	268.2	140	25	7	1	Positive	Qual
	5. Ergocristine	7.1	610.0	223.1	140	40	6	1	Positive	Quant
	6. Ergosinine	7.5	548.2	530.1	141	14	4	1	Positive	Qual
	6. Ergosinine	7.5	548.2	223.1	141	34	5	1	Positive	Quant
	6. Ergosinine	7.5	548.2	208.0	141	50	4	1	Positive	Qual
	7. Ergotamine	7.9	582.2	277.1	144	26	5	1	Positive	Qual
	7. Ergotamine	7.9	582.2	223.1	144	34	5	1	Positive	Quant
	7. Ergotamine	7.9	582.2	208.1	144	50	5	1	Positive	Qual
	8. Ergocornine	8.3	562.2	305.1	140	30	5	1	Positive	Qual
	8. Ergocornine	8.3	562.2	277.1	140	30	4	1	Positive	Qual

	A	B	C	D	E	F	G	H	I	J
	8. Ergocornine	8.3	562.2	223.1	140	38	5	1	Positive	Quant
	9. Ergocryptinine	8.9	576.2	305.1	138	30	5	1	Positive	Qual
	9. Ergocryptinine	8.9	576.2	291.1	138	30	4	1	Positive	Qual
	9. Ergocryptinine	8.9	576.2	223.1	138	38	5	1	Positive	Quant
	10. Ergocristinine	9.1	610.2	305.1	139	30	5	1	Positive	Qual
	10. Ergocristinine	9.1	610.2	268.0	139	24	7	1	Positive	Qual
	10. Ergocristinine	9.1	610.2	223.1	139	38	5	1	Positive	Quant
	11. Dihydroergocristine	6.6	612.8	350.1	178	26	4	1	Positive	Quant
	11. Dihydroergocristine	6.6	612.8	270.1	178	34	4	1	Positive	Qual
	11. Dihydroergocristine	6.6	612.8	253.1	178	38	4	1	Positive	Qual

21 **QQQ Time**

To minimize the matrix going into the spray chamber, the HPLC flow should be diverted into waste from 10 minutes to the end of the analysis.

	A	B	C	D	E	F	G
	#	**Start time**	**Scan type**	**Div Valve**	**Delta EMV (+)**	**Delta EMV (-)**	**Stored**
	1	0	Dynamic MRM	To MS	500	0	x
	2	10	Dynamic MRM	To Waste	0	0	x

DATA ANAYSIS

22 Anaylze data in mass hunter software.Note ISTD is run as analyte and not used in quantitation.

23 Analyze Batch.

24 The calibration curve is linear, ignore origin and weighed 1/x for all compounds.

25 Navigate thru Batch table to see compounds, curves and results.

26 Check integration of peaks and verify if compounds are correctly identified.

27 Check integration and response of the ISTD in each sample and standards.Verify that each sample has been injected.

28 Total Ergots is set up as a MRM compound in Mass Hunter Quant software Edit and is set up to add up all of the Ergots.It is shown in the quant results table and reported out as Total Ergot.

CALCULATIONS

29 Final extract is .0625 equivalence so the value from the curve needs to be multiplied by 16.

30 The standard curve is set to detect the following range: 16 ppb to 8000ppb.The detection limit is the lowest standard run.

31 LLOQ (Lower limit of Quantification): 20 ppb

32 ULOQ (Upper limit of Quantification): 8000 ppb

Protocol references

[1] Rapid and Sensitive Detection of Ergot Alkaloids in Wheat Using the Agilent 6460 Triple Quadrupole LC/MS with Jet Stream Technology. Agilent Technologies Application Note, Byrd, N. et all.2012.

[2] Evans TJ, Rottinghaus GE, Casteel SW. (2004) Ergot. In: K H Plumlee (ed). Clinical Veterinary Toxicology.St. Louis: Mosby, pp. 239-243.