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OPEN Efficiency of MALDI-TOF MS at identifying and discriminating immature stages of *cimex lectularius* and *cimex hemipterus* bed bugs

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Over the last two decades, an increase in bed bug infestations has been observed worldwide. Although their definitive role as vectors of infectious agents has not yet been demonstrated, bed bugs have a direct effect on human health through dermatological reactions to their bites and psychological disorders linked to domestic infestations. In this study, the effectiveness of using MALDI-TOF MS to correctly identify these two bed bug species at immature stages was assessed, as well as its effectiveness as discriminating between the immature stages (IS) of *C. lectularius* and *C. hemipterus* and their associated developmental stages. A total of 305 specimens were subjected to MALDI-TOF MS analysis, including 153 *C. lectularius* (28 eggs and 25 nymphs per stage from IS1 to IS5) and 152 *C. hemipterus* (27 eggs and 25 nymphs per stage from IS1 to IS5). MALDI-TOF MS analysis enabled us to obtain 84.97% (130/153) of high-quality MS spectra in terms of reproducibility and profile intensity. Twenty-four spectra including two per stage, from egg to IS5, and per bed bug species - were added to our in-house MS reference arthropod spectra database. All specimens were correctly identified at the species level, independently of the developmental stage, with log score values (LSVs) ranging from 1.75 to 2.79 (mean = 2.29 ± 0.12) and 1.81 to 2.71 (mean = 2.37 ± 0.03) for *C. lectularius* and *C. hemipterus*, respectively. MALDI-TOF MS correctly classified 53,33% (104/195) of the *Cimex* at the correct immature stage. Conversely, an accurate comparison of the profiles with a Genetic Algorithm model underlined that grouping the immature stages in two groups, early (IS1-IS2) and late (IS3-IS4-IS5), made it possible to obtain a cross validation (CV) and recognition capability (RC) greater than 92% and 94%, respectively, for both species. This study holds great promise for the management of bed bug infestations.

Keywords Bed bugs, *Cimex lectularius*, *Cimex hemipterus*, MALDI-TOF MS, Entomology

Bed bugs, including *Cimex lectularius* and *C. hemipterus*, the temperate and tropical bed bugs, respectively, are obligate hematophagous insects belonging to the Arthropoda phylum, order Hemiptera, family Cimicidae. These small, flattened, oval, brown, wingless insects are obligate parasites, laying eggs which emerge into five immature and one adult stage. Blood meals are necessary for their development cycle^{1,2}. *Cimex lectularius* is known to be a cosmopolitan species which is mostly frequent in temperate zones. *Cimex hemipterus* is widespread in tropical zones. However, a cohabitation of these two species exists and has already been observed in certain tropical and temperate regions including the United States³, South Africa⁴, West Africa⁶, and Asia^{7,8}.

In recent years, a sharp increase in bed bug infestations has been observed on almost all continents⁹. These infestations, which have become a significant public health problem, have also been accompanied by the description for the first time of certain species in areas not previously documented for their presence, it is the case of *C. hemipterus* in Italy¹⁰, France¹¹, Sweden¹², and Russia¹³. Despite the increasing numbers of bed

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bug infestations worldwide, and the various microorganisms that have been detected in these arthropods, the vectorial capacity to transmit disease agents to humans has not been yet demonstrated¹⁴. However, experimental studies have revealed the viable presence of *Bartonella quintana* and *Borrelia recurrentis* in *C. lectularius* faeces, suggesting the possibility of the stercoral transmission of these two bacteria by these bed bugs^{15,16}. In a study published in 2020, the identification of hepatitis C virus in a pool of *C. lectularius* immature stage, recently fed with blood from domestic residents, has also raised the question of transmission of this human virus¹⁷. Although there are currently no demonstrated cases of transmission of any infectious agents to humans by *Cimex* sp., bed bug infestations remain a public health scourge. In addition to skin lesions following bites, psychological disorders such as anxiety, nervousness, and insomnia in relation to home infestations are common^{2,18}. Determining appropriate measures to control such infestations depends upon the early identification of these species.

The morphological identification of bed bugs remains the main method of discriminating between these species. Although it is relatively easy to distinguish these two species at adult stage, at immature stages (IS), their correct distinction remains complex, even for qualified entomologists, as does discrimination between the immature stages. Recently published reports based on the morphometry of the head, antenna and pronotum of these IS have led to imperfect classification^{19,20}. Other relevant methods, such as molecular biology, can be used for species identification²¹. However, the cost of running the facilities and the time required to obtain molecular results are barriers to it becoming widely used²².

Since the beginning of the 2010s, the use of matrix-assisted laser desorption-ionization mass spectrometry (MALDI-TOF MS) has become a hot topic in entomological studies²³. This innovative proteomic tool has repeatedly demonstrated its ability and efficiency at reliably identifying different groups of arthropods at different development stages, including adult stages^{21,24–27}, immature stages^{28,29}, and eggs³⁰. One recent study demonstrated the reliability of this tool at distinguishing between the adult stages of two species of bed bugs, *C. lectularius* and *C. hemipterus*²⁶. More recently, another study reported the efficiency of this proteomic tool at differentiating between adult and immature stages of *C. hirundinis*³¹, another bed bug species.

Based on these previous studies, the aim of our work was to evaluate the effectiveness of MALDI-TOF MS at correctly identifying two human bed bug species, *C. lectularius* and *C. hemipterus*, at immature stages (nymphs and eggs). Subsequently, we wanted to see whether this tool could also discriminate between the stages of development within each species.

Materials and methods

Bed bug rearing and sampling of immature stages

The strains of *C. lectularius* (London) and *C. hemipterus* (Kenya) (eggs and immature stages from stage 1 (IS1) to stage 5 (IS5)) used in this study were obtained from Cimex Store (Chepstow, UK) and have been maintained in our laboratory since 2015 and 2018, respectively. These strains are reared in our laboratory in incubators, maintaining humidity at 70% ± 10% with a temperature of 25 °C ± 2 °C, and a photoperiod of 12 h of light and 12 h of darkness. The different stages of bed bugs were fed twice a week with human blood obtained from the *Etablissement Français du Sang* (EFS) in Marseille. Two millimetres of blood was placed in an artificial feeding machine (Hemoteck 5W1, Discovery Workshops, Washington, UK) covered with an artificial parafilm membrane (Sigma-Aldrich, St Louis, Missouri, USA), as previously described¹⁶. For egg production, upon each engorgement, three females and two males were transferred to a new tube labelled with the date of engorgement, and were monitored daily until egg laying. The eggs were then collected and transferred to another tube for daily monitoring of hatching. IS1 samples were transferred individually into a 15 mL Falcon tube, replacing the conical bottom with a net through which they were fed until the IS1 moulted into IS2, and then IS3, IS4 and IS5. To avoid potential bias associated to rearing or sample preparation, the experiments were repeated at two time independently, per *Cimex* species. At each moult, fasting IS were isolated from day three and stored at -20 °C for MALDI-TOF MS studies. Supporting photographs of each IS were taken by scanning electron microscope images (TM 4000Plus, Hitachi, Japan) and a digital microscope (Fig. 1A and B).

Sample preparation and MALDI-TOF MS analysis

The head and thorax of each specimen was separated from the rest of the body under a binocular loupe (Leica M80, Leica, Nanterre, France). After dissection, head and thorax of each specimen was immersed in distilled water for two-and-a-half minutes and then in a 200µL volume of homogenisation buffer, composed of a mixture of 70% (v/v) formic acid (Sigma-Aldrich, Lyon, France) and 50% (v/v) acetonitrile (Fluka, Buchs, Switzerland). Subsequently, were then homogenised in a 1.5mL Eppendorf tube containing 1.0 mm diameter glass beads³¹ and 15µL of the same mix solution, unlike the adult specimens whose heads were used, as described previously^{26–31}. Individual samples were homogenised using a TissueLyser (Qiagen, Hilden, Germany) with the parameter of three cycles of one minute per cycle at 30 Hz, and 1.0 mm diameter glass beads in 15µL of homogenisation. After homogenisation, the samples were centrifuged at 2000×g for one minute and 1 µl of the supernatant of each sample was deposited on the MALDI-TOF MS steel target plate in quadruplicate (Bruker Daltonics, Wissembourg, France). After air drying, 1µL of matrix solution, consisting of saturated α-cyano-4-hydroxycinnamic acid (Sigma-Aldrich, Lyon, France), 50% (v/v) acetonitrile, 2.5% (v/v) trifluoroacetic acid (Sigma-Aldrich, Dorset, UK), and HPLC grade water was added. To control the quality of the matrix (i.e. the absence of MS peaks due to impurities in the matrix buffer) and the performance of the MALDI-TOF MS apparatus, the matrix solution was loaded in duplicate onto each MALDI-TOF plate alone and homogenates from the legs of the laboratory specimen of *Aedes albopictus* were used as a positive control.

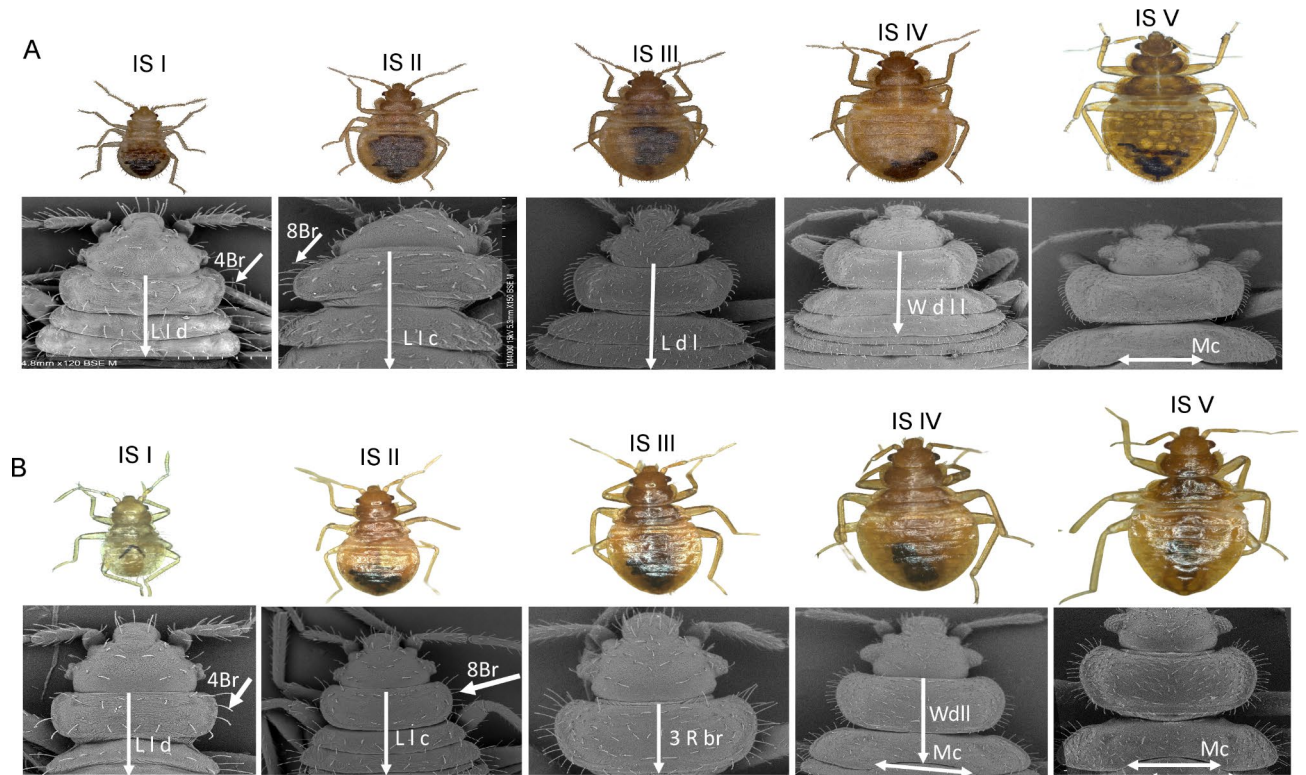


Fig. 1. Scanning electron microscope images (TM 4000Plus, Hitachi, Japan) and Digital microscope of the different immature stages (A): *Cimex lectularius* and (B): *Cimex hemipterus*. IS, Immature stage; Br, Bristles; Rbr, Rows of bristles; Mc, Curved mesonotum; Llc, Longitudinal line continuous; Lld, Longitudinal line discontinuous; Ldll, Less developed longitudinal line; Wdll, Well developed longitudinal line.

Analysis of MS spectra

Protein MS spectral profiles were obtained using a MALDI-TOF Microflex LT mass spectrometer (Bruker Daltonics, Germany), with linear mode detection of positive ions at 50 Hz laser frequency in the 2–20 kDa mass range. Acceleration voltage was 20 kV and extraction delay 200 ns. Each spectrum corresponds to the ions obtained from 240 laser shots taken in six regions of the same spot and acquired automatically using AutoXecute in Flex Control v2.4 software (Bruker Daltonics). The MS spectral profiles were first checked visually with the flexAnalysis v3.3 software (Bruker Daltonics). The MS spectra were then exported to ClinProTools v2.2 and MALDI-Biotyper v3.0. (Bruker Daltonics) for data processing (smoothing, baseline subtraction, peak detection). The reproducibility of the MS spectra was assessed by comparing the main spectrum profiles (MSP) obtained from the four spots from each sample with the MALDI-Biotyper v3.0 software (Bruker Daltonics). The reproducibility and specificity of the spectra were assessed by unsupervised statistical tests of the MS spectra, including principal component analysis (PCA), cluster analysis (MSP dendrogram) using the ClinProTools v2.2 and MALDI-Biotyper v3.0. software. Cluster analyses were carried out on the basis of the comparison of the MSPs given by the MALDI-Biotyper v3.0 software in order to visualise the level of heterogeneity of the MS spectra of the pre-immature and immature stages of these two species (hierarchical clustering of the mass spectra).

For the rest of the analysis, only reproducible spectra, specific to each stage and free of background noise, with an intensity ≥ 3000 a.u., were retained. To determine the evolutionary stage of each immature stage, using the ClinProTools v2.2 software, the mean spectra of the different stages of each species were superimposed to highlight the existence of specific peaks. The strategy consisted firstly in creating two comparison groups, Group I (IS1-IS2 versus IS3-IS4-IS5) and Group II (IS1-IS2-IS3 versus IS4-IS5), and comparing spectra between early and late immature stages by species, in order to detect discriminating peaks, using the statistical tests of ClinProTools v2.2 software (t-test). The software was used to generate a peak list for each group in the 2 to 20 kDa mass range and to identify discriminating peaks among the analyzed groups. To select peaks that could be used as biomarkers to distinguish immature groups, a ratio of mean intensity per group for each peak detected (in both groups) was calculated. The parameter sets in ClinProTools 2.2 software for spectra preparation were as follows: a resolution of 300; a noise threshold of 2.00; a maximal peak shift of 800 ppm and a match to calibrant peaks of 10%. The selected MS peaks were then included in the Genetic Algorithm (GA) model to determine the recognition capacity (RC) and cross-value percentages (CV) for discrimination of these immature stages and their developmental stage.

Data base creation and blind tests

Reference MS spectra were created from the spectra of eggs and of IS one to IS five of *C. lectularius* and *C. hemipterus* species using the MALDI-Biotyper v3.0 software (Bruker Daltonics). Prior to include MS spectra into the DB, intensity of MS profiles was controlled which should exceeded 3.000 a.u. and the reproducibility and specificity of selected spectra were also inspected by using MSP dendrogram tool (MALDI-Biotyper v3.0 software). Two spectrum per species and per stage were added to our homemade reference MS spectra database, containing 2212 spectra of various arthropods, including adults of *C. lectularius*, *C. hemipterus*, and *C. hirundinis* (head) as well as immature stages of fresh *C. hirundinis* (head and thorax)^{31,32}. Reference MS spectra were created using an unbiased algorithm and information on peak position, intensity and frequency. Blind tests were performed against the updated database using the remaining MS spectra. The reliability of species identification and, ultimately of eggs and immature stages, were estimated using LSV obtained from the MALDI-Biotyper software, which ranged from 0 to 3, LSVs greater than 1.8 were considered reliable for species identification, according to previous studies²⁵. One LSV was obtained for each spectrum of the samples tested.

Results

Specific MS spectra of cimex species eggs and immature stages

To assess the effectiveness of MALDI-TOF MS to differentiate between *Cimex* species, a total of 305 specimens, comprising 55 eggs (28 *C. lectularius* and 27 *C. hemipterus*) and 250 immature stages (IS), including 125 IS per *Cimex* species of 25 specimens per stage (IS1 to IS5) were subjected to MALDI-TOF MS analysis. One hundred and thirty (85.0%) *C. lectularius* and 135 (88.8%) *C. hemipterus* specimens produced high-intensity MS spectra which were visually reproducible per species and developmental stage (Fig. 2A and B). The comparison of MS spectra by GelView (Fig. 3A) supported the reproducibility of protein profiles among immature stages per species, which seems to distinguish between these two species. The application of PCA to spectra grouped by *Cimex* species distinguishing pre-immature from immature stages revealed a clustering of each group, suggesting a specificity of MS spectra between the immature and egg stages of each species (Fig. 3B). The MSP dendrogram confirmed this clustering by grouping these two developmental stages according to species (Fig. 3C). It is interesting to note that the distinction was first made between the eggs of each species and then according to the immature stages. These results clearly confirm a reproducibility of MS for the egg and immature stages, and a high specificity between these two *Cimex* species between these two stages. However, the intertwining which occurred among the five immature stages per species seems to indicate that no evident MS profile could separate them.

Data base creation and blind tests to assess the relevance of MS for the correct classification of Cimex species including immature stages and eggs

The 24 spectra used for the MSP dendrogram were added to our homemade arthropod reference spectra database which included per species two spectra per immature and egg stage. Of the 281 remaining spectra, 40 were considered as non-compliant, due to the low quality of their profiles (intensity < 3000 a.u.) and/or the high heterogeneity among the replicates (Table 1). Querying the 241 MS spectra from the different developmental stages of both species against the upgraded reference spectra database revealed that 100% of the pre-immature (egg) and immature stages were correctly identified at species level, with scores ranging from 1.75 to 2.79 (mean \pm standard deviation (SD): 2.29 ± 0.12), and from 1.81 to 2.71 (mean \pm SD: 2.37 ± 0.03), for *C. lectularius* ($n = 118$) and *C. hemipterus* ($n = 123$), respectively. Only one spectra from immature stage one (IS1) of *C. lectularius* had an LSV < 1.8. A boxplots distribution of LSV identification scores for each stage by species is shown in Fig. 4.

Efficiency of MALDI-TOF MS at distinguishing and classifying the eggs and the five immature stages per species

The high specificity of egg MS spectra compared to other immature stages for both species made it possible to obtain 100% correct classification for both species with LSVs ≥ 1.8 (Table 1). In contrast, at the immature stages (nymphs) the proportion of correct classification varied tremendously according to IS stage, with a mean of 56.38% (53/94) and 50.49% (51/101) classification at the correct IS level for *C. lectularius* and *C. hemipterus*, respectively. An accurate analysis of the identification stage revealed that IS mismatching occurred mainly with close immature stages (Supplementary Figure S1 and Supplementary Table S1). To investigate the possibility of distinguishing immature stages from one another, and classifying them according to their stage of evolution: early or late, we created two comparison groups, Group I (IS1-IS2 versus IS3-IS4-IS5) and Group II (IS1-IS2-IS3 versus IS4-IS5) as early vs. late evolutionary stage respectively. Their spectra were compared using ClinProTools v2.2 statistical tests (t-test) to detect discriminating peaks (Fig. 5). Peaks were considered discriminating if they were significantly distinct between the two immature groups and if the early/late immature ratio per species was greater than two or less than 0.5. In total, 26 and 17 discriminant peaks were detected in comparisons of the early and late immature stages from group I and II, respectively of *C. lectularius* (Supplementary Table S2). For *C. hemipterus*, three and seven discriminant peaks were detected in the early and late immature stage comparisons between groups I and II, respectively (Supplementary Table S3). To determine whether group I or group II produced the better classification of the spectra according to developmental stage, the GA model was applied using the respective selected peaks. The results recorded between the combination of the presence or absence of these peaks by stage showed that higher CV and RC values were obtained for group I for both *Cimex* species (Table 2). A CV and RC of 93.8% and 97.9%, respectively were obtained for *C. lectularius*, and of 92.9% and 94.09% for *C. hemipterus*. Therefore, the classification of immature stages according to group I i.e. (IS1-IS2) versus (IS3-IS4-IS5) seems to be required as the correct classification tool to distinguish early and late immature stages of these two *Cimex* species.

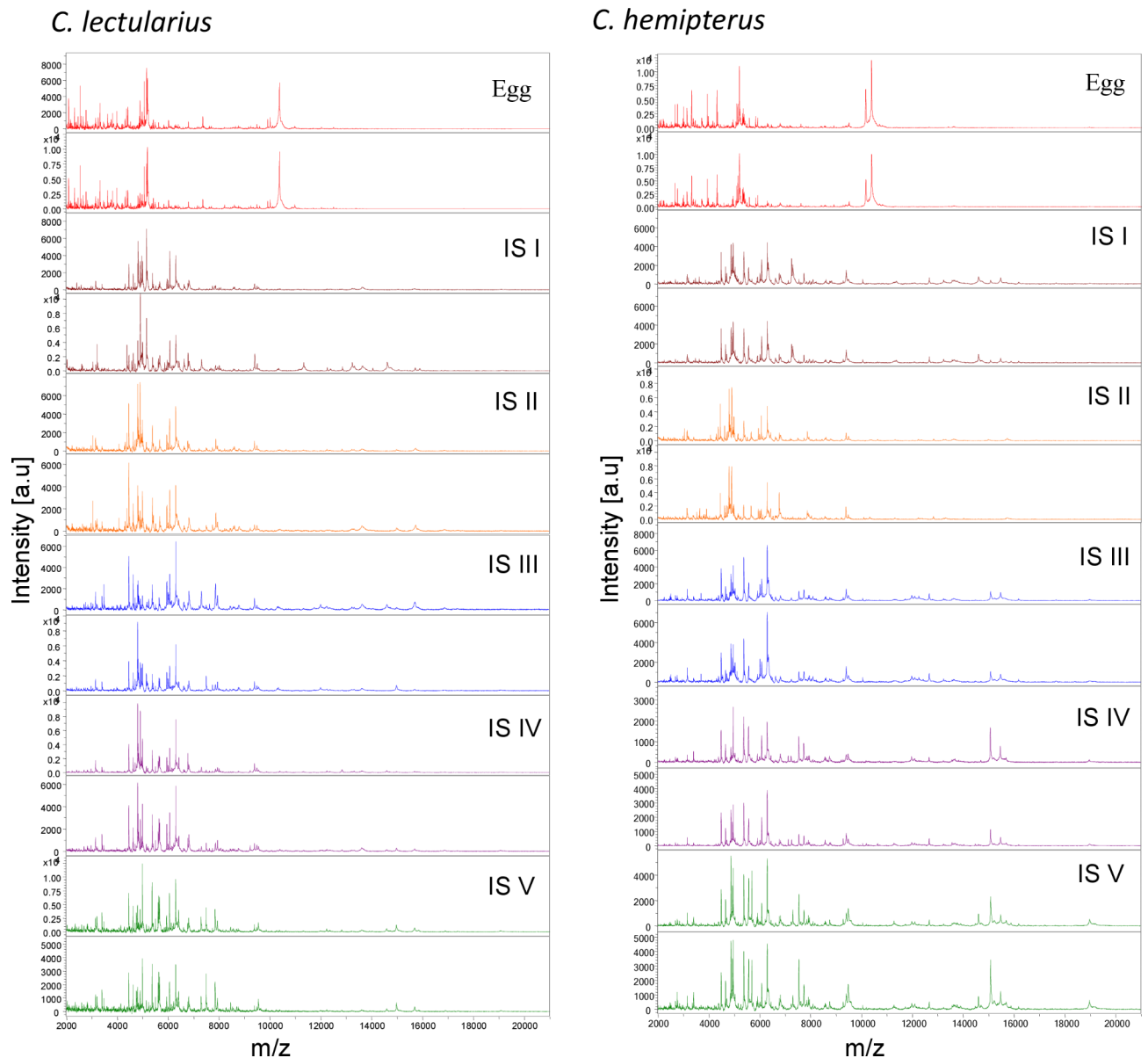


Fig. 2. MALDI-TOF MS spectra of two *Cimex* species at different developmental stages. The MS spectra of *C. lectularius* and *C. hemipterus*, at egg and immature stages, are indicated by the same colour code. a.u., Arbitrary units; *C.*, *Cimex*; m/z, Mass to charge ratio; IS, Immature stage.

Discussion

Misidentification can lead to serious problems, ranging from epidemics to moderate vector/pest resistance to insecticides, and inappropriate pest management strategies^{9,35}. The appearance of the sympatric cohabitation between *C. lectularius* and *C. hemipterus* species in certain areas requires increased surveillance. Studies have reported the resistance of these species to conventional insecticide treatments, with an emphasis on strong resistance on the tropical bug *C. hemipterus*³⁵. In terms of pest control and in order to understand the global spread of bed bugs, early identifications, specifying the *Cimex* species, is essential.

MALDI-TOF MS is a tool that was originally designed for the identification of bacteria, fungi or viruses. However, due to its speed, reliability and low cost, this innovative tool has been used for the identification or classification of other sample types, such as foods³⁶, blood sources³⁷, or for over a decade several arthropod families of medical and veterinary interest but also in other laboratories^{23,38}. Recently, MALDI-TOF MS biotyping has become a reliable alternative tool to overcome the limits observed with the morphological and molecular identifications of arthropods²³. This tool has shown its effectiveness in identifying adult bed bugs, but also in distinguishing between adult and immature stages^{26,31}. Despite this tool's recognised effectiveness at identifying arthropods, several factors and parameters can compromise the quality and reproducibility of MS profiles. Factors which could induce MS spectra changes include the method and duration of sample storing, the amount of mix buffer, and the parameters for sample grinding²⁶. The choice of the appropriate arthropod body

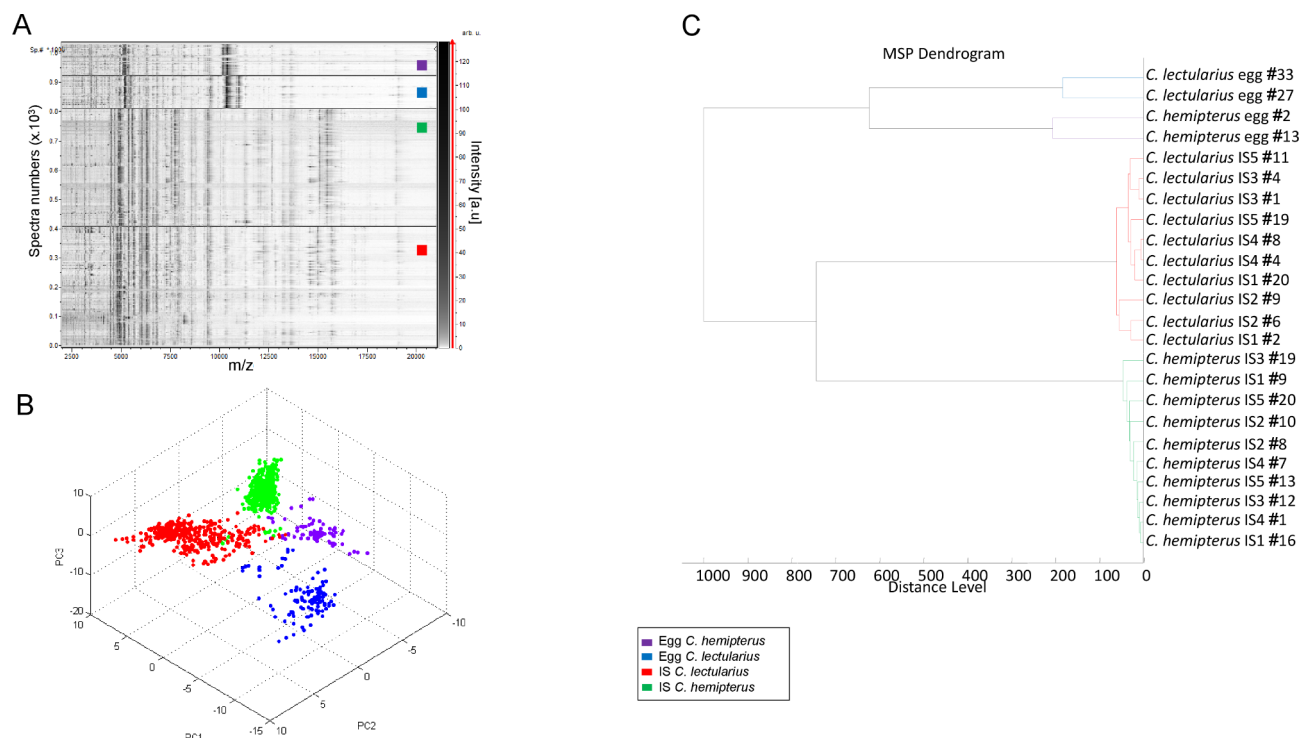


Fig. 3. Comparison of protein profiles of different developmental stages of *Cimex lectularius* and *Cimex hemipterus* using ClinProTools 2.2 software. (A) GelView representation of MS profiles of *C. lectularius* at egg (blue) and immature stages (red) and *C. hemipterus* at egg (purple) and immature stages (green). (B) Principal component analysis (PCA) showing the dimensional clustering of different developmental stages based on their MALDI-TOF MS profiles. (C) Dendrogram showing the clustering by branch of the stages of development obtained using Biotyper V3.0 software, and two specimens per stage at the immature stage and three specimens for the pre-immature stage (egg). a.u., Arbitrary units; m/z, Mass to charge; IS, Immature stage.

Species	Developmental stage	Number of samples tested	Number of non-compliant spectra	Number of spectra added to DB	Number of spectra blind tested	Proportion of correct species identified	Score range	Proportion of relevant identification at correct stage ^a (n)
<i>C. lectularius</i>	IS1	25	3	2	20		1.75–2.58	95.0% (19)
	IS2	25	4	2	19		1.89–2.59	57.9% (11)
	IS3	25	2	2	21	100% (94)	1.94–2.79	14.3% (3)
	IS4	25	6	2	17		1.95–2.55	82.4% (14)
	IS5	25	6	2	17		1.81–2.66	35.3% (6)
	Total ISs	125	21	10	94		1.75–2.79	56.4% (53)
	Egg	28	2	2	24	100% (24)	1.86–2.67	100% (24)
Total	153	23	12	118		1.75–2.79	65.3% (77)	
<i>C. hemipterus</i>	IS1	25	6	2	17		2.21–2.66	82.4% (14)
	IS2	25	4	2	19		1.84–2.59	63.2% (12)
	IS3	25	1	2	22	100% (101)	2.13–2.57	27.3% (6)
	IS4	25	3	2	20		2.11–2.66	35.0% (7)
	IS5	25	-	2	23		2.09–2.71	52.2% (12)
	Total ISs	125	14	10	101		1.84–2.71	50.49% (51)
	Egg	27	3	2	22	100% (22)	1.81–2.6	100% (22)
Total	152	17	12	123		1.81–2.71	59.3% (73)	

Table 1. Blind test results of *C. Lectularius* and *C. Hemipterus* according to their immature development stages. ^aLSV > 1.8 for relevant ID. C., *Cimex*; DB, Database; ID, Identification; IS, Immature stage; LSV, Log score value; n, Number of samples.

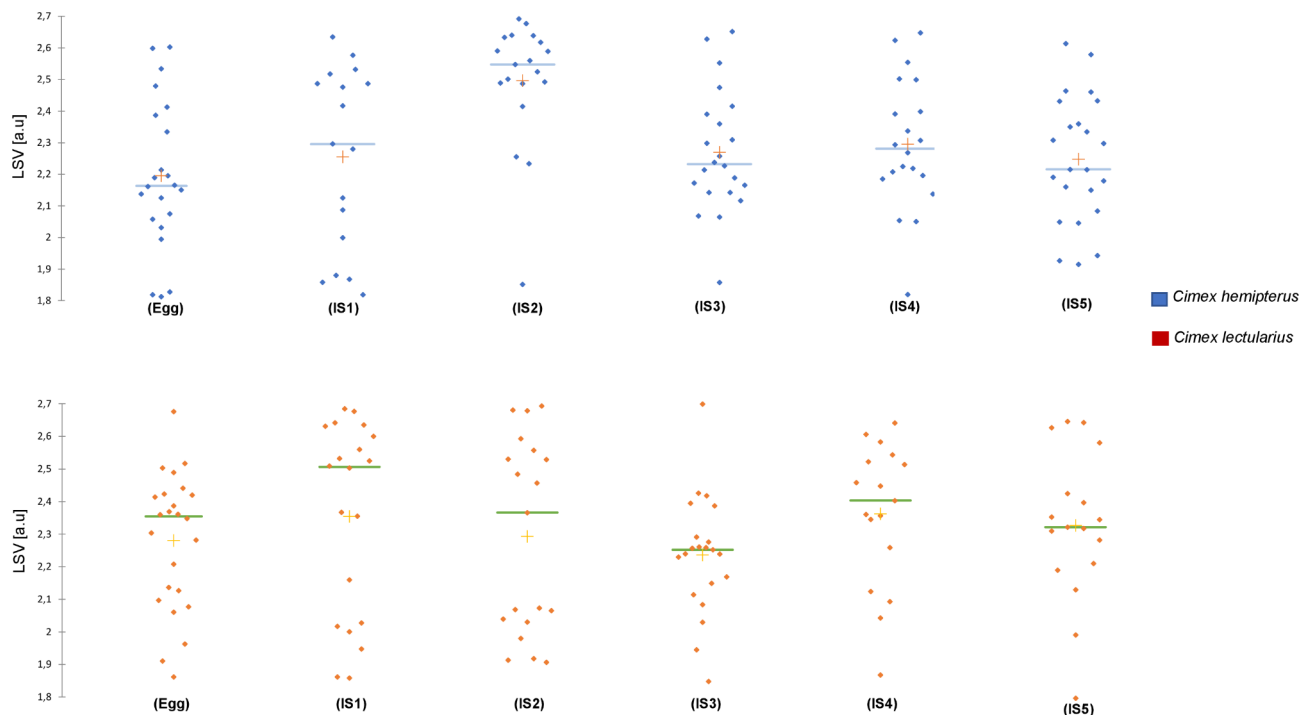


Fig. 4. Graphical representation of the distribution of LSV scores for identifying the different stages of development. In red, *C. lectularius*, and in blue, *C. hemipterus*. LSV, Log score value; a.u., Arbitrary unit.

part remains key to obtaining species-reproducible and specific MS spectra. Depending on the arthropod family, different body parts were selected, such as the legs for ticks³⁹, the thorax for adult phlebotomine sand flies⁴⁰, and the whole specimen for larval stages of mosquitoes²⁹. Furthermore, it has been reported that despite the choice of compartment, variations in MS spectra can be occurred among specimens from the same species but from distinct geographical origins. It was demonstrated repeatedly for mosquitoes at adult^{41,42} and at immature stages²⁹. Similarly, Benkacimi et al., reported also MS spectra changes among *Cimex* specimens from the same species at adult stage, but originated from distinct areas²⁶. For bed bug species, previous studies using the head for adult²⁶ and immature stages of *Cimex hemipterus*⁴³, and the head and thorax for immature stages of *Cimex hirundinis*³¹ are available.

In the present study, the use of MS on the head and thorax of fresh laboratory specimens from *C. lectularius* and *C. hemipterus* species at immature stages made it possible to obtain species-specific and reproducible MS spectra of high intensity. The decision to select the head and thorax rather than only the head, was based on the high reproducibility and intensity MS spectra obtained using these compartments for immature stages of the *C. hirundinis* species³¹. Similarly, a comparison of the protein profiles between these two anthropophilic species using whole eggs showed species reproducibility and the singularity of MS spectra. This specificity of eggs, making it possible to distinguish or discriminate between species, has also been observed when it comes to discriminating between the eggs of stink bug species³⁰, as well as mosquito species⁴⁴. The correct and relevant identification at the species level of 99.6% of the samples at both immature and egg stages confirmed the value of this tool for the identification of pest species and vectors. However, this requires use of an up-to-date database already containing reference spectra of the species stages in order to guarantee reliable results. We provide a DOI (<https://doi.org/10.35081/85dn-k285>) for the random selection of representative good quality spectra of the bed bugs studied here.

In this study, the specificity of the spectra of each stage used between the two species enabled us to distinguish between the two species with reliability and certainty. Similar results have already been observed in characterising two *Aedes* species from larval exuviae and stage 4 pupae²⁸, the precise identification of Culicidae mosquito species at the aquatic stage²⁹, and the identification of sand fly species at different stages³⁸, showing the robustness of this tool for classifying immature specimens of various species according to the specificity of their MS profiles.

Evaluating the effectiveness of this innovative tool in identifying the two anthropophilic species of bed bugs both immature and egg stages is an important step in managing infestations. During an infestation, eggs and immature stages are much easier to collect rather than adults because eggs and immature stages have a reduced mobility, unlike adults who have well-developed sense organs improving their ability to detect the presence of danger quickly.

In terms of pest control, determining the lethal concentration, corresponding to the concentration of a toxic product that causes the death of the target species over a given period of time, is an essential step. However, in paurometabolous insects such as bed bugs, the rigidity of the cuticle acquired during each moult can have a

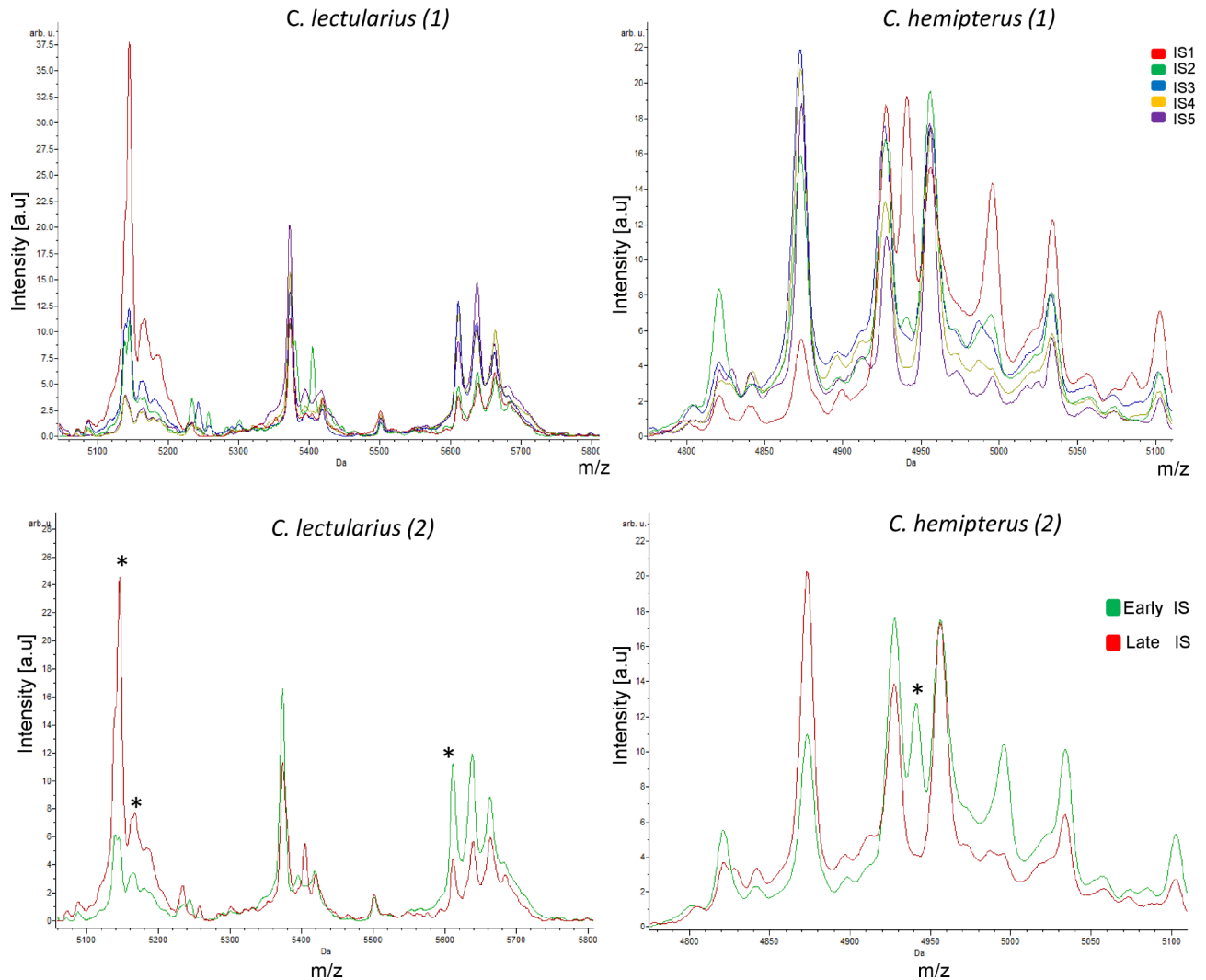


Fig. 5. Superimposed mean MS profiles of the immature stages of *Cimex lectularius* and *Cimex hemipterus*, respectively, obtained using ClinProTool 2.2 software. (1) Individual superimposed immature stages. (2): Superimposed evolutionary stages (early immature stage and late immature stage). Da, Daltons; m/z, Mass to charge; a.u., Arbitrary unit; *, Discriminant peaks selected by ClinProTools software and used for Genetic Algorithm analysis; IS, Immature stage.

negative impact, especially as most insecticides currently used to control bed bugs are not exclusively water-soluble products, and therefore cannot easily penetrate through their cuticle⁴⁵. Consequently, discriminating between immature stages is not only useful for determining the lethal concentrations of the species in order to avoid resistance⁴⁶, but also serves as an index for assessing the degree of infestation, as well as the origin of it. Previous studies have shown that eggs are resistant to certain insecticides⁴⁷, which means that even with treatment, a re-emergence of the species may be observed. In this case, the degree of infestation by early immature stages (IS1–IS2) may reflect a previous infestation that has already been treated, and the eggs from this infestation have resisted the products used, as opposed to an infestation dominated by advanced stages (IS3–IS4 and IS5).

In this study, the relative low rate by which stages could be identified may be explained by the lack of major variability observed within the MS profiles of these different immature stages, as was the case for sandflies where similar results were recorded³⁸. This similarity in MS profiles may be due to the use of the head and thorax rather than the whole body, showing a low abundance of stage-specific proteins and peptides that can be detected by this tool. This contrasts with other previous studies most of which used the whole body of the species to classify the different developmental stages^{29,38}. Furthermore, these incorrect identifications may be due to the fact that during the development of the immature stages, no real metamorphosis took place, only an increase in size, meaning that the protein signatures remained almost the same during the evolution of the stages. These hypotheses were confirmed by Genetic Algorithm analysis, the recorded results of which showed discriminant CV and RC values that were too low for *C. lectularius* and *C. hemipterus*, respectively. Interestingly, by classifying immature stages according to their evolutionary stage into two groups: group I (IS1–IS2 versus IS3–IS4–IS5)

Species		Number of peaks (ratio ≥ 2 or ≤ 0.5)*	Proportion of CV (%)	Proportion of RC
	Overall		93.83%	97.88%
	IS1–IS2	26	94.2%	98.62%
<i>C. lectularius</i>	IS3–IS4–IS5		93.45%	97.14%
	Overall		90.74%	95.83%
	IS1–IS2–IS3	17	86.97%	93.87%
	IS4–IS5		94.5%	95.83%
	Overall		92.93%	94.89%
	IS1–IS2	3	93.98%	93.43%
<i>C. hemipterus</i>	IS3–IS4–IS5		91.87%	96.36%
	Overall		72.81%	84.03%
	IS1–IS2–IS3	7	67.96%	83.44%
	IS4–IS5		77.66%	84.62%

Table 2. Results of genetic algorithm analysis to discriminate between early and late immature stage groups using specific peaks. *Peaks abundance significantly changed (t tests, $P < .05$) with fold-change > 2 or < 0.5 (see Supplementary Table S1 and S2 for details). CV, Cross validation; RC, Recognition capability.

and group II (IS1–IS2–IS3 versus IS4–IS5), we observed much higher discriminant CV and RC values between the two species. Similar results concerning the spectra of the L2–L3 larval stage versus the L4 nymphal stage of *Aedes albopictus* have already been reported, showing a similarity in the spectra of these two groups, as for L1–L4 larvae versus the pupae of *Anopheles gambiae*²⁹.

MALDI-TOF MS, a tool that has proven its effectiveness and robustness in identifying arthropods at all stages of development, offers many advantages over conventional methods such as molecular biology and morphological identification. Fast and inexpensive at the same time, this tool does not require a great deal of experience to be used, unlike molecular biology which is more expensive and chronophagous. The morphological identification is laborious, not easy for small immature stages and then requires skills.

In conclusion, the results obtained in this study confirm the robustness and reliability of the MALDI-TOF MS tool for entomological surveillance and the rapid identification of pest and vector species. However, discrimination of the actual immature stages of *Cimex* species remains problematic and is far from resolved. The results obtained show that this method is effective in separating adults and immature stages, but not really effective in separating the different immature stages themselves, although it is able to distinguish early immature stages from late immature stages. In the future, identification of these species using their exuviae will be of interest in order to optimise the chances of quickly and efficiently determining infesting species.

Data availability

The data presented in this study are freely available and additional information files are provided.

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Author contributions

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Declarations

Competing interests

The authors declare no competing interests.

Additional information

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