Quantitative Analysis of Ethyl Carbamate in Distillers Grains Co-products and Bovine Plasma by GC-MS

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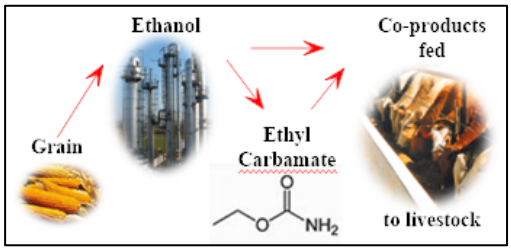
ABSTRACT : Ethyl carbamate (EC) is a fermentation byproduct in foods and beverages and classified as a Group 2A probable human carcinogen. Each year greater than 40 million metric tons of fermentation co-products from the U.S. ethanol industry are fed to food animals. A GC-MS assay was developed to analyze EC extracted from various distillers grains co-products with a limit of detection at 0.7 ng/g. EC was detected in all the distillers grains co-products surveyed in this study. Corn condensed distillers solubles contained the highest level of EC ranging from 1618 to 2956 ng/g. The levels of EC in the semi-solid co-products varied from 17 to 917 ng/g. Cattle fed on these fermentation co-products were found to contain 2-3 ng/mL of EC in blood plasma. No EC was detected in control animal blood plasma. The presence of EC in animal feeds and subsequently in animals may pose an animal health risk.



KEYWORDS: APGC-qTOF, bovine plasma, distillers grains co-products, ethyl carbamate, GC-MS

INTRODUCTION

Within the fed cattle industry, respiratory disease is still the number one health concern[1]. Acute interstitial pneumonia (AIP, also known as pulmonary edema and emphysema, atypical interstitial pneumonia) is one of the more costly respiratory diseases[2]. The economic costs of the disease are quite high in cattle because the majority of cases develop close to harvest and are usually fatal. A common factor in the pathogenesis of this disease in ruminants has been established. It is precipitated by the ruminal degradation of L-tryptophan, a common amino acid in lush forages and feed grains, to 3-methyl indole (3-MI)[3,4]. It is absorbed, and in high enough concentrations its metabolites cause damage to pulmonary alveolar cells and AIP can develop. In feedlot cattle being fed a high grain diet 3-methyl indole has been targeted as the causative agent of feedlot AIP, but past research has demonstrated inconsistent results[5,6]. The amount of l-tryptophan in high grain diets may not be high enough to create enough 3-MI to be toxic, leading the field to consider the cause of feedlot AIP to be multifactorial[7].

The formation of 3-MI toxic metabolites in pulmonary cells that leads to AIP is due to intracellular cytochrome P450 (CYP450) enzymes metabolizing 3-MI to 3-methyleneindoline (3-MEIN). Pulmonary pneumocyte CYP450 enzymes can catalyze metabolism of other substrates, one of which is ethyl carbamate[8]. The metabolism of ethyl carbamate in pulmonary cells by CYP450 enzymes produces its toxic metabolites[9]. Its pathogenesis is very similar to 3-MI toxicity[8].

Ethyl carbamate (EC) is an organic compound once used as an antineoplastic agent and as a solvent for delivering analgesics to humans[10]. When EC was found to be a pneumotoxin and carcinogen in laboratory animals its general use in human medicine ceased in the mid-1970's. In 2007 it was classified as a human Group 2A carcinogen by the International Agency for Research on Cancer[11]. In the 1980's EC

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was discovered to be a common contaminate of fermented spirits and foods and many countries established maximum limits for safe consumption[12]. There is renewed concern about ethyl carbamate being a food and environmental toxicant[13]. EC has several formation pathways. In its simplest formation it is the result from the reaction of ethanol, urea, heat, and time[14] (Figure1).

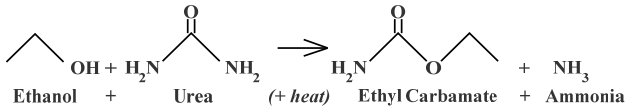

Figure 1. The primary reaction of ethanol and urea to form ethyl carbamate.

For over 50 years the co-products from the distilled beverage industry have been utilized for livestock feeds. During the past 3 decades there has been a dramatic increase in the production of distillers grains co-products with the increasing use of ethanol as a renewable fuel. The primary co-products utilized in animal feeds are condensed distillers solubles (CDS, syrup), wet distillers grains with solubles (WDGS) and dried distillers grains with solubles (DDGS)[15]. These co-products come from the fermentation of various grains such as corn, milo, and wheat for the production of ethanol. A condensed schematic on the production of these co-products is illustrated in Figure 2.

According to the Renewable Fuels Association as of 2018 there was >40 MMT of distillers grains co-products produced in the U.S. Over 75% of those co-products are fed to beef and dairy cattle. There are numerous reviews of the nutrient and chemical composition of distillers grains co-products, including the analysis of low molecular weight organic compounds[16-18]. To our knowledge there have been no reports of ethyl carbamate as a component of distillers grains co-products, despite the known occurrence as a fermentation by-product whose levels are regulated for human consumption. This lack of scientific literature prompted two questions, 1) is EC present in the co-products derived from the production of ethanol from corn or milo (sorghum)? 2) if so, does EC accumulate in animals fed fermentation co-products? Methods for EC quantification published in the past decade were mostly adapted from the AOAC method 994.07[19], the majority of which focused on alcoholic beverages and fermented foods[12]. Reports on EC quantification in biological samples were very limited[20,21]. Thus, analytical methods specific for distillers grains co-products and bovine blood/plasma are needed to assess how incorporating fermentation co-products into feeds have impacted animal nutrition with respect to the potential toxicity of EC. This report describes a GC-MS assay for sensitive and selective quantification of EC. This assay was then applied for analysis of EC from samples of distillers grains co-products and bovine plasma samples.

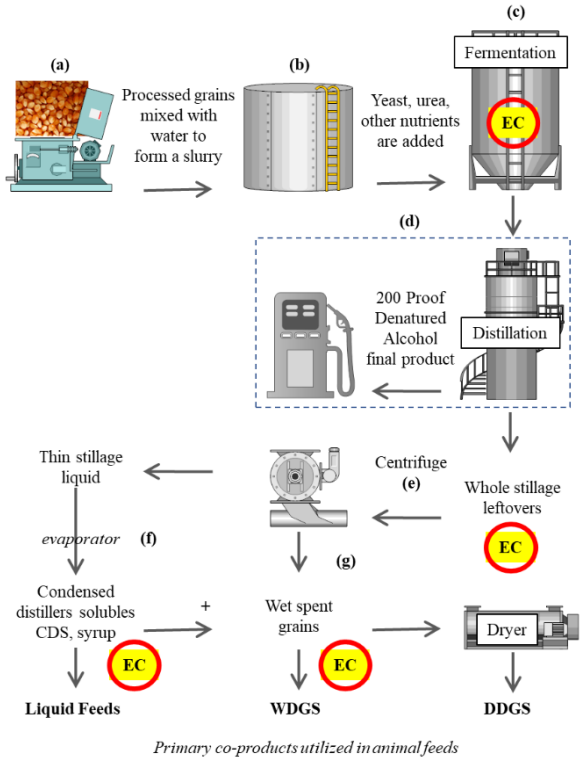

Figure 2. Schematic of the production of distillers grains co-products fed to livestock. (a) grains are processed to be (b) mixed with water, yeast, urea, and other ingredients; (c) fermentation produces the ethanol; ethyl carbamate formed at this stage; (d) the ethanol is distilled to the final product; the remaining solids and liquid is the whole stillage; (e) this is centrifuged to separate the thin stillage liquid and the wet spent grains; (f) the thin stillage is evaporated to produce the condensed distillers solubles that has the highest level of ethyl carbamate; (g) the CDS is blended back into the spent grains and is either dried to DDGS or fed directly as WDGS.

MATERIAL AND METHODS

Distillers Grains Co-products. Corn wet distillers grains with solubles (CWDGS), corn dry distillers grains with solubles (CDDGS), corn condensed distillers solubles, syrup (CCDS), and milo wet distillers grains with solubles (MWDGS) samples were collected from various Midwest ethanol plants and different feedyards during the fall of 2018. The details on collection period, post-manufacture residence days, and number of ethanol plant sources can be found in supplementary Table S1. Five samples were randomly chosen from each sample type to be assayed. Samples are only labeled 1-5 to protect the identity of the source. One liquid feed sample (LF) was acquired at a Midwest feed manufacturer and contained approx. 20% cane molasses, 20% CCDS, 20% Urea, minerals and other components. All grain and liquid samples were stored at -80°C until assayed.

Bovine Plasma. All blood samples were collected under the direct supervision by the veterinarian of record where a valid Veterinary Client Patient Relationship existed within the herd or feedyard. More details on the handling of livestock and collection of blood samples can be found in Supporting Information. Control bovine plasma samples were collected from control crossbred Angus calves (bulls and heifers) that were on pasture with their dams >90 days with no access to any distillers grains coproducts or liquid feeds. Plasma samples of three test groups were collected from crossbred Angus steers that were on feed in private feedyards with 19-38% of CWDGS and 0-6% of LF **(**Table S2). All plasma samples were stored at -80°C until assayed.

Chemicals. Ethyl carbamate (EC), 5-sulfosalcylic acid were purchased from Sigma-Aldrich (St.Louis, MO), and ethyl d5-carbamate (EC-d5) from C/D/N Isotopes (Quebec, Canada). Chem Elut S (20 g sample, 60 cc tube) was purchased from Agilent (Santa Clara, CA). LC grade solvents including methyl tert-butyl ether (MTBE), acetonitrile, hexane, and water were from Fisher Scientific (Pittsburgh, PA).

Liquid-liquid Extraction of CWDGS, MWDGS and CDDGS. Samples were first homogenized using a spatula, and then weighed (10 g) into 50 mL conical tubes, to which 15 mL of water for CWDGS and MWDGS, and 35 mL of water for CDDGS was added. Then EC-d5 (100 µL, 10 ug/mL in water) was added as internal standard (IS). Samples were vigorously mixed on a VX-2500 vortexer (VWR, Radnor, PA) at 4°C for 2 h and then centrifuged at 17000 *g* and 4°C for 30 min using an Eppendorf 5430 centrifuge (Hauppauge, NY). The supernatant was recovered and washed with hexane (5 mL) to remove neutral lipids. NaCl (4 g) was added to the de-oiled aqueous extract (10 mL) and extracted with MTBE in a solvent-to-sample ratio of 4:1 (by vol). After 1 min of vigorous vortexing, samples were centrifuged at 2000 *g* and 4°C for 10 min using an Allegra X-12R centrifuge (Beckman Coulter) and the top MTBE layer was recovered and dried under a gentle stream of nitrogen until about 100 µL remained. Cautions were taken not to let the extract completely dry. All the sample extracts were stored at -20°C until analyzed.

Supported-liquid Extraction of CWDGS. A method modified from AOAC Official Method 994.07[19] was tested on CWDGS samples. Samples were mixed with water and IS, and then washed with hexane as descried above. Then the de-oiled aqueous extract (10 mL) was loaded onto a Chem Elut S column. After 5 min equilibration, the column was eluted with 160 mL of MTBE. The MTBE eluent was collected and dried down to ~100 µL.

Liquid-liquid Extraction of CCDS and LF. The thick syrup like CCDS samples were first centrifuged at 17000 *g* and 4°C for 15 min. Then 0.1 g of viscous supernatant was added with EC-d5 (10 µL, 10 ug/mL in water) and 0.2 mL of acetonitrile. Samples were vigorously vortexed for 30 s and then centrifuged. The supernatant (0.2 mL) was mixed with 0.3 mL of hexane and 0.3 mL of water. After top hexane layer was removed, 1 mL of MTBE and 0.1 g of NaCl were added to the lower layer, and the samples were vortexed for 1 min and then centrifuged at 2000 *g* and 4°C for 10 min. The top MTBE layer was recovered and dried down to ~100 µL.

Liquid-liquid Extraction of Bovine Plasma. Thawed plasma samples (0.4 mL) were spiked with 20 µL of 500 ng/mL of EC-d5 in water, gently mixed, and incubated at 4°C for 10 min. Then 40 µL of 20% (w/v) 5-sulfosalcylic acid in water was added to induce protein precipitation and samples were vigorously mixed for 10 s, followed by 20 min of incubation at 4°C. The precipitated proteins were centrifuged at 17,000 *g* and 4°C for 15 min. Supernatant (0.35 mL) was recovered and washed with 0.3 mL of hexane to remove neutral lipids. After removal of hexane, the aqueous plasma extract was added with 120 mg of NaCl, and then 1.6 mL of ethyl acetate. Samples were vigorously mixed for 30 s and then centrifuged at 2000 *g* and 4°C for 10 min. Then upper organic layer was recovered, and the solvent was removed under a stream of nitrogen until about 60 µL remained.

Optimization of the Extraction of EC. To test how the process of solvent evaporation affected the yield of EC, EC (1 µg/mL) in MTBE or ethyl acetate was

added with methyl octadecanoate (abbr. C18:0 FAME, with a final concentration of 1.6 ug/mL). Then the mix solution of EC and C18:0 FAME was separately diluted with MTBE or ethyl acetate to a total volume of 2, 4, 10, 15 mL, dried under nitrogen until the remaining volume approximated to 100 µL. Undiluted sample was directly injected to GC-MS as a control. Two more undiluted samples were over-dried for 1 s and 10 min in separate vials, and then resuspended in 100 µL of corresponding solvent.

Treatments of plasma protein precipitation and the addition of NaCl were compared with a direct extraction from plasma without protein precipitation or salt addition. Bovine plasma from control groups where EC was not detected using the current method was spiked with authentic EC standard to obtain a final EC concentration of 1 µg/mL in plasma. The first treatment with protein removal and salt addition, referred to as [deprotein+salt], was carried out as described above. The second treatment with protein removal only, referred to as [deprotein], skipped the addition of NaCl. The third treatment, referred to as [control], applied MTBE extraction directly to the plasma as is. All the extractions started with 400 µL of spiked plasma and then used MTBE with a solvent-to-sample ratio of 4:1. Recovered MTBE extracts (1 mL) were spiked with 20 µL of 10 µg/mL of EC-d5 (dissolved in MTBE) before being dried down to ~100 µL.

Further, the solvent type, i.e., MTBE versus ethyl acetate, and solvent-to-sample ratio were examined for the best EC extraction yield. 5-Sulfosalcylic acid was added to the spiked plasma containing 1 µg/mL of EC to precipitate the proteins. The resulting supernatant was then extracted with MTBE or ethyl acetate using solvent-to-sample volume ratios of 1:1, 2:1, 4:1, 6:1, 8:1. After the mixing, 80% volume of the solvent extract was recovered and spiked with 20 µL of 10 µg/mL of EC-d5 dissolved in the corresponding solvent. Then the extracts were dried under nitrogen until about ~100 µL left. Salts were not added in this experiment.

Repeatability of the Extraction of EC. Control bovine plasma was spiked with authentic EC standard to obtain a final EC concentration of 24 ng/mL (medium level) and 48 ng/mL (high level) in plasma. The plasma sample combined from test group was used with no addition of EC and served as the low concentration level. For intra-day repeatability, samples of each level were assayed with six replicates. For inter-day repeatability, samples of each level were assayed with three replicates per day over a three-day period. The repeatability of distillers grains co-products was examined by repeatedly assaying a quality control (QC) sample that was generated by pooling a small portion of each experiment sample.

Calibration Curve, Limit of detection (LOD) and Limit of quantification (LOQ). Bovine plasma from control group where EC was not detected using the current method was spiked with 20 µL of EC standard solutions with concentrations ranged from 0 to 800 ng/mL in water to obtain a final EC concentrations in plasma at 0, 0.15, 0.3, 0.6, 1.2, 2.4, 4.8, 9.5, 19, 38 ng/mL, which were then extracted as described above. The calibration curves for distillers grains samples used pure water as the matrix. Limit of detection (LOD) and limit of quantification (LOQ) were calculated as 3 times and 10 times, respectively, of the standard deviation of the blank injections (i.e., contained IS but no EC) divided by the slope of the calibration curve's regression line.

GC-MS Analysis. Sample extracts were analyzed using a Trace 1310 gas chromatography coupled to a Thermo ISQ-LT mass spectrometer (ThermoFisher, Waltham, MA). Samples (1 µL) was injected onto a DB-WAXUI column (Agilent, 30 m x 0.25 mm x 0.25 µm) at a 5:1 split ratio. Helium carrier gas flow was held at 1.2 mL/min. The injector temperature was set at 220°C. The oven temperature program started at 100°C, ramped to 140°C at a rate of 10°C/min, then to 160°C at a rate of 5°C/min, to 250°C at a rate of 30°C/min with a final hold of 3 min. Temperatures of transfer line and ion source were both held at 250°C. Single Ion Monitoring was used at a rate of 10 scans/sec under electron impact mode for scanning the following *m/z* 44, 62, 64, 74, 76, 89, 94. Mass 62 and 64 were used as quantification ions for EC and EC-d5, respectively.

APGC-qTOF Analysis. The presence of EC in the extracts of plasma samples were further confirmed by accurate mass measurement using Waters Atmosphere Pressure Gas Chromatograph (APGC) coupled with a Xevo G2 QTOF time-of-flight mass spectrometer (Waters, Milford, MA). Samples (1 µL) was injected onto a DB-WAXUI column (Agilent, 30 m x 0.25 mm x 0.25 µm) with a pulsed splitless mode with pulse pressure held at 32 psi during the first 1.2 min. The oven was initially held at 50°C for 1.2 min, then ramped to 250°C at a rate of 20°C/min with a final hold of 1 min. Sample flow underwent atmospheric pressure ionization in positive mode with nitrogen gas temperature of 220°C at the interface. A 2-mL glass vial of methanol containing 10% acetic acid was situated on lower level of the source housing to supply polar vapor to assist ionization. Corona pin current was set at 3 µA, sampling cone at 5 V, and extraction cone at 4 V. Source temperature was set at 150°C.

Desolvation gas flow was 200 L/hr. The TOF analyzer was calibrated using Heptacosa, and operated under full scan mode with scan range 50-1200 Da and scan time 0.1 sec. Target enhancement was applied at 90.05 Da.

Statistical Analysis. The EC concentrations in bovine plasma of control and test groups were analyzed using one-way analysis of variance (ANOVA).

RESULTS AND DISCUSSION

Method Development and Optimization. The yield of liquid-liquid extraction depends on the partitioning of the compound between two immiscible solvents, i.e., the extraction solvent and the sample matrix. EC is a polar molecule and soluble in most solvents with medium polarity such as ethers to polar solvents such as water. Its partitioning between ethyl acetate and water is nearly 1:1 based on our preliminary data. The extraction yield of EC increased with the ratio of solvent-to-plasma and plateaued when the ratio was 4:1 or higher (Figure 3).

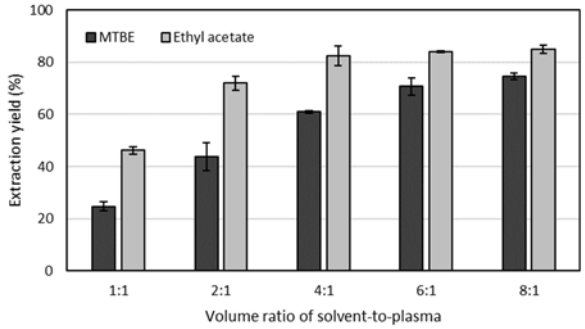

Figure 3. Effect of solvent-to-sample ratio on the recovery of EC from plasma. Based on these data we elected to use a solvent to plasma ratio of 4:1 or higher.

Ethyl acetate, a more polar solvent than MTBE, gave a higher recovery than MTBE, which agrees to the finding of Xu et al[22]. who compared ethyl acetate with diethyl ether and methylene chloride. Methylene chloride was not considered in this study due to its toxicity. Ethyl acetate was chosen for the extraction of EC in plasma. MTBE was used for the extraction of distillers grains samples because of its faster evaporation rate and the high levels of EC in distillers grains co-products.

A 32% increase of extraction yield was observed when NaCl was added to the deproteined plasma (Figure 4). Although the presence of plasma proteins showed no impact on the EC recovery, in order to utilize the salting-out effect, plasma proteins would

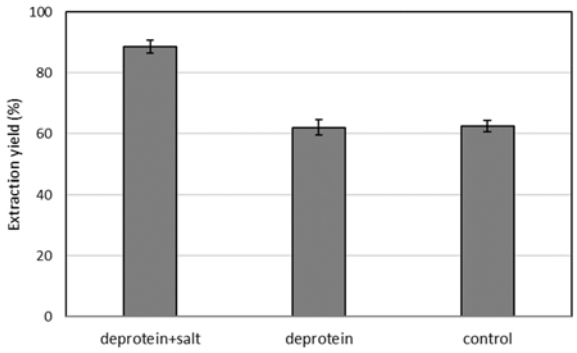

Figure 4. Effect of the addition of NaCl and protein removal on the recovery of EC from plasma. These data indicate that optimal extraction was achieved with a combination of deproteination using 5-sulfosalcylic acid and additional of 6M sodium chloride.

first need to be removed. Otherwise, upon mixing, a stable emulsion would form after NaCl was added. The same emulsion issue also occurred during hexane wash if the proteins were not previously removed. Hexane wash aimed to remove the neutral lipids which would be otherwise extracted with EC into the organic solvent and injected into the GC. The non-volatile nature of neutral lipids would shorten the GC column lifetime and requires more frequent cleaning of the GCMS system.

AOAC 994.07[19] and Nóbrega et al.[23] stated that precaution is needed to avoid complete solvent removal of the EC extract. Other reports specified a final solvent volume of the extract after evaporation[24,25]. These notes suggested that EC is recovered poorly after the solvent is completely removed. The recovery of EC during solvent evaporation was examined in the present study and no loss of EC was found during evaporation under nitrogen at ambient temperature for up to 2 h (Figure S1). However, as soon as the solvent was depleted, EC would be lost, presumably due to evaporation.

For analyzing distillers grains co-products, a supported-liquid extraction method modified from the AOAC method[19] was compared to the liquid-liquid extraction used in this study. The modified AOAC method used the Chem Elut S column with prepacked synthetic material instead of diatomaceous earth, and MTBE was used as elution solvent instead of methylene chloride. Although MTBE is a much lighter solvent, it still enabled the elution by gravity flow at a reasonable speed. The extraction yield of EC obtained from supported-liquid extraction as calculated using the peak intensity of EC-d5 was lower but within 6% of that obtained from traditional liquid-liquid extraction. The critical step when using the supported-liquid extraction with samples such as

WDGS and DDGS that contained fine particles in the aqueous extract would be sample clarification. Without prior removal of the particulates in the aqueous extract either by high speed centrifugation (>15000 g) or filtration, this column or the Chem Elut column recommended in AOAC method[19] would quickly become clogged. Repeatability tests are needed to establish a standard protocol of the modified AOAC method for distillers grains co-products.

Some studies suggested a higher pH for better yield or peak shape of EC[22,25]. Our experiments using EC dissolved in water that was adjusted to acidic, neutral, or alkaline pH did not show any difference in extraction yields (data not shown). Therefore, no pH adjustment was applied to the samples.

The extraction methods for distillers grains co-products were derived from the protocol established for bovine plasma. Although there were small variations in the sample preparations depending on the sample types, the principle was to 1) transfer EC from semi-solid sample martrix to aqueous extracts, 2) remove neutral lipids, 3) extract EC with organic solvent in the presence of NaCl.

Method Validation. The intra-day and inter-day repeatability of the quantification of EC in plasma measured as the coefficient of variation (CV, %) are presented in Table 1.

Table 1. Repeatability (CV, %) of liquid-liquid extraction of EC from bovine plasma

	Intraday	Inter-day			
EC spiking level	(n=6)	day 1 (n=3)	day 2 (n=3)	day 3 (n=3)	day 1 to 3
Low 2 ng/mL	5.8	2.8	3.2	5.2	6.4
Medium 24 ng/mL	2.3	2.3	0.9	2.7	2.8
High 48 ng/mL	2.8	2.8	2.0	3.6	3.3

All the CVs were below 6.5% with a slight increase at the low spiking level. The CV of QC samples of CWDGS (n=5) was 2.8%. The extraction yield of EC from plasma as calculated using the measured concentration divided by the spiked concentration is 86-90%. Peaks of EC and EC-d5 differed by 4.8 s on the Agilent's DBWAX-UI column but were not completely baseline separated (Figure 5, next page). The linear dynamic range of EC for plasma extraction (0-38 ng/mL) and for distillers grains co-products extraction (0-1600 ng/g) are shown in Figure 6 (next page). Despite that the calibration curve for plasma assay was obtained from samples with EC spiked in plasma matrix and that for distillers grains assay was obtained from samples with EC spiked in 100% water, their LOD and LOQ were very close, i.e., 0.8 and 2.7 ng/mL respectively for plasma assay, and 0.7 and 2.6 ng/g respectively for distillers grains assay. The detection limit in this study is lower than most of the published results[19,20, 24-26], but higher than the 0.4 ng/mL in wines[23] and 0.3 ng/mL in wines after BSTFA derivations[22].

The challenge to work with bovine plasma with low EC concentration was to confirm the EC peak identity because matrix noise significantly interfered with the confirmation ions *m/z* 44 and *m/z* 74 when EC <2 ng/mL. Parent ion $M^{+\cdot}$ *m/z* 89 is too weak under electron impact ionization to be used for confirmation. Thus, APGC-qTOF with soft ionization technology and high resolution mass spectrometer was used to obtain the accurate mass of the parent ion of EC to confirm its presence in the actual plasma samples. The corona pin current and cone voltages were optimized to improve EC ionization. A polar solvent methanol with 10% acetic acid was used as proton donor to assist the ionization and increase the signal intensity of $[M+H]^+$. The solution's vial position on the source housing, hot versus cold spot, was also optimized. A mass error of 2.2 ppm was obtained for $[M+H]^+$ *m/z* 90.056 of EC extracted from the test group bovine plasma (Figure S2), adding additional confirmation of the identity of EC in animal biofluids after consumption of distillers grains co-products.

EC Levels in Distillers Grains Co-products and Bovine Plasma. The sources of distillers grains co-products and bovine plasma samples were described in the method section and Table S1. These results are meant to illustrate the use of the developed methodologies for the detection of EC in distillers grains co-products and bovine plasma and give us more insight into future EC research in food animals.

Table 2 shows the EC results for the distillers grains co-products. Of interest is that there is a wide range of EC content in these co-products.

Table 2. EC concentration (ng/g or ppb) in distillers grains co-products[1]

Sample ID	Corn WDGS	Milo WDGS	Corn DDGS	Corn CDS	LF
1	26	19	134	2956	675
2	917	18	616	2895	
3	604	30	119	1618	
4	123	154	14	2411	
5	124	17	74	2889	

Notes on Table 2: The samples were sourced from different ethanol plants or different feedyards. There was no correlation of sources across sample type. When more than 5 samples were collected for any sample type, 5 samples were randomly chosen to be assayed. [1]Values of CCDS and LF were the averages of five replicates; Values of CWDGS, MWDGS, and CDDGS was from single measurement with its analytical variation being assessed using QC samples.

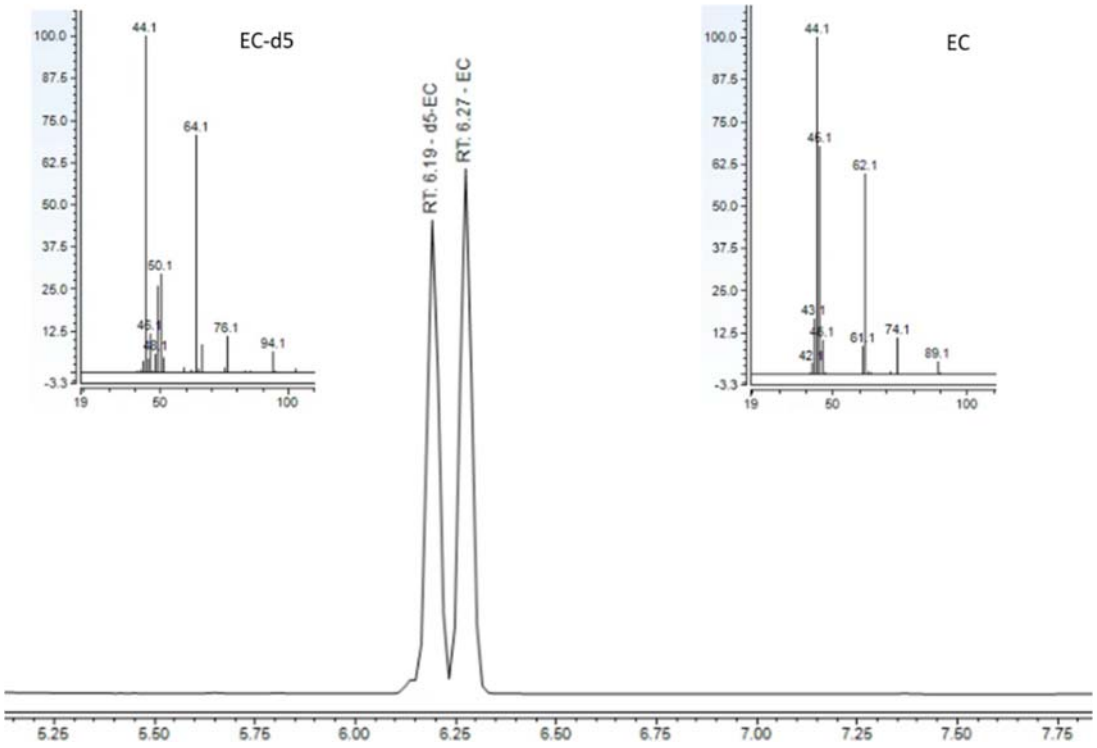

Figure 5. Gas chromatography extracted ion chromatogram and mass spectra of EC and EC-d5 standards.

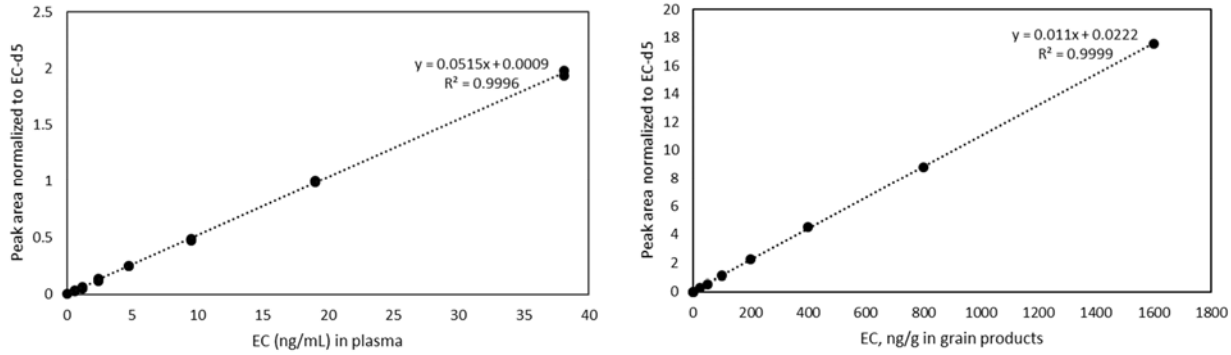

Figure 6. Linear dynamic range or calibration curves of EC in plasma (left) and in distillers grains co-products (right). Data depicted here represents a concentration range of 0 to 38 ng/mL for plasma assay and 0 to 1600 ng/g for distillers grains assay.

More details on typical manufacturing processes can be found elswhere in the ethanol manufacturing process (Figure 2). There would appear to be numerous variables that could influence the production of EC such as substrates, heat, yeast source, fermenting time, etc. After distillation of the ethanol when whole stillage is processed (centrifuged) to distillers grains co-products plus thin stillage, the evaporation process of thin stillage to produce the condensed distillers soubles (CDS) may have a variable amount of EC present. Additionally, the amount of CDS added back into the WDGS and DDGS will determine the amount of EC present in the livestock feed products. Milo (sorghum) WDGS appears to have lower EC content. It is not known why or if this is a consistent finding. Though one sample was significantly higher this may have been due to corn CDS being added back into the milo WDGS (personal communication with the sorghum industry). The LF sample contained approximately 20% CCDS and its EC content was consistent with our CCDS analysis. More research is needed to understand the variability of EC in these co-products and we will be able to do so with these new methodologies.

Figure 7 shows the results of EC in bovine plasma. It has been demonstrated in laboratory animals that orally administered EC is completely and rapidly absorbed from the gatrointestinal tract and evenly distributed in most tissues[27]. The fact that cattle are ruminants and have a "fermentation vat" as part of their digestive tract, it is not known if endogenous EC could be produced within the rumen.

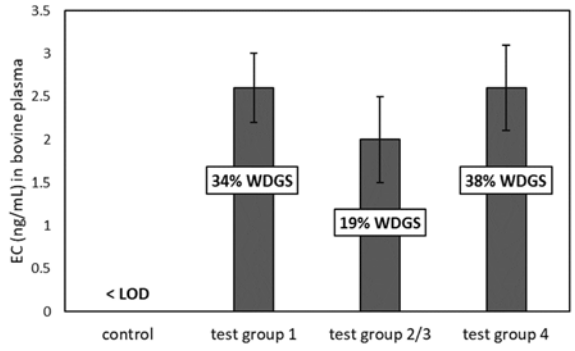

Figure 7. EC concentrations in bovine plasma. The error bars represent standard deviations of samples obtained from five animals (n=5). The percent WDGS fed to the cattle of test groups was labeled on the graph. EC was not detected in control bovine plasma. Each test group was significantly different from control group with $p<0.00001$. The three test groups were not significantly different from each other with $p=0.093$.

The plasma results suggest that plasma levels may be associated to the % of WDGS in the ration. More research and controlled studies are necessary to determine if there is a dose and time-related response to EC absorption and blood levels. It is important to note that EC itself is fairly innocuous as it is readily metabolized to ethanol, CO_2, and ammonia and these products excreted. It is the cytochrome P450 metabolites of EC, vinyl carbamate and vinyl carbamate epoxide that are toxic to cells[28].

Levels of EC are regulated in food products destined for human consumption, as the compound is listed as a Group 2A carcinogen[11].

This manuscript describes (1) a GC-MS assay for detection and quantification of EC in distillers grains co-products that are commonly used as animal feeds and (2) detection of EC in bovine plasma samples. Our data indicate that EC is found to circulate in bovine blood collected from animals fed with 19 to 38% of CWDGS on an as-fed basis. The hypothesis that was described in the introduction proposes that EC may contribute to AIP – the work presented here does neither confirms nor refutes this hypothesis, but does suggest that EC is circulating at levels sufficiently high that further research into the relationship between EC and AIP is warranted for animals consuming feed supplemented with appreciable amounts of distillers grains co-products.

A secondary implication raised by this work relates to the fact that cows are themselves food products for humans. Our data indicate blood plasma concentration of EC in the range of 2.0 to 2.6 ng/mL (ppb) in cattle fed distillers co-products supplemented feed. Extrapolating these measurements to dietary consumption is likely to be error prone. However, these values are comparable to reported from bread (Canada, 1-29 ng/g), soy sauce (China, 8-108 ng/g), and wine (U.S., <LOD – 254 ng/g)[29]. Our study does not report concentrations for meat (muscle) samples; therefore, it is difficult to estimate the amount of EC consumed.

Our future efforts will be directed toward (1) improving assay sensitivity through alternate sample preparation approaches (i.e., supported-liquid extraction for plasma) and analytical platforms such as LC-MS/MS (2) a broader exploration of the factors that influence EC levels in distillers grains co-products (3) quantification of EC from beef muscle and other tissues destined for human consumption and (4) exploration of the role EC may play in development of AIP and liver disease in cattle.

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Supporting Information

- Statement on the handling of livestock and collection of blood samples
- Table S1. Sample description-Distillers grains co-products
- Table S2. Sample description – bovine plasma samples.
- Figure S1. Loss of EC during solvent evaporation. It generally took about 2 h to evaporate 15 mL of solvent. These data suggest that solvent evaporation needs to be carefully controlled to avoid reaching complete dryness.
- Figure S2. Accurate mass of EC in bovine plasma, *m/z* 90.0557. Top: actual sample from test group; Bottom: theoretical prediction of the exact mass, *m/z* 90.0555.

Statement on the handling of livestock and collection of blood samples.

All blood samples were collected under the direct supervision by the veterinarian of record where a valid Veterinary Client Patient Relationship (VCPR) existed within the herd or feedyard.

All livestock were handled and all blood samples were obtained according to (1) Guide for the Care and Use of Agricultural Animals in Research and Teaching. Federation of Animal Science Societies, Third edition January 2010, available at https://aaalac.org/about/Ag_Guide_3rd_ed.pdf (abbreviated as the Ag-Guide), (2) BQA™ Cattle Care and Handling Guidelines, available at https://www.bqa.org/Media/BQA/Docs/cchg2015_final.pdf (abbreviated as the BQA™ Guide), and (3) generally accepted veterinary procedures for the acquisition of blood samples from live animals for routine diagnostic sampling. Additionally, to the best of our ability and knowledge, and based upon our

observations, these livestock facilities adhere to the Ag-Guide, the BQA™ Guide, and federal and state animal welfare regulations and guidelines for the handling, housing, nutrition, and health of the animals under their care.

When collecting blood sample animals are humanely restrained in a cattle chute or head-gate suitable for the blood collection procedure. When blood is collected from the jugular vein, a suitable halter is used to hold the head to one side allowing access to the right or left jugular vein. When blood is collected from the tail vein the restraining equipment will be appropriate to allow access to the tail in an elevated position exposing the tail vein collection site. Appropriate cleanliness and blood collection site preparation will be done per the veterinarian's directive. 10 ml Vacutainer™ EDTA (anti-coagulant) blood collection tubes were used to collect blood from each animal either via Vacutainer™ technique or separate sterile syringe and needle with blood transfer to the Vacutainer™ tube. Blood tubes with anti-coagulant were gently rotated at least eight times for thorough mixing. All blood tubes were placed on ice, or suitable refrigeration, after collection. Processing of the blood sample to collect plasma was done within 4 hours of collection. Samples were centrifuged at 1600 g, 4^0C for 15 minutes and plasma was transferred to 1.8 ml cryo-vials and placed in storage at -80^0C until assayed.

Table S1. Sample description-Distillers grain co-products.

Samples	Abbr.	Collection period (days)	Post-manufacture residence days	Number of ethanol plant sources[1]
Corn wet distillers grains with solubles	CWDGS	30	7	14
Milo wet distillers grains with solubles	MWDGS	30	7	3
Corn dry distillers grains with solubles	CDDGS	75	30	4
Corn condensed distillers solubles, syrup	CCDS	60	14	4
Liquid feed	LF	10	5	1

[1] If the number of ethanol plant sources was less than 5, then extra samples were collected from different feedyards or distribution centers.

Table S2. Sample description – bovine plasma samples.

Samples	Days on Feed (DOF)[1]	%WDGS in ration	%LF in ration
Controls	On pasture	Not fed	Not fed
Test Group 1	70	34	unknown
Test Group 2/3	82	19	5.6
Test Group 4	29	38	unknown

[1] DOF starts at the time of arrival at the feedyard.

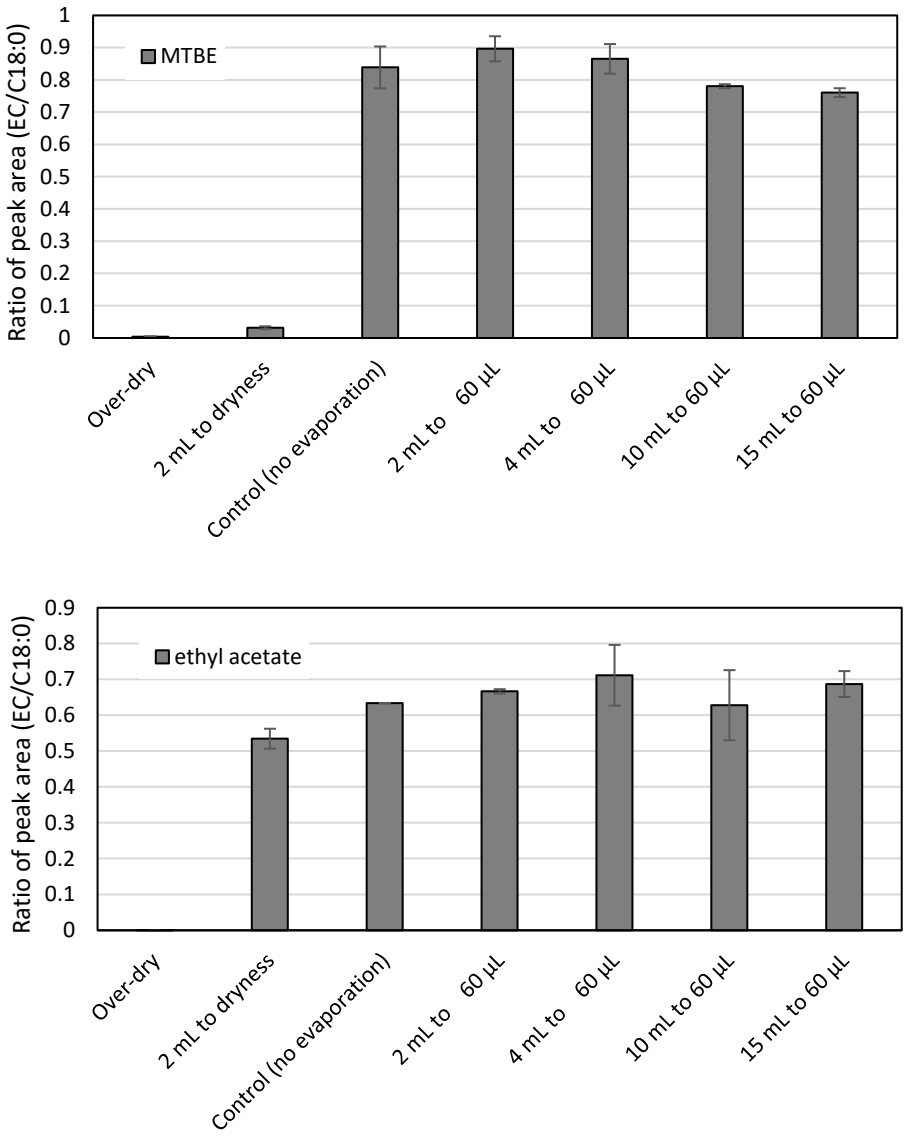

Figure S1. Loss of EC during solvent evaporation. It generally took about 2 h to evaporate 15 mL of solvent. These data suggest that solvent evaporation needs to be carefully controlled to avoid reaching complete dryness.

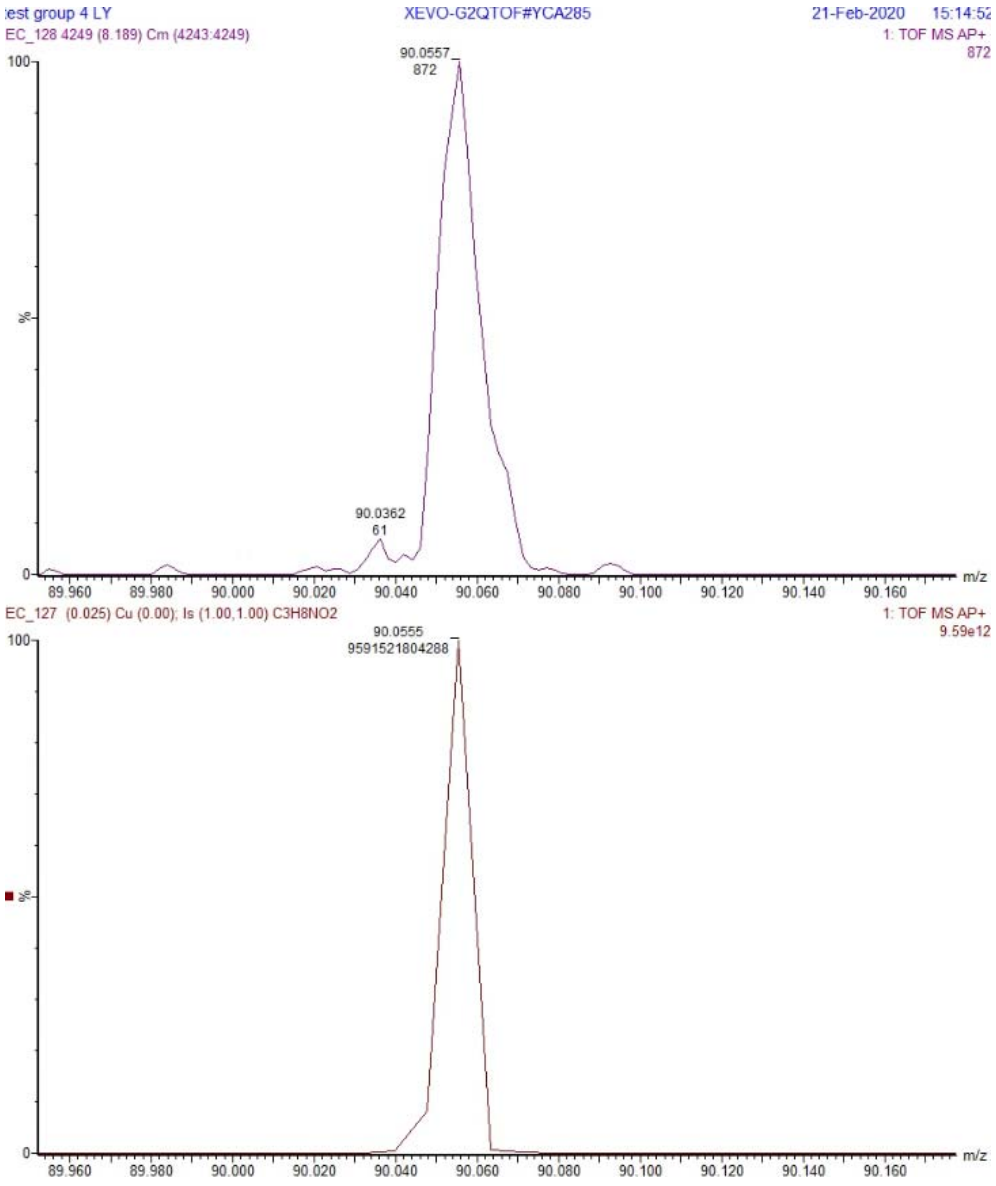

Figure S2. Accurate mass of EC in bovine plasma, *m/z* 90.0557. Top: actual sample from test group; Bottom: theoretical prediction of the exact mass, *m/z* 90.0555.