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Succinctus

Vasoconstriction induced by salivary gland extracts from ixodid ticks

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ABSTRACT

In their quest for blood, most haematophagous parasites secrete vasodilators in their saliva to counter the host haemostatic response of vasoconstriction. Surprisingly, salivary gland extracts from adult female *Dermacentor reticulatus* and *Rhipicephalus appendiculatus* ticks induced constriction in a rat femoral artery model; males induced vasoconstriction or vasodilation depending on the time of feeding. Based on comparative HPLC fractionation, the active compounds inducing vasoconstriction do not appear to be prostaglandins (which ticks normally use as vasodilators). Vasoconstriction may be unique to ixodid ticks, helping them control blood flow during their prolonged blood-feeding of up to 10 days or more.

Keywords: Tick, Salivary glands, Vasoactivity, Vasoconstriction, Vasodilation, Rat femoral artery

Haematophagy is a complex task compounded by the presence of haemostatic mechanisms used by the host to prevent blood loss (i.e. platelet aggregation, vasoconstriction and blood clotting). Haematophagous parasites respond to host haemostasis by secreting from their salivary glands a cocktail of anticoagulant, antiplatelet and vasodilatory compounds. The critical role of vasodilation in haematophagy is illustrated by the universality of vasodilatory molecules produced by blood-feeders. For example, ticks use prostaglandins (PGE_2, $PGF2_\alpha$, PGI_2) (Bowman et al., 1996), triatomes produce a nitric oxide binding protein (Champagne et al., 1995), tabanids produce vasotab peptide similar to Kazal-type protease inhibitors (Takáč et al., 2006), sand flies secrete maxadilan peptide that mimics pituitary adenylate cyclase activating peptide (PACAP) activity (Lerner et al., 2007), leeches use histamine (Hildebrandt and Lemke, 2011), and vampire bat submaxillary glands express PACAP and other potential vasodilators (Francischetti et al., 2013).

As part of our research to identify compounds responsible for anti-haemostatic activities of tick saliva, we investigated the vasoactive effects of salivary gland extracts (SGE) from two ixodid tick species, *Dermacentor reticulatus* and *Rhipicephalus appendiculatus*. Both species are important disease vectors: *D. reticulatus* transmits pathogenic bacteria (e.g. *Rickettsia slovaca, Francisella tularensis*) and protozoa (e.g. *Babesia canis*) (Salman and Tarrés-Call, 2013) and *R. appendiculatus* is the vector of *Theileria parva*, the cause of the devastating East Coast fever in cattle (Nuttall and Hindle, 1913). Surprisingly, we found most vasoactivity of the SGE was expressed as constriction rather than relaxation in a bioassay using pre-constricted rat femoral arteries.

To prepare SGE), adult ticks from laboratory colonies were fed in groups within retaining chambers glued to the shaved backs of guinea pigs (Trik strain, weight 250 – 300 g). To examine the dynamics of vasoactivity, SGE was prepared from unfed ticks and ticks fed on guinea pigs for 2, 6 or 9 days. Adult female *R. appendiculatus* take approximately 9 days to engorge, involving a 'slow phase' and a 'rapid phase'. At 2 days, the ticks are in the slow phase, preparing their cuticle for expansion to accommodate the extraordinary bloodmeal (≥200 × their unfed body weight); at

day 6 they transition to the rapid phase; and at day 9 the ticks are completing the rapid phase of feeding when 50% of their bloodmeal is consumed. Meanwhile, adult males feed intermittently and mate with feeding females on the host. Salivary glands were dissected under chilled 150 mM PBS, pH 7.2, pooled in Eppendorf tubes and immediately frozen at –70 °C. Prior to the assays, batches of salivary glands were quickly thawed, subjected to 80 °C for 5 min, homogenised, and the supernatants of the SGE preparations were pooled. The soluble protein concentration in the SGE was determined using the Bradford assay adapted to microplates with BSA as a standard. Vasoactivity of the SGE preparations was measured by myography using rat femoral artery preconstricted with phenylephrine (Takáč et al., 2006). Femoral arteries were obtained from laboratory rats (Wistar strain, males, 12 week old, weight 300 – 350 g). All laboratory animals were purchased from the Department of Toxicology and Dobrá Voda breeding station (Institute of Experimental Pharmacology, Slovak Academy of Sciences (SAS), Slovak Republic) and maintained according to guidelines for the care and use of laboratory animals (Act of the Government of the Slovak Republic 2003). All procedures and experimental protocols were approved by the Ethical Committee of the Institute of Normal and Pathological Physiology SAS, and conform to the European Convention on Animal Protection and Guidelines on Research Animal Use.

The arteries were cleaned of adherent connective tissue and cut into 1.5 mm ring segments. Two stainless steel wires were passed through the lumen of the artery segments and they were mounted on a myograph capable of measuring the isometric wall tension in a bath of Krebs-Ringer solution at 37 °C, pH 7.4, gassed with 95% O_2 and 5% CO_2. An initial tension of 13.3 kPa was applied and the preparation was allowed to rest for 30 min. After this period, 10 μM phenylephrine and 10 μM acetylcholine were added; if endothelium was present, acetylcholine induced relaxation of phenylephrine-induced constriction. After confirming the presence of endothelium, the segments were washed and then preconstricted by addition of phenylephrine. The plateau of the contractile response induced by 10 μM phenylephrine was taken as a measure of 100% contraction.

Vasodilatory and vasoconstrictive activities were expressed as the percentage decrease and increase, respectively, of phenylephrine-induced constriction. Differences in the vasoactivity of tick SGE derived from different feeding phases of individual species and sexes were evaluated by the Kruskal-Wallis test with post hoc paired comparisons; $P < 0.05$ was considered significant. Each experiment was repeated at least three times; a dose response was observed for most preparations.

SGE preparations from the two species showed similar vasoactive profiles (Fig. 1). All SGE preparations from adult females induced vasoconstriction whereas SGE from 6 day fed males induced vasodilation, while all other preparations caused constriction. In SGE derived from *R. appendiculatus* females, activity significantly increased with feeding, with the highest activity in 9 day fed ticks ($P < 0.05$). In contrast, the strongest vasoconstriction observed for female *D. reticulatus* was at day 6 of feeding ($P < 0.05$) (Fig. 1).

The different dynamics of SGE vasoactive activity between adult females and males observed for *D. reticulatus* and *R. appendiculatus* may well reflect their feeding and mating behaviour. Both species are members of the Rhipicephalinae subfamily, and show similar feeding and mating behaviour although their geographical distribution and hosts differ (Perry et al., 1991; Hillyard, 1996). They mate on the host, the male attaching and feeding for a short period before detaching, seeking a feeding female, mating and then reattaching adjacent to the mated female. Males secrete male-specific saliva proteins that help their female mate to feed, a form of molecular "mate guarding" (Wang et al., 1998). Possibly, the vasodilation observed with male SGE obtained at 6 days of feeding reflects the time when the male is reattaching to the host after mating and secreting mate-protecting saliva molecules. Vasoconstriction was also induced using SGE from unfed ticks of both species. However, 40 – 60 salivary glands were required to produce the 80 μg dose of unfed SGEs tested for activity whereas, for example, the same dose was achieved with only three salivary gland equivalents from female *R. appendiculatus* fed for 9 days.

Typically, ixodid ticks induce vasodilation by secreting prostaglandins in their saliva. Ticks of different genera have been shown to contain prostaglandins PGE_2, $PGF_{2\alpha}$ and PGI_2 (Bowman et al.,

1996). All are potent skin vasodilators; $PGF_{2\alpha}$ is the most potent prostaglandin to induce vasodilation in cattle (Kemp et al., 1983). In addition, prostaglandins aid the ectoparasites by modulating the host inflammatory and immune responses (Sá-Nunes et al., 2007). Prostaglandins of the 2-series (i.e. PGE_2, PGD_2, $PGF_{2\alpha}$, PGI_2) are synthesized from the arachidonic acid precursor via the cyclooxygenase pathway (Holtzman, 1991). Ticks cannot synthesize fatty acids with more than one double bond and lack the ability to desaturate or elongate dietary fatty acids. Consequently, all fatty acids with more than one double bond, including arachidonic acid, must be sequestered by ticks from their blood meal. Prostaglandins are synthesised from arachidonic acid sequestered at comparatively high levels in the salivary glands (Bowman et al., 1996). Although prostaglandins cause vascular smooth muscle to relax and blood flow to increase, at high concentrations they can induce vasoconstriction (Somova and Bojkov, 1983). Moreover, PGE_2 itself is a weak contractile agent but exhibits pronounced synergism with other contractile agents (Hung et al., 2006). To date, prostaglandins are the only vasoactive compounds identified in ixodid tick saliva.

To determine whether the observed vasoactivity might result from prostaglandin-like molecules, molecular sieving Reversed Phase – HPLC (RP – HPLC) was performed on SGE prepared from glands of females and males of both tick species, fed for 6 – 7 days, using 200 salivary gland pairs per sample. Following heat treatment and clarification (as described above), SGEs were resuspended in 500 µl of 10% acetonitrile (ACN) with 0.1% trifluoroacetic acid (TFA) and UV was monitored at 210 nm and 220 nm with a Beckman 126/168 DAD HPLC system. Fractions were assayed for vasoactivity. The first purification was performed with a Vydac C4 column, 4.6 mm inner diameter (ID) × 250 mm, 5 µm particle size. The purification was performed at 10 – 100% ACN gradient with 0.1% TFA, flow rate 1 ml/min and 1% ACN/min concentration gain. The second purification was performed with a Beckman Ultrasphere C18 column, 4.6 mm ID × 250 mm, 5 µm particle size and a gradient of 10 – 40% ACN with 0.1% TFA, flow rate 1 ml/min with 0.5% ACN/min increment. The third purification was performed with a Vydac C18 column, 4.6 mm ID × 250 mm,5 µm particle size, under the same conditions as described in the second

purification. Collected fractions were concentrated in a vacuum evaporator (Speed-Vac, Savant Instruments, USA). The fourth purification of the active fraction was performed with a Tosoh Biosep, TSKgel Super ODS column, 2.0 mm ID ×10.0 cm, 2 µm particle size and a 20–40% ACN gradient with 0.1% TFA. A prostaglandin HPLC mixture containing 6-keto prostaglandin $PGF_{1\alpha}$, $PGF_{2\alpha}$, PGE_2, and PGE_1 standards (Cayman Chemical Company, MI, USA), and a PGB_1 standard (Sigma-Aldrich Chemie GmbH, Germany) were used for comparative analysis of the elution spectra. These standards were analysed with a Tosoh Biosep, TSKgel Super ODS column, using the same RP-HPLC conditions as for the Step 4 (fourth purification as described above) analysis of the active SGE fraction.

HPLC fractions derived from SGE of both female and male *D. reticulatus* and *R. appendiculatus* fed for 6 – 7 days were tested for vasoactivity. Unlike the vasoactivity assays, there was considerable variation in the activity profiles of fractionated HPLC samples, both between species and between conspecific females and males. Furthermore, all the HPLC fractions obtained from male SGE showed vasoconstriction and none showed the vasodilation observed with unfractionated male SGE of both species fed for 6 days. The most potent fractions were obtained from SGE of female *D. reticulatus,* with a single peak at Step 1 of fractionation showing 785% constriction. This fraction was taken through to Step 4 when a peak at retention time 2.3 min still induced a relatively high level of constriction (130%). This peak showed maximum UV absorbance at 206 and 256 nm (Fig. 2). Comparison of the retention time and absorbance profile of the purified SGE fraction with those obtained with prostaglandin standards subjected to the same RP-HPLC conditions, revealed no similarities (Figs. 2, 3 respectively; Supplementary Fig. S1). Thus the relatively high vasoconstrictive activity of the purified fraction from 6 – 7 day fed female *D. reticulatus* SGE does not appear to be due to the presence of either $PGF_{1\alpha}$, $PGF_{2\alpha}$, PGE_2, PGE_1, or PGB_1. The apparent absence of prostaglandins in the fractions may have been the result of the method of preparing and processing SGE, and does not exclude the presence of prostaglandins in the salivary glands. Although prostaglandins normally have an antihypertensive action,

prostaglandin endoperoxide (PGH_2), thromboxane and isoprostanes can vasoconstrict blood vessels (Welch et al., 2007). However, none of these arachidonic acid derivatives has been reported in tick saliva. Thromboxane-scavenging saliva proteins have been reported from some argasid tick species, although their function is thought to be inhibition rather than induction of vasoconstriction (Mans and Ribeiro, 2008). Heat treatment of PGE_2, using the same conditions as those used in preparing the SGE samples, resulted in a 50% loss of vasoconstriction activity when tested in the rat femoral artery model. This contrasts with the relative heat stability of the purified female *D. reticulatus* vasoconstrictor. Heat treatment at 80 °C for 5 min of crude SGE followed by clarification is commonly used to inhibit protease activity and to remove proteins >20 kD. This procedure was used in the isolation of the 6.1 kD vasotab peptide from horse fly (*Hybomitra bimaculata*) SGE (Takáč et al., 2006). Overall, the results obtained for 6 – 7 day fed female *D. reticulatus* SGE are consistent with a small phenylalanine-rich peptide as the active ingredient in the purified fraction, however, further analysis using mass spectrometry is required to identify this vasoconstrictor.

The RP-HPLC results for *D. reticulatus* female and male SGE differed considerably, as was the case for *R. appendiculatus*, suggesting the presence of different vasoactive ingredients in females and males (Fig. 2, Supplementary Figs. S2 – S4). Sexual dimorphism at the level of bioactive salivary gland products has been reported previously for ixodid ticks (Wang et al., 1998). The comparatively low level of vasoactivity observed with fractions of 6 – 7 day fed female *R. appendiculatus* SGE indicate that peak vasoconstrictive activity is at around 9 days of feeding compared with 6 – 7 days for *D. reticulatus* (Fig. 1, Supplementary Figs. S3 – S4).

It appears counter intuitive that haematophagous parasites should induce vasoconstriction. However, ixodid ticks are exceptional in that a single blood meal takes days if not weeks to complete. The largest blood meal is acquired by adult females, where nutrients are converted into thousands of eggs and then they die, taking 2 weeks of continual attachment and feeding on a host to complete engorgement, and increasing their body weight ≥100-fold (Kaufman, 2007). Females acquire most of the bloodmeal (>50%) in the last 24 h of feeding (the rapid feeding phase),

197 spending much of the attachment period creating a feeding pool and preparing their exoskeleton for
198 enormous expansion. Hence vasoconstriction may be a means of regulating bloodflow and possibly
199 reducing inflammation during the prolonged feeding period of female ixodid ticks.
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Legends to figures

Fig. 1. Vasoactivity of salivary gland extracts (SGE) from unfed (80 µg only) and partially fed (2 day (2D), 6D, 9D) *Rhipicephalus appendiculatus* and *Dermacentor reticulatus* females and males. Rat femoral artery preconstricted with phenylephrine was treated with 20 µg, 40 µg or 80 µg of SGE. Values represent means ± S.E.M.

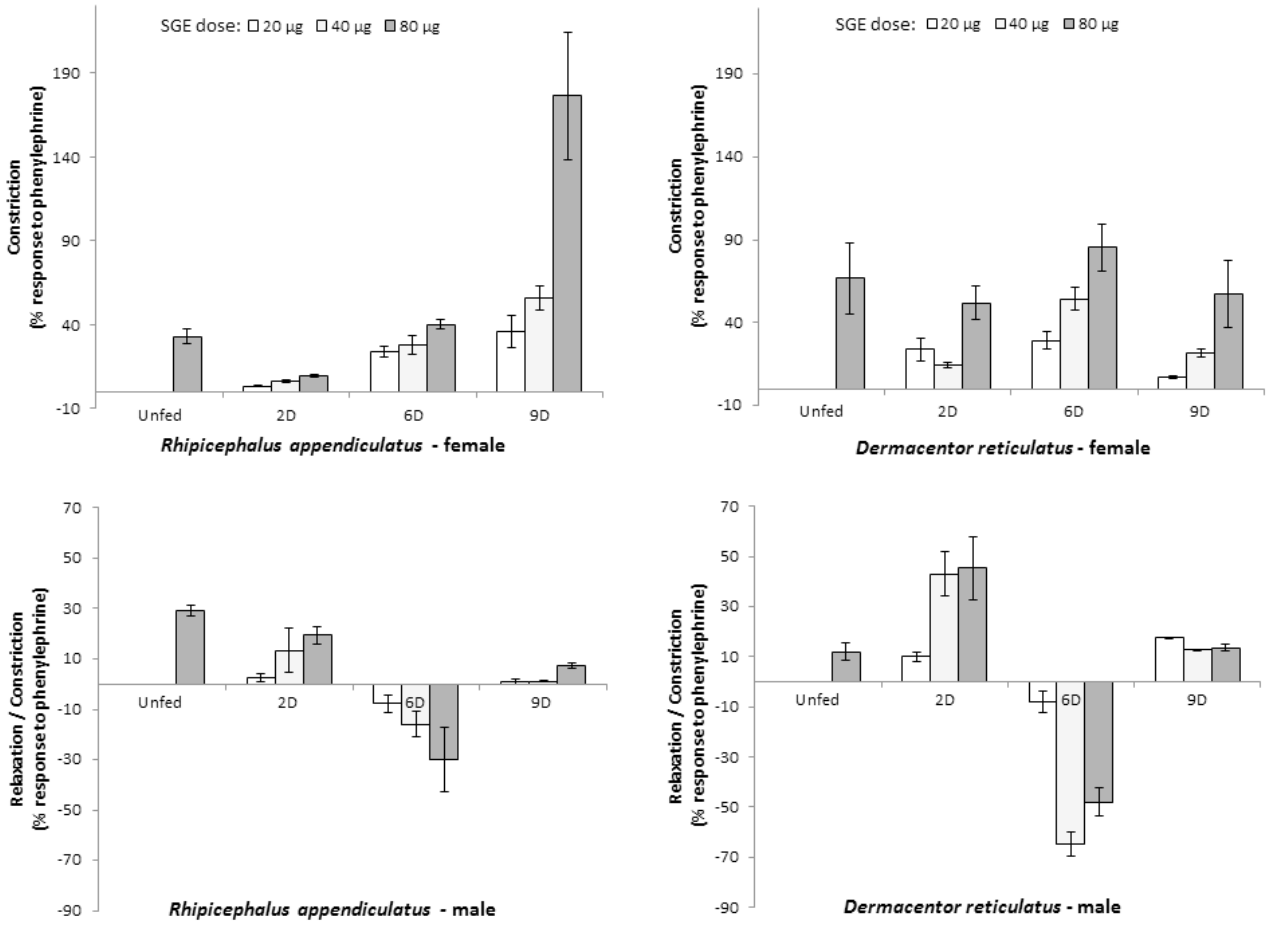
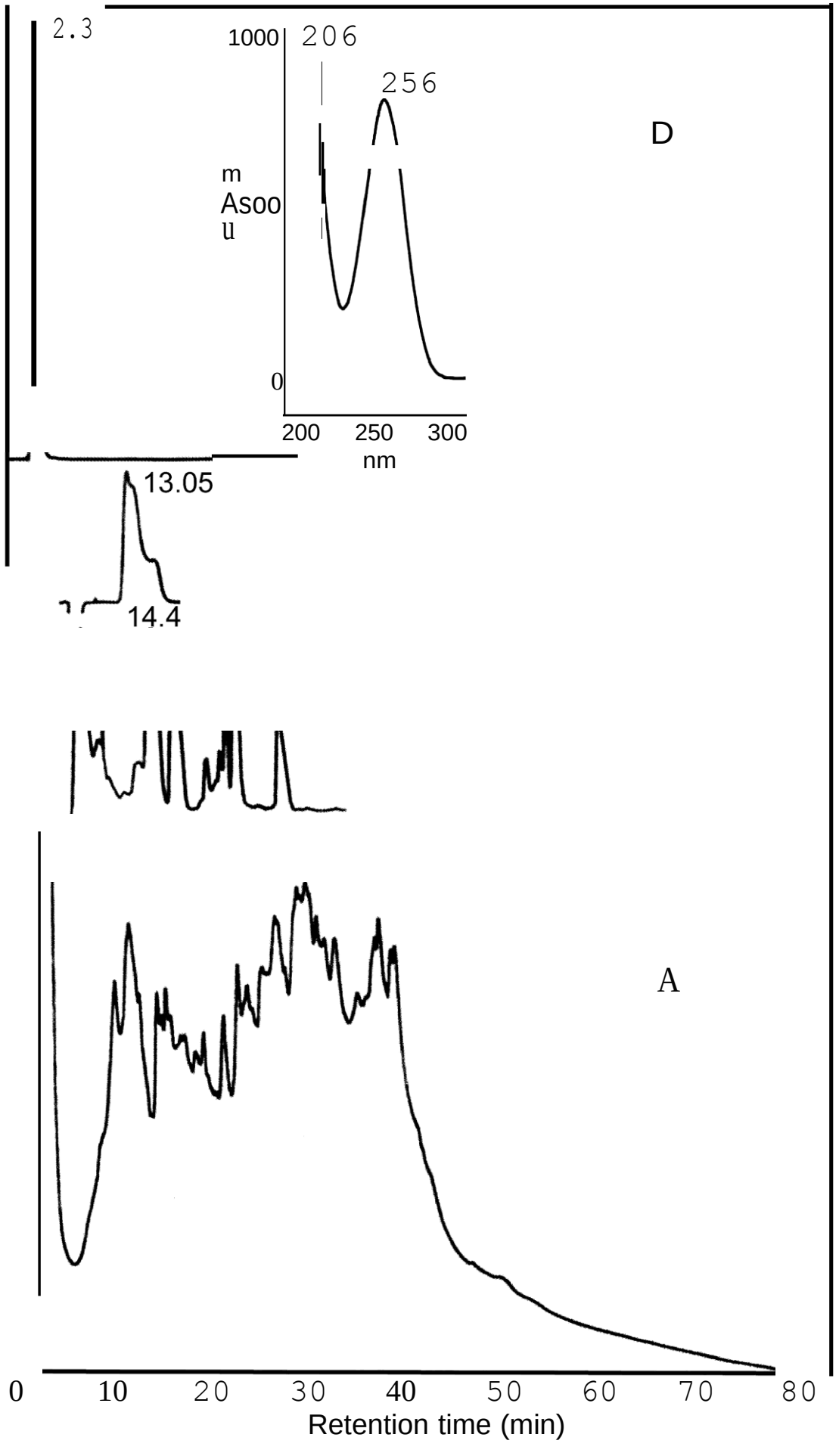


Fig. 2. Reversed Phase (RP)-HPLC chromatogram of salivary gland extracts from 6 – 7 day fed female *Dermacentor reticulatus*. The most potent fractions used for further purification are marked with retention times (min) on the graph. (A) First purification step active fraction at 3.5 min (785% constriction); (B) Second purification step using A-3.5 min gave active fraction at 14.4 min (273% constriction); (C) Third purification using B-14.4 min gave active fraction at 13.05 min (64% constriction); (D) Fourth purification step using C-13.05 min gave single active peak at 2.3 min (130% constriction). Inset: UV spectrum of D-2.3 min showing maximum absorbance (mAU, milli absorbance units) at wavelengths of 206 nm and 256 nm.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A
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10
20
30
40
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60
70
80
Retention time (min)

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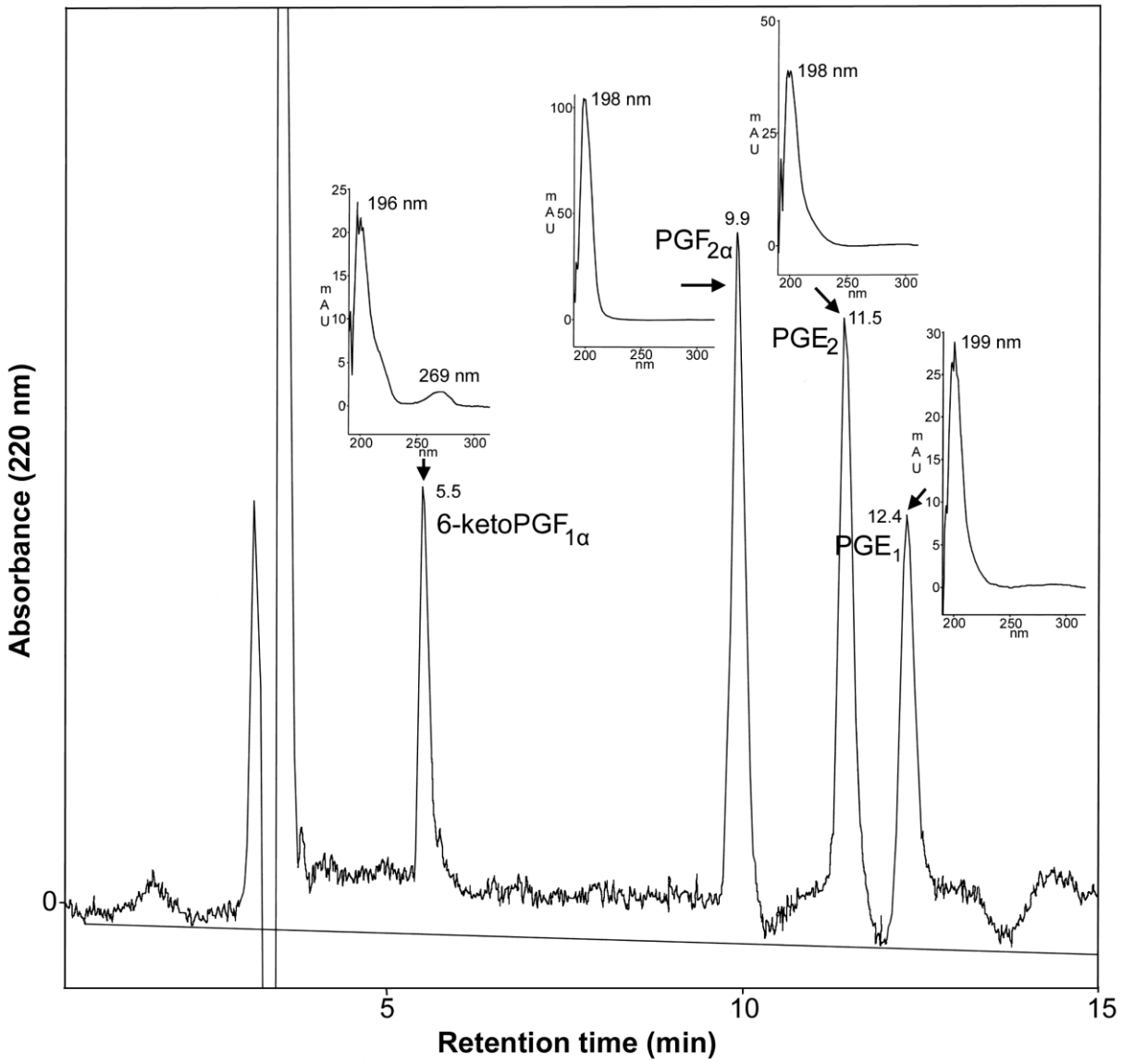
Fig. 3. Reversed Phase (RP)-HPLC chromatogram of HPLC prostaglandin mixture (6-keto $PGF_{1\alpha}$, $PGF_{2\alpha}$, PGE_2, PGE_1) showing UV spectra of prostaglandin standards at maximum absorbance (mAU, milli absorbance units) (insets) and acetonitrile gradient. The prostaglandin mixture was subjected to the same RP-HPLC conditions as used in the fourth purification step of salivary gland extracts shown in Fig. 2D.



281 # Supplementary data

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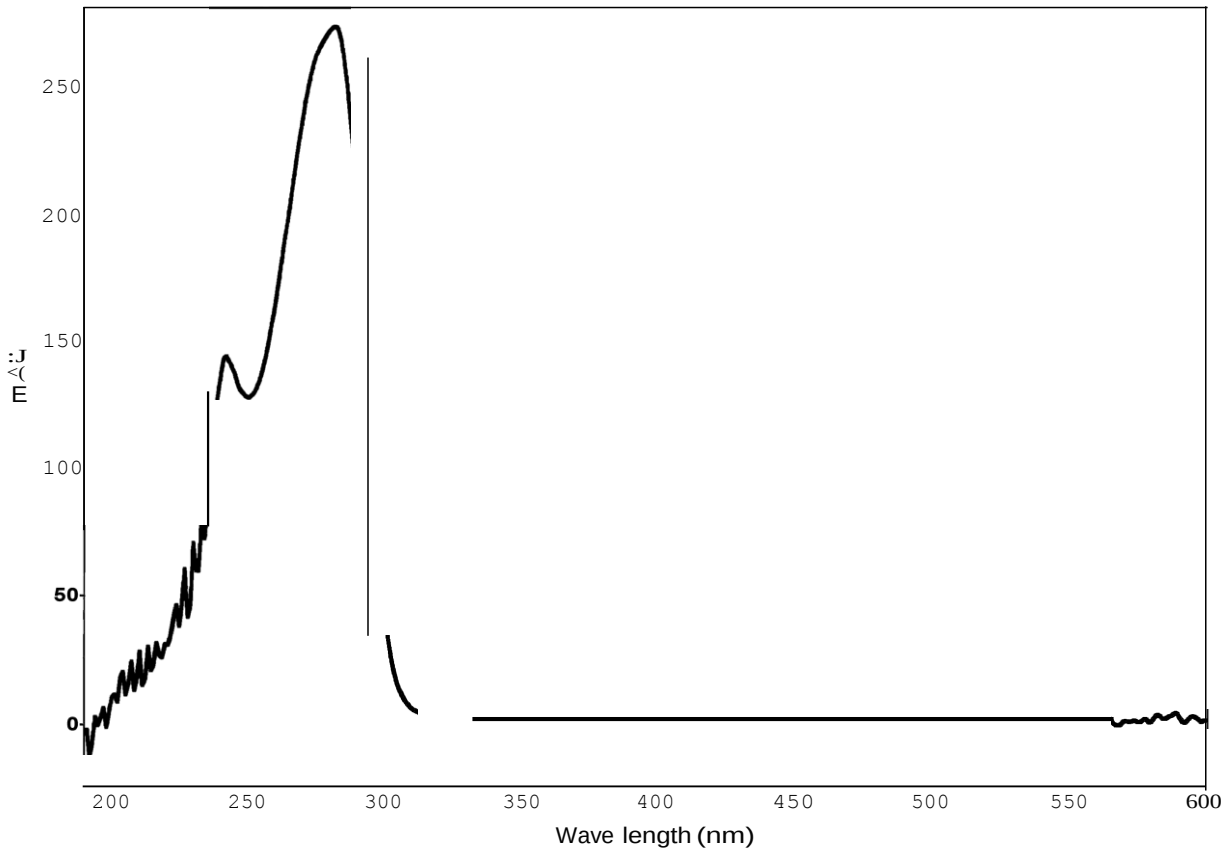
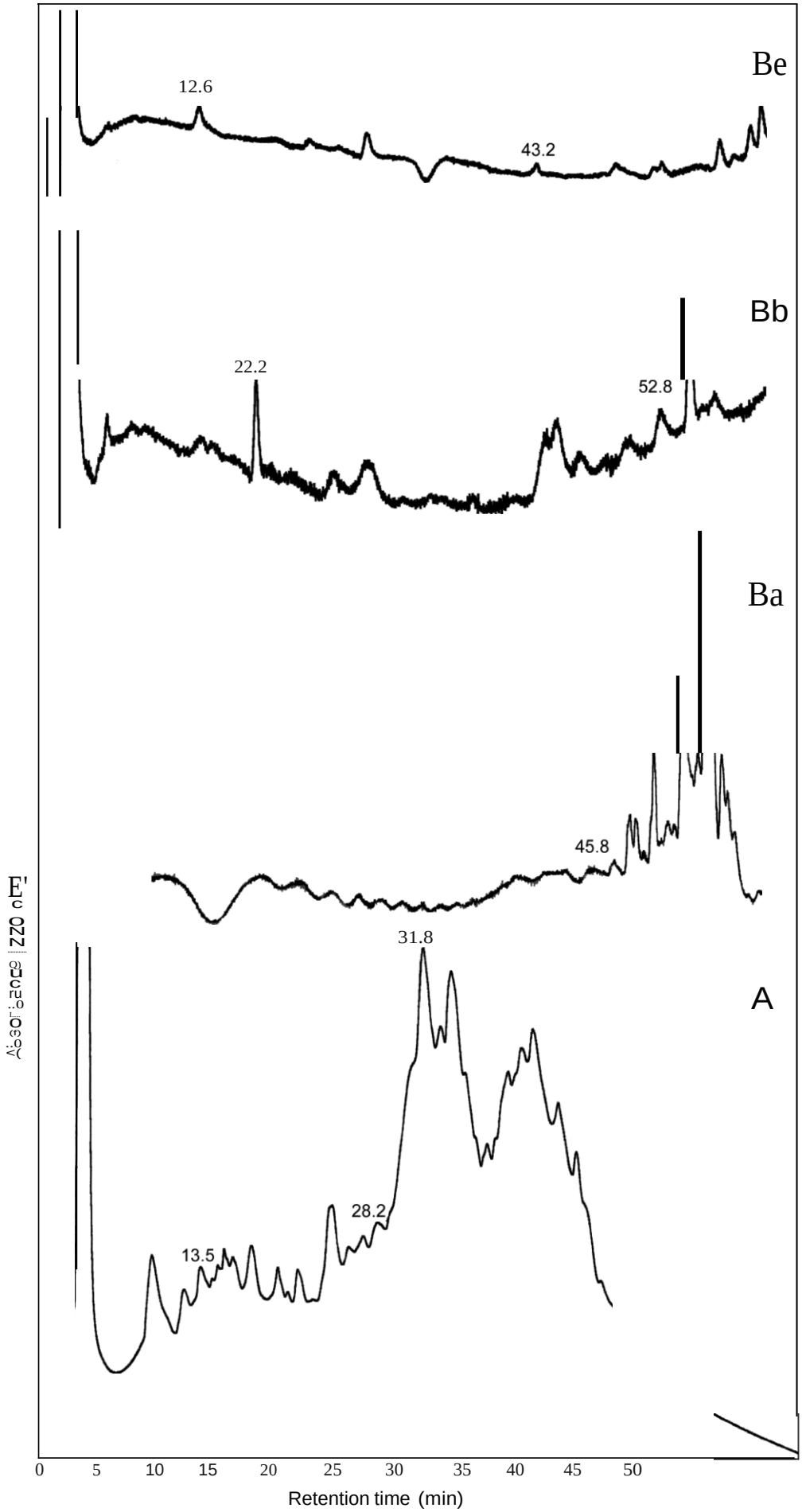
283 Supplementary Fig. St. UV spectrogram of prostaglandin PGB_1 standard (Sigma-Aldrich Chemie

284 GmbH, Germany) at maximum absorbance (mAU, milli absorbance units).



285

Supplementary Fig. S2. Reversed Phase (RP)-HPLC chromatogram of salivary gland extracts from 6 – 7 day fed male *Dermacentor reticulatus*. The most potent fractions used for further purification are marked with retention times (min). (A) First purification step active fractions at 13.5 min (152% constriction), 28.2 min (28% constriction) and 31.8 min (47% constriction). (Ba) Second purification step using A-13.5 min gave active fraction at 45.8 min (27% constriction). (Bb) Second purification step using A-28.2 min gave active fractions at 22.2 min (33% constriction) and 52.8 min (22% constriction). (Bc) Second purification step using A-31.8 min gave active fractions at 12.6 min (21% constriction) and 43.2 min (21% constriction).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Ba
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A
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20
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30
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45
50
Retention time (min)

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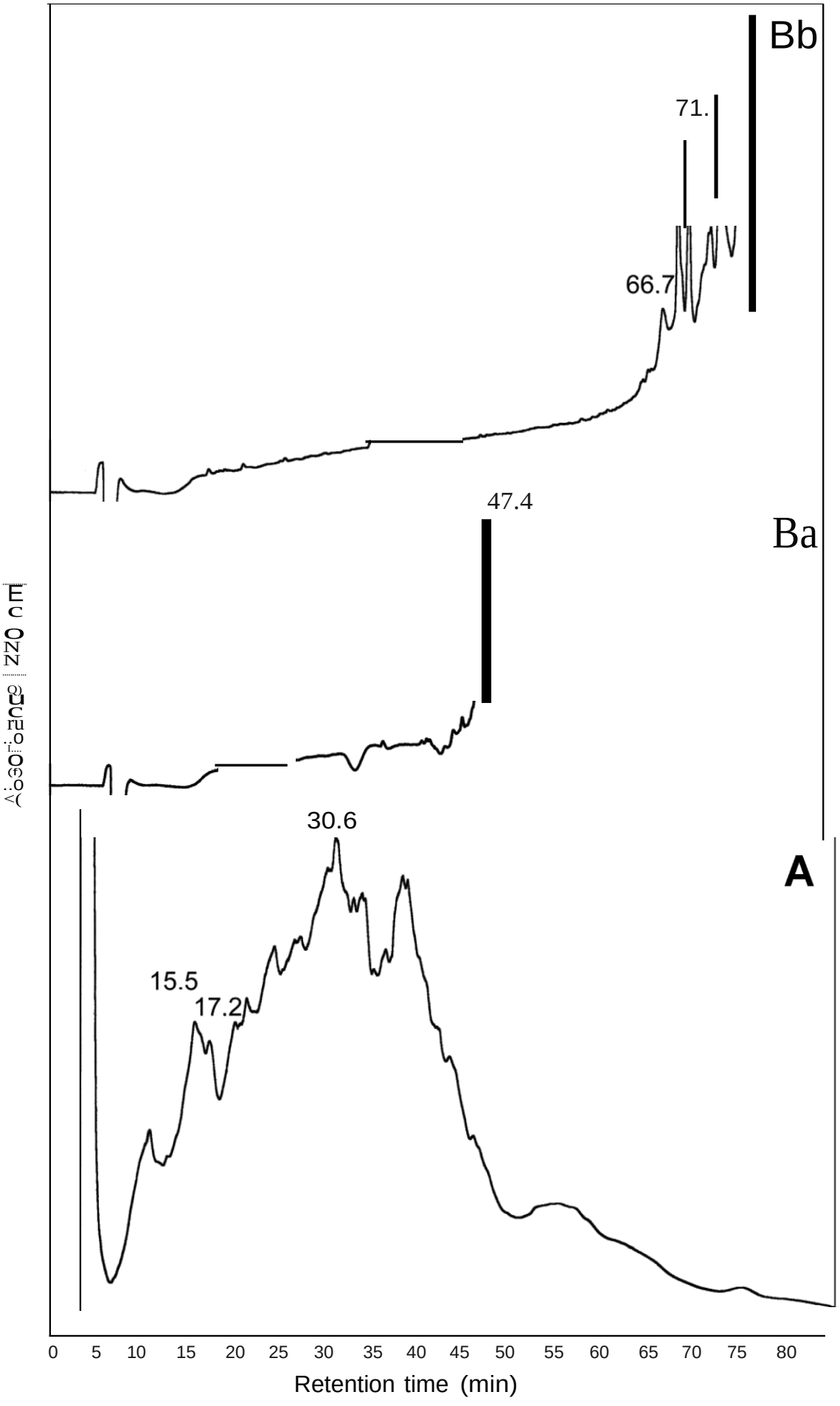
Supplementary Fig. S3. Reversed Phase (RP)-HPLC chromatogram of salivary gland extracts from 6 – 7 day fed female *Rhipicephalus appendiculatus*. The most potent fractions used for further purification are marked with retention times (min). (A) First purification step active fractions at 15.5 min (29% constriction), 17.2 min (32% constriction) and 30.6 min (15% constriction). (Ba) Second purification step using either A-15.5 min or A-17.2 min gave active fraction at 47.4 min (9% constriction). (Bb) Second purification step using A-30.6 min gave active fractions at 66.7 min (12% constriction) and 71.2 min (24% constriction).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15.5
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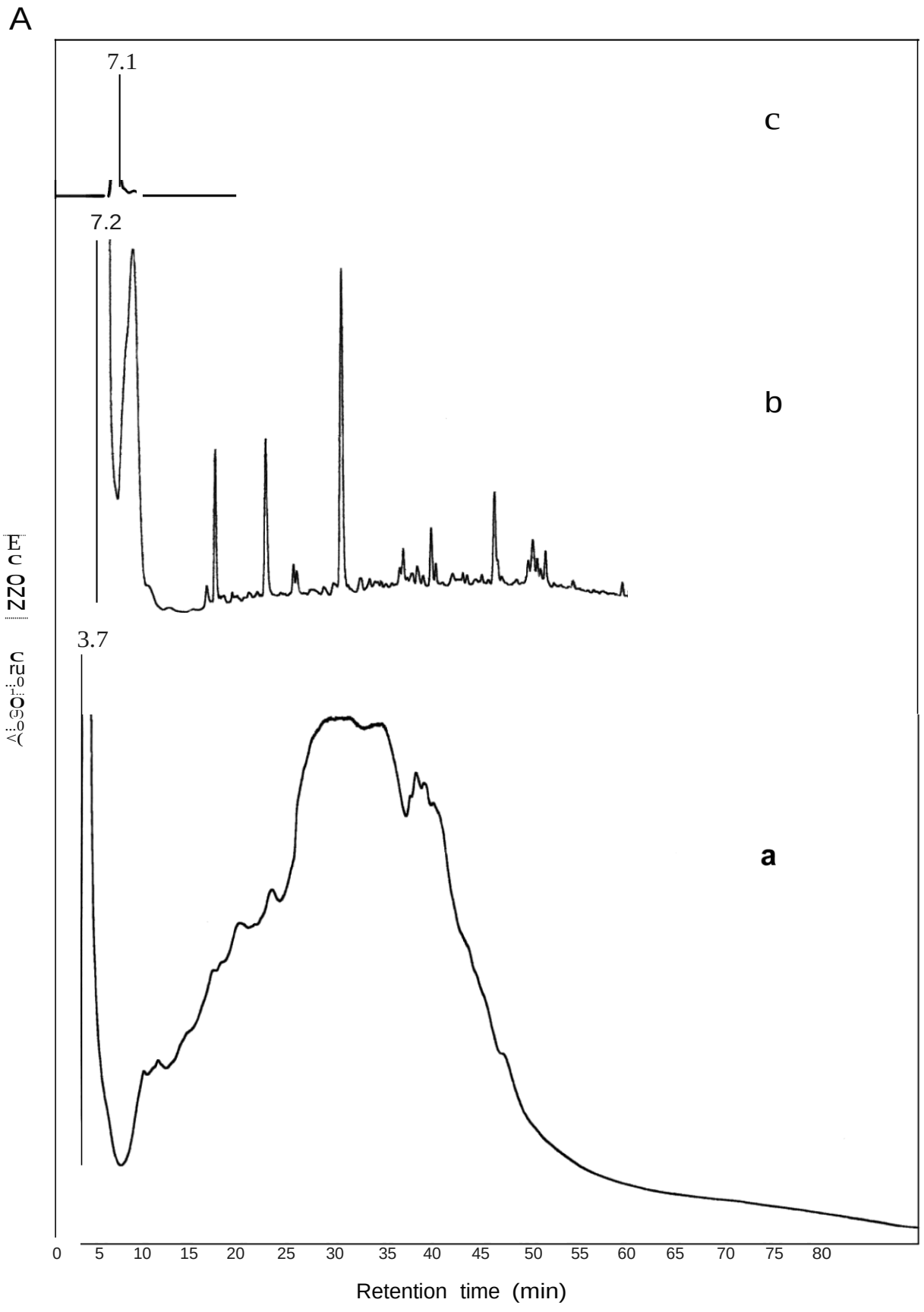
Retention time (min)

305

Supplementary Fig. S4. Reversed Phase (RP)-HPLC chromatogram of salivary gland extracts from 6–7 day-fed male *Rhipicephalus appendiculatus*. The most potent fractions used for further purification are marked with retention times (min). First purification step active fractions at (Aa) 3.4 min (14% constriction) and (Ba) 3.7 min (20% constriction). (Ab) Second purification step using Aa-3.4 min gave active fraction at 10.2 min (9% constriction). **(**Bb) Second purification step using Ba-3.7 min gave active fraction at 7.2 min (32% constriction). (Ac) Third purification step using Ab-10.2 min gave one active peak at 6.8 min (31% constriction). (Bc) Third purification step using Bb-7.2 min gave one major active peak at 7.1 min (40% constriction).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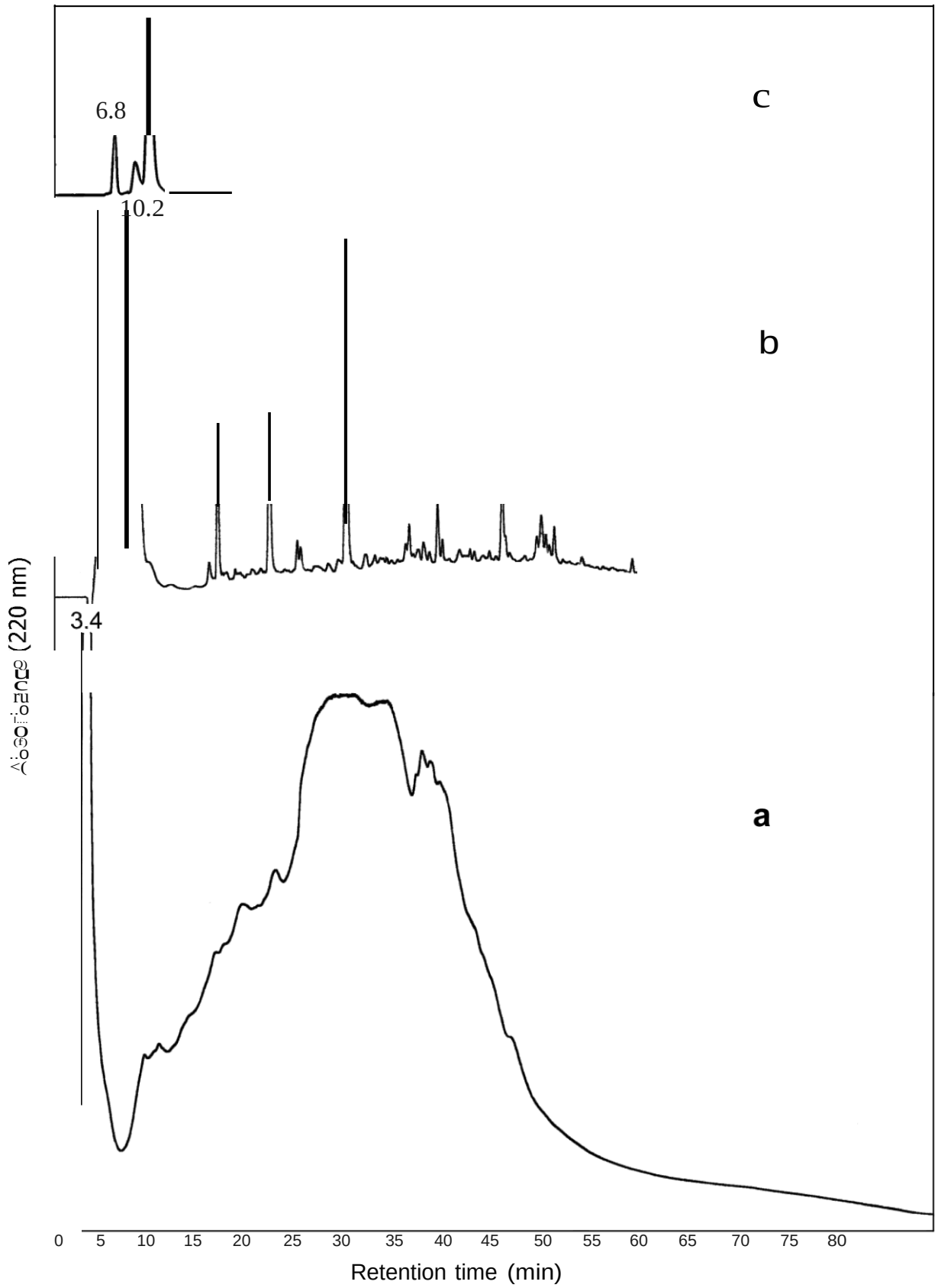
Retention time (min)

319

B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Retention time (min)

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