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Absorption, distribution and biliary excretion of cafestol, a potent cholesterol elevating compound in unfiltered coffees in mice.

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Absorption, distribution and biliary excretion of cafestol in mice.

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Non-standard abbreviations

ALT	alanine amino transferase
AST	aspartate aminotransferase
CMC	Carboxy methyl cellulose
EpRE	electrophile-response element
HPLC-RA	high-performance liquid chromatography with radioactive detection
LCMS	Liquid Chromatography mass spectrometry
Nrf2	NF-E2-related factor 2
SI	Small intestine
TUDC	tauroursodeoxycholic
QWBA	Quantitative whole body autoradiography

Abstract

Cafestol is a diterpene present in unfiltered coffees. It is the most potent cholesterol-elevating compound present in the human diet. However, the precise mechanisms underlying this effect are still unclear. In contrast, cafestol is also known as a hepatoprotective compound which is likely to be related to the induction of glutathione biosynthesis and conjugation. In the present study we investigated whole body distribution, biliary excretion and portal bioavailability of cafestol in mice. First, dissection was used to study distribution. Five hours after an oral dose with ^{3}H labeled cafestol, most activity was found in small intestine, liver and bile. These results were confirmed by quantitative whole body autoradiography in a time course study which also showed elimination of all radioactivity within 48 hours after administration. Next, radiolabeled cafestol was dosed i.v. to bile duct cannulated mice. Five hours post dose 20% of the radioactivity was found in bile. Bile contained several metabolites but no parent compound. After intestinal administration of radioactive cafestol to portal vein cannulated mice, cafestol was shown to be rapidly absorbed into the portal vein as parent compound, a glucuronide and an unidentified metabolite. From the presence of a glucuronide in bile that can be deconjugated by a bacterial enzyme and the prolonged absorption of parent compound from the GI tract we hypothesize that cafestol undergoes enterohepatic cycling. Together with our earlier observation that epoxidation of the furan ring occurs in liver these findings merit further research on the process of accumulation of this coffee ingredient in liver and intestinal tract.

Introduction

Cafestol (Figure 1) is a diterpene with a characteristic furan group which is present in unfiltered coffees such as French press coffee, Scandinavian boiled coffee, cafetiere coffee, and to a lesser extent espresso (Urgert and Katan 1997; Ranheim and Halvorsen 2005; Ricketts, Boekschoten et al. 2007). The compound is regarded as the major compound responsible for the increase in serum cholesterol observed after consumption of these coffee preparations (Weusten-Van der Wouw, Katan et al. 1994; Urgert and Katan 1997; Ricketts, Boekschoten et al. 2007). For example, three cups of French press (plunger pot) coffee, providing the equivalent of 10mg cafestol, consumed for 4 weeks are estimated to increase serum cholesterol by 0.13 mmol/l (Urgert and Katan 1996). In addition, cafestol transiently increases serum triglycerides and levels of liver alanine amino transferase (ALT), and aspartate aminotransferase (AST) (Urgert, Meyboom et al. 1996; Urgert, Essed et al. 1997; Boekschoten, Schouten et al. 2004). However, from a toxicological perspective cafestol shows a remarkable two-faced behavior. In addition to its deleterious effects on cholesterol levels and liver enzymes, cafestol has also been identified as an anti-mutagenic compound (Cavin, Holzhaeuser et al. 2002; Huber, Prustomersky et al. 2002; Huber, Scharf et al. 2002; Huber, Scharf et al. 2003; Huber, Teitel et al. 2004; Huber and Parzefall 2005). This potential beneficial effect was shown to be related to an induction of glutathione biosynthesis and conjugation, and a decreased activity of CYP450 enzymes involved in the bio-activation of some pro-carcinogens (Cavin, Bezencon et al. 2003; Huber, Teitel et al. 2004; Lee, Choi et al. 2007; Huber, Rossmanith et al. 2008). Recently we demonstrated that cafestol is able to induce an electrophile-response element (EpRE) *in vitro* after metabolic activation with S9 mix obtained from liver of male Sprague–Dawley rats treated with Aroclor-1254 (van Cruchten, de Haan

et al. 2009). The EpRE is a regulatory sequence mediating the coordinated transcriptional activation of genes associated with phase 2 biotransformation, protection against oxidative stress, and other cancer-chemo protective mechanisms (Kobayashi, Kang et al. 2006). The key regulator of EpRE-mediated gene expression is the transcription factor Nrf2 (nuclear factor erythroid 2-related factor 2) and, to a lesser extent, Nrf1 (Copple, Goldring et al. 2008). This Nrf2 activation may be responsible for the increase in glutathione biosynthesis and conjugation. We hypothesized that metabolites formed via epoxidation of the furan moiety are involved in this induction process. Indeed we were able to demonstrate the presence of cafestol epoxides and their epoxy-glutathione conjugates in bile of mice dosed with cafestol. In addition, we also found a glucuronide conjugate of cafestol in mouse bile (van Cruchten, de Haan et al., 2009).

The present studies were undertaken to study body distribution, portal bioavailability and biliary excretion of cafestol in mice in more detail. The selection of the mouse as a model was based on previous studies showing that among various animal models, the ApoE*3-Leiden transgenic mouse is the only model that responds to cafestol as humans do (Post, de Roos, et al. 2000).

Distribution after oral administration of radiolabeled cafestol was studied by liquid scintillation counting of dissected tissues, and by quantitative whole body autoradiography. Following the observation that the distribution of the radiolabel remained largely restricted to the liver and intestinal tract, the question was addressed whether enterohepatic cycling plays a role. This was first investigated by studying biliary excretion of cafestol and metabolites after i.v. administration to gallbladder cannulated mice. Finally, portal delivery of cafestol and potential metabolites was assessed after intestinal administration.

Material and methods

Chemicals

Chemicals were of analytical grade and purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.) unless otherwise stated. Solvable^{TM} (tissue solubilizing fluid) was purchased from Perkin and Elmer (Groningen, The Netherlands). Methanol was purchased from Baker (Mallinckrodt Baker, Deventer, The Netherlands).

Radio labelling;

^{3}H Cafestol (1mCi/mmol) was synthesized by RC Tritec (Teufen, Switzerland). The compound was made by hydrogenation of kahweol (Alexis Biochemicals, U.S.A) to cafestol under tritium gas atmosphere using $Pd/SrCO_3$ in EtOH. Labeling was on the C1 and C2 positions. Before each use, radiochemical purity (> 99 %) of ^{3}H Cafestol was verified by HPLC-RA. Exchange of the ^{3}H label with water was checked by collecting and counting water evaporating at 105 °C from a treated liver sample using a Carbolite furnace.

Animals

Pure bred wild-type adult (8-10 weeks), male (C57BL6/j) mice were purchased from Harlan (Horst, Netherlands). Mice were housed in a light- and temperature controlled facility and had free access to water and standard laboratory chow (CRME, SDS diets, The Netherlands). All animal studies were approved by the Local Committee for Care and Use of Laboratory Animals of Wageningen University, or the Amsterdam Medical Centre (for the studies with gallbladder and portal vein cannulated mice), Amsterdam, The Netherlands.

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^{3}H radiolabeled preparations for administration

Preparations for administration were made by mixing ethanol stock solutions of unlabeled cafestol with appropriate amounts of ^{3}H cafestol. These solutions were administered after mixing with either 0.1% of carboxymethyl cellulose in water (experiment I), olive oil (experiment II) or Clinoleic 20% emulsion (Baxter, IL, U.S.A, experiment III).

Experimental setup

Four experiments were performed. The aim of experiment I was to study distribution of the radiolabel after oral administration, using liquid scintillation counting of organs and tissues. Mice were fasted for 4 hours. Then 1.5mg of cafestol enriched with ^{3}H cafestol to an equivalent of 10μCi was given to each animal by oral gavage (mixed with a 0.1% solution of carboxymethyl cellulose in water). Five hours after dosing, mice were killed and organs and blood samples collected. In experiment II, quantitative whole body autoradiography (QWBA) was used to study tissue distribution after oral administration in more detail and at more points in time. Mice (eight in total) were dosed 1.5mg unlabeled cafestol in olive oil via oral gavage mixed with a trace amount of ^{3}H cafestol (1mCi/kg BW). At different time points (0.5, 1, 2, 4, 8, 12, 24 and 48 hours) mice were anesthetized with a mixture of isoflurane and oxygen and blood was sampled via orbital punction and puncture of the tail vein. Then mice were euthanized with a mixture of isoflurane and oxygen, fixated and subsequently frozen in a hexane-dry ice bath. Whole body autoradiography was performed according to standard operation procedures (Solon, Balani et al. 2002) as described below. Experiment III was performed to study biliary excretion of cafestol and its metabolites after i.v. administration of labeled cafestol. For this experiment, five mice were fasted overnight. Mice were anaesthetized,

subsequently their gallbladder was cannulated and bile collected (Kuipers, van Ree et al. 1996; Klett, Lu et al. 2004). To maintain a constant bile flow, mice were infused with a bile acid solution containing tauroursodeoxycholic (TUDC) acid (conc. 600nmol/100gramBW/min) according to standard procedures (Klett, Lu et al. 2004). After 60 minutes of infusion, bile flow was considered constant based on our previous experience and cafestol was injected through the tail vein. Every mouse was injected with 200µl of a mixture containing 12µg unlabeled compound and 10µCi ^{3}H cafestol dissolved in Clinoleic (20%) in the tail vein. Bile was sampled every 15minutes during the first hour and then every half hour. After 5 hours, blood samples were drawn from the systemic circulation by orbital puncture. Then mice were killed and liver, intestine and kidneys were isolated. Bile and plasma samples were immediately frozen at −80°C. Bile flow was determined gravimetrically assuming a density of 1g/ml for bile. Total radioactivity in the samples was determined by scintillation counting. These values were averaged for the number of mice. Organ samples were processed as described in the organ sample preparation section.

In the fourth experiment (experiment IV), the portal vein of C57Bl6 mice that had fasted for 4hours was cannulated. A solution containing cafestol mixed with 60µCi ^{3}Hcafestol was administered at a dose of 1.5 mg directly into the duodenum. Portal blood was sampled at different time points: 2, 5, 10, 20, 30, 40 and 50minutes. At the end (50 minutes post dose) a blood sample from the systemic circulation was collected by orbital puncture. Blood samples were centrifuged at 4000g for 10 minutes and plasma was stored at -80ºC until further HPLC analyses.

Organ sample preparation.

Organ samples were cut in pieces of approximately 25mg and incubated overnight at

room temperature with 1ml Solvable. After incubation, samples were decoloured with 300µl hydrogen peroxide (30%) (1000µl in case of spleen), and counted. Blood samples (30µl) were centrifuged and the radioactivity in plasma was determined. Five microliters of bile were diluted with 5ml scintillation fluid (Ultima Gold^{R}, Perkin and Elmer, Groningen, The Netherlands) and incubated at room temperature overnight in a dark environment. The radioactivity in all samples was measured using a liquid scintillation counter (model 3255, Packard Instrument Co., Downer's Grove, IL, U.S.A.), with a quench curve used for correction.

Quantative whole body autoradiography

The animals were rapidly frozen by total immersion in a hexane/solid carbon dioxide mixture (ca. -80°C) and retained at -80°C for quantitative whole body autoradiography. Following removal of the whiskers, legs and tail, each frozen carcass was placed in a block of carboxymethyl cellulose (1% aqueous solution, w/v) and mounted onto the stage of a Leica CM3600 cryomicrotome maintained at ca. -20°C. Sagittal sections (nominally 30µm) of each animal were subjected to whole-body autoradiography using procedures based on the work of Ullberg (Ullberg 1977), at five different levels of the animal body (to include as many tissues as possible). Three sections were taken at each level and freeze-dried. One section from each level was selected and, along with a ^{3}H-whole blood standard curve, placed in contact with FUJI imaging plates. The imaging plates were placed in light-tight cassettes and allowed to expose for 7 days. After exposure, and under subdued lighting, the sections were removed from the plates and the plates processed using a FUJI BAS 1500 Bio-image analyser (Fuji). These analyses were performed by Quotient Bioresearch (Rushden, UK).

HPLC analysis with radiochemical detection of cafestol and metabolites

Five microliters of bile sample were mixed with 45µl milliQ water. This sample was analyzed by a HPLC system equipped with a ChromSpher column (Varian, Middelburg, The Netherlands), 100*4.6 mm i.d, packed with 3µm omnisphere C18 material (Varian). The mobile phase was delivered using a system with Gynkotech pumps (Germering, Germany) at a flow rate of 0.4ml/min. Solvent A was 5mM phosphate buffer pH 6.3 and solvent B was 100% methanol. A linear gradient of 0 minutes 10%B to 20 minutes, 100%B was used to separate the components. Detection was performed using a flow scintillation analyzer (500TR series, Packard Instrument Co., Downer's Grove, IL, U.S.A., run in parallel with a absorbance detector (spectroflow 757, Kratos Analytical Instruments, Ramsey, USA).

Deconjugation of cafestol metabolites

Bile samples (5µl) were deconjugated by adding 10µl of E.coli derived β-glucuronidase solution. This mixture was incubated at 37°C for 1hour. HPLC analysis was performed with these samples as described.

Results

Tissue distribution of ^{3}H cafestol (metabolites).

Five hours after oral administration of ^{3}H labeled cafestol, the major amount of the radioactivity was found in the intestinal tract (Figure 2). Within the gastro intestinal tract, radioactivity was found both in the lumen of the small intestine (SI) (14%) and in the SI itself (10%), 34% was in the cecum (combined contents and mucosa), 6% in the colon lumen and 1% in the colon mucosa. After the intestinal tract, the largest fractions of radioactivity were found in liver (5%) and bile (2%). Approximately 1% of the radioactivity was detected in kidneys. No radioactivity was detected in esophagus, pancreas, spleen, adrenals, brain, heart, lung, white adipose tissue and muscle. No radioactivity was also detected in systemic blood and only 1 % of the dose was excreted per 100µl of urine. Total recovery of radioactivity was 98% (±31%). In this experiment it was not further investigated whether the radioactivity in these tissues was due to cafestol or to cafestol metabolites.

Quantitative whole body autoradiography

Every mouse was cut at 5 different levels of its body. From every level, 3 sections were made. Selected whole-body auto-radiograms are given in figure 3. QWBA confirmed the distribution pattern as obtained by dissection. Eight different time points (0-48 hours) were investigated by QWBA. Thirty minutes post dose, almost all radioactivity was found in liver and small intestines. Trace amounts were found in kidneys. Apart from liver and intestinal tract and the traces in kidney, no radioactivity was seen in any other organs or tissues that were analyzed. Blood samples were collected separately and consistent with the data from the dissection study, no

radioactivity was found. This relative distribution pattern remained essentially the same during the first 24 hours post dose. At 48hours post dose, no radioactivity was detected anymore.

Biliary excretion of cafestol metabolites

The biliary excretion of radioactive cafestol after i.v. administration is depicted in figure 4. Five hours after administration a cumulative amount of 20% of the administered dose of radioactivity was secreted in bile. In the blood samples taken at that moment by orbital puncture, no radioactivity was detected. HPLC analysis of bile showed that there was no parent compound detectable. Instead, several apparent cafestol metabolites were found. As can be seen in Figure 5a, bile samples contained multiple ^{3}H peaks. These peaks are designated as peak I, II, III, and IV, eluting at retention time 3.5, 10, 13 and 18 minutes, respectively. To further characterize these peaks, conjugation and deconjugation reactions were performed. Deconjugation with β-glucuronidase resulted in a decrease of peak IV. A new peak (V) eluted at retention time (RT) 22 minutes, which corresponds to the parent compound cafestol (Figure 5B). This was confirmed by conjugation reactions (data not shown) in which uridine 5- diphosphoglucoronic acid (UDPGA) and microsomes were added to radioactive cafestol. From the present study no direct structural information regarding the identity of the compounds can be derived. It is clear that the most abundant cafestol metabolite in bile is cafestol glucuronide conjugate (41%). The identity of the other three metabolites, representing the majority of the radioactive cafestol metabolites in bile (RT 3.5 minutes, 7%; RT 10 minutes, 32%, RT 13 minutes, 20%), was not further elucidated.

Analyses of cafestol and metabolites in portal blood

Very rapidly after duodenal administration three radio-active compounds appeared in portal blood. Figure 6 shows their relative abundance in time. At all time points, the most abundant radioactive compound in portal blood was found to be the parent cafestol (50% of total radioactivity). In addition, intestinal biotransformation led to two major metabolites. One of the two has the same retention time as the glucuronide found in bile suggesting that it is the glucuronide. At 10 min post dose 70% of parent compound is present in portal blood. After this the concentration of parent compound decreases (to 41%) whereas at 50 min post dose, the concentration parent compound in portal blood is increased up to 70% again. Taken together, absorption of parent compound and metabolites to the portal vein is prolonged, and apparently follows a cyclic pattern (figure 6). In the sample taken from the systemic circulation at t=50 min no radioactivity was detected.

Discussion

Results from both distribution studies indicate that cafestol and/or its metabolites strongly accumulate in the liver and gastro-intestinal tract. After oral administration, hardly any distribution to other parts of the body was found. This distribution pattern remains fairly constant for at least 24hours after administration. Both after i.v. and oral administration no radioactive fraction corresponding to the parent compound was found in bile. This confirms our earlier results using LC-MS, where we showed that cafestol is extensively metabolized by the liver to epoxy-glutathione conjugates, glutathione conjugates and glucuronide conjugates (van Cruchten, de Haan et al. 2009). These metabolites are subsequently excreted into the bile. Although the radioactive HLPC analyses in the present study do not provide direct structural confirmation, the conjugation-deconjugation experiment confirms our previous observations that one of the major metabolites in bile is a glucuronide. The same glucuronide is also found in portal blood (already 2min post dose), which indicates that glucuronidation also takes place in the intestinal epithelium. However, parent cafestol is also rapidly absorbed into the portal vein. Two minutes after dosing, the parent compound represented 50% of the total radioactivity present in portal blood. Remarkably, cafestol absorption continued during the next 50 minutes, still representing 70 % of the activity present in portal blood at 50min post administration. The presence of a glucuronide in bile found to be easily deconjugated by a bacterial enzyme, together with the prolonged absorption of parent compound from the GI tract suggests that cafestol undergoes enterohepatic cycling. It should be mentioned that the cafestol dose used in the oral studies, 1.5 mg per mouse, is rather high compared to the amount present in coffee. Depending on the brewing, coffees may contain up to 3.5 mg per cup of 100 ml (Ranheim and Halvorsen, 2005). If cafestol kinetics would be non-linear in mice, a relatively high concentration in the intestinal lumen might partly explain the prolonged

absorption and accumulation in intestinal tissue. Cafestol is the most potent cholesterol-elevating compound by weight present in the human diet (Urgert and Katan 1997). Several studies have shown that cafestol produces a clinical significant rise of serum cholesterol, which is a relevant risk for the development of cardio vascular diseases. Epidemiological studies in Scandinavians have shown that drinking large amounts of cafestol-rich coffee is indeed associated with an increased risk for coronary heart disease (Stensvold and Tverdal 1995; Urgert, Schulz et al. 1995; Stensvold, Tverdal et al. 1996; Urgert and Katan 1996). High intakes have also shown to increase serum levels of ALT and AST indicating liver damage (Urgert, Essed et al. 1997; Urgert and Katan 1997). In a previous study we proposed that epoxidation of the furan ring plays a role in these hepatotoxic effects of cafestol (van Cruchten, de Haan et al. 2009). We also showed *in vitro* induction by cafestol of an electrophile-responsive element (EpRE) derived from the human NQO1 regulatory region (van Cruchten, de Haan et al. 2009). Further studies are needed to elucidate in which form accumulation in the liver occurs. In combination with our earlier observations, it could be speculated that the accumulation of radioactivity is related to binding of reactive intermediates to cellular macromolecules. Data from the present study show that the liver is exposed to significant amounts of cafestol which can lead to the formation epoxides. This has been described for several other furan containing molecules, including furan, menthofuran, ipomeanine, 4-ipomeanol, furosemide and teucrin A (Khojasteh-Bakht, Chen et al. 1999; Alvarez-Diez and Zheng 2004; Baer, Rettie et al. 2005; Peterson, Cummings et al. 2005; Chen, DeRose et al. 2006). For some of these compounds, including the natural toxin ipomeanol, formation of furan-epoxides has indeed been associated with cellular toxicity. To the best of our knowledge, no evidence for the presence of either cafestol or its metabolites in human plasma is available. In the present study, we also found no cafestol in the peripheral circulation of mice that orally received

cafestol. The only human data on cafestol pharmacokinetics and metabolite formation in more detail have been published by De Roos et al (De Roos, Meyboom et al. 1998). These colleagues investigated cafestol disposition in healthy ileostomy volunteers. From the recovery of cafestol metabolites in the ileostomy effluent it was estimated that approximately 70% was absorbed from the GI tract. As only about 1% of the dose was recovered in urine, it was concluded from that study that cafestol is subject to extensive metabolism in the human body. These observations in humans are in line with those of the present study but our new data put the original conclusion in a slightly different perspective. Indeed, cafestol seems to be absorbed very rapidly and its passage to the systemic circulation is near to zero. However, in addition to direct biotransformation, accumulation in the liver and enterohepatic cycling play important roles in the kinetics of the compound. The causal relations between epoxide formation, liver accumulation and cholesterol elevation remain to be elucidated. The effect of cafestol on blood lipids in humans is unusually slow. It takes at least 4 weeks to reach new steady-state levels of blood lipids. Our data do not suggest that this is due to a slow accumulation of cafestol in some body pool, because cafestol does not seem to penetrate beyond the enterohepatic axis and is cleared fairly rapidly. Secondary changes in liver metabolism induced by cafestol may explain its unusually protracted effect on blood lipids.

In conclusion, data from the present study provide evidence that cafestol is efficiently absorbed and partially metabolized by the gut, cleared and further metabolized by the liver and excreted into bile. Because the main metabolite is a glucuronide and because the parent compound appears to be present in portal blood, we suggest that cafestol is likely to undergo enterohepatic circulation.

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Footnotes

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Competing interests: The authors have declared that no competing interests exist.

Figure 1: Structure of cafestol. Labeling was on the C1 and C2 positions. Before each use, radiochemical purity of ^{3}H Cafestol was verified (99%).

Figure 2: Organ distribution of cafestol in mice. Data show the levels of 3H cafestol(metabolites) 5 hours after oral administration to mice, expressed as percentage of administrated dose corrected for organ weight. Trace amounts of cafestol were detected in total blood volume (0.001%). No cafestol could be detected in; esophagus, pancreas, spleen, adrenals, brain, heart, lung, white adipose tissue and muscle. Values are expressed in averages ± SEM.

Figure 3: Quantitative whole body autoradiography (QWBA) of cafestol fed C56BL6/j mice. Thirty minutes post dose, almost all radioactivity is found in liver and small intestine. Trace amounts were found in systemic blood and kidneys. Apart from liver and intestinal tract, no radioactivity was detected in all other sections that were analyzed. At 48hours post dose, no radioactivity was detected anymore.

Figure 4: Cafestol metabolites are transported via bile in mice. This graph shows the ^{3}H cafestol (metabolites) present in bile at the different time points. Bile samples were taken at different time points and relative amounts ^{3}H cafestol were determined. Each value is the mean of 5 mice and variation is the SEM. Cafestol percentages are plotted cumulative.

Figure 5: Metabolite at retention time 18minutes is a cafestol glucuronide conjugate. In figure A is a bile sample of a mouse treated with cafestol at time point 5minutes. (y-as; DPM present in bile fraction); x-as; retention time (minutes). Figure 5b is the same bile sample but then deconjugated with β-glucuronidase.

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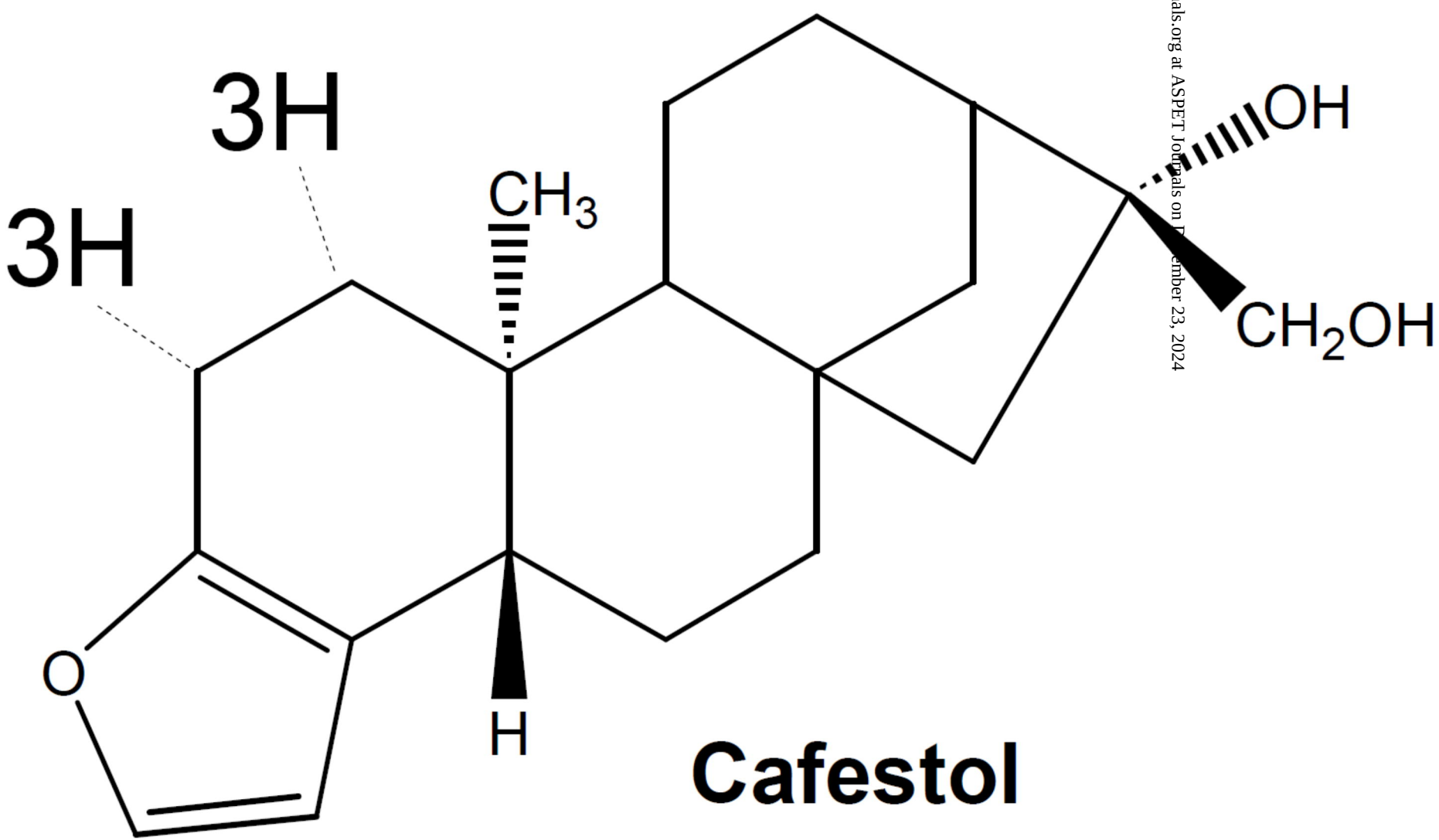
Figure 6: Cafestol and metabolites in portal blood. Data shows that after duodenal administration cafestol is absorbed and transported by portal vein. No cafestol is present in systemic blood. Most abundant in portal blood is the parent compound (±70%), however cafestol is also partly metabolized by intestine, resulting in two metabolites of which one is a glucuronide. (Black box, parent compound; Striped box peak 1, Grey box, cafestol glucuronide). Values are expressed in averages ± SEM.

Figure 1



Cafestol

Figure 2

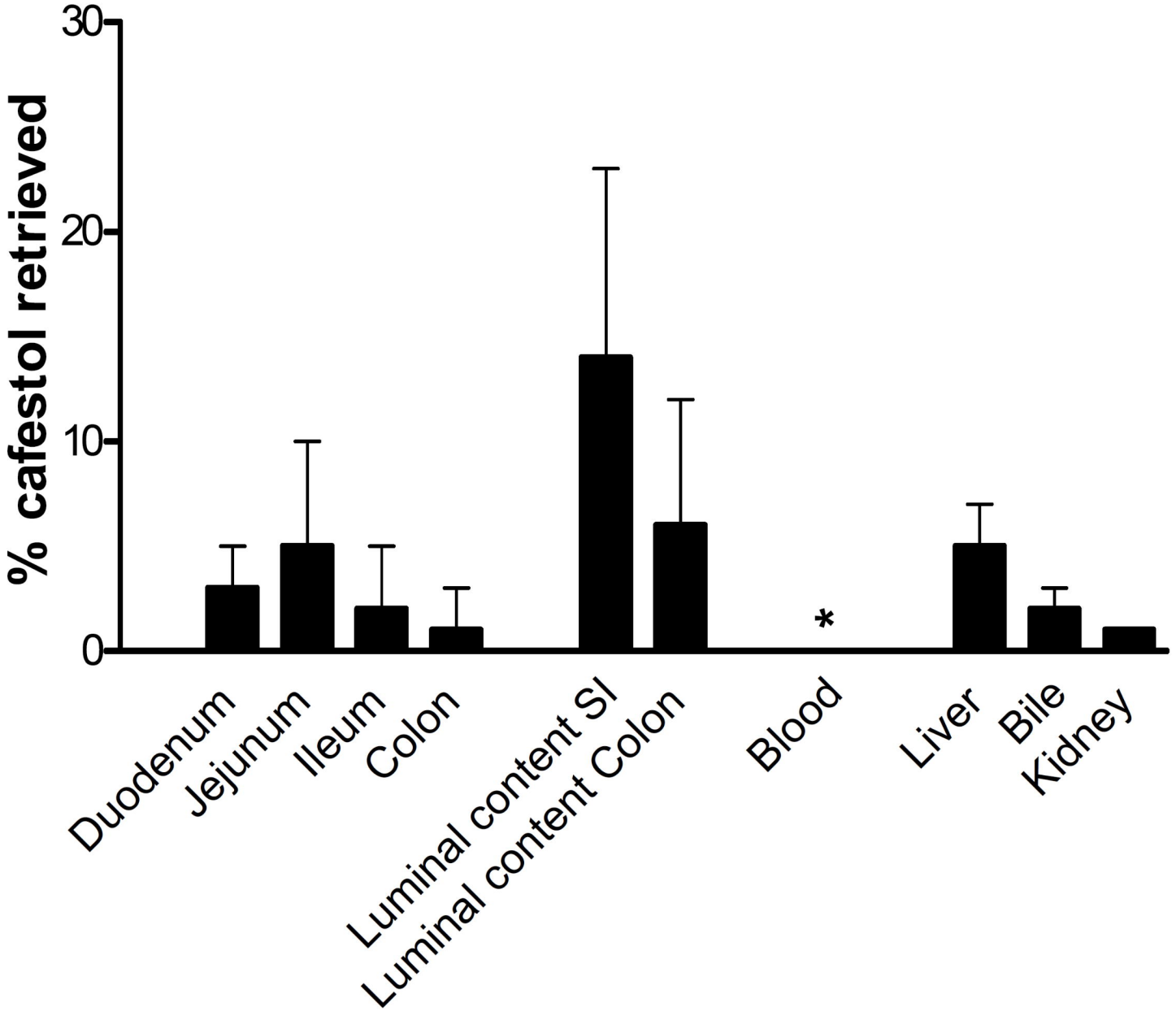

30
20
10
0
% cafestol retrieved
*
Duodenum
Jejunum
Ileum
Colon
Luminal content SI
Luminal content Colon
Blood
Liver
Bile
Kidney

Figure 3

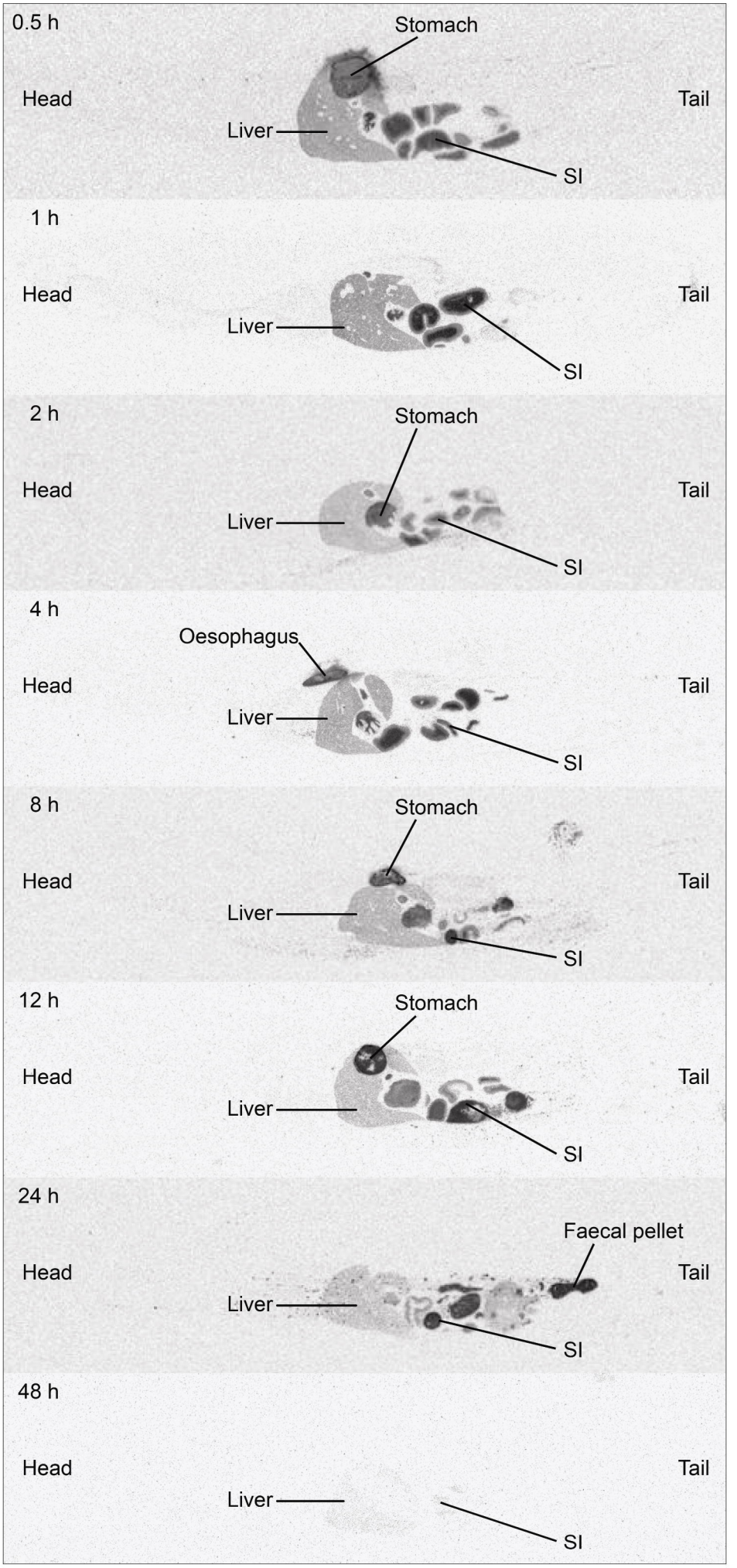

0.5 h
Stomach
Head
Tail
Liver
SI
1 h
Head
Tail
Liver
SI
2 h
Stomach
Head
Tail
Liver
SI
4 h
Oesophagus
Head
Tail
Liver
SI
8 h
Stomach
Head
Tail
Liver
SI
12 h
Stomach
Head
Tail
Liver
SI
24 h
Faecal pellet
Head
Tail
Liver
SI
48 h
Head
Tail
Liver
SI

Figure 4

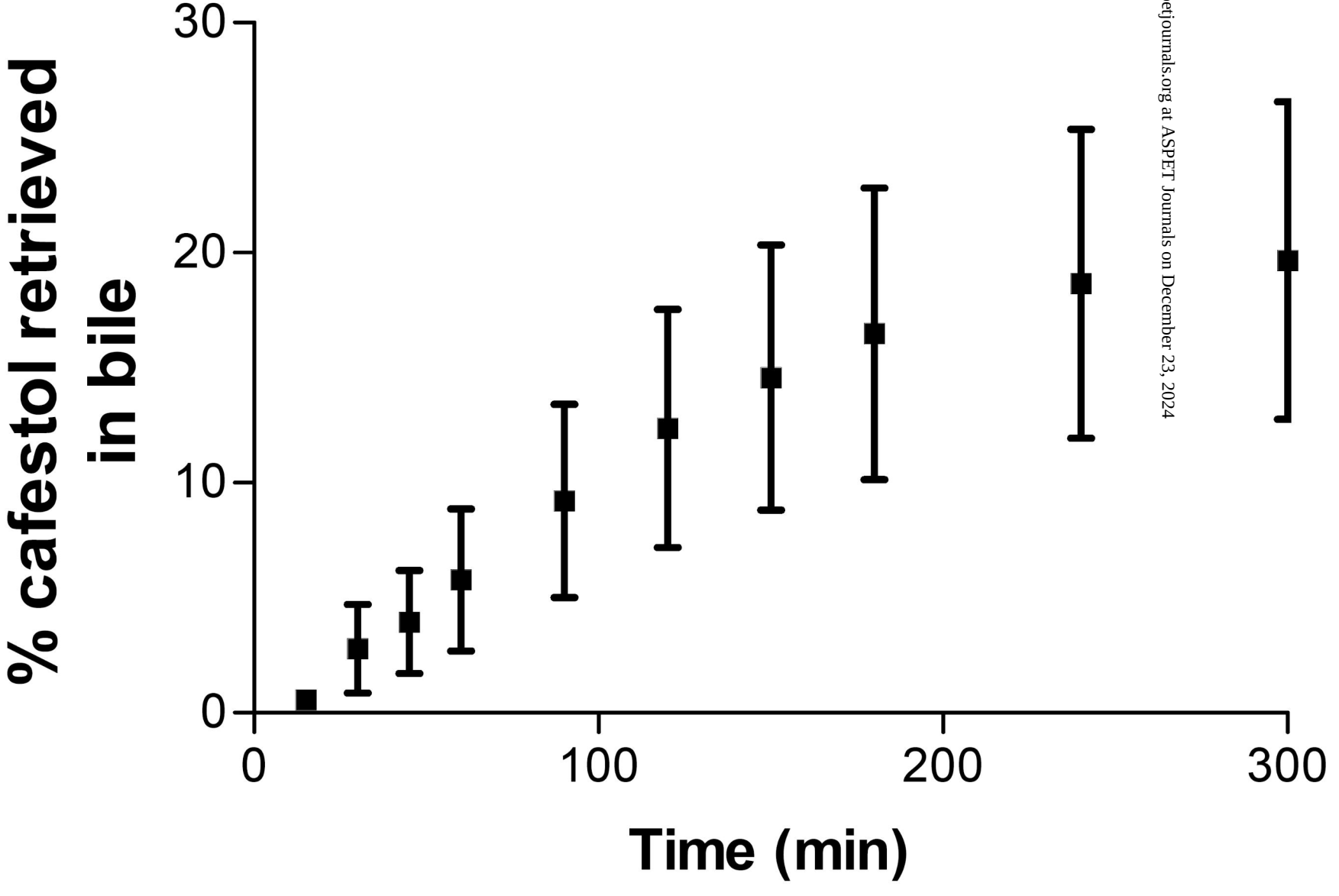


Figure 5

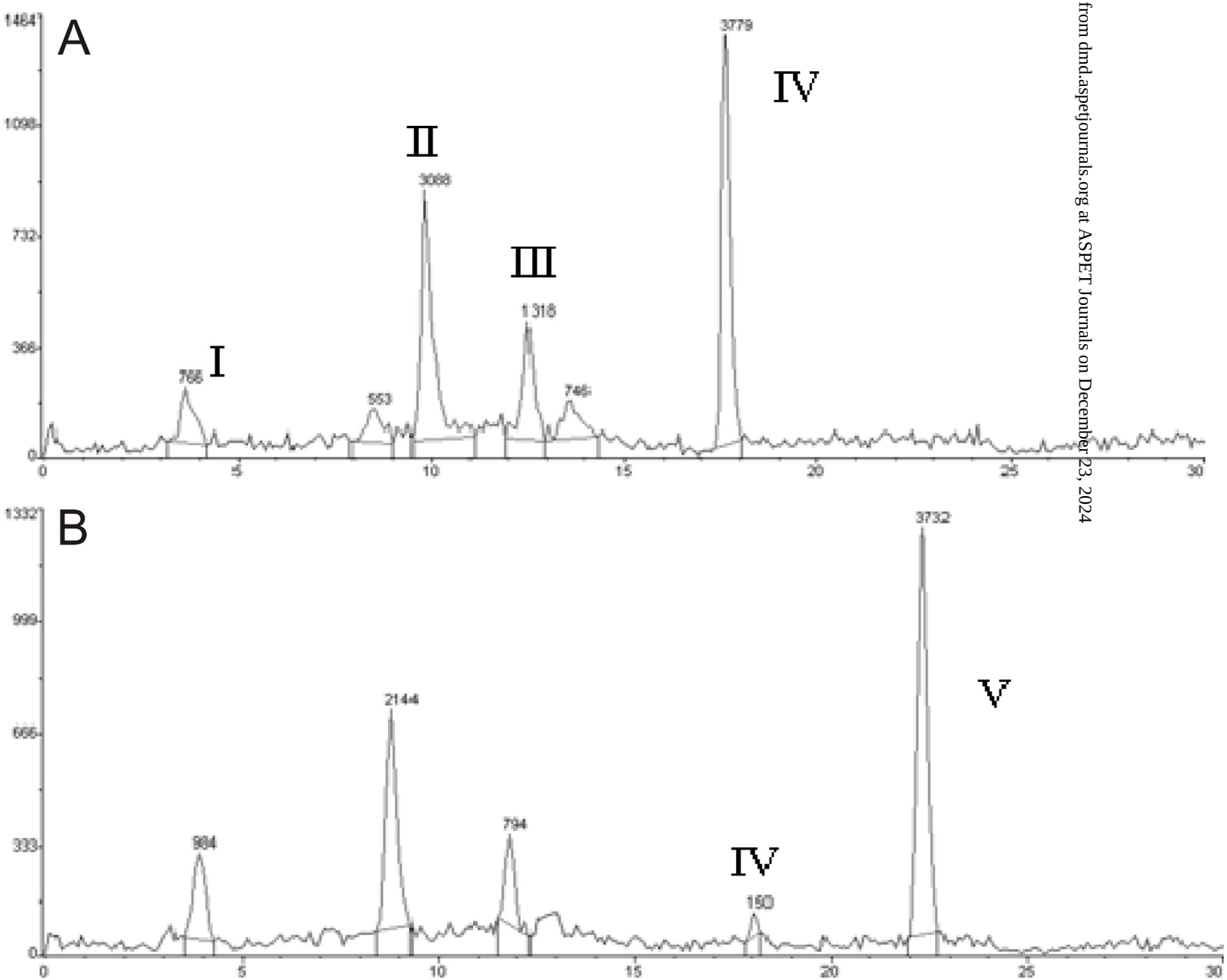


Figure 6