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Systematic antioxidant profiling reveals uric acid as an important antioxidant in polar extracts of selected edible insects and frass

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ABSTRACT

Edible insects are promoted as sustainable protein sources and reported as antioxidative. We aimed to identify previously unknown molecular drivers underlying this antioxidant capacity. We profiled hydrophilic-to-lipophilic extracts from eight edible insect samples (yellow mealworms, crickets, black soldier fly larvae), one frass sample, and two feeds using DPPH, ABTS, and FRAP. Targeted screening of known antioxidants using HPTLC and HPLC with UV or MS only partly explained measured capacities. HPTLC-bioautography with MS enabled identification of both known and previously unreported antioxidants. Uric acid was, for the first time, identified as a major antioxidant in the studied polar extracts. Its content in water extracts (0.97–18 mg g^{-1} dw in insects and 55 mg g^{-1} dw in frass) strongly correlated with DPPH ($\rho = 0.83$) and ABTS ($\rho = 0.76$) results, but may have potential negative health implications. This study advances understanding of insect and frass antioxidant profiles by linking antioxidant capacity to specific compounds using a transferable effect-directed identification framework.

1. Introduction

The European Food Safety Authority (EFSA) and the European Commission already adopted some insects as novel foods in the European Union. Yellow mealworms (*Tenebrio molitor* larvae) were the first insects to receive a positive EFSA opinion in 2021 (EFSA NDA Panel, 2021a). Migratory locusts (*Locusta migratoria*) (EFSA NDA Panel, 2021b), house crickets (*Acheta domesticus*) (EFSA NDA Panel, 2021c), and lesser mealworms (*Alphitobius diaperinus* larvae) (EFSA NDA Panel, 2022) were approved shortly after. Several applications for the authorization of other edible insect species are currently under review by EFSA and the European Commission. Efficient feed-to-biomass conversion, low water consumption, low greenhouse gas emission, and the ability to be reared on a wide range of feed materials illustrate the environmental benefits of insect farming. Nutritionally, insects provide high amounts of protein and essential amino acids, favorable lipids, vitamins and minerals (D'Antonio et al., 2021; Sun-Waterhouse et al., 2016). Beyond their nutritional value, insects also contain bioactive compounds, including antioxidant (AO) compounds (Aiello et al., 2023; Botella-Martínez et al., 2021; Di Mattia et al., 2019; Gumul et al., 2023; Navarro del Hierro et al., 2020; Nino, Reddivari, Ferruzzi, & Liceaga, 2021; Pyo et al., 2020; Tang et al., 2018; Zhang et al., 2022), which have undergone limited investigation. Dietary AO compounds can delay or prevent various diseases, including neurodegenerative, cardiovascular, and chronic inflammatory diseases, and may also exert chemopreventive effects (Sen & Chakraborty, 2011; Shahidi & Zhong, 2010). They act by preventing the formation of reactive oxygen species (ROS), scavenging free radicals, sequestering prooxidants, modulating the expression of genes encoding AO enzymes, and influencing cell signaling pathways (Finley et al., 2011; Sen & Chakraborty, 2011). For a mini-review of the research regarding AO capacity in insects, we refer the interested reader to (D'Antonio et al., 2021).

Specific research has examined the AO capacity of defatted insects (Botella-Martínez et al., 2021; Di Mattia et al., 2019; Son et al., 2020), lipophilic fractions (Di Mattia et al., 2019; Jiang et al., 2025) or protein preparations (Aiello et al., 2023; Gumul et al., 2023; Hoon Lee et al., 2024; Mokaya et al., 2024; Navarro del Hierro et al., 2020; Nino, Reddivari, Ferruzzi, & Liceaga, 2021; Pyo et al., 2020; Tang et al., 2018; Zhang et al., 2022). However, very little research has focused on crude insect extracts, where typically only a limited number of viable extraction solvents are evaluated (Muñoz-Seijas et al., 2025; Navarro del Hierro et al., 2020; Pyo et al., 2020; Tang et al., 2018; Vanqa et al., 2022;

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Zhang et al., 2022). AO results are often inconsistently reported, making meaningful comparisons across studies difficult. Limited qualitative analysis identifies polyphenols (Aiello et al., 2023; Nino, Reddivari, Ferruzzi, & Liceaga, 2021; Vehar et al., 2025), flavonoids (Nino, Reddivari, Ferruzzi, & Liceaga, 2021), and tocopherols (Gumul et al., 2023) as representative bioactive compounds contributing to AO capacity. However, specific compounds are rarely reported (Botella-Martínez et al., 2021; Di Mattia et al., 2019; Flores et al., 2020; Hall et al., 2018; Kurdi et al., 2021; Mintah et al., 2019; Navarro del Hierro et al., 2020; Pyo et al., 2020; Son et al., 2020; Tang et al., 2018; Zhang et al., 2022; Zielińska, Baraniak, & Karaś, 2017; Zielińska, Karaś, & Jakubczyk, 2017). Overall, while the AO capacity of edible insect extracts is often reported, the underlying contributors are seldom assigned. Only a few studies directly link the AO capacity of extracts to specific AO compounds, such as uric acid (UA; 7,9-dihydro-1*H*-purine-2,6,8(3*H*)-trione) in termites (*Reticulitermes speratus*) (Tasaki et al., 2017), and 4-(2-hydroxyethyl)-1,2-benzenediol and 4-ethylbenzene-1,3-diol in *Blaps rynchopetera* (Ji et al., 2024). In addition to insect biomass, insect frass may also represent a relevant source of AO compounds. A specific type of frass is consumed in Chinese culture as insect tea for its perceived health benefits (Xu et al., 2013). As insect farming is expected to expand to meet rising demand, frass production from farmed edible insects will also increase, highlighting the need to assess it as a potentially bioactive by-product.

This study systematically evaluates the AO capacity of several insect species. The selected insects include yellow mealworms (*Tenebrio molitor* larvae), black soldier fly (*Hermetia illucens*) larvae, as well as adult banded cricket (*Gryllodes sigillatus*), two-spotted cricket (*Gryllus bimaculatus*), house cricket (*Acheta domesticus*), and Jamaican field cricket (*Gryllus assimilis*). Compound-level explanations of measured AO capacity remain limited and inconsistent, making insects a relevant benchmark for the effect-directed approach in this study. Frass from laboratory-reared *T. molitor*, together with its corresponding rearing substrates, was also analyzed to assess whether AO compounds originate from feed, undergo metabolism, or are excreted, while evaluating frass as a potential source of bioactives. We applied several extraction solvents ranging from highly hydrophilic to lipophilic to cover AO compounds spanning a wide polarity range. We then assessed AO capacity of extracts with three spectrophotometric assays: the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay, 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical scavenging assay, and ferric reducing AO power (FRAP) assay. Subsequently, we screened the extracts for known classes of plant-derived AO compounds using chromatographic and mass spectrometric (MS) techniques. The primary aim of this study was to link the observed AO capacity of edible insect extracts to the specific compounds responsible. To achieve this, we combined multiple complementary approaches: measurement of overall AO capacity, targeted analysis of various AO phytochemical classes, and untargeted effect-directed analysis by coupling chromatography with *in situ* AO assays and MS.

2. Experimental

2.1. Chemicals and standards

For extraction, EtOH (absolute, anhydrous) was sourced from Carlo Erba (Val de Reuil Cedex, France). MeOH (HPLC grade, ≥99.9%) was obtained from Honeywell (Seelze, Germany). *n*-Hexane (p.a., ≥99%) and EtOAc (p.a., ≥99.5%) were procured from Merck (Darmstadt, Germany). Acetone (HPLC grade, ≥99.8%) was acquired from Sigma-Aldrich (Steinheim, Germany). Ultrapure water (18.2 MΩ•cm) was provided by a Milli-Q water purification system (Millipore, Bedford, MA, USA).

For AO capacity assays, ABTS diammonium salt (≥98%), DPPH, 2,4,6-tris(2-pyridyl)-*s*-triazine (TPTZ, ≥98%), ascorbic acid (≥99.5%), gallic acid (GA) monohydrate (≥98.0%), UA (≥98%), and potassium persulfate (≥99.0%) were purchased from Sigma-Aldrich. Iron(III) chloride hexahydrate (≥98.0%) and Trolox (≥98%) were obtained from Fluka Chemie (Buchs, Switzerland). MeOH (HPLC grade, ≥99.9%) was obtained from Honeywell. Acetic acid (100%), hydrochloric acid (37%), and sodium acetate anhydrous (p.a.) were procured from Merck.

For chromatographic analyses, acetonitrile (LC-MS grade, ≥99.9%), MeOH (HPLC grade, ≥99.9% and LC-MS grade, ≥99.9%), and formic acid (LC-MS grade) were obtained from Honeywell. Chloroform (p.a., ≥99.8%), dichloromethane (for gas chromatography), acetic acid (100%), formic acid (98–100%), methyl *tert*-butyl ether, 2-propanol (p.a.), toluene (≥99%), anisaldehyde (98%), 4-dimethylaminocinnamaldehyde, EtOAc (p.a., ≥99.5%), *n*-hexane (p.a., ≥99%), and sulfuric acid (95–97%) were purchased from Merck. Natural substance reagent (NST, 97%), 2-*tert*-butylhydroquinone (TBHQ, 97%), and 1-propanol (99.9%) were procured from Sigma-Aldrich. Polyethylene glycol 4000 was obtained from Fluka Chemie.

The following analytical standards were used for targeted screening analyses. Caffeic (≥98.0%), chlorogenic (95%), ellagic (95%), ferulic (98%), gallic (97.5–102.5%), *m*-coumaric (≥98.0%), *trans*-cinnamic (99%), ursolic (≥90%) acids, (−)-catechin (98%), (−)-epicatechin (≥90%), luteolin (98%), β-sitosterol (≥97%), (±)-α-tocopherol (≥97.0%), (+)-γ-tocopherol (≥96%), and (+)-δ-tocopherol (≥90%) were procured from Sigma-Aldrich. *o*-Coumaric, *p*-coumaric (≥98.0%), and syringic (98%; 4-hydroxy-3,5-dimethoxybenzoic acid) acids were obtained from Merck. (+)-Catechin (≥98%) and oleanolic acid (≥97%) were supplied by Carl Roth (Karlsruhe, Germany). Apigenin (95%; 4′,5,7-trihydroxyflavone), flavone (99%), kaempferide (99%), myricetin (≥96.0%), quercetin dihydrate (99%), protocatechuic (99%), and sinapic (99%) acids were sourced from Fluka Chemie. Aloe-emodin, emodin, aloin A, α-amyrin (≥98.5%), and β-amyrin (≥98.5%), betulinic acid (≥97%), cycloartenol (≥90%), cycloartenol acetate (≥90%), (−)-catechin gallate (≥98%), (−)-epicatechin gallate (≥97.5%), procyanidin B1 (≥90%), procyanidin B2 (≥90%), procyanidin B3 (≥95%), friedelin (≥99%), kaempferol (99%), lupenone (≥95%), lupeol (≥99%), lupeol acetate (≥95%), pinocembrin (95%), (+)-β-tocopherol (≥98%), β-carotene (≥98%), capsanthin (≥95%), lutein (≥95%), and zeaxanthin (≥98%) were purchased from Extrasynthèse (Genay, France). δ-Amyrin was isolated from tomato surface wax (Martelanc et al., 2009). Chrysin, hesperetin, naringenin (all research grade), and stigmasterol (≥99%) were purchased from Serva-Feinbiochemica (Heidelberg, Germany). Rosmarinic acid (98%) was acquired from CMS Chemicals (Compton, UK).

For quantification of UA, the UA assay kit (ab65344) was purchased from Abcam (Cambridge, UK).

2.2. Samples

Table 1 summarizes all samples, including a) insect biomass, b) frass, and c) feed, and their respective abbreviations used in this study along with the sample details. Laboratory-reared insects were obtained from Ghent University and terminated by freezing.

2.3. Sample preparation and extraction

Except for YM and DF, which were obtained in dried form, the other insects and carrots were freeze-dried. The dried insects and carrots were then frozen using liquid nitrogen and ground into a fine powder using a Mikro-Dismembrator S ball mill (Sartorius, Göttingen, Germany) at 1700 min^{-1} for 1 min. FR and DF were used in their intact form without grinding.

Extraction was performed by weighing the obtained materials directly into glass centrifuge tubes and adding the extraction solvents to obtain 20 mg mL^{-1}. The suspension was vortexed for 1 min, put in a sonication bath for 15 min, centrifuged at 1676×*g* for 5 min, and finally filtered through 0.20 μm hydrophilized polytetrafluoroethylene (H-PTFE) syringe filter (Macherey-Nagel, Düren, Germany). A wide range

Table 1
List of samples.

a)	Abbreviation	Sample content	Species and life stage	Origin and rearing conditions
	YM	yellow mealworm	*Tenebrio molitor,* larvae	obtained from EWL Naturprodukte (Ransbach-Baumbach, Germany)
	BEYM	yellow mealworm	*Tenebrio molitor,* larvae	reared; Insectus feed, carrots
	FYM	yellow mealworm	*Tenebrio molitor,* larvae	reared; Insectus feed, carrots, fasted for 24 h.
	BSF	black soldier fly	*Hermetia illucens,* larvae	reared; Insectus feed, carrots
	B	banded cricket	*Gryllodes sigillatus,* adult	reared*; chicken feed, water
	J	two-spotted cricket	*Gryllus bimaculatus,* adult	reared*; chicken feed, water
	H	house cricket	*Acheta domesticus,* adult	reared*; chicken feed, water
	S	Jamaican field cricket	*Gryllus assimilis,* adult	reared*; chicken feed, water
b)	**Abbreviation**	**Sample content**	**Sample description**	**Source**
	FR	frass of FYM	*Tenebrio molitor,* larvae frass	obtained from reared insects
c)	**Abbreviation**	**Sample content**	**Latin name**	**Source**
	DF	dry feed (Insectus feed)	/	obtained from Mijten (Bekkevoort, Belgium)
	C	carrots	*Daucus carota* subsp. *sativus*	obtained from a local market

* (Tzompa-Sosa et al., 2021)

of extraction solvents was used to span polarity and recover both hydrophilic and lipophilic AO compounds.

Extracts prepared with water, MeOH, 70% MeOH (aq), EtOH, 70% EtOH (aq), acetone, 70% acetone (aq), and EtOAc were used for the AO capacity assays (Section 2.4) and targeted screenings (Section 2.5). Hereafter, the designation "(aq)" will be omitted for the above-mentioned solvent mixtures and extracts for simplicity. Water was included to extract highly polar, water-soluble AO contributors, whereas neat organic solvents were used to access less polar constituents. The 70% aqueous mixtures were used as broadly effective solvents of intermediate polarity, commonly applied in AO studies. *n*-Hexane extracts were used for the targeted screening of tocopherols (Section 2.5). Except for the filter, all extraction consumables—including the centrifuge tubes, pipettes, syringes (Fortuna Optima from Poulten & Graf, Wertheim, Germany), and storage vials—were made of glass. A procedural blank (PB) was obtained by carrying out the entire extraction procedure with solvents only, while omitting the sample. The extracts were stored in amber glass storage vials at −20 °C until further use.

2.4. AO capacity assays

The AO capacity of the extracts was evaluated using DPPH, ABTS, and FRAP assays. Absorbances were measured using a Synergy H1 microplate reader from BioTek (Winooski, VT, USA). Assays were conducted in polystyrene flat-bottom 96-well tissue culture test plates (TPP Techno Plastic Products AG, Trasadingen, Switzerland), except for acetone and EtOAc extracts, which were measured in polypropylene flat-bottom 96-well plates (Greiner, Kremsmünster, Germany) due to their incompatibility with polystyrene.

To facilitate comparison of results with existing literature, results were expressed as standard equivalents of the following standards: 1) ascorbic acid, 2) gallic acid, 3) Trolox (excluding water extracts), and 4) UA (for water extracts only). The rationale for the selection of calibration standards is discussed in Section 3.1. Standard equivalents were used solely for reporting and comparison purposes and should not be interpreted as evidence for the presence of these compounds in the studied extracts. Standard stock solutions of ascorbic acid, gallic acid, Trolox, and UA (each at 0.1 mg mL^{-1}, except UA at 0.02 mg mL^{-1}) were prepared separately for each extract series on the day of the analysis by using the corresponding extraction solvent.

Calibration curves were constructed for each combination of standard compound (ascorbic acid, gallic acid, Trolox), extraction solvent (water, MeOH, 70% MeOH, EtOH, 70% EtOH, acetone, 70% acetone, EtOAc), and assay (DPPH, ABTS, FRAP) from standard stock solutions prepared by two-fold serial dilution directly in the microplate using the corresponding solvent. Calibration curves for UA were prepared only in water for all three AO assays. In addition to the standard stock solutions and extracts, the following solutions were prepared prior to analyses: reagents (DPPH, ABTS, and FRAP solutions), reagent blank (reagent solvent without the reactive component), and extraction solvent (blank). Each standard solution at the respective concentration and the extracts (hereafter referred to as [standard] and [extract]) were mixed directly in the wells as follows: [extract/standard + reagent] in triplicate and [extract/standard + reagent blank] in single replicate. Additionally, [blank + reagent] in triplicate as negative control and [blank + reagent blank] in single replicate as an instrument control were mixed on each microplate. Linear regression was used to determine AO capacity of samples according to calculations described in the Supplementary material (**SM**; Experimental → "Calculations of antioxidant (AO) capacity of samples"). Extracts were diluted to yield a calculated AO response within the linear range of the calibration curve. Detailed preparation and storage information for reagents used for the AO capacity assays can be found in the **SM**; Experimental → "Detailed preparation and storage information of reagents used for the AO capacity assays". The following remaining experimental conditions for specific assays were used.

The DPPH radical scavenging capacity assay was performed according to the method of (Jung et al., 2010). After pipetting 110 μL of the [reagent/reagent blank] and 80 μL of the [extract/standard/blank] directly into the wells, the plates were covered with a lid, kept in the dark for 30 min, and subsequently, the absorbance was read at 517 nm.

The ABTS radical scavenging capacity assay was performed using the method of (Re et al., 1999) with slight modifications. The prepared ABTS solution was diluted on the day of analysis at a 1:40 *v/v* ratio with the extraction solvent corresponding to the prepared extracts. However, for assays involving acetone extracts, 50% acetone (aq) was used as the diluent to prevent precipitation of the reagent constituent(s), and for assays involving EtOAc extracts, 50% MeOH (aq) was used as the diluent due to the immiscibility of EtOAc and water from the ABTS reagent. A mixture of water and the corresponding extraction solvent at a 1:40 *v/v* ratio was used as the blank reagent. After pipetting 280 μL of diluted [reagent/reagent blank] and 50 μL of [extract/standard/blank] into the wells, the plates were covered with a lid, left in the dark for 15 min, and afterwards scanned at 734 nm.

The FRAP assay was conducted according to the method of (Kurdi et al., 2021). [Reagent/reagent blank] (300 μL) was mixed with 20 μL of the [extract/standard/blank] directly in the wells. The plates were incubated at 37 °C in the microplate reader for 30 min with continuous shaking, then the absorbance was read at 593 nm without the lid to prevent condensation on the lids. FRAP assays of EtOAc and acetone extracts were not performed due to precipitation of the reagent constituents.

2.5. Targeted analyses of AO phytochemicals

Targeted analysis of phytochemicals was performed for different extracts of the samples listed in Table 1, using HPTLC, HPLC-UV, and LC-MS analyses. For targeted HPTLC analyses, extract selection was based on preliminary profiling of the BEYM sample. Several BEYM solvent

extracts were analyzed using the HPTLC screening protocols described below, and the extract(s) yielding the highest number and intensity of bands (data not shown) were selected for subsequent analysis of all remaining samples. Based on these preliminary data, 70% EtOH extracts were selected for screening for phenolic acids, flavonoids, flavan-3-ols and proanthocyanidins; MeOH extracts for anthraquinones, triterpenoids, and phytosterols; and EtOAc extracts for carotenoids. Additionally, *n*-hexane extracts of all samples from Table 1 were analyzed for tocopherols by HPLC-UV, while acetone and EtOAc extracts of frass were analyzed for carotenoids by LC-MS (details provided at the end of this section).

The detailed HPTLC protocols used for the screening of anthraquinones (Glavnik & Vovk, 2020), phenolic acids (Jug et al., 2018a), flavonoids (Jug et al., 2018b), flavan-3-ols and proanthocyanidins (Glavnik et al., 2017), carotenoids (Rodić et al., 2012), and triterpenoids and phytosterols (Martelanc et al., 2009), along with the full list of standards, are described in **Table S1**. Individual standards of phenolic acids, flavonoids, flavan-3-ols, and proanthocyanidins were prepared in MeOH at a concentration of 0.1 mg mL^{-1}, and anthraquinones at 0.2 mg mL^{-1}. Carotenoid standards were prepared as follows: lutein (90.6 μg mL^{-1} in EtOH), zeaxanthin (450 μg mL^{-1} in acetone), β-carotene (50.9 μg mL^{-1} in acetone), and capsanthin (15 μg mL^{-1} in acetone). A standard mix of triterpenoids and phytosterols was prepared in 1-propanol at concentration of 0.025 mg mL^{-1}.

Automatic TLC sampler 4 or Linomat 5, twin-trough developing chambers, TLC plate heater III, and VisionCATS version 3.1 software, all from Camag (Muttenz, Switzerland), were used for high-performance thin-layer chromatography (HPTLC) analyses. HPTLC plates (20 × 10 cm; with either silica or reversed phase (RP) adsorbents) were obtained from Merck. All extracts (Section 2.3) were applied onto the HPTLC plates along with the corresponding standard solutions. The developed plates were visualized under white light in transmittance, reflectance, and combined modes (White T, R and RT, respectively), as well as at 366 nm and 254 nm, before and after derivatization. The plate for carotenoid screening was not subjected to derivatization and was therefore imaged only after development.

Sample extracts (20 mg mL^{-1} in *n*-hexane) were directly screened for tocopherols using high-performance liquid chromatography (HPLC) with UV detection (SpectraSystem; Thermo Separation Products, Riviera Beach, CA, USA) on a Syncronis Silica column (150 mm × 4.6 mm i.d., 5 μm; Thermo Scientific, San Jose, CA, USA). The injection volume was 10 μL. Isocratic elution with 1% 2-propanol in *n*-hexane at 25 °C and a flow rate of 1 mL min^{-1} was employed for 10 min, and the compounds were detected at 292 nm. A standard mixture (20 μg mL^{-1} in *n*-hexane) of α-, β-, γ-, and δ-tocopherol was used for confirmation.

Acetone and EtOAc extracts of FR were additionally analyzed for carotenoids by LC-MS, using a modified method from (Metličar & Albreht, 2022), with full details provided in the **SM** (Experimental → "HPLC-MS analysis of carotenoids in frass of yellow mealworm").

2.6. Effect-directed (bioautographic) analyses

HPTLC analyses were carried out as described in Section 2.5, but in the case of carotenoids, addition of TBHQ into the developing solvent was omitted. After development, plates were dipped into the respective AO-detecting reagent (DPPH, ABTS, and FRAP) instead of undergoing the typical derivatization. A more concentrated DPPH solution (5 mM) was prepared in MeOH, ABTS stock solution was diluted 1:20 (*v/v*) with MeOH, while FRAP reagent was used without modification. After dipping for 3 s, the plates were dried under a stream of cold air using a hairdryer and left to stand in the dark for 30 min (DPPH) or 15 min (ABTS), whereas FRAP-treated plates were heated for 10 min at 40 °C. Finally, all plates were documented using the TLC Visualizer under White T. The hydrophobic surface of the RP plates repelled the aqueous FRAP reagent, preventing its use on RP-HPTLC plates for bioautographic analyses.

2.6.1. HPTLC-(AO)-MS^n

HPTLC analysis was carried out as described in **Table S1**, with a modified plate pre-development step to reduce the background chemical noise in the acquired mass spectra (Glavnik et al., 2017). Silica gel plates were pre-developed first with MeOH:HCOOH 10:1 (*v/v*), dried with a hairdryer, and subsequently pre-developed again with MeOH (Jug et al., 2018a). After the second pre-development, they were heated in an oven at 110 °C for 30 min. The pre-development of the RP plates with acetone remained unchanged. For MS analysis of the AO bands, only the edges of the plates were derivatized with the respective AO reagent (DPPH, ABTS, or FRAP). **Fig. S1** schematically exemplifies the HPTLC-(AO)-MS^n workflow, leading to the identification of the AO-reactive compounds in the samples.

Compounds were eluted from the plates using the TLC-MS Interface with an oval elution head (4 mm × 2 mm; Camag) through an in-line filter into the LTQ Velos mass spectrometer from Thermo Scientific. Xcalibur version 2.1 software was used for the evaluation of the mass spectra.

Two different sets of elution and MS parameters were used depending on the class of analyzed compounds. Carotenoids were eluted from the plates using acetonitrile (1 mL min^{-1}) and ionized by atmospheric-pressure chemical ionization (APCI) in positive mode. For other compounds, elution was performed with MeOH (0.2 mL min^{-1}) and ionization was carried out by electrospray ionization (ESI) in negative mode (Jug et al., 2021). Detailed MS parameters are provided in **Table S2**.

2.6.2. Effect-directed isolation and identification of the major AO compound

Effect-directed isolation was performed on PLC silica gel 60 plates from Merck, which were double pre-developed with 1) MeOH:HCOOH (10:1 *v/v*) and 2) MeOH (Jug et al., 2018a). 70% EtOH extract of YM (100 mg mL^{-1}, 960 μL) was applied on the plate as an 18 cm band. The plate was developed according to the initial screening protocol for flavan-3-ols and proanthocyanidins (**Table S1**). Two cm from the left and right edge of the 20 cm × 10 cm plate were derivatized with DPPH after development and the major AO fraction (R_F 0.45) was scratched from the underivatized part of the plate, suspended in approximately 20 mL of 70% EtOH, and filtered through a 0.2 μm H-PTFE filter. The procedure was repeated 15 times, and the isolates were collected in pre-weighed round-bottom flasks. The organic solvent was first removed using a rotary evaporator (Büchi, Switzerland), and the remaining water was freeze-dried. The resulting solid residues from each replicate isolation were redissolved in 500 μL 70% EtOH, filtered through a 0.2 μm H-PTFE filter and pooled together.

The final isolate (40 μL; 6 mm band) was subjected to HPTLC-MS^3 identification on a silica stationary phase using the same chromatographic method as for the PLC plates, with comparison against a UA standard (30 μL, 20 μg mL^{-1} in 70% EtOH; 6 mm band). The isolate was further subjected to additional compound confirmation by orthogonal LC-MS analysis on a Synergi Hydro-RP column (250 mm × 2 mm; 4 μm; Phenomenex, Torrance, CA, USA) at a flow rate of 0.2 mL min^{-1} using 10 mM aqueous ammonium acetate (pH 4.5) as the mobile phase. Both the LC-MS analysis and the MS detection of the HPTLC-MS workflow were carried out on an Accela 1250–LTQ system (Thermo) using ESI-MS in negative ionization mode, as described in **Table S2**. The autosampler and oven temperatures were set to 25 °C, and the injection volume was 5 μL. Compound confirmation was achieved using SIM (at *m/z* 167) and MS^2 (collision energy of 35%).

The chromatograms originally used for bioautography screening of flavan-3-ols and proanthocyanidins (Section 2.5 and 2.6) were analyzed by ImageJ (version 1.54 g, National Institutes of Health, Bethesda, MD, USA) to estimate the proportion of the bioautographic AO signal attributable to UA. The integrated density of each AO zone was obtained and corrected for background. The relative contribution of UA was calculated by dividing the corrected integrated density of UA by the sum

of all corrected integrated densities of all AO zones.

2.7. Quantification of UA

UA present in water extracts of insect biomass and frass was quantified using a UA colorimetric assay kit from Abcam (ab65344) according to the instructions provided by the manufacturer using the Synergy H1 microplate reader (BioTek). To ensure that the spectrophotometric measurements fell within the linear range of the kit, the extracts (as prepared for the measurement of the AO capacity; Section 2.3) were diluted with water prior to quantification as follows: two-fold for B, S, and J, five-fold for FYM, and ten-fold for H and FR. The YM, BEYM, and BSF samples were analyzed without dilution. Sample absorbance values used for the calculation of UA concentration were corrected by subtracting the background absorbance of the extract without added reagents.

2.8. Statistical analysis

Absorbance measurements for the determination of AO capacities were performed in triplicate and those for the quantification of UA using the assay kit were performed in duplicate. Results are reported as mean ± standard deviation. In cases where the calculated standard deviation equalled zero, results are reported as mean ± uncertainty calculated from the instrumental error of the microplate reader; these cases are explicitly labeled. Statistical analysis was conducted using R software (version 4.3.2, R Core Team, Vienna, Austria). Initially, the Shapiro-Wilk normality test was applied to assess data normality, which indicated non-normal distribution. Consequently, the data were log-transformed. The normality of distribution and homogeneity of variance in the log-transformed data were confirmed using additional Shapiro-Wilk and Levene tests, respectively. For independent two-group comparisons, Student's *t*-test was used when assumptions of normality and homogeneity of variance were met. Otherwise, the Mann-Whitney *U* test was applied. For paired comparisons, normality of paired differences was assessed using the Shapiro-Wilk test. Paired Student's *t*-test was used when the paired differences were normally distributed; otherwise, the Wilcoxon signed-rank test was applied. For planned multiple pairwise comparisons, values of p were adjusted using the Bonferroni correction. For comparisons involving more than two groups, one-way analysis of variance (ANOVA) followed by Tukey's HSD post hoc test was used when parametric analysis was justified. Otherwise, the Kruskal-Wallis test followed by Dunn's post hoc test with Bonferroni correction was used. Spearman rank correlation coefficients (ρ) and the corresponding values of p were calculated to assess correlations between variables. Correlations were interpreted as strong for $|\rho| > 0.7$, moderate for $0.3 \leq |\rho| \leq 0.7$, and weak for $0.1 \leq |\rho| < 0.3$. Variables were considered uncorrelated for $|\rho| < 0.1$ (Botella-Martínez et al., 2021). Values of $p < 0.05$ were considered statistically significant.

3. Results and discussion

3.1. AO capacity

While multiple studies have examined the AO capacity of insects (Andrade et al., 2025; Di Mattia et al., 2019; Hwang et al., 2019; Muñoz-Seijas et al., 2025; Navarro del Hierro et al., 2020; Vanqa et al., 2022), direct comparison across studies is often hindered by differences in data representation, assay conditions, and incomplete experimental details (de Menezes et al., 2021; Olszowy-Tomczyk, 2021). In this study, we address this gap by generating a dataset of AO capacities of the selected insect biomass, frass and feed samples using eight extraction solvents and solvent mixtures with various polarities (water, MeOH, 70% MeOH, EtOH, 70% EtOH, acetone, 70% acetone, EtOAc) per sample and three widely used assays (DPPH, ABTS, FRAP). To facilitate comparison of our data with the literature, AO capacities in this study were quantified against multiple commonly used reference standards in AO-related insect research—Trolox being the most frequently used, followed by ascorbic acid, and lastly, gallic acid. Additionally, due to findings described in the sections below, UA was also used for this purpose. Quantitative results, expressed as mg standard equivalent g^{-1} dry weight (dw), calculated against each standard, are gathered in **Table S3 (Sheet 1 and Sheet 2)**. For simplicity, results in the main text are reported primarily as ascorbic acid equivalents (AAE), which is the authors' reporting choice to facilitate comparability among different solvent systems, as ascorbic acid—unlike Trolox—is soluble across all extraction solvents used. For the same reason, numerical values in the main text are presented with the number of significant decimal places appropriate for each measurement, without standard deviations. Importantly, the standard equivalent notation is used only as a reporting style to facilitate comparison among samples and with diverse literature data. It should not be interpreted as the actual concentration of the AO compounds used as calibration standards, nor as a direct chemical equivalence between the standards and the AO compounds present in the extracts. The reported equivalent values should be understood as calibrated assay responses. Their interpretation is limited by differences in reactivity among AO compounds and by the assay-specific nature of responses.

This comprehensive dataset provides a valuable reference point for future research on insect-derived AO capacity while systematically mapping their AO capacity across extraction solvents of differing polarity, and guiding the subsequent effect-directed identification of AO constituents. Certain reagent–extract–standard combinations (as indicated in **Table S3**) were not measured due to their incompatibility. The supporting calibration data are provided in **Table S4**.

The DPPH assay is the most widely used method for evaluating AO capacity in insects, followed by ABTS, and lastly FRAP (D'Antonio et al., 2021, 2023). Therefore, all three assays were used in this study to facilitate comparison with literature data and to ensure the observed AO trends were not driven by a single assay. While ABTS is considered suitable for both hydrophilic and lipophilic compounds, DPPH is generally more appropriate for evaluating lipophilic AO compounds (Sadeer et al., 2020). Nevertheless, AO capacities were strongly positively correlated across assays (DPPH–ABTS: $\rho = 0.94$; DPPH–FRAP: $\rho = 0.73$; ABTS–FRAP: $\rho = 0.74$; all $p < 0.05$). The strong correlation between DPPH and ABTS is consistent with their shared radical-scavenging principle despite reported differences in submechanisms (Platzer et al., 2021). The strong correlations between FRAP and the radical-scavenging assays are not immediately intuitive because FRAP is based on a distinct redox mechanism; however, they align with previous observations in insect-derived matrices (Botella-Martínez et al., 2021).

Comparing the AO capacity of insects to other foods is not straightforward, not only because data are reported in different formats and assays vary, but also because the mode of consumption and consequent daily intake may vary. Nonetheless, to briefly contextualize the obtained results, Table 2 summarizes the AO capacities of selected reference foods (beef, carrot, Gala apple, and blackberry) in comparison with the insects and frass analyzed in this study. Beef was selected as a conventional protein reference, whereas insects represent emerging sustainable alternatives. Carrots, apples, and blackberries were chosen as model plant-based foods known for their AO properties. Although the dataset utilizes extraction solvents of similar polarity, methodological discrepancies between the cited studies necessitate that Table 2 be interpreted as a comparative guide rather than a precise quantitative meta-analysis of AO content. Overall, these comparisons indicate that insects—apart from being a protein source—could also be considered a relevant AO source.

AO capacity was evaluated using a range of solvents and solvent mixtures, from highly hydrophilic to lipophilic: water, MeOH, 70% MeOH, EtOH, 70% EtOH, acetone, 70% acetone, and EtOAc. Fig. 1 highlights the effect of solvent polarity on AO capacity of samples. Examination of insect and frass extracts with radical scavenging assays

Table 2
Illustrative comparison of antioxidant capacities of selected reference foods (beef, carrot, Gala apple, and blackberry), different insects, and frass, expressed as ascorbic acid equivalents (AAE) in mg g^{-1} dry weight. The table also indicates the assay type, extraction solvent, and data source. For easier comparison, numerical values are presented with the same number of decimal places and without standard deviations. Note that cross-study comparisons are approximate and assay-dependent.

Food	AAE (mg g^{-1} dry weight)	Assay	Extraction solvent	Reference
Beef	0.3–0.5*	ABTS	MeOH	(Santos et al., 2021)
Carrot	0.7 / 2.4 / 0.8	DPPH / ABTS / FRAP	water	Current study
Gala apple	12.1	ABTS	aqueous methanolic	(Kim et al., 2002)
Insects	**3.4–9.1 / 8.3–16.0**	**DPPH / ABTS**	**water**	**Current study**
Blackberry	29.8	ABTS	50% MeOH	(Pantelidis et al., 2007)
Frass	**25.6 / 61.0 / 30.9**	**DPPH / ABTS / FRAP**	**water**	**Current study**

* The reported range reflects variability among three beef samples.

(DPPH, ABTS) revealed strong positive correlations among the AO capacities of the various aqueous-organic extracts ($\rho \geq 0.85$), and moderate to strong correlation between water and aqueous-organic extracts ($\rho \geq 0.63$). In contrast, correlations between aqueous-organic and pure organic extracts were less consistent, being moderate in the case of ABTS ($0.3 \leq \rho \leq 0.67$) and ranging from moderately negative to strongly positive in the case of DPPH ($-0.4 \leq \rho \leq 0.82$). Strong correlations in FRAP capacity were identified between the following extract pairs: water and MeOH, EtOH and 70% EtOH, MeOH and 70% MeOH (Fig. 2). Only strong correlations were statistically significant. These correlation patterns reflect similar sample ranking of AO capacities across water and aqueous–organic extracts suggesting that the AO compounds responsible for the variation between samples are more consistently recovered under hydrophilic extraction conditions than under neat organic conditions.

Furthermore, the AO capacity of water extracts of all investigated insect and frass samples was significantly higher than that of extracts prepared with any other extraction solvent ($p < 0.05$; ANOVA with Tukey; conducted on all extracts of different insect species, analyzed separately for all three assays). The results ranged from 2.5 to 16 and from 25.6 to 61 mg AAE g^{-1} dw in insects and frass, respectively. In contrast, AO capacities of aqueous-organic and neat organic insect extracts only reached up to 5.1 and 1.39 mg AAE g^{-1} dw, respectively. Similarly, AO capacities of aqueous-organic and neat organic extracts of frass ranged from 0.45 to 9.6 and 0.07 to 1.0 mg AAE g^{-1} dw, respectively. Moreover, aqueous-organic extracts showed significantly higher AO capacity than the corresponding neat organic extracts in the DPPH and ABTS assays; for FRAP, the available 70% MeOH/MeOH pair showed the same trend (Bonferroni-adjusted $p < 0.05$; Wilcoxon signed-rank test for DPPH and paired Student's *t*-test for ABTS and FRAP). This pattern is consistent with previous reports showing that insect AO capacity is largely associated with water-soluble fractions (Di Mattia et al., 2019; Hwang et al., 2019; Navarro del Hierro et al., 2020). Taken together, these results further show that solvent choice systematically influences the measured AO capacity in insect and frass matrices and support the predominance of hydrophilic AO contributors.

No significant differences in AO capacity were detected among the insect species analyzed in this study ($p > 0.05$)—see **Fig. S2** and Supplementary results and discussion for details. Conversely, the analyzed frass (FR) of the reared yellow mealworm FYM showed significantly different AO capacities. Aqueous-organic FR extracts exhibited significantly higher AO capacity than the corresponding insect – FYM ($p < 0.05$; paired Student's *t*-test across aqueous-organic extracts and all three assays). The FR-to-FYM ratio of AO capacity ranged from 2.5 (ABTS assay in 70% acetone) to 7.5 (FRAP assay in 70% EtOH), indicating that frass has a markedly higher AO capacity than the corresponding FYM under aqueous-organic extraction conditions. In contrast, no significant differences were observed between the organic solvent extracts ($p > 0.05$; paired Student's *t*-test across organic extracts and all three assays), where most AO capacity ratios ranged from 0.7 to 1.6. Exceptions were noted in ABTS assays using EtOAc and acetone, where FR-to-FYM ratios reached 2.4 and 6.6, respectively. These findings suggest that, in the studied FYM/FR system, FR extracts (obtained from FYM) contain more potent hydrophilic AO compounds or higher concentrations of such compounds than the corresponding insect extracts. This observation points to the potential relevance of yellow mealworm larvae frass valorization. However, broader studies from different insect species and feeding regimes are needed before more general conclusions can be drawn.

We attribute the relatively high RSD values observed in a few cases (Fig. 1 and **S2**) mainly to two methodological factors. First, extracts exceeding the linear range of the calibration curve were reanalyzed after dilution, and the variability of the diluted measurements was propagated during the back-calculation to the final AO capacity. Second, some extracts exhibited low calculated AO response, where small differences between corrected reagent and sample absorbance values had a greater influence on the calculated result. In some cases (FYM in 70% acetone, along with H and FR in acetone), both methodological factors were present, as reflected in the larger error bars in the bar plots.

Our findings suggest that both insects and frass might contain hydrophilic AO compounds that are either absent from the feeds or present at much lower concentrations. The data suggest that feed-derived phytochemicals are unlikely to fully explain the observed AO activity. Water- and aqueous-organic extractions proved optimal for recovering these compounds. Overall, the results further highlight the potential of insects as AO-rich food. Notably, by quantifying AO capacity across eight extraction solvents and three assays, and reporting results against multiple common reference standards, this work provides comprehensive, directly comparable datasets on solvent-dependent AO capacity in selected edible insects and associated matrices (one frass and two standard feed samples), facilitating comparison across studies and promoting more standardized reporting.

3.2. Targeted analyses of AO phytochemicals

The notable AO capacity measured in the insect and frass samples prompted further identification of the corresponding bioactive species. Although our results (Section 3.1) indicated that the major AO compounds are not directly bioaccumulated in the insects from their plant-based feed, phytochemical screening was conducted to comprehensively characterize the chemical composition and assess whether plant-derived phytochemicals contribute, even at minor levels, to the observed activity. Since the targeted phytochemical classes are plant-derived, their occurrence in insect biomass is more likely related to dietary intake, accumulation, or transformation, rather than de novo synthesis by insects. Nevertheless, certain reports support both their presence in insects and their role in AO capacity (Aiello et al., 2023; Nino, Reddivari, Ferruzzi, & Liceaga, 2021; Vehar et al., 2025). Preliminary testing of various BEYM and FR extracts revealed that those obtained with 70% EtOH exhibited the most abundant chemical profiles, as determined by HPTLC analysis. Therefore, the 70% EtOH extracts were specifically selected for further targeted analyses of phenolic acids, flavonoids, flavan-3-ols and proanthocyanidins. MeOH extracts of the same samples were employed for the analysis of the less hydrophilic anthraquinones, triterpenoids, and phytosterols, while EtOAc extracts were used to screen for carotenoids.

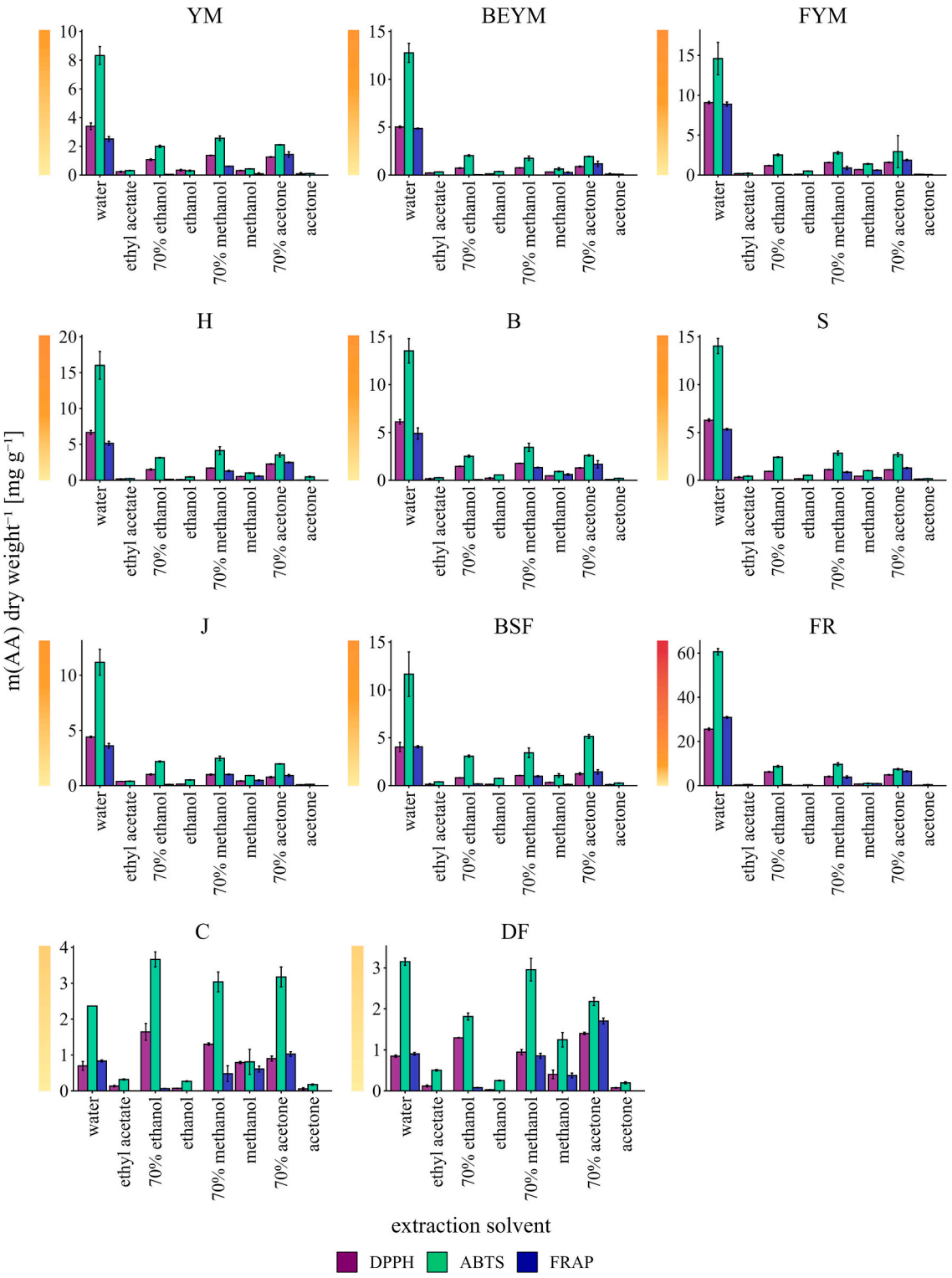

Fig. 1. Bar plots of antioxidant (AO) capacities (expressed as ascorbic acid (AA) equivalents) of individual insect (YM, BEYM, FYM, H, B, S, J, and BSF), frass (FR), and feed (C and DF) samples across different extraction solvents. The error bars represent measurement uncertainty. Heatmap strips to the left of each bar plot reflect the AO capacity range on the respective y-axes, with colour gradients taken from Sheet 2 of **Table S3**. The colors progress from yellow to red, where the deepest red indicates the highest AO capacity measured in this study. Sample abbreviations are defined in Table 1. Water extracts systematically showed the highest AO capacities in the analyzed insect and frass extracts, whereas this trend was not observed for the analyzed feed extracts. The highest AO capacities were generally observed in the analyzed FR extracts. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

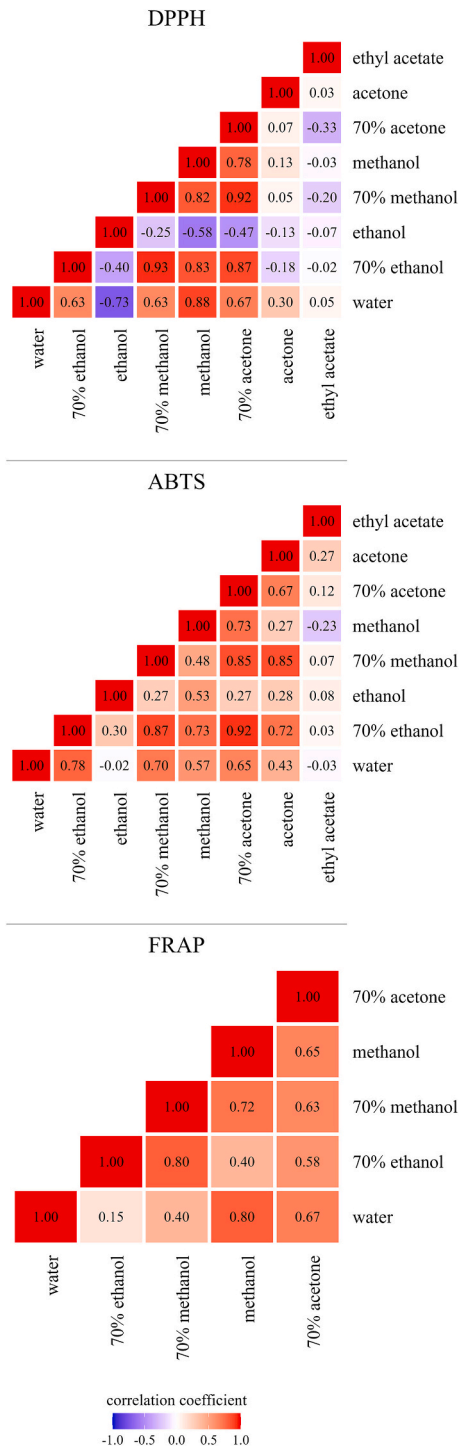

Fig. 2. Heatmaps depicting the Spearman's rank correlation coefficients (ρ) between pairs of antioxidant (AO) capacities of insect and frass extracts obtained using different extraction solvents, presented separately for DPPH, ABTS, and FRAP assays. Sample abbreviations are defined in Table 1. Correlations were interpreted as strong for $|\rho| > 0.7$ and are depicted in intense red or blue. Moderate correlations were assigned where $0.3 \leq |\rho| \leq 0.7$ and are shown in orange or purple. Weak correlations ($0.1 \leq |\rho| < 0.3$) are shown in pale orange or pale purple. Variables were considered uncorrelated for $|\rho| < 0.1$, and are depicted as off-white. Strong correlations between selected extraction solvents may reflect similarities in extracted AO compound profiles. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Of the 56 compounds screened, representing multiple classes of phytochemicals, only eight were detected during HPLC and HPTLC analyses; these included phenolic compounds, carotenoids, and tocopherols. A full list of compounds and the corresponding confirmation approaches is provided in Table 3. Notably, lutein and β-carotene were identified by HPTLC in C, as well as in FR, but not in the corresponding insect biomass FYM, implying that insects either metabolize or excrete them. The latter further supports potential valorization of frass as a source of bioactive compounds. Bands matching the identified compounds were generally of low intensity; however, additional bands were observed that did not correspond to the included standards (**Fig. S3**). Specifically, in triterpenoid and phytosterol screening, several additional bands showed similar R_F values and chromogenic responses to those of the reference compounds. In contrast, in the flavan-3-ol, proanthocyanidin, and carotenoid screenings, additional bands observed across all insect samples were distinct and did not correspond to any of the included standards. Those bands were also subjected to MS analysis, but could not be assigned due to the lack of corresponding literature data or available standards.

The apparent low abundance of phenolic constituents can reflect sample preparation and processing effects, as explained below. The insects, positively assessed by EFSA as novel foods, are terminated by either blanching (EFSA NDA Panel, 2021a, 2022) or freezing (EFSA NDA Panel, 2021c, 2021b). Because blanching can cause thermal degradation of polyphenols or their leaching into the blanching medium (Anuduang et al., 2020; Moongngarm et al., 2024; Nino, Reddivari, Osorio, et al., 2021), freezing was used as the termination method in this study to preserve polyphenol content. However, polyphenols in insects, most probably resulting from herbivorous feeding behavior, can exist in various forms (e.g., free, glycosylated, or incorporated into the cuticular matrix through a series of transformations) (Nino, Reddivari, Osorio, et al., 2021). Some of these transformations are enzyme–mediated and may persist even after insect termination. Termination of insects by blanching can permanently deactivate enzymes such as polyphenol oxidase (Andrade et al., 2025), whereas freezing only slows enzymatic activity without irreversible deactivation. Therefore, the freezing termination used in this study may have allowed enzymatic activity to persist, potentially influencing the compounds identified in both targeted and untargeted analyses. This may help explain the low and inconsistent occurrence of phenolic compounds observed among the samples, as phenolic compounds were detected only in selected insect extracts and at low intensity, rather than consistently across all samples. Further investigation of the effects of blanching versus freezing on polyphenol content in insects is warranted, including the role of specific enzymes and the stability of polyphenols during processing and long-term storage.

The limited number of compounds identified through our screenings did not intuitively explain the notable AO capacity measured in the extracts, and similar observations have been reported previously. For instance, several phenolic acids and flavonoids, including some targeted in this study, were identified in house cricket extracts. Nevertheless, their levels alone could not account for the observed AO capacity (Nino, Reddivari, Ferruzzi, & Liceaga, 2021). Moreover, qualitative phytochemical analyses of Tenebrionidae insects, including yellow mealworm, found no tannins or flavonoids despite measurable AO capacity (Flores et al., 2020). On the other hand, *Blaps rynchopetera*, another member of the same family, contained a wide array of phenolic constituents, which—apart from protocatechuic acid—were not targeted in this study (Xiao et al., 2017). Together, these case studies indicate that both the presence and identity of phenolic AO compounds can vary substantially among insect species and matrices, and that measurable AO capacity cannot be attributed solely to the commonly targeted phenolic classes. Consistent with this interpretation, previous studies have reported widely varying correlation strengths between AO capacity and total polyphenol content (TPC), ranging from weak (Pyo et al., 2020) to strong (Andrade et al., 2025; Botella-Martínez et al., 2021;

Navarro del Hierro et al., 2020).

Due to this variability, many authors suggest that the AO capacity in insects may not originate solely from phytochemicals but also from other sources, such as bioactive proteins and peptides (Zielińska, Baraniak, & Karaś, 2017). A tertiary source of AO compounds in insects has also been proposed (Di Mattia et al., 2019), comprising non-phenolic and non-peptide compounds. In addition, many reports of polyphenols in insects rely on non-specific spectrophotometric assays that may overestimate polyphenol levels due to interferences from non-polyphenol sources (Aiello et al., 2023). Taken together, these observations suggest that additional contributors may substantially affect AO capacity in insect and frass extracts, motivating the effect-directed characterization approaches described in the next section.

3.3. Effect-directed (bioautographic) identification of AO compounds

To reveal the AO constituents not covered in the targeted analysis (Table 3), we applied HPTLC-bioautography. This approach enabled selective visualization of HPTLC bands corresponding to AO compounds that produce a positive colorimetric reaction with the DPPH, ABTS and FRAP reagent. Positive reactions were characterized by a purple-to-yellow colour change in the DPPH assay, a green-to-colorless transition in the ABTS assay, or the formation of an intense blue colour in the FRAP assay. This untargeted approach, guided by DPPH, ABTS, or FRAP, and combined with MS^n analysis, enabled the identification of compounds responsible for the AO capacity of studied extracts. It is important to note that the bioautographic approach to AO screening is selective rather than exhaustive. As with the spectrophotometric AO capacity assays described in Section 2.4, detection remains limited to compounds capable of responding through the DPPH, ABTS, and FRAP reaction mechanisms. However, unlike in the spectrophotometric assays, where the entire extract reacts in solution, the bioautographic approach introduces additional requirements for detection. Compounds must remain stable during chromatography and derivatization, be able to react with the reagents on the HPTLC plate *in situ*, and produce a colorimetric response strong enough to exceed the visual detection limit. Therefore, the absence of a visible AO zone should only be interpreted as a lack of detectable AO activity under the applied bioautographic conditions, rather than as evidence for the absence of any AO compounds. The visual response on the bioautographic HPTLC plates may also be influenced by the possible synergistic or antagonistic effects of the potentially coeluting compounds.

The results from the HPTLC screening methods (**Table S1**), using different AO reagents (DPPH, ABTS, FRAP) applied *in situ*, are presented in Fig. 3. Generally, ABTS and DPPH reveal the same bands, with only minor differences, which is in accordance with the strong correlation calculated in Section 3.1. In contrast, the FRAP reagent detects fewer bands, which are distinct from those visualized by ABTS and DPPH. This observation is consistent with the overall AO capacity results for extracts measured in 70% EtOH, MeOH and EtOAc, where FRAP values were generally much lower than the ABTS and DPPH values. The incompatibility of the RP stationary phase with the purely aqueous FRAP reagent prevented effective interaction with the AO compounds, making the FRAP reagent unsuitable for bioautography on RP-HPTLC plates.

3.3.1. Identification of the primary AO contributor in the studied water and polar extracts

Interestingly, our bioautographic approach only faintly, if at all, highlighted the phytochemicals identified in Table 3. Instead, it uncovered additional compounds not detected in the targeted analysis (**Table S5** and **S6**), which are mainly discussed in the next subsection. The most prominent example was a previously undetected compound observed after derivatization with either DPPH or ABTS on the HPTLC plate developed according to the method for flavan-3-ols and

Table 3
Overview of phytochemicals identified in insect samples, frass and feed through targeted HPTLC and HPLC analyses.

Sample	Identified phytochemicals			
	triterpenoids and phytosterols in methanol extracts	tocopherols in *n*-hexane extracts	phenolic compounds in 70% ethanol extracts	carotenoids in ethyl acetate extracts
YM	triterpenoid-like and phytosterol-like compounds†	γ-tocopherol*	ellagic acid**, caffeic acid hexoside***, (−)-epicatechin gallate	–
BEYM	triterpenoid-like and phytosterol-like compounds†	α-tocopherol*, β-tocopherol*, γ-tocopherol*, δ-tocopherol*	ellagic acid**	–
FYM	triterpenoid-like and phytosterol-like compounds†	α-tocopherol*, γ-tocopherol*	–	–
BSF	triterpenoid-like and phytosterol-like compounds†	α-tocopherol*, γ-tocopherol*, δ-tocopherol*	–	–
B	triterpenoid-like and phytosterol-like compounds†	α-tocopherol*, γ-tocopherol*, δ-tocopherol*	–	–
J	triterpenoid-like and phytosterol-like compounds†	α-tocopherol*, β-tocopherol*, γ-tocopherol*	–	–
H	triterpenoid-like and phytosterol-like compounds†	α-tocopherol*, γ-tocopherol*, δ-tocopherol*	–	–
S	triterpenoid-like and phytosterol-like compounds†	α-tocopherol*, γ-tocopherol*	–	–
FR	triterpenoid-like and phytosterol-like compounds†	β-tocopherol*, γ-tocopherol*	–	β-carotene*, lutein*
DF	–	–	–	–
C		α-tocopherol*, β-tocopherol*		β-carotene, lutein

* additionally confirmed with HPLC-UV (tocopherols) or HPLC-MS (carotenoids), compared with a reference standard.
** additionally confirmed with HPTLC-MS^2, compared with a reference standard.
*** additionally confirmed with HPLC-MS^3, compared with literature data.
† tentative identification based on HPTLC R_F values and chromogenic responses that are consistent with triterpenoids or phytosterols.

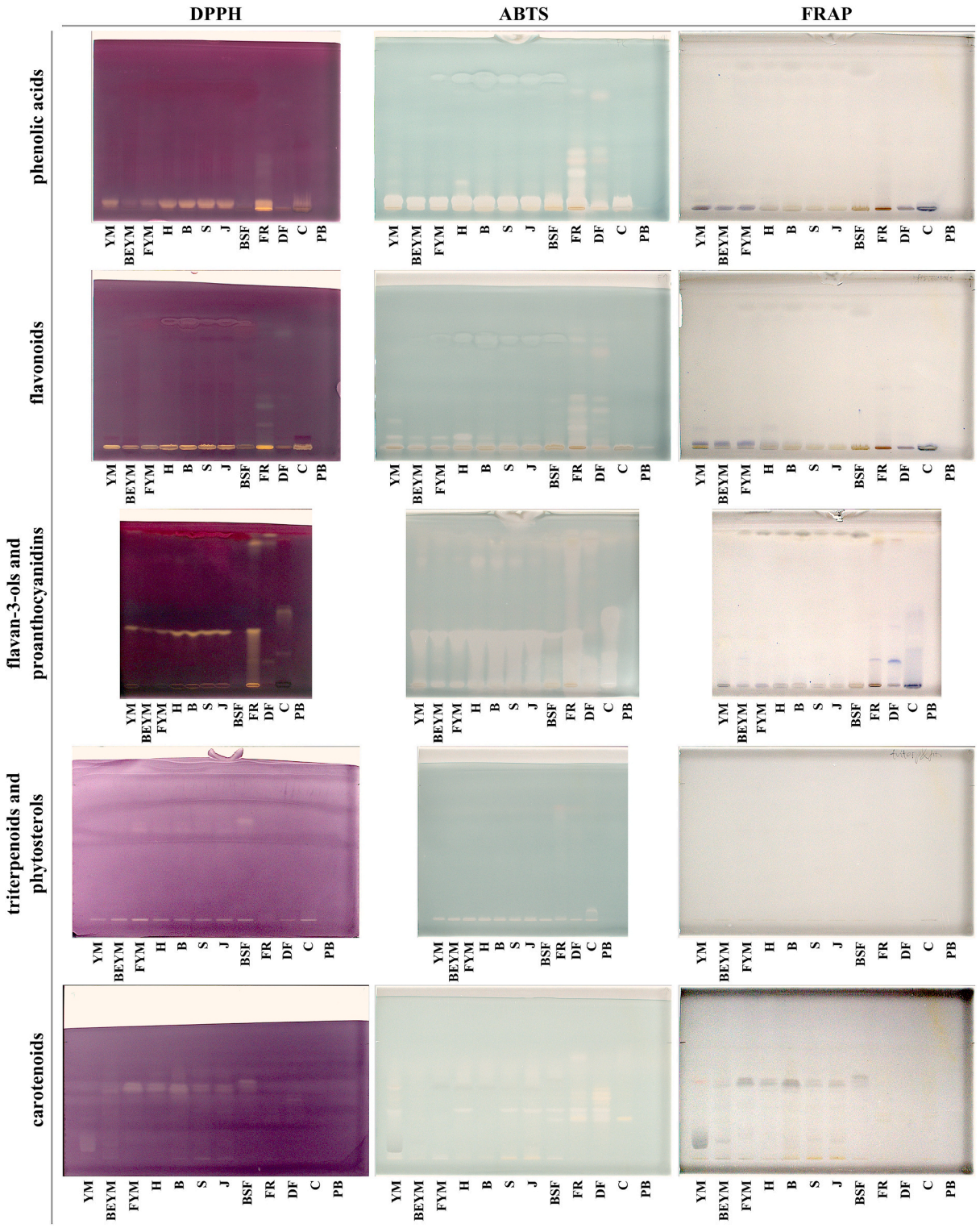

Fig. 3. HPTLC plates developed according to the original screening protocols targeting phenolic acids, flavonoids, flavan-3-ols and proanthocyanidins, triterpenoids and phytosterols, and carotenoids, derivatized with DPPH, ABTS and FRAP reagents, and captured under WhiteT. Antioxidants in the extracts are visible as yellow (DPPH), discolored (ABTS), or blue (FRAP) bands on the derivatized plates. Sample abbreviations are defined in Table 1. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

proanthocyanidins. This compound produced a strong AO band in all insect and frass 70% EtOH extracts, except in BSF, where the band was noticeably weaker (Fig. 3). These bioautography results further support the notion that phytochemicals are not the primary contributors of AO capacity observed in the tested aqueous insect extracts. The band's identity was confirmed by matching our HPTLC-MS^3 results with literature data and further confirmed with a reference standard as UA. The fraction of the HPTLC chromatogram corresponding to the band of interest was isolated from the plate and subjected to further LC-MS analysis using an orthogonal RP method for additional confirmation of analyte identity. Matching retention times, MS and MS^2 spectra of the isolate and the UA standard additionally confirmed the presence of UA

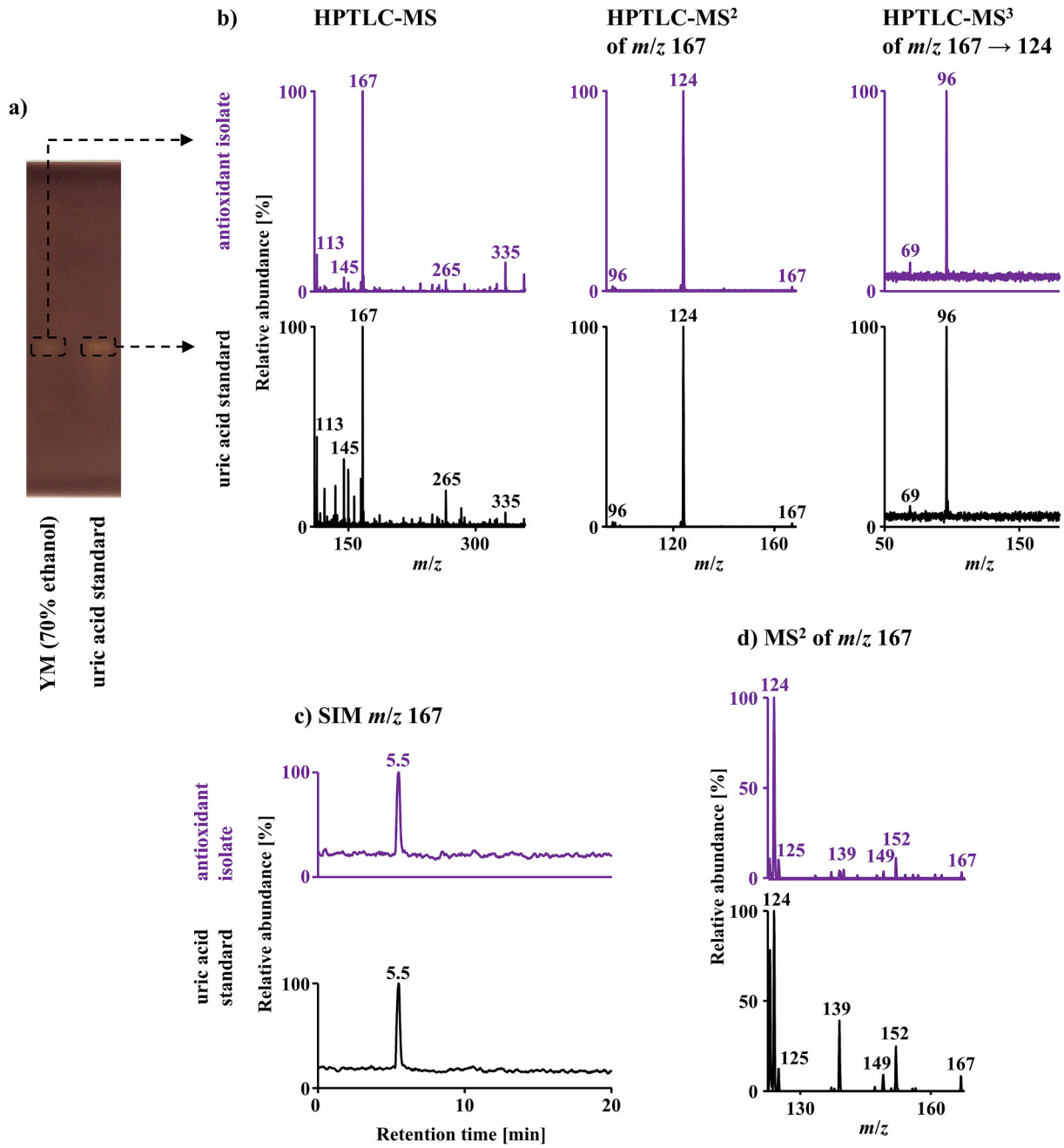

Fig. 4. HPTLC plate developed using the development solvent for the flavan-3-ol and proanthocyanidin screening (**Table S1**) and derivatized with DPPH, demonstrating matching R_F values of antioxidative bands (yellow) in the YM 70% ethanol extract and the uric acid reference standard (a); MS, MS^2 and MS^3 analyses of the antioxidative bands, acquired via HPTLC-MS^n confirm the presence of uric acid in the YM extract (b); selected ion monitoring (SIM) chromatograms at *m/z* 167 display matching retention times ($t_R = 5.5$ min) of the isolated antioxidative band from the YM extract and the uric acid reference standard (c); and the corresponding MS^2 spectra, further confirming the presence of uric acid in the YM antioxidative isolate (d). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

in the DPPH-positive (yellow) fraction (Fig. 4). Based on the band intensity, UA appeared to be the key contributor to AO capacity in 70% EtOH extracts of insects. Using HPTLC-bioautography for phenolic acids and flavonoids, UA appeared at the start of the plate with strong intensity and was confirmed by LC-MS (**Table S5**), showing it is also a major contributor to the AO effect observed in this method. The bioautographic plates used to screen for flavan-3-ols and proanthocyanidins were subjected to ImageJ densitometry to estimate the relative contribution of UA to the total bioautographic AO signal of all 70% EtOH extracts (**Table S7**). Densitometry results should be considered only as estimates, as they can be affected by concentration effects, reaction kinetics, radical-specific reactivity, and coeluting compounds. Nevertheless, ImageJ results indicate that UA accounted for 84–97% of the DPPH-derived signal and 56–72% of the ABTS-derived signal in all insect extracts except BEYM and BSF, where its respective contributions were 42% and 17%. Similarly, UA accounted for more than half of both DPPH- and ABTS-derived signals in FR (DPPH: 61%; ABTS: 76%). The estimated UA contribution varied among samples and between DPPH- and ABTS-derived signals. This variability most likely reflects both sample-specific differences in UA abundance and the presence of additional AO constituents, in addition to the densitometry-related factors discussed above. The low contribution in the ABTS-derived signal of

BEYM and BSF suggests a relatively higher contribution of other AO compounds in these samples (see Section 3.2). However, overall, the consistently high contribution of UA to the sum of AO signals across plates indicates that UA is a major AO constituent in the 70% EtOH extracts and provides support for its prominent role in the measured AO capacity.

UA is a poorly soluble metabolite of purines and can be synthesized de novo by insects. The role of UA and urate in insects is described in detail in other works (Sabolová et al., 2021; Weihrauch & O'Donnell, 2021). Most importantly, they can serve as AO compounds and as nitrogen storage compounds during periods of protein shortage. Due to its important physiological roles, UA can account for as much as 11% of the dw of silkworm (*Bombyx mori*) larvae (Weihrauch & O'Donnell, 2021) and up to 45% in termites after prolonged captivity (Potrikus & Breznak, 1980). A study (Tasaki et al., 2017) analyzed Japanese termite (*Reticulitermes speratus*) extracts by HPLC and confirmed the AO capacity of UA fraction by a post-column DPPH assay. Evidence from termites and silkworms indicates that UA contributes to oxidative stress tolerance. However, termite studies suggest that excessive UA-mediated quenching of ROS may increase susceptibility to infections (Konishi et al., 2025; Matsuo & Ishikawa, 1999).

AO systems in insects were described as early as 1995 (Felton & Summers, 1995). They list several known AO compounds in insects, ranging from enzymatic components to small molecules, such as carotenoids, ascorbic acid, various forms of vitamin E (e.g. tocopherols), UA, glutathione, and others. Except for glutathione, these compounds were examined through targeted and untargeted screenings using bioautography, but no major contributions to the overall AO capacity were observed. Notably, no band on the HPTLC plates exhibited AO capacity as consistently and intensely as the UA band. Importantly, it should not be implied that all observed AO capacity is fully explained by UA alone (see Section 3.3.2). The better solubility of UA in aqueous-based solvents likely contributed to the higher AO capacity observed in the more polar insect extracts, an observation consistent with other studies (Di Mattia et al., 2019; Navarro del Hierro et al., 2020).

3.3.2. Identification of minor AO compounds

Selected minor AO bands observed by bioautography (Fig. 3) across multiple extracts were further analyzed by HPTLC-(AO)-MS^n. The identification of these compounds offers a preliminary overview rather than an exhaustive characterization; due to the appreciable total size of AO bands across the plates, not all extracts were individually subjected to MS analysis. In the FR sample, identification of several compounds was based on literature data, while UA, syringic acid and apigenin were also detected by LC-MS^n and subsequently confirmed with a reference standard (**Table S5)**. Syringic acid and apigenin were not observed in our targeted HPTLC screenings, likely due to their low concentrations combined with the limited sensitivity of the method. Experimental data for the remaining samples are provided in **Table S6**. Our effect-directed approach reduced the number of HPTLC zones selected for identification. However, the MS analysis remained untargeted and was conducted on crude extracts with complex matrices using a low-resolution MS instrument. Additionally, compound identities were assigned only when both the precursor ion and MS^n fragmentation pattern could be confirmed using reference standards or peer-reviewed literature data. When standards were not available or no suitable literature match was found, compounds were marked as unidentified ("x"). For these reasons, several compounds in Table S5 and S6 remained unidentified.

Besides UA (discussed above), several compounds identified in frass in this study have also been reported in whole insects in previous studies. For instance, tocopherols, glutaric acid and adipic acid were found in silkworms (*Bombyx mori*) fed on mulberry leaves (Dong et al., 2017), while glutaric acid was also found in greater wax moth (*Galleria mellonella*) larvae (Gołębiowski et al., 2021). Higher concentrations of apigenin and syringic acid were observed in nut bars containing yellow mealworm compared to insect-free bars (Gumul et al., 2023), and apigenin was determined in stink bugs (*Encosternum delegorguei*) (Musundire et al., 2016). Squalene and α-tocopherol were determined alongside other compounds in yellow mealworm oil (Son et al., 2020), while α-tocopherol acetate was identified in the cuticle of soybean pod armyworm (*Spodoptera cosmioides*) (Fronza et al., 2013). The occurrence of several of the same compounds across different insect species reared under different conditions indicates that these compounds are not unique to a single species or substrate. However, their contributions to the measured AO capacity, and origins (dietary carryover, diet-derived but insect-metabolized, or endogenously produced) remain unresolved and warrant further controlled rearing studies combined with metabolism-focused analyses. This study reports, for the first time, the presence of secoisolariciresinol, glutaric acid, 4-hydroxybenzoic acid, 4-hydroxycoumarin, 1α,25-dihydroxyvitamin D_3 (calcitriol), 25-hydroxyvitamin D_2 (ergocalciferol) and ergone in frass, all supported with literature data, thereby expanding the known chemical profile of this material.

3.4. UA quantification

Water extracts of insect biomass and frass samples were analyzed for UA content because they exhibited the highest AO capacity and at the same time directly provided a suitable medium for enzymatic quantification. The obtained UA content ranged from 0.97 to 18 mg g^{-1} dw (Table 4), with BSF and H extracts showing the lowest and highest values, respectively. Yellow mealworm larvae (YM, BEYM and FYM) extracts, and cricket (H, B, S, and J) extracts contained approximately 4 to 13-fold, and 11 to 19-fold more UA than BSF, respectively. UA content in frass (FR) was more than four-fold higher than in the corresponding insect biomass (FYM).

UA content within the same species can vary vastly due to methodological factors (extraction and quantification methods), but it can also be influenced by rearing conditions and even developmental phase (Sabolová, Kulma, Škvorová, et al., 2023) and sex (Sabolová et al., 2021). Among yellow mealworm samples analyzed in this study, FYM contained approximately three-fold more UA than the other yellow mealworm samples (YM and BEYM), while the latter two did not differ significantly. YM and BEYM results fell within the reported range of 0.7 (Sabolová, Kulma, Petříčková, et al., 2023) to 4.7 (Bednářová et al., 2014) mg UA g^{-1} dw, whereas FYM exceeded these values by a factor of 2.7 to 18. Similarly, reported UA content values also vary in crickets. Values range from 3.9 (Sabolová, Kulma, Petříčková, et al., 2023) to 22.2 (Bednářová et al., 2014) mg g^{-1} dw for Jamaican field crickets, and from 4.8 (Sabolová, Kulma, Petříčková, et al., 2023) to 12.9 (Sabolová et al., 2021) mg g^{-1} dw for house crickets. In our study, the Jamaican field cricket (S) fell within the reported range, whereas the house cricket (H) exceeded previous reports by a factor of 1.4 to 3.7.

UA content in water extracts correlated strongly with AO capacities measured by DPPH ($\rho = 0.83$, $p = 0.015$) and ABTS ($\rho = 0.76$, $p = 0.037$), but only moderately and non-significantly with those measured by FRAP ($\rho = 0.64$, $p = 0.096$). These results are consistent with

Table 4
Uric acid (UA) content in the water extracts of the analyzed insects and frass. Values with different letters indicate significant differences ($p < 0.05$; ANOVA followed by Tukey's test).

Sample	UA content [mg g^{-1} dry weight]
YM	3.8 ± 0.1^{b}
BEYM	4.5 ± 0.2^{b}
FYM	12.6 ± 0.9^{c}
H	18 ± 2^{d}
B	11.8 ± 0.3^{c}
S	10.4 ± 0.1^{c}
J	11.6 ± 0.2^{c}
BSF	0.97 ± 0.02^{a}
FR	55 ± 2^{e}

bioautography on the flavan-3-ols and proanthocyanidins plate, where DPPH yielded an intense yellow band, ABTS showed discolored bands, and FRAP was negative for UA (Fig. 3). Overall, our results indicate that UA is indeed the main contributor to AO capacity in the water extracts of insects and frass, particularly for DPPH and ABTS measurements. **Fig. S4** visually assesses the correlation between UA content and AO capacity by plotting AO (expressed as UA equivalents) versus UA content in the samples. All samples fit the theoretical curve well, except for BSF, BEYM and H. BSF and BEYM water extracts showed AO capacities exceeding predictions from their UA content, especially with ABTS (9- and 2-fold higher, respectively), indicating contributions from other compounds. BSF had the lowest UA content, increasing susceptibility to matrix effects, while H extract showed lower-than-expected AO values, likely due to suppressing or interfering compounds. Although the kit is reported to be specific for UA in biological samples, such interactions cannot be entirely ruled out in complex matrices.

Humans lack uricase, making UA the final product of purine metabolism, mainly excreted via urine. Elevated serum UA (hyperuricemia) is a key risk factor for gout (Hafez et al., 2017; Pasalic et al., 2012) and is also associated with other health conditions (Pasalic et al., 2012). However, hyperuricemia does not invariably lead to gout, and gout can occur without consistently elevated serum UA, supporting the view that UA is a marker rather than the sole determinant of disease (Pasalic et al., 2012; W. Z. Zhang, 2021). UA also has AO properties and has been associated with potential neuroprotective effects (Pasalic et al., 2012; Schlesinger & Schlesinger, 2008; Tovchiga & Shtrygol", 2014; Zhang, 2021). Therefore, identifying UA as a major AO constituent in polar extracts of edible insects is relevant for dietary exposure considerations, but its health implications cannot be inferred from UA content alone.

Based on current literature, there is no compelling evidence to support the exclusion of edible insects from the human diet solely due to their UA content. In fact, in animal studies, partial substitution of the feeds of quail (Sarica et al., 2020) and broilers (Bovera et al., 2015) with yellow mealworms significantly reduced their serum UA levels (Sarica et al., 2020). In summary, current evidence on UA is contradictory; although elevated UA is linked to gout and metabolic disorders, it also possesses AO properties and may exert protective effects under certain conditions. Moreover, to our knowledge, the absorption of UA from edible insects has not yet been directly studied.

4. Conclusions

This study presents a systematic evaluation of AO capacity in insect and frass extracts, integrating multiple extraction solvents, AO assays, targeted screenings, and bioautography. This integrated strategy provides a large, cross-study comparable dataset, particularly for measured AO capacities. It also enabled identification of the compound(s) driving AO capacity in the studied samples. UA was the only AO compound consistently detected across all insect and frass samples, particularly in polar extracts, and the strong, statistically significant correlation between its content and AO capacity measured by DPPH and ABTS provides compelling evidence that it is the primary contributor to the observed effects. Other compounds were also detected, several of which were reported for the first time in specific samples, thereby expanding the relatively unexplored AO profiles of the studied insects and frass. This work highlights the value of integrating multiple analytical approaches to link chemical composition with functional activity. Future studies could examine the bioavailability, stability, and potential synergistic effects of UA and minor AO compounds, and explore strategies to enhance samples' AO capacity through improved sample preparation, rearing conditions or species selection. Overall, these findings expand the chemical and functional understanding of insect and frass AO compounds and may inform future functional-food research.

CRediT authorship contribution statement

Eva Korenčič: Writing – review & editing, Writing – original draft, Visualization, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Alen Albreht:** Writing – review & editing, Formal analysis. **Katerina Naumoska:** Writing – review & editing, Supervision, Project administration, Methodology, Formal analysis, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2026.150461.

Data availability

All data needed to evaluate the conclusions in this paper are included within the article and its Supplementary material. The raw data supporting this article will be made available by the authors upon request. References cited in the Supplementary material are listed in the article's reference list.

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