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Metabolomic analysis of secondary metabolites from Caribbean crab gills using comprehensive two-dimensional gas chromatography - time-of-flight mass spectrometry—New inputs for a better understanding of symbiotic associations in crustaceans

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ABSTRACT

Secondary metabolites are bioactive compounds produced by living organisms that can be indicative of symbiotic relationships in nature. Prior studies have demonstrated putative symbiotic interactions between decapod crabs and epibiotic bacteria in their environment. This study presents a sample preparation protocol for metabolomics analysis of lyophilized crab gill samples from four brachyuran crab species inhabiting different regions of Guadeloupe, Lesser Antilles. Sample extracts were prepared using a two-step methoximation-trimethylsilylation derivatization protocol and analyzed by comprehensive two-dimensional gas chromatography coupled to time-of-flight mass spectrometry (GC×GC-TOFMS). Three main secondary metabolites identified in the crab gills and discussed herein include cycloserine, trigonelline, and amphetamine. Cycloserine is a bacterial-produced metabolite with antibiotic properties, and along with the antimicrobial activity of trigonelline, these compounds may influence the microbial community of the crabs and/or protect them from bacterial infection in the frame of the symbiotic relationships. Amphetamine is a psychostimulant that has been previously detected in crab muscles, and has been found in crab gills in this study. Cycloserine was present in the gills of *Ucides cordatus* and *Percnon gibbesi*, trigonelline was present in all crabs except *Ucides cordatus*, and amphetamine was detected in all crab gill samples. Further research into these secondary metabolites is facilitated with the sample preparation protocol and can elucidate the various symbiotic interactions of crabs and their microbiome; potentially for other crustaceans as well.

1. Introduction

Symbiotic associations are common in the living world. These associations generally confer a benefit to at least one actor involved in such an association. The most described roles concern either nutrition or an ecological advantage for the host. For example, the jellyfish *Cassiopea xamachana* lives in association with unicellular algae belonging to the genus *Symbiodinium* which transfers 95% of the carbon it fixes by photosynthesis to the host [1]. The association between the tropical bivalve *Codakia orbicularis* and sulfur-oxidizing bacterial gill-endosymbionts allows this mollusk to colonize an ecological niche initially toxic for other eukaryotic organisms and access to 90% of the carbon fixed by its symbionts [2] as for all lucinids described. Crustaceans represent the least-studied group in terms of symbiosis. Some interactions are studied in shrimp and crabs from hydrothermal vents [3,4] even if the role of the symbiotic association is not always easy to identify. Concerning the crab *Shinkaia croisnieri* the presence of ectosymbionts is not fully justified [4] even if the authors assume a role in nutrition. Recent studies describe symbiotic relationships between crabs from African and Asian areas and epibiotic bacteria [5]. Bacterial ectosymbionts are also present on the surface of gill lamellae of two species of crabs living in the Caribbean [6]. This phenomenon seems to arise in several species but the role of such bacterial symbionts remains unknown. Secondary metabolites may be the key to understanding the role of these bacterial symbionts and their

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influence on the host. These bioactive molecules were previously considered non-essential [7,8], and although they do not pertain to primary metabolism, they have been found to play important roles in symbiotic relationships [7–9]. Interactions between marine invertebrates and symbiotic microbes have inspired further research into antibiotic discovery as several secondary metabolites produced by these microbes have antimicrobial properties [10–14]. Despite the growing attention, research on the source of these metabolites and their importance in symbiosis is limited or not widely available [15].

Gas chromatography (GC) has been widely used for studying metabolites in biological samples [16,17]. In cases when samples are not complex, metabolite separation can be optimized for GC analysis. The metabolite profiles of many biological samples, including crab gills, are far more complex, containing thousands of compounds, and thus making adequate one-dimensional separation challenging [18–21]. Comprehensive two-dimensional gas chromatography (GC×GC) is far superior compared to conventional one-dimensional GC with enhanced sensitivity, resolution, and separation power [19,20], making it an ideal technique for analyzing primary and secondary metabolites from crab gills. Metabolites from crab gill samples have been analyzed by GC coupled to mass spectrometry (MS) detection, following a chemical derivatization protocol [22–26].

Derivatization is used to increase the volatility, chromatographic performance, and thermal stability of polar metabolites [16,27]. Of the various types of derivatization methods available for GC-based analyses, the two-step methoximation-trimethylsilylation is the most widely used for metabolomics as it provides a greater coverage of metabolites containing hydroxyl, carboxyl, amine, amide, and thiol functional groups [16,17,27]. Herein, we compare the standard crab gill preparation protocol to a similar liquid extraction and chemical derivatization protocol developed for this study. We also evaluate the methods for analysis of crab gills by GC×GC coupled to time-of-flight mass spectrometry (TOFMS).

The objective of this study was to investigate the primary and secondary metabolites produced in the gills of a few crabs from a mangrove environment and a marine species living in reefs using GC×GC-TOFMS. Having an idea of the compounds associated with the gills would give information on the true relationship existing between the two partners of the association (mutualism or parasitism).

2. Materials and methods

2.1. Sites of collection

Four species of brachyuran crabs were collected at different locations in Guadeloupe (Table 1, Fig. 1), Lesser Antilles. Three species were semi-marine crabs from mangrove environment. *Aratus pisonii* (Milne Edwards,1837), a tree-climbing crab living on branches/roots of the mangrove tree *Rhizophora mangle* (Linneaus, 1753), *Minuca rapax* (Smith, 1870), and *Ucides cordatus* (Linnaeus 1763), both running and/or burrowing on mangrove sediment. The last species studied was *Percnon gibbesi* (H. Milne-Edwards, 1853) an exclusive marine crab living in coral reefs associated with the sea urchin *Diadema antillarum* (Philippi, 1845).

Table 1
Species studied and their location.

Crab species	Site of collection	Samples
Aratus pisonii (AP) ★	Manche à Eau (AP-ME)	3
	Marina (AP-M)	3
	Pointe Sable (AP-PS)	3
Minuca rapax (MR) ▲	Ilet Cochon (MR-IC)	3
	Pointe Sable (MR-PS)	3
	Manche à Eau (MR-ME)	3
Ucides cordatus (UC) ■	Goyave (UC-G)	3
Percnon gibbesi (PG) ●	Port-Louis (PG-PL)	3

2.2. Sample preparation and analysis

At least three individuals per site were used and cold anesthesia was applied to all individuals before gill dissection from the cavity with forceps. The gills of each individual were weighed (minimum of 0.1 g of gill tissue) before being placed into microtubes. For small species, two individuals were used to reach the minimum weight of 0.1 g of gill tissue. All gill samples were then lyophilized and stored at 4 °C for 128 days prior to extraction for analyses by GC×GC-TOFMS. A preliminary study was conducted to compare the current standard crab gill sample preparation method [22,23,28] and a method inspired by metabolomic analyses of other aquatic organisms [29–32] in order to maximize the metabolite coverage (Supplementary Information 1). The selected method is described below. In total 24 samples were analyzed in this study in addition to a pooled quality control (QC) sample, and quality assurance (QA) samples, including sample replicates, instrument blanks and reagent blanks. Fig. 2 shows the sample preparation workflow diagram.

2.3. Chemicals

HPLC grade methanol (>99.9%, Millipore Sigma, Canada), ACS grade chloroform (Fisher Scientific, Canada), and 18.2 MΩ deionized MilliQ water from an Elga PURELAB Flex 3 system (VWR International, Canada) were used for the extraction solvent mixture. Internal standard (IS) was 4^{13}C methylmalonic acid (Millipore Sigma, Canada). Cycloserine (Millipore Sigma, Canada) was used for positive identification of this metabolite (SI1). Anhydrous sodium sulfate (Millipore Sigma, Canada) was used to dry HPLC grade toluene (Millipore Sigma, Canada) prior to derivatization. The derivatization reagents were methoxyamine hydrochloride (Millipore Sigma, Canada) prepared in HPLC grade pyridine (Millipore Sigma, Canada), and N-Methyl-N-(trimethylsilyl) trifluoroacetamide + 1% trichloromethylsilane (MSTFA + 1% TMCS) (Fisher Scientific, Canada).

2.4. Extraction

The 24 lyophilized crab gill samples were weighed (11–12 mg) into 2 mL Eppendorf SafeLock tubes (Eppendorf Canada Ltd., Canada) for extraction. Three beads (2.38 mm metal beads, Qiagen, Canada) and 30 µL of the internal standard 4^{13}C methylmalonic acid (100 mg/L in water) were added to each sample prior to extraction. The extraction solvent for each sample consisted of 600 µL MeOH:H_2O (4:1 v/v) and 120 µL of $CHCl_3$. The samples were homogenized by bead beating for 2 min after addition of the solvents, followed by vortexing for 2 min and sonication for 10 min to extract. The extracts were centrifuged at 10,000 g for 10 min, and 300 µL of the supernatant was transferred to 2 mL vials (Chromatographic Specialties Inc., Canada) and dried at 40 °C under nitrogen (099A EV2412S Glas-Col Heated Analytical Evaporator, Cole-Parmer, Canada) until dry, followed by storage at −80 °C prior to derivatization.

2.5. Derivatization

A 100 µL aliquot of toluene dried with anhydrous sodium sulfate was added to each of the extracts which were then dried under nitrogen at 50 °C to remove any traces of water. The samples were derivatized in two steps: methoximation followed by trimethylsilylation. Methoximation was performed by adding 50 µL of methoxyamine hydrochloride (20 mg/mL in pyridine) into each sample vial and vortexing for 10 s. Samples were incubated for 1 h at 60 °C then cooled for 5 min. Trimethylsilylation was achieved by adding 80 µL of MSTFA + 1% TMCS to each sample and vortexing for 10 s, samples were incubated for 1 h at 40 °C. After a 5 min cooling, the derivatized samples containing a white precipitate (due to salt) were transferred to 300 µL glass insert vials (Chromatographic Specialties Inc., Canada) and centrifuged at 1000 g

Fig. 1. Sites of collection of the four studied crab species. Map of Guadeloupe indicating major landmarks and locations where crabs were obtained.

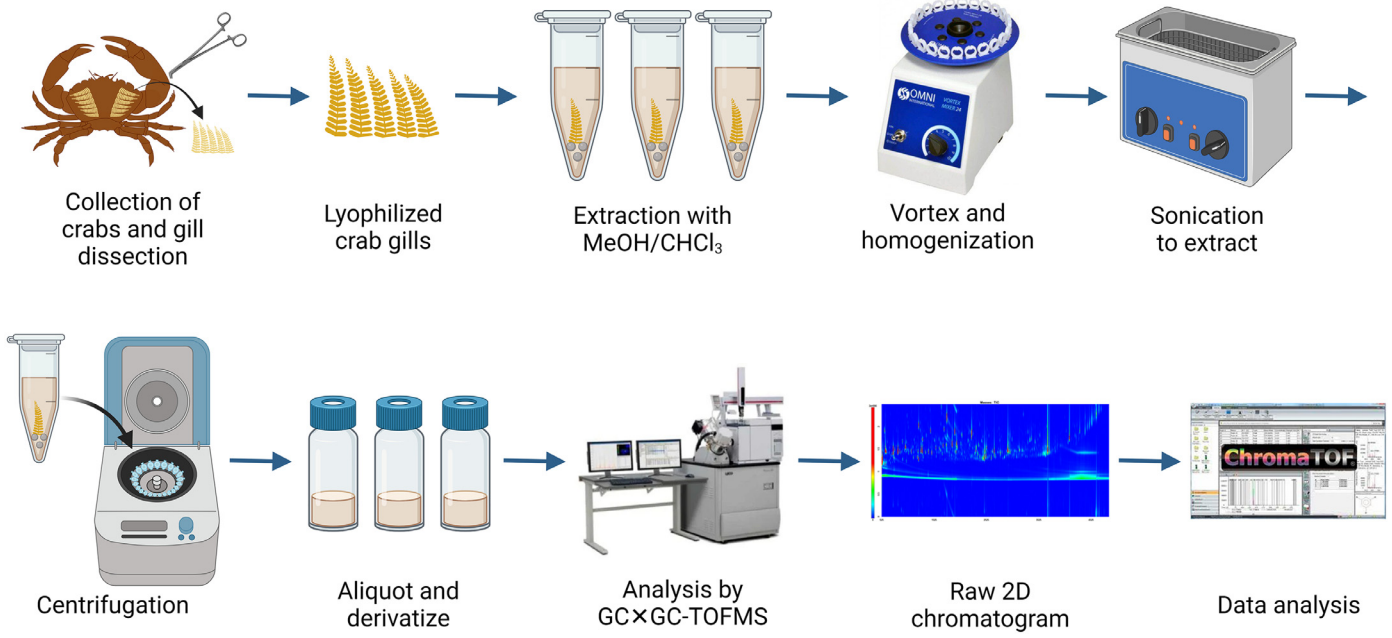


Fig. 2. Workflow diagram of the lyophilized crab gills analyzed by GC×GC-TOFMS. Created with BioRender.com

for 1 min. The supernatants were transferred to new glass insert vials for analysis.

2.6. GC×GC-TOFMS method

The analyses were performed using a LECO Pegasus 4D GC×GC-TOFMS (LECO Instruments, USA) with a cooled injection System (Gerstel, USA) and liquid injection using a MultiPurpose Sampler (MPS; Gerstel, USA). The first-dimension column was a 60 m × 0.25 mm × 0.25 µm Rxi-5SilMS, and second-dimension a 1.2 m × 0.25 mm × 0.25 µm Rtx-200MS (Chromatographic Specialties, Canada). Ultra-pure helium (5.0 grade; Praxair Canada Inc., Canada) was used as the carrier gas, with a constant flow rate of 2.0 mL/min. Injection was splitless, using a split/splitless liner (Chromatographic Specialties, Canada), and an injection volume of 0.5 µL with the inlet temperature of 250 °C. The instrument conditions were used as described by Nam et al. for the GC and MS methods [33]. Briefly, the temperature program of the primary oven began at 80 °C, held for 4 min, followed first by a ramp of 3.5 °C/min to a temperature of 315 °C held for 10 min. The secondary oven and modulator temperature offset were constant at +10 °C and +15 °C respectively. The modulation period was 2.5 s. Mass spectra were collected at an acquisition rate of 200 Hz over a mass range between 40 and 800 m/z. The detector voltage was 1700 V with an electron impact energy of −70 eV. The ion source temperature was 200 °C and the transfer line temperature was 225 °C.

2.7. Data processing and analysis

GC×GC-TOFMS data were processed using ChromaTOF® (v.4.72; LECO Instruments, USA). The baseline offset was set to 0.9 above the middle of the noise. The minimum S/N ratio for base- and sub-peaks were set at 100:1 and 6:1, respectively, and the mass spectral match re-

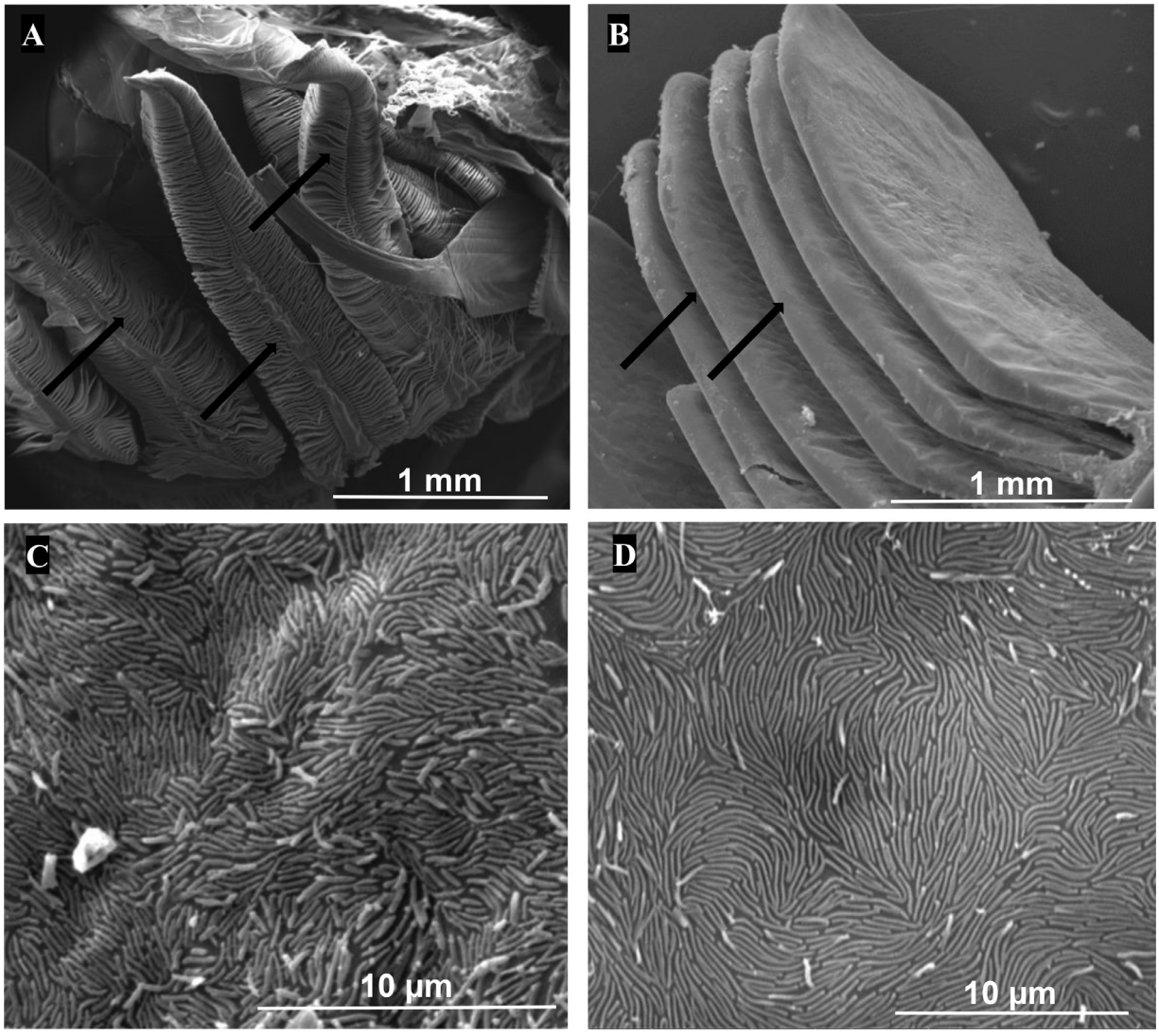


Fig. 3. Ultrastructure of the gills. SEM views of phyllobranchia and bacterial biofilm covering the gill lamellae of *Percnon gibbesi* and *Ucides cordatus.* **A.** Low magnification of several phyllobranchia (black arrows) of *Percnon gibbesi* **B.** Gill lamellae of *Ucides cordatus* with a different shape (black arrows). Epibiotic bacteria covering the gill filaments are represented mainly by rod-shaped bacteria either on *Percnon gibbesi* (**C**) or on *Ucides cordatus* (**D**).

quired for the sub-peaks to be included in the auto-smoothed peak was set at 650. Expected peak widths throughout the entire chromatographic run were assumed to be approximately 12 s in the first dimension and 0.15 s in the second dimension. Chromatograms were processed using a data processing method to find all peaks with S/N > 100 and all chromatographic peaks were searched against the NIST-MS 2017 Libraries.

Statistical Compare features of ChromaTOF® were used to align the peak tables based on retention times and mass spectra. The retention time match criteria were set to 5 modulation periods apart in the first dimension (12.5 s) and 0.2 s in the second dimension to allow for minor shifts in retention times occurring between samples. The minimum similarity spectral match to combine sub-peaks was set to 600 for all m/z values with abundance greater than 1% of the base ion. The peak tables present the values of S/N and area for each peak. Peak areas were normalized by sample weight. ChromaTOF® scripting tools were also used to process the chromatograms to find families of compounds.

Additional metabolomic data analysis was conducted using Microsoft Excel 2013 and consisted of selecting metabolites of interest.

The samples were evaluated by the number of peaks, common peaks, total peak area (TPA), total useful peak area by class (C-TUPA), and tentative identifications of metabolites were based on mass spectral information and first-dimension retention index (RI). The number of peaks refers to the number of identified compounds in the samples after data alignment, and the common peaks were determined by sample class. The TPA provides a sum of all the signals for each sample, while TUPA is the sum of all the signals obtained from common peaks in all the samples [33]. Class-TUPA was used for these samples where a class was assigned for each crab species, as well as each crab species and the location they inhabit, and the sum of signals was obtained from the common signals within each class [34].

2.8. Ultrastructural analysis of gill tissues

Gills from *P. gibbesi* and *U. cordatus* were prepared as described by Béziat et al. [6]. Gill samples were extracted from the gill cavity and fixed in 2.5% glutaraldehyde in 0.8x PBS buffer (pH 7.2). They were dehydrated in a series of acetone solutions (30°, 50°, 70°, 90°, and 3× 100°), then dried to the critical point at 31 °C and 74 bars. The dried gills were sputter-coated with gold before observations with a FEI Quanta 250 electron microscope at 20 kV.

Table 2
Comparison among sample locations.

			Number of peaks		TPA		C-TUPA	
Class	Number of samples	Common peaks	Average	RSD (%)	Average	RSD (%)	Average	RSD (%)
AP-ME	3	1738	2589	8	1.26E+08	26	8.94E+07	28
AP-M	3	1500	2325	5	8.63E+07	15	5.59E+07	16
AP-PS	3	1268	2240	15	9.04E+07	33	5.41E+07	37
MR-IC	3	1583	2237	4	8.36E+07	9	5.99E+07	5
MR-PS	3	1636	2350	4	9.89E+07	4	7.17E+07	5
MR-ME	3	1920	2506	2	1.03E+08	8	8.26E+07	1
UC-G	3	1705	2280	2	1.08E+08	12	9.43E+07	12
PG-PL	3	1323	2094	7	7.71E+07	16	4.78E+07	16

Table 3
Comparison among sample species.

			Number of peaks		TPA		C-TUPA	
Class	Number of samples	Common peaks	Average	RSD (%)	Average	RSD (%)	Average	RSD (%)
AP	9	854	2384	11	1.01E+08	30	4.41E+07	32
MR	9	1089	2364	6	9.51E+07	11	4.89E+07	13
UC	3	1705	2280	2	1.08E+08	12	9.43E+07	12
PG	3	1323	2094	7	7.71E+07	16	4.78E+07	16

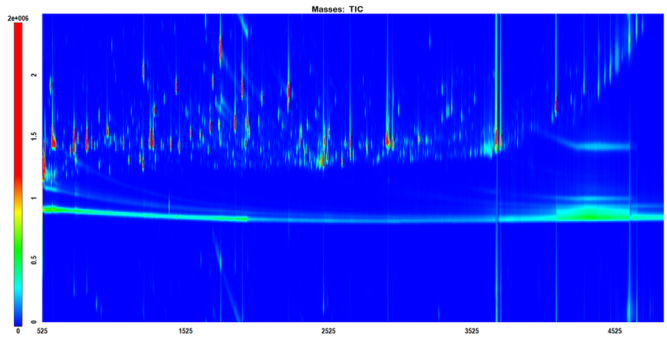


Fig. 4. GC×GC-TOFMS contour plot of a derivatized crab gill sample.

3. Results

3.1. Gill structure

Phyllobranchia (gill type of brachyuran crabs) of *Ucides cordatus,* the semi-marine crab from mangrove, and *Percnon gibbesi,* the exclusively marine crab, were checked for the presence of bacteria. The presence of rod-shaped bacteria on the gill lamellae surfaces for both species was confirmed (Fig. 3), similarly to the already described species *A. pisonii* and *M. rapax* [6].

3.2. Metabolomics results

GC×GC-TOFMS is a powerful tool used in metabolomics because of its superior ability to separate and identify compounds in complex samples, thus it was chosen as the platform to perform the analysis of the crab gills. In this analysis, more than 2000 peaks were detected in a typical derivatized crab gill chromatogram (Fig. 4). The majority of the peaks were primary metabolites, including amino acids, sterols, fatty acids, and sugars (SI2 Table 4). Several secondary metabolites, mainly terpenoid compounds, were tentatively identified using the mass spectral information from the peaks, the library match, and retention indices (SI2 Table 1).

After peak table alignment, the 24 samples presented a total of 3797 aligned peaks; the number of peaks having increased due to the difference in metabolites in each sample. Table 2 shows the results for each group of samples by location. The average number of peaks varied from 2094 to 2589 peaks, with common peaks representing between 57% and 71% of the total number of peaks. The lowest number of peaks was presented by *AP-Pointe Sable* samples along with the higher RSD% (15%). The locations that presented lower RSD% in TPA and TUPA were Ilet Cochon, Pointe Sable and Manche à Eau where the species *Minuca rapax* (MR) was found. On the other hand, the highest RSD% were observed for Manche à Eau, Marina and Pointe Sable for the species *Aratus pisonii.*

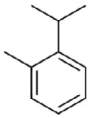
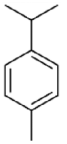
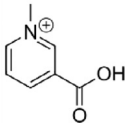
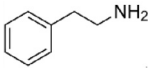
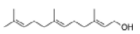
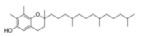
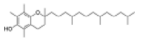
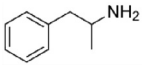
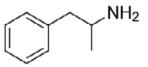
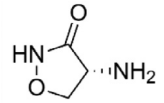
Table 3 shows the results by crab species. The average number of peaks was also calculated for the total number of detected peaks, TPA and TUPA. Comparing the average number of peaks detected by species, no significant differences were observed between the four species. However, the common peaks presented differences from 854 common peaks (*AP*) to 1705 peaks (*PG*). These results can be explained by the high variability presented by the *Aratus pisonii* samples amongst the different locations. These crab samples presented the higher RSD% in TPA and TUPA. The results were consistent with Table 2. The lowest RSD% for TPA and TUPA was obtained with the *Ucides cordatus* samples.

Crab gills are complex samples with a wide variety of metabolite families. The objective of this study was to develop a method to extract, identify and relatively quantify secondary metabolites of crab gills, because of their importance in symbiotic relationships [35,36]. Using the method described above, we tentatively identified 17 secondary metabolites using mass spectral information from each peak and RI, and positively identified cycloserine in the crab gills by injection of a pure standard. Table 4 shows average retention times from the secondary metabolites in the first- and second-dimensions, RI, and library match. The peak areas normalized by weight for each sample are included in the supplementary information (SI2 Table 1). Furthermore, the variability by location of 10 secondary metabolites was analyzed. Fig. 5 shows scatter plots of these 10 metabolites plotted against the log (for visualization) peak areas normalized by weight.

4. Discussion

A couple of recent studies [5,6] showed the presence of ectosymbiotic bacteria covering gill lamellae surface as observed here for *P. gibbesi* species and *U. cordatus* (Fig. 3). Hence, nothing is known yet about possible exchanges of metabolites between the crustacean's host and its bacteria. In order to explore the interaction between the host crabs and the bacteria, the present manuscript describes a method to analyze the metabolites of crab gill samples allowing us to identify a high number of different primary and secondary metabolites (≈2000). Different families

Table 4
Selected tentative secondary metabolites of crab gills.

Metabolite	Type	Structure	1st Dimension Time (s)	2nd Dimension Time (s)	Library RI	Exp. RI	Library match
o-Cymene	monoterpenoid		618.50	1.3525	1015	1016	799
p-Cymene	monoterpenoid		673.056	1.31667	1043	1044	832
Trigonelline TMS derivative	alkaloid		1725.33	1.74804		1521	808
Phenethylamine 2TMS derivative	monoamine alkaloid		1840.87	1.43978	1568	1579	858
10C monoterpenol*	monoterpenol		1849.17	1.30083		1583	
10C monoterpenol*	monoterpenol		1911.07	1.27643		1614	
10C monoterpenol*	monoterpenol		1919.76	1.27476		1619	
10C monoterpenol*	monoterpenol		2017.95	1.31182		1671	
10C monoterpenol*	monoterpenol		2018.57	1.30333		1671	
10C monoterpenol*	monoterpenol		2041.62	1.27412		1683	
Farnesol TMS derivative	acyclic sesquiterpenol		2267.14	1.30143	1800	1807	694
15C sesquiterpenol	sesquiterpenol		3337.25	1.4635		2522	
γ-Tocopherol TMS derivative	tocopherol		3927.50	1.565	2987	2952	726
α-Tocopherol TMS derivative	tocopherol		4083.53	1.6481	2999	3054	790
30C pentacyclic triterpenoid	pentacyclic triterpenoid		4329.91	2.03554		3208	
Amphetamine TMS derivative	Amine		1176.47	1.31966	1298	1268	708
Amphetamine 2TMS derivative	Amine		1414.05	1.475	1394	1374	721
Cycloserine 3TMS derivative	bacterial metabolite, broad spectrum antibiotic		1409.50	1.48833	1445	1372	717

* The monoterpenols are based on borneol or farnesol, but the retention index did not agree. The mass spectra had some similarities, but ultimately did not correspond to the same molecules.

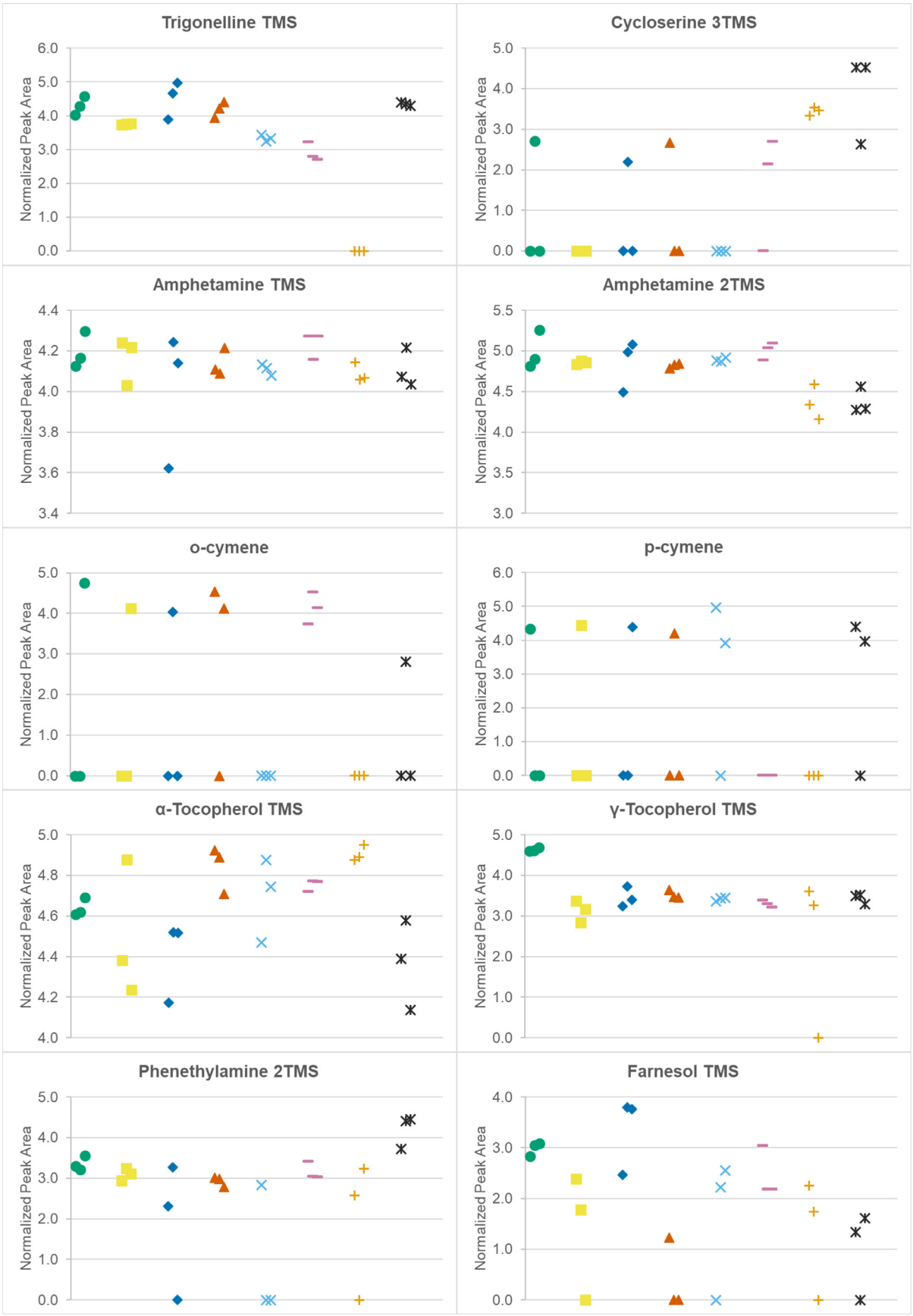


Fig. 5. Scatter plots of secondary metabolites. Normalized peak areas are plotted on logarithmic scale. ●AP-ME, ■AP-M, ◆AP-PS, ▲MR-IC, ✕ MR-PS, – MR-ME, + UC-G, ✱ PG-PL. Normalized peak area values are available in SI Table 1.

of primary metabolites were identified, including amino acids, sterols, fatty acids, and sugars.

Our research focuses on the secondary metabolites and their importance in symbiotic relationships [35,36]. This study detected and tentatively identified the presence of several bioactive secondary metabolites in the different crab gill samples. These secondary metabolites included terpenoids (*o*- and *p*-cymene) [37], derivatives of vitamin D (α- and γ-tocopherols) [38], etc. However, in the present manuscript we focused our attention on three bioactive secondary metabolites: cycloserine, trigonelline and amphetamine. The interest in amphetamine is justified by previous studies describing the biosynthesis of this compound by crabs [39].

Cycloserine is an antimicrobial molecule produced by bacteria belonging to the *Streptomyces* genus and by strains of *Pseudomonas fluorescens* Migula [40] used for the treatment of tuberculosis disease (*Mycobacterium tuberculosis*). It is has been isolated from *Streptomyces orchidaceus* [41] and, according to Li et al. [42], it is more effective against gram-positive than gram-negative bacteria. Cycloserine inhibits bacterial cell wall synthesis. *Pseudomonas* are gram-negative bacteria belonging to Gamma-proteobacteria group while *Streptomyces* are gram-positive bacteria belonging to the Actinobacteria group. These genera were not detected from *A. pisonii* and *M. rapax* communities according to recent metabarcoding analysis [6]. Main bacteria on gills of these two crab species are mostly a community of gram-negative bacteria composed of Alphaproteobacteria, Bacteroidetes and Actinobacteria [6]. The composition of bacterial communities colonizing the gills of the two other species, *Percnon gibbesi* and *Ucides cordatus,* are not yet known. Although the bacteria are not yet identified, they are likely the only source of the cycloserine secretion in gill filaments of crustaceans. Nearly no cycloserine (or in very low levels) could be detected from individuals of *Aratus pisonii* and *Minuca rapax,* from Marina and Pointe Sable sites respectively. This may be related to a weak production or decreased bacterial presence on the gill filaments from these two sites. However, it was found present in UC-G and PG-PL, this last one with a higher level relative to UC-G. Since genus *Pseudomonas* and *Streptomyces* were not present in certain crab species (*A. pisonii* and *M. rapax*), it will be necessary to identify the bacteria responsible for cycloserine production in these species.

The second antibiotic compound, trigonelline, was present in all AP, MR and PG individuals regardless of the sites. Samples from UC did not show the peak. AP individuals showed the highest amount, with individuals from the Pointe Sable location having the highest abundance as well as the largest variation. AP-M has the most consistent level of trigonelline. PG samples showed the second highest level and the MR samples the third. This compound is naturally produced by the plant *Trigonella foenum-graecum* [43], and has been found to be naturally present on crustaceans as in the shrimp *Sergestes lucens* and *Penaeus japonicus* [44]. It is also described as a urinary metabolite from the marine crab *Callinectes sapidus* and present in the soft tissues of the mud crab *Panopeus herbstii* [45]. In the present paper, regardless of the biotope, it is found on all four species analyzed: the arboreal crab *A. pisonii*, the mud crabs *M. rapax* and *U. cordatus,* and the exclusively marine crab *P. gibbesi*. It has been reported that trigonelline has an antibacterial effect on some bacterial strains [43] and antifungal activity [46] on *Candida albicans*. Hence, we suspect the production of this compound could have an impact on the selection of the microbial community of each crab species. In fact, each species of crab seems to have their own community [6].

Finally, amphetamine was found in all species and locations. Studies have demonstrated that such a compound has psychostimulant effects on invertebrates such as crayfish [47] and could be naturally present on crab muscle as volatile compounds [39]. This compound can also be found in the environment through the release in sewage waste water [48]. Amphetamine was found in crabs from all sites regardless of the ecology of the crabs studied. It is unlikely that amphetamine is present at all collection sites, and we suppose that this compound comes from the same biosynthetic pathway that Yuan and collaborators described for the muscles [39]. The only difference found was UC-G and PG-PL presented a lesser amount of amphetamine compared to the rest of the samples. Currently, this hypothesis cannot be confirmed because we have no data concerning the analysis of wastewater or other bodies of water around Guadeloupe for this or other drugs and their metabolites. Further research will aim to identify the origin of the amphetamine by analyzing other crab tissues, including crab muscle, hepatopancreas, and kidneys. The presence of amphetamine in some of these other tissues could be indicative of its production by the crabs, or suggestive of bioaccumulation if it is found in all organs of the crabs.

5. Conclusions

This study presented a method for metabolomics analysis of lyophilized crab gill samples using GC×GC-TOFMS. The developed sample preparation protocol is applicable for analyses of crustacean samples by GC-based methods and will allow for more in depth analyses on their physiology focusing on primary and secondary metabolites, and their role in symbiotic relationships. Using this protocol, further analyses on crab tissue samples can be conducted to reveal the localization of bioactive secondary metabolites within the crab tissues and further enhance our knowledge of the many symbiotic interactions between crabs and bacteria.

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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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.jcoa.2022.100069. The raw and processed data files associated with this work will be publicly available on the Federated Research Data Repository at https://doi.org/10.20383/103.0657.

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