

Analytical confirmation of *Xanthium strumarium* poisoning in cattle

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Christo J. Botha,¹ Dries Lessing, Magda Rösemann, Erna van Wilpe,
 June H. Williams

Abstract. *Xanthium strumarium*, commonly referred to as “cocklebur,” rarely causes poisoning in cattle. When mature, this robust, annual weed bears numerous oval, brownish, spiny burs. Only the seeds in the burs and young seedlings (cotyledonary leaves) contain the toxic principle, carboxyatractyloside. In the Frankfort district of the Free State Province of South Africa, a herd of 150 Bonsmara cows were allowed to graze on the banks of a small river, where mature cocklebur was growing. Four cows died while grazing in this relatively small area. Clinical signs ranged from recumbency, apparent blindness, and hypersensitivity to convulsive seizures. During necropsy, burs completely matted with ingesta were located in the rumen content. The most distinctive microscopic lesions were severe, bridging centrilobular to midzonal hepatocyte necrosis and hemorrhage. Ultrastructurally, periacinar hepatocytes were necrotic, and novel electron-dense cytoplasmic needle-like crystals were observed, often in close association with peroxisomes. Carboxyatractyloside concentrations were determined using liquid chromatography–high-resolution mass spectrometry (LC-HRMS). Carboxyatractyloside was present in rumen contents at 2.5 mg/kg; in burs removed from the rumen at 0.17 mg/kg; in liver at 66 ng/g, and was below the limit of quantitation in the kidney sample, estimated at approximately 0.8 ng/g. Based on the presence of the plants on the riverbank, the history of exposure, the clinical findings, the presence of burs in the rumen, and the microscopic and ultrastructural lesions, *X. strumarium* poisoning in the herd of cattle was confirmed and was supported by LC-HRMS.

Key words: Bovine; carboxyatractyloside analysis; cocklebur; hepatotoxicity; *Xanthium* spp.

Xanthium species poisoning, a relatively rare occurrence throughout the world, has been reported in domesticated livestock, with pigs more often affected than cattle and sheep.^{7,8} The organs primarily targeted are the liver and to a lesser extent myocardium and kidneys.⁷ *Xanthium* spp. are robust annual weeds that are troublesome pioneers on disturbed soils such as road verges, reaped lands, along streams, and on floodplains in Australia, Brazil, certain parts of the United States, and in southern Africa.^{1,7,8,10,11} *Xanthium strumarium* L., commonly referred to as “cocklebur,” grows up to 2 m high and has large, broad leaves with irregular toothed margins.^{1,7,8} The mature brownish burs are 2–2.5 cm long and covered with hooked spines.^{1,7,8}

The phytotoxin contained by *X. strumarium* has been purified and identified as carboxyatractyloside, a diterpene glycoside.³ The toxic principle is primarily located in the seeds inside the burs and, after germination, in the cotyledonary leaves.³ Very young seedlings in the cotyledon or 2-leaf stage have been reported as particularly dangerous to swine.³ The concentration of the toxin diminishes rapidly at the appearance of the first true leaves (4-leaf stage).³ Carboxyatractyloside inhibits a translocase enzyme required to transport adenosine diphosphate across the mitochondrial membrane, thus preventing the synthesis of adenosine triphosphate.⁹ The inhibition of oxidative phosphorylation results in an intracellular energy “crisis,” and cell death ensues.⁹

Clinical signs of *Xanthium* spp. poisoning appear abruptly within hours of ingestion of the burs or cotyledonary leaves.^{1,11}

In general, anorexia, abdominal pain, depression, weakness, ataxia, hypersensitivity, blindness, opisthotonus, and convulsions have been reported in cattle.^{8,10,11,14} Gross lesions include hepatomegaly with lobular accentuation, edema of the gallbladder wall, hepatic and renal congestion, and fluid accumulation in the body cavities.^{10,11,14} The liver shows the most distinctive microscopic lesions, which are characterized by acute diffuse necrosis of the centrilobular hepatocytes accompanied by congestion and hemorrhage.^{10,11,14} Other histological lesions include degenerative changes in the myocardium and kidneys, neuronal degeneration in the brain, and cerebral edema.^{7,11,14}

There is a dearth of information with respect to the detection of carboxyatractyloside in animal tissue and/or analytical confirmation of *X. strumarium* poisoning in cattle. In the only published report, thin layer chromatography was used to determine carboxyatractyloside concentration in rumen contents and urine of a group of calves following cocklebur intoxication.¹⁴

From the Department of Paraclinical Sciences, Faculty of Veterinary Science, University of Pretoria, South Africa (Botha, Williams); Frankfort Animal Clinic, Frankfort, South Africa (Lessing); National Horseracing Authority of Southern Africa, Johannesburg, South Africa (Rösemann); and Department of Anatomy and Physiology, Faculty of Veterinary Science, University of Pretoria, South Africa (van Wilpe).

¹Corresponding Author: Christo J. Botha, Department of Paraclinical Sciences, Faculty of Veterinary Science, Private Bag X04, Onderstepoort, 0110, South Africa. christo.botha@up.ac.za



Figure 1. *Xanthium strumarium* burs removed from the rumen content of a cow that died: matted with ingesta (left) and after rinsing with water, demonstrating the characteristic hooked spines (right).

The concentration was estimated to be 100–200 parts per million (ppm) in the rumen contents and 0.05–0.1 ppm in the urine.¹⁴ A competitive enzyme-linked immunosorbent assay was developed to detect atractyloside, the toxic principle contained in *Callilepis laureola* (impila) used in Zulu traditional medicine, and it was concluded that the assay has diagnostic potential to confirm mortalities in human beings.² The current report describes an outbreak of *X. strumarium* poisoning in cattle in South Africa and the laboratory workup and analytical procedures required to confirm poisoning.

In August 2013, due to a lack of grazing, a herd of 150 Bonsmara cows were allowed to graze on the banks of a small river in the Frankfort district of the Free State Province of South Africa. Owing to the low water level, the cows were watched by a few herdsman to prevent them from crossing the river. Consequently, the cows were not allowed free range and were kept crowded in a small area, thus forcing them to consume most of the plant material growing there. On the riverbank, mature *X. strumarium* weeds were present. After some animals showed clinical signs, a private veterinarian was consulted who observed sternal recumbency, apparent blindness, and hypersensitivity in a cow. When touched, the animal reacted violently, jumped up, but collapsed again and exhibited a convulsive seizure. Four cows died and 2 were necropsied. Gross findings included some postmortal autolysis, hepatitis, pulmonary edema, and myocardial hemorrhages; burs were palpable in the rumen content. Some of the spiny burs, completely matted with rumen ingesta, were removed and rinsed with water to aid identification (Fig. 1). A provisional diagnosis of *X. strumarium* poisoning was made.

Samples of the brain, lung, liver, kidney, and small intestine were placed in 10% buffered formalin and submitted for histology. The tissue samples were routinely processed, embedded in paraffin wax, sectioned, and stained with hema-

toxylin and eosin. In addition, formalin-fixed liver and kidney tissue samples were further processed for transmission electron microscopy using standard techniques. Briefly, the samples were postfixed in 1% osmium tetroxide in Millonig phosphate buffer and dehydrated through a series of graded ethanols before infiltrating with propylene oxide and epoxy resin. Ultrathin (50–90 nm) sections were collected onto copper grids, stained with Reynolds lead citrate and an aqueous saturated solution of uranyl acetate,⁶ and examined in a transmission electron microscope^a operated at 80 kV.

Fresh samples of the liver, kidney, and rumen content, as well as some of the burs present in the rumen content were collected for toxicological and chemical analyses. Routine analyses of rumen content for pesticides by gas chromatography–mass spectrometry (GC-MS) and kidney for lead concentration by atomic absorption spectrometry (AAS) were performed at the Toxicology Laboratory, Onderstepoort Veterinary Institute to rule out other common neurotoxicities.

Xanthium strumarium leaves and mature burs were collected from a control site near Lethlabile, North West Province (–25.476501, 27.838068). Some of these burs were planted and, after germination, the cotyledonary leaves removed. The plant material was dried and milled prior to analysis and served as positive control samples.

Carboxyatractyloside concentrations were determined using liquid chromatography–high-resolution mass spectrometry (LC-HRMS). Carboxyatractyloside^b was purchased and used as reference material. The procedure to extract the plant samples is briefly described. Dried, milled cabbage leaves were used as a blank matrix for the preparation of calibrators. A calibration curve consisting of 1 blank plus 5 calibrators, covering the range of 1–10 µg carboxyatractyloside, was prepared. The plant samples and calibrators (5 g) were weighed into 50-ml centrifuge tubes and homogenized^c with 15 ml of methanol:acetonitrile^d (5:95). Calibrators were spiked and vortex mixed, and samples and calibrators were centrifuged at 3,000 × g for 15 min. The supernatant was decanted into a second set of 50-ml centrifuge tubes, and the extraction was repeated with 10 ml of methanol:acetonitrile (5:95). The supernatants were combined, 10 ml of hexane^d was added to each tube, and the tubes were vortex mixed and centrifuged for 5 min at 3,000 × g to remove excess chlorophyll. The hexane layer was discarded, and the samples were dried on an evaporator^e at 50°C under nitrogen. The extracts were reconstituted in 200 µl of methanol.

For the analysis of the liver and kidney samples, separate calibration curves were prepared for each matrix. Each curve consisted of 1 blank plus 4 calibrators covering the range of 20–200 ng of carboxyatractyloside. Samples (2 × 10 g) and calibrators (5 g) were weighed into 50-ml centrifuge tubes, and the process as described for the plant extraction, except the chlorophyll removal step, was repeated.

The high-performance liquid chromatography instrument^f was fitted with a C18 column^g (150 mm × 2.1 mm, 5 µm particle size). The column temperature was 40°C, and the flow rate was 350 µl/min. The mobile phase was a gradient of water to acetonitrile, both containing 5 mM ammonium acetate^b and 0.1%

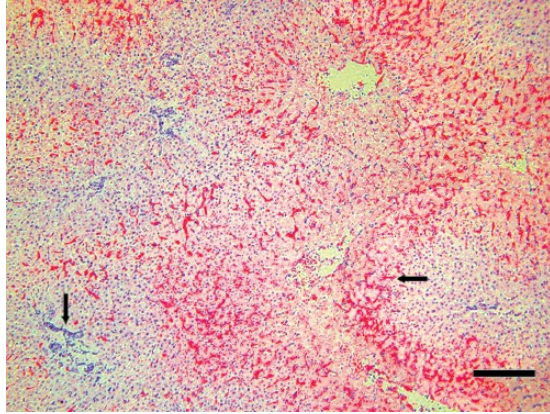


Figure 2. Micrograph; liver; bovine. Bridging centrilobular to midzonal hepatocyte necrosis with hemorrhage (horizontal arrow) and mild portal bile duct proliferation (vertical arrow). Hematoxylin and eosin. Bar = 200 μ m.

formic acid.^b The linear gradient started at 2 min from 2% to 98% acetonitrile at 20 min, kept at 98% to 26 min, followed by a return to initial conditions at 28 min. The column was allowed to recondition until 35 min when the next injection started.

Mass spectrometric analysis employed a high-resolution mass spectrometer^h controlled by the manufacturer's software.^h The scan range was from 350 to 1,000 m/z units with a resolution of 70,000 with continual positive/negative switching for qualitative analysis and 140,000 resolutions in negative mode for quantitative analysis. The AGC (automated gain control) target was set to 3e6, and the maximum injection time was set to 200 msec. The following tune file settings were used: heater temperature at 400°C, capillary temperature at 250°C, sheath gas flow rate at 50, aux gas flow rate at 10, and sweep gas was switched off. The source voltage in positive ion mode was 3.5 kV (negative ion mode -2.5 kV), and the S-lens was set at 55 V. No pesticides, including the organophosphorus, carbamate, and organochlorine compounds, were detected in the rumen content; renal cortical lead concentration was within the normal reference range.

Microscopic examination of the liver revealed severe, widespread, bridging centrilobular to midzonal necrosis and hemorrhage (Fig. 2) with the occasional presence within these areas of neutrophils and a few macrophages. Mild portal bile duct hyperplasia was present with a few bile ducts containing necrotic leukocyte debris. Occasional portal triads were associated with the presence of a few neutrophils and mononuclear leukocytes. Periportal hepatocytes showed some cytoplasmic protein droplets and mitochondrial swelling and, at the borders of the necrotic zone, scattered hepatocytes contained cytoplasmic lipid vacuoles. Histological lesions in the kidney included flattened epithelium with mild distension of some cortical tubules, occasional glomeruli with globular protein leakage into Bowman spaces and associated convoluted tubular lumens, and some tubules contained granular pink pigment casts. Occasional cortical

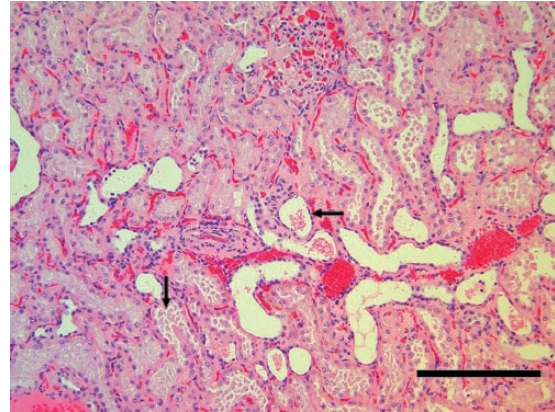


Figure 3. Micrograph; kidney cortex; bovine. Mild tubular dilatation and flattening of epithelium, as well as multifocal intraluminal granular pigment casts (horizontal arrow) and globular protein casts (vertical arrow). Hematoxylin and eosin. Bar = 200 μ m.

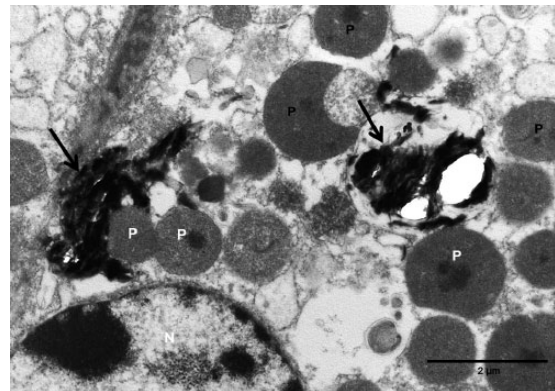


Figure 4. Transmission electron micrograph; liver; bovine. Electron-dense crystalline cytoplasmic inclusions (arrows) in hepatocyte. Note close association of peroxisomes (P) with the inclusions. N = hepatocyte nucleus. Bar = 2 μ m.

tubular cells contained intracytoplasmic bile pigment. Scattered mineralized casts and numerous granular pigment casts, sometimes-associated with amorphous protein, were found in the renal medullary tubules (Fig. 3). Severe diffuse pulmonary congestion and proteinaceous alveolar edema alternated with areas of alveolar emphysema or partial atelectasis. Diffuse lymphoid tissue in small intestinal mucosa and solid lymphoid tissue in ileal Peyer patches appeared reactive. No significant brain lesions were identified.

Ultrastructural examination of the hepatocytes reflected the widespread necrosis, noticed with light microscopy, with the loss of most organelles, but peroxisomes remained prominent. Conspicuous electron-dense cytoplasmic inclusions consisting of plate-like or needle-like crystals (Fig. 4) were present, often in close association with peroxisomes.

These inclusions were also present in Kupffer cells and neutrophils. The renal tubules displayed intraluminal amorphous debris of medium electron density, comparable to the pigment casts seen by light microscopy, with only 1 structure found resembling the crystalline inclusions as seen in the hepatocytes.

In the LC-HRMS analysis, carboxyatractyloside eluted at approximately 11 min. Direct infusion of carboxyatractyloside (50 ng/ml in methanol:water) revealed a strong signal in negative mode corresponding to a $[C_{31}H_{46}O_{18}S_2-H]^-$ ion with mono-isotopic mass of 769.20449 atomic mass units (amu). The mono-desulfate ion $[C_{31}H_{46}O_{15}S-H]^-$ with mono-isotopic mass of 689.24799 amu co-eluted indicating that the ion was formed post-column, most likely in the source. The mono-desulfate was 10 times less prominent than the carboxyatractyloside, and quantitation was done using carboxyatractyloside response (Fig. 5). The calibration curve was linear ($R^2 = 0.98$) in the plant matrix over the range of 0–10 μ g. Retention times in the plant samples were slightly shorter compared to the reference standard. The carboxyatractyloside concentrations in the plant material analyzed are given in Table 1. For confirmation, tandem mass spectrometry (MS/MS) experiments were performed on the *Xanthium* spp. cotyledonary leaf extract, and it was compared with the MS/MS fragments of the reference standard. The MS² fragments matched those of the standard. The carboxyatractyloside concentration in the sample of rumen contents was calculated using the calibration curve of the plant matrix. Carboxyatractyloside was present in the rumen contents collected from a dead cow at 2.5 mg/kg (Fig. 6). The calibration curve was linear in the liver and kidney matrices ($R^2 = 0.99$ and $R^2 = 0.98$, respectively) over the range of 0–200 ng. Carboxyatractyloside was determined in the liver sample at 66 ng/g, but was below the limit of quantitation (20 ng/g) in the kidney sample and estimated at approximately 0.8 ng/g.

The current report describes a LC-HRMS method to detect carboxyatractyloside in rumen content and tissue samples of poisoned animals that have died. Analytical confirmation of *X. strumarium* poisoning is a challenge as it is a relatively rare intoxication, and diagnostic analysis is not performed routinely. The carboxyatractyloside concentration determined in the rumen contents (2.5 mg/kg) in the current study was considerably lower when compared to a concentration of 100–200 mg/kg reported previously in a case of cocklebur poisoning; however, these authors of the previous study estimated the concentration in the rumen content based on a thin layer chromatography method, which calls for some subjectivity.¹⁴ The authors also reported a urinary carboxyatractyloside concentration ranging from 0.05–0.1 mg/kg and concluded that metabolism probably occurs prior to urinary excretion or there are other major routes of excretion.¹⁴ In the present study, the kidney carboxyatractyloside concentration was estimated at only 0.0008 mg/kg, which supports the contention that either biotransformation occurs or there

are other excretory pathways. The available literature reports a wide variation for carboxyatractyloside concentration in burs, ranging from 0.1 to 4,570 mg/kg.^{3,14} In the present study, the burs removed from the rumen content and those collected from the control site contained 0.17 and 1.4 mg/kg carboxyatractyloside, respectively. The lower concentration in the burs removed from the rumen is expected and probably reflects leaching of carboxyatractyloside from the ingested burs. After germination and sprouting, the carboxyatractyloside concentration of the cotyledonary leaves was 48.6 mg/kg, which is considerably lower than 1,200 mg/kg previously reported for seedlings.³

In South Africa, there are several hepatotoxicities where centrilobular hepatic necrosis is a common finding. The histologic hepatic lesions of acute *X. strumarium* poisoning in cattle are similar to those reported in acute poisoning with other hepatotoxic plants, such as *Cestrum* species and the pyrrolizidine alkaloid-containing plants (*Senecio* and *Crotalaria* species), and intoxication with cyanobacteria, for example *Microcystis aeruginosa*.^{7,13} During the current outbreak, which occurred toward the end of winter, there was no cyanobacterial bloom in the low flowing river and no possibility of prior eutrophication of the water. *Senecio inaequidens* poisoning has been recorded in the same district⁵; however, the most important differential diagnosis is probably *Cestrum* poisoning.¹³ *Cestrum parqui* (willowleaf jessamine) contains the toxic kaurene glycosides, parquin and carboxyparquin, which are similar to atractyloside and carboxyatractyloside¹² and explains the similarity in the pattern of liver necrosis. Nonetheless, there was no exposure to any of the other potential hepatotoxic plants.

In the present case, the diagnosis was aided by locating burs in the rumen content, by the clinical picture, and by pathological lesions consistent with previous reports. Although intoxication in cattle is more common after ingestion of very young seedlings that still contain the cotyledonary leaves,^{3,8,10,11} cocklebur toxicosis in calves has been reported following ingestion of hay contaminated with mature plants with numerous burs.¹⁴ The excitatory nervous signs such as hyperesthesia, apparent blindness, and convulsive seizures observed during *Xanthium* spp. poisoning in cattle^{8,10,11,14} are most likely a result of hypoglycemia induced by carboxyatractyloside,⁴ and can probably not be attributed to hepatic encephalopathy when the acuteness of onset of clinical signs is taken into consideration and no spongy changes (*status spongiosis*) of the white matter in the brain were observed.^{7,11} The hepatic crystalline inclusions and their close association with peroxisomes, as observed with transmission electron microscopy, probably indicate a detoxification process by the peroxisomal enzymes, with subsequent accumulation and precipitation of the metabolic derivatives in the cytosol.

After due consideration of other potential hepatotoxicities in this district of the Free State Province of South Africa, the diagnosis of *X. strumarium* poisoning in the herd of cattle,

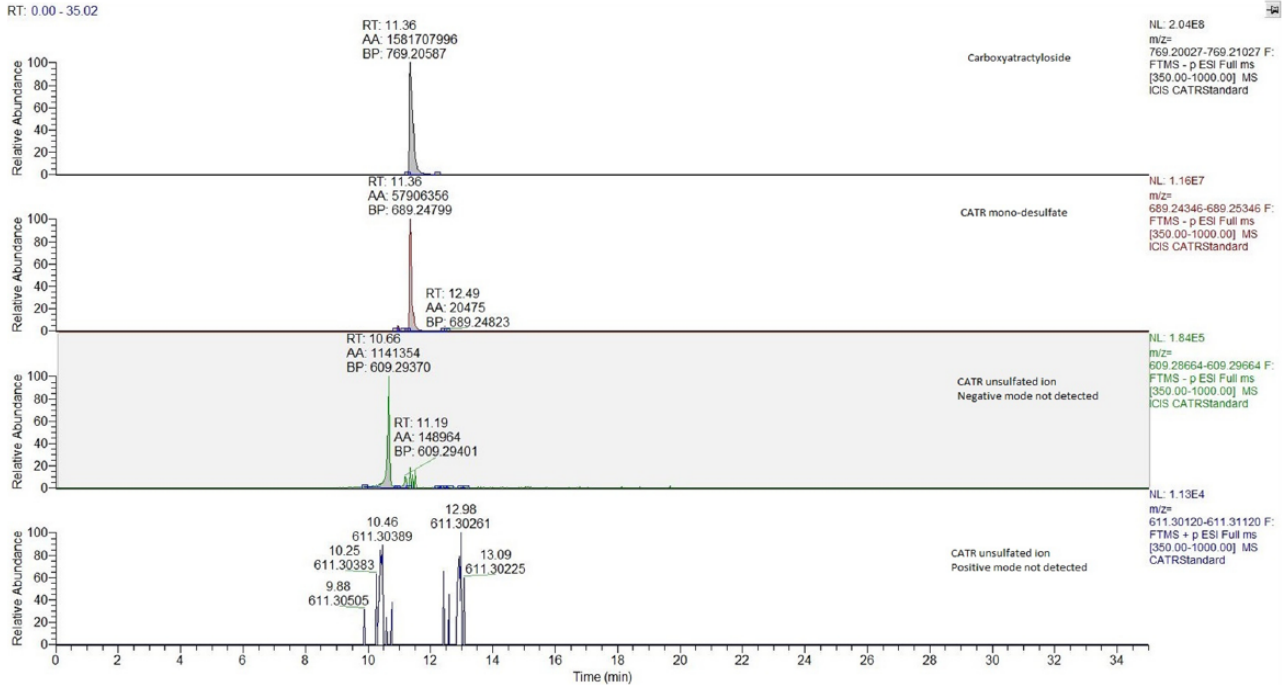


Figure 5. Liquid chromatography–high-resolution mass spectrometry results revealed the carboxyatractyloside (CATR) ion and a mono-desulfated ion in the reference standard.

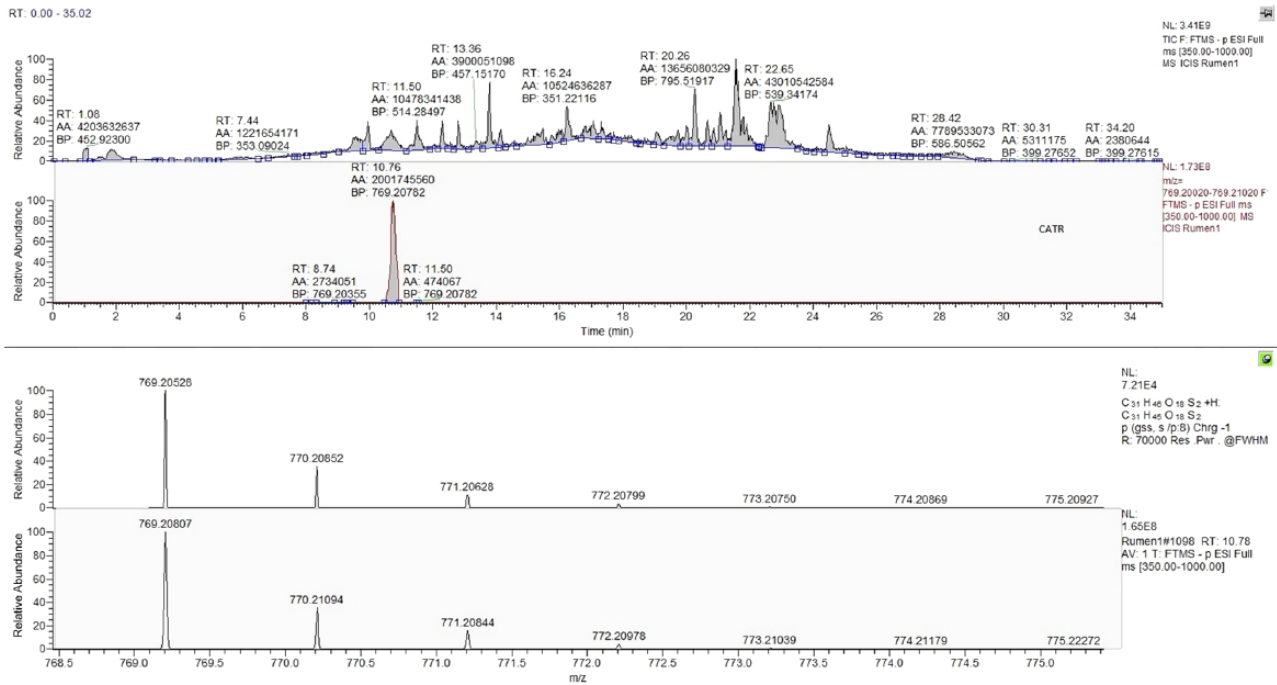


Figure 6. Liquid chromatography–high-resolution mass spectrometry analysis. Chromatogram demonstrating carboxyatractyloside (CATR) in the rumen contents and the isotope ratios matching the theoretical values.

Table 1. Comparative concentrations of carboxyatractyloside in the dried, milled *Xanthium strumarium* plant material analyzed in the current study.

Plant material	Carboxyatractyloside ($\mu\text{g/g}$)
<i>Xanthium</i> burs (removed from rumen content)	0.17
<i>Xanthium</i> burs (collected at control site)	1.4
<i>Xanthium</i> cotyledonary leaves (germinated from burs collected at control site)	48.6
<i>Xanthium</i> leaves (collected at control site)	<0.01

based on the presence of the plants on the riverbank, the history of exposure, the clinical findings, the presence of burs in the rumen, and the microscopic and ultrastructural lesions, was confirmed and supported by LC-HRMS.

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- Philips CM10 TEM, Philips Electron Optical Division, Eindhoven, The Netherlands.
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- Ultra-Turrax homogenizer, IKA, Staufen, Germany.
- Burdick & Jackson, Muskegon, MI.
- Zymark TurboVap, McKinley Scientific, Sparta, NJ.
- 1260 Infinity instrument, Agilent Technologies Inc., Santa Rosa, CA.
- XSelect CSH, Waters Corp., Milford, MA.
- QExactive, Xcalibur software, Ion Max API source and HESI-II probe; Thermo Fisher Scientific, Waltham, MA.

Declaration of conflicting interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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