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Short communication

# Speedy milking of fresh venom from aculeate hymenopterans

Eduardo G.P. Fox [a, *], Meng Xu [b], Lei Wang [a], Li Chen [b], Yong-Yue Lu [a, **]

[a] Red Imported Fire Ant Research Centre, South China Agricultural University, Guangzhou, 510642, PR China
[b] Chinese Academy of Sciences, Institute of Zoology, State Key Laboratory of Integrated Management of Pest Insects and Rodents, Beijing, 100101, PR China

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ABSTRACT

A straightforward method for extracting aculeate arthropod venoms by centrifugation is described, based on adapting a glass insert containing a piece of metal mesh or glass wool into a centrifuge tube. Venom apparatuses are centrifuged for 30 s intervals at ≈2000–6000 g, with samples being dislodged between cycles. Venom from fire ants, honeybees, and a social wasp were extracted within minutes. The method is suited for small-scale bioassays and allows for faithful descriptions of unmodified toxin cocktails.



A main impediment to the advancement of research into the topic of arthropod toxins has been the difficulty associated with obtaining sufficient pure venom for robust analyses, particularly regarding bioassays (Benli and Yigit, 2008; Touchard et al., 2014; Fox, 2014). Obtaining venom from small arthropods typically involves manually dissecting or stimulating individual animals, which apart from being labor intensive also relies on specific equipment (for discussions see Fox, 2014, Aili et al., 2017). Moreover, mass extraction methods and even the dissection of venom sacs in solvents such as water or organic liquids will inevitably change/bias the biochemical composition of the obtained extracts (e.g. Chen and Fadamiro, 2009; Fox et al., 2013). There can also be considerable loss and cross contamination between tissues when dissecting venom apparatuses open and manually separating them from their secretions.

Herein we describe a straightforward method to obtain crude arthropod venom directly by conventional centrifugation. The trivial steps involve minimal structure that can be adapted to the local conditions and focal species. As per example fresh milligram-amounts of crude fire ant, wasp, and bee venoms were herein obtained from freshly-captured alive specimens within less than 30 min.

Materials needed: (1) a centrifuge capable of reaching at least 3000 g at room temperature (the one used was Eppendorf 5417R); (2) either glass wool or a fine metal screen (e.g. mesh size 60); (3) 2.0-mL polypropylene centrifuge tubes; (4) 250-μL glass inserts; (5) fine scissors; (6) fine tweezers; (7) living venomous arthropods.

The procedure starts by fitting either a clean square piece of metal mesh or glass wool into a glass insert as to form a basket-shaped stopper (find example pictures at Fox et al., 2018a,b). The glass insert is then inserted, ideally on some cushioning, inside the centrifuge tube. Freshly dissected living arthropod venom apparatuses are amassed in the insert basket in the tube. Finally the tubes are centrifuged at ≈2000–6000 g for 30–60 s at room temperature. The centrifuge should be opened and fine tweezers or some pin used to dislodge the venom-containing tissues inside the stopper basket. Centrifuge again. The procedure is repeated until no more venom collects at the bottom of the glass insert (5–8 cycles with our samples). Venom yield per individual was estimated from dividing the total net venom yield (in μg) as obtained from centrifuged sets of venom apparatuses collected into the glass inserts, based on the weight difference of the inserts prior to and after centrifugation, as obtained with a digital scales SARTORIUS BS 224S (max. precision 0.0001 g).

The following venomous hymenopterans were tested for this

* Corresponding author.
** Corresponding author.
*E-mail addresses:* ofoxofox@gmail.com (E.G.P. Fox), luyongyue@scau.edu.cn (Y.-Y. Lu).

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**Table 1**
Estimated amounts of crude venoms collected by centrifugation at 3000–5000 rpm of venom apparatuses of aculeate hymenopteran insects using an adapted stopper basket inside a glass tube insert.

| Venom Sampled Arhropod | Estimated venom (μg) obtained per individual | Replications |
|---|---|---|
| Fire ant gyne | 70–100 | 5 |
| Fire ant major worker | 75–110 | 5 |
| Fire ant minor worker | 10–35 | 3 |
| Asian hornet | ca. 450 | 3 |
| European Honeybee | 110–160 | 5 |
| Asian Honeybee | 120–200 | 3 |

protocol:

Red imported fire ants, *Solenopsis invicta* Buren. Fire ants were anesthesized with $CO_2$ to prevent them from dispensing venom during manipulation. Major workers (i.e. >4 mm long) were selected, from which 10 venom glands along with reservoirs were dissected dry, by pulling out and then separating from the stinger, on a glass slide. Extracted venom glands were amassed into a metal basket and centrifuged to ≈3000 g.

Asian wasps, *Polistes jokahamae jokahamae* Radoszkowski. Worker wasps were anesthesized with ethylacetate fumes for 1 min and their venom apparatuses gently pulled out by the stinger. Five venom apparatuses were amassed in a metal basket.

European honeybees *Apis mellifera* L. Worker bees were anesthesized with $CO_2$ and dissected as described above. Venom glands of 3 individuals were amassed into a glass wool basket.

Chinese Honeybees, *A. cerana* L. Same procedure as above.

To qualitatively assess the obtained venoms, 0.5–1.0 μL of each were injected directly into an Agilent 7890B GC-MS system following the settings described in Fox et al. (2017) (method and raw chromatogram files available in Fox et al., 2018b). When injecting fire ant venom a split ratio of 1:100 was used to avoid sample overload (alkaloids represent >95% w/w). Additionally, fire ant venom was assessed for contaminants using a micro-spectrophotometer AllSheng Nano-300, following the same principle of routine purity checks of DNA extracts (Wilfinger et al., 1997) through UV absorbance within the range 220–340 nm. It was known that solenopsin alkaloids have absorbance peaks around OD232 (unpublished results of EGPF), while proteins peak at OD280 (Wilfinger et al., 1997). Thus the overall purity of venom obtained by centrifugation was assessed by comparing obtained OD232/OD280 ratios relative to synthetic isosolenopsin A (WuXi AppTec purity >98%) and other ant fluids. The OD232/OD280 ratio was also estimated for fire ant hemolymph and a 'dirty venom extract'. Hemolymph was obtained from alitrunks (i.e. ant thorax minus petiole) of males, and dirty extracts were from integral gasters with the petiole, both separately centrifuged in metal baskets as described to ≈10,000 g. Obtained hemolymph was also directly injected for GC-MS analysis as described.

Conspicuous amounts of venom (Table 1) were obtained within minutes from the few individuals tested in all cases, which could be directly collected into capillary tubes and syringes for posterior bioassays (not shown). Pictures of the collected venoms and accompanying chromatograms can be found in Fox et al. (2018a, 2018b).

Among the tested species *S. invicta* stands out as a particularly problematic model for venom studies (Fox, 2014). Fig. 1 shows chromatograms obtained for crude venom collected from the venom gland reservoirs of fire ant major workers. Re-injection in splitless mode (Fig. 1 lower panel) revealed clear minor peaks identified as methyl-L-isoleucine, geraniol, and α-farnesene, based on synthetic compounds (find raw chromatogram files in Fox et al., 2018b). The same compounds were detected from supplementary analyses of venom from other castes (Figures S4, S5 in Fox et al., 2018a).

While venom reservoirs are bound to exude crude venom when centrifuged, it is possible that some hemolymph gets extracted as well, and these compounds would be external contaminants. Towards testing for contamination we analysed high-centrifugation extracts of whole-gasters and thoraces containing hemolymph and gut contaminants. The samples were scanned by UV spectrophotometry and compared with pure synthetic alkaloids (Figure S6 in Fox et al., 2018a), which revealed similar spectra with high OD232/OD280 ratios. Moreover crude injection of hemolymph extract does not reveal the same compounds (not shown, raw chromatograms in Fox et al., 2018b). Geraniol was originally detected from whole-body extracts of fire ants by Sarmiento (1969) and recently Chen (2017) recorded the presence of the closely-related compound geranial as a volatile. Chen (2017) also identified L-isoleucine methyl ester as a volatile of thawing fire ant bodies. Our present observations with crude venom locate these compounds to the venom apparatus for the first time. The detection of geraniol and methyl-L-isoleucine in the venom apparatus of fire ants is particularly interesting as they are described as precursors for the biosynthesis of alkaloids (Aniszewski, 2015). It remains to date a mystery how ants produce their venom alkaloids (Fox, 2014; Touchard et al., 2016). Farnese isomers are recorded as trail pheromones in the fire ants (Vander Meer et al., 1981).

Other extracts showed potentially important differences in the venom composition of two different species of honeybees (Fig. S1 in Fox et al., 2018a) where the main compound eicosenol is known to be a venom component secreted into the venom reservoir by the Dufour's gland (Martin et al., 2007). The venom of the Asian hornet (Fig. S2 in Fox et al., 2018a) is a complex mixture, largely confirming compounds previously reported by several authors (for a review see Turillazzi, 2006) however also including unreported conspicuous amounts of water-insoluble fatty acids (mainly hexanoic and oleic acids; see Supplementary Materials). Likely these compounds derive of the denser, lower phase of the extracted venom (left inset on Fig. S2 in Fox et al., 2018a) which was surely discarded in previous analyses by authors along with manually dissected gland tissues , because of high viscosity and relative insolubility in saline. The venom of such wasps remains largely unstudied (Turillazzi, 2006) thus the matter should be pursued further.

Novel compounds are continuously identified from venoms even though research with arthropods being delayed by difficulties involved in obtaining sufficient and/or pure venom. As the main example illustrated herein, a fire ant *S. invicta* worker will typically deliver around 0.7 nl of crude venom per sting, out of a total volume of roughly 22 nl (Haight and Tschinkel, 2003) present inside the venom reservoir. Not much is known about fire ant venom toxins beyond the presence of the most abundant alkaloids and some allergens (for an overview see Fox, 2014). Previous methods for obtaining fire ant venom involved either milking individual ants from the stinger into a capillary tube (e.g. Blum et al., 1958) or soaking whole ants in solvents to partition major fractions (e.g. Fox et al., 2013). Therefore while milking crude fire ant venom is labour intensive and dependent on specialised handling, solvent extractions are bound to select for classes of compounds based on their relative solubility, in addition to result in losses during later evaporation. Such limitations likely precluded the direct identification of methyl-L-isoleucine, geraniol, and farnesene from fire ant venom secretions by previous authors.

Regarding other hymenopterans, the methods of choice of previous researchers have been either manual dissections or physical stimulation to collect venom for bioassays (Benli and Yigit, 2008; Aili et al., 2017). Apart from being labor intensive, venom secretions obtained as such are bound to be modified by contact with

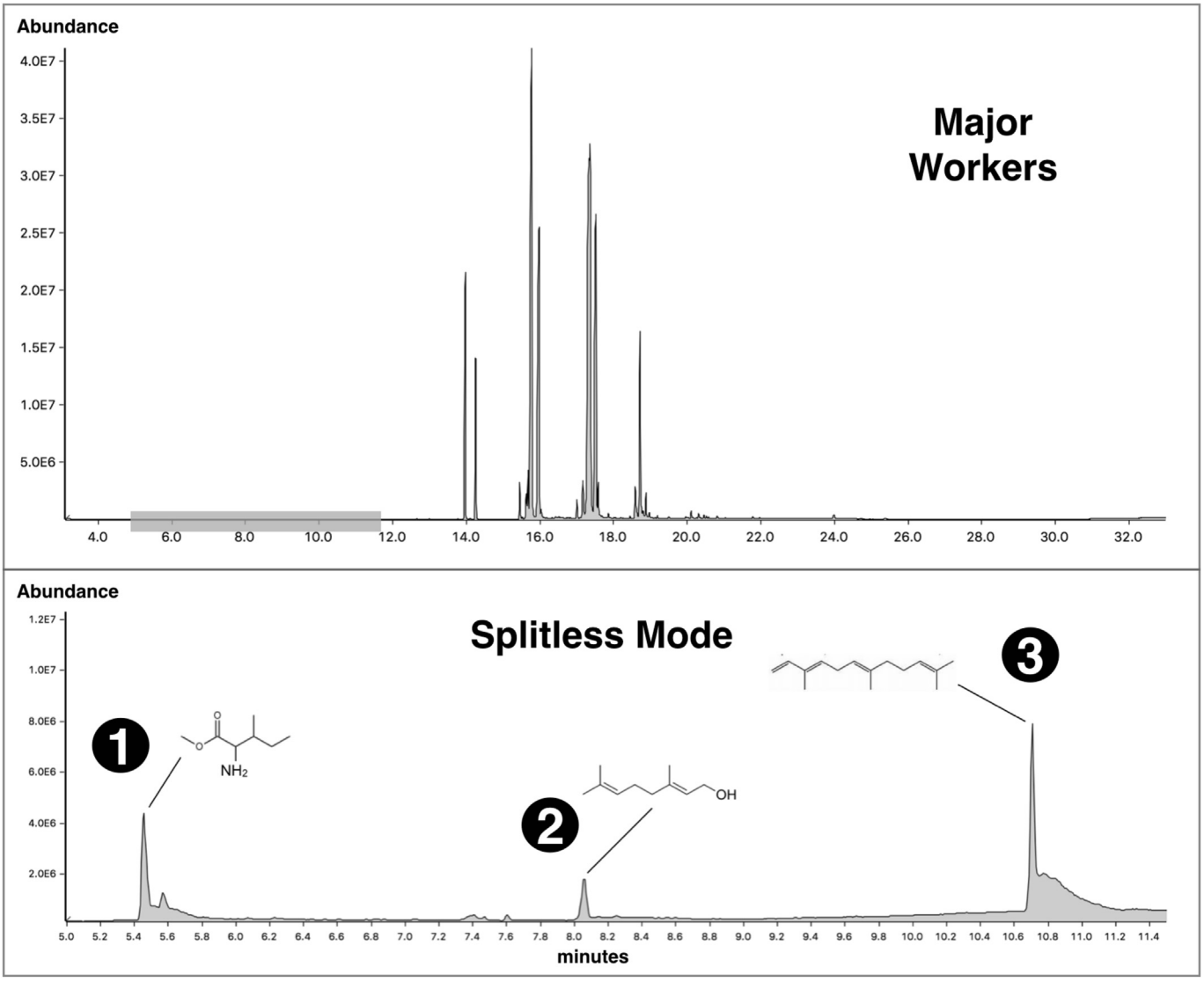


**Fig. 1.** Fire ant venom collected by centrifugation, as viewed by GC-MS chromatograms. Upper panel: Crude venom chromatograms of *S. invicta* major workers acquired in 1:100 split ratio. Lower panel: Magnified chromatogram from the shaded time interval in the upper panel, as obtained by splitless mode injection. Numbered peaks are minor compounds included in pure venom, tentatively identified as: (1) L-isoleucine methyl ester; (2) geraniol; (3) α-farnesene. For details on the spectra of all compounds find the original chromatogram files in Fox et al. (2018b).

saline solution and surrounding dissected tissues. As the method herein described relies on gravity separation of secretions directly from the venom sac, there is no need for liquid solvents or invasive manipulation.

Immediate applications of the proposed venom extraction method pertain bioassays and volatile re-analyses of crude venoms. The venom extraction method herein described is expected to be compatible with other venomous animals, which will be tested subsequently.

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## Transparency document

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