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Gas Chromathography as a Tool in Quorum Sensing Studies

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Dedicated to Professor Carmenza Duque

1. Introduction

Since cell differentiation was studied cell-to-cell signaling and cell regulated cycles were considered exclusive of eukaryotic organisms, prokaryotic organisms were regarded as isolated cells without cooperative behaviors. It took us more than 30 years, after Nealson et al., (1970) explained bioluminescence as an auto-induced regulated mechanism of bacterial groups, to assume in microbiology research that bacteria can synchronize group behaviors. In consequence, it was possible to explain that inter-cell signaling regulates some bacterial phenotypes and this phenomenon was called Quorum Sensing (QS). QS is one of the most revolutionary mechanisms discovered in the past 15 years. It involves the cell control of bacterial population by communication using chemical signals and a complex network of genetic circuits with a positive feedback regulation (for review see: Waters et al., 2005). Sensing these chemical signals bacteria can respond as groups and detect the "quorum" of a population in order to regulate different phenotypes.

In 1970 Neaslson et al., described light production in the marine organism *Vibrio fischeri* in response to secreted signal molecules, depending on the cell density. After that, 10 years later, Eberhard et al., (1981) identified those signal molecules as *N*-acyl Homoserine Lactones (AHLs) synthesized by the enzyme LuxI and distributed into the media by Simple Diffusion (Eberhard et al., 1981). Auto-induction occurs when AHLs accumulates in the media (in a high cell density) and binds the target in the protein LuxR, a transcriptional activator for the expression of many genes including *luxI*, generating positive feedback loop (Figure 1A). Fuqua et al., in 1994, explaining the regulation of gene expression by a cell density manner, and called this phenomenon "Quorum Sensing" (QS) (Fuqua et al., 1994; Stauff et al., 2011). Before, it was considered a peculiarity of few Gram-negative bacteria since AHLs were not found in Gram-positive ones, nowadays it is known that Gram-positive bacteria are using oligopeptides to communicate with each other. In this case, peptide transporters secrete these autoinducing peptides (AIPs) because the cell membrane is not permeable to them, and the sensing systems do not occur inside the cell as in Gram negatives. Instead, two component transduction systems are used for the detection of the

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AIPs and the activation of the QS system (Figure 1B) (for review see: de Kievit & Iglewski, 2000; Miller & Bassler, 2001; Thoendel et al., 2011).
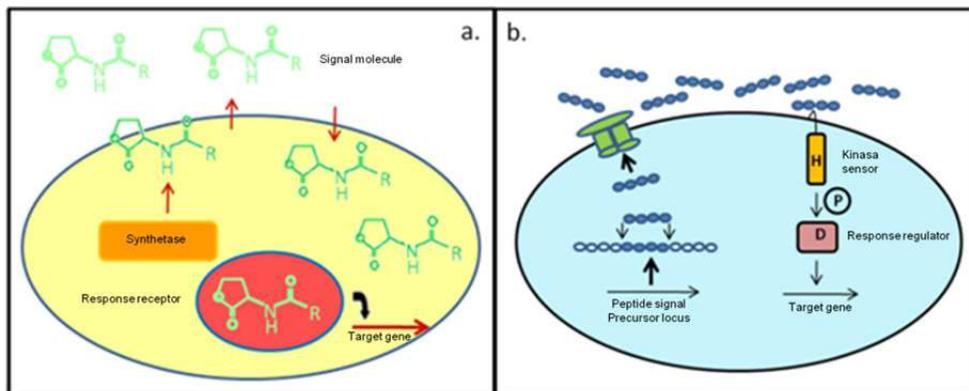

Fig. 1. General Quorum Sensing Systems: **a**. Gram-negative bacteria, **b**. Gram-positive bacteria (from Pamela Zorro, 2011).

In most Gram-negative bacteria QS circuits work as it has been described for *Vibrio fischeri.* Light production is coordinated by the *lux* regulon that comprises: (1) the *lux*ICDABE operon consisting of the *lux*I gene, encoding the 3-oxo-C6-HSL synthase, followed by the *lux*CDABE, the structural genes necessary for bioluminescence, and (2) the *lux*R gene encoding the transcriptional activator protein LuxR. At low cell densities, *lux*I is transcribed at a basal level leading to a low concentration of 3-oxo-hexanoil-homoserine lactone (3-Oxo-C_6-HSL). AHLs (Figure 2) are freely diffusible and accumulate in the medium with increasing cell density. When 3-Oxo-C_6-HSL reaches a critical threshold concentration, it binds to the LuxR-receptor protein. This complex in turn binds to a 20 bp palindromic DNA promoter element, the lux-box, and activates the transcription of *lux*ICDABE resulting in the bioluminescent phenotype and in a positive autoregulation of *lux*I (a positive feedback loop) (Engebrecht & Silverman, 1984; Kaplan & Greenberg, 1985). This was the first QS circuit described and the name of the cupple LuxI/LuxR remains as a symbol for every LuxI type protein or LuxR type protein in Gram-negative bacteria.

For Gram-positive bacteria, the circuits in general work as in the *Streptococcus pneumoniae* (ComD/ComE) Competence System. The signal molecule is the Competence Stimulating Peptide (CPS) (Figure 2), it is a processed peptide of 17 aminoacids secreted by the type 1 secretion system Com AB, an ABC-transporter. At high cell density, CPS accumulates and is detected by ComD, a sensor kinase protein. CPS induces autophosphorylation of ComD and the phosphorylation cascade until the phosphoryl group is transferred to the transcriptional activator ComE. Once ComE is activated by phosphorylation, it induces the transcription of the *com*X gene, responsible of the competence (ability to acquire exogenous DNA molecules) in *S. pneumoniae* (Miller & Bassler, 2001).

Currently different QS signaling molecules are recognized in Gram negative or Gram positive bacteria as well as the ones used for inter-species communication, as the autoinducer 2 (AI-2) to mention one (Bassler et al., 1997). However, AHLs, AI-2 and CPS are

not the only nature of sensing molecules, there are diesters, butyrolactones, quinolines, branched and unsaturated fatty acids, diketopiperazines (Figure 2.), among others detected by the same mechanisms or by a hybrid of both as de QS circuit of *V. harveyi*. The hybrid QS circuits works with molecules, similar to AHLs or synthesized using similar pathways, freely diffusible through the bacterial membrane but those molecules are sensed by a kinase protein just like in Gram positive bacteria, using a cascade of phosphate to induce the expression of regulated phenotypes. The AI-2 from *Vibrio harveyi* was the first molecule described for the hybrid QS circuits, it is a furanosyl borate diester (Figure 2) synthesized by the enzyme LuxS. Interesting, this enzyme has been detected in a wide diversity of bacteria including Gram positive and Gram negative cells, in that sense AI-2 has a role of universal detection that may give the rule of crosstalk between organisms (Bassler *et al.*, 1997; Miller & Bassler, 2001; Warren et al., 2011).
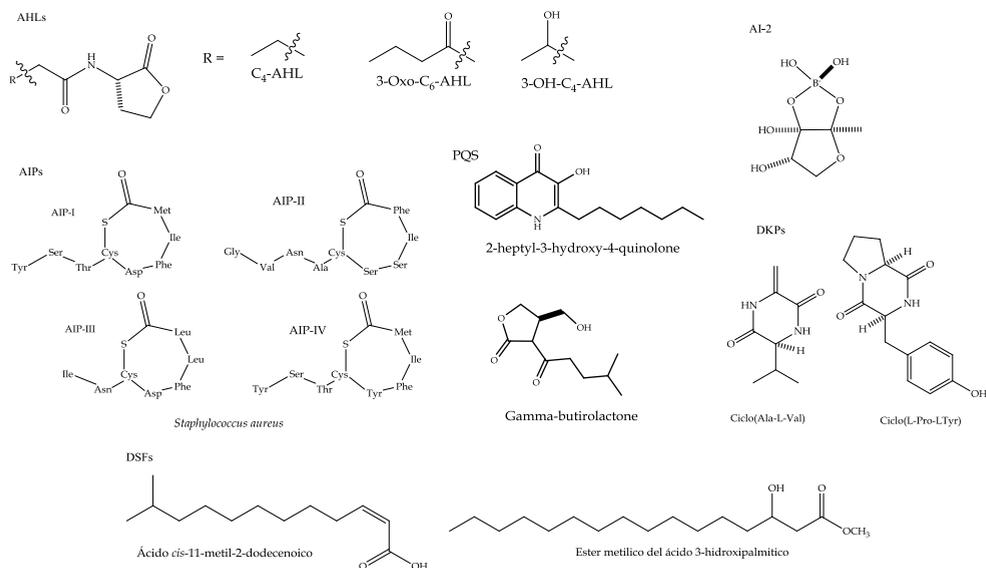

Fig. 2. Chemical structures of some Quorum Sensing signaling molecules. General structure of N-acyl-homoserine lactones (AHL), autoinducer 2 (AI-2), autoinducing peptides (AIP), *Pseudomonas* quinolone signal (PQS), Difusible signal factors (DSFs), and Diketopiperazines (DKPs).

In many bacteria Quorum Sensing regulates a variety of phenotypes including bioluminescence, transfer of tumor-inducing plasmids (Ti plasmids), antibiotic production, swarming motility and specially biofilm maturation and production of virulence factors. These last have been the most studied by pharmaceutical or chemical researches for their human health implications. It has been demonstrated that many bacteria do not express virulence factors until the population density is high enough to overwhelm host defenses and to establish the infection (Greenberg, 2003). However, further than virulence, QS allows bacteria to coordinate group behaviors therefore it has a unique ecological role in ecosystems development, bacteria can communicate sensing these molecules between the same species but also between different species. The species specificity of signaling

molecules allows bacteria to be responsive only to their own cell density even when other species are present. Nevertheless, cross-species communication mechanisms have been described. In some cells it occurs because LuxR-type proteins have a range of activity with similar AHLs molecules, but the classical cross-species talk is done using signal molecules as the AI-2 (Bassler et al., 1997; Riedel et al., 2001).

This interplay has a pivotal role in development and differentiation of bacterial biofilms, therefore in the conformation of bacterial communities on biotic or abiotic surfaces. However, the interaction between organisms colonizing a surface goes beyond inter-bacteria communication, there have been reports about communication between kingdoms and it has been shown how AHLs have a role in immunomodulation, and apoptosis, as well as in development and metamorphosis of some eukaryotic organisms (Hughes & Sperandio, 2008; Joint et al., 2007). Biofilm of bacterial populations involved in the microbiome of an organism are structured by sensing diffusion gradients of QS signaling molecules, among other factors. These chemical gradients allow the community to shape up the physiological role of each population by differential regulation of gene expression in order to coordinate metabolic pathways and phenotypes (McLean et al., 2005; Valle et al., 2004). Every signaling molecule present in a community is responsible of a behavior that should be in agreement with host signaling and requirements. The influence of bacterial communication in macro-organisms surfaces has been describe for nitrogen fixation, nutrients disposition and defense that includes the production of antimicrobials and Quorum Sensing Inhibitor molecules (Knowlton & Rohwer, 2003; Shnit-Orland & Kushmaro, 2009).

Clinical implication of QS has taken this phenomenon into the pharmaceutical industry with the aim of developing new molecules able to control bacterial pathogenesis since virulence factors of bacteria are mostly QS regulated. Usage of QS Inhibitors is interesting in order to avoid virulence without release bacterial resistance to antibiotics, therefore QS became a pharmaceutical target as an important subject for anti-virulence therapies development (Köhler et al., 2010). Due to the role on human health of bacterial virulent phenotypes regulated by QS systems, there have been plenty of reports for the Quorum Sensing inhibition (QSI) activity of molecules isolated from different organisms. This will be discussed in further section, but it is important to mention that this QSI activity is a target for pharmaceutical industries nowadays since QS regulates development of biofilm formation and expression of virulence factors, both involved in chronic infection progress and medical treatments failures, in general. On the other hand, compounds with QSI activity found application in the antifoulant technologies, because macro-organisms are able for sensing bacterial QS signaling molecules to settle and metamorphose, selecting like that the proper biofilm conditions for its development. Following these concepts it is possible to assume that bacterial biofilm and the signaling molecules present in a surface, may influence the colonization of surfaces and the development of phenomena such as biofouling (Joint et al., 2007; Marshall et al.,2006; Tait et al., 2009).

Nevertheless, for further remarks, it is important to keep in mind that QS regulated phenotypes are a two-edged sword. Even if clinical role of virulence factors or biofilm development on surfaces are adverse for human civilization; researchers may not forget the functional role of QS in fitness and stability of different ecosystems. Normal associated bacteria in an organism have different functions including nutrients availability, defense, essential molecules for metabolic pathways in the host and selection of other

microorganisms present in the community, among others. In general, all those interactions give the homeostasis required for an organism and changes in the composition or structure of natural communities could lead to diseases development or any other undesirable conditions in ecosystems. Furthermore, some health problems are now better undertanded like changes in the bacteria community.

The first reports about structural characterization was from the *N*-acyl homoserine, being consequently the most studied molecules up to now although its detection is made routinely using biosensors (Steindler & Venturi, 2007). These techniques are limited to biological variations and mutants transformation; moreover, it is almost impossible to have a biosensor for every existent signaling molecule and there are examples where these molecules used for cross-communication cannot be detected by the habitual biosensor cross streaking technique and require more specific techniques to be detected; this field is still under development. Likely, one of the reasons, is the urgent necessity of standardize more reproducible and robust methodologies for detection and quantification as the chromatographic methods developed by gas chromatography GC and high performance liquid chromatography HPLC coupled to many of the available range of compatible detectors that includes mass detectors. In the next section this issue is reviewed.

2. Analysis of signaling molecules involved in cell communication by GC

In this section we will focus on the analytical uses (detection, identification and quantification) by GC for signaling molecules like acyl homoserine lactones (AHLs), diketopiperazines (DKPs), diffusible signal factors (DSFs), autoinducer type 2 and type 3 molecules (AI-2 and AI-3), pseudomonas quinolone signals (PQS), and gamma-butirolactones. The GC methods have been used in a wide range of studies that usually search for signaling molecules in strains of different origin (i.e. phatogenics, marine, soil, food, among others). However, is remarkable how the study of crop-pathogenic bacterial strains is one of the fields that lead to the discovery of many of the signaling molecule families here mentioned. In general terms, there is an abscense of information about GC analysis of peptide signaling molecules of the Gramm positive bacteria, due to its incompatibility with gas chromatography systems by the low volatility of the molecules.

2.1 Extraction, structural and stereochemical analysis by GC of signaling molecules

The signaling molecules are usually found at low concentrations; due to that, several culture media and extraction techniques are used. In order to obtain these signaling molecules form bacterial cultures, the most used growth media is the Luria-Bertani (LB) broth, together with other complex non defined culture media such as nutrient broth (NB), sea water agar (SWA), marine agar and in general other minimal media. The choice of the culture media depends on some bacterial growth requirements. The volume of culture used in these studies ranges from 5 to 10 mL in the bottom line, up to 20 L scale cultures, depending on the extraction method and if the aims of the conducted research is analysis-detection in mixture or isolation, respectively.

As the direct injection of the samples may affect the separation or may clog the chromatographic columns due to the low volatile components of culture media, homogenates, or environmental samples, a pre-treatment that involves extraction and purification steps of the sample containing signaling molecules is highly recommended.

The bacterial culture is centrifuged an the supernatant is extracted with common solvents such as ethyl acetate, chloroform and dichloromethane to obtain the signaling molecules such as AHLs, DKP and PQS (Pesci et al., 1999; Pomini et al., 2005; Wang et al., 2010). In order to prevent degradation of AHLs the extractive solvent contains acetic acid, formic acid or TFA acid in concentration from 0.05 to 1.0 % (v/v) (Götz et al., 2007; Huang et al., 2007). For DSF the most employed solvent is hexane, a common solvent for free fatty acid and their methyl esters extraction and analysis; however, as the DSFs are consider weak acids, its extraction can be favored carring out the extraction from an acidfied (pH 4.0) supernatant with the refered organic solvents (Deng et al., 2011). The extraction of high polar AI-2 family signal molecules was achieved by a mixture of dichloromethane/methanol (1:1) (Sperandio et al., 2003). One of the main disadvantages of solvent extraction is the low selectivity of the solvents, even more if the extraction is conducted over the complex matrix of the culture media. Other disadvantages are the low yields of organic layers extractions, the concentration process that includes heating and low pressure rotatory evaporation that can lead to recovery looses for thermal degradation, artifacts formation, and evaporation. Due to that, concentration under a current of nitrogen at room temperature is commonly used and fractionation steps of the extract are required before its chromatographic analysis. The use of neutral interchange resins, such as XAD-16 and HP-20 and HP-21 for the extraction step is also reported (Shaw et al., 1997) due to its higher selectivity compared to that of the organic solvents, low solvent consume and possibility of recycling. Usually resins are added at the begin of the cultivation step, and further purification of the eluted extracts is often required, because of the high amount of culture broth absorption into the resins. The use of solvents and/or resin extraction is observed for both, small and large scale fermentations.

Solid phase extraction (SPE) is one of the most used techniques for extraction of signaling molecules, due to several advantages as higher selectivity than the above mentioned methods, high removal of culture broth, salts and other impurities, pre-concentration of the target molecules, the possibility of gradient elution that produce semi-purified fractions in a single procedure and the requirement of small samples (usually from 5 to 10 mL). All these advantages reduce detection and quantification limits at the chromatographic analysis step enhancing sensitivity and robustness of the used methods (Li et al., 2006; Shupp et al., 2005). From the wide range of options for the choice of SPE supports, reverse phase SPE cartridges are preferred over the normal phase chromatographic supports, because of the wide range of polarity of the signaling molecules to be analyzed and the strong interactions of the high polarity analyzed molecules with polar stationary phases. For AHLs, SPE cartridges with ion-exchange or H-bridge activity supports have been also tested, but there are examples where cartridges loaded with strong or mild ion-exchange abilities (basic and acid supports) retained high amounts of AHLs (Li et al., 2006), leading to looses of the usually low-concentrated molecules. The preferred SPE cartridges for AHLs were the end-capped ODS cartridges and the PSDVB cartridges, washed with water and methanol/water (15:85, v/v) to remove broth media components without target molecules looses, and further elution with isopropanol or with mixtures of hexane 25%, toluene 50%, tetrahydrofuran 75% with isopropanol, and isopropanol 100% (Li et al., 2006).

Once the extracts have been obtained detection techniques that include mutant bacterial strains used as biosensors and chromatographic techniques such as TLC, HPLC and GC are employed in order to detect, identify and quantify the signaling molecules (Charlton et al., 2000; Li et al., 2006; Shaw et al., 1997). Biosensors are used due to their high selectivity and

low detection limits. However, chromatographic techniques are preferred over the bioassay detections, because of biosensors responses depends on culture conditions such as growth and pH, and the biosensors are only able to recognize a specific and, by that, a limited number of the signaling molecules.

The sample analysis has been usually conducted by GC-MS as well as by HPLC-MS methods, or even by the combination of both techniques, taking advantage of the high sensibility of the mass spectrometry (MS) detectors, the versatility of the ionization sources (EI and ESI) that allows the ionization of molecules chemically and structurally different, and the tandem techniques of the MS detectors that provides structural information that leads to the identification and differentiation of the signaling molecules (Teplitski et al., 2003; Li et al., 2006). Characteristic fragmentation patterns for signaling molecules as AHLs, DKPs and DSFs have been reported by EI and ESI analysis (Cataldi et al., 2007; Charlton et al., 2000; Morin et al., 2003; Pomini et al., 2005). In this sense, GC-MS combines the high chromatographic resolution and the specificity and sensibility of the mass detectors, required for the analysis of molecules involved in quorum sensing. Even when HPLC-MS has risen as other very interesting and widely used technique in the analysis of bacterial signaling molecules, its use in the analysis of such molecules will not be discused here attending the scope of this book.

Injection of the samples into GC chromatograph has been made using split (Cataldi et al., 2007, Pomini et al., 2005) or in a splitless mode for AHLs and DSFs (Charlton et al., 2000; Colnaghi et al., 2007). Degradation of some AHLs at the injection port has been proposed, due to the high temperatures of the liners, leading to low sensitivity in some GC methods that analyze the sample directly (Schupp et al., 2005). This degradation could be avoided and an increase of the sensibility for the analysis of AHLs is achieved by the use of derivatives that, with the help of on-column injections, reduce the risk of thermal degradation, increase the thermal stability and the volatility of the AHLs (Charlton et al., 2000). Usually low polarity DB-1 or DB-5 columns, and equivalent columns, are employed for these chromatographic separations. Even when detection of signaling molecules has been reported by FID detectors (Soni et al., 2008) and electron capture detectors (ECD), the most used detector is the single quadrupole MS detector, in both positive and negative detection modes. In addition, for increase the sensibility of the methods for previously identified signaling molecules, selected ion monitoring (SIM) detection modes are preferred over the full SCAN detection mode. The SIM mode allows detecting signaling molecules at femtomolar scales (Charlton et al., 2000).

As was previously mentioned, chemical derivatives help to deal whit thermal stability and the volatility required for the GC chromatographic analysis of the signaling molecules. AHLs can be directly analyzed by GC-MS (Pomini et al., 2005), but an increase in sensitivity and accurateness of the GC-MS method can be reached by the use of pentafluorobenzyloxime PTFBO derivatives (Charlton et al., 2000). Other derivatives are employed for DSFs as the formation of the fatty acid methyl esters FAMES (Wang et al., 2010). However, in order to confirm molecular weight, methylation patterns and double bond position for of these DSFs other derivatives as beta-picolinic esters or *N*-acylpyrrolidides or dimetyldisufide (DMDS) must be prepared for the GC-MS analysis (Huang & Lee-Wong, 2007; Thiel et al., 2009). AI-2 type signaling molecules were analyzed as an equilibrium mixture transformed to quinoxaline derivatives (Thiele, 2009b). Due to his

volatility, other signaling molecules such as gamma-butirolactones, DKPs and PQSs signaling molecules have been analyzed without derivatization.

AHLs possess two quiral centers, one at the carbon C-3 in the lactone ring for all AHLs and the other at the hydroxylated position at C-3´ of the side chain for the 3-OH-AHLs. The absolute configuration assignment at position 3 (C-3) in AHLs, was completed by a GC-FID method using beta-cyclodextrin as chiral stationary phase using coinjection with synthetic standards (Pomini et al., 2005; 2006; Pomini & Marsaioli, 2008).On the other hand, the absolute configuration assignment at position C-3´ for the 3-OH-AHL was completed by the analysis of the FAMEs obtained by treatment of the AHLs from the crude extract with sulphuric acid in methanol (it maintains the stereocentre at C-3´ in the acyl chain) and further chromatographyc analysis employing a beta-cyclodextrin column and coinjection with enantiomerically pure FAMEs standards (Thiel et al., 2009a). Double bond configuration for AHLs was also established by GC-MS injection and comparison with synthetic standards (Thiel et al., 2009a). This approach seems to be compatible for the stereochemical analysis of the substituted fatty acids of the DSFs. The identification of L- and D-DKPs isomers was carried out by GC-MS using a 5% pheyl column due to that DKPs corresponds more to a diasteroemeric mixtures of the cyclic dipeptides than the enantiomeric mixtures (Wang et al., 2010). Even when there are not examples abot the absolute configuration analysys for other signaling molecules it seems realiable to conduch such analysis by the use of quiral columns.

The quantification of signaling molecules by GC has been proposed only for AHLs, AI-2 and DKPs. As the *N*-heptanoilhomoserine lactone had not be detected as signaling molecule it was consider a non natural AHL and its use as internal or external standard for AHLs quantification was accepted (Cataldi et al., 2007). However, nowdays it is known that this molecule is produced by several bacterial species (Pomini et al., 2005; Pomini & Marsaioli, 2008; Thiel et al., 2009a) and its use for quantification proposes is no longer recommended. Other quantification method was proposed for 3-oxo-AHLs, usingthe PTFBO derivatives of ^{13}C-AHLs as internal standard for quantitative analysis (Charlton et al., 2000). The main advantage of this method is that the internal standard undergoes the same losses during work up as does the analyte. Moreover, the isotopes displays the same chemical behavior differing only slightly from the analyte in terms of mass and its volatility, reactivity and distribution coefficient and chromatographic behavior are the same as those of the unlabeled compounds, with the exception of minor and negligible isotope effects. This method showed a high sensitivity (signal noise ratios of 10/1 for 1 ng), and high accurateness. Its applicability was validated in the quantification of 3-oxo-AHLs from supernatants, cell fractions and biofilms. Following the same approach for the isotope dilution quantification of AI-2 type molecules, the [5,6,7,8-^{2}H]1-(3-methylquinoxalin-2-yl)ethane-1,2-diol was used, finding again high linearity, sensitivity and accurateness showing S/N of 5 and detection limits of 0,7 ng/mL. Diketopiperazines have been quantified by the use of a L-cyclo (Phe-Pro) as external standard (Wang et al., 2010).

Extraction, detection, and quantification analysis of signaling molecules produced by bacteria, and other organisms from their culture media are of great interest and the development of novel analyitic strategies can provide information of their biological role in cell-cell communication. In the following lines advances in their structure identification and activity, obtained in studies that used GC will be presented.

2.2 AHLs

The most widely studied signaling molecules are the AHLs, the main QS modulators for Gram negative bacteria. Some of its structural features are the presence of hydroxyl or carbonil substitution at C-3 in the side-chain, the presence of double bonds and the carbon chain length, that ranges commonly from C_4 up to C_{18} and are mainly even; however, there are examples of odd AHLs as the C_7, C_{13} and C_{15} –AHLs that have been found in *Erwinia psiidi* (Pomini et al., 2005), *Rhizobium leguminosarum* (Horng et al., 2002), *Pantoea ananatis* (Pomini et al., 2006), *Edwardsiella tarda* (Morohosi et al., 2004), *Serratia marcescens* (Lithgow et al., 2000) and in marine alphaproteobacteria (Wagner-Döbler et al., 2005). The odd and even AHLs biosynthesis is suggested to be made by the same enzymes (Pomini & Marsaioli, 2008). Double bond position and geometry assignation (Thiel et al., 2009a), presence or absence of carbonyl and or hydroxyl groups at the position C-3 and its C-3 absolute configuration (Pomini et al., 2005; 2006; Pomini & Marsaioli, 2008) have been assigned by GC analysis, using both chromatographic and spectroscopic criteria. Fragmentation pattern analysis shows as characteristic fragments the ion at m/z 143 most likely due to the McLafferty rearrangement, and the ion at m/z 102 probably formed after an acylic side chain cleavage and H rearrangement (Cataldi et al., 2007; Cuadrado, 2009).

In one attempt for develop a GCMS method for AHLs analysis, the bacteria fish pathogens *Aeromonas hydrophila*, *A. salmonicida*, and the opportunistic human pathogens *Pseudomonas aeruginosa*, *P. fluorescens*, *Yersinia enterocolitica* and *Serratia liquefaciencs*, were studied (Cataldi et al., 2007). The bacteria growth medium were extracted with solvents and these extracts analized by GCMS. The series of AHL´s from 4 to 14 all even, 3-oxo-C_6-AHL and 3-oxo-C_{12}-AHL were detected in SIM mode by EI, searching for the prominent ion at m/z 143, from the crude cell free supernatant. Full scan mode did not allowed AHLs detection. The Quantification was conducted by external standard using C_7-AHL. This method allowed detecting signaling molecules that were not detected previously by the use of biosensors or other chromatographyc techniques (Cataldi et al., 2007). However, that study failed at detection of underivatized 3-oxo-AHLs which are heat labile compounds.

Brazilian strains of the crop-pathogen bacteria *Erwinia psidii*, *Pantoea ananatis* and *Pantoea* sp. were studied for its production of AHLs by an analysis protocol that involves solvent extraction, fractionation by column chromatography, identification by direct GC-MS and absolute configuration analysis by GC-FID with chiral column and synthetic standards, together with biosensor detection with *Agrobacterium tumefaciens* NTL4 (pZLR4) strain (Pomini et al., 2005; 2007; Pomini and Marsaioli, 2008). The studies allowed to detect (*S*)-(-)-C_6-AHL and the rare (*S*)-C_7-(-)-AHL with a 92% enatiomeric exces in *E. psidii*; (*S*)-(-)-C_6-AHL, C_7-AHL, and (*S*)-C_8-AHL from *P. ananatis*; and finally (*S*)-C_4-AHL, and traces of (*S*)-C_6-AHL in *Pantoea* sp. Analysis of the activity of *R*- and *S*-AHLs showed that enantiomers were both equally active against *Bacillus cereus*, while the racemic mixture was less active than the pure enantiomers (Pomini & Marsaioli, 2008)

The way how AHLs can induce the settlement of the fouler polychaete *Hydroydes elegans* was evaluated using seven bacterial strains that were originally isolated from natural biofilm that effectively induced larval settlement of the *H. elegans* and synthetic C_6-AHLs, C_{12}-AHL and 3-oxo-C_8-AHL (Huang et al., 2007). AHLs and bacterial biofilms were tested for settlement activity and characterized by GC-MS. The GC-MS analysis of the biofilms

solvent-extracts showed that biofilms can produce C_6-AHLs, C_{12}-AHL. In the settlement assays the tree synthetic AHLs, but specialy C_{12}-AHL, induced some initial larval settlement behaviors such as reducing swimming speed and crawling on the bottom.

In a study using C_6- and C_8- and C_{10}-AHLs that attempted for the evaluation of the uptake, degradation, and chiral discrimination of N-acyl-D/L-homoserine lactones by barley (*Hordeum vulgare*) and yam bean (*Pachyrhizus erosus*) using UPLC, FTICRMS and tritium labeled AHLs, and chiral separation by GC-MS revealed that both plants discriminated D-AHLs stereoisomers to different extents. These results indicate substantial differences in uptake and degradation of different AHLs at the tested plants (Götz et al., 2007).

Two strains of the nitrogen-fixing bacterial symbiont *Sinorhizobium meliloti* were analyzed to determine the production of AHLs (Teplitski et al., 2003). Both strains produce C_6-AHL, 3-OH-C_6-AHL, C_8-AHL, 3-oxo-C_8-AHL, 3-OH-C_8-AHL, C_{10}-AHL, 3-oxo-C_{10}-AHL, 3-OH-C_{10}-AHL, C_{14}-AHL, 3-oxo-C_{14}-AHL, C_{16}-AHL, $C_{16:1}$-AHL, 3-oxo-$C_{16:1}$-AHL, characterized by a protocol that includes solvent extraction, SPE fractionation and HPLC-MS GC-MS analysis. The differences in AHLs produced suggest that significant differences in their patterns of quorum-sensing regulation could exist.

In a study that search in more than 100 bacterial isolates from various marine habitats for AHLs production by *Pseudomonas aeruginosa* and *Vibrio fischeri* biosensors, 39 Alphaproteobacteria isolates induced fluorescence in either one or both of the used sensor strains (Wagner-Dobler et al., 2005). AHLs were identified by GC-MS analysis and shown to have chain lengths of C_8, C_{10}, C_{13}–C_{16}, and C_{18}. One or two double bonds were often present, while a 3-oxo or 3-OH group occurred only rarely in the side chain. Most strains produced several different AHLs. $C_{18:1}$-HSL and $C_{18:2}$-HSL were produced by *Dinoroseobacter shibae*. 7(Z)-$C_{14:1}$-AHL, which has previously been detected in *Rhodobacter sphaeroides*, was produced by *Roseovarius tolerans* and *Jannaschia helgolandensis*. The same research group identified for the first time in an *Aeromonas culicicola* strain AHLs with acyl chains carrying a methyl branch, by means of a GC-MS method that allowed distinguish these compounds from unbranched isomers. In the same publication, a strain of the marine bacterium *Jannaschia helgolandensis*a shows to produce a doubly unsaturated AHL, identified as (2E,9Z)-C16-AHL (Thiel et al., 2009b). The position and configuration of the double bonds was proven by MS spectrometric analysis and by synthesis. Absolute configuration of the detected AHLs was determined by mild cleavage with sulphuric acid and chiral chromatographic analysis.

A preconcentration methodology was developed for the analysis of AHLs using single drop microextraction or liquid-liquid microphase extraction in toluene for their analysis using GC-MS (Kumar-Malik et al., 2009). The performance of the method was determined and discussed for the chiral separation of these autoinducers using a β-cyclodextrin chiral column. A remarkable fact of this study is the demonstration, that *Burkholderia cepacia* LA3 produced D-C_{10}-AHL besides the L-C_{10}-AHL and the L-C_8-AHL enantiomers.

GC has proved to be helpful in the characterization of AHLs from the extreme acidophile *Acidithiobacillus ferrooxidans* produce, that produced C_{12}-, C_{14}-, and C_{16}-AHLs detected by CG-MS (Rivas et al., 2007). In that work, different assays with mutant strains make evident that the AHLs production was controlled by two different quorum sensing systems (Rivas et al., 2007). Other study showed that the ammonia oxidizing bacteria *Nitrosomonas europea* was a producer of C_6-, C_8- and C_{10}-AHLs characterized by independent methods including

biosensors and GC-MS (Burton et al., 2005). Additionally, other study used SPE combined with GC-MS in order to detect AHLs from environmental samples such as the dried tissue of the marine sponge *Stylinos* sp. (Schupp et al., 2005). Even when the C_6-AHL and 3-oxo-C_6-AHL used in this study could be detected by GC-MS, the applied method was less sensitive than the biosensor *Agrobacterium tumefaciens* A136. This result compared to the other here mentioned shows the importance of conditions adjustment for through the method development process.

2.3 AI-2

AI-2 is an important interspecies signaling molecule, produced by both Gram-positive and Gram-negative bacteria: It is well established that AI-2 mediates intra- and interspecies communication among bacteria. The cleavage of S-ribosylhomocysteine yields homocysteine and (*S*)-4,5-dihydroxy-2,3-pentanedione ((*S*)-DPD), the metabolic precursor of AI-2. This (*S*)-DPD exist as an equilibrium mixture of several compounds that can be formed by cyclation, hydration and borate formation reacctions. Different bacterial species recognize different signals within this AI-2 pool. (Reading and Sperandio, 2006; Sperandio et al., 2003). AI-3 has been identified as a signaling molecule related to epinephrine and norepinephrine (Sperandio et al., 2004). Its molecular weight was stablished as 212 u, but its structure has nos been yet published.

These compounds are usually analyzed qualitatively by means of bioluminescent biosensors. Recently, a chromatographic methodology for AI-2 type molecules detection and quantification was developed, using a quinoxaline derivative, formed directly from the cultura media by the quantitative reaction of 1,2-phenylenediamine with 1,2-dicarbonyl compounds in water bufered at pH 7.2 (Thiel et al., 2009b). The quinoxaline derivative was then reacted with *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide (MSTFA) in order to obtain a polar quinoxaline derivative accesible for GC–MS analysis by formation of its trimethylsilyl derivative. This derivative was easily recognized by its characteristic fragmentation such as the ion at m/z 73 $[(CH_3)_3Si]^+$, a significant molecular ion M^+ at m/z 348, a typical fragment ion resulting from the loss of a methyl group $[M-CH_3]^+$ at m/z 333, and the characteristic ion at m/z 245 $[M-CH_2OSi(CH_3)_3]^+$. Quantification was conducted by isotope dilusion analysis using the deuterium-labeled standard $[5,6,7,8-^2H]$-phenylendiamine reaching detection limits of 0,7 ng/mL and quantification limits of 2,1 ng/mL. The (*S*) configuration of the natural DPD was confirmed by the coninjection of natural and both synthetic enantiomer derivatives using beta-cyclodextrin chiral columns for this analysis.

2.4 DFS´s and other signaling molecules

The role of diffusible signal factors (DSFs) as signaling molecules was originally identified in *Xanthomonas campestris* pv *campestris*, the causal agent of black rot of cruciferous plants. However, there are evidences that these signals are widespread (Deng et al., 2011). The DSFs are generally fatty acids with chain-lenghts of C-10, C-12, C-13 and C-14, with different structural patterns such as unsaturations usually at C-2 and C-9; and chain branches (i.e. iso and anteiso branch). The biological activity of DSF-family signals depends on not only their structural features, but the bacterial species on which they act.

Analysis of DSFs has been conducted by ethyl acetate extraction, solid-phase extraction with an Oasis HLB cartridge, and a combinaton of HPLC-ESI-MS and GC-MS from the culture supernatants of *Stenotrophomonas maltophilia* (Huang & Wong, 2007). The isolated compounds that facilitate the bacteria translocation were the identified as cis-Δ^2-11-methyl-dodecenoic acid and 11-methyl-dodecanoic acid, together with other six structural related fatty acids. Their structures were established based on their FAMEs and pyrrolidide derivatives. The patogenic bacteria *Xylella fastidiosa* was found to be the producer of 12-methyl-tetradecanoic acid as DSFs, based on its endoglucanase restoration activity in a mutant strain of *Xanthomonas campestris* pv *campestris*. The active fraction was obtained by solvent extraction from its culture media with hexane and further derivatized to form the FAMEs. These derivatives were analyzed by GC-MS and the fragment at m/z 74 characteristic for McLafferty rearrengement in FAMEs and other important losses were used for the structural identification of the DSF (Colnaghi et al., 2007).

Diketopiperazines (DKPs) are aminoacid dimmers commonly found in Gram negative bacteria, and usually analyzed by GC-MS. The idenfiyed molecules were the diasteromeric couples (D- and L-) cyclo-(Ala-Val), cyclo-(Pro-Phe), cyclo-(Pro-Leu) cyclo-(Pro-Val) and cyclo-(Pro-Tyr) in *Burkholderia cepacia* (Wang et al., 2010) and *Pseudomonas putida* (Degrasi et al., 2002). These molecules have been extracted with ethyl acetate and detected by GC-MS in both SIM and SCAN modes following their M^+ ions and the characteristic ions at m/z 70, 153, 154. However, these molecules can also be culture media artifacts produced by heating as demonstrated for the cyclo-(Pro-Phe), but in minor amounts than the DKP produced by the bacteria (Wang et al., 2010). In this way, the methodologies used for DKPs analysis must involve a careful evaluation of its production by the bacterial strains.

Farnesol showed to be a signaling molecule that regulates yeast-mycelium conversion in *Candida albicans* without a depletion of its growth rate (Hornby et al., 2001). Its activity as signaling molecule was determined in six different *C. albicans* strains and different culture media and growth conditions. As this signaling molecule was extracted in ethyl acetate and analyzed in a preliminar way by TLC, it was further analyzed by GC-MS by CI and EI and its BSTFA derivative (Hornby et al., 2001). Nerolidol, a farnesol isomer showed activity but in a leser extent than farnesol.

The Pseudomonas quinolone signal has been analyzed by TLC and HPLC (Pesci et al., 1999), but there are not reports of its analysis by GC methods even when these molecules seem to be succeptible of GC analysis. The analysys by GC of signaling molecules of the gamma-butirolactone family as the A-Factor found in *Sptreptomyces* species has not been described. Other signaling molecules as 2,3-diamino-2,3-bis(hydroxymethyl)-1,4-butanediol from *Streptomyces natalensis* ATCC27448, (Recio et al., 2004) result to be non compatible with GC analysis due to its high polarity. These molecules have been analysed by HPLC isolation, and NMR structural elucidation.

In this context, gas chromatography proved to be very useful and versatile in the detection, structural analysis and quantification of signaling molecules. There are still challenges to resolve as the reduction of detection limits for signaling molecules, the detection or even the discovery of new signaling molecules, the development of quantification methods for linear AHLs that do not envolve the use of C_7AHL as internal standar and quantification methods for 3-hydroxyl AHLs, among other issues, or GC methods for PQS signaling molecules.

3. Quorum sensing inhibitors analysis

The antibiotic therapy has a lot of problems, which includes the emergence of drug-resistant bacteria both in hospital and in community-acquired infection, and the slow progress in developments of new antibiotics with novel modes of action. All this problems makes necessary the development of new strategies to control bacterial infections. In this area the quorum sensing inhibitors are emerging as a novel and potentially useful strategy. Is noteworthy to mention antifungal and cancer therapies are currently exploring the use of QS inhibitors compounds as a new strategy of control (Dembitsky et al., 2011; Chai et al., 2011). The QS inhibitors may act in four different ways in bacterial systems. First inhibiting the signaling molecules biosynthesis (i.e. AHLs); second inducing degradation of the signaling molecules (Dong et al., 2002), where the AHLs lactonolysis degradation could be followed by GC-MS or HPLC-MS analysis; third blocking specific bind sites of AHLs to LuxR type proteins; and finally, by inhibition of DNA transcription (Dobrestov et al., 2009). Inhibition of QS systems regulating the expression of virulence factors as well as biofilm formation is a highly attractive field for developing novel therapeutics, because the biofilm provides to bacteria a large resistance to antibiotics. A quorum sensing inhibitor, suppress specific genetic expression of bacteria without cause of death. Since survivable mechanisms of bacteria are not induced by QSI compounds, bacterial resistance is not developed, thus it has also been considered since years for the pharmaceutical industry as a promising strategy for the design and development of antipathogenic compounds, useful in controlling microbial chronic infections (Rasmussen et al., 2006).

The GC analysis has been used for the characterization of some compounds with QSI activity, however for the understanding of mechanism of action this technique lacks of value, and the use of biosensor and *in silico* investigation is the current option. Due to volatility restrictions for GC analysis, only the QSI inhibitors from essential oils, free volatile compounds and furanone analogues will be discussed here. The no volatile compounds with QSI properties, like some flavonoids, malyngamide (Kwan et al., 2010), *S*-ribosyl-L-homocysteine analogs (Shen et al., 2006) and macromolecules (Amara et al., 2011), are not revised. For a general revision Chan et al., 2004; Dobrestov et al, 2009; Konaklieva & Plotkin, 2006; McDougald et al., 2007; Ni et al., 2009, Rasmussen et al., 2006 and the issue number 1 of 111th volume of The Chemical Reviews are suggested.

The dependence of QS on small molecule signals has inspired organic chemists to design non-native molecules that can intercept these signals and thereby perturb bacterial group behaviors. The main investigated QS inhibitors are the AHLs analogues. Lots of analogues have been synthetized, including aromatic and sulfur derivatives (Galloway et al., 2011). The use of GC in the analysis of AHLs compounds was provided in the previous section.

3.1 Furanones and analogues

The most interesting natural compounds for its QSI activity are the bromofuranones from the red alga *Delisea pulchra* (Figure 3a), these compounds are able of inhibit QS-regulated virulence genes, including the production of antibiotics, the bacterial motility and the biofilm formation (Ren et al., 2001; Steinberg & De Nys, 2002). However, most of them have not yet qualified as chemotherapeutic agents because of its toxicity, high reactivity and instability. These facts make evident the necessity of finding new, more potent and safe

compounds with QSI properties. For that reason the synthesis of furanone compounds has being explored as well as its structure-relationships (Persson et al., 2005; Martinelli et al., 2004; Wright et al., 2006). However, few examples of the use of GC for furanones analysis in the search of QSI are available in the bibliography in spite of its potential as analytical tool.

Kim et al., in 2008 synthesized new furanone derivatives (Figure 3b) with structural similarities to patulin, a *Penicillium* QSI compound, in order to develop *P. aeruginosa* QS inhibitors and biofilm controlling agents. Each of the six synthesized compounds was confirmed using techniques as NMR and GC-MS. All compounds could remarkably inhibit both *Pseudomonas* QS signaling and biofilm formation. Additionally, the authors for the profound understanding about inhibition mechanisms estimate the binding energy between QS receptor, LasR, and the synthesized compounds in silico modeling systems, which showed good agreement with the experimental results. The most important goal of this work was the structural modeling which can be used as a tool to design the QS inhibitors or some other kinds of enzyme modulators (Kim et al., 2008).

a. Some *Delisea pulchra* furanones   b. Furanone derivatives related to Patulin

c.   Examples of the most active QSI compounds identified by Kim et al., 2008
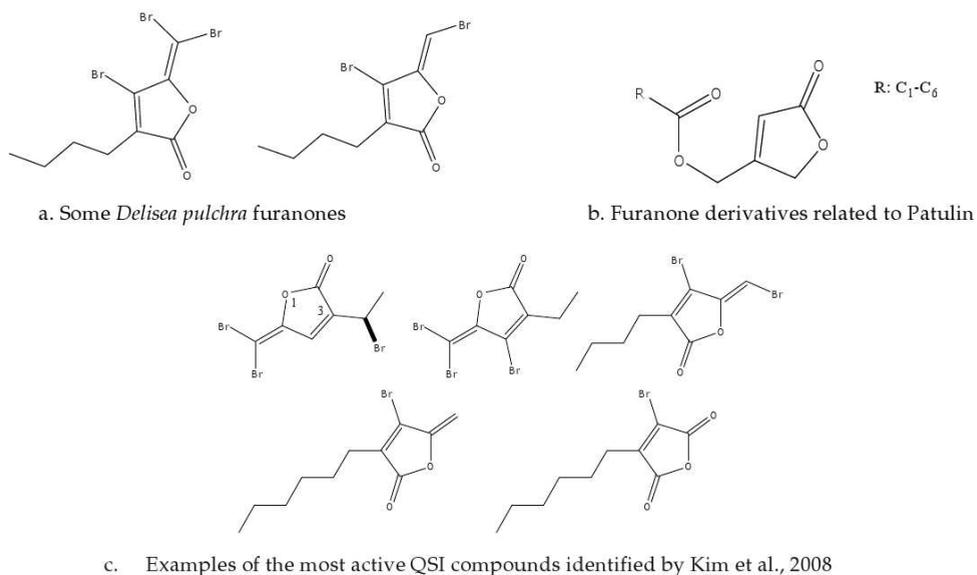
Fig. 3. Some furanone derivatives with quorum sensing inhibition properties

Other example that used GC-MS in the characterization of synthetic compounds in the searching of new QS inhibitors is the work of Steenackers et al., 2010, who synthesized a library of 25 1'-unsubstituted and 1'-bromo or 1'-acetoxy 3-alkyl-5-methylene-2(*5H*)-furanones and two 3-alkylmaleic anhydrides (Figure 3c). The compounds were evaluated for the antagonistic effect against the biofilm formation by *Salmonella enterica* Typhimurium, an important pathogen for humans, and the bioluminescence of *Vibrio harveyi*, which is quorum sensing regulated. *Vibrio* species are important pathogens in the intensive rearing of marine fish and invertebrates like penaeid shrimp. Because multi-resistant *Vibrio* strains have emerged and antibiotics are no longer effective in the treatment of luminescent vibriosis, these marine industries require new alternatives for vibriosis controls, and the QSI

represents an important option in this field. In this way Kim et al., found a drastic influence of 3-alkyl chain, bromination pattern and the ring structure on the biological activity of the compounds. Moreover, molecules without a 3-alkyl chain were shown to be highly toxic for both, *Salmonella* and *Vibrio*, while the 1'-unsubstituted furanones with a long 3-alkyl chain did not reduce biofilm formation (octyl chain and longer) nor bioluminescence (dodecyl chain). However, the 1'-unsubstituted furanones with ethyl, butyl or hexyl side chains inhibited biofilm formation at low concentrations, without affecting the planktonic growth at these concentrations. Similarly, the 1'-unsubstituted furanones with a butyl to decyl side chain inhibited bioluminescence without affecting the planktonic growth of the bacteria at the same concentrations.

On the other hand, the introduction of a bromine atom on the first carbon atom of the alkyl side chain drastically improved the activity of the furanones in both tested systems. The introduction of an acetoxy function in this position did in general not improve the activity. The main goal of this work was the identification of the potential of the (bromo) alkylmaleic anhydrides as a new and chemically easily accessible class of biofilm and quorum sensing inhibitors.

3.2 Microorganisms as source of QSI compounds

Many marine or terrestrial bacteria produce volatile compounds, but the specific function of these compounds keeps unknown for many cases. These volatiles could be used either in intra- or inter-specific communication and/or in the chemical defense against other organisms. Schulz et al., evaluated the antiproliferative activity of 52 volatile compounds released from bacteria characterized by GC-MS. The results showed that octanoic acid is active against hyphal fungi and yeasts, as are some *N*-phenethyl amides. Gamma-butyrolactones similar to the signaling molecules are active against fungi, yeasts, and bacteria, with a large influence of a double bond in the lactone ring. Furthermore, the expansion of the ring was found to reduce QSI activity. Pyrazines as well as ketones are largely inactive with the exception of (*Z*)-15-methylhexadec-12-en-2-one, showing broad activity. *S*-Methyl benzothioate is the only sulfur containing compound with activity against fungi and yeast, while all others are inactive. In contrast, the compounds common to many bacteria (i.e. 3-methylbutanol, 2-phenylethanol, (2*R*,3*R*)-butanediol, acetoin, geosmin, (+)-*R*-methylisoborneol, (-)-*S*-methylisoborneol and dimethyl disulfide) showed no inhibitory activity. Additionally, all compounds were investigated for their activity in AHLs mediated bacterial communication systems. The test were performed using *E. coli* MT102 (pJBA132) and *Pseudomonas putida* F117 (pKR-C12) as biosensors. The first strain shows the highest sensitivity for (3-oxo-C_6-AHL), while the second is very sensitive to C_{12}-AHL. Schulz et al., observed that 25% of the compounds were able to reduce activity of *P. putida* sensor F117, while 19% showed inhibitory activity against *E. coli* MT102 biosensor, and 13% enhanced its activity. Most of the gamma- and delta-lactones inhibited the response of the C_{12}-AHL sensor. Especially the delta-lactone of Figure 4a was highly active. In contrast, the *E. coli* MT102 biosensor showed a different behavior. In particular some gamma-lactones stimulated this sensor. This influence may be due to the structural similarity between the lactones and the AHLs. Some aliphatic ketones also proved to be active as well some related alcohols (Figure 4b). The 2-hexylpyridine (Figure 4c) showed inhibitory activity. The common compounds 3-methylbutanol, 2-phenylethanol, (+)-*R*-methylisoborneol and (-)-*S*-methylisoborneol showed some activity,

reducing the effectivity of the 3-oxo-C6-AHL sensor. In summary, the observed that some compounds interfered with the quorum-sensing-systems, especially the γ-lactones while the pyrazines showed to have only low intrinsic activity. (Schulz et al., 2010).

An interesting example of the complementarily of GC-MS analysis and the use of *Chromobacterium violaceum* CV017 in the QSI bioassay was published by Dobretsov et al., in 2010. In this study the analysis of 25 marine cyanobacteria ethyl acetate/methanol (1:1) extracts collected in different locations of the world (South Florida (USA), Belize and Oman) during different seasons, showed that 19 extracts inhibited violacein pigment production of *C. violaceum*. The minimal inhibitory amount of extract varied from 1.2 to 66.4 μg per disk, these values indicated a strong QSI activity of these organic extracts. The most active extract was separated by classic chromatographic methods; finally, 0.9 mg of the pure QS inhibitory compound was isolated from 213.8 g (dry weight) of *L. majuscula* indicating a very low concentration of the active compound in the cyanobacterial extracts. The compound was identified as malyngolide (MAL) (Figure 4d) by NMR and MS analysis. All the others crude extracts were analyzed by GC-MS, using a Shimadzu GC17-A gas chromatograph coupled to a Shimadzu QP5000 mass spectrometer. A high-resolution gas chromatography column (HP-5 MS, 30 m x 0.25 mm) was used. The Injections were performed in splitless mode at 70°C. The injection port was held at 250°C with a 70°C to 290°C temperature ramp at 10°C/min. Scan mode was used to analyze ions characteristic of malyngolide, particularly the peak at *m/z* 239, which is characteristic for malyngolide, and is due by the loss of angular CH_2OH group. Production of MAL appears to be widespread in *Lyngbya* spp. from different locations sampled over the time, with reports of this compound from different geographic regions as diverse as Florida, Hawaii, and Guam. The QSI assays allowed to establish that active MAL concentrations ranged from 0.07 to 0.22 mM with EC_{50} = 0.11 mM, even at the highest concentration MAL doesn't effects the *C. violaceum* growth. In order to understand the mechanisms of action of MAL, Dobretsov et al. in 2010 used in addition of violacein production of CV017 the QS-dependent β-galactosidase activity of the *A. tumefaciens* NTL4 (pZLR4) (Cha et al., 1998), *Escherichia coli* JM109 strains (pSB1075; pMT505 pTIM5211; and pTIM2442) and *P. aeruginosa* PAO1. The results indicated that MAL was not recognized as an AHL signal-mimic by the reporter and does not function as an antagonist of QS in *A. tumefaciens*. Additionally, the results suggested MAL may exert its inhibitory effect on QS by reducing or partially blocking the expression of *lasR* but not by interference with the bacterial AHL-binding domain. This mode of action has not been previously characterized in any of the naturally occurring QS inhibitors and could open a new way for inhibition of bacterial QS.

On the other hand, MAL has several ecological functions in the marine environment, including feeding deterrents to opistobranch mollusks (Nagle et al., 1998) and coral reef fishes (Thacker et al., 1997) as well as an antibacterial compound (Cardellina et al., 1979; Babler et al., 1980). However, Dobretsov et al., in 2010 found that this compound was not toxic to *C. violaceum* CV017 or *E. coli* at QS inhibitory concentration, this observation allowed them to conclude that MAL activity is possibly similar to some other antibiotics, which at sublethal concentrations inhibit QS in bacteria. In order to establish if the MAL is released by *L. majuscula* into seawater, the presence of MAL in it was estimated using GC-MS, founding hat MAL could be released and accumulated at the surrounding sea water. These results suggested that MAL can block bacterial QS and help cyanobacterium to control growth of heterotrophic bacteria (Dobretsov et al., 2010).
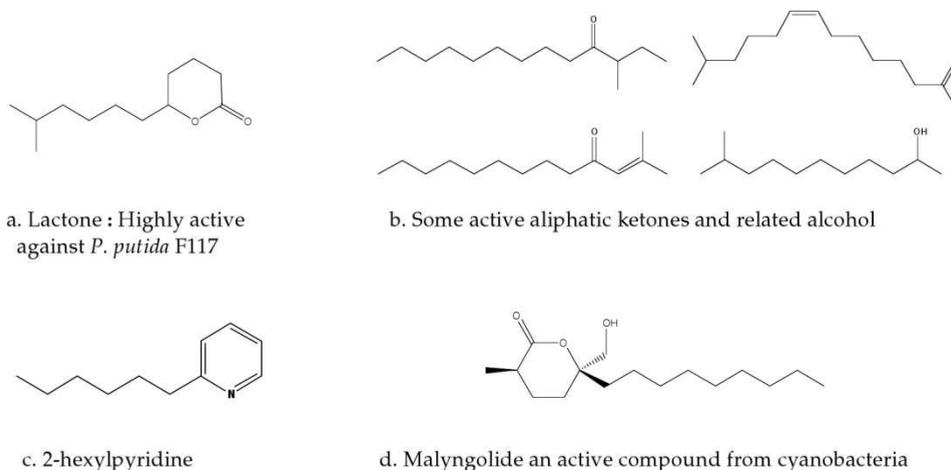
a. Lactone : Highly active against *P. putida* F117

b. Some active aliphatic ketones and related alcohol

c. 2-hexylpyridine

d. Malyngolide an active compound from cyanobacteria

Fig. 4. Some volatiles compounds with QSI activity isolated from bacteria

3.3 Food as source of QSI

Recently, researches observed certain inhibition of the autoinducer AI-2 by food matrixes; however, the understanding of bacterial cell signaling in relation to foodborne pathogens and food spoilage organisms is limited. Widmer et al., showed that poultry meat wash (PMW) inhibited the QS of *Vibrio harveyi* BB170 strain reporter. The PMW was separated using bioguided methodology employing molecular size exclusion and reverse phase column chromatography. This procedure allowed identifying the hydrophobic fraction as the responsible for the QSI activity of the PMW. The mixture of fatty acids from the PMW were converted in their FAMEs, and extracted with hexane. This extract was further analyzed by gas chromatography, using a FID detector and passed through a fused silica capillary column, according to the AOAC oficial method Ce 1h-05. Six FAMEs were identified and quantified. After that the mayor fatty acids: linoleic acid, oleic acid, palmitic acid, and stearic acid were tested for inhibition as pure compounds, finding that all samples expressed AI-2 inhibition. Then, the fatty acids combined in concentrations equivalent to those detected by GC analysis expressed inhibition. The combined fatty acids at 100-fold natural concentrations did not demonstrate a substantial decrease in colony plate counts, despite presenting high AI-2 inhibition. The inhibition by the combined fatty acid samples was, however, significantly ($P \leq 0.05$) lower than that observed in the PMW control samples, this could be due to the absence of minor fatty acids, and other compounds, in the artificial combined fatty acids sample. These fatty acids, through modulating quorum sensing by inhibition, may offer a unique means to control for food-borne pathogens and reduce microbial spoilage (Widmer et al., 2007).

Examples of inhibitors of AI-2 are not common as the AHL quorum sensing systems inhibitors (for revision Dembitsky et al., 2011); however, some AI-2 inhibitors have been designed and synthesized. These compounds usually contain ribosyl and organic acid moieties (Alfaro et al., 2004; Shen et al., 2006). Some of these inhibitors were shown to coordinate with the metal Co^{2+} ion of LuxS using a ketone oxygen and a hydroxyl group of

the inhibitor, and they may cause inhibition by preventing the hydroxamate from approaching the metal ion properly (Shen et al., 2006). The fatty acids identified by Widmer et al., 2007 did not have a similar structure to AI-2. However; the acid moiety may be binding LuxS, impeding its function. Another mode of action of these fatty acids could be the interference of transport systems employed by the bacteria for taking up AI-2 from the environment.

3.4 Essential oils as source of QSI

The essential oils have been identified as an interesting source of compounds able to disrupt the bacterial quorum sensing. The analysis of these oils is usually done by chromatographic methods; however, the isolation and identification of the compounds responsible for the QSI activity is yet an objective for the natural products researchers. The preparative GC could play an important role in this area.

Essential oils have been investigated by its QSI activity. These kinds of extracts are usually analyzed by GC-MS. This is the case of Clove, cinnamon, lavender and peppermint oils that showed activity against the biosensors *Chromobacterium violaceum* (CV12472, CVO26 and CV31532) and *Pseudomonas aeruginosa* (PAO1). Another 17 essential oils were tested without positive results; these essential oils included *Citrus* spp and *Cymbopogon* spp among others. The clove oil had the strongest activity (zones of pigment inhibition 19 mm), and its activity was found to be concentration-dependent. At sub-MICs of clove oil, 78% reductions on violacein production over control and up to 78% reduction on swarming motility in PAO1 over control were observed. The GC-MS analysis was conducted on a HP-1 Column (30 m X 25 mm X 0.25 μm). The GC–MS analysis of clove oil allowed establishing the presence of eugenol (74%), and other minor constituents identified as α-caryophyllene (4%), iso-caryophyllene (6%), caryophyllene oxide (2.4%), β-caryophyllene (5%), napthalene, 1,2,3,5,6,8a-hexahydro-4,7-dimethyl-1-(1-methyl ethyl) (7%) and 1,6-Octadiene-ol-,3,7-dimethyl acetate (1%). However, when the eugenol was assayed it did not exhibit QS inhibition activity. Additionally, neither major constituent like eugenol nor minor constituents like α-caryophyllene and α-caryophyllene share structural similarity with AHLs or known QS inhibitors like halogenated furanones (Khan et al., 2009).

A Hungarian research Group studied nine essential oils for its QSI properties, the essential oils were purchased to Phoenix Pharma Ltd. (Hungary, Budapest) in quality according to the requirements of Hungarian pharmacopoeia. As biosensors the strains *Chromobacterium violaceum* CV026, *Escherichia coli* ATTC 31298 and the partially characterized Ezf 10/17 isolated from a grapevine crown gall tumor were used. The last two strains were used as AHLs producers in order to induce pigmentation of CV026 and therefore they can be used to monitor AHLs-induced pigment production by *C. violaceum* CV026. 5-fluoro-uracil and acridine orange were selected as positive controls by its inhibition CV026 violacein production properties. The rose, geranium, lavender and rosemary oils had potent QS inhibition activity in different biological model test. Eucalyptus and citrus oils moderately reduced pigment production by *C. violaceum* CV026, whereas the chamomile, orange and juniper oils were ineffective (Szabó et al., 2010). However, in this work the authors didn't identify the responsible compounds for QSI activity from the essential oils.

Farnesol is a sesquiterpene (Figure 5a) present in many essential oils (i.e. *Pluchea dioscoridis*, *Zea mays* and *Pittosporum undulatum*), and able to inhibit the growth of some

microorganisms, such as the human pathogens *Staphylococcus aureus* and *Streptococcus mutans*, and the plant pathogenic fungus *Fusarium graminearum*. Farnesol also enhances microbial susceptibility to antibiotics, indicating a putative application as an adjuvant therapeutic, and a possible role as chemical defense in the plant. This sesquiterpenoid was also identified as a quorum sensing molecule produced by the dimorphic fungus *Candida albicans*, where it prevents the fungal transition from yeast to mycelium, and disrupts biofilm formation. Some studies showed that farnesol increases the virulence of *C. albicans* in a mouse infection model and this fungus uses it in order to reduce competition with other microbes. Derengowski et al., tested the effects of farnesol on *Paracoccidioides brasiliensis* growth and morphogenesis. This fungus is the etiologic agent of paracoccidioidomycosis (PCM), a systemic human mycosis geographically confined to Latin America. The results indicated that farnesol acts as a potent antimicrobial agent against *P. brasiliensis*. The fungicide activity of farnesol on this pathogen is probably associated to cytoplasmic degeneration, in spite of the apparent cell wall integrity, as observed by transmission and scanning electron micrographs. In concentrations that did not affect fungal viability, farnesol retards the germ-tube formation of *P. brasiliensis*, suggesting that the morphogenesis of this fungal is controlled by environmental conditions (Derengowski et al., 2009).

Another well known compound present in essential oils like cinnamon and cassia is the cinnamaldehyde (Figure 5b). This compound is effective at inhibiting two types of AHLs mediated QS, and also AI-2 mediated QS. The effect of cinnamaldehyde on 3-OH-C_4-AHL and AI-2 mediated cell signalling was determined using *V. harveyi* BB886 and BB170 strains. The effect of cinnamaldehyde on LuxR-mediated transcription from the P_{luxI} promoter, which is induced by 3-oxo-C_6-AHL, was evaluated using the destabilized green fluorescent protein-based bioreporters *E. coli* ATCC 33456 pJBA89 and *E. coli* ATCC 33456 pJBA113. Niu et al., in 2006 proposed that the three carbon aliphatic side chain of cinnamaldehyde interferes with the binding of the smaller 3-hydroxy-C4- and 3-oxo-C6-HSLs to their cognate receptors, but was not sufficiently long enough to substantially reduce the binding of 3-oxo-C_{12}-HSL to LasR. They also observed that cinnamaldehyde significantly reduced AI-2 mediated signalling. Its concentrations in common cinnamon-containing foods range from 4 to 300 ppm. Consequently, the potential influence of it on 3-OH-C4-, 3-oxo-C6-HSL and AI-2 mediated quorum sensing could affect bacterial activity, and may be relevant to food ecology. Brackman et al., in 2008 studied some derivatives of cinnamaldehyde as quorum sensing inhibitors, the mechanism of QS inhibition was evaluated by measuring the effect on bioluminescence in several *Vibrio harveyi* mutants. The compounds were also evaluated in an *in vivo* assay measuring the reduction of *Vibrio harveyi* virulence towards *Artemia* shrimp.

Cinnamaldehyde and several derivatives were shown to interfere with AI-2 based QS by decreasing the ability of LuxR to bind to its target promoter sequence. These compounds, used in sub-inhibitory concentrations, did not only affect *in vitro* the production of multiple virulence factors and biofilm formation, but also reduced *in vivo* the mortality of *Artemia* shrimp exposed to *Vibrio harveyi* BB120. In addition, cinnamaldehyde reduced the ability to cope with stress factors like starvation and exposure to antibiotics. Since inhibitors of AI-2 based quorum sensing are rare, and considering the role of AI-2 in several processes these compounds may be useful leads towards antipathogenic drugs. These results indicated that cinnamaldehyde and cinnamaldehyde derivatives are potentially useful antipathogenic lead compounds for treatment of vibriosis, which is a major disease of marine fish and shellfish and is an important cause of economic loss in aquaculture (Brackman et al., 2008).

a- farnesol   b. cinnamaldehyde

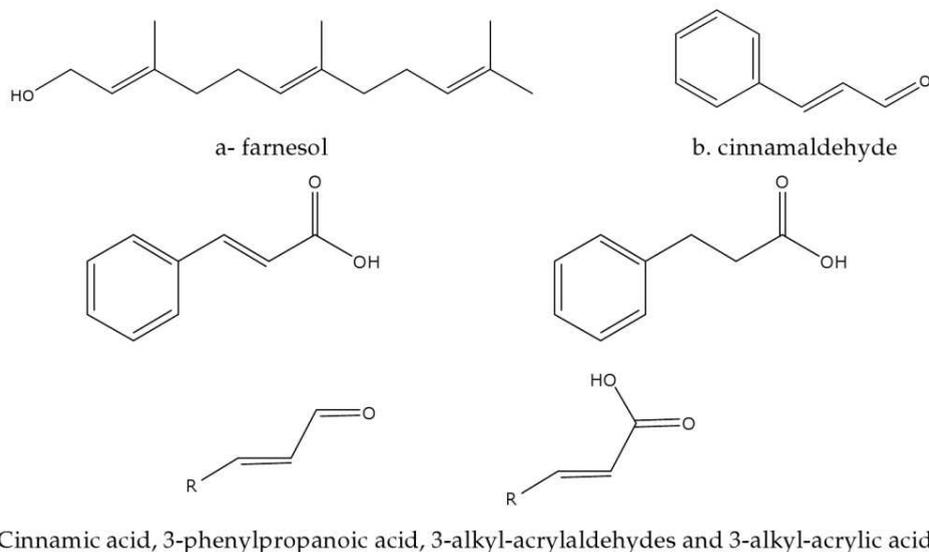c. Cinnamic acid, 3-phenylpropanoic acid, 3-alkyl-acrylaldehydes and 3-alkyl-acrylic acids

Fig. 5. Some compounds present in essential oils with QSI activity.

In 2011 Brackman et al., studied 42 cinnamaldehyde analogs, including cinnamic acids, 3-phenylpropanoic acid derivatives, 3-alkyl-acrylaldehydes and 3-alkyl-acrylic acids derivatives (Figure 5c). These included an α,β-unsaturated acyl group capable of reacting as Michael acceptor connected to a hydrophobic moiety and a partially negative charge. Cinnamaldehyde and most analogs did not affect the growth of the different *Vibrio* strains when they were used at concentrations up to 250 mM. Several new and more active cinnamaldehyde analogs were discovered and they were shown to affect *Vibrio* spp. virulence factor production *in vitro* and *in vivo*. These compounds significantly increased the survival of the nematode *Caenorhabditis elegans* infected with *Vibrio anguillarum*, *Vibrio harveyi* and *Vibrio vulnificus*. In addition, the most active cinnamaldehyde analogs were found to reduce the *Vibrio* species starvation response, to affect biofilm formation in *V. anguillarum*, *V. vulnificus* and *V. cholerae*, to reduce pigment production in *V. anguillarum* and protease production in *V. anguillarum* and *V. cholera*. However, the mechanism of action of these compounds is not clear at all; the chemical structure of cinnamaldehyde analogs and their effect on the DNA binding ability of LuxR, led authors to purpose that cinnamaldehyde analogs can act as LuxR-ligands, thereby changing the DNA-binding ability of LuxR (Brackman et al., 2011). Compounds capable of participating in a Michael-type addition reaction were found to be active, while replacement of the aldehyde group by a carboxylic acid moiety resulted in less active compounds. Compounds lacking conjugated double bond were found to be inactive. In this way, nucleophilic amino acid side chains (e.g. thiol groups of cysteine residues) in LuxR possibly react with the electrophilic beta-position to form irreversible cinnamaldehyde-receptor conjugates.

3.5 Marine organism

The marine organisms are well known as a source of bioactive compounds, particularly cytotoxic; however, a few examples of its potential as source of quorum sensing inhibitory compounds are described in the literature, in spite of the active bromo furanones were isolated from the marine red algae *Delisea pulchra*. Nowadays, examples of systematic bioprospection of this resource are more common than five years before (Skindersoe et al., 2008; Dobrestov et al., 2010).

Peters et al., in 2003 studied the North Sea bryozoan *Flustra foliacea*. The GC-MS analysis of the dichloromethane extract of the bryozoan allowed identifying 11 compounds. GC-MS analysis were conducted using a Perkin-Elmer PE-1 column (30 m x 0.32 mm) and He (2 ml/min) as the carrier gas, the temperatura gradient program was used: increase from 90°C (at zero time) to 160°C at a rate of 6°C/min and increase from 160 to 300°C at a rate of 10°C/min. Preparative HPLC of the extract yield one diterpene and 10 bromo-tryptamine alkaloids (Figure 6). All of these compounds were tested in order to determine their activities in agar diffusion assays against bacteria derived from marine and terrestrial environments. Additionally, using the biosensors *P. putida* (pKR-C12), *P. putida* (pAS-C8), and *E. coli* (pSB403) the antagonistic effect on AHL dependent quorum-sensing systems was investigated. The most active compounds caused reductions in the signal intensities in these bioassays ranging from 50 to 20% at a concentration of 20 μg/ml. At higher concentrations, however, the compounds had additional biocidal effects.
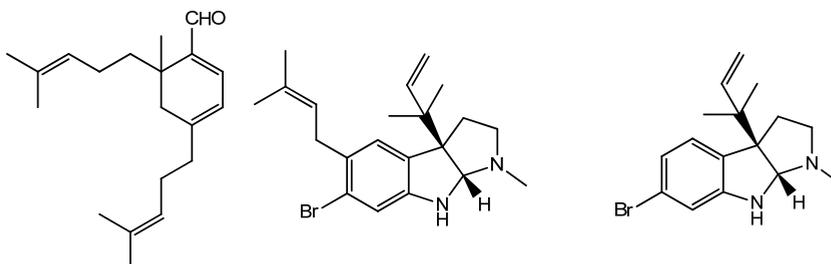

Fig. 6. Diterpene and the most active prenylated alkaloids isolated form bryozoan *Flustra foliacea*.

4. Conclusion

Studies in quorum sensing involve both, understanding of quorum sensing molecular pathways as well as signaling molecules characterization. The use of GC has shown to be one of the more versatile tools for detection, identification and quantification of many types of signaling molecules involved in quorum sensing. Additionaly, GC allowed characterizing several quorum sensing inhibitors from different organisms including bacteria, fungi, terrestrial and marine macroorganisms. The success of this chromatographic technique in characterize quorum sensing molecules (inducers and inhibitors) is supported with the development of other extraction and fractionation techniques that allowed reducing detection limits and improve accurateness, making the GC methods as sensible as the biosensors. Further improvements should be maked for detection, identification and quantification of known and unknown signaling molecules.

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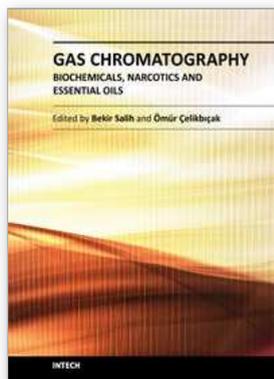
Gas Chromatography - Biochemicals, Narcotics and Essential Oils

Edited by Dr. Bekir Salih

Gas Chromatography involves the study of various vaporizable molecules in chemistry and the other related research fields. This analytical method has a number of features and advantages that make it an extremely valuable tool for the identification, quantification and structural elucidation of organic molecules. This book provides detailed gas chromatography information to applications of biochemicals, narcotics and essential oils. The details of the applications were briefly handled by the authors to increase their comprehensibility and feasibility. This guide should be certainly valuable to the novice, as well as to the experienced gas chromatography user who may not have the enough experience about the specific applications covered in this book. We believe this book will prove useful in most laboratories where modern gas chromatography is practiced.

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